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Overexpression of GDNF can Effectively Maintain the Pluripotency of Buffalo Spermatogonia *In Vitro*

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KHL, XGY, TTL and SSG conceived and designed the study. TTL, SSG, LX, HYX, ALL, PWZ and HY acquired the data. KHL, XGY and TTL analyzed and interpreted the data. KHL, XGY and TTL wrote the article.

Key words
Endogenous, GDNF gene, Buffalo Sertoli cells, Spermatogonia.

ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor cloned from mouse brain tissue by Lin *et al.* (1993) and can also be secreted by Sertoli cells. GDNF has a variety of physiological functions and plays important roles in the nervous system and in the reproduction of spermatogonial stem cells (SSCs). To date, commercialized murine and human GDNF have typically been used for the *in vitro* culture of SSCs from domestic animals. However, whether endogenous GDNF is superior to xenogenic GDNF in SSC culture *in vitro* remains unclear. To answer this question, the buffalo GDNF gene was cloned and integrated into the PiggyBac eukaryotic expression vector, which was stably expressed in Sertoli cells, thus establishing a PB-GDNF-Sertoli cell line. Quantitative real-time polymerase chain reaction (qRT-PCR) results showed that the GDNF gene expression in the PB-GDNF-Sertoli cell line was significantly increased compared with that in the control Sertoli cell line ($p<0.01$). Enzyme-linked immunosorbent assay (ELISA) results showed that GDNF gene secretion was also significantly increased in PB-GDNF-Sertoli cells compared with that in control Sertoli cells ($p<0.05$). We further compared two types of spermatogonia cultured on either PB-GDNF-Sertoli cells or control Sertoli cells, revealing that the use of PB-GDNF-Sertoli cells as a feeder layer could significantly increase the expression levels of DDX4 ($p<0.01$), PLZF ($p<0.01$) and NANOS2 ($p<0.05$) in buffalo spermatogonia *in vitro*. These results suggested that overexpression of GDNF could effectively maintain the pluripotency of buffalo spermatogonia *in vitro*, laying the foundation for improving the *in vitro* culture of buffalo spermatogonia.

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor cloned from mouse brain tissue (Lin *et al.*, 1993) that plays a key role in several important nervous system cells, such as dopaminergic neurons, central and peripheral nervous system neurons, sensory neurons, and autonomic neurons. Moreover, GDNF can also promote the regeneration of neurons and protect them from external damage (Henderson *et al.*, 1994; Buj-Bello *et al.*, 1995).

As research on GDNF has continued, important physiological functions of GDNF in spermatogonial stem cells (SSCs) have gradually been discovered, elucidating its involvement in the self-renewal and differentiation of SSCs (Bennett *et al.*, 2000; Meng *et al.*, 2000; Kubota *et al.*, 2004; Schmidt *et al.*, 2009). Therefore, GDNF has been identified as an essential growth factor for the *in vitro* proliferation of SSCs (Kubota *et al.*, 2004).

In previous studies examining buffalo SSCs, murine and human GDNF were mostly used as trophic factors during cell culture (Kubota *et al.*, 2004; Kanatsu-Shinohara *et al.*, 2005; Kala *et al.*, 2012; Kadam *et al.*, 2013; Rafeeqi and Kaul, 2013; Yu *et al.*, 2014; Feng *et al.*, 2016). However, differences among the proteins of different species exist, which could result in new destabilizing factors. Whether endogenous GDNF is superior to xenogenic GDNF in SSC culture *in vitro* remains unclear.

Due to differences in the sequences and structures of GDNF among species, we hypothesized that endogenous GDNF can more effectively maintain the characteristics of SSCs. To verify this hypothesis, we constructed a GDNF vector and introduced it into buffalo Sertoli cells to establish a cell line stably expressing GDNF. Using this cell line as a feeder layer, we elucidated the effects of GDNF overexpression on the expression of pluripotency-and reproductive-related genes in SSCs, providing a reference

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for optimizing the *in vitro* culture of buffalo SSCs.

MATERIALS AND METHODS

Animal ethics

All animal procedures used in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed., National Research Council, USA) and approved by the Institutional Animal Care and Use Committee of Guangxi University.

Collection of buffalo brain tissue

Buffalo brain tissue was collected from the local slaughterhouse and cryopreserved in liquid nitrogen for long-term storage.

Separation of buffalo testicular single-cell suspension

Testes were washed twice with 75% alcohol for 5 min each time and then washed with PBS containing penicillin-streptomycin (PS, 10×, Gibco, USA, Cat No. 15070063). The tunica albuginea was removed, and the tissue was placed in an Eppendorf (EP) tube for cutting. Then, the tissue was transferred to a dish and digested with collagenase IV and DNase I (Worthington Biochemical Corp., LS002138) at a 1:1 ratio in an incubator. After 15 min, the tissue was transferred to a centrifuge tube and centrifuged at 2000 rpm for 5 min. After discarding the supernatant, the tissue was re-suspended in PBS and digested with 0.25% trypsinase (Gibco, 25200056) for 5 min; the digestion was halted by the addition of medium containing foetal bovine serum (FBS; HyClone, 30070.03). The digestion product was collected and centrifuged at 2000 rpm for 5 min, the supernatant was discarded, and the sample was resuspended in culture medium. Debris was removed by a cell strainer.

Purification of buffalo spermatogonia and Sertoli cells

The obtained single-cell suspension of buffalo testicular cells was seeded on a gelatine-coated dish and cultured in stem cell culture medium overnight in a 37°C incubator. The stem cell culture medium was composed of Iscove's Modified Dulbecco's Medium (IMDM), KnockOut Serum Replacement (KSR) Solution (cat. no. 10828028, Gibco), fetuin (CAT. NO. F2379, Sigma), MEM Non-Essential Amino Acids Solution (NEAA, 100 ×, Cat. No. 11140050, Gibco), Chemically Defined Lipid Mixture 1 (Cat. No. L0288, Sigma), GDNF (Cat. No. CYT-305, ProSpec), GFRα1 (Cat. No. 560GR, R&D Systems), bFGF (Cat. No. CYT-218, ProSpec), β-Me (cat. no. M0482-100ML, Sangon Biotech), B27 (Cat. No. 12587-010, Gibco) and LIF2010 (Millipore). The suspended cells were collected, transferred to a collagen protein-coated dish and cultured for 4 h in a 37°C incubator. Then, the adherent cells were collected as Sertoli cells. After 4 h, the suspended cells in the collagen protein-coated dishes were collected as spermatogonia.

Immunofluorescence staining of buffalo Sertoli cells

Purified Sertoli cells were counted, seeded in a cell culture dish, and incubated overnight in a 37°C incubator. Then, the cells were fixed with a 4% paraformaldehyde solution at room temperature for 15 min, blocked with 6% horse serum for 1 h, incubated with WT1 (ab89901) and GDNF (ab18956) primary antibodies for 1 h at room temperature and then washed with PBS^{++} 3 times for 5 min each time. Next, the cells were incubated with the secondary antibodies goat-anti rabbit IgG H&L (Alexa Fluor® 594, 1:500, ab150080) and goat-anti rabbit IgG H&L (Alexa Fluor® 488, 1:200, ab150077) for 1 h at room temperature and then washed with PBS^{++} 3 times for 5 min each time. A drop of ProLongTM Gold antifade reagent with DAPI (1825386, Life Technologies) was added to the dish for 5 min. Fluorescence images were captured with an NIS Elements image system (Olympus TH4-200) and then processed and analysed with Photoshop CS5 software (Adobe Systems Inc., USA).

Culture of Sertoli cells and mouse embryonic fibroblasts (MEFs) with buffalo spermatogonia

Approximately 9×10^4 Sertoli cells and mouse embryonic fibroblasts (MEFs) were seeded into a 96-well plate and treated with mitomycin C (HaiZheng, 1 μg/μl) for 2 h for preparation as two feeder layers. The purified buffalo spermatogonia were then seeded on the two feeder layers, and the culture medium was changed every 2-3 days.

Primer design and synthesis

According to the published messenger RNA (mRNA) sequence of the cattle GDNF gene published in GenBank (NM_001192849.1), Primer 5.0 software was used to design the following primers: upstream primer (ATGAAGTTATGGGATGTCGT) and downstream primer (TCAGATACATCCACACCTTTTAGC).

We chose Bam HI and Xba I as insertion sites based on analysis of the GDNF fragments and PiggyBac 513B-1 (PB, purchased from System Biosciences, SBI) plasmid sequence sites, respectively. PCR was used to insert the enzymatic cleavage site using the following primers: upstream primer (5'-TCTAGAATGAAGTTATGGGATG-3') and downstream primer (5'-GGATCCTCAGATAC-ATCCA-3').

Cloning of the GDNF gene

Buffalo brain tissue was ground into powder in liquid nitrogen, and total RNA was extracted using precooled TRIzol Reagent according to the total RNA extraction procedure described in the "Molecular Cloning Experiments Guide". Reverse transcription was performed immediately after the extraction, and PCR was performed using cDNA as a template. The reaction system (20 µl) consisted of the following: PrimeSTAR mix, 10 µl; forward and reverse primers, 1 µl; cDNA, 200 ng; and H_2O, 7 µl. The reaction conditions were 98°C for 3 min; 34 cycles of 98°C for 10 sec, 55°C for 5 sec and 72°C for 5 sec; and 72°C for 5 min.

The PCR product of the PrimeSTAR mix was blunt-ended, and the master mix was added at a ratio of 1:1 at 72°C for 25 min to add the A tail. Next, 1% agarose gel electrophoresis was performed at a constant 90 V for 25 min, and the gel was observed on a gel imager.

T vector binding and PiggyBac recombinant plasmid construction

PEASY T1 (1 µl) was reacted with GDNF gel (4 µl, approximately 150 ng) for 10 min at room temperature, and the product was transformed into DH5α competent cells on ice. After resistance selection, the clone was picked and identified by PCR. PCR-positive clones were amplified and cultured, and the monoclonal bacterial solution was sent to a company (Huada, China) for gene sequencing.

The GDNF-positive strain was amplified and then subjected to plasmid extraction, which was used for PCR. A 1 µg DNA fragment recovered from the gel was digested with Bam HI (5' end) and Xba I (3' end) at 37°C for 5 h and then digested at 65°C for 10 min to stop the reaction. The product was stored at -20°C.

Additionally, 1 µg of the PB 513B-I plasmid was digested with Bam HI and Xba I at 37°C for 5 h, and the enzyme digestion was halted by incubating the mixture at 65°C for 10 min. Electrophoresis was performed on a 1.5% agarose gel, and the gel was recovered. The product was stored at -20°C.

The T4 ligation reaction was carried out with 50 ng of the digested PB 513B-I product and 300 ng of the digested GDNF gel. The following reaction mixture was incubated overnight at 16°C: 1 µl of T1 ligase buffer, 0.5 µl of T4 ligase, 300 ng of DNA, and 50 ng of plasmid.

The T4-ligated product was transformed into competent cells, which were incubated at 37°C in a table concentrator for 1 h and then plated and cultured in a full-humidity incubator at 37°C for 14 to 16 h. Then, the monoclonal strain was selected to identify positive strains. The positive strains were amplified, and endotoxin-free plasmids were extracted. Spe l and Hind III were used to perform a double restriction enzyme digestion prior to transfection.

Stable transfection of PB-GDNF plasmids

P0 Sertoli cells were seeded on 4-well plates and cultured in DMEM/F12 to maintain 60 to 70% cell confluency after transfection. The PB vector (1 µg or 0.4 µg) was added to OPTI-MEM Optimized Medium (Gibco, Cat. No. 31985062) as M1 medium, and Lipofectamine 2000 (3 µl) was added to 50 µl of OPTI-MEM culture medium for 5 min at room temperature as M2 medium. Then, the M1 and M2 media were gently mixed and incubated at room temperature for 30 min. Next, the mixed solution was added dropwise to the Sertoli cell culture medium, which was mixed gently by shaking, incubated at 37°C for 6 h, and then replaced with normal culture medium. The culture medium was changed every 3 days after transfection, and fluorescence was observed under a fluorescence microscope.

Screening of Sertoli cells after transfection of the PB-GDNF plasmid

According to preliminary experiments, the puromycin concentration most optimal for screening Sertoli cells was determined to be 2µg/ml.

The cells were passaged into a 35 mm dish after transfection for 48 h, and the medium was replaced with fresh medium containing 2µg/ml puromycin 12 h later. The puromycin-containing medium was removed and replaced with fresh medium every 2 to 3 days, and the state of cell growth was observed daily. The transfected cells expressed green fluorescent protein (GFP) so that we could visually observe whether the cells were successfully transfected. After 14 days, all the cells expressed GFP, and the stable transfection of Sertoli cells was completed. The screened Sertoli cells were expanded and cultured.

Quantitative real-time PCR (qRT-PCR) and ELISA identification of PB-GDNF-Sertoli cells and Sertoli cells

The P4 generations of the PB-GDNF-Sertoli cells and Sertoli cells were collected, and a PrimeScriptTM RT reagent kit (TaKaRa) with gDNA Eraser was used to synthesize cDNA from these cells. The genomic DNA was removed by gDNA Eraser for 2 min at 42°C. Next, the samples were incubated at 4°C in a 10 µl reaction mixture comprising 1 µl of PrimeScript RT Enzyme Mix I, 1 µl of RT Primer Mix, 4 µl of 5 × PrimeScript Buffer 2 (for RT-PCR) and 4 µl of RNase-free ddH_2O at 37°C for 15 min, incubated at 85°C for 5 sec and, finally, stored at 4°C. We performed q-PCR in an RT-PCR thermocycler (CFX96 Touch; BIO-RAD, USA). The primer pairs used to amplify mRNA are listed in Table I. Each experiment

was replicated three times, and we determined the relative expression level of each mRNA using the $2^{-\Delta\Delta Ct}$ (normalized expression ratio) method.

Table I.- Sequences of the primers used in the qRT-PCR experiments and the expected product lengths (PL).

Gene	PL (bp)	Primer sequence
GAPDH	221	F: 5'-CGTGGAGGGACTTATGACCAC-3' R: 5'-CCACAACAGACACGTTGGGA-3'
DDX4	123	F: 5'-AACAGCGTCAGACCCTTATG-3' R: 5'-CTCTACATGCTCCACCCACT-3'
ZBTB16	125	F: 5'-GCAACAGCCAGCACTATACTCTG3' R: 5'-AGCGGCATACAGCAGGTCA-3'
POU5F1	184	F: 5'-AAGCTGGACAAGGAGAAGCT-3' R: 5'-TAGTCGTTTGGCTGAACACC-3'
NANOS2	143	F: 5'-ATGGAGAGTCTCGCAACGTG-3' R: 5'-AAGTGGGCAGTACTTGAGCG-3'
GDNF	132	F: 5'-ACTTGGGTTTGGGCTACG-3' R: 5'-GTCACTCACCAGCCTTCTAC-3'

After generating the Sertoli and PB-GDNF-Sertoli cells as described above, the cells were counted by trypan blue staining and inoculated at equal densities into 35 mm cell culture dishes. After 36 h, the supernatants of both cell types were collected and centrifuged at 2000 × rpm for 20 min; the levels of GDNF in these samples were determined. The experiment was carried out according to the manufacturer's instructions (Bovine GDNF ELISA KIT, China). Finally, the absorbance (OD value) was measured by a microplate reader at a wavelength of 450 nm. The concentration of the standard was the abscissa, and the OD was the ordinate. The standard curve was drawn on coordinate paper, and the equation was calculated. The concentration was then calculated based on the OD value of the sample.

Buffalo spermatogonia cultured with PB-GDNF-Sertoli cells or control Sertoli cells (with human GDNF)

Approximately 9×10^4 PB-GDNF-Sertoli cells and 9×10^4 control Sertoli cells were seeded onto a 12-well plate overnight and then treated with 1 μg/μl mitomycin C for 2 h to prepare the feeder layers. Afterwards, the purified buffalo spermatogonia were cultured on PB-GDNF-Sertoli and control Sertoli cells at equal densities. Both groups of cells were cultured in stem cell culture medium, but 0.775 μl of diluted human GDNF was added to the control group. The cells were cultured in a 5% CO_2 atmosphere at 37°C, and the culture medium was changed every 2 days.

Statistical analysis

All statistical analyses were performed with GraphPad Prism5 software (USA). The results are presented as the mean ± the standard error of the mean (SEM). Statistical analysis was performed by Student's t-test or one-way analysis of variance (ANOVA). Differences were considered significant at p values less than 0.05.

RESULTS

Effects of different feeder layers on the cultivation of spermatogonia

In previous studies, MEFs were often used as a feeder layer for the *in vitro* culture of mouse SSCs (Kanatsu-Shinohara *et al.*, 2003; Hamra *et al.*, 2005). However, for the *in vitro* culture of buffalo spermatogonia, we considered whether the Sertoli cells of buffalo could be used as a feeder layer to better support SSC proliferation *in vitro*. Therefore, we compared the effects of culturing MEFs and Sertoli cells from buffalo spermatogonia to select a suitable feeder layer.

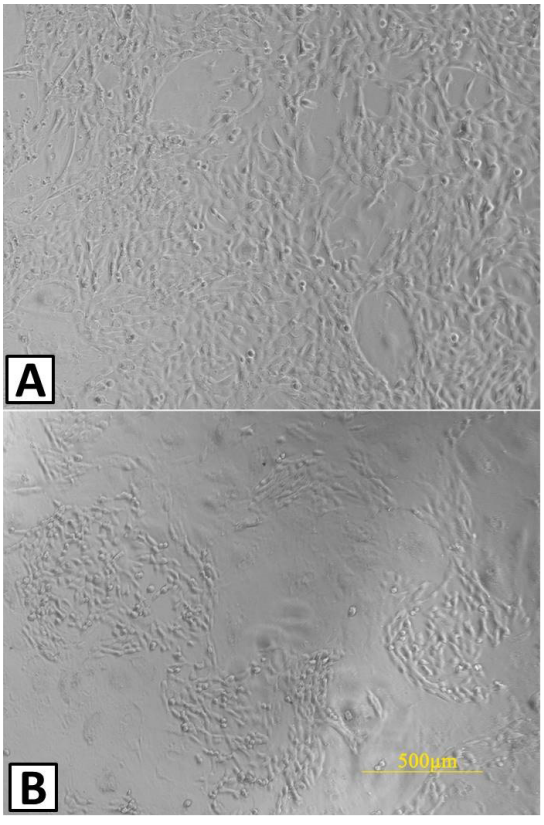

Fig. 1. MEFs and Sertoli cells were used as feeder layers to culture buffalo spermatogonia. Buffalo spermatogonia cultured on MEFs (A) and on Sertoli cells (B). Scale bars=500 μm.

The enriched spermatogonia were cultured on either MEFs or Sertoli cells that were treated with mitomycin C. Regarding morphology, the spermatogonia cultured on MEFs did not form cell clones, while the spermatogonia cultured on Sertoli cells formed numerous cell clones (Fig. 1). Therefore, the Sertoli cells were cultured with spermatogonia as the feeder layer in our study.

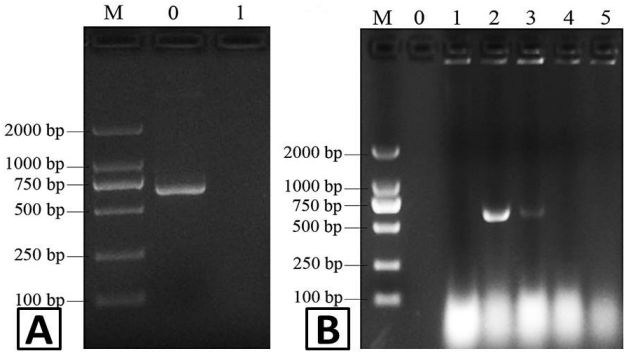

Fig. 2. Cloning of the buffalo GDNF gene and construction of the T vector. **A**: M, DL2000; 0, cDNA; 1, H2O; **B**: M, DL2000; 0, H2O; 1-5, monoclonal colonies.

Cloning the GDNF gene and identification of the GDNF monoclonal sequence

Total RNA was extracted from buffalo brain tissue, and RT-PCR amplification was performed using reverse-transcribed cDNA as a template. The buffalo GDNF gene (636 bp) was successfully cloned from buffalo brain tissue (Fig. 2A).

The GDNF fragment was obtained by gel extraction and then ligated into the T vector (pEASY T1, full-form gold). After plating, positive colonies were identified (Fig. 2B), and PCR-positive strain 2 was amplified and sent for sequencing (HuaDa Gene, China).

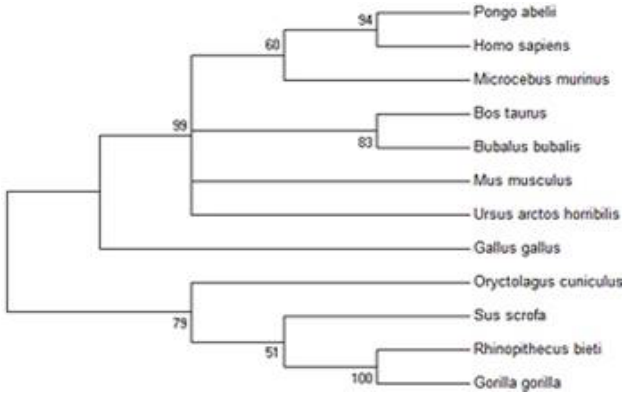

Fig. 3. Phylogenetic tree of the GDNF sequences in various mammalian species.

The GDNF sequence obtained by cloning was 99.5% similar to the buffalo GDNF sequence published in the NCBI database (JQ326280.1) and 99% homologous to the cattle GDNF sequence. However, the sequence was 91% and 86% homologous to the human and mouse GDNF sequences, respectively, indicating that the GDNF fragment is highly conserved (Fig. 3).

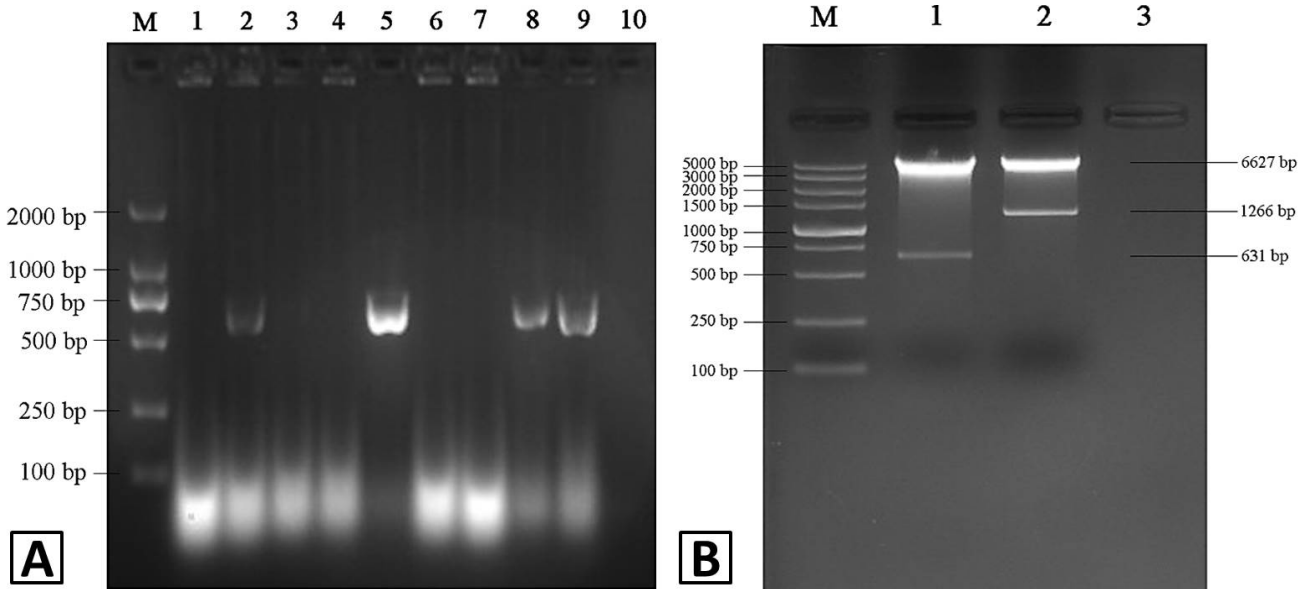

Fig. 4. Construction of the PB-GDNF plasmid. **A**: M, DL2000; 1-9, monoclonal colonies; 10, H2O; **B**: M, DL5000; 1, PB 513B-I plasmid; 2, PB-GDNF plasmid; 3, H_2O; 1-5, monoclonal colonies.

Construction of the PiggyBac vector carrying the buffalo GDNF gene

In this study, Bam HI and Xba I were selected as insertion sites based on analysis of the GDNF fragment and the PB 513B-1 plasmids (purchased from SBI), and the two insertion sites were connected to the 5' and 3' ends of the GDNF fragment, respectively, by PCR. Then, the PCR product and PB empty plasmid were simultaneously double-digested, and the T4 ligation reaction was carried out to integrate the GDNF fragment into the PB vector.

After the T4 ligation, strain no. 5 of 4 GDNF-positive strains (Fig. 4A) was amplified and subjected to endotoxin-free plasmid extraction as well as Spe 1 and Hind III digestion. The expected fragment sizes of the digested empty vector product were 6627 bp and 631 bp. The sizes of the GDNF-positive cloned fragment were 6627 bp and 1266 bp, which was consistent with our expectations (Fig. 4B). The above results indicate that we successfully constructed a recombinant GDNF plasmid with the PB transposon.

Isolation, purification and identification of buffalo Sertoli cells

Buffalo testes were digested by a two-step enzymatic digestion method to obtain the testicular single-cell suspension, and Sertoli cells were purified by the differential plating method. To verify the purity of the obtained testicular Sertoli cells, the cells were stained with antibodies against WT1 (ab89901, Abcam) and GDNF (ab18956, Abcam), specific genes expressed in Sertoli cells (Morohoshi *et al.*, 2019). The rates of $WT1^+$ (Fig. 5A) and $GDNF^+$ (Fig. 5B) cells were greater than 90%, and these cells were thus used in subsequent experiments.

Overexpression of the GDNF gene in buffalo Sertoli cells

The PB transposon eukaryotic expression vector carrying GDNF was constructed to verify the sustained expression of buffalo GDNF in eukaryotic cells. We transfected the PB transposon vector into eukaryotic cells. The general map of the PB 513B- transposon recombinant GDNF plasmid (Fig. 6) is as follows: the CMV promoter regulates the expression of GDNF, and the EF1 promoter regulates the expression of copGFP. Both of these promoters are persistently and strongly expressed; thus, the GDNF gene and GFP are expressed in the successfully transfected cells.

Sertoli cells of passage 1 were transfected by Lipofectamine 2000. After 24 h of transfection, the Sertoli cells (passage 2) expressed GFP, and the transfection efficiency was approximately 15%. After 15 days of transfection, almost all the cells expressed GFP (Fig. 7A), indicating that the PB vector integrated the target fragments (GDNF and GFP) into the genomic DNA of Sertoli cells and that the GDNF eukaryotic expression vector was continually expressed in eukaryotic cells. To further verify the expression of buffalo GDNF in Sertoli cells before and after transfection, we examined the transcription level of GDNF. The GDNF gene expression in PB-GDNF-Sertoli cells was significantly higher than that in control Sertoli cells (Fig. 7B).

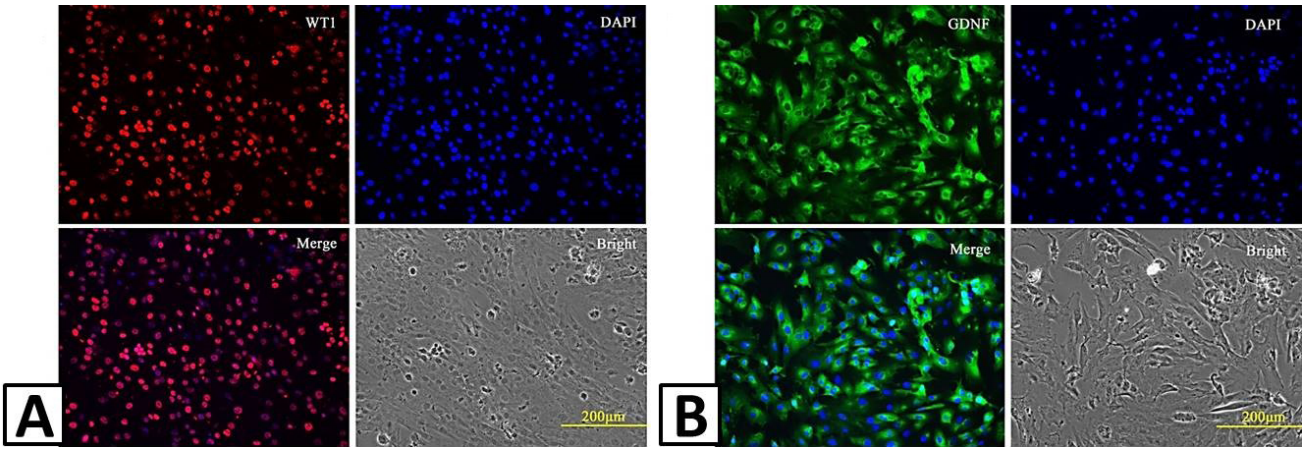

Fig. 5. Immunofluorescence staining of Sertoli cells using WT1 marker (A) and GDNF marker (B) antibodies. Scale bars=200 μm.

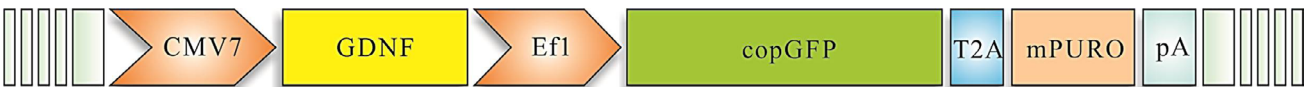

Fig. 6. Brief map of the PB-GDNF plasmid.

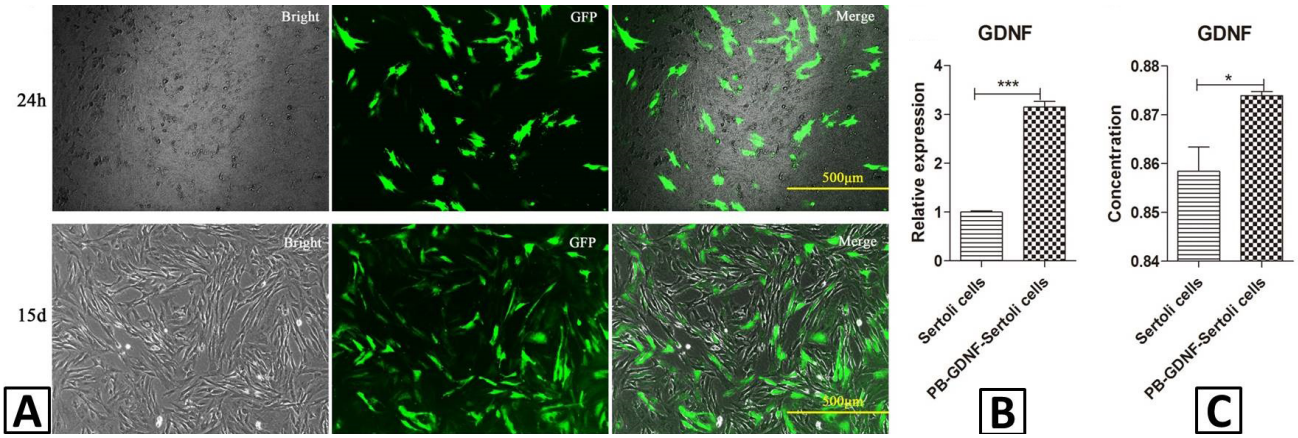

Fig. 7. Overexpression of the GDNF gene in buffalo Sertoli cells. After 15 days of transfection, almost all cells expressed green fluorescent protein (A). The GDNF gene expression in PB-GDNF-Sertoli cells was significantly higher than that in control Sertoli cells (B). The GDNF gene secretion in PB-GDNF-Sertoli cells was significantly higher than that in control Sertoli cells (C). Scale bars=500 μm.

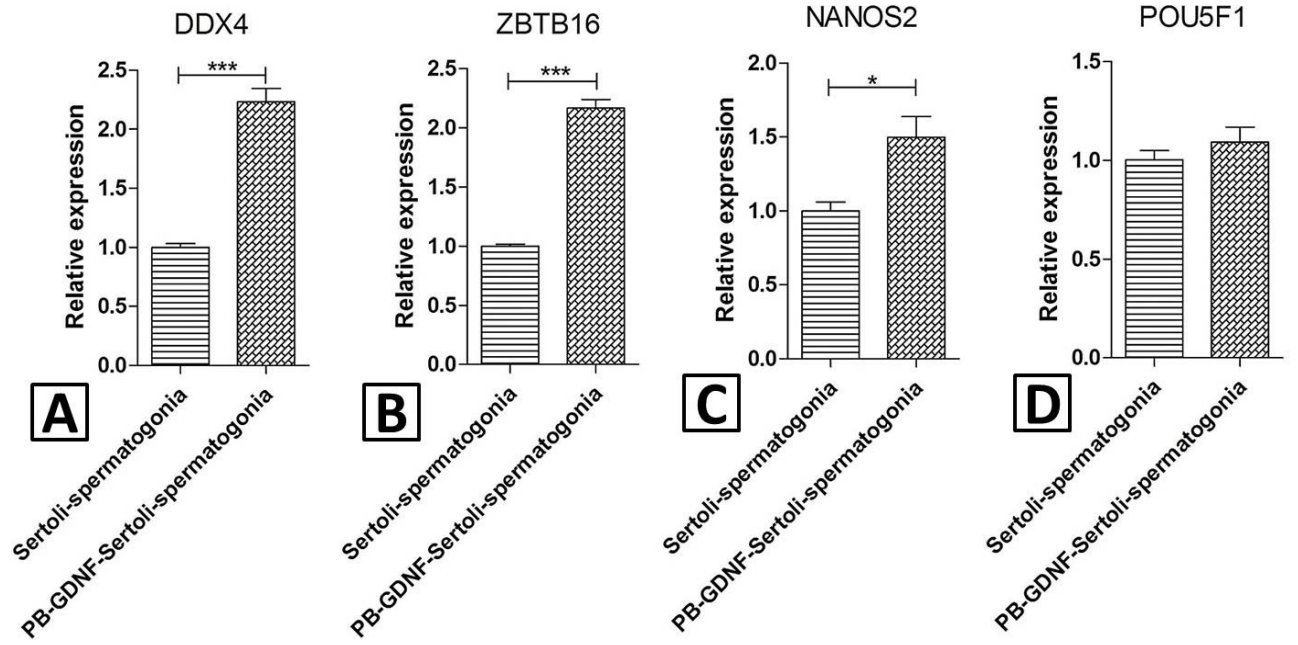

Fig. 8. Quantitative-PCR analysis of buffalo spermatogonia cultured on either experimental group cells (PB-GDNF-Sertoli) or control group cells (Sertoli with human GDNF) *in vitro*. Histograms showing the quantitative-PCR analysis of the germline-related marker DDX4 (A), the buffalo spermatogonia marker ZBTB16 (B), the inchoate buffalo spermatogonia marker NANOS2 (C) and the pluripotency marker POU5F1 in buffalo spermatogonia cultured on either PB-GDNF-Sertoli Cells or control Sertoli Cells (with human GDNF).

The concentrations of GDNF in the supernatants of PB-GDNF-Sertoli and control Sertoli cells were calculated by the standard curve (Y=0.146 X+0.131, p=0.0366, $0.01<p<0.05$), which indicated that the level of GDNF gene secretion in PB GDNF-Sertoli cells was also significantly higher than that in control Sertoli cells (Fig. 7C).

Comparison of the effects of buffalo spermatogonia cultured on PB-GDNF-Sertoli cells and control Sertoli cells (with human GDNF) in vitro

PB-GDNF-Sertoli cells and Sertoli cells treated with mitomycin C were used as feeder layers to culture buffalo spermatogonia of the same density. After cultivation *in*

vitro, qRT-PCR was used to compare the spermatogonia cultured on both feeder layers (Fig. 8). The expression levels of the inchoate buffalo spermatogonia markers NANOS2 (Saba *et al.*, 2014) and ZBTB16 (Costoya *et al.*, 2004) as well as those of the reproductive-related gene DDX4 (Kim *et al.*, 2015) were significantly higher in the spermatogonia cultured in the experimental group (PB-GDNF-Sertoli cells) than in that cultured in the control group (Sertoli cells with human GDNF). The expression level of the pluripotency-related gene POU5F1 (Mahla *et al.*, 2012) in the spermatogonia cultured in the experimental group was also higher than that in the spermatogonia cultured in the control group.

DISCUSSION

Currently, commercial GDNF is usually added for the *in vitro* cultivation of SSCs (Kanatsu-Shinohara *et al.*, 2005; Kala *et al.*, 2012; Kadam *et al.*, 2013; Rafeeqi and Kaul, 2013; Yu *et al.*, 2014; Feng *et al.*, 2016). However, whether endogenous GDNF is superior to xenogenic GDNF in SSC culture *in vitro* remains unclear. Therefore, we herein adopted buffalo Sertoli cells overexpressing GDNF as the feeder layer to observe the effect of endogenous GDNF on spermatogonial cell proliferation and pluripotency.

To efficiently obtain Sertoli cells overexpressing the GDNF gene, we used the PB transposon as a backbone to construct a transfection plasmid. The PB dual promoter (PB 513B-1) is a plasmid used for mammalian cell transposition experiments that is located at the multiple cloning site (MCS) downstream of the CMV promoter, making it easier to clone the gene or microRNA of interest. Moreover, the downstream EF-1α core promoter activates the expression of GFP. The PB transposase system is highly efficient in mammals (Ding *et al.*, 2005; Chen *et al.*, 2010) and has been widely used in the study of a variety of transgenic animals (Woltjen *et al.*, 2009; Park and Han, 2012). An important feature of the PB transposon is that it almost always accurately excises its own fragments during enzymatic cleavage without leaving fragments (Fraser *et al.*, 1996). Studies have confirmed that the transposition activity of PB in mammalian cell lines is significantly higher than that of Sleeping Beauty (SB) and Tol2 (Wu *et al.*, 2006); therefore, we used PB 513B-1 as the transfection plasmid to establish a Sertoli cell line (PB-GDNF-Sertoli cells).

When experimental (PB-GDNF-Sertoli) and control group (Sertoli with human GDNF) cells were separately cultured with spermatogonia, we ensured that the GDNF contents in the two groups were consistent. However, the results showed that the experimental group could better maintain the buffalo spermatogonia biomarkers *in vitro* (Fig. 8), which indicated that buffalo PB-GDNF could not only maintain SSC pluripotency but also enhance buffalo SSC proliferation.

Mounting experimental evidence suggests that functional differences exist between different species of the same gene, which may be due to differences in its mRNA sequence or posttranslational modification of its proteins (Heemskerk *et al.*, 2011; Nunomura *et al.*, 2010; Schnerwitzki *et al.*, 2014). The current research results showed differences in the functions of buffalo- and human-derived GDNF. Endogenous GDNF secreted from genetically modified buffalo Sertoli cells could promote the proliferation of buffalo spermatogonia and transcription of specific marker genes more effectively than human-derived GDNF. However, the similarity of the buffalo and human GDNF genes was high (91%), and whether the functional differences in the buffalo endogenous GDNF and human GDNF were caused by sequence differences or by different posttranslational modifications needs further confirmation. Based on these experimental results, improving the efficiency of SSC culture *in vitro* by constructing Sertoli cell lines overexpressing GDNF as the feeder layer is feasible.

In conclusion, by cloning the buffalo GDNF gene, we found that GDNF is highly conserved among bovine animals. The PB transposon allowed the GDNF gene to be stably integrated into buffalo Sertoli cells for sustained expression, and we successfully constructed a Sertoli cell line (PB-GDNF-Sertoli cells) that stably expressed endogenous GDNF. Compared with that in the control group, the GDNF gene expression in PB-GDNF-Sertoli cells was significantly increased at both the transcriptome and protein levels. In the cocultivation stage, the appropriate phenotype, self-renewal and proliferation of buffalo spermatogonia *in vitro* were relatively better maintained by endogenous GDNF than by xenogenic GDNF. Endogenous GDNF was superior to xenogenic GDNF in the culture of spermatogonia *in vitro*.

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Statement of conflict of interest

The authors declare no conflict of interest.

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