FSH acts on the proliferation of type A spermatogonia via Nur77 that increases GDNF expression in the Sertoli cells

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ABSTRACT

The molecular mechanism responsible for the regulation of GDNF in Sertoli cells remains largely unknown. In the present study, FSH induced the expression of Nur77 and GDNF in mouse testis tissue and human fetal Sertoli cells. Moreover, FSH increased the number of A spermatogonia co-cultured with Sertoli cells. In the additional assays, Nur77 was observed to directly regulate *GDNF* transcription. Furthermore, overexpression of Nur77 and siRNA-mediated knockdown of Nur77 affected levels of *GDNF* mRNA and protein in primary human fetal Sertoli cells. These results indicate that FSH-induced Nur77 regulates the expression of GDNF in Sertoli cells to stimulate the proliferation of A spermatogonia in vitro.

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1. Introduction

In a male testis, A spermatogonia are germ stem/progenitor cell population including spermatogonial stem cells (SSCs), that can self-renew and continuously differentiate to become germ cells, then spermatozoa [1]. During the process of spermatogenesis, Sertoli cells (one somatic cell type within the seminiferous tubules), secrete many types of cytokines in order to appropriately regulate the proliferation of SSCs and their differentiation. In particular, glial cell line-derived neurotrophic factor (GDNF) is a key factor [2,3]. Moreover, sufficient levels of GDNF have been shown to cause large clumps of SSCs to form in the seminiferous tubules, while low levels of GDNF can induce the differentiation of SSCs [4,5]. In *GDNF* knockout mice, the seminiferous epithelium was observed to be depleted, concomitant with a significant decrease in SSCs [6].

Despite these valuable insights, however, regulation of GDNF signaling in Sertoli cells remains incompletely characterized. It is known that follicle-stimulating hormone (FSH) can induce GDNF expression in primary cultures of mouse Sertoli cells, and in prepuberty testis [7]. Rat oligonucleotide microarray analysis has also revealed that expression of nerve growth factor inducible gene B (NGFI-B, also known as Nur77) is up-regulated in FSH-treated rat Sertoli cells [8]. As a member of the nuclear receptor superfamily, and one of the immediate-early response genes, Nur77 is widely expressed in several tissues, including testis, ovary, muscle, adrenal gland, and brain [9]. In response to many types of stimulation, Nur77 has been shown to recognize a specific nucleotide sequence, TGACCTTC, referred to as a NGFI-B response element (NBRE), that is present in a subset of genes [10,11]. Nur77 can bind as a monomer, homodimer, or heterodimer with RXRs, and contributes to the regulation of a set of genes involved in steroidogenesis and cell survival [10,11].

In the present study, FSH stimulation is shown to promote the expression of Nur77 and GDNF in mouse prepuberty testis, and in human fetal Sertoli cells. Moreover, exogenous FSH is shown to facilitate colony formation and the proliferation of mouse primary A spermatogonia co-cultured with Sertoli cells. The capacity for Nur77 to directly bind a novel NBRE site in the *GDNF* promoter is also demonstrated, which results in an increase in promoter activity and GDNF expression. In combination, these results support the hypothesis that the FSH/Nur77/GDNF signaling pathway in the Sertoli cells plays an important role in the proliferation and maintenance of A spermatogonia.

2. Materials and methods

2.1. Mice

Eighty male ICR mice of different ages were purchased from the Model Animal Research Center of Nanjing University. In addition, another 80 one-week-old male ICR mice were obtained that received a single intraperitoneal injection of 5 IU human recombinant FSH (Gonal-F, Merck Serono). At different time intervals, the mice were sacrificed and the testes were removed for quantitative real-time PCR analysis of *Nur77* and *GDNF*. All of the experiments involving animals were performed according to the guidelines of the Experimental Animals Management Committee (Jiangsu Province, China).

2.2. Human fetal Sertoli cells and mouse A spermatogonia

With the approval of the Drum Tower Hospital Medical Ethics Committee, human primary Sertoli cells were isolated from the testes of an aborted twenty-week-old fetus that did not have a cerebellum. Briefly, the decapsulated testes tissue was exposed to enzymatic digestion with Dulbecco's modified eagle's medium (Gibco) containing 0.1% collagenase I (Sigma-Aldrich) and DNase I (Sigma-Aldrich) at 37 °C for 15 min. During this incubation, the digested tissue was blown 2–3 times to enhance the digestion of the tissue. For high pure (>95%) Sertoli cells, cell suspension was plated down on laminin-coated culture dishes. The single cells obtained were then cultured in high-glucose DMEM supplemented with 1 mM sodium pyruvate (Gibco), 10% fetal bovine serum (HyClone), 50 IU/ml penicillin (Gibco), and 50 μg/ml streptomycin (Gibco). After 4 d, endogenous germ cells were further removed by osmotic shock (10 mM Tris–HCl), and human recombinant FSH (rFSH) (20 ng/ml) was added at various timepoints as indicated.

Cultures of self-renewing mouse A spermatogonia were established from 6 to 8 d postpartum (dpp) donor DBA male mice (Model Animal Research Center of Nanjing University) as previously described [12]. By the procedure in [13], the enriched lamininbinding germ cells populations contained more than 90% undifferentiated type A spermatogonia. The isolated A spermatogonia (>90% pure, $\sim 1 \times 10^4$ cells/cm^2) were then plated into a 3.5 cm culture plate containing feeder layers of irradiated mouse Sertoli cells (98% pure, $\sim 5 \times 10^4$ cells/cm^2) in 2 mL SSC culture medium. The SSC culture medium was StemPro-34 SFM (Gibco), supplemented with StemPro supplement (Gibco) containing 1% Insulin-Transferrin-Selenium (v/v, Gibco), 1 μl/ml DL-lactic acid (Sigma-Aldrich), 5 mg/ml bovine albumin (Sigma-Aldrich), 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, minimal essential medium (MEM) vitamin solution (Gibco), MEM non-essential amino acid solution (Gibco), 10^{-4} M ascorbic acid, 10 mg/ml d-biotin, 150 ng/ml GFRα1 (Sigma-Aldrich), and 1 ng/ml basic fibroblast growth factor (bFGF) (Gibco). Human rFSH (20 ng/ml) was added into the culture medium as indicated.

In the spermatogonia-Sertoli cells co-culture experiment, Herbimycin A (Sigma-Aldrich) was used at a concentration of 10 mM to inhibit the GDNF-specific intracellular signals in the spermatogonia [14]. The number of spermatogonia co-cultured with Sertoli cells was counted by FITC-Oct3/4 staining. Cells were analyzed with a FACS-Calibur system (BD Biosciences).

2.3. siRNA transfection and adenovirus infection

Mouse Sertoli cells were seeded on a culture plate and precultured overnight. Before transfection, the medium was changed to fresh medium. SiRNA-nur77 mixed with Attractene transfection reagent (Qiagen) was added to the culture medium at a final concentration of 50 nM according to the manufacturer's instructions. SiRNA-m-nur77 sequence (sense): 5′-UCCCUGGCUUCAUUGAGCUdTdT-3′.

Adenoviruses containing Nur77 fused to a FLAG-tag epitope (Ad-Nur77-FLAG), Nur77-targeted siRNA (Ad-si-Nur77), or a control Ad-LacZ construct (Ad-LacZ), were generated as described previously [15]. Primary human fetal Sertoli cells were then infected with these adenoviruses after being cultured in serum-free medium overnight.

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol® reagent (Invitrogen) and treated with DNase I (Promega) to remove any contaminating genomic DNA. The quality of the RNA obtained was evaluated using spectrophotometry and denaturing agarose gel electrophoresis. One μg of RNA was reverse-transcribed using oligo (d)T priming and SuperScript II reverse transcriptase (Invitrogen). Expression levels of specific genes were then measured using SYBR® *Premix Ex Taq*™ II (Takara) and the iCycler iQ™ system (Bio-Rad). Melt curve analyses were conducted to ensure the specificity of the amplification. All primer sequences (Table 1) were designed using Primer Express 3.0 software (Applied Biosystems). Quantitative comparisons were made by normalizing the expression of each gene of interest to expression of ribosomal protein 18S. Relative transcript abundance was determined according to $2^{-\Delta\Delta CT}$ [16].

2.5. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay kit (Upstate Biotechnology) was used with some modifications to the recommended protocol [17]. Briefly, human Sertoli cells (70–80% confluence) were infected with Ad-LacZ and

Table 1
Primers used for qRT-PCR assays.

Species	Gene	Primer sequence 5′-3′
Mouse	FSHR	GGC CAG GTC AAC ATA CCG CTT G
		TGC CTT GAA ATA GAC TTG TTG CAA ATT G
Mouse	Nur77	GCACAGCTTGGGTGTTGATG
		CAGACGTGACAGGCAGCTG
Mouse	18S RNA	ATG GCC GTT CTT AGT TGG TG
		CGG ACA TCT AAG GGC ATC AC
Mouse	GDNF	GACTTGGGTTTGGGCTATGA
		AACATGCCTGGCCTACTTTG
Human	Nur77	GGCATGGTGAAGGAAGTTGT
		CGGAGAGCAGGTCGTAGAAC
Human	GDNF	CCAACCCAGAGAATTCCAGA
		CAACATGCCTGCCCTACTTT
Human	18S RNA	CGGCTACCACATCCAAGGAA
		CTGGAATTACCGCGGCT
Human	CK18	CACAGTCTGCTGAGGTTGGA
		GAGCTGCTCCATCTGTAGGG
Human	AMH	TCCGAGAAGACTTGGACTGG
		CAGGTCACTTCCTCCAGGTG

Ad-Nur77-FLAG (50 MOI) for 72 h were washed with PBS then crosslinked with 1% formaldehyde for 15 min at RT. Cross-linking was stopped with the addition of glycine (0.125 M final concentration) for 10 min. Cells were washed twice with cold PBS, harvested in lysis buffer A (20 mM Tris–HCl (pH 8.0), 85 mM KCl, 1 mM EDTA, 0.5 mM EGTA, 0.5% Nonidet P40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A), then pelleted by centrifugation. Cells pellets were then lysed in nuclear lysis buffer B (50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% SDS, and protease inhibitors as described above). Cell samples were ultrasonicated on ice to yield genomic DNA fragments of 500–1000 bps. Precleared sonicates were then incubated with 2 μg FLAG antibody (Santa Cruz) and rotated overnight. Protein A/G beads (Upstate Biotechnology) were added for 3 h, and collected beads were washed extensively. Immunocomplexes were eluted following an incubation of samples at 65 °C for 30 min, then at RT for 15 min with fresh elution buffer (1% SDS; 0.1 M NaHCO$_3$). Crosslinks were reversed with an incubation of the samples at 65 °C for 5 h, at a final concentration of 0.3 M NaCl. Eluates were then incubated with proteinase K, and DNA was purified using phenol/chloroform extraction and ethanol precipitation. Finally, the purified DNA fragments were used as a PCR template for the amplification of Nur77-binding sequences present in human fetal Sertoli cells. Primers designed to amplify putative NBRE sites (spanning −2579 to −2344 bp) included: 5′-cagcgagagaggagctgaat-3′ and 5′-actgtggaaggaaggct-gac-3′.

2.6. Avidin–biotin conjugate DNA precipitation assay

For avidin–biotin conjugate DNA precipitation (ABCD) assays, double stranded oligonucleotides designed to bind wild type human GDNF (hGDNF), a reverse sequence of hGDNF, and a mutated version of hGDNF were designed. The sequence of these primers included: 5′ biotin–cctgaa*tgaccttc*cagttcctgaacttc-3′ for hGDNF WTF (from −2473 to −2434 bp); 5′ biotin–cctgaag*tcaagga*cag-ttcctgaacttc-3′ for hGDNF MUF; and 5′ biotin -aaactaatgtgtgt*aaaggt-ca*gaaagcg-3′ for hGDNF REVF, respectively. All of these primers were also labeled with biotin at the 3′ end of each sense strand. These double-stranded DNA primers were immobilized on streptavidin-conjugated agarose beads suspended in binding buffer for 3 h at 4 °C. The beads were then washed four times with binding buffer. human Sertoli cells infected with Ad-LacZ and Ad-Nur77-FLAG (50 MOI) for 72 h were lysed with lysis buffer (10 mM Tris–Cl [pH 7.8], 1 mM EDTA, 150 mM NaCl, 0.1% NP-40) containing protease inhibitors at 4 °C for 1 h. Whole-cell lysates were clarified by centrifugation, mixed with the 50% slurry of avidin beads, and pelleted at 5000 rpm. Supernatants were added to the beads-DNA complex and rotated at 4 °C for 1 h to mix. The beads were collected by centrifugation at 5000 rpm and were washed successively in the binding buffer. Bound proteins were resolved by SDS–PAGE. Following the transfer of the proteins onto polyvinylidine fluoride (PVDF) membranes, FLAG-conjugated proteins were detected using anti-FLAG antibodies, which were subsequently bound by goat anti-rabbit secondary antibodies and visualized using an enhanced chemiluminescence kit (Amersham Biosciences) [17].

2.7. Luciferase reporter assay

Approximately 2.7 kb of the GDNF promoter sequence (−2512/+230) was amplified by PCR using 5′-ggaggtacctgaggggggcagcgaga-taacc-3′ and 5′-gacagatctcacccgcgacgcaggcaccac-3′ primers. The resulting PCR product was subcloned into pGL3-basic (Promega) using Kpn I and Bgl II sites and sequenced to confirm the resulting vector. Preconfluent (75–80%) hSC cells in 6-well plates were infected with Ad-Nur77-FLAG, Ad-si-Nur77, and Ad-LacZ (50 MOI in each case) in duplicate for 48 h. Cells were then transfected with

luciferase reporter constructs using Lipofectamine 2000 (Invitrogen). After 48 h, cell lysates were assayed for luciferase activity using the dual Luciferase Assay System (Promega).

2.8. Western blotting

Protein lysates were prepared and separated by SDS–PAGE as previously described [18]. Immunoblotting was performed using primary antibodies raised against Nur77 (1:200 dilution; ab13851, Abcam), GDNF (1:400 dilution; SC-32551, Santa Cruz) and β-actin (1:5000 dilution; Sigma-Aldrich). Goat anti-rabbit (1:5000, BA1054, Boster, China), donkey anti-goat (1:5000, SC-2020) and goat anti-mouse (1:5000, BA1050, Boster) secondary antibodies conjugated to horseradish peroxidase were used and an enhanced chemiluminescence kit was used to visualize antibody binding.

2.9. Immunofluorescence analysis

Human fetal Sertoli cells, or mouse A spermatogonia, were plated in 24-well plates and fixed with cold acetone and absolute alcohol (3:2 v:v) for 30 min. Cells were then washed with PBS and blocked with 10% FBS/PBS (Hyclone). After 2 h, cells were incubated with rabbit anti-Oct3/4 (1:200, #SC-9081), rabbit anti-SCF (1:400, #SC-9132), mouse anti-Vimentin (1:400, #SC-6260) (Santa Cruz), and rabbit anti-Nur77 (1:200, ab13851, Abcam) overnight at 4 °C. Cells were then washed three times with PBS and incubated with Alexa-488 goat anti-mouse (A11029), Alexa-488 goat anti-rabbit IgG antibodies (A11008), or Alexa-546 goat anti-rabbit IgG antibodies (A11010) (1:1000; Molecular Probes) and for 45 min at 37 °C in the dark. Cells were washed again with PBS then covered with a drop of glycerin and observed using a fluorescence microscope (Carl Zeiss).

2.10. Statistical analysis

All values are expressed as the mean ± standard error (S.E.). Differences between mean values were analyzed using a two-tailed Student's *t*-test.

3. Results

3.1. FSH stimulation induces the expression of Nur77 and GDNF in mouse prepuberty testis

In mouse prepuberty testis, the level of *Nur77* mRNA increased during the first week postpartum, then decreased quickly in the following weeks. *GDNF* mRNA exhibited a similar pattern of expression (Fig. 1A). Western blot analysis further demonstrated that the increase in mRNA levels correlated with an increase in levels of Nur77 and GDNF protein in postpartum testis tissue (Fig. 1B).

One-week-old mice were then stimulated with human rFSH and testis tissue was analyzed. FSH treatment resulted in a 2.5-fold increase in *Nur77* mRNA at 12 h, and a 3.2-fold increase in *GDNF* mRNA at 48 h (Fig. 1C). Furthermore, protein levels of Nur77 and GDNF were observed to increase from 12 to 48 h, with a peak at 24 h after FSH treatment (Fig. 1D). In combination, these results indicate that FSH can regulate the transcription of *Nur77* and *GDNF* in mouse testis.

3.2. FSH promotes the proliferation of mouse A spermatogonia co-cultured with Sertoli cells

Signaling by the FSH is important for Sertoli cells and the spermatogonial cell population, and has been shown to restore the spermatogonial number in impaired testes of hamsters and humans [19]. To further identify the effect of FSH on the proliferation
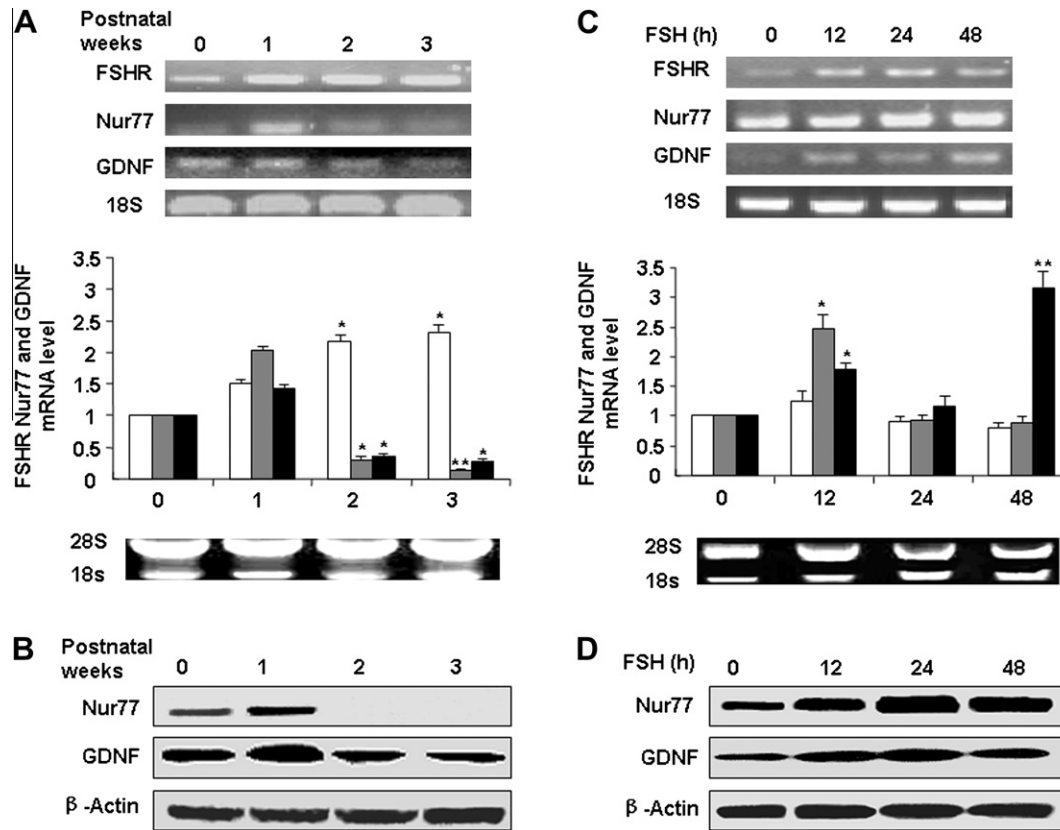
Fig. 1. Expression of Nur77 and GDNF in prepuberty testis tissue during development and following stimulation with rFSH. (A, C): The mRNA levels of FSH receptor (*FSHR*) (white bars), *Nur77* (gray bars), and *GDNF* (black bars) were quantified. (B, D): Levels of Nur77 and GDNF protein during development and following rFSH stimulation were also determined by western blot assays. $^*p < 0.05$; $^{**}p < 0.01$.

of spermatogonia, mouse A spermatogonia were co-cultured with mouse Sertoli cells in serum-free medium. When 20 ng/mL rFSH was added to the medium daily, the total number of spermatogonia detected by flow cytometry was significantly higher than that of unstimulated co-cultures after 7 d, which was following with higher expression of Nur77 and GDNF (Fig. 2A-a, A-b and B). After 14 d, the A spermatogonia cultured in the presence of rFSH were observed to proliferate quickly (Fig. 2A-c). In contrast, A spermatogonia cultured in the absence of rFSH proliferated slowly, and exhibited loose arrangement in line (Fig. 2A-d).

Furthermore, when Nur77 was knocked down in Sertoli cells, the expression of GDNF was reduced (Fig. 2C). A spermatogonia co-cultured with Nur77-knock down Sertoli cells proliferated slowly compared to those co-cultured with scramble siRNA-treated cells although FSH was added (Fig. 2C). A spermatogonia co-cultured with Sertoli cells in the presence of FSH maintained the characteristics of undifferentiated germ cells include expression of Oct 3/4, CD49f (Integrin alpha 6), and CD133 (Prominin 1) (Fig 2D and E).

FSH has been shown to induce the expression of Nur77 and GDNF in rodent Sertoli cells [7,8], and GDNF is a key regulator of mouse SSC proliferation in vitro [2]. When GDNF pathway was inhibited by herbimycin A, spermatogonia showed decreased proliferation (Fig. 2C). The A spermatogonia-Sertoli cells co-cultures demonstrated that FSH promotes the proliferation of A spermatogonia via Nur77 that maintain their undifferentiated qualities in vitro.

3.3. FSH stimulation increases the expression of Nur77 and GDNF in human fetal Sertoli cells

Previous studies have shown that FSH receptors within the seminiferous epithelium are localized to Sertoli cells [20]. The expression of FSH receptors also increases after birth concurrent with the prolif-

eration of Sertoli cells and SSCs [20]. Therefore, Sertoli cells represent the major target of FSH in regulating normal spermatogenesis. In this study, primary human Sertoli cells were successfully isolated (Fig. 3A), and were characterized by positive expression of SCF, Vimentin (Fig. 3B), and CK18 (Fig. 3C). When these cells were subsequently stimulated with rFSH, a rapid increase in *Nur77* mRNA levels was detected after 30 min, and these mRNA levels further increased up to 1.5 h following stimulation before starting to decrease (Fig. 3D). FSH stimulation was also found to increase the level of *GDNF* mRNA. For example, a 7.8-fold increase in *GDNF* mRNA was detected 6 h post-stimulation, and these elevated levels were maintained up to 24 h following stimulation (Fig. 3E).

Expression of Nur77 induced by FSH in human fetal Sertoli cells was also detected by immunofluoresence. Compared with the control group, Nur77 protein expressed in human Sertoli cells was localized to both cytoplasts and nuclei, and a significant increase in protein levels was detected 3 h after stimulation (Fig. 3F). It has been hypothesized that Nur77 is regulated post-transcriptionally, mainly via dephosphorylation. In this study, Nur77 expression was observed to decrease 9 h after FSH stimulation, yet remained primarily localized in the nuclei of Sertoli cells (Fig. 3F). It has previously been reported that Nur77 enters the nucleus after being dephosphorylated in Ser^{354} [21,22]. Based on these data, it would appear that Nur77 has the opportunity to act as a transcription factor to regulate target genes of FSH in Sertoli cells after dephosphorylation.

3.4. Nur77 increases the expression of GDNF by binding to its promoter in human fetal Sertoli cells

To determine whether NBRE sequences (i.e., TGACCTTC) within a *GDNF* promoter fragment could be bound by Nur77, ChIP-PCR as-
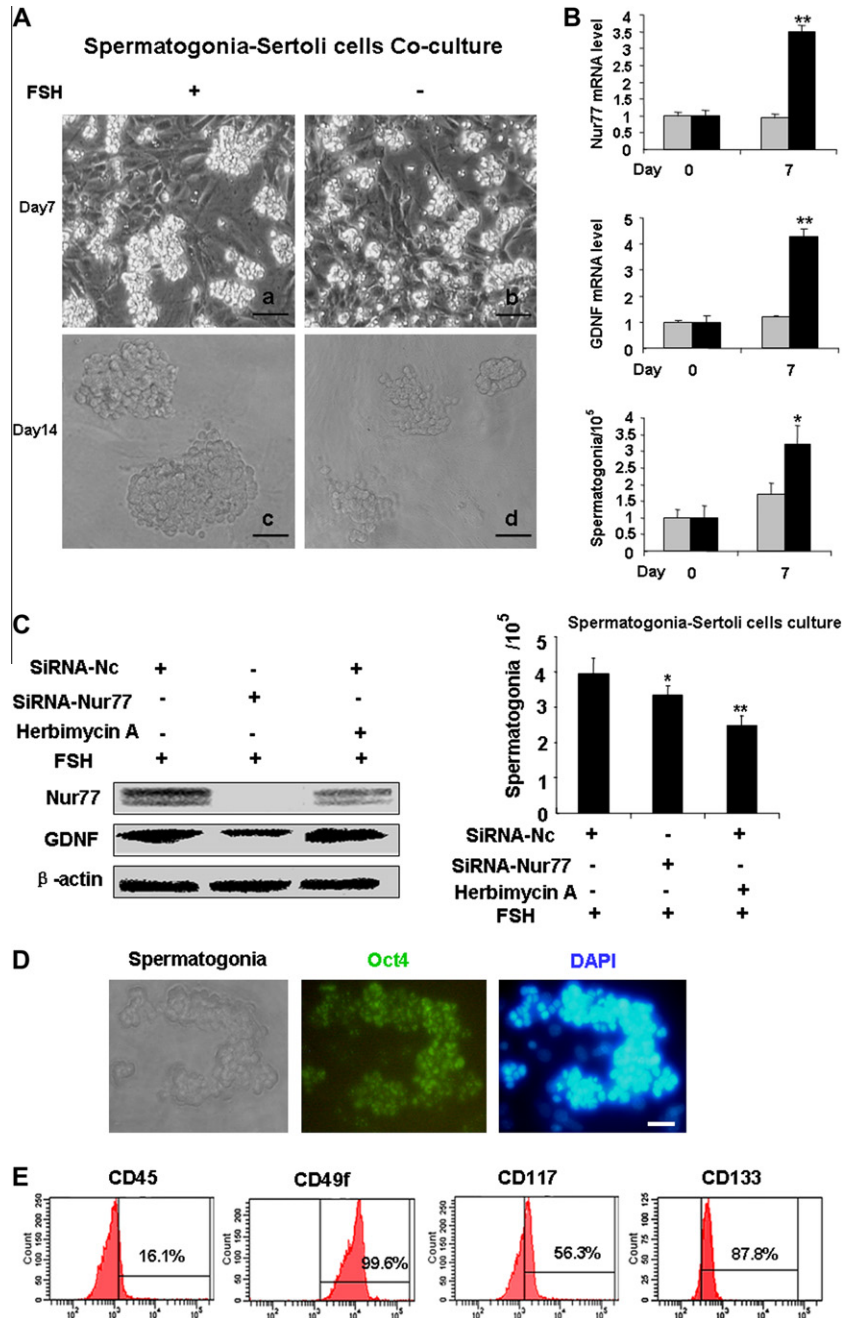
Fig. 2. FSH enhances the proliferation of mouse A spermatogonia in vitro. (A): Isolated primary mouse A spermatogonia co-cultured with Sertoli cells 7 d after treatment with 20 ng/mL rFSH (a), in the complete absence of rFSH for 7 d (b), 14 d after treatment with 20 ng/mL rFSH (c), in the complete absence of rFSH for 14 d (d). Scale bar: 100 μm. (B): The expression of Nur77 and GDNF in the spermatogonia-Sertoli co-culture cells was detected by qRT-PCR. And the number of A spermatogonia was determined on d 7 by randomly choosing three 35 mm plates to analyze with flow cytometry. A spermatogonia co-cultured with Sertoli cells with 20 ng/mL rFSH (black bars) or without FSH (gray bars). (C) GDNF expression decreased in the mouse primary Sertoli cells after Nur77 was knocked down with specific siRNA. The number of A spermatogonia co-cultured with Nur77 knock-down Sertoli cells with 20 ng/mL rFSH also decreased compared to the negative control. Herbimycin A was used to specifically inhibit GDNF-induced signaling pathways to decrease the proliferation of A spermatogonia co-cultured with Sertoli cells as positive control. (D): Immunostaining of undifferentiated A spermatogonia stimulated with rFSH. From left to right: visible light imaging, anti-Oct-4 staining (green), and DAPI staining (blue). Scale bar: 100 μm. (E): The gated population of A spermatogonia analyzed by flow cytometry with anti-CD45, anti-CD49f, anti-CD117 and anti-CD133 antibodies. $*p < 0.05$; $**p < 0.01$.

says were performed. Cell lysates were immunoprecipitated with an antibody specific for Nur77-FLAG, or protein A/G, and were PCR amplified using primers specific for the NBRE regions of the promoter. In Fig. 4A, the *GDNF* promoter is shown to be efficiently recovered from immunoprecipitates of Nur77, but not from immunoprecipates of LacZ (Fig. 4A). In combination, these data demonstrate that Nur77 is able to associate with chromatin-associated *GDNF* promoters in human Sertoli cells.

To determine whether NBREs are required for the binding of Nur77 to the *GDNF* promoter, three GDNF promoter oligonucleotides were prepared: wild type, mutate type and reverse type. ABCD assays were performed and included whole cell lysates extracted from intact human Sertoli cells that were incubated with each of the three oligonucleotides immobilized on streptavidin-agarose beads. Based on the results of these assays, Nur77 was
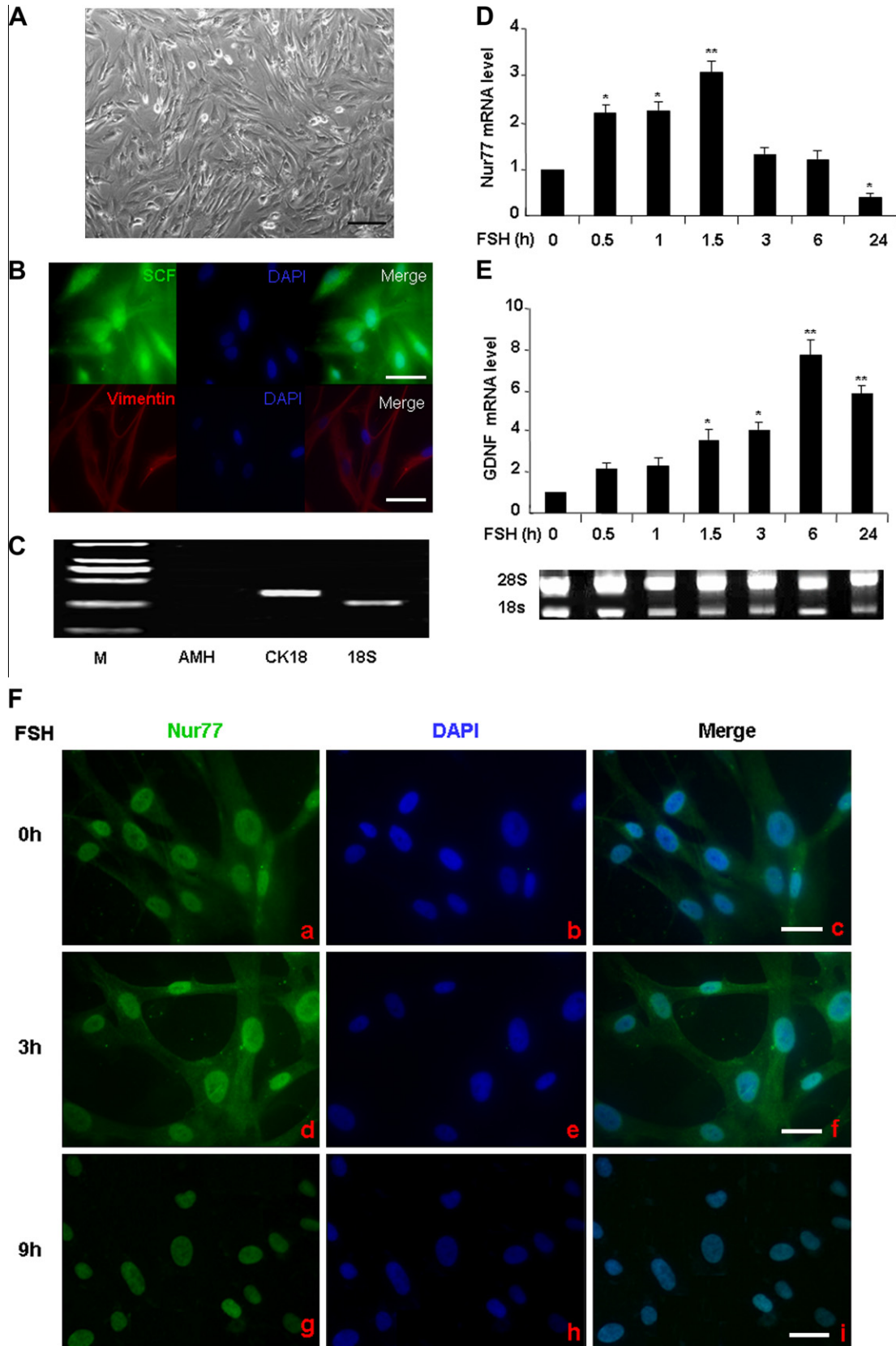
Fig. 3. FSH stimulation increases the expression of Nur77 and GDNF in human fetal Sertoli cells. (A): Cultured primary human fetal Sertoli cells. Scale bar: 50 μm. (B): Immunofluorescent staining of cultured primary human fetal Sertoli cells with anti-SCF or anti-vimentin antibodies. Scale bar: 25 μm. (C): RT-PCR assays to detect mRNA levels of AMH and CK18. (D, E): Levels of *Nur77* and *GDNF* mRNA were quantified by q-RT-PCR at various timepoints after treatment with rFSH. (F): Immunofluorescence analysis of Sertoli cells prior to (a–c), and up to 9 h after (d–i), stimulation with rFSH. Expression of Nur77 was compared with DAPI staining of cell nuclei. Scale bar: 25 μm. *$p < 0.05$; **$p < 0.01$.

found to strongly bind both the GDNF WT and GDNF REV oligonucleotides, but not the GDNF MU oligonucleotide (Fig. 4B).

Additional experiments to investigate the functional role of Nur77 binding to the *GDNF* promoter were performed, which included an analysis of *GDNF* promoter activity in primary Sertoli cells. When Nur77 was overexpressed, luciferase activity increased ~67% compared to control cells infected with Ad-LacZ. In contrast, knockdown of endogenous Nur77 by Ad-si-Nur77 was associated with approximately a 30% decrease in luciferase activity (Fig. 4C).

Additional characterization of Nur77 overexpression and Nur77 silencing in Sertoli cells were determined by qRT-PCR and ELISA, respectively. Overexpression of Nur77 was observed to markedly enhanced endogenous levels of *GDNF* mRNA by ~60% in Sertoli cells (Fig. 4D), while GDNF secretion increased ~0.7-fold following Ad-Nur77 infection (Fig. 4E). In the latter case, knockdown of endogenous Nur77 also decreased mRNA and protein levels of GDNF by ~25% (Fig. 4D and E). Overall, these data confirm that Nur77 binds to the GDNF promoter and enhances GDNF promoter activity to up-regulate GDNF expression.

4. Discussion

A significant advance in the study of germ stem cells has been the ability to isolate and culture SSCs that can be maintained as stable, multipotent cell lines [23]. The ability to cryopreserve and transplant SSCs further enhances the possibility that additional therapeutic clinical measures will be discovered [24]. However, at this point, the molecular mechanisms responsible for the proliferation of germ stem/progenitor cells need to be elucidated. Until now, this process has remained unclear due to the contributions made by both the hormone system and the interactions between Sertoli cells and germ cells within the seminiferous tubules.

FSH has been shown to play a crucial role in spermatogenesis. For example, FSH is able to activate the proliferation of Sertoli cells, influence the mitotic activity of spermatogonia, and enhance cellular differentiation [25]. Similar results have been reported in rats following FSH withdrawal, indicating that FSH, not testosterone, is an important hormone for restoring the initial phases of spermatogenesis, which includes A spermatogonia [26]. Although male FSH knockout mice retain their fertility, 46% reduction in spermatogonia is detected [27]. In combination, these results indirectly demonstrate that FSH affects the survival and proliferation of A spermatogonia. In the present study, FSH stimulation induced the expression of Nur77 and GDNF in the testis and Sertoli cells of pre-puberty mice. Furthermore, FSH increased the number of A spermatogonia present in co-culture with Sertoli cells, without affecting their phenotype. These observations would appear to confirm that FSH is the main regulatory hormone of A spermatogonia proliferation and self-renewal.

It has previously been demonstrated that GDNF is an important regulator of the proliferation and differentiation of mammalian SSCs, especially during testis development [4,6,28]. Although stimulation by FSH has previously been shown to induce the expression of GDNF in Sertoli cells or cell line TM4 [7,29], the genes directly
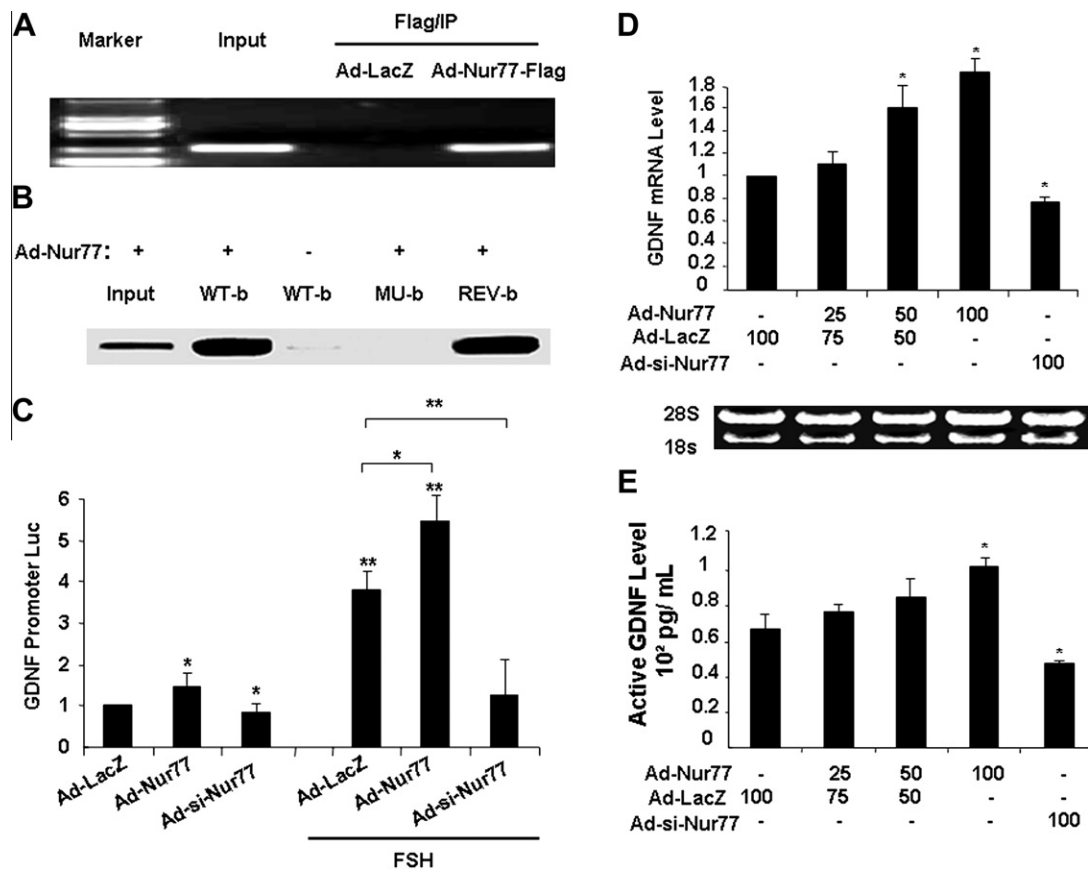
Fig. 4. Nur77 increases GDNF expression by directly binding the *GDNF* promoter in human fetal Sertoli cells. (A): ChIP-PCR amplification using primers against the human *GDNF* promoter region. (B): ABCD assays were performed using biotinylated, double-stranded GDNF WT (WT-b), GDNF MU (MU-b), and GDNF REV (REV-b) oligonucleotides and Sertoli cell extracts after infected with Ad-Nur77-FLAG. (C): The Sertoli cells transfected with GDNF promoter reporter constructs with different Nur77 level were incubated with or without rFSH for 48 h before cells lysates were assayed for luciferase activity. (D): Primary human fetal Sertoli cells infected with various combinations of Ad-Nur77, Ad-LacZ, and Ad-si-Nur77 were assayed for mRNA levels of *GDNF*. (E): Secretion of GDNF by primary fetal Sertoli cells infected with various combinations of Ad-Nur77, Ad-LacZ, and Ad-si-Nur77 was assayed by ELISA. $^*p < 0.05$; $^{**}p < 0.01$.

responsible for regulating the expression of GDNF have not been identified. However, in this study, it is demonstrated that Nur77 can directly bind a NBRE site in the GDNF promoter, and this binding is associated with an increase in the expression of GDNF in the Sertoli cells. Furthermore, when Nur77 was induced or knocked down in the co-culture of spermatogonia and Sertoli cells, the level of GDNF and the number of spermatogonia were also changed. These results indicate that Nur77 has an important role in regulating the expression of GDNF and spermatogonia proliferation. However, it is not the only factor involved. For example, when expression of Nur77 was targeted by siRNA, expression of GDNF was not completely inhibited. Furthermore, since other N4RA subfamily proteins, such as Nurr1, are expressed in Sertoli cells (data not shown). It is possible that these family members are able to rescue the function of Nur77 through compensatory mechanisms [22].

Nur77 and GDNF expression in the testis increase during the late fetal and early postnatal period [30]. During this stage, the proliferation of Sertoli cells and SSCs is very active and their mitotic divisions stop following the decrease of GDNF in vivo [30]. GDNF plays an important role in the proliferation of Sertoli cells and SSCs in the early postnatal period of rat testis development [28,31]. We found that FSH stimulation induced Nur77 expression in the fetal Sertoli cells, which affected the secretion levels of GDNF in the Sertoli cells to directly influence the proliferation of SSCs in vitro. Therefore, FSH/Nur77/GDNF pathway may regulate the proliferation of SSCs and Sertoli cells during the late fetal and early postnatal period in vivo.

Acknowledgments

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