Identification of NR0B1 as a novel androgen receptor co-repressor in mouse Sertoli cells

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Abstract. Nuclear receptor subfamily 0 group B member 1 *(Nr0b1)* is an atypical member of the nuclear receptor family that is predominantly expressed in mouse Sertoli cells (SCs). Mutations of *NR0B1* in humans cause adrenal failure and hypogonadotropic hypogonadism. The targeted mutagenesis of *Nr0b1* in mice has revealed a primary gonadal defect characterized by the overexpression of aromatase and cellular obstruction of the seminiferous tubules and efferent ductules, leading to germ cell death and infertility. The transgenic expression of *Nr0b1* under the control of the Müllerian-inhibiting substance promoter (MIS-*Nr0b1*), which is selectively expressed in SCs, improves fertility. Testicular androgen receptor (AR) was also expressed in SCs. Many genes are directly regulated by androgen and its AR, which are involved in spermatogenesis and male infertility. As the association between NR0B1 and AR remains unclear in mouse SCs, we decided to further explore the relationship between them. In the present study, we have identified NR0B1 as a novel AR co-repressor in mouse SCs. Using RT-qPCR and immunofluorescence, we determined that NR0B1 was mainly expressed in mouse SCs in an age-dependent manner from 2-8 weeks of age postnatally. The inhibition of the effects of AR on AR target genes by NR0B1, in an androgen-dependent manner, was further demonstrated by western blot analysis and RT-qPCR in TM4 cells, a mouse Sertoli cell line. Finally, *in vitro* luciferase and co-immunoprecipitation assays validated that NR0B1, as an AR co-repressor, significantly inhibited the transcriptional activation of its target genes. These results suggest that novel inhibitory mechanisms underlie the effects of NR0B1 in modulating androgen-dependent gene transcription in mouse SCs.

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Introduction

Nuclear receptor subfamily 0 group B member 1 *(Nr0b1)* is an orphan nuclear receptor expressed in the ventromedial hypothalamus, pituitary gonadotropes, the adrenal cortex, the testis and the ovary (1,2). The duplication of a region of the X-chromosome spanning *NR0B1* results in dosage sensitive, male-to-female sex reversal (3). Mutations of *NR0B1* cause an X-linked form of adrenal hypoplasia congenita in males, who usually present with an adrenal crisis during the first year of life (4). The disorder is limited to males and is characterized by neonatal adrenal insufficiency and failure to undergo puberty as a result of hypogonadotropic hypogonadism. It has been demonstrated that the targeted disruption of *Nr0b1* in male mice results in infertility, decreased testicular size, and degeneration of germinal epithelium (5). A thorough histological examination of the male reproductive tract from *Nr0b1* knockout (*Nr0b1*-KO) mice revealed that the rete testis, the passageway for sperm to leave the testis, is obstructed by ectopic clusters of Sertoli cells (SCs) (6). Despite these findings, the function of *Nr0b1* in the male reproductive system remains unclear.

Androgen and its receptor, androgen receptor (AR), play important roles in spermatogenesis and male fertility (7-9). The AR belongs to a family of nuclear transcription factors that mediate the action of androgens. It contains an N-terminal transactivation domain, a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). Cytoplasmic AR, when bound by androgens, translocates to the nucleus and binds to the androgen response elements (AREs) on target genes. Testicular AR has been detected in Leydig cells, peritubular cells and SCs (10). Androgens affect spermatogenesis indirectly through AR-expressing somatic cells, such as SCs or peritubular myoid cells (10). AR mutations cause a spectrum of hereditary disorders, including androgen insensitivity syndrome and male infertility (11). Furthermore, male total AR-KO mice exhibited a typical female external appearance, which was similar to a human

androgen-insensitivity syndrome or testicular feminization mutation in mice (12).

In light of accumulating data regarding the function and expression of NR0B1, we became interested in studying the possible interactions between NR0B1 and AR. The AR is a member of the steroid hormone receptor branch of the nuclear receptor superfamily and, as a mediator of androgen signaling, it plays important roles in coordinating gene expression in male reproductive tissues (13-16). A number of distinct co-regulatory factors are involved in the regulation of AR signaling (17). Although many of these factors function as bona fide co-activators or co-repressors by directly communicating with chromatin and the transcription machinery, additional co-regulators may exist that function in an antagonistic manner by preventing, disrupting or redirecting interactions with bona fide co-activators and co-repressors.

In the present study, we have identified NR0B1 as an inhibitory co-regulator of AR. To the best of our knowledge, we have provided evidence of previously uncovered aspects of the mechanisms of action of NR0B1 in mouse SCs. The data strongly suggest that NR0B1 antagonism plays a physiological role in modulating AR-dependent gene regulation in male reproductive tissues.

Materials and methods

Reagents and medium. Rabbit polyclonal anti-NR0B1 antibody (ab60144), anti-AR antibody (ab74272) and mouse monoclonal anti-GAPDH antibody (ab8245) were purchased from Abcam (Cambridge, UK). Anti-HA antibody (H3663) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-SOX9 antibody was purchased from Millipore Corp. (Billerica, MA, USA). Anti-rabbit-Cy3, anti-rabbit-Alexa Fluor 488, anti-mouse-Cy3 and anti-mouse-Alexa Fluor 488 were purchased from Invitrogen (Carlsbad, CA, USA). All restriction endonucleases were purchased from New England BioLabs, Inc. (Ipswich, MA, USA). The Dual-Luciferase reporter assay system and the pGL4.15 plasmid, which was used to construct two recombinant plasmids [pUBE2B(-882/-343)-LUC and pHSF1(-625/-390)-LUC], were obtained from Promega Corp. (Madison, WI, USA). Lipofectamine 2000 was obtained from Invitrogen.

Animals and tissue collection. One hundred mice (C57BL/6) were purchased from the Southern Medical University Animal Center (Guangzhou, China). All animals were treated according to the National Research Council Guide for the Care and Use of Laboratory Animals. The study was approved by the Ethics Committee of Peking University Shenzhen Hospital (Shenzhen, China).

The mouse testes were individually collected from the mice at 1, 2, 3, 4, 6 and 8 weeks, and 6 months of age after sacrifice by cervical dislocation. Five mice were sacrificed in each group at each time-point. Other organs, namely the brain, heart, lung, liver, kidney, spleen, epididymis and bladder, were obtained from adult mice at 8 weeks of age.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from mouse tissues and TM4 cells using TRIzol (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized using oligo(dT) primers (K1622; Fermentas, Waltham, MA, USA). The primers specific for mouse *Nr0b1* were 5'-CACAGAGCAGCCACAGATG-3' (forward), and 5'-AATG TTCAGACTCCAGCACTTG-3' (reverse). The primers for Ube2b were 5'-GCAGCTGCGGAGCATGTCGA-3' (forward), and 5'-CAGATGGGGCGCCACTGACC-3' (reverse); The Hsf1 primers were 5'-CTGGTCCGTGTCAAGCAA-3' (forward), and 5'-GGCTACGCTGAGGCACTT-3' (reverse). Mouse *Gapdh* was used as an internal control and the primers for *Gapdh* were 5'-AGTGGCAAAGTGGAGATT-3' (forward), and 5'-GTGG AGTCATACTGGAACA-3' (reverse). RT-qPCR was performed using the SYBR® Premix EX Taq™ II PCR kit (RR820A; Takara, Dalian, China) according to the manufacturer's instructions on the Roche LightCycler 480 Real-Time PCR system (Mannheim, Germany). The data were calculated according to the Applied Biosystems comparative (Ct) method (18).

Western blot analysis. The proteins were extracted from mouse tissues and TM4 cells and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 10% (w/v) low-fat milk in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20). Following incubation with the anti-NR0B1 antibody (1:1,000; #ab60144; Abcam), anti-UBE2B antibody (1:2,000; #10733-1-AP; Proteintech, Chicago, IL, USA) and anti-HSF1 antibody (1:2,000; #ABE1044; Millipore Corp.) overnight at 4˚C, the membranes were treated with HRP-labeled secondary antibody (ab6721; Abcam) for 1 h at room temperature. Positive bands were detected using an ECL kit (Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence analysis. The mouse testes were fixed in Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA, USA) and sectioned with an ultra-thin semiautomatic microtome (CUT4062; Leica Microsystems, Bensheim, Germany). After being blocked in 10% goat serum, incubated with anti-NR0B1 polyclonal antibody (1:50), anti-AR antibody (1:50) and anti-SOX9 antibody (1:100) overnight at 4 ˚C, the appropriate FITC- or TRITC-conjugated secondary antibodies were used, and the slides were counterstained with Hoechst 33258 and mounted with ProLong Gold antifade reagent (both from Invitrogen). Primary antibody pre-incubated with neutralizing peptide was used as a negative control. The results were observed under a laser scanning confocal microscope (Zeiss, Oberkochen, Germany) and analyzed using Image-Pro Plus 5.1 software.

Plasmid construction and cell culture. The full length of *Nr0b1* cDNA was amplified by PCR with the primers, 5'-CCG GAATTCATGGCGGGTGAGGACCACC-3' (forward), and 5'-CCGCTCGAGTCACAGCTTTGC ACAGAGCATCTCC-3' (reverse), and then inserted into pCDNA3.1/HA plasmids using *Eco*R1 and *Xho*l. The PCR products were cloned and sequenced. Three cell lines, TM4, 15P1 and 293T, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies,

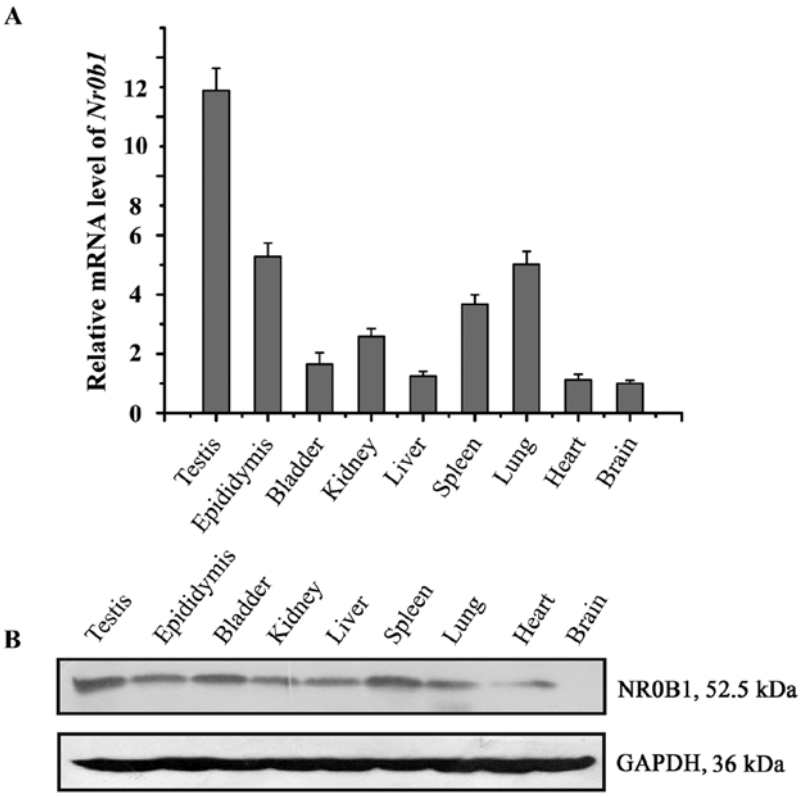

Figure 1. Analysis of the distribution of *Nr0b1* mRNA and its protein in mouse tissues. (A) The mRNA expression of *Nr0b1* in different tissues of adult mice was detected by RT-qPCR. After normalizing to *Gapdh*, the relative expression level of *Nr0b1* in different tissues was compared with that in the brain. Data are expressed as the means ± SD (n=5). (B) The protein expression of NR0B1 was detected by western blot analysis. The molecular weight of each protein is shown on the right. GAPDH was used as an internal control. All experiments were performed three times by independent individuals.

Rockville, MD, USA) supplemented with 10% fetal bovine serum at 37˚C in a humidified atmosphere with 5% CO_2.

RNA Interference. The siRNAs targeting mouse Nr0b1 (siNr0b1, 5'-UAUCUGAAAGGGACCGUGCTCTT-3') were obtained from GenePharma Co., Ltd. (Shanghai, China). The TM4 cells were seeded in a 6-well plate and transfected with 200-pmol pools of siNr0b1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Co-immunoprecipitation (Co-IP). The cell cultures were prepared as described above. NR0B1-pCDNA3.1/HA was transfected into 293T cells together with AR-pCDNA3.1$^+$ or the vector control using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. The cells were harvested 48 h after transfection, washed with TBS (50 mM Tris-HCl (pH 7.4) and 150 mM NaCl), and then the cells were lysed on ice for 30 min with lysis buffer [50 mM Tris-HCl (pH 7.4), 2 mM EGTA or $CaCl_2$, 150 mM NaCl, 1% (v/v) NP-40, protease inhibitor cocktail (P8340; Sigma-Aldrich), and 1 mM PMSF]. The lysates were centrifuged at 13,000 x g for 15 min at 4˚C. The supernatants were incubated with nProtein A Sepharose 4 Fast Flow (GE Healthcare, Little Chalfont, UK) pre-bound with anti-HA or anti-AR antibody at 4˚C overnight with gentle rotation. The beads were washed four times with lysis buffer and prepared with SDS-PAGE sample buffer for western blot analysis.

Luciferase assay. Two mouse SC lines, TM4 ($6x10^4$ cells/well) and 15P1 ($8x10^4$ cells/well), were seeded into a 24-well plate and transfected with 200 ng AR-pCDNA3.1$^+$, 200 ng pMMTV-LUC or pUBE2B(-882/-343)-LUC or pHSF1(-625/-390)-LUC reporter, and increasing amounts (50, 100, 200 and 400 ng) of NR0B1-pCDNA3.1/HA. The cells were treated with 10 nM testosterone (T; Sigma-Aldrich) or ethanol vehicle for 24 h prior to performing the luciferase assay. *Renilla* and firefly luciferase activities were measured using the Dual-Luciferase reporter assay system. Firefly luciferase data were normalized to *Renilla* luciferase data. After normalization for transfection efficiency, the induction factors were calculated as ratios of the average value of the luciferase value of the T-stimulated samples vs. ethanol vehicle-treated samples.

Statistical analysis. All experiments were repeated at least three times. Statistical analysis was calculated using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). The data values are expressed as the means ± SD. The Student's t-test was used to compare the difference between two groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of NR0B1 in different mouse tissues. To assess the expression pattern of NR0B1 in adult mouse tissues, NR0B1 expression was examined in different mouse tissues using RT-qPCR and western blot analysis. The results showed that *Nr0b1* mRNA and its protein were highly expressed in the testes compared with that in the other tissues (Fig. 1).

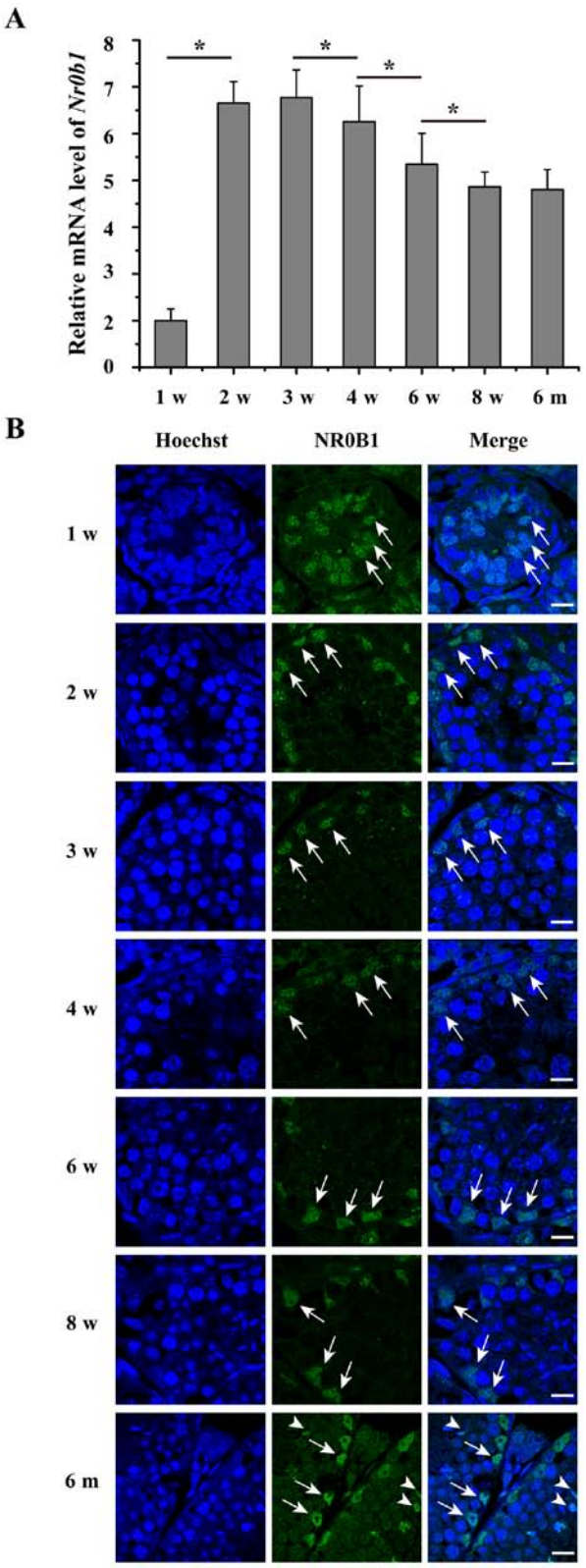

Figure 2. Age-dependent expression of NR0B1 in mouse testes. (A) The mRNA expression of *Nr0b1* in mouse testes at the indicated ages was determined by RT-qPCR. After normalizing to *Gapdh*, the relative expression of *Nr0b1* in testes at different ages was compared to that at week (w) 1. (B) The expression of NR0B1 at different developmental stages were examined by immunofluorescence analysis. The localization of NR0B1 was labeled with NR0B1 antibody (green). The cell nuclei were labeled with Hoechst 33258 (blue). Scale bar, 10 μm. White arrows indicate Sertoli cells (SCs) and the white arrowheads indicate spermatids. Data are expressed as the means ± SD (n=5), and the bars marked with asterisks indicate significant differences ($P<0.05$). We repeated each experiment three times by independent individuals. m, month.

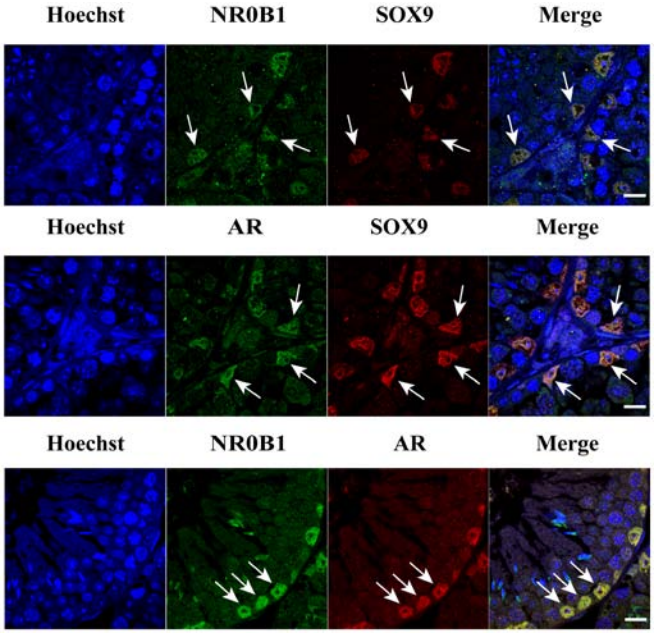

Figure 3. Co-location of NR0B1 and AR in mouse Sertoli cells (SCs) from 8-week-old adult mouse testes. The subcellular location of NR0B1 or androgen receptor (AR) was revealed with specific antibodies (green). SOX9 was used as a marker of SCs (red). Hoechst 33258 was used to stain the nuclei (blue). White arrows, SCs. Scale bar, 10 μm.

Temporal expression profile of NR0B1 during mouse testicular development. To detect the expression of NR0B1 during testicular development, RT-qPCR and immunofluorescence analysis were performed. The results showed that NR0B1 was expressed in the testes during all the stages and decreased in a time-dependent manner from 2-8 weeks postnatally (Fig. 2A). As shown in Fig. 2B, NR0B1 was mainly located in the SCs at weeks 1-8, and was also expressed in the spermatids at 6 months.

Co-location of NR0B1 and AR in mouse SCs. Previous studies have established that NR0B1 expression is associated with testis cord development in mice (19,20). To further test the hypothesis that AR function may be inhibited by NR0B1 expression, it was necessary to determine whether NR0B1 and AR were co-located in mouse SCs, as the AR is known to be expressed in developing mouse SCs (21-23). We next performed immunostaining of 8-week-old adult mouse testes. We observed that NR0B1 and AR were co-located in the mouse SCs (Fig. 3), as determined by co-immunostaining with a SC marker, SOX9.

NR0B1 inhibits the effects of AR on the expression of AR target genes in an androgen-dependent manner. To determine whether NR0B1 is capable of inhibiting AR target gene expression, NR0B1-pCDNA3.1/HA and AR-pCDNA3.1$^+$ vectors were transfected into TM4 cells, following treatment with 10 nM T for 24 h. We determined-the expression of two AR target genes, ubiquitin-conjugating enzyme E2B (UBE2B) and heat shock transcription factor 1 (HSF1) using RT-qPCR and western blot analysis. The results showed that the overexpression of NR0B1 inhibited UBE2B expression and promoted HSF1 expression, while the knockdown of NR0B1 expression by siNr0b1 exerted opposite effects (Fig. 4), which indicated

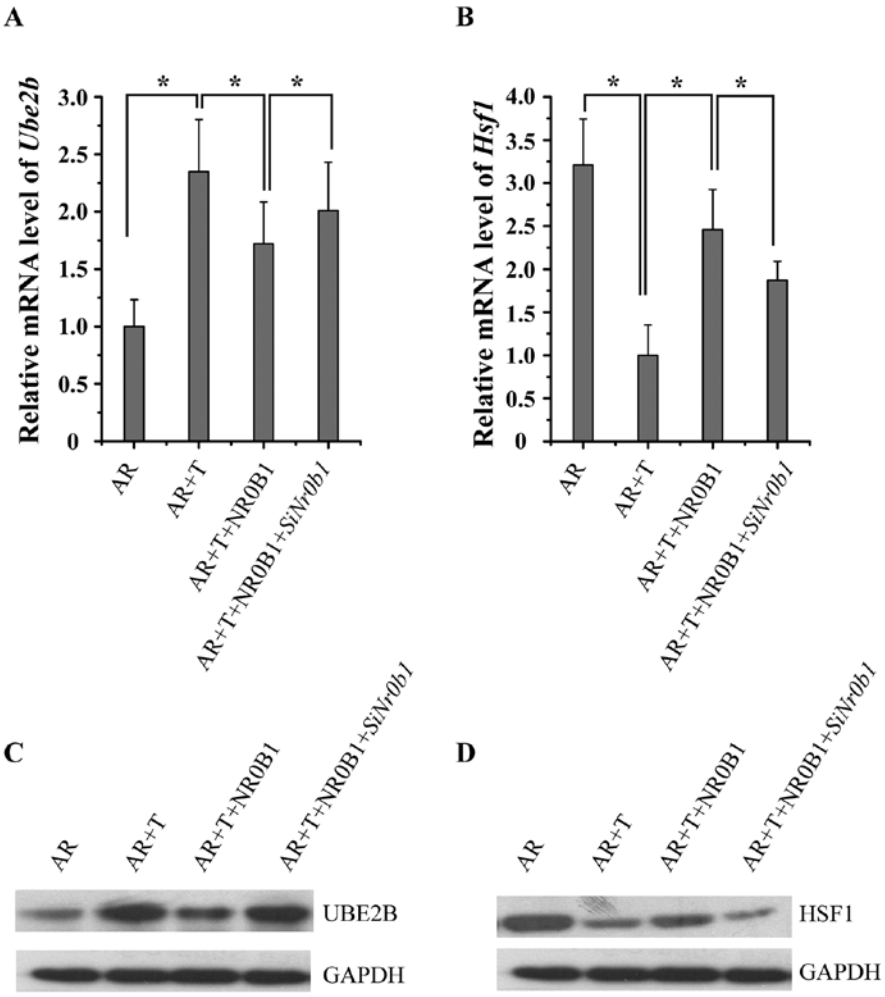

Figure 4. Inhibition of androgen receptor (AR) target genes by NR0B1 in an androgen-dependent manner. TM4 cells were transfected with AR- and NR0B1-overexpressing plasmids. Following treatment with 10 nM testosterone (T) for 24 h, the expression of ubiquitin-conjugating enzyme E2B (UBE2B) and heat shock transcription factor 1 (HSF1) in the TM4 cells was examined by (A and B) RT-qPCR and (C and D) western blot analysis. Data are expressed as the means ± SD (n=5), and bars marked with asterisks indicate significant differences ($P<0.05$). All experiments were performed three times by independent individuals. AR, only AR-overexpressing plasmid; AR+T, AR-overexpressing plasmid and T; AR+T+NR0B1, AR-overexpressing plasmid and T and NR0B1-overexpressing plasmid; AR+T+NR0B1+SiNr0b1, AR-overexpressing plasmid and T and NR0B1-overexpressing plasmid and siRNA-mediated knockdown NR0B1.

that NR0B1 reversed the effects of AR on the expression of its target gene.

NR0B1 inhibition of transcriptional AR activation. To determine whether NR0B1 inhibits the transcriptional activity of AR, we performed transient transfection studies using mouse SC lines (TM4 and 15P1). We used androgen-responsive luciferase reporter constructs, namely pMMTV-LUC containing mouse mammary tumor virus long terminal repeat (LTR), in which there are AREs in front of a TATA box. In the absence of NR0B1, AR activated pMMTV-LUC reporter in an agonist-dependent fashion. The co-expression of increasing amounts of NR0B1 decreased AR activity in a dose-dependent manner, and 200 ng NR0B1/well typically resulted in up to 70% inhibition of AR activity (Fig. 5A). Furthermore, NR0B1 did not directly inhibit the pMMTV-LUC reporter in the absence of AR. These results indicate that NR0B1 potently inhibited ligand-dependent transcriptional AR activation.

Transcriptional profiling studies with AR knockout mouse models searching for androgen-regulated genes relevant to spermatogenesis have identified many candidate target genes of ARs, including *Rhox5*, *Gpx5*, *Lcn5*, *Tubb3*, *Crisp1* and *Eppin* (24-28). In our previous experiment, we have identified *Ube2b* and *Hsf1* as two critical target genes of the AR in mouse SCs. To further demonstrate that NR0B1 may inhibit the transcriptional activity of AR, we also performed transient transfection studies using TM4 and 15P1 cell lines. We used two other androgen-responsive luciferase reporter constructs, namely pUBE2B(-882/-343)-LUC and pHSF1(-625/-390)-LUC, containing an ARE in front of a TATA box (29,30). In the absence of NR0B1, AR activated pUBE2B(-882/-343)-LUC reporter in an agonist-dependent fashion (Fig. 5B) and activated pHSF1(-625/-390)-LUC reporter in an antagonist-dependent fashion (Fig. 5C). The co-expression of increasing amounts of NR0B1 decreased AR activity in a dose-dependent manner, and 200 ng of NR0B1/well typically resulted in up to 55% inhibition of the AR activity (Fig. 5B). Furthermore, NR0B1 did not directly inhibit pUBE2B(-882/-343)-LUC and pHSF1(-625/-390)-LUC reporters in the absence of AR. All of these results strongly suggest that NR0B1 antagonism plays a important role in modulating AR-dependent gene regulation in mouse SCs.

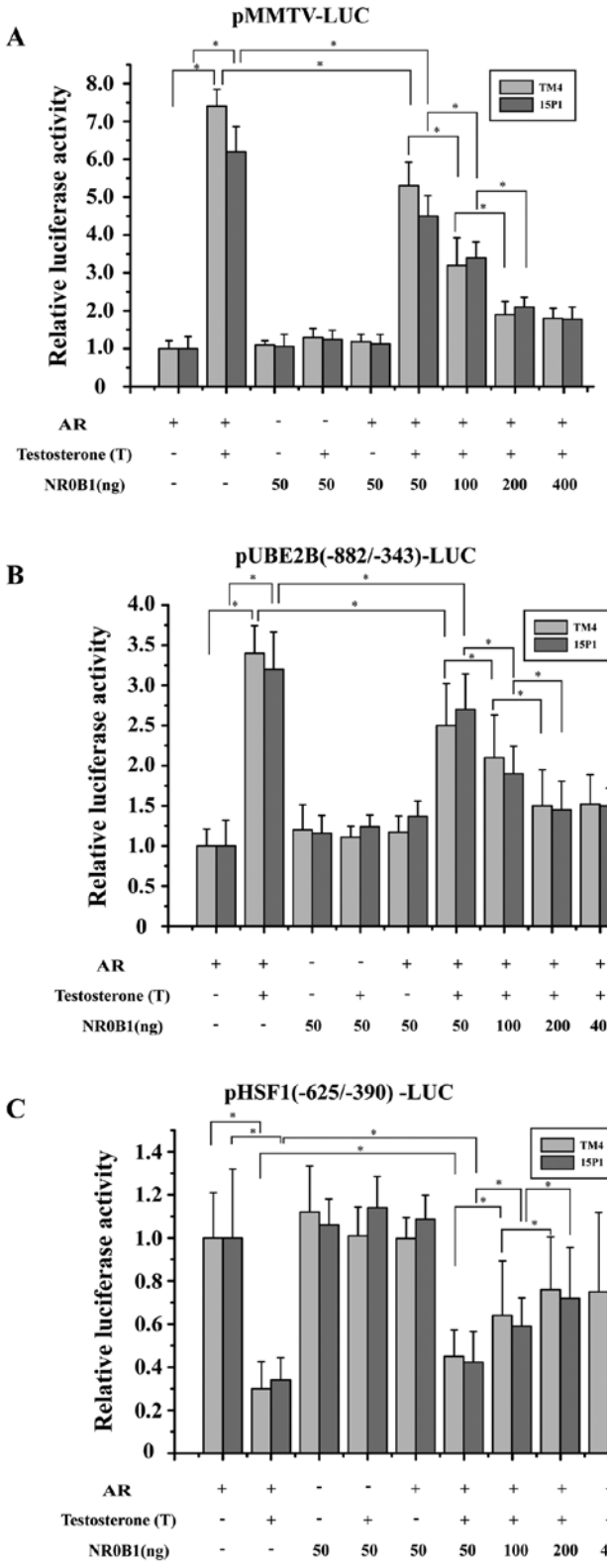

Figure 5. Inhibition of androgen receptor (AR)-dependent transcription by NR0B1 in mouse Sertoli cell lines. TM4 and 15P1 cells, cultured on 24-well plates, were transfected with 200 ng AR-pCDNA3.1$^+$, 200 ng pMMTV-LUC (A) or pUBE2B(-882/-343)-LUC (B) or pHSF1(-625/-390)-LUC (C) reporter, and increasing amounts (50, 100, 200 and 400 ng) of NR0B1-pCDNA3.1/HA and treated with or without 10 nM testosterone (T). Firefly luciferase data have been normalized to *Renilla* luciferase data. After normalization for transfection efficiency, induction factors were calculated as the ratios of the average value of the luciferase value of the T-stimulated samples vs. ethanol vehicle-treated samples. Data are expressed as the means ± SD (n=5), and bars marked with asterisks indicate significant differences (P<0.05).

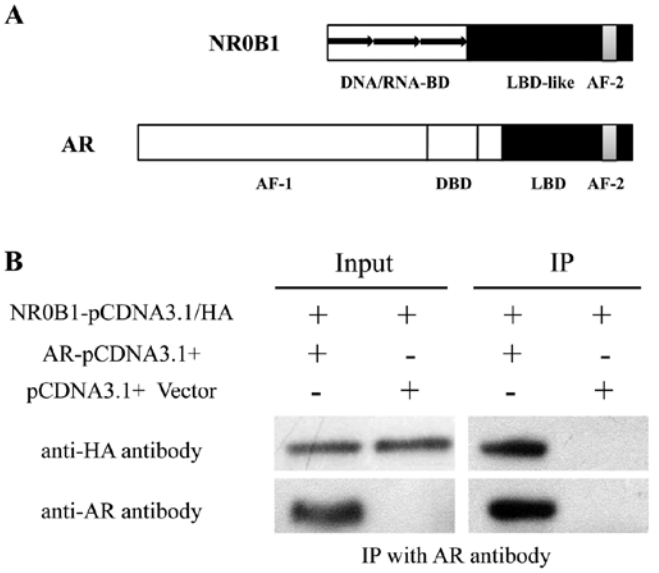

Figure 6. Interaction between NR0B1 and androgen receptor (AR) *in vitro*. (A) Schematic representation of the NR0B1 and AR domain structure. (B) NR0B1-pCDNA3.1/HA was transfected into 293T cells together with AR-pCDNA3.1$^+$ or the vector control. NR0B1 was immunoprecipitated using an anti-AR antibody. Anti-HA antibody (upper panel) or anti-AR antibody (lower panel) were used for western blot analysis. Expression of NR0B1-pCDNA3.1/HA and AR-pCDNA3.1$^+$ in 293T cells was indicated in the lanes labeled 'Input'. The immunoprecipitation products indicated that NR0B1-pCDNA3.1/HA was pulled down in the presence only of AR-pCDNA3.1$^+$ but not the vector control. DBD, DNA-binding domain; LBD, ligand-binding domain.

Interaction of NR0B1 and AR in vitro. *Nr0b1* has been classified as an orphan member of the nuclear receptor superfamily (31,32). The NR0B1 domain structure has an amino-terminal domain (DNA/RNA-BD), a carboxy-terminal domain (LBD-like) and an AF-2 transactivation domain (Fig. 6A). The amino-terminal domain has a novel structure consisting of 3.5 alanine/glycine-rich repeats of a 65-70 amino acid motif that has no known homology to any other proteins, with the exception of the related nuclear receptor superfamily member, small heterodimer partner (SHP), encoded by NR0B2 (33). ARs (also known as dihydrotestosterone receptors) are nuclear hormone receptors of the NR3C class. The AR domain structure has a AF-1 transactivation domain, a DBD, a LBD and an AF-2 transactivation domain (Fig. 6A).

In our previous experiment, we found that NR0B1 inhibits the transcriptional activation of AR, and NR0B1 and AR were co-located in mouse SCs. From these results, we speculated that NR0B1 interacts with AR and then inhibits its transcriptional activation. The interaction between NR0B1 and AR was determined using an *in vitro* co-immunoprecipitation assay. The AR and HA-fused NR0B1 vectors were transcribed as described above and transfected into 293T cells. We found that HA-fused NR0B1 protein was pulled down by anti-AR antibody, which demonstrated that NR0B1 interacted with AR in mouse SCs (Fig. 6B).

Discussion

Nr0b1 is an atypical member of the nuclear receptor superfamily that is predominantly expressed in mouse testes. In a previous study aiming to evaluate the role of *Nr0b1* in mice,

Nr0b1 was expressed by the Müllerian-inhibiting substance promoter (MIS-*Nr0b1*) in the genetic background of the *Nr0b1*KO model (34). The expression of a *Nr0b1* trans-gene was sufficient to partially rescue the primary testicular defect of the male *Nr0b1*-deficient (KO) mouse. Even so, the function of *Nr0b1* in mouse SCs remains unclear.

Androgen signaling via the AR may be represented as a multistep cascade involving the dissociation of cytoplasmic chaperone/heat shock protein complexes upon ligand-binding, nuclear localization, DNA binding, and the association of the AR with various bona fide co-activators, such as histone acetyltransferases [p160s, cAMP response element binding protein (CREB)-binding protein (CBP), p300, p300/CBP-associated factor (PCAF), Tat-interacting protein 60 (TIP60)] and a number of unrelated proteins, including protein inhibitor of activated STAT (PIAS) proteins (35), AR-interacting protein (ARIP)/small nuclear ring finger protein (SNURF)/RNF4s, and AR-interacting nuclear protein kinase (ANPK) (17). Considerably less is known about the mechanisms by which androgen-dependent transcription is inhibited, and candidate co-repressors have only recently been identified. They include the amino-terminal enhancer of split, a member of the Groucho/transducin-like enhancer of split family of co-repressors, that is not associated with histone deacetylases, but instead functions through direct contact with the basal transcription factor, TFIIE (36). Other repressors of androgen action include cyclin D1, which directly antagonizes the acetyltransferase, PCAF (37); SMAD3, an intracellular mediator of the TGF-β pathway (38); and the protein kinases, PAK6 and AKT, which presumably repress AR activity through direct phosphorylation (39,40). Moreover, a novel covalent modification of the AR by the attachment of small ubiquitin-related modifier 1 in certain contexts inhibits the transcriptional activity of the AR (41).

In this study, we have demonstrated that NR0B1 interacts with AR and inhibits the transcriptional activation of AR in mouse SCs. The enforced expression of NR0B1 suppresses the expression of target genes of AR, while the silencing of NR0B1 promotes the expression of these target genes. This is consistent with our observation that a significant amount of NR0B1 co-localized with AR in the nuclei of mouse SCs. Although the precise mechanisms responsible for these phenomena remain unclear, NR0B1 most likely interferes with the events required for AR activation in mouse SCs. This may include interference with the association and dissociation of chaperones or interference with nuclear import by masking the nuclear localization signals of AR. In support of the general application of this mechanism, there is preliminary evidence from several studies demonstrating that NR0B1 is also able to repress steroidogenic factor 1 (SF-1) and estrogen receptor (ER)-mediated transactivation (42-45).

AR function is often altered in humans with reproductive abnormalities, as well as in prostate cancer, due to mutations within the LBD (15,46). While some of these mutations have been demonstrated to affect the ligand-binding capacity and specificity, others are proposed to affect inter-domain communication or direct interactions with co-activators (47-49). Similarly, multiple *NR0B1* mutations have been detected that primarily target the putative LBD of *NR0B1* (50-52). One of these mutations (*NR0B1* R267P) has been found to be less potent in ER inhibition (42). However, as this mutation presumably affects several features of the *NR0B1* LBD, further investigations are required in order to determine whether mutated *NR0B1* displays changes with respect to intracellular tethering, co-activator competition or co-repressor recruitment. AR and NR0B1 are the only two reproductive nuclear receptors in which high numbers of natural mutations have been detected in human males (53). Notably, the genes for both *AR* and *NR0B1* are located on the X-chromosome. Thus, all mutations affecting the function yield a phenotype. Interactions between NR0B1 and AR may be important for the proper development of the male reproductive system.

In conclusion, we have identified NR0B1 as a new AR co-repressor in mouse SCs. We have provided evidence, for the first time to the best of our knowledge, for the novel roles of NR0B1 as an inhibitory co-regulator of the AR in mouse SCs. These data strongly suggest that NR0B1 antagonism plays a important role in modulating AR-dependent gene regulation in the male reproductive system.

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