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G protein-coupled receptor 48 upregulates estrogen receptor α expression via cAMP/PKA signaling in the male reproductive tract

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SUMMARY

The epididymis and efferent ducts play major roles in sperm maturation, transport, concentration and storage by reabsorbing water, ions and proteins produced from seminiferous tubules. *Gpr48*-null male mice demonstrate reproductive tract defects and infertility. In the present study, we found that estrogen receptor α (ERα) was dramatically reduced in the epididymis and efferent ducts in *Gpr48*-null male mice. We further revealed that ERα could be upregulated by Gpr48 activation via the cAMP/PKA signaling pathway. Moreover, we identified a cAMP responsive element (*Cre*) motif located at –1307 to –1300 bp in the *ERα* promoter that is able to interact with Cre binding protein (Creb). In conclusion, Gpr48 participates in the development of the male epididymis and efferent ducts through regulation of ERα expression via the cAMP/PKA signaling pathway.

KEY WORDS: Gpr48, ERα (Esr1), Creb, Epididymis, Infertility, Mouse

INTRODUCTION

Spermatozoa, which are produced and differentiated in seminiferous tubules, pass through rete testis and efferent ducts to the epididymis. The efferent ducts and the epididymis play major roles in reabsorbing water, ions and proteins produced from the seminiferous tubules (Clulow et al., 1998; Ilio et al., 1994; Brooks et al., 1983; Hinton et al., 1995). The epididymis, derived from the anterior Wolffian or mesonephric duct, elongates, expands and folds into a highly organized structure that includes the caput, the corpus and the cauda segments. This elaborate process consists of many events including conversion of mesenchymal cells to ductal epithelia, formation of mesonephric ducts, elongation of the Wolffian duct and regionalization of the epididymal duct (Avenel et al., 2009). Androgen and estrogen play crucial roles in the proliferation, differentiation and function of the epididymis and efferent ducts (Meistrich et al., 1975; Hess et al., 1997). Androgen withdrawal by orchidectomy causes a decrease in epididymal weight and luminal diameter (Robaire et al., 1977). Epididymal epithelial cells cultured in vitro also require testosterone to maintain their normal morphology and functions (Wider et al., 2003). Mice lacking global androgen receptors show azoospermia and infertility because of incomplete germ cell development (Yeh et al., 2002). Recent studies have demonstrated that estrogen also participates in the development of male reproductive tracts. Estrogen receptor alpha (ERα; Esr1 – Mouse Genome Informatics) is abundantly expressed in male reproductive tracts, mainly in the epididymis and efferent ducts. ERα is essentially responsible for maintaining epithelial cytoarchitecture and testicular fluid reabsorption in the efferent ducts and epididymis (Couse and Korach, 1999). *ERα*-null male mice show dilated efferent ducts and disrupted spermatogenesis due to fluid accumulation in association with a reduction in sodium/hydrogen exchanger 3 (NHE3; Slc9a3 – Mouse Genome Informatics) expression in the efferent ducts (Zhou et al., 2001), whereas *ERβ*-null mice are fertile and have normal sexual differentiation (Krege et al., 1998). However, the regulation of androgen and estrogen receptor expression in male reproductive tracts is poorly understood.

G protein-coupled receptor 48 (Gpr48; Lgr4 – Mouse Genome Informatics), a newly identified orphan receptor, exhibits a classic seven transmembrane spanning (TMS) structure like other G protein-coupled receptor family members and contains several leucine-rich repeats at its N-terminus (Hsu et al., 1998; Loh et al., 2001). Gpr48 functions by activating heterotrimeric Gα proteins to elevate intracellular cAMP levels (Gao et al., 2006a; Gao et al., 2006b). Moreover, Gpr48 is widely expressed in human and mouse tissues, suggesting a crucial role at normal organ development stages (Weng et al., 2008; Song et al., 2008; Loh et al., 2000; Van et al., 2005). Previous studies have reported that mice lacking Gpr48 exhibit intrauterine growth retardation coupled with embryonic and perinatal lethality (Mazerbourg et al., 2004). *Gpr48*-null male mice are infertile owing to impaired integrity of the reproductive tracts (Mendive et al., 2006; Hoshii et al., 2007). Moreover, an underdeveloped epididymis, with dilated and much less convoluted ducts and a flattened epithelium,

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is observed in *Gpr48*-null mice. The expression of ERα, NHE3 and aquaporin 1 (Aqp1) is reduced in the proximal segment of the efferent ducts.

In the present study, we investigated the role of Gpr48 in the regulation of ERα expression and further explored the molecular mechanism underlying this regulation.

MATERIALS AND METHODS

Mice

Gpr48-null male mice were housed at 21±1°C with a humidity of 55±10% and a 12-hour light-dark cycle. Food and water were available ad libitum. For genotyping analysis, genomic DNA was isolated from tail biopsy and PCR was carried out using three primers: upstream primer 5′-CCAGTCACCACTCTTACACAATGGCTAAAC-3′; and downstream primers 5′-GGTCTTTGAGCACCAGAGGAC-3′ and 5′-TCCCGTAG-GAGATAGCGTCCTAG-3′. With regard to the male fertility assay, each $Gpr48^{+/+}$, $Gpr48^{+/-}$ and $Gpr48^{-/-}$ adult male aged 12 weeks was housed with two $Gpr48^{+/+}$ females. The females were examined daily for vaginal plugs. The litter number was counted immediately after parturition. The animal protocol was reviewed and approved by the Animal Care Committee of Shanghai Jiao Tong University School of Medicine.

Ligation operation

Twenty-four mice at 3 weeks of age received bilateral efferent ductile ligation (EGL; *n*=8), bilateral vasoligation (VGL; *n*=8) and sham operations (*n*=8). For EGL, the efferent ductule was doubly ligated close to the epididymis and away from the epididymal and testicular vasculature and vas deferens. For VGL, bilateral vasa deferentia were doubly ligated. The mice receiving EGL, VGL or sham operations were observed daily to ascertain a normal descending of the testes.

Immunofluorescence and immunohistochemical staining

Animal tissues were fixed overnight in Bouin's solution, dehydrated in ethanol, embedded in paraffin and sectioned at 5 μm. Immunofluorescence and immunohistochemical staining were performed according to a standard protocol. In brief, the sections were de-paraffined, progressively rehydrated and treated with 3% hydrogen peroxide in methanol for 30 minutes to quench endogenous peroxidase activity. The pretreated sections were then blocked in PBS containing 2.5% horse serum for 1 hour (Vector Laboratories) then incubated with primary antibodies in a humidified chamber at 4°C overnight. The following primary antibodies were used: anti-NHE3 (1:100, Chemicon), anti-Aqp9 (1:200, Chemicon), anti-Na^+/K^+-ATPase α1 (1:200, Upstate Biotechnology), anti-ERα (1:500; Santa Cruz Biotechnology) and anti-Ar (androgen receptor; 1:500; Santa Cruz Biotechnology). Images were acquired using an Olympus BX51 microscope.

Cell culture and transfection

Primary mouse embryonic fibroblast cells (MEFs) were obtained from $Gpr48^{+/+}$ and $Gpr48^{-/-}$ male mice at embryonic day (E) 16.5. HEK293T, MEF, MCF-7 and CHO cells were cultured in DMEM and DMEM/F12 (Invitrogen). All media were supplemented with 10% fetal bovine serum (Gibco) as well as 100 U/ml penicillin and 100 μg/ml streptomycin. For the estrogen response element (ERE)-luciferase reporter assay, MCF-7 cells were cultured in estrogen-free conditions that contained Phenol-Red-free DMEM with 5% charcoal-treated fetal bovine serum. All the transient transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

siRNA, plasmids and luciferase reporter assay

Mouse Gpr48 siRNA regents were purchased from Dharmacon (Thermo Scientific). The *ERα* promoter was amplified from the mouse genomic DNA template and inserted into pGL4.15 empty vector (Promega). Mutant *Cre* motif was generated using a PCR mutagenesis kit (Toyobo) with primer (mutation sites underlined) 5′-GGCTTGATGAGTGTGCTAGCAT-TGTTGACCTACAGGAG-3′ and a reverse complement primer. Cells were seeded in 24-well plates for the luciferase reporter assay and transfected with 1.0 μg Gpr48 plasmids and 0.2 μg reporter vectors. pRL-TK-expressing renilla luciferase (Promega) was used to normalize the luciferase activity. Cells were harvested 48 hours after transfection and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assays

A chromatin immunoprecipitation (ChIP) assay kit was used (Upstate Biotechnology). DNA was sheared to fragments of 200-1000 bp by several 10 second sonications. The chromatins were incubated and precipitated with antibodies against Creb or phosphorylated Creb at 4°C overnight. The promoter fragment containing the *Cre* motif was amplified with the following primers: forward 5′-CCCCTGAGATGATACCTG-3′ and reverse 5′-AAAGATTGCTAACCCTTTG-3′.

RNA isolation and real-time PCR

Total RNA was isolated from tissues or cells using TRIzol (Invitrogen) according to the manufacturer's instructions. In order to quantify the transcripts of genes of interest, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Shiga, Japan) with an Applied Biosystems 7300 Real-Time PCR machine. The primers used are shown in Table S1 in the supplementary material.

Western blotting

Tissues and cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM NaF, 1% NP40 and 0.1% SDS. The cell lysates were loaded onto 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 10% nonfat milk and then incubated with different primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The proteins were visualized with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Little Chalfont Buckinghamshire, UK) according to the manufacturer's protocol.

RESULTS

Malformation of the reproductive tract and infertility in *Gpr48*-null males

Gpr48 homozygous mutant mice ($Gpr48^{-/-}$) were generated by microinjecting gene trap-mutated *Gpr48* ES cells into blastocysts of C57BL/6 mice (Weng et al., 2008). Fifty-two percent (52%) of *Gpr48*-null mice died within 28 hours after birth and no fetal death was observed (data not shown), which is consistent with previous reports (Mazerbourg et al., 2004).

To observe the fertility of *Gpr48*-null mice in both sexes, we housed adult males with females (1:2) and examined daily for vaginal plugs. None of the 15 $Gpr48^{-/-}$ males inseminated and made females pregnant over 25 weeks. $Gpr48^{-/-}$ females also demonstrated severe infertility. Only 10% of $Gpr48^{-/-}$ females became pregnant while mating with a $Gpr48^{+/+}$ male (Table 1A,B).

Table 1A. Fertility test of $Gpr48^{-/-}$ male mice

Test			
Male	Female	Pairs	No. of pups
+/+	+/+	10	10.8±1.03
+/–	+/+	10	10.2±1.32
–/–	+/+	15	None

Table 1B. Fertility test of $Gpr48^{-/-}$ female mice

Test			
Male	Female	Pairs	No. of pups
+/+	+/+	10	10.8±1.03
+/+	+/–	10	10.0±2.45
+/+	–/–	10	0.50±1.58

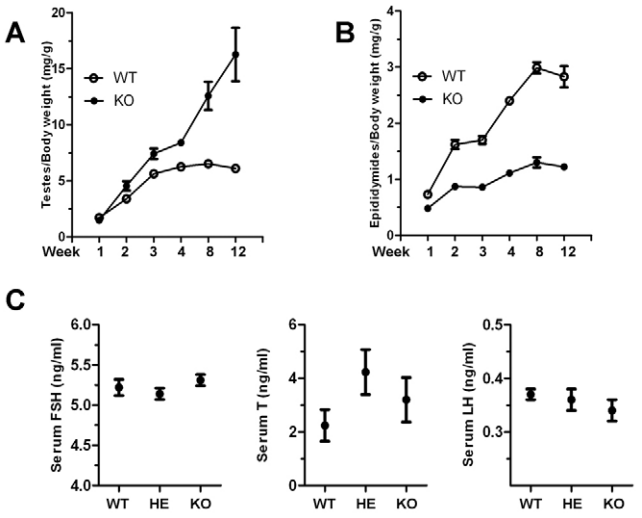

Fig. 1. Testes and epididymis weights and serum hormone levels in *Gpr48* wild-type and knockout mice. (**A**,**B**) The weight change of the testes and epididymis normalized to body weight in *Gpr48*$^{+/+}$ (WT) and *Gpr48*$^{-/-}$ (KO) mice from 1 week of age to 12 weeks (*n*=13-16). (**C**) Serum follicle-stimulating hormone (FSH), testosterone (T) and luteinizing hormone (LH) levels in wild-type (WT), heterozygous (HE) and homozygous (KO) *Gpr48* mice (*n*=16-22). Data are expressed as mean±s.e.m.

To explore the causes of male infertility, we examined the integrity of the male reproductive organs. We found that the testes were enlarged and the epididymis was smaller as a ratio of body weight in *Gpr48*$^{-/-}$ mice starting from 2 weeks of age (Fig. 1A,B). However, serum follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) levels in *Gpr48*$^{-/-}$ mice were the same as those in *Gpr48*$^{+/+}$ mice (Fig. 1C).

We further examined the microscopic morphology of the testes, efferent ducts and epididymis of *Gpr48*-null mice. The rete testis was excessively dilated and accumulated large amounts of sperm cells and liquid in adult *Gpr48*$^{-/-}$ males (data not shown). The seminiferous epithelium was thinned, with seminiferous tubules enlarged and degenerative spermatocytes detached from the lumen epithelium (Fig. 2A). Efferent ducts and three sections of the epididymis ducts were dramatically reduced and the lumens markedly dilated in adult *Gpr48*$^{-/-}$ mice compared with *Gpr48*$^{+/+}$ mice (Fig. 2B,C). Spermatozoa could not be found in the lumens of the epididymis in *Gpr48*$^{-/-}$ males. To determine whether the epididymal malformations occurred during reproductive tract development, we further examined the epididymis morphology perinatally. The defects occurred before birth (E18.5) and gradually became apparent after birth in *Gpr48*$^{-/-}$ males (see Fig. S1 in the supplementary material).

To verify the causal relationship of epididymal defects to a relative obstruction of epididymal ducts and the dilation and liquid accumulation of the upstream rete testis, we performed a bilateral efferent ductile ligation (ELG) (Nicander et al., 1983) and bilateral vasoligation (VLG) (Preslock et al., 1985) in *Gpr48* wild-type mice at 3 weeks of age. At 12 weeks of age, the seminiferous epithelium in mice with either ELG or VLG demonstrated similar changes to those in *Gpr48*$^{-/-}$ adult males (Fig. 3). Nevertheless, the efferent ducts and epididymis retained normal morphology in ELG and VLG mice, differing from *Gpr48*$^{-/-}$ mice. The ligation results favored a causal relationship of epididymal malformation to *Gpr48*$^{-/-}$ males.

In situ hybridization with mouse *Gpr48* extracellular domain probes and *lacZ* staining showed that *Gpr48* was expressed in the caput, corpus and cauda epididymis, as well as in the epithelium of seminiferous tubules and efferent ducts (see Fig. S2 in the supplementary material).

Reduced expression of water and ion transporters in reproductive tracts of *Gpr48*$^{-/-}$ males

Fluid reabsorption in the epididymis depends on crucial water and ion transporter proteins located on the surface of epithelial cells, including Na^+-K^+-ATPase, NHE3 and aquaporin 9 (Aqp9) (Bahr et al., 2006; Leung et al., 2001; Pastor et al., 2001). Na^+ transportation is actively linked to H^+ secretion and fluid reabsorption (Wong and Yeung et al., 1978; Chew et al., 2000). Immunofluorescence showed that the Na^+-K^+-ATPase α1 subunit, NHE3 and Aqp9 were markedly reduced in the efferent ducts and caput epididymis in adult *Gpr48*$^{-/-}$ mice (Fig. 4A,B,C). The dramatic reduction of these transporters was also seen in the corpus and cauda epididymis (data not shown). However, levels of E-cadherin and β-catenin, key molecules associated with cell adhering junctions in the blood-testis barrier (Wu et al., 1993; Wong et al., 2004), were not impaired in *Gpr48*$^{-/-}$ reproductive tracts (see Fig. S3 in the supplementary material).

Disruption of ERα and Ar expression in the epididymis of *Gpr48*$^{-/-}$ mice

NHE3 and Aqp9 expression in the male reproductive tract is under the control of estrogen signaling, whereas the Na^+-K^+-ATPase α1 subunit is regulated by circulating androgens (Oliveira et al., 2005; Ruz et al., 2006). As serum hormone levels are not changed in *Gpr48*$^{-/-}$ mice, we examined ERα and androgen receptor (Ar) expression in the epididymis and efferent ducts. We found that ERα and Ar expression was dramatically reduced in epithelial cells of the

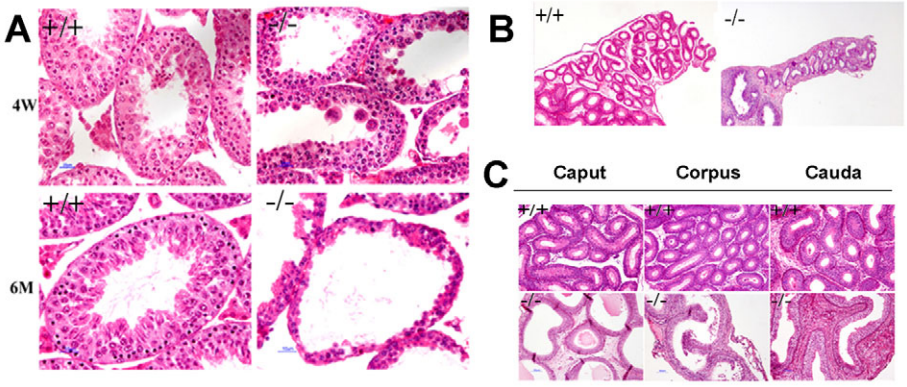

Fig. 2. Histology of testes and reproductive tracts in *Gpr48*$^{+/+}$ and *Gpr48*$^{-/-}$ mice. (**A**) The seminiferous tubules at 4 weeks of age and 6 months (magnification: 400×). (**B**,**C**) The efferent ducts (B) and epididymis tracts (C) were significantly reduced and the epididymal lumen enlarged in *Gpr48*$^{-/-}$ mice at 4 weeks of age (magnification: 200×).

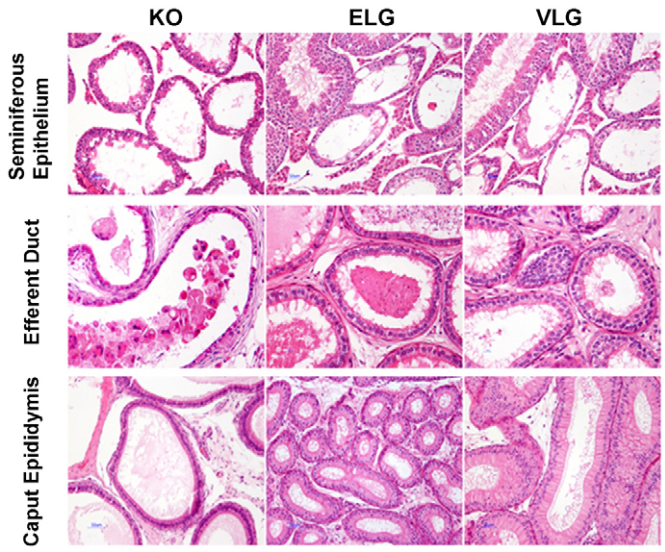

Fig. 3. Histology of the seminiferous epithelium, efferent ducts and epididymis in C57BL mice receiving efferent ductile ligation (ELG) and vasoligation (VLG) and *Gpr48*$^{-/-}$ mice (KO). Seminiferous epithelium, 200× magnification; efferent ducts and epididymis, 400× magnification. Scale bar: 100 μm.

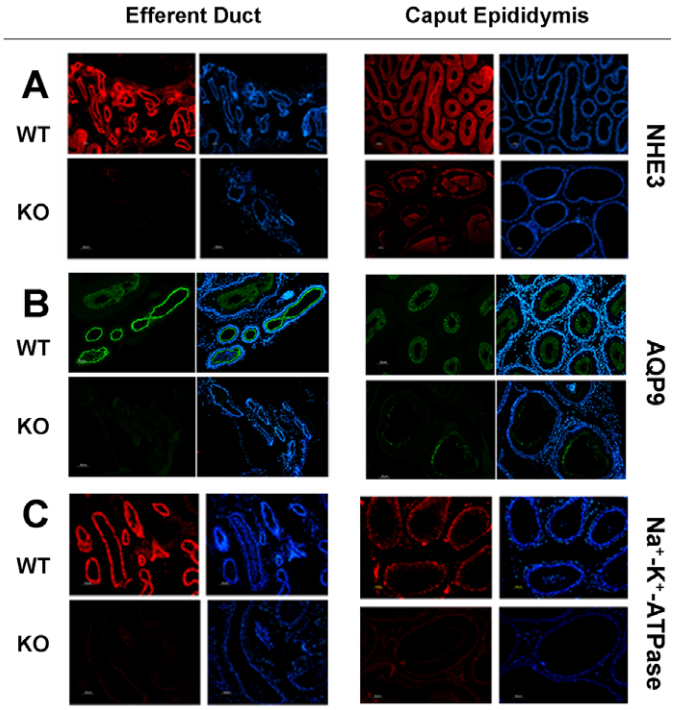

Fig. 4. Immunofluorescence staining of NHE3, Aqp9 and Na$^+$-K$^+$-ATPase α1 subunit in the efferent ducts and caput epididymis. NHE3 (**A**), Aqp9 (**B**) and Na$^+$-K$^+$-ATPase α1 (**C**) expression was markedly reduced in the efferent ducts and caput epididymis in *Gpr48*$^{-/-}$ mice. Blue, DAPI staining.

epididymis and efferent ducts in *Gpr48*$^{-/-}$ males (Fig. 5A,B) by immunohistochemistry. Moreover, western blotting and real-time PCR showed that *ERα* and *Ar* expression was markedly declined in the epididymis at different ages (Fig. 5C,D,E).

Decreased ERα expression can be recovered by cAMP/PKA activation in *Gpr48*$^{-/-}$ MEFs

We isolated mouse embryonic fibroblast cells (MEFs) at E16.5 and found that ERα expression was also markedly reduced in *Gpr48*$^{-/-}$ MEFs (Fig. 6A,B,C). As reported above, Gpr48 activated adenylate cyclase and increased intracellular cAMP levels. We examined whether the ERα reduction in *Gpr48*$^{-/-}$ MEFs could be restored by the cAMP agonist forskolin. Our results showed that forskolin could successfully upregulate *ERα* mRNA levels in a dose-dependent manner (Fig. 6D,E). Moreover, ERα expression in *Gpr48*$^{+/+}$ MEFs was clearly reduced by knockdown of Gpr48 using its small interfering RNA (siRNA) (Fig. 6F). Therefore, we speculated that Gpr48 directly regulates *ERα* transcription via the cAMP/PKA signaling pathway.

Gpr48 regulates ERα expression through a *Cre* binding site in its variant C promoter

To investigate whether Gpr48 directly regulates ERα expression, we analyzed the transcriptional activity of the *ERα* promoter. It is known that there are at least six *ERα* mRNA transcript variants (A, B, C, F1, F2 and H) in mice based on differential splicing (Kos et al., 2000). Variants C and F are the major mRNA transcripts in mice. *ERα* variant C is highly expressed in the uterus, testis, brain and aorta, whereas variant F is expressed predominantly in the liver. We analyzed the promoter of variant C and identified a *Cre* motif (TGACATCA) located at –1300 to approximately –1307 using an online promoter scanning system (http://www.cbil.upenn.edu/cgi-bin/tess/tess) (Fig. 7A). We further generated a construct containing the variant C promoter from position –2500 to +1 (transcription start site of variant C). The luciferase assay showed that the transcriptional activity of the variant C promoter was dramatically upregulated by constitutively activated Gpr48-T755I and Gpr48-T755A (Fig. 7B). The transcriptional activity of the truncated variant C promoter (–1200 to +1), lacking this *Cre* motif, was not upregulated by Gpr48 (Fig. 7C). Furthermore, the full-length variant C promoter (–2500 to +1), with the *Cre* motif mutation (TGACATCA to TGctAgCA), also resulted in a marked reduction of transcriptional activity (Fig. 7D). However, the variant C promoter from –2500 to –1200 was still actively regulated by Gpr48 and its activation was lost when the *Cre* motif was mutated (see Fig. S4A,B in the supplementary material). Furthermore, the Grp48-mediated transcriptional activity of variant C could be suppressed by PKA inhibitor H-89 (Fig. 7E). The same results were also observed in CHO cells (data not shown). In addition, we observed that the transcriptional activity of the *ERα* promoter (–2500 to +1) was greatly reduced in *Gpr48*$^{-/-}$ MEFs compared with that in *Gpr48*$^{+/+}$ MEFs, whereas there was no difference in the transcriptional activities for the *Cre*-mutated promoter (see Fig. S4C,D in the supplementary material).

In addition, we performed a ChIP assay to analyze whether Creb proteins could bind to this *Cre* motif and be phosphorylated by Gpr48 activation. Creb proteins were clearly shown to bind to the *Cre* motif in the *ERα* promoter in *Gpr48*$^{+/+}$ MEFs (Fig. 7F). Phosphorylated Creb protein (Ser133 pCreb) was also shown to bind to the *Cre* site in *Gpr48*$^{+/+}$ MEFs but not in *Gpr48*$^{-/-}$ MEFs (Fig. 7G). Hence, these results strongly implicated that the *Cre* motif in the *ERα* promoter was functional and that the transcriptional activity could be upregulated by Gpr48 protein via cAMP/PKA signaling.

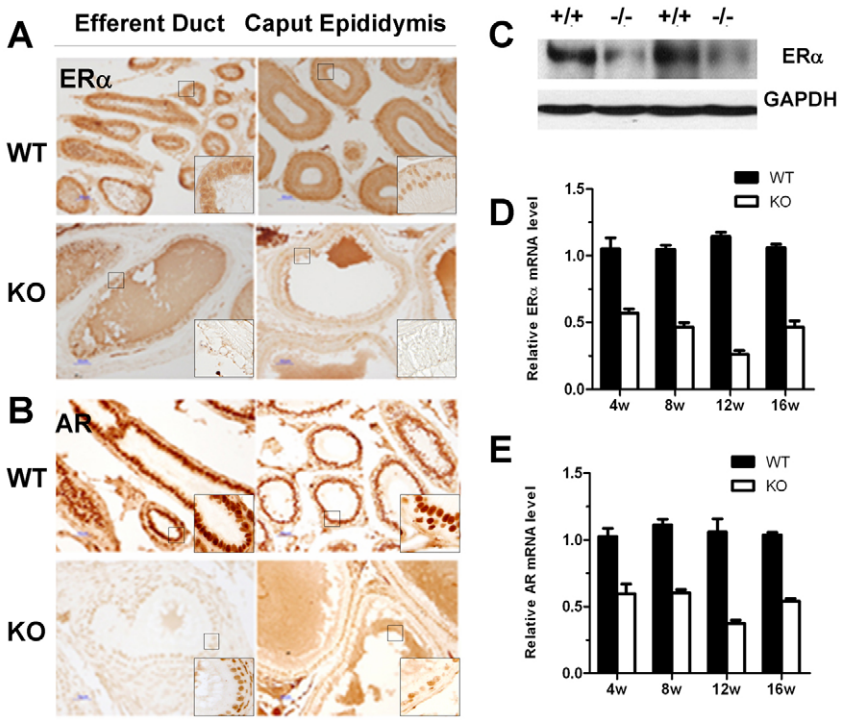

Fig. 5. *ERα* and *Ar* expression in the efferent ducts and epididymis. *ERα* and *Ar* were markedly reduced in the efferent ducts and epididymis in *Gpr48*$^{-/-}$ mice as shown by immunohistochemistry (**A**,**B**), western blot (**C**) and real-time PCR (**D**,**E**) at 4-16 weeks of age.

In addition, to explore the relationship between Gpr48 and Ar, we examined the mouse *Ar* promoter region and found a semi-*Cre* binding site (CGTCA) located at position –255 relative to the transcription start site. Luciferase assays proved that Gpr48 could elevate the activity of this proximal promoter region (from position –350), whereas H-89 dramatically downregulated this activation by Gpr48. However, the promoter region from position –150 that lost this *Cre* binding site showed no activation when co-transfected with Gpr48 constructs (see Fig. S5A,B in the supplementary material). These results demonstrated that *Ar* is also a direct target gene modulated by Gpr48 and that a *Cre* motif located at position –255 is essential for this activation.

Endogenous ERα is upregulated by Gpr48 in MCF-7 cells

Finally, to investigate whether endogenous ERα expression could be regulated by Gpr48, we transfected MCF-7 cells with constitutively activated Gpr48. *ERα* variants A and C are predominantly expressed in MCF-7 cells (Kos et al., 2001). Real-time PCR and western blot showed that endogenous ERα was dramatically upregulated by Gpr48 (see Fig. S6A,B in the supplementary material). To further affirm this regulation, we examined ERα activity using a luciferase reporter containing the estrogen responsive element (ERE) promoter in MCF-7 cells. MCF-7 cells were transfected with constitutively activated Gpr48 and pGL3-ERE-luciferase plasmids and then treated with estradiol (E2). As expected, active Gpr48 induced a robust increase of ERE activities in the presence of estrogen compared with vehicle vector (see Fig. S6C in the supplementary material). This result further proved the upregulation of endogenous ERα by Gpr48.

DISCUSSION

The precursor of the epididymis, known as the Wolffian duct, arises from the urogenital ridge during embryogenesis. In mouse embryos, the initiation of the Wolffian duct begins at E10, when it undergoes a transition of mesenchymal cells to ductal epithelia and tubulogenesis to form the coiled caput epididymis at E14.0-18.0 (Avenel et al., 2009). Although previous studies showed that Gpr48 is expressed in whole mouse embryos as early as E7.0 and peaks at E15.0 (Loh et al., 2001), our investigation demonstrated a normal division of the epididymis and efferent ducts in *Gpr48*-null male mice. This suggests that Gpr48 plays little role in regulation of the morphogenesis of the Wollfian duct and subsequent formation of the epididymal duct. However, we observed the hypoplastic and dilated convoluted ducts of the epididymis and efferent ducts in *Gpr48*-null

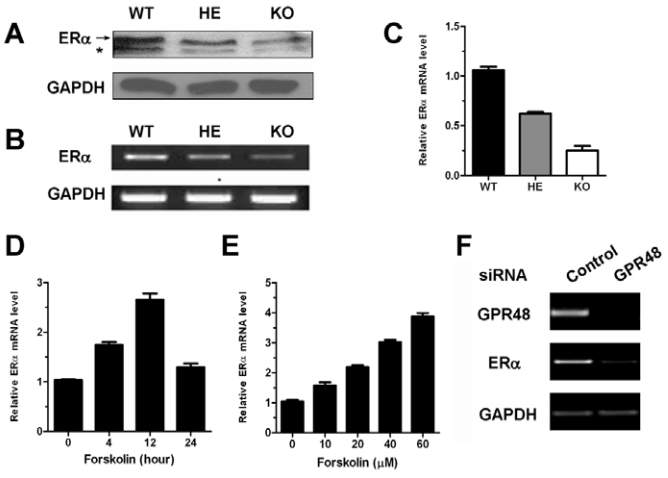

Fig. 6. *ERα* expression in MEFs. (**A-C**) *ERα* expression was markedly reduced in *Gpr48*$^{-/-}$ MEFs as detected by western blot (A), RT-PCR (B) and real-time PCR (C). (**E**,**F**) *ERα* expression was upregulated by forskolin (20 μM) in a time-course (**D**) and dose-dependent manner (E) in *Gpr48*$^{-/-}$ MEFs. (F) Knockdown of *Gpr48* using siRNA in *Gpr48*$^{+/+}$ MEFs led to a marked reduction of *ERα* mRNA levels. Asterisk in A, non-specific band.

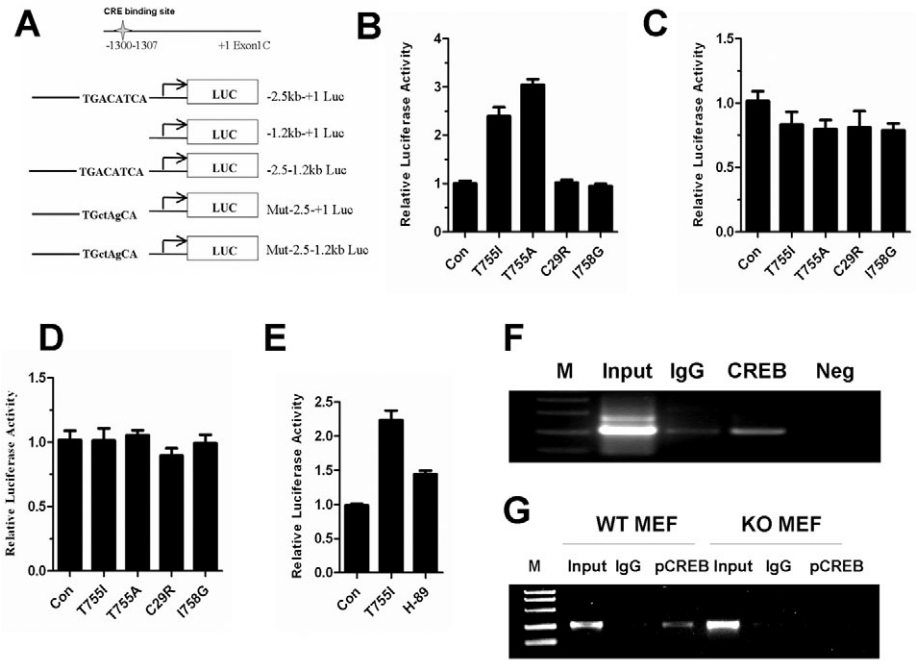

Fig. 7. The transcriptional activity and functional *Cre* motif of the *ERα* promoter. (**A**) The *ERα* promoter constructs containing a potential *Cre* motif (–1307 to –1300). Point mutations were induced in the *Cre* motif (TGACATCA to TGctAgCA). (**B-E**) The transcriptional activity of the *ERα* promoter. The full-length *ERα* promoter (–2500 to +1) was upregulated by constitutively activated Gpr48 (B). The truncated *ERα* promoter (–1200 to +1) was unregulated by constitutively activated Gpr48 (C). The full-length *ERα* promoter with *Cre* motif mutation abolished the Gpr48-mediated transcriptional activity (D). The full-length *ERα* promoter activity was suppressed by H-89, a PKA inhibitor (E). (**F**) ChIP assays of Creb and the *Cre* motif. Creb proteins were bound to the *ERα* promoter in *Gpr48*$^{+/+}$ cells. (**G**) Phosphorylated CREB (Ser133) was solely bound to the *ERα* promoter in *Gpr48*$^{+/+}$ MEFs (WT) but not in *Gpr48*$^{-/-}$ MEFs (KO). M, DNA marker; Neg, no DNA.

mice at E18.5 when the epididymis and efferent ducts started to bend and coil. Therefore, ducts were poorly developed in the epididymis in *Gpr48*-null mice, accompanied by reduced surface areas of epithelia, more severely dilated lumens and swollen testes.

It is known that the epididymis and efferent ducts play pivotal roles in reabsorbing luminal fluids, including water, ion and proteins flowing down from the rete testes. The epithelial lining of the epididymis also secretes several ions and proteins and creates a specialized luminal environment for maturation of testicular spermatozoa. We observed a relative obstruction in the epididymis and efferent ducts of *Gpr48*-null males that could fully explain the destruction of the seminiferous epithelium. However, the cause for the malformation of the epididymis and efferent duct remains poorly understood. We further performed ligation operations upstream and downstream to the epididymis to determine the causal effect of reproductive tract obstruction on seminiferous epithelium defects and to localize the obstruction sites. Our results show that both EGL and VGL ligations result in seminiferous epithelium deterioration but do not cause epididymal defects, indicating a primary developmental defect of the epididymis itself.

Circulating steroid hormones and their receptors are key molecules involved in the development of the epididymis and efferent ducts. Estrogens and androgens participate in regulation of water and ion transport proteins, such as NHE3 and Aqp9, and are responsible for fluid reabsorption in male reproductive tracts. Moreover, *ERα* knockout male mice showed a dilated epididymis and efferent ducts, and a reduction of NHE3 expression (Zhou et al., 2001). Males lacking global androgen receptors are also infertile owing to incomplete germ cell development and lower serum testosterone levels (Yeh et al., 2002). Hence, we examined the expression of AR and ERα in male reproductive tracts including the efferent duct and the caput, corpus and cauda of the epididymis and found that expression levels of both AR and ERα were dramatically disrupted in *Gpr48*$^{-/-}$ mice. However, the phenotype of reproductive tract defects in *Gpr48*$^{-/-}$ male mice was more like that in *ERα*$^{-/-}$ male or *NHE3*$^{-/-}$ mice, thus our investigation focused on the relationship between Gpr48 and ERα. In MEFs cultured in vitro, we also found reduced expression of ERα due to a lack of Gpr48; therefore, we speculated that Gpr48 could directly modulate *ERα* transcription.

It has been reported that orphan receptor Gpr48 activates Gα proteins, increases intracellular cAMP and upregulates gene expression (Weng et al., 2008; Song et al., 2008). In this study, we found that ERα expression was dramatically reduced in vivo and in vitro. It is known that the mouse *ERα* gene codes for at least six different transcript variants (Kos et al., 2000). Multiple promoters have also been identified in human, rat and chicken that are associated with tissue-specific expression and different developmental stages (Kos et al., 2001). We determined that only transcript variants C and F are expressed in the epididymis and efferent ducts using primer-specific RT-PCR (data not shown). We then identified a potential cAMP responsible element (TGACATCA) in the mouse *ERα* variant C promoter. We further proved that this *Cre* motif was functional and essential for Gpr48 regulation of ERα expression.

Previous studies have reported that ERα expression could be modulated by activin in the mouse ovary (Kipp et al., 2007) and also by prolactin in the rat corpus luteum via the Jak2-Stat5 pathway (Frasor et al., 2001; Frasor et al., 2003). In human, ERα expression in osteoblast cells was upregulated by PKC/c-Src (Longo et al., 2006), and in breast carcinoma cells by ERF-1 (also known as TFAP2C), a member of the AP2 transcription factor family (McPherson et al., 1997). In this study, our results first demonstrate that *ERα* gene expression could be upregulated by Gpr48 via the cAMP-PKA-Creb pathway.

In conclusion, our results demonstrate that Gpr48 participates in the development of the male epididymis and efferent ducts through regulation of ERα and Ar expression via the cAMP/PKA signaling pathway.

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Supplementary material

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