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Targeted Deletion of the Epididymal Receptor HE6 Results in Fluid Dysregulation and Male Infertility

Ben Davies,[1]* Claudia Baumann,[1] Christiane Kirchhoff,[2] Richard Ivell,[2] Reinhard Nubbemeyer,[3] Ursula-Friederike Habenicht,[3] Franz Theuring,[1]† and Ulrich Gottwald[3]†

Center for Cardiovascular Research-Institute for Pharmacology and Toxicology, Charité-University Medicine, Berlin,[1] and Schering AG,[3] Berlin, and Institute for Hormone and Fertility Research, Hamburg,[2] Germany

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Human epididymal protein 6 (HE6; also known as GPR64) is an orphan member of the LNB-7TM (B_2) subfamily of G-protein-coupled receptors. Family members are characterized by the dual presence of a secretin-like (type II) seven-transmembrane (7TM) domain and a long cell adhesion-like extracellular domain. HE6 is specifically expressed within the efferent ductules and the initial segment of the epididymis, ductal systems involved in spermatozoon maturation. Here, we report that targeted deletion of the 7TM domain of the murine HE6 gene results in male infertility. Mutant mice reveal a dysregulation of fluid reabsorbtion within the efferent ductules, leading to a backup of fluid accumulation in the testis and a subsequent stasis of spermatozoa within the efferent ducts. The fertility phenotype of HE6 knockout mice identifies this receptor as a potential nonsteroidal, nontesticular target for future male contraceptives and identifies an in vivo function for a member of this unusual gene family.

In the last few years, molecular biology has made substantial advances in understanding how sperm are made and how fertilization occurs. However, disappointingly little of this new information has translated into relevance for the clinic, particularly in terms of novel contraceptive agents for men.

Spermatozoa, produced in the seminiferous tubules of the testis, are passed via the rete testis through the efferent ductules to the epididymis. It is within this organ that the spermatozoa acquire their capabilities for forward motility and oocyte fertilization (19, 33, 44). Although the molecular basis of this maturation process is as yet poorly understood, analyses of gene expression within the epididymis have revealed the identities of many secretory proteins (18, 22) that potentially interact with and modify the sperm surface, enabling the mature spermatozoa to develop. The proximal epididymis and efferent ductules have a further function in the reabsorption of testicular fluid, concentrating the spermatozoa and establishing an adequate milieu for maturation of the spermatozoa (37). The epididymis, therefore, represents an alternative target organ for male contraception (5) and circumvents the potentially disadvantageous testicular steroidal regulation which is currently a favored approach (21).

A seven-transmembrane (7TM) domain protein, human epididymis-specific protein 6 (HE6/GPR64) has been found to be highly expressed within the proximal epididymis (34) and recently within the efferent ductules (32). The gene encoding HE6 has been localized to the X-chromosomal region XF4, and orthologous highly conserved genes have been identified in the rat, mouse (32), and puffer fish (3). Sequence homology suggests that HE6 is a member of the newly defined LNB-7TM (B_2) subfamily of G-protein-coupled receptors (GPCRs) (13, 42). Family members are characterized by a conserved secretin-like (type II) 7TM domain and a long N-terminal extracellular domain with an array of protein motifs considered to be involved in cell adhesion and protein-protein interactions. Family members have been implicated in diverse biological processes, such as immune response (12, 43), synaptic function (24), and vascularization (31, 39), and a recent study has implicated this receptor family in neuronal development (35).

The extracellular domain of HE6 is hypothesized to form a highly glycosylated mucin-like rod structure and is separated from the 7TM domain by a cysteine-rich GPCR proteolysis site motif. The GPCR proteolysis site motif is common to virtually all members of this subfamily (9, 10) and has been shown to be the site of endoproteolytic cleavage (1, 23, 43). Cleavage of LNB-7TM receptors appears to occur intracellularly at an early stage in the biosynthetic pathway, is necessary for correct protein folding and trafficking (1, 23), and is not associated with the unveiling of the receptor ligand, as is the case with the protease-activated receptor family (PAR1-4) (38). Processing of HE6 into the mucin-like ectosubunit and the 7TM endosubunit has recently been confirmed (32). Analogously to other LNB-7TM receptor family members, it appears that the resulting HE6 subunits remain noncovalently associated at the cell membrane.

The exact function of HE6 in male reproductive tissue is not known, although the dual structural motifs of a cell adhesion domain and a signal transduction domain perhaps suggest a role in monitoring and controlling the extracellular milieu. Considering the importance of the proximal epididymis and efferent ductules in spermatozoon maturation, HE6 represents an interesting pharmacological target for potential male contraceptives. To explore these possibilities, we have developed mutant mice in which the 7TM domain within the murine HE6 gene has been deleted. The hemizygous mutant male mice reveal age-dependent infertility caused by a defect in luminal fluid reabsorption, resulting in an accumulation of spermato-

* Corresponding author. Present address: Murinus GmbH, Falkenried 88, 20251 Hamburg, Germany. Phone: 49 40 42803 1660. Fax: 49 40 42803 1699. E-mail: b.davies@murinus.com.

† F.T. and U.G. contributed equally to this work.

zoa within the efferent ducts and a buildup of fluid within the testis.

MATERIALS AND METHODS

Gene targeting in ES cells. Mouse genomic lambda clones corresponding to the murine HE6 gene were obtained from a 129Sv mouse genomic library (Stratagene) using a mouse cDNA probe corresponding to the 7TM domain (34). For the 5′-homology arm, a 3.85-kb genomic fragment corresponding to a region between exons 16 and 21 was amplified by PCR (High Fidelity PCR System; Roche) using the primers 5′-TGCAGCCCAAGACCCAACAAAT-3′ and 5′-TGATGAAAGCCCACAGCCAAT-3′ and was subcloned into a pBluescript KS vector 5′ of a 5-kb reporter-selection cassette, comprising the LacZ reporter coding sequence prefixed by a viral internal ribosome entry site (IRES) element, a neomycin phosphotransferase gene under independent promoter control (MC1), and a polyadenylation sequence (30). The IRES element enables bicistronic translation of the reporter gene (*lacZ*) from the transgenic HE6 transcript, allowing the expression pattern of HE6 mRNA to be assessed by using histochemical staining (28). The 3′-homology arm, corresponding to exon 26 and the surrounding intronic sequence, was derived as a 2.4-kb EcoRI-EcoRV fragment subcloned from the above-mentioned lambda clones. This fragment was cloned 3′ of the reporter-selection cassette, and the targeting construct was completed by the inclusion of two copies of a herpes simplex virus thymidine kinase gene, serving as a negative selection cassette (41). The completed targeting vector was transfected into E14-TG2a embryonic stem (ES) cells, and the resulting neomycin-ganciclovir-resistant colonies were screened for homologous recombination within the 3′-homology region by PCR. A 2.5-kb amplification product was obtained from homologously combined clones using the primers 5′-CGCATCGCCTTCTATCGCCTTCTT-3′, a forward primer hybridizing to the simian virus 40 polyadenylation signal contained within the reporter-selection cassette, and 5′-CAATGTGCCCCTTACTTACTGTCA-3′, a reverse primer hybridizing to intron 27 immediately 3′ of the 3′-homology region used. Positively recombined ES cell clones were then investigated by Southern analysis to confirm that homologous recombination had occurred within the 5′- and 3′-homology arms.

Generation of knockout mice. Two independently targeted ES cell clones (A78 and A85) were injected into the blastocysts of C57BL/6 mice, and the resulting chimeric males were mated to C57BL/6 females. Genotyping of transgenic progeny was performed by PCR analysis of tail tip DNA using a forward primer hybridizing to intron 20 (5′-GCTCCTCCACTTTCTAGCT-3′) and two reverse primers, one hybridizing to the a region of intron 27 deleted in the knockout allele (5′-CTGCTGGTGACATACTAGGCA-3′) and the other hybridizing to the 5′ region of the knocked-in reporter-selection cassette (5′-ATCCACTAGTTCTAGAGCGGCC-3′). Using these primers, a 560-bp band was amplified from the wild-type allele and a 290-bp band was amplified from the transgenic allele, thus enabling both alleles to be detected using a single PCR (30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 30 s). Alternatively, mice were genotyped by Southern analysis using an EcoRV genomic digest probed with an exon 26-specific cDNA probe. An 11-kb EcoRV fragment corresponded to the wild-type allele, and a 6-kb EcoRV fragment corresponded to the knockout allele. The mice were systematically backcrossed to C57BL/6 animals. To prepare hemizygous knockout males, heterozygous females were mated with wild-type C57BL/6 males. The results presented in this study are exclusively from the A78 line; the A85 line revealed the same phenotype.

Northern and Western analysis. Total RNA, isolated from freshly prepared epididymides by using Trizol (Invitrogen), was electrophoretically separated on a 1% agarose gel in the presence of 18% formaldehyde and was transferred to Hybond N membranes (Amersham). Hybridization according to standard conditions was performed with ^{32}P-radiolabeled probes corresponding to nucleotides 1372 to 1712 (exons 16 to 18) and 2237 to 2980 (exons 24 to 25) of the murine HE6 cDNA (accession no. NM_178712).

The mN2 antibody, recognizing an epitope on the ectodomain of murine HE6 protein, and the mA23 antibody, recognizing an epitope on the murine HE6 endodomain, were prepared as described previously (32). Epididymal membrane suspensions were prepared from pooled epididymides collected from at least four animals per group, as described previously (32). For the analysis of the ectosubunits, membrane proteins were denatured in Laemmli sample buffer and separated on 8 or 10% Laemmli polyacrylamide gels. For the analysis of the endosubunits, membrane preparations were solubilized for 1 h at ambient temperature in 7 M urea, 2 M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 1% Triton X-100, 1% dithiothreitol, 20 mM Tris, pH 9.5, and complete protease inhibitor cocktail (Roche). After the addition of β-mercaptoethanol (2% end concentration), samples were separated on 12% urea (7 M) gels (36). Immunodetection of the transferred proteins was carried out by standard procedures as described previously (32).

Fertility studies. Knockout (n = 13 to 20/age group) and wild-type littermate control (n = 10 to 16/age group) male animals were mated at different ages with 8-week-old virgin NMRI female mice in a ratio of 1:1. Breeding pairs were maintained together for a maximum of 4 nights or until successful mating had been observed, as determined by the observation of a vaginal plug. The female mice were sacrificed 12 days postcoitum, and the number of embryos was determined.

Analysis of spermatozoa. Spermatozoa were collected from the cauda epididymis of knockout (n = 17) and wild-type (n = 15) mice between the ages of 15 and 20 weeks. The cauda region from one epididymis was cut open and incubated for 10 min in phosphate-buffered saline at 37°C to allow the spermatozoa to emerge. A homogenous suspension was then diluted and spread over a microscope slide, and the spermatozoa were counted and assessed. The motility was defined as the percentage of motile spermatozoa present per defined volume. Mobility was visually assessed, and an arbitrary scale was used, in accordance with the World Health Organization scaling (45) (the scale runs from a [normal motion] to d [no motion]).

Histology. Tissues were removed and fixed for 24 h in buffered formalin and stored in 70% ethanol until they were processed. The tissues were dehydrated, paraffin embedded, and sectioned (5 μm thick). Hematoxylin and eosin staining was performed according to standard procedures. β-Galactosidase staining was performed on 10-μm-thick cryosections of epididymis and testis with the chromogenic substrate BLUO-GAL (Invitrogen) as described previously (2). The sections were counterstained with 1% neutral red.

RESULTS

Disruption of the mouse HE6 gene. To address the role of HE6 in male reproductive function, the murine gene encoding this protein was inactivated by homologous recombination in mouse ES cells. Exons 22 to 25, encoding the 7TM domain, were deleted and replaced with a neomycin resistance gene, translation termination codons in all three reading frames, and a β-galactosidase reporter gene under the control of an IRES, allowing translation of the reporter gene from the transgenic transcript (Fig. 1A). Targeted ES cells were injected into blastocysts, and the resulting chimeras were mated with C57BL/6 female mice, yielding heterozygous female and wild-type male animals, in accordance with the X-chromosomal location of HE6. The heterozygous females were crossed with wild-type C57BL/6 mice, yielding hemizygous knockout male mice and wild-type control littermates for further analysis. The disruption of the murine HE6 gene was confirmed by genomic Southern blot analysis (Fig. 1B).

Northern blot analysis of caput RNA hybridized with an HE6 7TM-specific probe (exons 24 to 25) confirmed the absence of mRNA encoding this transmembrane region in hemizygous knockout mice (Fig. 1C). Further Northern analysis with a probe corresponding to the murine exons 16 to 18 revealed the presence of two weakly expressed mutant transcripts: a shorter transcript of ~2 kb corresponding to a truncated mutant mRNA encoding the 5′ exons (exons 1 to 20) and a larger transcript of ~7.5 kb corresponding to the above-mentioned truncated mRNA fused to the β-galactosidase transcript (Fig. 1C). This larger transcript also hybridized with a *lacZ*-specific probe (data not shown).

To confirm a lack of HE6 protein in the knockout mice, Western analysis was performed using two different antibodies (Fig. 1D). The mN2 antibody, which recognizes an epitope in the HE6 ectoprotein (32), identified the previously described 180-kDa band in wild-type caput membrane preparations, along with a smaller nonspecific band present in all samples

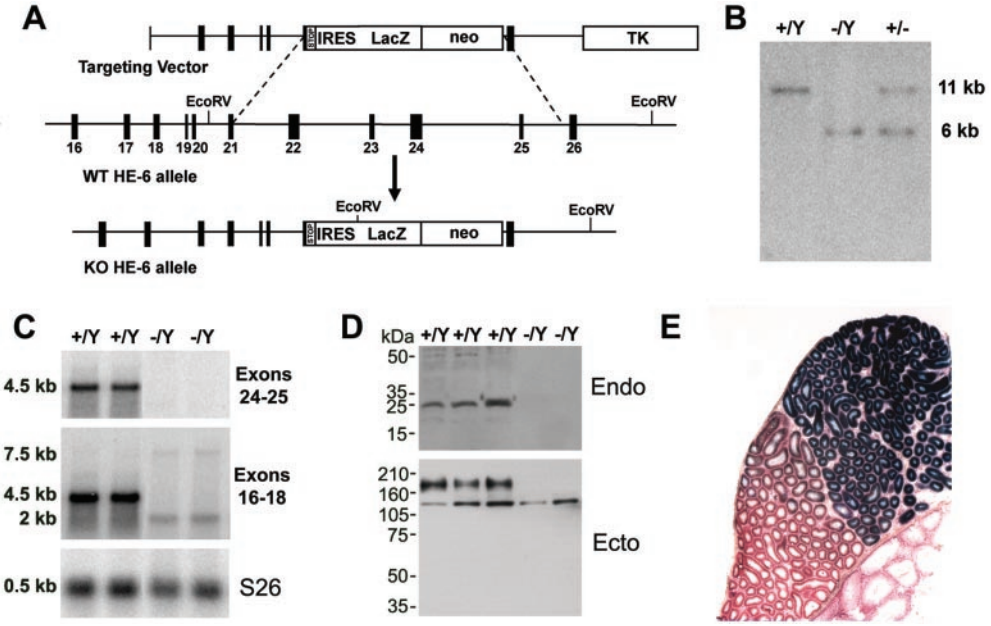

FIG. 1. (A) Targeting strategy for the deletion of exons 21 to 25, encoding the 7TM domain. A selection cassette (neo) and a reporter cassette (IRES LacZ) were inserted. Two copies of a herpes simplex virus thymidine kinase (TK) gene were included in the vector for selection against nonhomologous integration. WT, wild type; KO, knockout. (B) Southern blot analysis of tail genomic DNA from wild-type male (+/Y), heterozygous female (+/−), and hemizygous knockout male (−/Y) animals demonstrating disruption of the HE6 gene. The probe corresponding to exon 26 hybridizes to an 11-kb EcoRV wild-type fragment and a 6-kb EcoRV fragment from the targeted allele. (C) Northern blot analysis of initial-segment total RNA from HE6 mutant animals (−/Y) and wild-type littermates (+/Y) hybridized with cDNA probes corresponding to the 7TM domain (exons 24 to 25) (top) and an extracellular domain (exons 16 to 17) (middle). The bottom blot shows an S26 ribosomal protein loading control. (D) Western blot analysis of epididymal membrane suspensions from HE6 mutant (−/Y) and wild-type (+/Y) littermates using the mN2 antibody recognizing an epitope within the ectodomain (Ecto) (bottom) and the mA23 antibody recognizing an epitope within the endodomain (Endo) (top). (E) Photomicrograph of a BLUO-GAL-stained section through the epididymis and testis of an HE6 mutant animal showing the activity of the β-galactosidase gene.

analyzed. The 180-kDa band was not detectable in knockout caput samples, and apart from the smaller, nonspecific band present in all samples, no signal representing a potential truncated HE6 protein was detected. The mA23 antibody recognizes an epitope of the HE6 endoprotein within the 7TM domain deleted in the knockout (32). Consequently, this antibody detected the HE6 endoprotein in wild-type caput membrane preparations while no specific signal was obtained for knockout caput. These results confirm the creation of a null allele and demonstrate that no protein is made from the expressed ectodomain-encoding mRNA. The chromogenic activity of the inserted β-galactosidase reporter cassette was used to examine the expression pattern of HE6. A high level of reporter activity was seen in the proximal epididymis (Fig. 1E), recapitulating the reported endogenous expression (34).

No significant difference between the numbers of hemizygous knockout and wild-type males obtained from matings of heterozygote females with wild-type males was found (knockout, 161; wild type, 210; chi-square test = 3.25 [degree of freedom = 1]; $P > 0.05$), indicating that loss of HE6 function does not lead to embryonic lethality. Furthermore, hemizygous knockout males and homozygous knockout females showed no apparent developmental or behavioral abnormalities in comparison with wild-type littermates.

Hemizygous knockout males show reduced fertility. To establish the role of HE6 in male reproductive physiology, hemizygous knockout males 6 to 20 weeks of age were mated with 8-week-old wild-type NMRI females, a mouse strain well known for high fecundity. There was no significant difference observed between the mating behaviors of hemizygous knockout males and their wild-type littermates, as judged by the presence of vaginal plugs.

Hemizygous knockout males, however, revealed severely decreased fertility in comparison to wild-type littermates, which manifested itself both in the number of successful matings that did not lead to pregnancy and in the number of embryos produced by a successful mating (Fig. 2). The fertility of hemizygous knockout males was found to decline with age, resulting in almost complete infertility at the age of 15 weeks.

To further investigate the basis of this infertility, spermatozoa were isolated from the cauda region of knockout and wild-type epididymides from mice between 15 and 20 weeks of age. HE6 knockout mice were found to have a considerably reduced number of sperm in comparison with wild-type littermates, and those sperm present were frequently morphologically abnormal. Knockout animals possessed an increased number of acephalic spermatozoa, and spermatozoa with a sharp angulation of the flagellum were frequently observed (Fig. 3). The proportion of motile spermatozoa and their absolute mobility were also significantly decreased in HE6 knockouts in comparison with wild-type control mice (Table 1). Accordingly, the epididymides of HE6 knockout mice appeared to be reduced in size and were found to be considerably lighter in weight in comparison with those of wild-type littermates

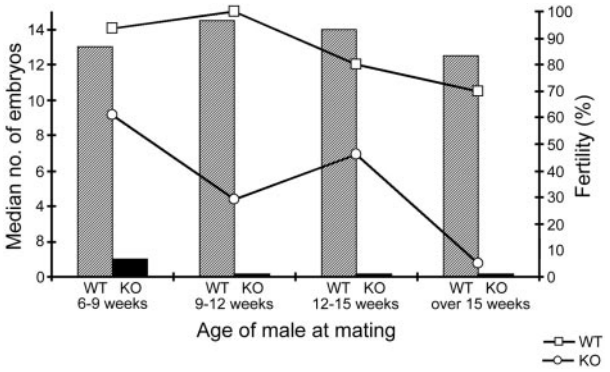

FIG. 2. Fertility of HE6 knockout and wild-type control males at various ages, as measured by the median number of embryos resulting from a successful mating (left axis and line graph; WT, wild type; KO, knockout) and the fertility (percentage of successful matings resulting in pregnancy) (right axis and bar chart).

(Table 1). Representative views of spermatozoa at equal concentrations collected from the cauda epididymis of wild-type and knockout males are shown in Fig. 3.

Hemizygous knockout males reveal a dysregulation of fluid reabsorption and sperm accumulation within the efferent ductules. Histological sections through the efferent and epididymal ductules obtained from knockout and wild-type males were compared by light microscopy. In the efferent ductules, where HE6 is found to be highly expressed (32), the ductules were found to be greatly dilated in knockout mice, and the lumen of the ductules was frequently obstructed by a large accumulation of spermatozoa (Fig. 4A and B). The prevalence of this pathology increased with age and was observed to be more pronounced within the distal region of the ducts.

Within the initial segment (Fig. 4C and D) and caput (Fig. 4E and F), the epididymal epithelium was found to be macroscopically indistinguishable between knockout and wild-type mice. The only obvious histological difference observed was the reduction of spermatozoa in the lumen of the knockout ductules, consistent with a proximal obstruction. The corpus and cauda epididymis were also indistinguishable between knockout and wild-type mice, with the exception of a trend toward increased clear-cell activity in the cauda region (data not shown). The overactivity of these scavenging cells is indicative of a proximal obstruction, as the clear cells are stimulated by the resulting increased luminal protein.

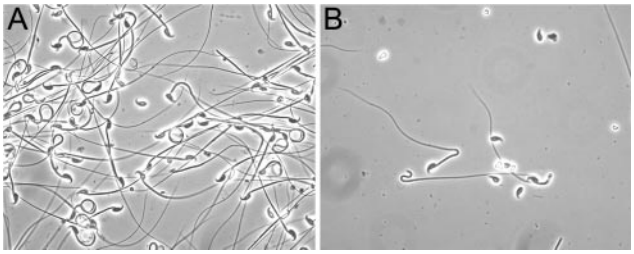

FIG. 3. Photomicrographs of caudal sperm preparations from equal volumes obtained from the cauda epididymis of wild-type (A) and knockout (B) mice at 12 weeks of age. The knockout animals reveal a drastically reduced number of spermatozoa, and those present show frequent morphological abnormalities. The results shown are representative of analyses of 17 knockout and 15 wild-type spermatozoon samples.

TABLE 1. Epididymal weights and spermatozoan parameters for HE6 knockout mice and wild-type littermate controls[a]

HE6 genotype (*n*)	Normalized epididymis wt (mg)	Sperm count (10^6)/cauda	Motility (%)	Mobility	
				%	Rank
Wild type (15)	118.8 ± 18.5	9.38 ± 3.47	94.7 ± 1.2	86	a
				14	b
Knockout (17)	88.9 ± 20.7[b]	0.93 ± 1.34[b]	9.1 ± 15[b]	65	d
				23	c

[a] Data are from animals between 15 and 20 weeks of age. Motility is defined as the percentage of motile spermatozoa. Mobility (the extent or degree of movement) was assessed using an arbitrary scale (a, normal, to d, no movement).
[b] $P < 0.01$.

The reduction in fertility and the lack of spermatozoa appear to be caused by the accumulation of spermatozoa within the efferent ductules. Since these tubules, which connect the testis and epididymis, are responsible for the absorption of >90% of testicular fluid, the testes of wild-type and knockout mice were investigated. Consistent with the accumulation of spermatozoa within the efferent ducts, a buildup of fluid was observed within the testis, resulting in a vastly expanded rete

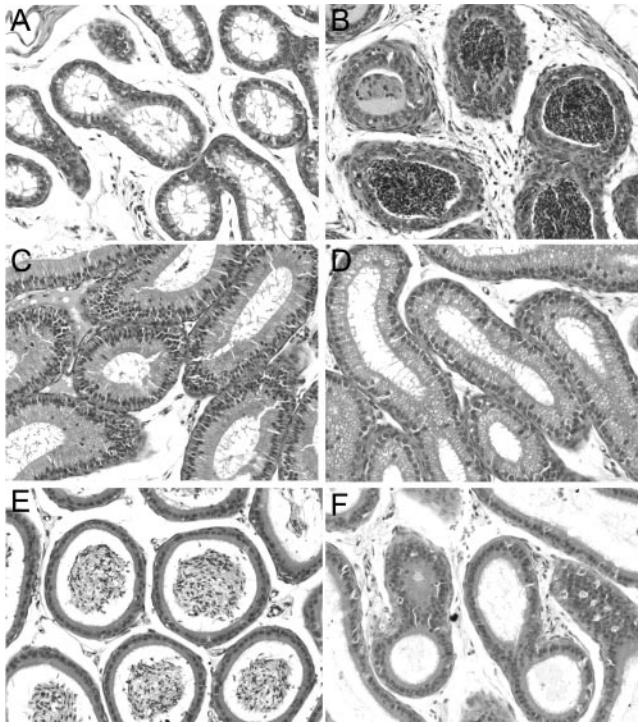

FIG. 4. Photomicrographs (magnification, ×100) of hematoxylin- and eosin-stained sections through the efferent ductules of wild-type (A) and knockout (B) mice, the epididymal initial segment of wild-type (C) and knockout (D) mice, and the caput epididymis of wild-type (E) and knockout (F) mice. An abnormal accumulation of spermatozoa in knockout efferent ductules (B) and subsequent reduction in spermatozoa in the lumen in the knockout initial segment and caput epididymis (D and F) were observed. The results shown are representative of histological analyses of 17 knockout and 15 wild-type 12-week-old mice.

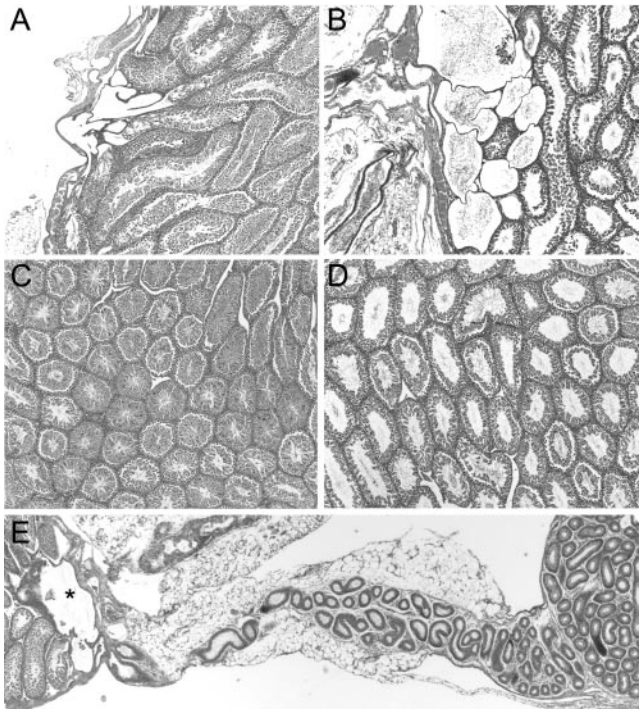

FIG. 5. (A to D) Representative photomicrographs (magnification, ×100) of hematoxylin- and eosin-stained sections through the rete testis of wild-type (A) and knockout (B) 12-week-old mice and the seminiferous tubules of wild-type (C) and knockout (D) 12-week-old mice showing dilation and expansion of these testicular structures in knockout mice. (E) Photomicrographs (magnification, ×25) showing expanded rete testis (*) and unoccluded efferent ducts in 2-week-old knockout mice.

testis in knockout mice (Fig. 5A and B). Seminiferous tubules from knockout mice were also found to be dilated in comparison with those of wild-type littermates, and reduced spermatogenesis was observed in many tubules (Fig. 5C and D). Closer examination revealed that all stages of spermatogenesis were present in knockout animals, but the level of differentiation of the germ layer epithelia was significantly retarded, with a selective loss of later spermatogenesis stages. Although the testicular phenotype was found to be more exaggerated with increasing age, no evidence of complete atrophy was found, even when 9-month-old knockout mice were investigated.

To establish the temporal onset of the observed phenotype, knockout mice were analyzed at 2 weeks of age, before spermatozoon production had begun. Interestingly, an enlarged rete testis was already observable in the absence of spermatozoon accumulation within the efferent ducts (Fig. 5E), suggesting that the primary effect of HE6 deletion is a defect in fluid reabsorption, with the accumulation of spermatozoa occurring as a result of this imbalance.

DISCUSSION

The HE6 receptor is expressed at high levels within the efferent ductules and the initial segment of the epididymis (32, 34), regions of the reproductive tract which are well characterized as being responsible for the absorption of >90% of the fluid produced by the testis (4). The concentration of sperm delivered to the ductus epididymis is determined by the net fluid reabsorption across the efferent duct epithelium (4, 20). Considering the abnormal accumulation of spermatozoa within the efferent ductules observed in HE6 knockout mice, a role for this receptor in the regulation of fluid balance is suggested.

Fluid reabsorption within the efferent ductules is primarily a passive process that occurs in parallel with the establishment of an Na^+ gradient across the nonciliated cells of the epithelium (17). The hydration of CO_2 by carbonic anhydrase II within the nonciliated cells results in the production of H^+ and HCO_3^-. H^+ is exchanged for luminal Na^+ by the apically located sodium-hydrogen exchanger 3 (NHE3), resulting in a net influx of Na^+ into the epithelium. This process occurs in tandem with the exchange of HCO_3^- for luminal Cl^- by the apically located DRA exchanger, an exchange balanced by the apically located CFTR Cl^- channel. A basolaterally located Na^+ or K^+ ATPase removes the Na^+ from the epithelia, resulting in a net movement of Na^+, and subsequently water, from the lumen through the epithelia. In addition to this passive transport, active uptake of luminal fluid through water channels (aquaporins) (11) and through endocytosis also occurs.

Knockout studies have shown that the stability of this electrochemical gradient is essential for controlled fluid reabsorption within the efferent ductules. Decreasing the Na^+ gradient through the genetic elimination of carbonic anhydrase II or NHE3 has been shown to result in reduced fluid absorption (47). Both lines of knockout mice revealed dilated efferent ductules, expanded rete testis, and a reduction in fertility. A similar phenotype was also observed in the estrogen receptor alpha (ERα) knockout mice in which the resulting buildup of fluid resulted in almost total testicular atrophy (14). The phenotype of ERα-deficient mice is partially explained by the resulting reduced expression of NHE3 (47) and increased expression of the chloride transporters DRA and CFTR (25), which presumably leads to a depressed electrochemical gradient and a subsequent reduction in fluid influx. Furthermore, the endocytotic apparatus of the nonciliated cells, which also contributes to luminal fluid uptake, appears to be disorganized in ERα-deficient mice, exacerbating the fluid reuptake phenotype (29).

The dilated efferent ductules, the buildup of fluid within the testis, and the infertility seen in these mice are also common in HE6 knockout mice. Specific to the HE6 knockout phenotype, however, is the accumulation of spermatozoa within the efferent ductules. Such ductal occlusions are often indicative of an increased rate of fluid absorption, which results in increased luminal viscosity and subsequent spermatozoon stasis, blocking the passage of water out of the testis (15). Similar luminal blockages have been reported in toxicological studies examining the effects of environmental toxins on the male reproductive system. Treatment of rats with the fungicides benomyl and carbendazim (16), for example, results in severe and rapid occlusions within the efferent ductules and subsequent backpressure testicular atrophy.

The phenotype observed in these studies differs from the experimental models in two important ways. First, the accumulation seen in this study leads to only a partial blockade within the distal efferent ductules, allowing a residual quantity of spermatozoa to be collected from the knockout caudal ep-

ididymis. Second, in premature males, the expansion of the rete testis is observed in the absence of spermatozoon accumulation, suggesting that a dysregulation in fluid reabsorption is the primary effect of HE6 mutation.

The molecular basis of HE6 receptor interaction with fluid homeostasis is as yet unclear. However, some functions can be hypothesized by a comparison with other members of this unusual GPCR gene family. Recently the LNB-7TM gene family has been revealed to encompass at least 30 individual members in humans (10). The family members consist of a secretin-like 7TM domain, together with a long extracellular N-terminal structure containing a variety of cell adhesion-like domains, for example, laminin, immunoglobulin, lectin, epidermal growth factor, and thrombospondin domains (7). LNB-7TM receptor family members have been implicated in the modulation of immune and inflammatory responses (12, 43), in synaptic function (24), in vascularization (31, 39), and in the determination of cell polarity during development (8, 6). A recent report has demonstrated a role of the related receptor GPR56 in human forebrain development, where the long extracellular domain of this receptor, together with its GPCR domain, is hypothesized to control cortical neuronal patterning (35). Mutations in this receptor are associated with bilateral frontoparietal polymicrogyria, a genetic disease characterized by disorganized cortical lamination.

The extracellular domain of HE6 consists of highly glycosylated mucin-like domains—serine- and threonine-rich regions considered to play a role in cell-cell or cell-matrix adhesion. These extracellular motifs, in combination with a GPCR motif, suggest a possible role for the HE6 receptor in directly monitoring and communicating the state of the luminal environment to the cellular machinery determining fluid uptake. Interestingly, fluid reabsorption within the efferent ducts has been shown to be tightly regulated by the second messenger cyclic AMP (27). It is thus tempting to speculate that the HE6 receptor represents the start of a signal transduction cascade, exerting transcriptional effects on the genes through a cyclic-AMP-mediated second-messenger system controlling the electrochemical gradient and thus influencing fluid absorption.

Another possible role of HE6 in regulating fluid absorption is at the level of exo- and endocytosis. Interestingly, other family members have also been implicated in secretory epithelia. GPR56, for example, which has a mucin-like extracellular domain similar to that of HE6, is expressed within the thyroid in the cuboidal epithelia of only the more actively secreting follicles (26). Furthermore, the calcium-independent receptors of α-latrotoxin (CIRL1-3) also play a role in exocytosis, instigating a massive neurotransmitter release when bound to their nonphysiological ligand α-latrotoxin (24). A role for HE6 in the maintenance of endo- and exocytotic apparatuses within the highly active absorbent and secretory efferent ductules and epididymis would thus not be unexpected.

Interestingly, the obstructive phenotype seen in this study is entirely restricted to the efferent ductules, and no apparent histological abnormalities were observed within the initial segment, despite a high level of HE6 expression within this region of the epididymis. Although it also plays a role in fluid reabsorption, studies of c-*ros* knockout mice and mice overexpressing simian virus 40 within the initial segment have revealed a more predominant role for this region in conveying the ability of sperm to regulate their volume (46, 40). The caudal spermatozoa obtained from HE6 knockout males showed the same flagellar angulation revealed by the above-mentioned transgenic models, indicative of a failure to regulate volume during the resulting osmotic imbalance. However, the effects of HE6 mutation on spermatozoon maturation within the initial segment are difficult to assess independently of the proximal obstruction within the efferent ducts.

Irrespective of the molecular function of HE6, it is clear that the receptor has an important function in the regulation of the luminal environment within the efferent ductules and proximal epididymis. The modulation of HE6 receptor function within these tissues appears to have a dramatic effect on male fertility and identifies this molecule as a potential target for nontesticular male contraceptives.

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