Effect of nerve growth factor on the proliferation in newborn bovine testicular Sertoli cells

Qiaoge Niu, Maosheng Cao, Chenfeng Yuan, Yuwen Huang, Zijiao Zhao, Boqi Zhang, Xin Wang, Yameng Wei, Wenjing Fan, Xu Zhou and Chunjin Li

College of Animal Sciences, Jilin University, Changchun, Jilin, China

Correspondence should be addressed to X Zhou or C Li; Email: xzhou65@vip.sina.com or llcj158@163.com

Abstract

Nerve growth factor (NGF) has been proved to play important roles in male reproductive physiology, but the molecular mechanisms of NGF action remain unclear. In this study, the effects of NGF on the growth of newborn bovine testicular Sertoli (NBS) cells and the related signaling pathways were investigated. The NBS cells were treated *in vitro* with NGF (100 ng/mL) for 18 h. The expression levels of cell proliferation related genes, *INHBB*, and cytoplasmic specialization related gene were determined using real-time PCR and Western blot. The roles of PI3K/AKT and MAPK/ERK pathways in NGF-induced cell proliferation were investigated. It was found that NGF regulates proliferation and function of NBS cells via its receptor *NTRK1* by activating the PI3K/AKT and MAPK/ERK signaling pathways. The study will help to further understand the role of NGF in male reproduction and provide new therapeutic targets for reproductive dysfunctions in male animals.

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Introduction

In the testes, Sertoli cells act as spermatogenic mother cells, which function to nourish, protect, support, directly control the microenvironment of spermatogenesis, and guide the process of primordial germ cells in the seminiferous tubules toward sperm development (Griswold 1998). *INHBB* that is mainly secreted by Sertoli cells is used as a marker of testicular cell maturation, and it has a negative feedback regulation effect on follicle stimulating hormone (FSH) secretion (Petersen *et al.* 1999, Jain & Halder 2012). Clinically, serum *INHBB* expression is used as an endocrine marker for spermatogenesis and testicular function that can be correlated with male fertility. (Illingworth *et al.* 1996, Hipler *et al.* 2001, Pierik *et al.* 2003).

The formation of mature junctions in testicular Sertoli cells is a prerequisite for spermatogenesis, and adjacent Sertoli cells are joined into a complex that forms a typical adhesion junction and ectoplasmic specializations (ES). ES is an atypical type of adhesion junction in the testis that connects actin and transmembrane molecules together by vinculin (VCL), which is localized to the actin filament layer (Pfeiffer & Vogl 1991, Wong *et al.* 2008).

Long-term studies have shown that Sertoli cells in mammals after puberty are in a terminally differentiated state, without division or proliferation. However, studies have also isolated human primary Sertoli cells from adult

testes and found that primary Sertoli cells can be rapidly proliferated and have corresponding cellular functions under optimal conditions *in vitro* (Chui *et al.* 2011). In this pilot study, we isolated functional newborn bovine testicular Sertoli cells. *In vitro* proliferation of Sertoli cells provides an important reference for the study of male infertility and reproductive toxicology.

Nerve growth factor (NGF) is one of the first neurotrophic factors to be found. It is a protein that acts mainly by binding to its high-affinity receptor tyrosine kinase receptor A (*NTRK1*) and maintains neuronal differentiation and synaptic stability in the peripheral and central nervous systems (Zhang *et al.* 2000, Attiah *et al.* 2003). NGF is thought to promote cell proliferation and differentiation and regulate angiogenesis. In inflamed organs, NGF can help recruit inflammatory cells by increasing adhesion factors (Raychaudhuri *et al.* 2001, Cantarella *et al.* 2002). PI3K/AKT and MAPK/ERK signaling pathways are important molecular pathways in cells and both play important roles in cell proliferation. NGF can promote the proliferation and differentiation of rat myoblasts *in vitro* by activating the PI3K/AKT and MAPK/ERK signaling pathways (Rende *et al.* 2000).

In the testis, the widespread presence of NGF can be detected in the epididymal sperm, and studies have used immunohistochemistry to obtain positive staining for NGF in the seminal vesicle epididymis and testis of male Japanese macaques (Jin *et al.* 2006). Gene expression of *NGF* and *NTRK1* was detected in the reproductive organs

of male rabbits (i.e. the testes, prostate and seminal vesicles). The prostate may be the main source of this neurotrophin in the rabbit reproductive system. NGF is involved in testicular development and spermatogenesis in rabbits. In addition, large amounts of NGF in semen may induce ovulation (Maranesi *et al.* 2015). Studies have also shown that the content of NGF and its receptor, NTRK1, in semen determines the semen quality. NGF expression levels are highly positively correlated with the mitochondrial membrane potential of pure semen samples, and higher NGF expression levels indicate better semen quality (Parthipan *et al.* 2017). Reports have also directly indicated that NGF regulates sperm acrosome reactions and activates sperm motility, which is important in bovine sperm motility and apoptosis (Jin *et al.* 2010, Li *et al.* 2010). In addition to its expression in testicular Sertoli cells, NGF, as an important testicular growth factor, is expressed in various other testicular cells, such as Leydig cells, spermatogonia and spermatocytes (Li *et al.* 2005). However, the specific mechanism of NGF action on a single type of cells in the testis is not well understood.

The aim of this study was to investigate the role and mechanism of NGF in testicular Sertoli cells. In our preliminary tests, we successfully detected the expression of NGF and its receptor, NTRK1, using immunofluorescence. This study explored stimulation of cell proliferation and cell function by NGF, focusing on two molecular pathways, PI3K/AKT and MAPK/ERK. To further explore whether NGF affects Sertoli cells via the PI3K/AKT or MAPK/ERK pathway, we first determined whether NGF activates AKT or ERK phosphorylation. The results of this study will help further explain male testicular hypoplasia and spermatogenic disorders.

Materials and methods

Newborn bovine testicular Sertoli cell culture

The Sertoli cells used in this experiment were isolated by Shanghai Fu Life Industry Co., Ltd from newborn Holstein bovine testes using a differential adhesive method under sterile conditions. After being subcultured and amplified, vials of the cells were frozen in a liquid nitrogen cryofreezer for later use.

The cells were isolated as follows. Testicular tissue was obtained from newborn calves, rinsed twice with normal saline, and quickly rinsed three times with phosphate buffered saline (PBS). After removing the white film, the seminiferous tubules were cut into 1 mm³ sections and placed in 15 mL centrifuge tubes. Next, 5 mL of 1.0 mg/mL collagenase IV/DNase solution (Sigma) was added using the thermostatic shaker set at 34°C, shaking 110 times/min for 20 min. The tissues were then washed with Dulbecco's modified Eagle's medium (DMEM)/high-glucose (HyClone, South Logan, UT, USA), then 2.5 mg/mL trypsin was added and the tissue digested at 34°C for 20 min. This process continued with the shaker under the same

conditions. After digestion, the mixture was filtered through a 100 µm filter and washed with DMEM/high-glucose, followed by centrifugation at 250 g for 10 min. The cells were then resuspended in complete medium, collected and inoculated into a petri dish. The complete medium contained 10% inactivated fetal bovine serum (Gibco, Life Technologies) and 1% penicillin streptomycin (HyClone), the rest of the ingredients is DMEM/high-glucose medium. Cells were stored at 38.5°C with saturated humidity, 5% CO₂, and 95% air. After 1.5 h, the culture medium with cells suspended in the petri dish was collected and re-seeded into a new petri dish. The medium was changed after cultivating for more than 4 h to obtain relatively pure support cells (Wang *et al.* 2019a,b).

The NBS cells were cultured in complete medium, and when they reached 60% confluence, they were cultured for 4 h in fresh, prewarmed, serum-free high-glucose medium for starvation, then treated with NGF (Recombinant Human beta-Nerve Growth Factor, Novoprotein, Shanghai, China) at 100 ng/mL for 18 h. The mitogen-activated protein kinase (MAPK) activity inhibitor PD98059 (Beyotime Biotechnology, Shanghai, China; 10 µM), the phosphoinositide 3 kinase (PI3K) activity inhibitor LY294002 (Beyotime Biotechnology, Shanghai, China; 5 µM) and NTRK1 inhibitor K252α (Invitrogen, Life Technology; 100 ng/mL) were also used. These inhibitors were administered 30 min before NGF was added, and the medium was not changed throughout NGF treatment of the cells, leaving the inhibitors work throughout the period. After 18 h, cells were harvested for quantitative real-time PCR (qRT-PCR) and Western blotting (WB), with three replicates per treatment. All experiments were repeated at least three times.

Cell proliferation assays

Cell Counting Kit-8 (CCK-8; Beyotime Biotechnology) was used for the cell viability assay. NBS cells were seeded in 96-well plates and cultured in complete medium for 12 h, at which time the cells were completely adherent and had reached 30–40% confluence. The cells were then incubated in fresh, prewarmed, serum-free medium for 4 h to complete the starvation treatment, and the cells were treated with different concentrations of NGF for 12, 18, and 24 h. When the cell confluence was about 90%, 10 µL of CCK-8 reagent was added to each well and the plates were incubated for 2 h at 37°C in darkness. Cell OD (optical density) values were measured using a plate reader ELX 800 Universal Microplate Reader (BioTek) with a wavelength that was set at 450 nm. At this time, the cell viability was directly proportional to OD450. The experiment was repeated at least three times.

Giemsa staining experiment

The cells were seeded into 12-well plate and stained with Giemsa. Giemsa staining solution (AR-0751, Beijing Dingguo Changsheng Biotechnology Co. Ltd.) was used, and the Giemsa staining procedure was performed in accordance with the manufacturer's instructions. Images were taken under an inverted microscope (Olympus).

Immunofluorescence

The cells were washed twice with phosphate buffered saline (PBS, HyClone, South Logan, UT) for 5 min each at room temperature. The cells were then fixed with 4% paraformaldehyde for 15 min and washed three times with PBS for 5 min each. Triton X-100 (0.1%) was added for 10 min to permeabilize the cells, which were then washed three times with PBS for 5 min each. The cells were then blocked with 5% goat serum for 30 min. The primary antibody FSHR/NGF/ NTRK1 (1:200, diluted with primary antibody dilution, Bioworld, Nanjing, China) was added and incubated for 16 h at 4°C. The cells were then washed three times in PBS for 5 min each. The fluorescein (FITC)-labeled secondary antibody (1:500, diluted in PBS, Bioworld, Nanjing, China) was then added and the cells were incubated at room temperature for 1 h in darkness. The cells were then washed three times with PBS for 5 min each. PI counterstaining was then performed (1:1000, diluted in PBS) for 10 min, followed by washing three times in PBS for 5 min each. The same volume of PBS was added to each well, and the cell images were taken using an inverted fluorescence microscope (IX71; Olympus).

Transfection with small interfering RNA

siRNA for NGF and negative control siRNA (siNC) were purchased from Shanghai GeneBio Co., Ltd. (Shanghai, China). The NGF siRNA sequences were 5'-CCAUGUUGUUCUACACUCU-3' and 5'-AGAGUGUAGAACAACAUGG-3'. The siNC sequences were 5'-UUCUCCGAACGUGUCACGU-3' and 5'-ACGUGACACGUUCGGAGAA-3'. NBS cells were seeded in 12-well plates and cultured in complete medium to achieve 60% confluence. NGF siRNA was transfected with FuGENE® HD (Roche) at a concentration of 50 nM in accordance with the manufacturer's protocol. After 36 h, cells were harvested for continued experiments.

RNA preparation and qRT-PCR analysis

Total RNA was obtained using an RNA isolation system (Sigma-Aldrich), and then the previously mentioned extracted RNA was reverse transcribed using a PrimeScript RT kit with gDNA removal function (Takara Bio). For cDNA, the experimental procedure was performed in strict accordance with the manufacturer's instructions. The total qRT-PCR reaction system

was 20 µL, containing 10 µL SYBR (Takara Bio), 0.5 µM for the forward primer, 0.5 µM for the reverse primer, 5 ng/µL for the cDNA, and 20 µL supplemented with sterile ultrapure water. The reaction conditions were as follows: 95°C for 2 min, 95°C for 15 s, and 60°C for 30 s for 40 cycles. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of genes. The primers were synthesized from KuMei, Changchun, China (Table 1).

Western blotting analysis

Total protein was extracted using WBIP lysis buffer (Cell lysis buffer for Western blot and immunol precipitation) (Beyotime Biotechnology) containing 1 mM of PMSF. The total protein concentration of the samples was measured using a BCA (Bicinchoninic acid) Protein Assay Kit (Beyotime Biotechnology), and the concentration of each protein sample was adjusted to be uniform, based on the measured total protein concentration. The sample total protein was separated by 12% SDS-PAGE, then transferred to a polyvinylidene difluoride (PVDF, Roche; 0.45 µm) membrane using a semi-wet transfer system, and the PVDF membrane was blocked with 5% BSA for 1 h. BSA (Albumin Bovine V, Guangzhou saiguo biotech Co., Ltd, Guangzhou, China) was diluted with TBST (Monad Biotech Co., Ltd, Shanghai, China). The monoclonal antibodies CCNE2 (1:1000, Bioworld), PCNA (1:1000, Bioworld), NGF (1:1000, Bioworld), NTRK1 (1:1000, Bioworld), INHBB (1:1000, Bioworld), CCND1 (1:1000), VCL (1:1000, Bioworld), t- ERK (1:1000, Bioworld), p-ERK (1:1000, Bioworld), AKT (1:1000, Bioworld), P-AKT (1:1000, Bioworld), and GAPDH (1:8000, Bioworld) were diluted with the quick-block Western primary antibody diluent (Beyotime Biotechnology). The membrane was incubated with the diluted specific antibody for 16 h at 4°C. Then, the PVDF membrane was washed four times with TBST for 9 min each. The PVDF membrane was incubated with HRP-labeled secondary antibody (1:10,000, Bioworld) for 1 h at room temperature. The secondary antibody was diluted with the quick-block Western secondary antibody diluent (Beyotime Biotechnology). Subsequently, the membrane was washed four times with TBST for 9 min each. The BeyoECL Plus kit (Beyotime Biotechnology) was used for protein development. Finally, the relative protein levels were analyzed by grayscale scanning using a Tanon gel imaging system (Tanon, Shanghai, China).

Table 1 Primer sequences used in qRT-PCR.

Names	Forward sequences (5'→3')	Reverse sequences (5'→3')
<i>GAPDH</i>	ACGGCAGCTCAAGGCAGAG	GTGATGGCGTGGACAGTGGT
<i>GDNF</i>	GCAGCCGAAACAATGTACGA	AAGGCGATGGGTCTGCAA
<i>SCF</i>	ATTGGTGGCAAATCTTCCCA	TGCACTCCACAAGGTCATCAA
<i>CCNE2</i>	GCTGCTGCTGCCTTATGC	TCTGTATGTTGTGTCTGTCTTCC
<i>PCNA</i>	GTAATGTTGATAAAGAGGAGGAAG	GTAGGAGACAGTGGAGTGG
<i>CCND1</i>	AACGGCTTCTCTCTATC	TCCTCTCTCTCTCTTCC
<i>INHBB</i>	CTCATCCCTCTCTTCTCACTC	ATGTTCTGCCCATCGTCATAG
<i>VCL</i>	GCCTCCATAGACTCAAACCTG	CACCAACTTTTCCAGCTTCATC
<i>NGF</i>	TCAACAGGACTCACAGGAGCAA	ACCTCTCCCAGCACCATCAC

CCND1, cyclin D1; *CCNE2*, cyclin E2; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *GDNF*, glial cell line-derived neurotrophic factor; *INHBB*, Inhibin subunit beta B; *NGF*, nerve growth factor; *PCNA*, proliferating cell nuclear antigen; *SCF*, stem cell factor; *VCL*, vinculin.

Statistical analysis

All experiments were repeated at least three times. All data were analyzed using a one-way ANOVA and independent-sample *t*-tests. IBM SPSS Statistics 22.0 software (version X, IBM) was used for the analysis, and the difference between the control and treatment groups was tested using Dunnett's multiple comparison. The data from the previously mentioned analysis were compiled using GraphPad Prism 6.0 software. Data are expressed as the mean $\pm$ s.d. of three independent processes. Additionally, '*' means $P < 0.05$, which denotes a significant difference; '**' denotes $P < 0.01$, and '***' means $P < 0.001$, which indicates that the difference is extremely significant.

Results

NGF and TrkA expression in NBS cells

Giemsa staining revealed the presence of satellite chromocenters in the NBS cells nuclei (Fig. 1A). Additionally, NBS cells were verified by two genes (GDNF, glial cell line-derived neurotrophic factor and SCF, stem cell factor) biomarkers used for Sertoli cells (Yao *et al.* 2016) (Fig. 1B).

FSHR expression was demonstrated in NBS cells using immunofluorescence (Fig. 1C). Counting the FSHR-expressing cells in the sample (approximately 100 cells per area counted) showed that $96.0 \pm 1.0\%$ of the

Figure 1 Identification of NBS cells and NGF and NTRK1 expression in cells. (A) Giemsa staining showed the presence of chromocenters around the nucleolus. (B) GDNF and SCF molecular markers were detected in Sertoli cells. (C) The expression of FSHR, NGF and NTRK1 in NBS cells was identified by Immunofluorescence. Scale = 50 μ m. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; SCF, stem cell factor; FSHR, follicle-stimulating hormone receptor; NGF, nerve growth factor; NTRK1, tyrosine kinase receptor A; PI, Propidium iodide; FITC, Fluorescein isothiocyanate isomer.

Table 2 Data analysis of positive cells.

	Total cells	Positive cells	Percentage of positive cells (%)
Sample 1	83	80	96.4
Sample 2	102	99	97.1
Sample 3	93	89	95.7
Sample 4	115	109	94.8
Mean (%)			96.0
S.D.			1.0

cells were FSHR-expressing Sertoli cells (Table 2). All the previously mentioned experiments confirmed that the cells we used are bovine testicular Sertoli cells that can be used for the following experiments. Further, the expression of NGF and NTRK1 in NBS cells was shown using immunofluorescence (Fig. 1C).

Effect of NGF on NBS cells proliferation

NGF affected cell viability in a concentration-dependent manner. The largest increase in cell proliferation was observed with treatment with NGF at 100 ng/mL for 18 h (Fig. 2A). The cell proliferation-related genes *CCNE2*, *PCNA*, and *CCND1* were up-regulated after NGF treatment, and *INHBB*, which was secreted by the cells, and *VCL* were also up-regulated (Fig. 2B and C).

The effect of NGF knockdown on NBS cells proliferation

The results from qRT-PCR and WB indicated that the level of endogenous NGF expression in the cells was effectively reduced with siRNA of NGF (siNGF) (Fig. 3A and B). The cell viability was detected by CCK-8, showing that the cell viability was significantly reduced

after adding siNGF (Fig. 3C). Changes in expression of genes for *CCNE2*, *PCNA*, and *CCND1*, *INHBB*, and *VCL* after NGF reduction were detected in the levels of mRNA and protein using qRT-PCR and WB, respectively, and it was found that expression levels of all these genes decreased significantly (Fig. 3D and E).

Effect of NGF on cell proliferation via NTRK1

The results showed that the cell viability was significantly reduced after K252 α treatment, but the viability of the cells in the group that NGF was added 30 min after K252 α addition (NGF-K252 α group) was increased compared with the K252 α group (Fig. 4A). Additionally, the expression levels of *CCNE2*, *PCNA*, and *CCND1*, *INHBB* and *VCL* were significantly decreased after the inhibitor treatment. But in the NGF-K252 α group, the gene expression levels were relatively increased (Fig. 4B and C).

PI3K/AKT signaling pathway participates in the NGF induced cell proliferation, *INHBB* secretion and ectoplasmic specialization of NBS cells

NBS cells were treated with NGF for different time periods, and the proportion of phosphorylated cells in the treated cells was assessed (Fig. 5A). Results showed that NGF significantly induced AKT phosphorylation. Then we selected the PI3K/AKT signaling pathway inhibitor LY294002 to treat NBS cells. NBS cells were treated with LY294002, NGF, or both (Fig. 5B), and the viability of cells treated with LY294002 alone was significantly lower compared with the negative control group. The cell viability of the combined treatment group was significantly lower compared with the NGF treatment

Figure 2 Effect of exogenous NGF on NBS cells proliferation. (A) The effect of NGF on the viability of NBS cells is concentration dependent. (B) The mRNA levels of *CCNE2*, *PCNA*, *CCND1*, *INHBB*, *VCL* were significantly increased after NGF treatment. (C) NGF treatment significantly increased the protein levels of *CCNE2*, *PCNA*, *CCND1*, *INHBB*, *VCL* in NBS cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3 The effect of si-NGF on NBS cells proliferation. (A) The expression of NGF in cells is significantly reduced at the mRNA level. (B) Western blot analysis of siNGF transfected NBS cells significantly reduced the amount of NGF protein in cells. (C) Knockdown NGF in NBS cells reduced the cell viability. (D) The mRNA levels of *CCNE2*, *PCNA*, *CCND1*, *INHBB*, and *VCL* were significantly decreased at mRNA level after siNGF transfection of NBS cells. (E) Western blot showed that the expression of *CCNE2*, *PCNA*, *CCND1*, *INHBB* and *VCL* protein decreased significantly after transfected siNGF in NBS cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

alone. This indicated that LY194002 successfully inhibited the intracellular PI3K/AKT signaling pathway.

The expression changes of cell proliferation-related genes *CCNE2*, *PCNA*, and *CCND1*, *INHBB*, and the ES-related gene *VCL* were investigated with qRT-PCR and WB (Fig. 5C, D and E). The expression levels of *CCNE2*, *CCND1*, *INHBB*, and *VCL* were significantly decreased after the PI3K/AKT signaling pathway was inhibited. However, the *PCNA* expression was not changed (Fig. 5D).

The MAPK/ERK signaling pathway is involved in the effect of NGF on cell proliferation

To determine whether MAPK/ERK is involved in the regulation of proliferation and cell function by NGF in NBS cells, NBS cells were treated with NGF for different times and the changes in p-ERK and t-ERK expression levels were detected (Fig. 6A). NGF activated MAPK/ERK phosphorylation and increased p-ERK expression. Figure 6 shows that the level of cell phosphorylation

Figure 4 Addition of NTRK1 inhibitor K252α to detect effect of NGF on NBS cells proliferation. (A) Treatment of cells with the NTRK1 inhibitor K252α significantly reduced cell viability. (B) The mRNA levels of *CCNE2*, *PCNA*, and *CCND1*, *INHBB* and *VCL* were significantly decreased after K252α treatment. (C) Western blot showed that K252α significantly reduced the expression of *CCNE2*, *PCNA*, *CCND1*, *INHBB*, and *VCL*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5 PI3K/AKT is activated by NGF and involved in the regulation of NBS cells proliferation induced by NGF. (A) NGF activated AKT phosphorylation at a time-dependent manner. (B) PI3K/AKT signaling pathway inhibitor LY294002 attenuated the cell viability induced by NGF. (C) LY294002 decreased the mRNA levels of CCNE2, CCND1, INHBB, and VCL regulated by NGF. (D) The expression of PCNA at the mRNA level was not reduced by the addition of LY294002. (E) Western blot analysis showed that the blockade of PI3K/AKT signaling pathway significantly reduced the expression of CCNE1, CCND1, INHBB, and VCL regulated by NGF. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

was significantly increased after 15 min or 30 min of NGF treatment. Then PD98059 (a MAPK inhibitor) was selected to treat the cells and cell viability was significantly reduced (Fig. 6B). Using NGF alone or in combination with PD98059, the expressions of *CCNE2*, *PCNA*, *CCND1* and *INHBB* showed a significant decrease resulting from the addition of the inhibitor, but the expression of *VCL* did not change significantly after signal pathway inhibition (Fig. 6C, D and E).

Discussion

NGF, the first discovered member of the neurotrophins family, is expressed in multiple systems other than the nervous system. In previous studies, immunohistochemistry results showed the presence of NGF and its high-affinity receptor NTRK1 in various cells in the testis during the breeding and non-breeding

seasons (Jin *et al.* 2006, Wang *et al.* 2011, Zhang *et al.* 2015). The quantitative results showed that the NGF in the human testis was 5.4 ng/g and that NGF affected the morphology and function of the seminiferous tubules that were cultured *in vitro* (Seidl & Holstein 1990). Sertoli cells secrete NGF as a growth factor which maintains the vitality of Sertoli cells and promotes the normal development of the perinatal male fetal testes (Seidl *et al.* 1996, Skinner 2005). *In vitro* studies have shown that exogenous NGF can increase cell viability and sperm motility. Thus, NGF may participate in regulating sperm function by paracrine way (Naraballoh *et al.* 2010, Jiang *et al.* 2018).

NGF promotes cell proliferation in neuronal cells, such as Müller cells and oligodendrocytes, and in many other types of cells, such as vascular endothelial cells, myoblasts, and keratinocytes (Althaus *et al.* 1992, Wang *et al.* 2016). Moreover, most published studies report that NGF promotes cell proliferation through

Figure 6 MAPK/ERK signaling pathway is activated by NGF and involved in the NGF-induced NBS cells proliferation. (A) Western blot results showed that NGF could activate ERK phosphorylation in NBS cells. (B) PD98059 (a MAPK inhibitor) attenuated the NGF-induced cell viability. (C) PD98059 decreased the mRNA level of *CCNE2*, *PCNA*, *CCND1*, and *INHBB* induced by NGF. (D) qPCR results showed that vinculin expression was not decreased after blocking of ERK signaling pathway in NBS cells. (E) Western blot showed that *CCNE2*, *PCNA*, *CCND1*, and *INHBB* were significantly decreased in NBS cells after PD98059 treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

its high affinity receptor NTRK1 (Rende *et al.* 2000, Raychaudhuri *et al.* 2001, Paus *et al.* 1994). While investigating the NGF mechanism of action on Sertoli cells, we screened the proliferation-related genes *CCNE2*, *CCND1*, and *PCNA*. *CCNE2* (cyclin E2) and *CCND1* (cyclin D1) are cyclins that regulate the transition from G1 to S phase in the mitotic cell cycle and promote cell proliferation (Chen *et al.* 2019). *PCNA* (proliferating cell nuclear antigen) is a hallmark of cell proliferation and it is closely related to cellular DNA synthesis (Dietrich 1993, Mcneal *et al.* 1995). Both mRNA and protein levels of genes associated with cell proliferation were detected after NBS were treated with exogenous NGF or transfected with siRNA of NGF (siNGF). It was found that NGF promoted expression of the proliferation genes, *CCNE2*, *CCND1*, and *PCNA*. To demonstrate whether the effect of NGF on NBS cells occurs through its receptor NTRK1, the NTRK1 inhibitor K252 α was used

to treat the cells. The results showed that NGF promoted NBS cells proliferation through the NTRK1 receptor.

Sertoli cells are thought to contain unique satellite chromocenters in their nuclei (Raychoudhury & Kubinski 2003, França *et al.* 2016). The mouse satellite DNA sequence was located in the centromeric chromosomal heterochromatin. In the adult Sertoli cells of most mouse strains, all centromere heterochromatin is concentrated in two chromocenters on the sides of the nucleolus. Chromocenters are the hallmark structures of the interphase nuclei of constitutive heterochromatin (Pardue & Gall 1970, Jean *et al.* 1983, Kuznetsova *et al.* 2016). Evidence also suggests that satellite DNA is present in bovine cells (Streeck and R. 1981, Pages & Roizes 1982). In this experiment, satellite chromocenters were successfully detected in NBS cells.

In males, *INHBB* is mainly derived from testicular Sertoli cells, which can negatively regulate pituitary

Figure 7 Flowchart of overall description of the effect of NGF on NBS cells proliferation. NGF activates two molecular signaling pathways, PI3K/AKT and MAPK/ERK, through its high-affinity receptor NTRK1. Activation of the AKT signaling pathway affects the expression of CCNE2, CCND1, INHBB and VCL and promotes the cell proliferation. Meanwhile, activation of MAPK/ERK promotes the expression of CCNE2, PCNA, CCND1 and INHBB and increased the cell proliferation and inhibin secretion.

FSH release and coordinate sexual development. When the function of Sertoli cells is impaired, there will be an increase in FSH and a decrease in inhibin. Therefore, serum INHBB levels have now been used as an indicator of normal testicular function in males (Mather *et al.* 1992, Klingmuller & Haidl 1997). Studies have shown that a variety of growth factors in testis are involved in regulating the proliferation and functions of Sertoli cells. It has been proven that NGF can nourish Sertoli cells and increase cell viability (Chen *et al.* 1997). However, the interaction between NGF and INHBB remains to be elucidated. Based on previous studies, NGF can stimulate FSHR expression in follicles in female ovaries (Julio-Pieper *et al.* 2006), and it is known that follicular growth is controlled by FSH that is secreted by the pituitary. FSH stimulates granulosa cells in the follicle to produce estradiol and inhibin A and B. Subsequently, the accumulation of estradiol and inhibin A and B in the follicle increased. Negative feedback also regulates the production of pituitary FSH (Hale *et al.* 2014). Therefore, we speculate that there may be a regulatory relationship between NGF and inhibin A and B in females. In this experiment, we explored the effect of NGF on INHBB in Sertoli cells and found that NGF can positively regulate INHBB.

In the testes, there are many forms of adhesion junctions in Sertoli cells. ES is an atypical cell junction, which is observed by VCL and actin immunohistochemical colocalization and can link Sertoli cells to Sertoli cells or Sertoli cells to spermatids (Pfeiffer & Vogl 1991). VCL is a common cytoskeletal protein that is expressed in a variety of cells. In previous studies, VCL was found to be

involved in neuronal synapses and elongation in PC12 cells and it can be phosphorylated and recombined in response to NGF regulation (Halegoua 1987, Varnum-Finney & Reichardt 1994). The data from the present study showed that NGF promotes the expression of VCL gene at the mRNA and protein levels via the receptor NTRK1 and that this process requires the involvement of the MAPK/AKT signaling pathway.

The PI3K/AKT pathway is an important molecular pathway in cells, and it is involved in the regulation of cell proliferation and apoptosis. Studies have shown that the PI3K/AKT pathway is involved in the proliferation of adult hippocampal neural progenitor cells (Peltier *et al.* 2007). Our results indicate that the AKT signaling pathway is involved in the regulation of CCNE2, CCND1, INHBB, and VCL by NGF, while the regulation of PCNA by NGF does not depend on the AKT pathway (Fig. 7). When a cell develops a proliferative phenotype, another pathway in the cell that is closely related to proliferation also comes to mind: the MAPK/ERK pathway. When ERK is activated, many factors in the cell become phosphorylated, induce early gene production, and promote cell proliferation (Lewis *et al.* 1998). In NBS cells, NGF-induced expression of CCNE2, PCNA, CCND1, and INHBB was regulated by ERK pathway (Fig. 7). Studies have reported that NGF can affect cell proliferation by activating AKT and ERK pathways in different cell types. NGF promotes fibroblast proliferation to accelerate skin wound healing (Chen *et al.* 2014). NGF accelerates cell cycle progression in human corneal epithelial cells by regulating D-type cyclins and promotes corneal wound healing and regulates the proliferation of retinal Müller cells (Hong *et al.* 2012, Wang *et al.* 2016).

In conclusion, the results showed that NGF activates PI3K/ATK and MAPK/ERK signaling pathways via its receptor NTRK1, promotes the expression of cell proliferation related genes *CCNE2*, *PCNA*, *CCND1* and cell function related genes *INHBB* and *VCL*, and promotes the proliferation of NBS cells (Fig. 7). This study will help to understand the role of NGF in male reproduction and provide a potential therapeutic target for diseases such as testicular hypoplasia and spermatogenesis disorders caused by abnormal testicular Sertoli cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability statement

There is no data source for data used in this article and the data involved are original and available on reasonable request.

Author contribution statement

The study was designed by Chunjin Li, Xu Zhou and Qiaoge Niu and was performed by Qiaoge Niu, Maosheng Cao, Chenfeng Yuan, Yuwen Huang, Zijiao Zhao, Boqi Zhang, Xin Wang, Yameng Wei and Wenjing Fan. All authors read and approved the final version of the manuscript.

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