Activation of the GFRa1/NCAM pathway stimulates Sertoli cell proliferation in vitro

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ABSTRACT. The proliferation and final density of Sertoli cells in the testis are regulated by hormones and local growth factors. Glial cell line-derived neurotrophic factor (GDNF), a distantly related member of the transforming growth factor-β (TGF-â) superfamily, and its receptor components: GFRα1, Ret and neural cell adhesion molecule (NCAM) have been recently reported to be expressed in the testis and to be involved in the proliferation regulation of immature Sertoli cells. However, the number of the reports is very low, and the signalling pathways have not been well characterized. In the present study, we used purified Sertoli cell cultures from 4- to 5-day-old mouse testis to detect the expression of GDNF, to examine the effect of GDNF on Sertoli cell proliferation and possible signalling pathways mediating such effect. GDNF expression in mouse Sertoli cells was detected at both RNA and protein levels by RT-PCR and immunocytochemistry methods. The expression was up-regulated by FSH. GDNF stimulated the proliferation of Sertoli cells and synergized with FSH to promote the proliferation to a higher level. GFRα1/Ret and GFRα1/NCAM are two receptor complexes for GDNF. As GFRα1 and NCAM not Ret were expressed in cultured Sertoli cells, we proposed that the stimulatory effect of GDNF on Sertoli cell proliferation was through pathways initiating from the GFRα1/NCAM receptor. This proposition was supported by the fact that the stimulatory effect of GDNF was significantly reduced by anti-NCAM antibody.

KEY WORDS : Sertoli cells; proliferation; glial cell line-derived neurotrophic factor; neural cell adhesion molecule

INTRODUCTION

GDNF signals through two independent pathways of GFRα1/Ret and GFRα1/NCAM to support several types of neurons in central and peripheral nervous systems (ARENAS et al., 1995; LIN et al., 1993; PARATCHA et al., 2003; ROUSSA & KRIEGLSTEIN, 2004; SARIOLA & SAARMA, 2003), and plays different roles in other mammalian tissue development such as the development of kidney (FUKUDA et al., 2003; SHAKYA et al., 2005), and the self-renewal and differentiation of spermatogonial stem cells (SSCs) in the testis (KANATSU-SHINOHARA et al., 2005; KUBOTA et al., 2004; OGAWA et al., 2004; RYU et al., 2005). Previous studies showed that GDNF was secreted by Leydig cells, Sertoli cells, some spermatocytes, round spermatids and smooth muscle cells in both human and mouse testis, and that GFRα1 was expressed in Sertoli and Leydig cells (DAVIDOFF et al., 2001; GOLDEN et al., 1999; MASURE et al., 1998). It has been reported that NCAM was expressed in fetal or immature Sertoli cells (LASLETT et al., 2000), and downregulated in the rat during maturation of Sertoli cells (ORTH et al., 2000; ORTH & JESTER, 1995). Moreover, it has been found that GDNF exerted a proliferation-promoting effect on Sertoli cells during mouse embryonic development and in the early postnatal period of rat testis (HU et al., 1999; WU et al., 2005). However, the mechanism and roles of GDNF in immature mouse Sertoli cells were not clearly understood. The aims of the present study were just to clarify the roles of GDNF on the proliferation of immature mouse Sertoli cells by using serum-free medium and purified Sertoli cell cultures, and to examine which signalling pathway mediated such effects.

MATERIALS AND METHODS

Reagents and experimental animals

Recombinant rat GDNF was obtained from R&D Systems. Ovine FSH, testosterone and β-estradiol were purchased from Sigma-Aldrich. One mouse line of ICR was used and obtained from Beijing Weitong River Laboratory Animal Inc., China. Animals were housed under 16h light, 8h dark schedule with food and water *ad libitum*, and treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the protocols were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

Preparation and culture of Sertoli cells

Sertoli cells of 4~5 days old mice were prepared by following the two-step enzymatic digestion protocol (OGAWA et al., 2004) with some modifications. Briefly, decapsulated testis tissue was treated with 10 volumes of Digestion solution I (2mg/mL collagenase type IV and 200mg/mL DNAse (Sigma-Aldrich) in Ca^{2+}- and Mg^{2+}-free PBS) at room temperature for 3 to 5 minutes with gentle agitation followed by 3 washes in 10 volumes of PBS. Collected specimens were then treated with 5 volumes of Digestion solution II (2mg/mL collagenase type

IV, 200mg/mL DNAse and 2mg/mL hyaluronidase (Calbiochem) in serum-free Dulbecco's Modified Eagle Medium (DMEM) at room temperature for 2 to 5 minutes with vigorous agitation until tubular clumps were invisible, followed by 3 washes in PBS. The dissociated cell suspension was filtered through a nylon mesh with 60mm pore size. Cells were washed twice by centrifugation at 200g for 5 minutes in DMEM medium containing 10% FBS. The pellet was resuspended in DMEM/F12 (Sigma-Aldrich) culture medium supplemented with 10% FBS, 2mM L-glutamine (Sigma-Aldrich), 100unit/mL penicillin and 100mg/mL streptomycin (Invitrogen), plated on 0.2% (w/v) gelatin (Sigma-Aldrich) -coated tissue culture flasks with a density of $2x10^5$cells/cm^2. Cells were cultured at 37°C in an atmosphere of 5% carbon dioxide in air for 1 hour. After gentle agitation, floating cells were removed as the medium was changed, and the cells attaching to the bottom were again incubated overnight. The cells were detached with trypsin-EDTA solution (0.25% (w/v) trypsin-0.04% (w/v) EDTA in Ca^{2+}- and Mg^{2+}-free PBS) (Sigma-Aldrich). After counting, the cells were seeded out again on 13mm round plastic cover slips pre-coated with 0.2% (w/v) gelatin in 24-well plates with 95,000 cells per well in a volume of 1mL medium. Following incubation at 37°C for another 24 hours in serum-free medium, the cells were washed and incubated with test material for 48 hours. In some experiments such as immunocytochemistry and RT-PCR analysis of gene expression, Sertoli cells of a higher concentration were used (125,000 cells per well) in order to get confluent cells in culture.

Cell viability assessment by MTT supravital staining

Cell viability was assessed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) calorimetric assay. The principle of the assay is that MTT, a nontoxic pale yellow substrate, is taken up by living cells but not dead cells to yield a dark blue formazan product that can be quantified spectrophotometrically at 570nm absorbance. The absorbance is directly proportional to the number of viable cells. The assay was performed as reported by Mosmann (Mosmann, 1983). Briefly, MTT (Sigma-Aldrich) was added to cultures in 24-well plates with an amount of 0.2mg/well 44 hours after the test reagents were added. Four hours later, cells in each well were lysed with 360mL 10% SDS in 0.01M HCl and incubated at 37°C for 4 hours. The absorbance at 570nm was measured in a microtiter plate reader. Triplicate cultures were used and each experiment was repeated 3 times.

Cell proliferation monitor by BrdU labelling

Cells cultured on cover slips were pulsed with 5-bromo-2'-deoxyuridine (BrdU) (BD Biosciences) at a final concentration of 10μM in culture medium for 4 hours. Cells were then fixed in ice-cold acetone–ethanol for 10 minutes at -20°C. Cell staining was performed by using BrdU in-situ detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, fixed cells on slides were washed 2 times in PBS for 5 minutes each time, and permeabilized by incubation with dilution buffer for 30 minutes. After two washes, slides were incubated with 0.3% H_2O_2 in PBS for 10 minutes to block endogenous peroxidase activity, followed by washes. The slides were placed in a jar containing the working solution of BD Retrievagen A and heated to 89°C for 10 minutes, and then allowed to slowly cool down to room temperature. Following 3 washes, the slides were incubated for 1 hour at room temperature with 100mL/slide of biotinylated anti-BrdU antibody diluted in the dilution buffer (1:10). After another wash, the slides were incubated with 100mL/slide of Streptavidin-HRP for 30 minutes at room temperature followed by 3 washes in PBS. Colour of BrdU-positive cells was developed by diaminobenzidine (DAB), and counterstaining was performed with hematoxylin. BrdU-positive cells were counted within at least three randomly chosen areas on each cover slip, with about 200 total cells in each area, and the experiment was repeated 3 times.

Immunocytochemistry

The primary antibodies used included rabbit anti-human GDNF polyclonal antibody, rabbit anti-human GFRα1 polyclonal antibody (Santa Cruz biotechnology), mouse anti-human CD56 (NCAM) monoclonal antibody (BD Biosciences), mouse anti-human Ret polyclonal antibody (R & D systems), goat anti-human vimentin polyclonal antibody (chemicon) and mouse anti-á smooth muscle actin monoclonal antibody (Sigma-Aldrich). The secondary antibodies included FITC-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, FITC-conjugated mouse anti-goat IgG (Santa Cruz biotechnology), TRITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich).

For immunofluorescent staining, the cultured cells on slides were stained according to the standard procedure. Briefly, the cells were washed 2 times in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Following two washes in PBS, cells were blocked with PBS containing 1% BSA, 0.1% Triton X-100 and 2% normal goat serum at room temperature for 45 minutes. The samples were then incubated overnight at 4°C with primary antibody diluted as described by manufacturers. The slides were washed 3 times with PBS containing 1% BSA, incubated with diluted secondary antibody for 1 hour at room temperature in the dark followed by another 3 washes. Nuclei of the cells were stained with Hoechst 33258 or Propidium iodide (Sigma-Aldrich). Colour of the slides was visualized and captured under Leica confocal microscope. For negative controls, the cells were incubated with non-immune rabbit serum.

RT-PCR analysis of gene expression

Total RNA was extracted from 8×10^6Sertoli cells by using Trizol reagent (Invitrogen) according to the manufacturer's instructions and was reverse transcribed into cDNA with SuperScript™ II reverse transcriptase (Invitrogen) and oligo (dT) primer. Then the cDNAs were amplified by PCR with Recombinant Taq DNA Polymerase (Takara) according to the manufacture's instruction. For semi-quantitative RT-PCR, equal amounts of RNA extracts

were used to generate first-strand cDNAs. Primer pairs specific for mouse GDNF, GFRα1, Ret, sulfated glycoprotein 2 (SGP2, a Sertoli cell marder) (COLLARD & GRISWOLD, 1987) and luteinizing hormone receptor (LH-R, a Leydig cell marker) cDNAs are listed in Table 1. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as normalization control. To perform PCR amplification, reaction mixtures were first denaturalized at 94°C for 3 minutes, 30 cycles with the following conditions were then carried out: 1 minute of denaturalization at 94°C, 40 seconds of annealing at 58°C (65°C for Ret), 40 seconds of extension at 72°C. Subsequently, the reaction was incubated at 72°C for 7 minutes. The PCR products were verified by agarose gel electrophoresis.

TABLE 1

Primer sequences and PCR product size

Genes	Forward primer	Reverse primer	Product size
GDNF	5'-TCACTGACTTGGGTTTGGGCTAT-3'	5'-TCAGACGGCTGTTCTCACTCCTA-3'	477 bp
GFRα1	5'-ACTCCTGGATTTGCTGATGTCGG-3'	5'-CGCTGCGGCACTCATCCTT-3'	193 bp
Ret	5'-CTGCCGCTGCTAGGAGAAGCCCCAC-3'	5'-CTTCACACTGATGTTGGGACAAAGGAA-3'	555 bp
SGP2	5'-GACAATGAGCTCCA(G/A)GAA(A/C)TG-3'	5'-CAGGCATCCTGTGGAGTT(G/A)TG-3'	806 bp
LH-R	5'-AATCCCATCACAAGCTTTCAG-3'	5'-TGCCTGTGTTACAGATGC-3'	214 bp
G3PDH	5'-ACCACAGTCCATGCCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'	450 bp

Statistical analyses

All the experiments were repeated at least three times if not otherwise stated. Differences between groups were analyzed for statistical significance by using one-way ANOVA and Tukey's post hoc test on raw data. $P<0.05$ was considered as significant.

RESULTS

Purity and morphology of immature mouse Sertoli cell cultures

In order to study the proliferation of immature mouse Sertoli cells, we set up an isolation and culture procedure as described in the Materials and Methods section by which highly purified Sertoli cell cultures could be established from 4~5 days old mice. The Sertoli cells in culture were frequently spindle-shaped, or occasionally elliptic (Fig. 1a). It is known that vimentin is specifically expressed in Sertoli cells, and smooth muscle á-actin in peritubular myoid cells in testis (PARANKO et al., 1986; TUNG & FRITZ, 1990). We, therefore, used immunocytochemical assay to assess the purity of our cultures. After immunocytochemical staining, the numbers of vimentin-labelled cells and propidium iodide-stained nuclei were counted respectively in each of three distinct areas per slide, with approximately 400-500 total cells per area. The purity of Sertoli cell cultures was 98%±1.2% as indicated by the percentage of vimentin positive cells to the propidium iodide-stained nuclei (i.e. total cells) (Fig. 1b). The 2% contaminated cells were the peritubular myoid cells as indicated by using antibody to α-actin, which were elongated and spindle-shaped (data not shown).

GDNF expression and hormonal regulation in cultured mouse Sertoli cells

As expected, our results indicated that basal level expression of GDNF mRNA and protein in cultured Sertoli cells was detected by RT-PCR and immunocytochemistry (Fig. 2). In order to determine whether the GDNF expression was regulated by FSH, we performed time-dependent and dose-dependent experiments. In the time-dependent experiments, the analysis was performed every 1 hour before 3 hours (data not shown) and then every 3 hours after treatment. In the dose-dependent experiments, 10ng/mL, 20ng/mL, 30ng/mL, 40ng/mL, 50ng/mL, 100ng/mL, 150ng/mL and 200ng/mL FSH were used respectively. As indicated in Fig.2, the GDNF expression was up-regulated by FSH in a time- and dose-dependent manner. The maximum mRNA expression was detected 3 hours after FSH treatment and dropped significantly starting from 6 hours (Fig. 2A-a), and the GDNF mRNA expression started to level off when the dose of FSH reached 50ng/mL and thereafter (Fig. 2A-b) (data for FSH treatment of less than 50ng/mL are not shown). A similar pattern was also observed in the immunocytochemical assays (Fig. 2B). Estrogen and androgen had no effects on the expression of GDNF (Fig. 2A-c and 2A-d).

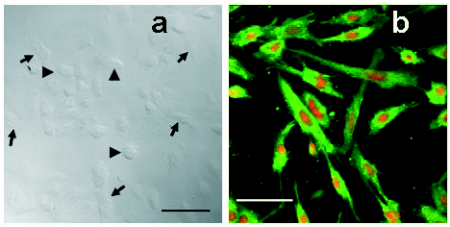

Fig. 1. – Morphological and immunocytochemical analysis of cultured Sertoli cells. a) Phase contrast microscopic image of cultured Sertoli cells, noting that the Sertoli cells were spindle-shaped (arrow) or elliptic (arrowhead); b) Immunofluorescent staining of cultured Sertoli cells. The cytoplasm of Sertoli cells was stained by using the goat anti-human vimentin polyclonal antibody and the FITC-conjugated mouse anti-goat IgG. The nuclei were stained by using propidium iodide. Images were acquired under a Leica confocal microscope. Scale bar: 50mm.

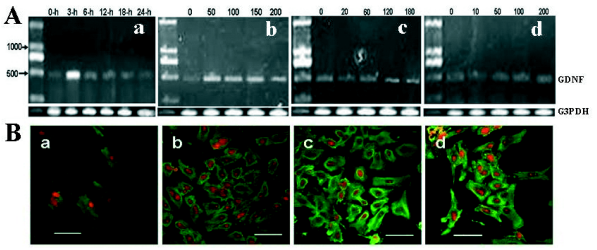

Fig. 2. – Semi-quantitative RT-PCR and immunocytochemical analysis of GDNF expression in cultured Sertoli cells. A: Semi-quantitative RT-PCR analysis of GDNF expression with FSH, estrogen and androgen treatment. a) The time-dependent expression of GDNF stimulated by 50ng/mL FSH. The expression gradually increased before 3 hours (data not shown) and was maximum 3 hours after treatment. b) The dose-dependent expression of GDNF detected 3 hours after FSH treatment. Expression increased gradually with the increase of FSH concentrations (data for FSH treatment of less than 50ng/mL were not shown) and levelled off with doses starting from 50ng/mL c) GDNF expression 4 hours after the addition of different concentrations of estrogen. d) GDNF expression 4 hours after the addition of different concentrations of androgen. B: Immunocytochemical analysis of GDNF expression. Sertoli cells were cultured for 20 hours and either untreated (a) or treated with 10ng/mL (b), 50ng/mL (c), or 100ng/mL FSH (d), and then fixed and stained with rabbit anti-human GDNF polyclonal antibody followed by FITC-conjugated goat anti-rabbit IgG for GDNF expression (green) and propidium iodide for nuclei (red). The staining was performed with the same protocol and same antibody dilution at the same day. The pictures were taken with identical Leica confocal microscope settings. The unit of numbers above figure: ng/ml; Scale bar: 50mm.

Sertoli cell viability and proliferation stimulation by GDNF

The viability of Sertoli cells was first monitored by MTT supravital staining. As shown in Fig. 3-A, GDNF at doses of both 10ng/mL and 20ng/mL stimulated the viability of Sertoli cells conspicuously compared with the cells without any treatment, although the effects of these two doses were not different significantly. As a positive control, 50ng/mL FSH also stimulated Sertoli cell viability significantly. Moreover, 10ng/mL GDNF in combination with 50ng/mL FSH increased the absorbance unit to a higher level, indicating a synergistic effect of these two factors or other mechanisms such as the up-regulation of GDNF expression by FSH. The stimulatory effect of GDNF and FSH on Sertoli cell proliferation was confirmed by BrdU-labelling assay (Fig. 3-B). Statistical analysis showed that significant differences could be identified between 10ng/mL GDNF treatment group and control group (DMEM/F12), and between group of 10ng/mL GDNF treatment and that of the combination of 10ng/mL GDNF with 50ng/mL FSH treatment. Although no statistical differences could be seen between 10ng/mL GDNF treatment group and 20ng/mL GDNF treatment group, and between group of 50ng/mL FSH and that of this dose in combination with 10ng/mL GDNF treatment, the proliferation-promoting effects of GDNF and FSH were obviously identified from Fig. 3-B. Also, in the presence of both GDNF and FSH, the number of BrdU-positive cells in total cells was increased compared with those under other treatments—clumps of BrdU-positive cells were frequently seen, probably resulting from a higher proliferation rate of the cells (Fig. 4).

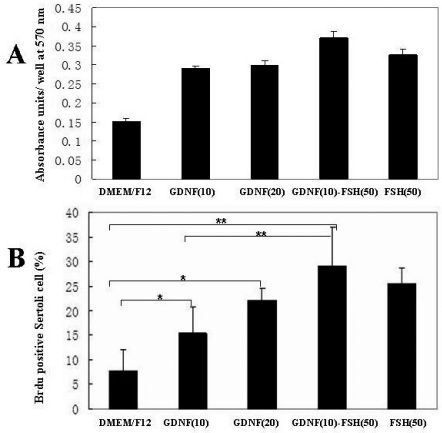

Fig. 3. – Enumeration of viable and proliferating Sertoli cells by supravital staining and BrdU labelling. A: Estimation of living Sertoli cells by MTT staining. The Sertoli cells were first plated and incubated in 24-well plates overnight, and again cultured and treated with GDNF, FSH separately or in their combination for 44 hours, then stained with MTT for 4 hours. Each bar represent 570nm absorbance units (AU) per well (mean±S.E.M. n=3). B: Enumeration of Sertoli cells in S-phase by BrdU labelling. For each experiment at least 3 areas under microscope (approximately 200 total cells) were chosen to count BrdU-positive cells. Values were represented as % Brdu-positive cells over total number of cells from three independent experiments (mean±S.E.M.; n=3). Statistical difference was analyzed by using Tukey's post hoc test. *, $p<0.05$; **, $p<0.01$. The unit of numbers in parentheses: ng/mL.

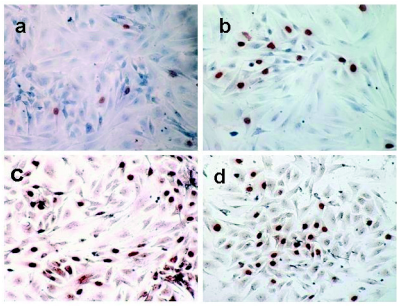

Fig. 4. – Light microscopic image of cultured Sertoli cells labelled by BrdU. a) no treatment; b) treated with 10ng/mL GDNF; c) treated by 10ng/mL GDNF plus 50ng/mL FSH; d) 50ng/mL FSH only.

Contribution of GFRα1/NCAM signalling to GDNF-induced Sertoli cell proliferation

We would like to know, as the first step to elucidating the molecular mechanism of the stimulatory effect of GDNF on mouse Sertoli cell proliferation, whether its receptor subunits were present on the membrane of mouse Sertoli cells. As shown in Fig. 5, the ligand binding subunit GFRα1 was detected both by RT-PCR and immunocytochemistry. Interestingly, Ret, which represented the first identified GDNF signalling pathway, was not expressed, while NCAM, which represented the alternative pathway, was expressed at both RNA and protein levels, suggesting that the stimulatory effect of GDNF on Sertoli cells was most likely mediated by signalling pathways starting from the GFRα1/NCAM receptor complex. This proposition was supported by the observation that anti-NCAM antibody (4mg/mL) reduced the stimulatory effect of GDNF significantly in serum-free culture (Fig. 6). The percentage of BrdU-positive cells in the total number of cells under GDNF treatment was decreased from 23.6% to 10.8% (P=0.028) by anti-NCAM antibody.

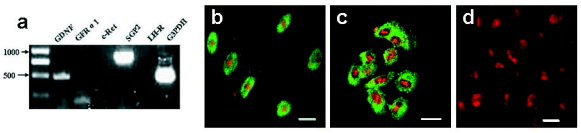

Fig. 5. – GDNF receptor subunit expression in immature mouse Sertoli cells. RT-PCR analysis (a) indicated that GFRα1 but not Ret was expressed in cultured immature Sertoli cells. SGP2 and G3PDH were included as positive controls while LH-R was used as a negative control. Immunocytochemical analysis of GFRα1 (b), NCAM (c) and Ret (d) on cultured Sertoli cells. Marker proteins were stained green while the nuclei were stained red by propidium iodide. Scale bar: 20mm.

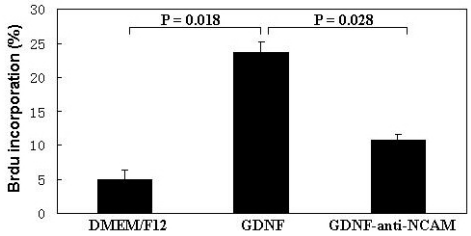

Fig. 6. – Reduction of stimulatory effect of GDNF on Sertoli cell proliferation by anti-NCAM antibody. Values were expressed as the percentages of BrdU-positive cells in total cells from results of three independent experiments (mean±S.E.M; n=3). Statistical differences were analyzed through Tukey's post hoc test. Concentrations of GDNF and anti-NCAM antibody were 10ng/mL and 4mg/mL respectively.

DISCUSSION

The proliferation of Sertoli cells has been reported to be stimulated by hormones, such as FSH (Griswold et al., 1977) and estrogen (Sharpe et al., 1998), and other paracrine growth factors including insulin-like growth factors I and II (IGF- I and IGF- II) (Borland et al., 1984), fibroblast growth factor (FGF) and transforming growth factor-α (TGF-α) (Petersen et al., 2001). Recently, studies reported that the stimulatory effect of GDNF on Sertoli cell proliferation was observed in cultured neonatal rat seminiferous tubules and in Sertoli cells from fetal mouse testis (Hu et al., 1999; Wu et al., 2005). In the present study, we used Sertoli cell cultures prepared from neonatal mice to examine whether and how GDNF stimulates the proliferation of Sertoli cells. To this end, highly purified Sertoli cell cultures were first established. The purity of our cultures was assessed by immunocytochemistry for cell markers. Vimentin and smooth muscle α-actin have been shown to be specific markers for Sertoli cells and peritubular myoid cells in the testis, respectively (Paranko et al., 1986; Tung & Fritz, 1990). The results of immunocytochemical assay verified that about 98% of the cultured cells were Sertoli cells with the remaining 2% being contaminating peritubular myoid cells, indicating that our Sertoli cell cultures, together with the serum-free medium, should be sufficient for elucidating the effects of GDNF on immature mouse Sertoli cells and signalling pathway mediating such effects.

A previous study indicated that FSH but not testosterone up-regulated GDNF mRNA expression (Tadokoro et al., 2002). This observation was confirmed in our present study by means of RT-PCR and immunohistochemistry. We also observed that estrogen had no effect on the expression of GDNF. Two studies indicated that GDNF stimulated the proliferation of immature Sertoli cells from newborn rats and fetal mice (Hu et al., 1999; Wu et al., 2005). Here we also confirmed the proliferation-promoting effects of GDNF on Sertoli cells in culture prepared with newborn mouse testes. However, the stimulatory effect of GDNF was only observed in the presence of FSH according to one study (Hu et al., 1999), a phenomenon not observed in another previous report (Wu et al., 2005) or in our study. The discrepancy was probably caused by the different cultures used – seminiferous tubule fragments and homogeneous cells were used respectively in the types of studies. Besides, the different animals (rat versus mouse) used in these studies should also be taken into account, and we suggested that the mechanisms of proliferation-promoting effects of GDNF on Sertoli cells in rat should slightly differ from those in mouse.

Our study also showed that GDNF in combination with FSH had more potent effects on Sertoli cell proliferation than GDNF alone, which was probably caused by accumulation of proliferation-promoting effects of the two factors on Sertoli cells. It has been known that FSH was a strong mitogenic hormone of Sertoli cells (Griswold et al., 1977; Meachem et al., 1996), and the expression of GDNF was up-regulated by FSH discussed as above, so the action of additional GDNF secreted by Sertoli cells should also be considered. Alternatively,

other possibilities might exist. For example, one factor could render the cells more sensitive to the action of the other one.

GDNF signals through the receptor GFRα1, which activates Ret (Durbec et al., 1996; Treanor et al., 1996). GDNF in complex with GFRα1 also signals through the NCAM independent of Ret (Paratcha, et al., 2003). However, the GDNF signalling in Sertoli cells had not yet been characterized. In the present study, we demonstrated for the first time that NCAM but not Ret was expressed in cultured mouse Sertoli cells at both RNA and protein levels. However, a previous study showed that the Ret mRNA was present in Sertoli cells from 20- and 55-day-old rats (Fouchecourt et al., 2006), which contradicts our results of this study. We suggest that this discrepancy was probably caused by the differences in experimental materials used: not 100% pure Sertoli cell fraction from 20- and 55-day-old rats in Fouchecourt's report, and purified Sertoli cell cultures from 4- to 5-day-old mice in our studies were used, indicating that the GDNF signalling might be divergent between rat and mouse. Therefore, we still propose that the stimulatory effect of GDNF on mouse Sertoli cell proliferation was mediated by pathway(s) starting from the NCAM, but not Ret subunit. This was supported by the fact that anti-NCAM antibody significantly reduced the proliferation-promoting effect of GDNF on Sertoli cells. The activation of downstream components of the NCAM pathway(s) should be addressed in future studies.

CONCLUSIONS

In conclusion, our data confirmed that GDNF, an autocrine growth factor, was produced by Sertoli cells and regulated by FSH, and played an important role in Sertoli cell proliferation via GDNF/NCAM pathway in the testis development of mouse.

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