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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.

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].

Abbreviations: FSH, follicle-stimulating hormone; rFSH, recombinant FSH; GDNF, glial cell line-derived neurotrophic factor; NGFI-B, nerve growth factor inducible gene B; SSCs, spermtaogonial stem cells; NBRE, NGFI-B (Nur77)-response element; ChIP, chromatin immunoprecipitation; ABCD, avidin-biotin conjugate DNA precipitation

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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 laminin-binding 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