Sodium–hydrogen exchanger NHA1 and NHA2 control sperm motility and male fertility

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Our previous work identified NHA1, a testis-specific sodium–hydrogen exchanger, is specifically localized on the principal piece of mouse sperm flagellum. Our subsequent study suggested that the number of newborns and fertility rate of NHA1-vaccinated female mice are significantly stepped down. In order to define the physiological function of NHA1 in spermatozoa, we generated Nha1FxFx, Zp3-Cre (hereafter called Nha1 cKO) mice and found that Nha1 cKO males were viable and subfertile with reduced sperm motility. Notably, cyclic AMP (cAMP) synthesis by soluble adenylyl cyclase (sAC) was attenuated in Nha1 cKO spermatozoa and cAMP analogs restored sperm motility. Similar to Nha1 cKO males, Nha2FxFx, Zp3-Cre (hereafter called Nha2 cKO) male mice were subfertile, indicating these two Nha genes may be functionally redundant. Furthermore, we demonstrated that male mice lacking Nha1 and Nha2 genes (hereafter called Nha1/2 dKO mice) were completely infertile, with severely diminished sperm motility owing to attenuated sAC-cAMP signaling. Importantly, principal piece distribution of NHA1 in spermatozoa are phylogenetically conserved in spermatogenesis. Collectively, our data revealed that NHA1 and NHA2 function as a key sodium–hydrogen exchanger responsible for sperm motility after leaving the cauda epididymidis.

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As many as 15% of human couples are infertile, and male infertility is about half of these cases.1 To fertilized egg, spermatozoa from the cauda epididymis must travel a long journey in the female reproductive tract to reach ampulla of uterine tube. Interestingly, in most mammalian species examined, the sperm journey experiences a natural increase in Na+/HCO₃⁻ concentration and pH value (pH < 7, Na⁺ < 25 mM, HCO₃⁻ < 1 mM in cauda epididymis, whereas pH ~ 7.4, Na⁺ > 100 mM, HCO₃⁻ > 10 mM in female reproductive tract).2,3 It is thus clear that intracellular pH (pHi) regulation is of the utmost importance for sperm physiology, including motility, maturation and the acrosome reaction.4 The maintenance of sperm pHi is kept through the involvement of several mechanisms, among which is included the sodium–hydrogen (H⁺) exchangers (NHEs).5

NHEs, also known as Na+/H⁺ antiporters (NHAs), are integral membrane proteins that catalyze the exchange of Na⁺ for H⁺ across lipid bilayers and are ubiquitously distributed in almost all living organisms.6 The SLC9 gene family encodes NHEs and can be divided into three subgroups (reviewed in Martins et al.). The SLC9A subgroup encompasses plasmalemmal isoforms NHE1–5 (SLC9A1–5) and the predominantly intracellular isoforms NHE6–9 (SLC9A6–9). The SLC9B subgroup consists of two recently cloned isoforms, NHA1 and NHA2 (SLC9B1 and SLC9B2, also known as NHEDC1 and NHEDC2). The SLC9C subgroup consists of a sperm-specific plasmalemmal NHE (SLC9C1, also known as sNHE) and a putative NHE, SLC9C2, for which there is currently no functional data.

Four Na⁺/H⁺ exchangers (NHE1,8 NHE2,9 sNHE10 and NHA11) are reported to be expressed in spermatozoa. However, normal sperm motility is maintained in Nhe1-null mice, suggesting that Nhe1 gene is male fertility independent.12 Tests histology, sperm numbers and morphology are normal, but sNhe null males are completely infertile with severely diminished sperm motility.10 Further study suggests that cyclic AMP (cAMP) metabolism is impaired in spermatozoa lacking sNHE.13 A recent study showed that NHE8 is highly expressed in the Leydig cells and male mice lacking Nhe8 gene are infertile through its effect on modifying luteinizing hormone receptor (LHR) function.14 Second messenger cAMP has been reported to be essential for sperm function, including activation of motility, hyperacervation and acrosome reaction, mainly via activation of holoenzyme protein kinase A (PKA).15 In mammalian spermatozoa, cAMP is synthesized by a soluble isoform of the adenylyl cyclase (sAC) family.16,17 There are two alternative splicing products, which independently encode full-length sAC (sACα) and truncated forms of sAC (sACβ).18 sAC-null male mice are infertile because of a severe defect in sperm motility.19,20 In addition, as HCO₃⁻ directly regulates sAC, this enzyme is able to translate pH changes into cAMP levels.21,22

The sperm flagellum-specific NHE identified by Liu et al.23 in our laboratory in 2010 is now classified into a new family of...
NHE, NHA1 (SLC9B1, also known as NHEDC1). Our subsequent study demonstrates that anti-NHA1 antibody reduced sperm motility and the rate of in vitro fertilization.23 Therefore, NHA1 is proposed to regulate sperm motility. The critical role for NHA1 in human male fertility is highlighted by the finding that NHA1 expression is either reduced or absent in patients with teratozoospermia.24

In order to define the physiological function of NHA1 in spermatozoa, we generated Nha1 cKO, Nha2 cKO and Nha1/2 dKO male mice. Although single conditional knockouts for Nha1 or Nha2 were subfertile, male double knockout mice exhibited completely infertile with severely diminished sperm motility. cAMP synthesis by sAC was attenuated in cKO and dKO spermatozoa. Furthermore, the sperm motility defects could be rescued by the addition of cell-permeable cAMP analogs. In addition, the number of newborns and fertility rate of Nha1/2-vaccinated female mice were significantly stepped down, suggesting NHA1 and 2 may be an excellent target molecules for developing a novel male contraceptive.

**Results**

**NHA1 and NHA2 expression in sperm.** Our previous reports suggest that NHA1 is exclusively expressed in mouse testis.11,23 We further found that NHA1 was specifically localized to the sperm tail during late spermatogenesis (Figures 1a and b) and sperm storage in cauda epididymis (Figures 1c and d). High-magnification immunofluorescence of isolated sperm from cauda epididymis further demonstrated that the localization of NHA1 was confined to the principal piece of flagella (Figures 1e and f). Nha1 seems most closely related to Nha2 (Figure 1g). We further found that NHA2 is specifically localized in the principal piece of sperm tail, which is similar to NHA1 expression pattern (Figures 1h-j).

**Generation and analysis of Nha1 knockout mice.** Our previous study suggests that polyclonal antibody to transmembrane region of NHA1 significantly reduced the in vitro sperm motility and fertilization.11 To elucidate the physiological function of NHA1, we generated Nha1 knockout mice by

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**Figure 1** NHA1 and NHA2 were specifically expressed in the principal piece of sperm tail. (a-f) Immunofluorescence staining of NHA1 in mouse testis (a and b), cauda epididymis (c and d) and sperm from cauda epididymis (e). Note the intensive green signal at principal piece of sperm tail. NHA1 antibody staining in the presence of competing immunogen (f). The nuclei are counterstained with DAPI (blue). (g) Phylogenetic tree displays the relationship between the NHA1 and the other NHEs. The tree was generated with GeneBee aligning the predicted open reading frames of each NHE. (h-j) Immunofluorescence staining of NHA2 in mouse testis (h), cauda epididymis (i) and sperm from cauda epididymis (j). Scale bar in a, c, h and i, 100 μm. Scale bar in b and d, 50 μm. Scale bar in e, f and j, 20 μm.
homologous recombination technology. LoxP sites flank exon 4 of the Nha1 allele, and recombination of the loxP sites using Cre recombinase resulted in the removal of exon 4 (Figure 2a). The successful acquisition of Nha1 cKO mice was determined by polymerase chain reaction (PCR) amplification (Figure 2b). We confirmed that NHA1 was successfully depleted in both testis and spermatozoa in Nha1 cKO males (Figure 2c).

In Nha1 cKO male mice, there were no overt abnormalities in testicular weight (123 ± 4 mg in wild-type and 110 ± 9 mg in cKO), epidiymal weight (40 ± 1 mg in wild-type and 36 ± 2 mg in cKO) and sperm count (1.4 ± 0.2 x 10^6/ml in wild-type and 1.4 ± 0.3 x 10^6/ml in cKO) (Figures 3a-c). No overt abnormalities in spermatogenesis and epididyism of Nha1 cKO mice (Figure 3d). Isolated sperm from cauda epididyism exhibited typical morphology (Figure 3d) and flagella structure (Figure 3e).

**NHA1 is essential for sperm motility and male fertility.** By breeding assay, we found that although Nha1 cKO males copulated, the pregnancy rate (92% in wild-type and 52.5% in cKO) and litter size (11.5 ± 0.3 in wild-type and 5.2 ± 0.4 in cKO) were markedly lower compared with their control littersmates (Figures 4a and b). Acrosome reaction, a prerequisite for spermatozoa to fertilize eggs, occurred normally in Nha1 cKO spermatozoa (acrosome rate: 58 ± 1% in wild-type and 61 ± 1% in cKO) (Figure 4c). However, percentage motility of Nha1 cKO spermatozoa after release from the caudal epididyism was significantly lower than those of control spermatozoa (82 ± 3% in wild-type and 38 ± 2% in cKO) (Figure 4d). Moreover, fewer spermatozoa of Nha1 cKO males reached the female oviduct 4–6 h after coitus, compared with those of controls (Figures 4e and f).

**cAMP analogs rescue the motility defects of Nha1 cKO spermatozoa.** Our previous study suggests that sperm pH descends after treatment with the NHA1 antisera.11 Given that cAMP synthesis by sAC is closely related to sperm pH regulation and sperm motility, we detected the levels of cAMP and sAC in Nha1 cKO spermatozoa. Basal cAMP concentrations were significantly lower in Nha1 cKO spermatozoa compared with wild-type sperm cells (76 ± 3 fmol/10^6 cells in wild-type and 34 ± 2 fmol/10^6 cells in cKO) (Figure 5a). The protein level of sAC δ, a cAMP synthetase, was greatly diminished in Nha1 cKO spermatozoa (Figure 5b). Moreover, transfection of NHA1 enhanced sAC δ protein expression in HEK293F cells (Figure 5c). Notably, addition of membrane-permeable cAMP analogs, such as Sp-cAMP, 8-Br-cAMP and AM-cAMP, almost fully recovered the sperm motility of Nha1 cKO sperm (Figure 5d).

**Generation of Nha2 cKO and Nha1/2 dKO mice.** Nucleotide sequence is very similar between Nha1 and Nha2, indicating two Nha genes may be functionally redundant. Accordingly, we generated Nha2 cKO and Nha1/2 dKO mouse mice. LoxP sites flank exon 3 of the Nha2 allele, and recombination of the loxP sites using Cre recombinase resulted in the removal of exon 3 (Supplementary Figures S1a-c). Afterward, we generated Nha1/2 dKO mice (Supplementary Figure S1d).

Similar to Nha1 cKO, no overt abnormalities in testicular weight (120 ± 3 mg in wild-type and 112 ± 4 mg in cKO), epidiymal weight (38 ± 1 mg in wild-type and 36 ± 2 mg in cKO) and sperm count (1.3 ± 0.2 x 10^6/ml in wild-type and 1.3 ± 0.3 x 10^6/ml in cKO), spermatogenesis, epididyism and epididyism sperm structure of Nha2 cKO males (Supplementary Figures S2a-f). The pregnancy rate (91.6% in wild-type and 65% in cKO) and litter size (11.5 ± 0.2 in wild-type and 7.2 ± 0.3 in cKO) of Nha2 cKO males were markedly lower compared with their control littersmates (Supplementary Figures S3a and b). Nha2 deletion significantly reduced the percentage of motile sperm (84 ± 2% in wild-type and 56 ± 3% in cKO) (Supplementary Figure S3c) and the cAMP synthesis by sAC (Supplementary Figures S3d and e).

In addition, the mRNA level of Nha2 was significantly increased in the Nha1 cKO sperm (Supplementary Figure S4a). Similarly, significant upregulation of Nha1 level was observed after deletion of Nha2 (Supplementary Figure S4b). Collectively, these two Nha genes are functionally redundant.
Nha1/2 dKO males are infertile. No obvious behavioral and gross anatomical differences were noted when comparing adult Nha1/2 dKO males and wild-type littersates. Also, no relevant changes in testicular weight (110 ± 4 mg in wild-type and 104 ± 6 mg in dKO), epididymal weight (37 ± 1 mg in wild-type and 36 ± 2 mg in dKO) and sperm count (1.3 ± 0.2 × 10⁶/ml in wild-type and 1.2 ± 0.2 × 10⁶/ml in dKO) were observed in Nha1/2 dKO males (Supplementary Figures S2g-i). Elongated spermatids were present in testes and spermatozoa were present in the epididymis (Supplementary Figures S2j-k). Moreover, isolated sperm from cauda epididymis exhibited typical morphology (Supplementary Figure S2l).

Mating behavior of male Nha1/2 dKO mice was normal because vaginal plugs were observed in the females. However, mating of Nha1/2 dKO male mice with wild-type females for 2 months did not produce any pregnancies (Figure 6a). Motility analysis indicated that sperm motility was drastically reduced in Nha1/2 dKO males when compared with control littersates (84 ± 2% in wild-type and 5 ± 1% in dKO) (Figure 6b). Nha1/2 dKO sperm produced only a slightly trembling movement of the flagella. Significant reduced levels of cAMP (80 ± 2 fmol/10⁶ cells in wild-type and 17 ± 2 fmol/10⁶ cells in dKO) and sACII (< 20-fold) were observed in Nha1/2 dKO sperm cells (Figures 6c and d).

Contraceptive function of pCR-Nha1/2 vaccine. Oral vaccination of female mice with pCR-Nha1/2 significantly reduced both the pregnancy rate (90% in control and 13% in vaccinated group) and the number of newborns (10.3 ± 0.2 in control and 3.2 ± 0.2 in vaccinated group), as compared with the pCR3.1 mock plasmid (Figures 6e and f). Sperm motility was statistically reduced when sperm were mixed with vaginal wash from the pCR-Nha1/2 orally vaccinated females (80 ± 2% in control and 27 ± 1% in vaccinated group) (Figure 6g). Notably, sperm agglutination was only observed as the cross-linking or clumping together when sperm were treated with vaginal wash from the pCR-Nha1/2-vaccinated female mice (Figure 6h).

Sperm distribution of NHA1 are present across mammalian species. To determine whether sperm distribution of NHA1 is phylogenetically conserved in spermatogenesis, we performed immunolocalization of NHA1 in testes or sperm from diverse mammalian species, including rat, monkey, goat, bull, mouse and human (Figure 6i). As expected, NHA1 was specifically localized to the sperm tail during late spermatogenesis and confined to the principal piece of flagella.

Discussion
Fertilization is a fundamental and convoluted process. However, we are still far from fully understanding the gamete intracellular molecular dialog necessary for fertilization. Furthermore, it is needed to tackle increasing male infertility and to provide safer male gamete-based contraceptives.
Figure 4  Nha1 cKO male mice are subfertile. (a) Pregnancy rate was calculated as the ratio of the number of females with pregnancy to the number of females with successful mating. (b) When calculating average litter size, only the females that generated pups were included. (c) The percentage of sperm that underwent acrosome reaction (AR) after A23187 induction was counted. (d) Percentage motility of Nha1 cKO and wild-type spermatozoa after release from the cauda epididymis. Data in a–d are expressed as the means ± S.D., *P < 0.05. (e) Oviduct was collected from females mated with Nha1 knockout and wild-type males 4–6 h after coitus. Sperm tails were staining with AQP3 antibody (green). The nuclei are counterstained with DAPI (blue). Note that Nha1 cKO sperm were seldomly observed in the oviduct. Scale bar in d, 50 μm. (f) The sperm position was calculated in uterus or oviduct of females mated with Nha1 cKO and wild-type males 4–6 h after coitus.

Figure 5  sAC-mediated cAMP signaling is compromised in Nha1 cKO spermatozoa. (a) Sperm from wild-type and Nha1 cKO mice were collected in a bicarbonate-free medium. cAMP content was measured by using RIA. Data represent the mean ± S.D. (n = 3). (b) Percentage motility of null spermatozoa after a 60-min incubation with cAMP analogs plus 100 μM IBMX. Sp-cAMP (1 mM); 8-Br-cAMP (1 mM); AM: AM-cAMP (50 μM); Rp-cAMP is the biologically inactive cAMP analog (1 mM). Data in a and b are expressed as the means ± S.D., *P < 0.05. (c) Immunoblot of total sperm cell lysate (2 × 10⁶ cells) from wild-type or Nha1 cKO mice with anti-sAC antibody, which detected two major proteins: a sACg and a truncated sAC (sACt1). (d) HEK293F cells were transfected with pcDNA-Nha1 or empty pcDNA3.1 vector. Total cell lysates were used for immunoblotting with anti-NHA1 or anti-sAC antibody. The protein level was normalized and plotted against β-tubulin.
Pathological and pharmacological studies of the specific ion channel from spermatozoa, in the species where it can be done (i.e., null mice), will yield clear evidence of the role of the channel in sperm physiology and final fertilization. In this study, we showed that sodium–hydrogen exchangers, NHA1 and NHA2, were essential for sperm function as their elimination caused infertility in male mice because of a severe defect on sperm motility.

Several unique sperm ion transporters and enzymes whose elimination caused infertility in male mice because of a severe defect on sperm motility.

Several unique sperm ion transporters and enzymes whose elimination causes infertility are either pH-dependent or somehow related to pH regulation. Among them are: CatSper, a Ca\(^{2+}\) channel; Slo3, a K\(^+\) channel; Na\(^+\)/H\(^+\) exchanger and the sAC (reviewed in Nishigaki et al.\(^{4}\)). An increase in bicarbonate (HCO\(_3\)-) during the transit from the epididymis to the female genital tract (HCO\(_3\)-1 mM in cauda epididymis, whereas HCO\(_3\)-4 10 mM in female reproductive tract) activates sAC.\(^{19,20,25}\) HCO\(_3\)-induced cAMP synthesis by sAC stimulates PKA and promotes sperm maturation, motility and capacitation in the female genital tract.\(^{26-28}\) In addition to PKA, cAMP is able to bind and regulate other proteins containing cyclic nucleotide binding domain (CNBD).\(^{29}\) A CNBD protein known to be present in mouse sperm is a specific member of the mammalian NHE superfamily of Na\(^+\)/H\(^+\) exchangers, sNHE.\(^{10}\) Targeted deletion of sNhe gene results in infertility because of severely diminished sperm motility. Interestingly, sperm from sNe null mice lack motility but either permeable cAMP analogs or NH\(_4\)Cl rescues this phenotype.\(^{10}\) Later report from the same group showed that sNHE is necessary for the full-length expression of sAC suggesting that these two proteins form a protein complex involved in sperm pH control.\(^{13}\) Thus, it is proposed that cAMP affects the sperm pH by controlling the sNHE through its CNBD.\(^{30}\)

The phenotype of Nha1/2 dKO males is somewhat similar with sNhe null males as follows. First, male (sub)infertility is caused by severely diminished sperm motility. Second, their cAMP synthesis by sAC is highly diminished. Third, addition of membrane-permeable cAMP analogs almost fully recovered the sperm motility of null sperm. Quite distinct from NHA1 and NHA2, sNHE contains a consensus sequence for a putative CNBD near the C terminus, implying that sNHE function could be regulated by cyclic nucleotides. However, we did not observe any CNBD in the nucleotide sequences of Nha1 or Nha2 gene.
Based on knockout evidence, together with previous work in vitro studies, we can infer that NHA1 and NHA2 are functional Na+/H+ exchangers, and thus, Nha1 and Nha2 deficiency causes sperm pH reduction and immotility most likely via attenuating sAC-mediated cAMP synthesis. However, how NHA1 and NHA2 regulate sAC and the exact correlation between pH and cAMP level are still unclear. Further experiments, such as proteomic analysis and co-immunoprecipitation, will be useful to reveal whether NHA1 and/or NHA2 are the sAC-associated proteins.

Besides, the defect of the Nha1-null sperm is clinically related, because NHA1 expression is either reduced or absent in patients with teratozoospermia, a male infertility condition characterized by the presence of abnormally shaped sperm in the semen (microarray data; Pubmed: GDS2697/1555142_at/SLC9B1). Understanding the mechanisms regulating the expression of NHA1 gene is of great importance. A recent study by Kumar et al. identifies and characterizes regulatory DNA elements in the 5′ end of the human NHA1 gene and suggests that DNA methylation at these elements can regulate its expression.

Previous studies identify several genes, which could be served as the candidate contraceptive targets, given their sperm-specific expression and absolute requirement for fertility. We are currently suggesting that male mice lacking Nha1 and Nha2 genes, sperm Na+/H+ exchangers, are infertile, with seriously reduced sperm motility owing to attenuated sAC-mediated cAMP synthesis. Our study not only unveils a genetic basis for the role of Na+/H+ exchangers in sperm motility, but also provides an attractive contraceptive targets in humans. Given that NHA1 and NHA2 exhibit a high degree of homology among different species, Nha1/2 DNA vaccine may be a good strategy for developing an immun contraceptive vaccine for human and animal use. As shown in this study, the number of newborns and fertility rate of Nha1/2-vaccinated female mice were significantly stepped down. However, the contraceptive efficiency of orally delivered Nha1/2 DNA vaccine remains to be improved.

Materials and Methods

Ethics statement. All animal procedures were in accordance with the Animal Care and Use Committee (IACUC) of Institute of Zoology, Chinese Academy of Sciences.

Antibody production. We produced anti-NHA1 polyclonal antisera obtained from the immunized rabbits using the purified NHA1 recombinant protein according to our previous study. Briefly, the selected partial cDNA sequence (encoding 62 amino acids) of the NHA1 was inserted into pGEX-4T -1 (Invitrogen, Carlsbad, CA, USA). The primer pair for cloning was: forward/BamHI (5′-TTGGATCCCATGGATGTTTACCACTGGACTGTTT-3′) and reverse/SaI (5′-AAGTGACCGCAGATCCACACCTGGTTT-3′). The preparation of recombinant protein (GST plus NHA1 fragment) and immunization of rabbits were performed as described. Six rabbits of each group were immunized and the antisera were harvested from arteriae caroiae. We produced anti-NHA2 polyclonal antisera according to the above method. The primer pair for cloning (encoding 83 amino acids) of NHA2 was: forward/BamHI (5′-TTGGATCCCATGGATGTTTACCACTGGACTGTTT-3′) and reverse/SaI (5′-AAGTGACCGCAGATCCACACCTGGTTT-3′).

Generation of Nha1 and Nha2 knockout mouse model. A targeting vector containing Nha1 exon 4 or Nha2 exon 3 flanked by a loxP site and a loxP-Neo cassette was constructed and introduced into mouse embryonic stem (ES) cells (AB1, 129/SvEv) by electroporation. Next, ES cell clones containing the targeted Nha1 or Nha2 construct were injected into C57BL/6 (B6) blastocysts to generate chimeras. The resultant male chimeras were identified by coat color and mated with wild-type females to obtain germline transmission. Tail biopsies of agouti-pigmented F1 animals were genotyped using a primer set specific to the neo cassette. Nha1+/− and Zp3Cre transgene mice (Cre recombinase expressed in the developing oocyte and in each cell of the resulting embryos) to generate Nha1 knockout mice. We used similar mating strategy to obtain Nha2 knockout mice. The DNA isolated from tail biopsies was used for genotyping. The presence of the Fx allele and Cre transgene was determined by PCR amplification. Mice were genotyped by PCR analysis using primers (5′-CCAGCATCTCTCTCAAAACC-3′ and 5′-AGACTGAAACAGGCGGCC-3′) to identify theloxP locus, primers (5′-TTTTCTCATTCTTTTACACTACA-3′ and 5′-AGGCTCTAATTCAGGCTTTAGGACG-3′) to identify the exon 4 deleted locus for Nha1 targeting. Mice were genotyped by PCR analysis using primers (5′-TTTGTGCGCTTCTTTTGGCCTGCATC-3′) and (5′-CACAGGTTGGTGATCTCATA-3′) to identify theloxP loci, primers (5′-TCGGAAGAAGGATCCGGAATG-3′ and 5′-AGTGCGCTTACGTTGACG-3′) to identify the exon 3 deleted locus for Nha2 targeting.

Breeding assays. Eight to 12 weeks Nha1 cKO (n=10), Nha2 cKO (n=5), Nha1/2 dKO (n=5) males and their control littersmates males (n=5 each) were used for the breeding assay. Each male mouse was caged with two adult wild-type females (7–8 weeks) that were checked for vaginal plugs every morning. Once a vaginal plug was identified, another female was placed in the cage for another round of mating. The plugged female was separated and single caged, and the pregnancy records were recorded.

Sperm count and motility assays. The cauda epididymal sperm from 3-month-old male mice were placed in 1 ml modified HTF media and backflushing was used to obtain spermatozoa. Spermatozoa were incubated with DAPI (1 : 1000, Sigma-Aldrich, St. Louis, MO, USA) for 2 min. Samples were placed in Leja counting chambers (Orange Medical, Rotterdam, The Netherlands), and sperm concentration was evaluated, using the IVOS sperm analyzer (Hamilton Thorne Bioscience, St. Louis, MO, USA). Cauda epididymal spermatozoa in the media were treated with or without 50 μM to 1 mM cAMP analogs, and 100 μM IBMX (all purchased from Selleck, Shanghai, China). Motility of knockout spermatozoa after a 0- to 60-min incubation was analyzed by computer-assisted sperm analysis using the IVOS sperm analyzer (Hamilton Thorne Bioscience) and the motility rate (%) was measured.

Hematoxylin and eosin (H&E) staining. Testes and epididymis were fixed in Bouin’s solution for 24 h. Following dehydration through an ethanol series, the fixed testes were embedded in paraffin and then sectioned. Paraffin sections (5-μm-thick) were stained with H&E (Zhong Shan Technology, Beijing, China). Staining were examined under light microscopy (Nikon DS-R1, Tokyo, Japan).

Immunofluorescence on tissue sections and epididymal sperm. Testes, cauda epididymis and oviduct were fixed in 4% paraformaldehyde (PFA) for 24 h, dehydrated and embedded in paraffin. The sections (5-μm-thick) were deparaffinized and rehydrated; heat-induced antigen retrieval was performed in 10 mM sodium citrate buffer. Mouse sperm were collected from cauda epididymides using the backflushing method, washed in PBS, fixed in 4% PFA for 30 min at room temperature and air dried on poly-l-lysine-treated slides. Sections were blocked using a blocking buffer (5% donkey serum, 0.3% Triton X-100 in PBS) and incubated with primary antibodies against NHA1 (1: 200, our laboratory) or NHA2 (1: 200, our laboratory) or AQPI (1: 400, kind gift from Dr. Qi Chen) overnight at 4°C. Sections were washed and incubated with anti-rabbit FITC-conjugated secondary antibody (1: 200, Jackson Immuno Research, West Grove, PA, USA) for 1 h and counterstained with DAPI. Images were captured using a fluorescence microscope (Nikon Eclipse 80i).

Western blotting. Western blot analysis was performed as described previously. Briefly, testes and sperm were lysed in a radioimmune precipitation assay lysis buffer. The proteins were electrophoresed under reducing conditions on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were incubated with the primary antibody overnight at 4 °C and then with the secondary antibody (anti-rabbit Dye 800CW, LI-COR, St. Louis, MO, USA) for 1 h at room temperature. The specific signals and the corresponding band intensities were evaluated using an Odyssey Infrared Imaging system (Odyssey, Berlin, Germany). The protein level was normalized and plotted against β-tubulin. The following
antibodies were used in this study: rabbit anti-NHA1 (1:2000, our laboratory), rabbit anti-NHA2 (1:2000, our laboratory), rabbit anti-sAC (1:2000, Abgent, Cambridge, UK, AP5862c) and rabbit anti-\( \beta \)-tubulin (1:3000, Abcam, Cambridge, UK, UK, AP5862c). The reaction was run in triplicate in three independent experiments. The CT values for the samples were normalized to the corresponding Gapdh CT values, and relative expression levels were calculated using the \( \Delta \Delta C_{T} \) method. The primer pair for Nha1 forward (5′-TGGGACAATTGAGTAAAGAAC-3′) and reverse (5′-CAAGCTTTCAGACATCTCAAA-3′) and the primer pair for Nha2 forward (5′-GGCGACTTCTGGTTCTG-3′) and reverse (5′-CACCTCATGGCTGTAGGA-3′).

**Immunization with Nha1/2 DNA vaccine.** The purified PCR product was inserted into pCR3.1 (Invitrogen, San Diego, CA, USA) according to the manufacturer’s instructions. The primer pair for cloning Nha1 is: forward/HindIII (5′-GGCGGAAGCTTATGAGGGACAAGTTTG-3′) and reverse/EcoRI (5′-GGCGGATCTTATGAGGGACAAGTTTG-3′). The primer pair for cloning Nha2 is: forward/HindIII (5′-GGCGGAAGCTTATGAGGGACAAGTTTG-3′) and reverse/EcoRI (5′-GGCGGAAGCTTATGAGGGACAAGTTTG-3′). The primer pair for pcDNA-Nha1 vaccine or pCR3.1 mock plasmid dissolved in 30 μl of saline was used for RIA.

**Conflict of Interest**

The authors declare no conflict of interest.

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