Disruption of NHE8 expression impairs Leydig cell function in the testes

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Xu H, Chen H, Li J, Zhao Y, Ghishan FK. Disruption of NHE8 expression impairs Leydig cell function in the testes. Am J Physiol Cell Physiol 308: C330–C338, 2015. First published December 4, 2014; doi:10.1152/ajpcell.00289.2014.—Multiple sodium/hydrogen exchanger (NHE) isoforms are expressed in the testes, and they play various roles in cell volume regulation, intracellular pH regulation, and fluid absorption. NHE8, the most recently characterized NHE family member, is detected in the Leydig cells in humans and mice in great abundance by immunohistochemistry in the current study. Male mice lacking NHE8 expression were infertile. Despite having intact male reproductive organs, male NHE8−/− mice have smaller testes and lacked spermatozoa in the seminiferous tubules and the epididymis. At the age of 39 wk, few spermatogonia were seen in the testis in NHE8−/− mice. Although male NHE8−/− mice have normal serum levels of luteinizing hormone and follicle-stimulating hormone, serum testosterone level was significantly reduced. These mice have decreased expression of luteinizing hormone receptor in the testes. In NHE8−/− small-interfering RNA-transfected mouse Leydig cells (MLTC-1), silencing of NHE8 decreased the expression of luteinizing hormone receptor by ~70%. Moreover, loss of NHE8 function in Leydig cells resulted in disorganized luteinizing hormone receptor membrane distribution. Therefore, male infertility in NHE8−/− mice is at least partially due to the disruption of luteinizing hormone receptor distribution and consequent low testosterone production, which leads to Sertoli cell dysfunction. Our work identified a novel role of NHE8 in male fertility through its effect on modifying luteinizing hormone receptor function.

sodium/proton exchanger 8; testosterone; luteinizing hormone receptor; testis

SODIUM/HYDROGEN EXCHANGERS (NHEs) belong to solute carrier family 9 and consist of 10 members (NHE1-10) that have broad tissue distribution in mammals. They are integral plasma membrane proteins and are typically involved in the exchange of intracellular H+ with external Na+ according to the concentration gradient. NHEs regulate intracellular pH, cell volume, acid-base balance, and neutral NaCl absorption (43, 44). Their activity also facilitates cellular adhesion, migration, and proliferation (34). Before this study, NHE1, 2, 3, 8, and 10 have been detected in the testes. NHE1, a ubiquitously expressed isoform, is detected in virtually all cell types in the testis. NHE2 and NHE3 are expressed on the apical membrane of epithelial cells in the efferent and epididymidal ducts (6, 14, 29, 46). NHE8 mRNA was detected in mouse testis (12). NHE10 is a spermatozoa-specific NHE isoform (38). Of these NHEs, only the functions of NHE3 and NHE10 were known to directly affect male fertility (6, 24, 38). The testes of mice lacking NHE3 have increased fluid volume, so sperm concentration was diminished, which leads to subfertility (46). Loss of NHE10 in mice resulted in male infertility due to severely diminished sperm motility (38). Recently, we found that loss of NHE8 expression in mice also resulted in male infertility (42). Male reproductive function requires a high level of cooperation in a delicately balanced network. Problems arising from any stage of reproductive organs and sperm development can result in infertility. Worldwide, approximately one in five couples of reproductive age are infertile, and male infertility contributes to 30–40% of these cases (5, 15, 30, 32, 35, 36, 40). While most male infertility cases displayed low sperm counts and/or poor sperm quality, only 15% of them displayed no sperm (8, 21, 32, 40). The factors contributing to male infertility range from hormonal imbalance and reproductive component defect to transporter malfunction in the male reproductive system (14, 16, 18, 31). Lack of luteinizing hormone (LH) or LH receptor (LHR) expression has been linked to infertility (26, 45). Some ion transporters expressed in the male reproductive system also contribute to male fertility. In addition to NHE3 and NHE10, cystic fibrosis transmembrane conductance regulator (CFTR) and downregulated in adenoma (DRA) could also contribute to male infertility. Similar to NHE3 and NHE10, CFTR and DRA are expressed in the male reproductive system in humans and mice. Mutation in CFTR in humans results in the absence of the vas deferens (2). Loss of DRA function in humans contributes to male subfertility due to defective Cl−/HCO3− transport function in the efferent ducts (16). Because we noticed that male NHE8−/− mice are infertile, we conducted the current study to identify the localization of NHE8 in the testis and the role of NHE8 in male reproductive function using NHE8−/− mice.

MATERIALS AND METHODS

Animals. NHE8−/− mice were generated from NHE8+/− breeding pairs with mixed genetic background (129/Swiss Webster) as previously described (42). Only male mice were used in this study. All animal work was approved by the University of Arizona Institutional Animal Care and Use Committee.

Cell culture. Mouse Leydig cells (MLTC-1) were purchased from ATCC (Manassas, VA) and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin according to ATCC guidelines. Cells were grown at 37°C in a shaking incubator (80 strokes/min). Digested testes were dispersed in 2 ml dispersal medium (DMEM-F-12 containing 1 g/l BSA, 1.2 g/l sodium bicarbonate, and 0.4 mg/ml collagenase) for 15 min at 34°C in a shaking incubator (80 strokes/min). Digested testes were

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filtered through a 100-μm sieve to separate interstitial cells from tubules. Tubules left on the sieve were washed with 5 ml collection medium (DMEM-F-12 containing 1 g/l BSA and 1.2 g/l sodium bicarbonate). The interstitial cells were pelleted at 300 g for 5 min at 4°C and then resuspended in 1 ml collection medium. To enrich Leydig cells, the 1-ml cell suspension was layered over a discontinuous Percoll gradient and centrifuged at 800 g for 20 min at 4°C. Enriched Leydig cells, which have a density between 30 and 45% gradients, were collected and then washed three times in 7 ml collection medium and centrifuged at 300 g for 10 min at 4°C. Cells were resuspended in culture medium (DMEM-F-12 with 1.2 g/l sodium bicarbonate, 3% FBS, and 1% penicillin-streptomycin) and cultured at 37°C in an incubator supplied with 95% air and 5% CO2. The purity of Leydig cells was assessed by histochemical localization of 3β-HSD performed according to the method of Aldred and Cooke (1). Over 95% of the cells isolated with this procedure were Leydig cells (27). DMEM-F-12 medium and sodium bicarbonate solution were purchased from HyClone. BSA and collagenase were purchased from Sigma-Aldrich (St. Louis, MO). Percoll was purchased from GE Healthcare Life Sciences.

**Tissue histological observation and immunohistochemistry.** Human testis tissue sections were purchased from Abcam (Cambridge, MA). Mouse testis tissues were collected and fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, and embedded in paraffin. Sections of 8 μm thick were cut and stained with hematoxylin and eosin (H&E) at the pathology services laboratory (University Animal Care, Tucson, AZ). H&E-stained sections were reviewed under a Zeiss Axiosplan microscope equipped with ×20 objective. To detect NHE8, LHR, early endosome antigen 1 (EEA1), and the Golgi apparatus, immunohistochemical labeling was performed using rabbit polyclonal NHE8 antibody (41), rabbit polyclonal LHR antiserum (sc-25828; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal EEA1 antiserum (sc-6414; Santa Cruz Biotechnology), and goat polyclonal 58K Golgi protein antibody (Abcam). Alexa Fluor secondary antibodies were purchased from Molecular Probes (Carlsbad, CA). Briefly, primary antiserum was incubated with sections overnight at various dilutions (1:400 for NHE8 and 1:100 for LHR, EEA1, and anti-58K Golgi). The sections were subsequently incubated with secondary antiserum at a 1:400 dilution for 1 h. Antibody-labeled sections were visualized using an MRC-1024ES laser-scanning confocal microscope (Bio-Rad, Hercules, CA). DAB kit (Vector Laboratories, Burlingame, CA) was also used to detect NHE8 in human and mouse testis tissues.

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**Fig. 1.** Morphological assessment of male reproductive system in NHE8−/− mice. Male reproductive organs were collected from wild-type (WT) and NHE8−/− (KO) mice. Tissues were sectioned, and hematoxylin and eosin (H&E) staining was used to assess the histology. A: male reproductive organ comparison from 8-wk-old WT and KO mice. B: testis tissue H&E staining of 8-wk-old WT and KO mice. C: testis tissue H&E staining from 4-, 8-, 17-, and 39-wk-old WT and KO mice. Scale bar in the images is 100 μm.
RNA isolation and PCR amplification. Tissues were harvested and total RNA were isolated using Trizol reagent (Invitrogen). Total RNA (500 ng) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), and 10% of the RT reaction was used for PCR analysis. For Real-Time PCR, TaqMan technology was used to determine the expression levels of NHE8 gene. TATA-binding protein gene was used as an endogenous reference to normalize expression levels. All TaqMan probes used for this study were purchased from Applied Biosystems (Foster City, CA). Resulting data were analyzed using the comparative cycle threshold (Ct) method. The target gene Ct values are adjusted relative to a calibrator (normalized Ct value obtained from control groups) and expressed as 2^ΔΔCt (Applied Biosystems User Bulletin no. 2: Rev B “Relative Quantitation of Gene Expression”).

Protein preparation and western blot detection. Tissues and cells were lysed in a small volume of RIPA buffer on ice. Resulting lysates were centrifuged at 15,000 rpm for 10 min at 4°C. Supernatants were collected and used for Western blot. NHE8 antibody (1:2,500 dilution) (36), β-actin antiserum (1:5,000 dilution; Sigma-Aldrich), and LHR antibody (1:1,000 dilution, Santa Cruz Biotechnology) were used to detect NHE8, β-actin, and LHR protein abundance, respectively. NHE8 antibody detected NHE8 protein at 65 kDa. β-Actin antibody detected β-actin protein at 43 kDa. LHR antibody detected LHR protein at 85 kDa. A ratio of target protein intensity over β-actin protein intensity was used for protein expression quantification.

Detection of testosterone, LH, and FSH. Serum samples were collected from mice. Testosterone level was measured using a mouse testosterone EIA kit (Cayman Chemical, Ann Arbor, MI). LH level was measured using a mouse LH ELISA kit (NovaTeinBio, Cambridge, MA). Follicle-stimulating hormone (FSH) level was measured using a mouse FSH ELISA kit (NovaTeinBio).

LH stimulation, forskolin stimulation, and cAMP measurement. Enriched mouse Leydig cells and MLTC-1 cells were seeded in a 24-well plate (1 × 10^5/well in 1 ml medium) and cultured for 24 h for isolated Leydig cells or 72 h for MLTC-1 cells before they were treated with a maximal stimulating concentration of LH (100 ng/ml) for 4 h at 37°C according to previously established methods (22, 23) or 10 μM forskolin (Cell Signaling Technology, Danvers, MA) for 4 h at 37°C (17). After removal of culture medium, cells were washed and then lysed with 0.2 ml 0.1 M HCl for 10 min at room temperature. Supernatants were stored at −20°C for future assay. LH was purchased from Sigma-Aldrich. The direct cAMP ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY) and was used to measure cAMP concentration in cell lysates. The relative change of cAMP production was used to determine the magnitude of cAMP induction in response to LH or forskolin compared with untreated cells.

Statistical analysis. Unpaired t-test and one-way ANOVA followed by Fisher’s PLSD post hoc for multiple pairwise comparison were used to compare values of the experimental data. P values ≤0.05 were considered significant.

Fig. 2. Testicular function assessment in NHE8−/− mice. A: testis weight comparison between wild-type (WT) and NHE8−/− (KO) mice. One-way ANOVA followed by Fisher’s PLSD post hoc was used to compare results among groups. Data are presented as means ± SE from various numbers of WT and KO mice in each age group (n = 19 mice for the 4-wk-old age group, 20 mice for 8- to 10-wk-old age group, 16 mice for 13- to 17-wk-old age group, and 6 mice for 39-wk-old age group). *P < 0.01 for 4-wk-old WT mice and 4-, 8-, 12-wk-old KO mice vs. 8- to 10-, 13- to 17-, and 39-wk-old WT mice. B: serum testosterone concentration measurement. One-way ANOVA followed by Fisher’s PLSD post hoc was used to compare results among groups. Data are presented as means ± SE from various numbers of WT and KO mice in each age group (n = 13 mice for the 4-wk-old age group, 13 mice for 8-wk-old age group, and 12 mice for 12-wk-old age group). *P < 0.01 for 8- and 12-wk-old WT mice vs. 4-wk-old WT and 4-, 8-, 12-wk-old KO mice. #P < 0.01 for 8- and 12-wk-old KO mice vs. 4-wk-old KO mice and 4-, 8-, 12-wk-old WT mice. C: serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentration measurement. One-way ANOVA followed by Fisher’s PLSD post hoc was used to compare results among groups. Data show the summary results (means ± SE) from 10 mice in each age group.
Fig. 3. NHE8 expression characterization in the testes. A: NHE8 mRNA (top) and protein expression (bottom) in the testes in WT mice. Testis samples were collected from 4-, 8- and 12-wk-old WT mice. RNAs were extracted and were used for Real-Time PCR assay. Tissue lysates were prepared in RIPA buffer and were used for Western blot. Protein (40 μg) was separated on 8% SDS-PAGE and immunoblotted with NHE8 antibody (1:2,500 dilution) and β-actin antibody (1:5,000 dilution). β-Actin is used as internal control to quantitate NHE8 expression level. One-way ANOVA followed by Fisher’s PLSD post hoc was used to compare results among groups. Data show the summary results (means ± SE) from 9 mice in each group. B: NHE8 immunohistochemical labeling in human and mouse testis tissues. Testis tissue sections from adult human and 8-wk-old mouse were reacted with NHE8 antibody (1:400 dilution) overnight. DAB kit was used for color detection by incubating tissue sections with substrate solution at room temperature for 45 s. Arrows indicate Leydig cells. Scale bar in the images is 100 μm. C: NHE8 localization in 10-wk-old mouse testis tissue. Mouse testis section was reacted with NHE8 antibody (NHE8) (1:400 dilution) and 58K Golgi antibody (Golgi) (1:100 dilution). Immunofluorescence labeling was analyzed by MRC-1024 laser-scanning confocal microscope. Scale bar in the images is 20 μm.
RESULTS

NHE8−/− mice have normal morphology of the male reproductive system. We have previously reported that NHE8−/− mice had normal growth and were indistinguishable from their wild-type littermates after weaning, and male mice were infertile (42). Male NHE8−/− mice displayed normal mating behavior such as libido and copulation. Because male NHE8−/− mice are infertile, we examined the morphology of the male reproductive organs to check for any abnormalities. No obvious alterations were seen in male organs of the NHE8−/− mice. Similar to male wild-type mice, testes were descended in the scrotum in male NHE8−/− mice, and the male reproductive components were also intact in these mice. The overall appearance of testis, epididymis, vas deferens, and seminal vesicles in NHE8−/− mice was normal (Fig. 1A). However, significant difference was seen in the seminiferous tubules at the histological level. No sperm was found in the seminiferous tubules and the epididymis in 8-wk-old male NHE8−/− mice, but few elongate spermatids were found (Fig. 1B). When male NHE8−/− mice grew older, almost complete absence of spermatogenesis and vacuolar appearance was observed in seminiferous epithelium. These are likely the result of severe seminiferous tubules atrophy (Fig. 1C).

NHE8−/− male mice have smaller testes and reduced serum testosterone levels. To determine why male NHE8−/− mice have no sperm, we compared testis weight and the level of male hormones between wild-type and NHE8−/− mice. As shown in Fig. 2A, testis weight was the same between wild-type and NHE8−/− mice at 4 wk of age. At 8 wk of age, growth in the testis reached the peak in the wild-type mice, but it was arrested in NHE8−/− mice (Fig. 2A). The serum testosterone level was also significantly lower in NHE8−/− mice at the age of 8 and 12 wk than that of wild-type mice, whereas no difference was seen at the age of 4 wk between NHE8−/− mice and wild-type mice (Fig. 2B). Contrary to serum testosterone level, serum LH and FSH levels were similar between male NHE8−/− mice and wild-type mice (Fig. 2C).

NHE8 is highly expressed in Leydig cells in the testes. NHE8 mRNA has been detected in the testis tissue by Northern blot in mice (12) and in humans (unpublished observation), but the precise localization of NHE8 in the testes was not known. As shown in Fig. 3A, results from Real-Time PCR and Western blot indicated that NHE8 was indeed expressed in the testis and that the expression of NHE8 in the testis remained unchanged from 4 wk of age to 12 wk of age. Immunohistochemical labeling determined that NHE8 is highly expressed in the Leydig cells and not the Sertoli cells of human and mouse testis (Fig. 3B). In fact, in mouse testis, NHE8 was expressed in the intracellular compartments, and some NHE8 signals were overlapped with Golgi in Leydig cells (Fig. 3C).

Male NHE8−/− mice have impaired LH-stimulated cAMP production. Testosterone production is regulated by LH via LHR activation in the testis. Because male NHE8−/− mice have low serum testosterone levels but normal LH levels, we hypothesized that LHR expression and/or LHR activation is impaired in NHE8−/− Leydig cells. Thus we compared LHR expression in testis tissue lysates in 12-wk-old wild-type and NHE8−/− mice. We also isolated Leydig cells from 8- to 10-wk-old mice to study LHR activation by measuring the amount of LH-stimulated cAMP production. As shown in Fig. 4A, LHR protein expression was significantly reduced by 45% in the NHE8−/− testis compared with that of in the wild-type testis. In isolated Leydig cells, the basal cAMP concentration was similar between wild-type and NHE8−/− Leydig cells (2.69 ± 0.42 pmol/ml in NHE8−/− cells vs. 2.80 ± 0.28 pmol/ml in wild-type cells). LH-stimulated cAMP production was significantly increased in wild-type Leydig cells, but not in NHE8−/− Leydig cells (Fig. 4B).

NHE8 siRNA treatment in MLTC-1 cells results in reduced LHR expression and LH-stimulated cAMP production. To confirm the changes in LHR expression observed in male NHE8−/− mice were the direct result of loss of NHE8 function, NHE8 siRNA was used to transfect cultured mouse Leydig cells (MLTC-1). Immunohistochemical labeling detected that the endogenous NHE8 is expressed in the intracellular compartments, and some of them are overlapped with Golgi apparatus in MLTC-1 cells (Fig. 5A). In NHE8 siRNA-transfected
cells, the expression of NHE8 was reduced by 68%, and LHR expression was reduced by 65% (Fig. 5B). The basal level of cAMP was similar between NHE8 siRNA-transfected cells and the control cells (2.90 ± 0.59 pmol/ml in siRNA-treated cells vs. 3.53 ± 0.72 pmol/ml in control cells). Upon LH stimulation, cAMP production in siRNA-treated cells was only 57% of the LH-stimulated cAMP production of control cells. Forskolin-stimulated cAMP production in NHE8 siRNA-treated MLTC-1 cells was exposed to 100 ng/ml LH or 10 μM forskolin for 4 h and then were harvested for cAMP assay. The relative change was calculated by cAMP production in LH- or forskolin-treated cells over cAMP production in nontreated cells. One-way ANOVA followed by Fisher’s PLSD post hoc test was used to compare results among groups. Data are presented as means ± SE from 3 to 6 experiments. *P < 0.05 for cAMP production in LH- or forskolin-stimulated cells vs. that in nonstimulated cells.

**DISCUSSION**

NHEs are a large family of NHE proteins that are expressed in a variety of cells in the body. These proteins serve important functions in regulating intracellular pH, cell volume, acid-base balance, and neutral NaCl absorption (43, 44). NHE8 joins NHE1, NHE2, NHE3, and NHE10 as NHE isoforms detected in the testis (6, 14, 38). Previously, we detected NHE8 mRNA in human testis (unpublished observation), and others detected NHE8 mRNA in mouse testis (12). In the current study, we confirmed the presence of NHE8 protein in mouse and human testes by Western blot and immunohistochemical labeling. Interestingly, the expression of NHE8 in the testis remains constant through development, whereas intestinal NHE8 expression decreases with age (41). Another difference in the expression of NHE8 in the testes is its location. We and others have shown that NHE8 protein in the intestine and the kidney is located at the apical membrane of the epithelial cells (11, 41), but, in testes, NHE8 protein is not located at the plasma membrane in Leydig cells (Fig. 3). Thus NHE8 expression and localization is subject to tissue-specific regulation.

Like NHE3 and NHE10 (38, 46), deficiency in NHE8 function directly affects male fertility. We found that male NHE8−/− mice were infertile. However, we were surprised to find that male NHE8−/− mice have an overall normal reproductive system. Whereas infertility in NHE3−/− mice is due to too low concentration of sperm and in NHE10−/− mice is due to nonfunctional sperm, no sperm was detected in the seminiferous tubules and the epididymis of male NHE8−/− mice. In tracking the developmental change of the testis tissue, we found that testis growth was arrested at the age of 4 wk in male NHE8−/− mice.
NHE8−/− mice. Serum testosterone level increased only 4.8-fold from 4 to 8 wk of age in NHE8−/− mice compared with an increase of 15-fold in wild-type mice. The changes in serum levels of testosterone, LH, and FSH in NHE8−/− mice are different from LHR knockout (LuR−/−) mice and LH β-subunit knockout (LHβ−/−) mice. Similar to LuR−/− mice and LHβ−/− mice, NHE8−/− mice have small testis and low testosterone levels. However, the serum LH and FSH levels in
NHE8<sup>−/−</sup> were normal compared with elevated LH and FSH levels in male LuR<sup>−/−</sup> mice and increased LH level in male LHβ<sup>−/−</sup> mice (26, 45). LuR<sup>−/−</sup> mice and LHβ<sup>−/−</sup> mice also displayed Leydig cell hypoplasia (26, 45), but this was not observed in male NHE8<sup>−/−</sup> mice. Because male NHE8<sup>−/−</sup> mice have different changes in LH and FSH than in LuR<sup>−/−</sup> mice and LHβ<sup>−/−</sup> mice, the effect of NHE8 on male reproductive function is likely independent of LH and FSH. Because NHE8 mRNA is detected in the brain (12), it is plausible that the hypothalamus-pituitary-testicular axis might also be affected at the central nervous system level in NHE8<sup>−/−</sup> mice, although more studies will have to be performed to address this issue. Adult male NHE8<sup>−/−</sup> mice display lower testosterone levels compared with wild-type male mice, but these mice still make testosterone and display normal mating behavior. Early studies have shown that there was no relationship between plasma testosterone levels and sexual behavior, including the ability to engage in sexual activity (mounting, ejaculating, etc.) (9). Similar data were reported in adult male humans or ability to engage in sexual activity (mounting, ejaculating, etc.) (37). Therefore, the infertility observed in male NHE8<sup>−/−</sup> mice is not the result of abnormal sexual behavior.

An important function of Leydig cells is the production of testosterone, a critical hormone for male reproductive function, upon the stimulation of LH (33, 39). LH binding onto its receptor, which is located on the plasma membrane of Leydig cells, signals testosterone production through activation of the cAMP pathway (13, 19). Adult male NHE8<sup>−/−</sup> mice displayed low testosterone level in spite of normal LH and FSH levels, which suggests that LH-stimulated testosterone production in NHE8<sup>−/−</sup> mice may be impaired. Indeed, LH treatment failed to stimulate cAMP production in isolated NHE8<sup>−/−</sup> Leydig cells. Thus there is a link in male NHE8<sup>−/−</sup> mice between reduced testosterone production and lack of LH-stimulated cAMP production. Because defect in LH-stimulated cAMP production might be a result of reduced LHR expression in the testis, we analyzed LHR expression in adult male NHE8<sup>−/−</sup> mice. As expected, LHR protein expression was reduced ~50% in NHE8<sup>−/−</sup> testis compared with in wild-type testis. This reduction was not detected in NHE8 mRNA in NHE8<sup>−/−</sup> testis (data not shown). Therefore, NHE8 contributes to testosterone production though its effect on LHR protein expression in Leydig cells.

To confirm whether these in vivo observations were solely due to the lack of functional NHE8 protein, we used NHE8 siRNA to inhibit endogenous NHE8 expression in cultured mouse Leydig cells (MLTC-1). NHE8 localization in MLTC-1 cells was the same as that in native Leydig cells in the testis. When MLTC-1 cells were transfected with NHE8 siRNA, the expression of NHE8 was reduced by 70%, and the expression of LHR was down to 35% compared with that in the control cells. Upon LH stimulation, cAMP production in NHE8 siRNA-transfected cells was only 57% of control cells. Interestingly, cAMP production induced by forskolin treatment was similar between control and NHE8 siRNA-treated MLTC-1 cells. These observations suggest that reduced cAMP production in the absence of NHE8 is the result of reduced LHR expression and not deficiencies in the cAMP pathway. Therefore, NHE8 indeed regulates LHR expression in Leydig cells.

Under normal physiological conditions, LHR protein is expressed at the plasma membrane of Leydig cells. Upon binding with LH, LHR are internalized via coated pits, coated vesicles, or multivesicular bodies to lysosomes (10). A recent study showed that only a small amount of LHR could be detected in early endosome after LH stimulation (20). Our studies showed that plasma membrane-bound LHR, which has molecular mass of ~85 kDa (3, 7), was decreased in NHE8<sup>−/−</sup> Leydig cells, and some of these mature LHR were detected in the early endosome compartment in NHE8<sup>−/−</sup> Leydig cells (Figs. 3 and 6). In NHE8 siRNA-transfected MLTC-1 cells, the majority of the LHR protein was trapped around the nuclei in the cells, and some of the LHR were detected in the early endosome. Because other studies have shown that NHE8 was expressed in mid- to trans-Golgi in COS-7 and HeLa M cells to regulate Golgi compartment pH and protein trafficking (25, 28), we suspected that NHE8 may also participate in protein maturation/trafficking, particularly LHR protein, in the Golgi apparatus in Leydig cells.

In conclusion, our study showed for the first time that NHE8 is highly expressed in Leydig cells and that loss of NHE8 expression resulted in impaired LHR function in these cells. This observation sheds light on a novel role of NHE8, where it affects male fertility via its effect on modifying LHR expression. Thus a focus on NHE8 may provide important diagnostic information for male fertility clinics.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: H.X., H.C., and F.K.G. conception and design of the study; H.X., H.C., J.L., and Y.Z. performed experiments; H.X., H.C., J.L., and Y.Z. analyzed data; H.X., H.C., and J.L. interpreted results of experiments; H.X., H.C., J.L., and Y.Z. prepared figures; H.X. and H.C. edited and revised manuscript; H.X. and F.K.G. approved final version of manuscript.

REFERENCES
Lack of NHE8 results in male infertility


