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Full Length Research Paper

Construction of a mammalian cell expression vector pAcGFP-FasL and its expression in bovine follicular granulosa cells

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Fas ligand (FasL) is a cytokine that may be secreted or expressed as a transmembrane ligand at the cell surface, and induces apoptosis by binding to the Fas. Ovarian follicular atresia and luteolysis are thought to occur by apoptosis. To reveal the intracellular signal transduction molecules involved in the process of follicular development in the bovine ovary, the Fas ligand gene was cloned using RT-PCR. By deleting the stop codon, the amplified Fas ligand gene was directionally cloned in frame into the eukaryotic expression vector pAcGFP-N1. The pAcGFP-bFasL recombinant plasmid was then transfected into bovine follicular granulosa cells by using lipofectamine 2000. Expression of AcGFP was observed under fluorescent microscopy and the transcription and expression of *Fas ligand* was detected by RT-PCR and Western-blot. The results show that the pAcGFP-bFasL recombinant plasmid was successfully constructed. AcGFP expression was detected as early as 24 h after transfection and the percentage of AcGFP positive cells reached about 68%. As expected, a 847 bp fragment was amplified by RT-PCR and a 59 kD target protein was detected by Western-blot from the transfected cells. This study will thus serve as a valuable tool in understanding the mechanism of regulation of *Fas ligand* on bovine oocyte formation and development.

Key words: Fas ligand, apoptosis, follicular granulosa cell.

INTRODUCTION

Morphological and biochemical studies have shown that the demise of both somatic and germ cells in the ovary is mediated by apoptosis (Morita et al., 1999). Coordination between oocyte and granulosa cells is an essential prerequisite to normal follicular development (Quirk et al., 2001). Studies in rat and bovine granulosa cells have demonstrated that cell–cell contact plays a vital role in inhibiting granulosa cell apoptosis and regulating proliferation (Lai et al., 2000). Hence, a role for gap and tight junctions between granulosa cells and oocytes in preventing granulosa cell apoptosis has been proposed.

Apoptosis is an important process that maintains appropriate cell numbers by killing excess cells (Gjorret et al., 2005). The Fas ligand (FasL)/Fas pathway is an important pathway of apoptosis that controls cell proliferation and tissue remodeling (Hsu and Kuo, 2008; Vij et al., 2004). Fas is a transmembrane protein of the TNF/nerve growth factor super family that is expressed on both immune and non-immune cell types (Porter et al., 2000). Fas, when bound by FasL, activate a signal transduction pathway that eventually results in apoptosis of the cell.

Bovine Fas ligand (FasL) is a 31 kDa type II membrane protein of 277 amino acids and belongs to the tumor necrosis factor ligand family (Taniguchi et al., 2002; Townson et al., 2006). In the bovine ovary, the Fas/FasL system may be regulated by gonadotropin-dependent mechanisms and may play a role during attrition, follicular

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regression, and atresia, as evidenced by the expression of Fas antigen in oocytes from fetal and adult ovaries and in granulosa cells during the luteal phase. FasL also mediates apoptosis in human granulosa luteal cell cultures when these cells are pretreated with the Th1 cytokine, interferon-gamma (IFN-g) (Chen et al., 2005). Hence, it can regulate the development of oocytes, to maintain the equilibrium state of follicular development (Moniruzzaman et al., 2007; Tourneur et al., 2003). This reveals that Fas ligand plays an important role in the regulation of oogenesis.

In the present study, we have inserted the cloned *Fas ligand* gene into the eukaryotic expression vector pAcGFP-Nl, successfully constructed fusion protein recombinant plasmid pAcGFP-bFasL and transfected it into follicular granulosa cells. It could provide technical support for the basic research on the regulation of Fas ligand on bovine oogonium development, and also important for further research.

MATERIALS AND METHODS

Collection of bovine ovaries

Bovine ovaries were collected at a local abattoir and froze rapidly in liquid nitrogen and then brought back to the laboratory.

Extraction of total RNA and cDNA synthesis

Total RNA was extracted from bovine ovary using a TRIzol kit (Intrivogen Corporation, Carlsbad, California, USA), OD values were measured by the use of UV spectrophotometer (PGeneral, Beijing, China) and the RNA (OD_{260} / OD_{280} > 1.8) was chosen and then reverse-transcribed using cDNA synthesis reverse transcription kit (Takara, Dalian,China) to synthesize cDNA.

Gene cloning and sequence analysis

According to the *Fas ligand* gene total length sequence (GenBank accession number: BankIt 1468310 Seq1 JN380921), a pair of primers was designed: forward 5'TCTGGCCTTTGACA CCTG 3' and reverse 5'CCTCCTGGTTCATGTCTTCG 3'. PCR amplification cycles were performed as follows: 94°C for 90 s; 35 cycles of 94°C for 30 s, 55.5°C for 30 s, and 72°C for 1 min; and a final extension period at 72°C for 10 min. PCR products were run by electrophoresis in 1.5% (w/v) agarose gels and stained with ethidium bromide. Next the PCR products were purified and recovered using the agarose gel DNA recovery kit (Tiangen, Beijing, China). The purified *Fas ligand* genes were ligated with pMD19-T vector (Takara, Dalian, China) and then the ligation products were transformed into DH5α competent cells. The positive clones were picked out and shaken overnight at 37°C and then a random analysis of 8 clones with PCR and sequencing was conducted at SinoGenoMax Company (Beijing, China).

Construction of a mammalian cell expression vector for pAcGFP - bFasL fusion protein

According to the restriction enzyme mapping of ORF fragments of bovine *Fas ligand* and multiple cloning sites of pAcGFP-N1 vector (Clontech, Mountain View, CA, USA), *Bgl*II and *Eco*RI were chosen as cloning sites Primers at the two ends of the Fas ligand open reading frame were designed with a BglII restriction site and four protective bases inserted before the ATG start codon in the upstream primer. A Kozak sequence was also included to increase the inserted gene expression level in eukaryotic cells. The forward primer was designed as follows: 5'ACTAAGATCTGCCACCAT GCAGCAGCCCTTGA A3' (AGATCT is *Bgl*II enzyme site, while GCCACCATG is Kozak sequence). For the reverse primer, the stop codon TAA was deleted and an EcoRI restriction site was inserted. The *Fas ligand* open reading frame should be in frame with the downstream AcGFP gene sequence to ensure coexpression with the fusion protein. The reverse primer was designed as follows: 5'ACTAGAATTCCGAGTTTATATAAGCCAAA 3' (GAATTC is *Eco*RI enzyme site).

In order to improve the amplification efficiency, the full length coding region of the bovine *Fas ligand* gene was amplified by touchdown PCR (TD-PCR) from the plasmid template, and PCR cycles were performed as follows: 94°C for 90 s; 5 cycles of 94°C for 30 s, 69°C for 30 s, and 72°C for 1 min; 5 cycles of 94°C for 30 s, 67°C for 30 s and 72°C for 1 min; 28 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; and a final extension period at 72°C for 10 min.. The PCR product was recovered and cloned into pMD19-T Simple vector, and then it was transformed into DH5α competent cells. The positive clones were picked out and shaken overnight at 37°C. Plasmids were extracted from sense colonies using TIANprep Mini Plasmid Kit (Tiangen, Beijing, China) and digested with *Bgl*II and *Eco*RI enzymes (Takara). A cDNA fragment of 847 bp was recovered and directly ligated to the AcGFP-N1 eukaryotic expression vector that was previously digested with *Bgl*II and *Eco*RI enzymes, and transformed into DH5α competent cells. The positive clones were picked out and shaken overnight at 37°C.

Identification of recombinant plasmid pAcGFP-bFasL

After random analysis of 10 clones with PCR, plasmids were extracted from sense colonies and digested with *Bgl*II and *Eco*RI enzymes to confirm the expression of the bovine Fas ligand. The DNA sequence of the ORF was determined using an automatic DNA sequencer (ABI Prism 310, Foster, CA, USA). All of these procedures were performed according to the manufacturer's instructions. The Recombinant Plasmid pAcGFP-bFasL was amplified in DH5α cells, and then the EndoFree Plasmid was extracted from the sense colonies using the EndoFree Plasmid Kit (Tiangen), and stored at -20°C.

G418 cytotoxicity test for follicular granulosa cells

Follicular granulosa cells were obtained from the Cell Center of Chinese Academy of Medical Sciences. The cells were plated on 24-well culture plates (Falcon, Franklin Lakes, NJ, USA) and incubated in a CO_2 incubator (Thermo, Marietta,Ohio,USA) at 37°C for 24 h, with 5% CO_2 in the air. After 24 h of culture, the DMEM medium (GIBCO, Invitrogen, Carlsbad, California, USA) supplemented with 10% (V/V) fetal bovine serum (GIBCO) and 1% (V/V) L-glutamine (GIBCO) was replaced with DMEM medium containing different concentrations of G418 (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µg/ml; Sigma, St. Louis, MO, USA). Cells were incubated at 37°C in 5% CO_2, and the media was replaced every 72 h for two weeks of observation. The optimum concentration of G418 as a selection agent for follicular granulosa cell was found to be the lowest concentration, under which all of the cells were killed 10 to 14 days after culture in DMEM with G418. We determined this concentration to be 600µg/ml. After the cells spread out fully, positive clones were reselected using 600 g/ml of G418.

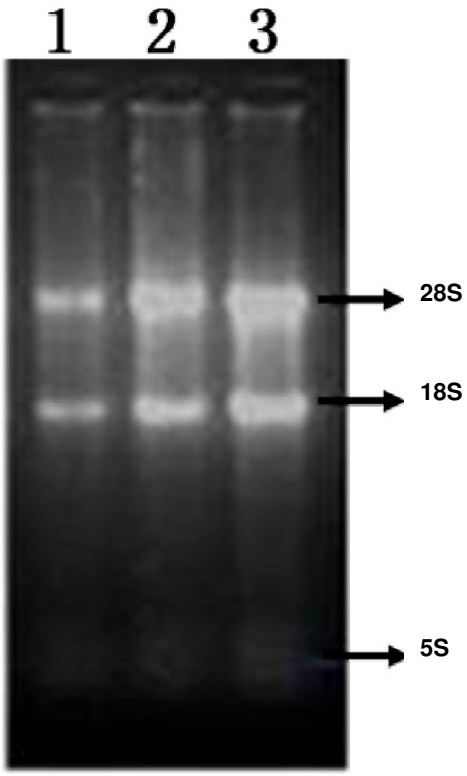

Figure 1. The result of total RNA from bovine ovary.

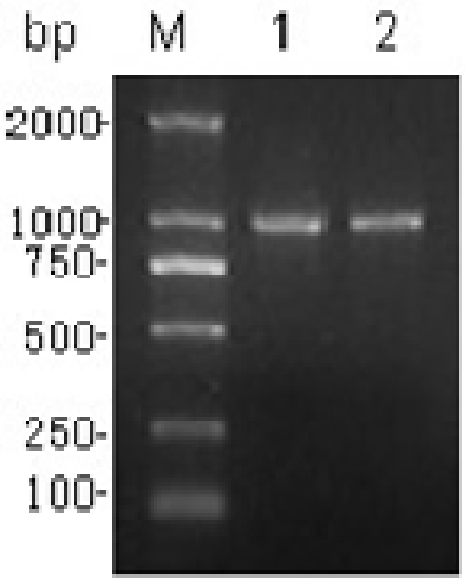

Figure 2. The product of bovine *Fas ligand* gene. The cDNA from bovine ovary acted as template, *Fas ligand* forward primer and FasL reverse primer were used to amplify the FasL fragment. Total volume of the reaction was 20 µL. A 1037 bp fragment was detected by electrophoresis on 1.2% agarose gel. M, DNA Marker DL 2000; lanes 1 and 2: cDNA of bovine *Fas ligand*.

Finally, cell clones which could stably express bovine *Fas ligand* gene were chosen for subsequent analysis.

Transfection and fluorescence detection of fusion protein

One day before transfection, 0.5-2 × 10^5 follicular granulosa cells were plated in 500 µL of growth medium without antibiotics per well of a 24-well culture plate (Falcon). When the cells reached more than 90% confluency, the growth medium (10% (V/V) fetal bovine serum, 100 U/ml Penicillin-Streptomycin (GIBCO) and 1% (V/V) L-glutamine) was replaced by Opti-MEM serum-free media (GIBCO). For transfection, DNA was diluted in 50 µL Opti-MEM serum-free media, and then mixed gently with Lipofectamine™ 2000 (GIBCO) before use, and the appropriate amount was diluted in 50 µL of Opti-MEM serum-free media and incubated for 5 min at room temperature. After 5 min of incubation, the diluted DNA was combined with diluted Lipofectamine™ 2000 (total volume = 100 µL) and was mixed gently and incubated for 20 min at room temperature. 100 µL DNA-lipofectamine 2000 mixture was added to each well containing the cells and medium. The cells were incubated at 37°C in a CO_2 incubator for 4 to 6 h, and then the medium was changed to growth medium. The cells were put in a 1:10 or higher dilution of fresh growth medium 24 hours after transfection. The positive cell clones were screened using G418. Twelve hours later, the expression of AcGFP in the cells was observed under a fluorescence microscope (NikonTE2000, Japan) and the numbers of AcGFP-positive cells were counted under high power magnification every 24 h.

Analysis of bovine Fas ligand by RT-PCR and western-blotting

To confirm the insertion of a bovine *Fas ligand* open reading frame, the cells were harvested after a stable transfection screening with G418. mRNA was extracted from the cells using Quickprep Micro mRNA Purification Kit (Invitrogen), and then reverse-transcribed to synthesize the cDNA. The primer for amplification of partial cDNA sequence of bovine Fas ligand was designed as follows: forward 5' ACTAAGATCTGCCACCATGCAGCAGCCCTTGAA 3' and reverse 5' ACTAGAATTCCGAGTTTATATAAGCCAAA 3'. PCR cycles were performed as follows: 94°C for 90 s; 5 cycles of 94°C for 30 s, 69°C for 30 s, and 72°C for 1 min; 5 cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 1 min; 28 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; and a final extension period at 72°C for 10 min.

The other cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), treated with 10% (V/V) trichloro acid (Wako Pure Chemical Industries, Osaka, Japan) at 4°C for 30 min, and scraped off. These cells were then suspended in UTD buffer (9 mol/L urea (Wako), 2% (V/V) triton X-100 (Sigma), and 1% (W/V) (±)-dithiothreitol (Wako)) and 2% (W/V) lithium dodecyl sulfate (Wako). The whole cell lysate was separated by 15% (W/V) gradient sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Red laboratories Inc, USA). The PVDF membranes were stained with a 0.2% (W/V) Ponceau-S solution (Sigma) at 25°C for 1 min and then immersed in blocking solution (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% (V/V) Tween-20 containing 5% (W/V) skim milk (Sigma)) for 30 min. They were then incubated with rabbit anti-bovine Fas ligand polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at 4°C for 12 h. After a wash with blocking solution, they were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Golden Bridge, Beijing, China) at 25°C for 1 h. Chemiluminescence was visualized using an ECL system (Applygen Technologies Inc, Beijing, China) according to the manufacturer's direction.

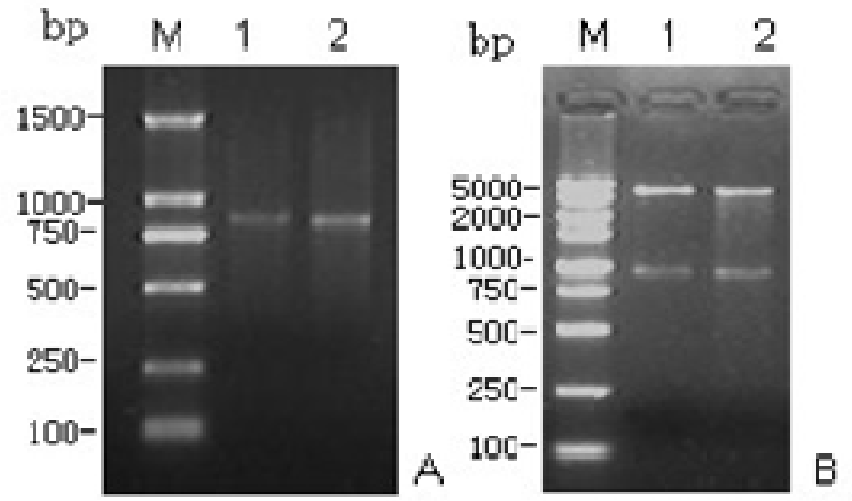

Figure 3. Construction and identification of pMD19T-bFasL. The pT-bFasL plasmid acted as template, specificity primers were used to amplify the *Fas ligand* coding region with *Bgl*II / *Eco*RI site. A 847 bp fragment was detected by electrophoresis. The *Fas ligand* coding region was cloned into the pMD19-T Simple vector, then transformed into DH5a and the plasmids were extracted from positive clones and digested with *Bgl*I and *Eco*RI enzymes (Takara) for 6 h at 37°C following the supplier's direction. **A:** Result of bovine *Fas ligand* gene with *Bgl*I and *Eco*RI cloning sites by PCR (M, DNA Marker DL 2000; lanes 1 and 2 represents cattle *Fas ligand*). **B:** Identification of pT-bFasL (M, DNA Marker DL 5000; lanes 1 and 2, pT-bFasL plasmid digestion by restrictive enzyme *Bgl*II / *Eco*RI).

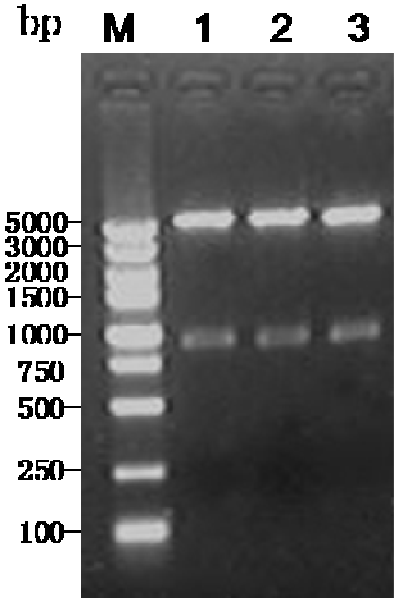

Figure 4. Identification of recombinant plasmid pAcGFP-bFasL by restriction enzyme digestion. The restriction fragments of *Bgl*I / *Eco*RI was cloned into the pAcGFP-N1 vector then transformed into DH5a,the plasmids were extracted from positive clones and digested with *Bgl*I and *Eco*RI enzyme(Takara) for 6 hours at 37°C following the supplier's direction. M, DNA Marker DL 5000; lanes 1, 2 and 3 represent pAcGFP-bFasL digestion by restrictive enzyme *Bgl*I / *Eco*RI.

RESULTS

Bovine Fas ligand gene cloning and sequence analysis

The experimental results showed that a cDNA fragment with a molecular size of about 1037 bp was obtained by RT-PCR amplification, consistent with the expected fragment size (Figures 1 and 2). Using T/A cloning, positive clones were randomly chosen and the double stranded cDNAs were sequenced. The length of one clone was 1037 bp, which contained an ORF of 834 bp (277 amino acids). The alignment results showed that the amplified sequence for bovine Fas ligand had 100% homology with that reported in GenBank (NCBI).

Construction and identification of a eukaryotic expressing vector of fusion gene bFas-pAcGFP

The 847 bp coding region of the *Fas ligand* gene was amplified from pT-bFasL plasmid with specific primers by TD-PCR (Figure 3). The expected fragments were obtained by complete digestion of the PMD19-T-FasL plasmid, which was extracted from the transformed positive clones and digested using *Bgl*II and *Eco*RI. The target gene fragment was successfully connected to the 5' end of the AcGFP cDNA, which was confirmed to have the *Fas ligand* reading frame aligned with AcGFP. The 847 bp fragments were obtained by complete digestion of

Table 1. Cytotoxicity test of G418 to cultured follicular granulosa cells for 12 days.

G418 concentration (µg/ml)	100	200	300	400	500	600	700	800	900	1000
Survival rate (%)	+++	++	++	+	+	-	-	-	-	-

+++ = Survival rate of 80%; ++ = survival rate of 50%; + = survival rate of 30%; - = survival rate of 0%.

the recombinant plasmid pAcGFP-bFasL, which was extracted from the transformed positive clones using *Bgl*II and EcoRI (Figure 4).

The sequence analysis showed that the bovine *Fas ligand* gene was successfully cloned into *Bgl*II / *Eco*RI site of the pAcGFP-N1 vector. The authors confirmed that the *Fas ligand* coding region sequence and the AcGFP gene sequence had the same reading frame. This was achieved through deleting the stop codon TAA and inserting the C base, such that the target gene and fusion protein gene could be expressed at the same time. The reconstructed plasmid was named the pAcGFP-bFasL vector.

Determination of the minimum dose of G418 for follicular granulosa cells

After three days of selection with different concentrations of G418, the cells were found to be in various degrees of death, with the number of floating and broken cells increasing in treatments supplemented with higher than 600 µg/ml G418. Peak mortality was in the eighth to tenth day exposure duration, and cells treated with 600 µg/ml G418 or more were dead by the tenth day. The concentration of 600 µg/ml was therefore considered as the minimum dose of G418 for follicular granulosa cells to cause cell death (Table 1).

Transfection of follicular granulosa cells with pAcGFP-bFasL plasmid and G418 selection

The positive charge of the cationic liposome's surface and the phosphate backbone of pAcGFP-bFasL plasmid DNA stably combine by electrostatic interaction to form the DNA-liposome complex. The complex is adsorbed to the cell membrane with the negative charge and then the DNA complex transfers into the cells and forms the inclusion bodies in the cytoplasm by fusion, osmosis of cytomembrane and endocytosis. The DNA-liposome complex transfers into cells and the anionic lipid of the membrane diffuses into the complex because the membrane loses its electrostatic balance. The anionic lipid of the membrane then combines with the positive ions of cationic liposomes, forming the neutral ion pair, so that the pAcGFP-bFasL plasmid DNA break away from the DNA-liposome complex, enter the cytoplasm, and then enter the nucleus through the nuclear pore. Finally, the bovine *Fas ligand* gene encoding protein is produced by transcription and expression in the nucleus.

Cells transfected with the pAcGFP-bFasL plasmid by lipofectamine 2000 were screened with G418 up to the fourteenth day. The negative control cells were all dead, but cell clones formed in experimental conditions. Subsequently, the maintaining dose of G418 (600 ug/ml) was used to the 18th day, when all cell degeneration and necrosis disappeared and the resistant cells formed positive clones and gradually proliferated. The expression of AcGFP was detected in the plasma and nucleus using fluorescent microscopy (Figure 5). More also, detection of green fluorescence in the cells showed that the untransfected cells appropriately lacked fluorescence, whereas expression of AcGFP was discretely observed in the nuclei of follicular granulosa cells transfected with pAcGFP-bFasL; uniform cellular distribution of AcGFP expression was detected in the pAcGFP-N1 transfection group (Figure 6).

RT-PCR analysis of monoclonal cell strains following selection with G418

The RNA of the monoclonal cells screened by G418 was extracted. A bright 847 bp fragment was amplified in the pAcGFP-bFasL transfected follicular granulosa cells by RT-PCR, whereas the 847 bp strap was weak in the pAcGFP-N1 transfected cells and the negative control cells (Figure 7). The results show effective expression of *Fas ligand* in the pAcGFP-bFasL transfected follicular granulosa cells, suggesting that the pAcGFP-bFasL successfully transfected the follicular granulosa cell.

Evaluation of expression product by SDS-PAGE electrophoresis and Western blot analysis

SDS-PAGE analysis indicated that the fusion protein of AcGFP-bFasL was expressed in pAcGFP-bFasL transfected cells and its molecular weight was about 59 kD (Figure 8a, lanes 3 and 4). No expression of the fusion protein of AcGFP-bFasL was detected in pAcGFP-N1 transfected cells or negative control cells (Figure 8A, lanes 1 and 2). These results serve as preliminarily evidence that follicular granulosa cells transfected with AcGFP expression vectors of the bovine *Fas ligand* gene are capable of expressing fusion target proteins. The expressed fusion protein showed specificities of FasL polyclonal antibody, as proved by Western blot, and further proved to be an immunocompetent protein (Figure 8B). Fas ligand is known to induce apoptosis of ovarian granulosa cells and then make the follicular atresia, so it

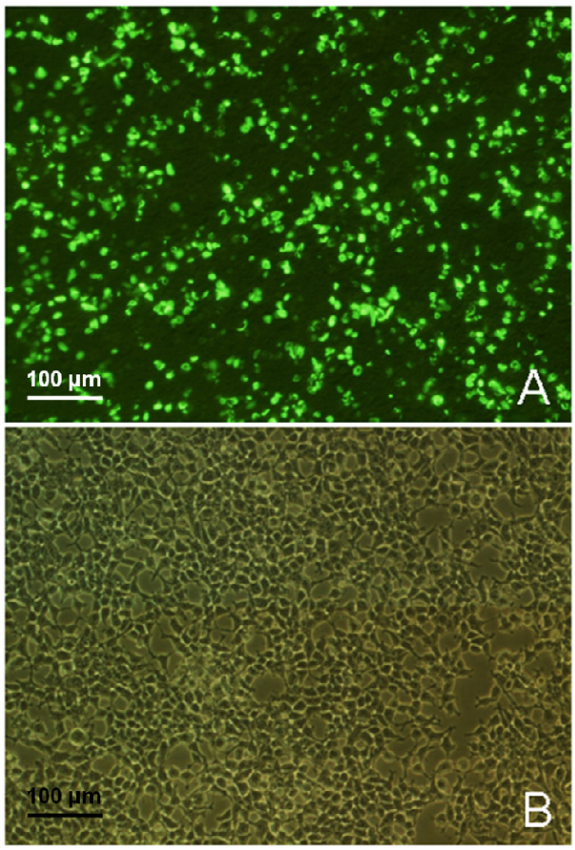

Figure 5. Sequence analysis of recombinant expression vector pAcGFP-bFasL. The pAcGFP-bFasL plasmid was transfected into follicular granulosa cells mediated by Lipofectamine 2000. After transfection, green fluorescent was observed by fluorescent microscopy. The expression rates of green fluorescence in follicular granulosa cells was 68% at 24 h after transfection. **A:** Transfected follicular granulosa cells by pAcGFP-bFasL under fluorescent microscope. **B:** Transfected follicular granulosa cells by pAcGFP-bFasL under visible light. Scale bar 100 μm.

could maintain the equilibrium state of bovine follicular development.

DISCUSSION

Apoptosis is an important phenomenon involved in cell survival and death during differentiation and development (Yamauchi et al., 2007; Yan et al., 2001). The death ligand and receptor systems are considered to be apoptosis-inducing factors (Arican and Ilgar, 2009). Apoptosis can be mediated by caspase-8 activation via the extrinsic or death receptor- mediated pathway, resulting in formation of the death-inducing signaling complex (DISC) containing the adapter molecule FADD and procaspase 8 (Whitley et al., 2006; Yang et al., 2008).

A previous study performed in our lab using an analyzing expression map showed that bovine *Fas ligand* mRNA is highly expressed in lymphoid tissue, ovaries and testes, compared to other tissues. This suggests that *Fas ligand* expression in the lymphoid tissue plays an important role in keeping the bovine immune environment stable. Fas in testicular germ cells and ovary oocytes interact with *Fas ligand* in sertoli cells and follicular granulosa cells, an interaction that could keep the spermatogenesis and oogenesis balanced. During the development of the bovine oocytes, the Fas/FasL pathway induces apoptosis of ovarian granulosa cells by initiating an apoptosis signal, leading to follicular atresia. Gene mutation or abnormal expression of *Fas ligand* in the reproductive system can lead to an internal environment disorder and abnormal spermatogenesis and oogenesis, which could subsequently cause

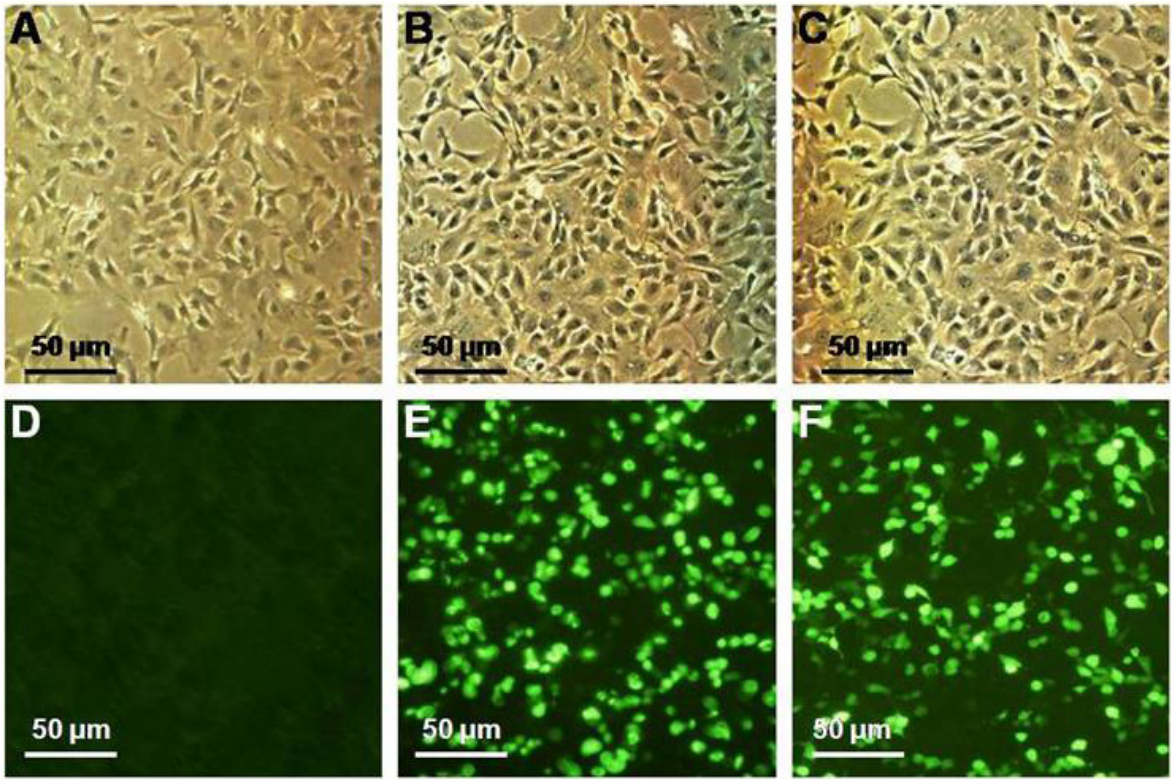

Figure 6. The green fluorescence positive cells after transfected with pAcGFP-bFasL plasmid. After transfection, the green fluorescence could be detected in follicular granulosa cells transfected by pAcGFP-bFasL and pAcGFP-N1 plasmid, while there was no AcGFP expression in follicular granulosa cells untransfected by any plasmid. AcGFP could be observed in the nucleus and its lateral region in pAcGFP-bFasL transfection group and uniform distribution throughout on whole cell in pAcGFP-N1 transfection group. **A, B** and **C** shows transfected follicular granulosa cells under visible light, while **D, E** and **F** shows transfected follicular granulosa cells under fluorescent microscope. **A** and **D** represent the control group; **B** and **E:** pAcGFP-bFasL transfection group; **C** and **F:** pAcGFP-N1 transfection group. Scale bar 50 μm.

oligzoospermous or aspermia in bulls and reduce a cow's ovulation and conception rate.

When the authors constructed the eukaryotic expression vector for the pAcGFP-bFasL fusion protein, the authors took advantage of directional cloning, by introducing *Bgl*II(AGATCT) and *Eco*RI(GAATTC) at two sites in the upstream primer and downstream primer, respectively. These two restriction enzymes produced different 3'cohesive ends, which allowed the target gene to be directionally connected to vector. The benefits of this method are as follows: i) the vector fragment could not be cyclized since the vector's two cohesive ends did not complement each other, so there were few false positive recombinant clones; ii) because the foreign bovine *Fas ligand* gene was inserted into recombinant plasmid in one direction, it was not necessary to screen for the right connection; and iii) restriction enzyme sites were preserved, which was beneficial for further identification. In addition, the Kozak sequence was introduced after the upstream primer's *Bgl*II site to promote transcription and translation efficiency of the *Fas ligand* gene in the recombinant plasmid (Michelon et al., 2003; Moshfegh et al., 2000).

pAcGFP-bFasL was transfected into follicular granulosa cell, with a transfection efficiency reaching 68%. After screening for two weeks using 600 μg/ml of G418 (Vanhamme et al., 2007), the positive clones emitted fluorescence, indicating that the bovine *Fas ligand* gene was completely inserted into the follicular granulosa cell genome and the fusion protein was stably expressed. The molecular weight of the green fluorescent protein was 28 kD and the bovine Fas ligand's molecular weight was 31 kD, so the fusion protein's molecular weight was about 59 kD, consistent with the detection using SDS-PAGE electrophoresis and Western blotting. Furthermore, Fas ligand's antibody binding to the NC membrane showed a specific reaction with the fusion protein, indicating that the follicular granulosa cells transfected with pAcGFP-bFasL highly expressed the immune-competent Fas ligand protein. Additional variables, such as decreasing the concentration of colored solution, increasing the rinse time, increasing the buffer volume, and shortening exposure time, could improve the protein immunoblotting ECL development effect.

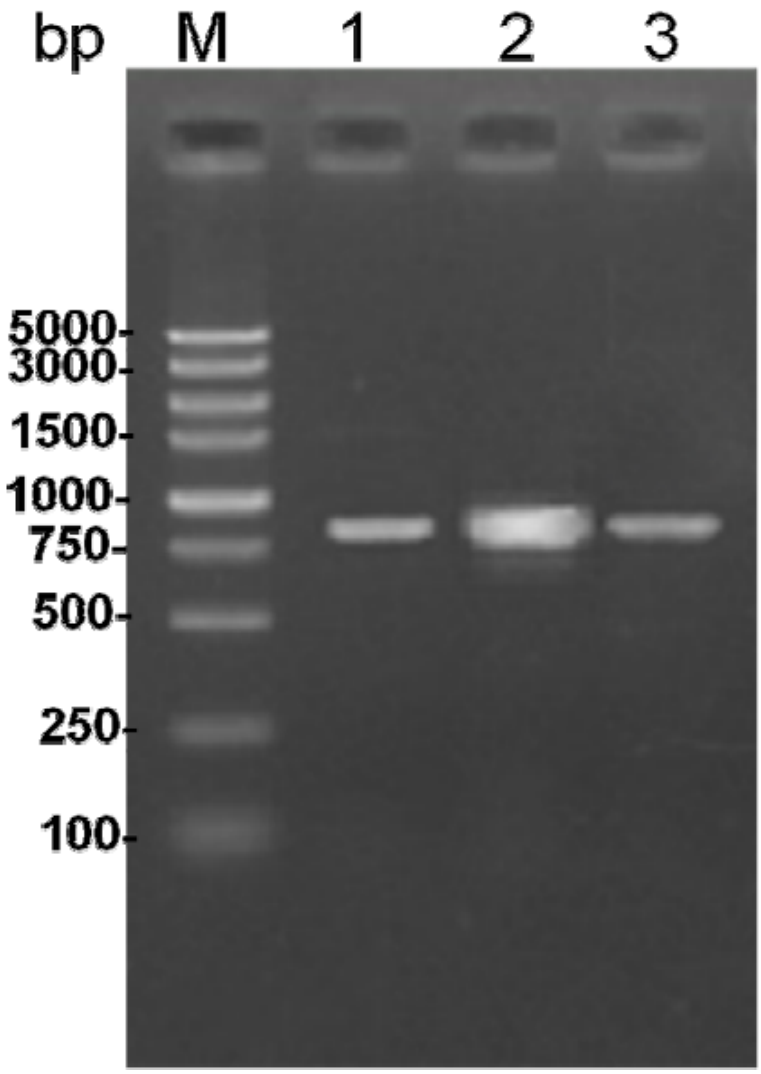

Figure 7. The expression of bovine *Fas ligand* mRNA on follicular granulosa cells determined RT-PCR.Total RNA was extracted from follicular granulosa cells and cDNA was prepared using universal primer. Specificity primers were used to amplify the *Fas ligand* sequence and a bright 847 bp fragment was detected by electrophoresis on 1.2 % agarose gel in pAcGFP-bFasL transfection group. M, DNA Marker DL 5000; lane 1 represents control group; lane 2: pAcGFP-bFasL transfection group and lane 3 represents pAcGFP-N1 transfection group.

This research was designed to study the mechanism of bovine oogonium's proliferation and differentiation. *Fas ligand* was inserted into pAcGFP-N1's N end and the fusion protein was expressed in the pAcGFP-N1 vector driven by the CMV promoter, which is thought to improve the expression level of *Fas ligand* in eukaryotic cells while keeping its structure and function unchanged. The AcGFP reporter protein can be detected 8-12 h after transfection, and fluorescence detection is stable for a long time (Itoh et al., 2010). AcGFP, as pAcGFP-bFasL's reporter gene, may improve transfection efficiency and reduce cell death. It may also be beneficial for environment regulation and simulation of oocyte gene expression *in vivo*, which could be applied to study the regulation of *Fas ligand* on differentiation and proliferation of oogonium at the gene level.

In conclusion, by fusing the bovine *Fas ligand* gene to the AcGFP gene, the mammalian expression vector of the pAcGFP-bFasL fusion protein was constructed and found to be highly expressed in transfected follicular granulosa cells. This method could provide technical support for basic research on the regulation of Fas ligand on bovine oogonium development and become important for further research in the field of bovine development

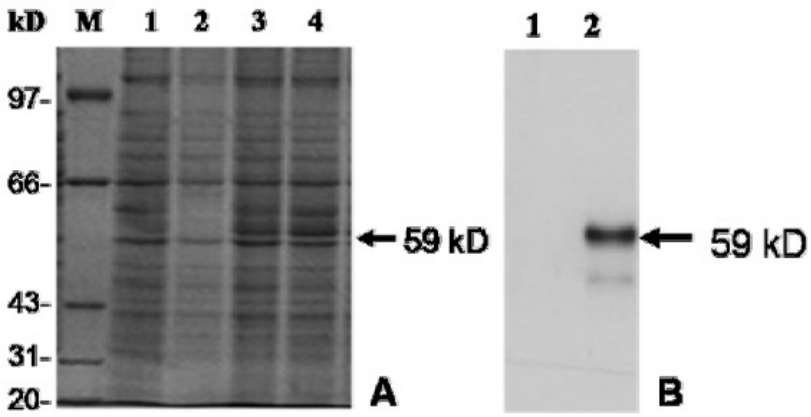

Figure 8: Figure.8 The expression of AcGFP-bFasL fusion protein and AcGFP protein in follicular granulosa cells after transfection. Protein sample were loaded onto 15% SDS-PAGE to separate protein and transferred to nylon cellulose membrane. The membrane was probed with anti bovine Fas ligand polyclonal antibody and then was probed with perxidase-conjugated goat anti-rabbit polyclonal antibody as the second antibody.Bound antibodies were detected with the enhanced chemiluminescence(ECL) method.**Figure 8A:** M. Protein molecular weight marker(MW marker);1.cell lysate of follicular granulosa cells of control group; 2.cell lysate of follicular granulosa cells of pAcGFP-N1 transfection group;3,4. cell lysate of follicular granulosa cells of pAcGFP-bFasL transfection group; **Figure 8B:** 1.western blot analysis of pAcGFP-N1 transfection group;2.western blot analysis of pAcGFP-bFasL transfection group.

and reproduction.

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