Asian-Aust. J. Anim. Sci.
Vol. 23, No. 3 : 401 - 409
March 2010

www.ajas.info

Construction of Mammalian Cell Expression Vector for pAcGFP-bFLIP(L) Fusion Protein and Its Expression in Follicular Granulosa Cells*

Run Jun Yang[1, 2], Wu Feng Li[3], Jun Ya Li[1], Lu Pei Zhang[1], Xue Gao[1], Jin Bao Chen[1] and Shang Zhong Xu[1, **]

[1] Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Yuanmingyuan West Road no.2, Haidian District, Beijing 100193, China

ABSTRACT : FLICE inhibitory protein (FLIP) is one of the important anti-apoptotic proteins in the Fas/FasL apoptotic path which has death effect domains, mimicking the pro-domain of procaspase-8. To reveal the intracellular signal transduction molecules involved in the process of follicular development in the bovine ovary, we cloned the c-FLIP(L) gene in bovine ovary tissue with the reverse transcription polymerase chain reaction (RT-PCR), deleted the termination codon in its cDNA, and directionally cloned the amplified c-FLIP(L) gene into eukaryotic expression vector pAcGFP-N1, including AcGFP, and successfully constructed the fusion protein recombinant plasmid. After identifying by restrictive enzyme *Bgl*II/*Eco*RI and sequencing, pAcGFP-bFLIP(L) was then transfected into follicular granulosa cells, mediated by Lipofectamine 2000, the expression of AcGFP observed and the transcription and expression of c-FLIP(L) detected by RT-PCR and Western blot. The results showed that the cattle c-FLIP(L) was successfully cloned; the pAcGFP-bFLIP(L) fusion protein recombinant plasmid was successfuly constructed by introducing a *Bgl*II/*Eco*RI cloning site at the two ends of the c-FLIP(L) open reading frame and inserting a Kozak sequence before the start codon. AcGFP expression was detected as early as 24 h after transfection. The percentage of AcGFP positive cells reached about 65% after 24 h. A 1,483 bp transcription was amplified by RT-PCR, and a 83 kD target protein was detected by Western blot. Construction of the pAcGFP-bFLIP(L) recombinant plasmid should be helpful for further understanding the mechanism of regulation of c-FLIP(L) on bovine oocyte formation and development. (**Key Words :** c-FLIP(L), pAcGFP-N 1, Recombinant Plasmid, Follicular Granulosa Cell)

INTRODUCTION

FLICE inhibitory protein (FLIP) is one of the important antiapoptotic proteins that have been shown to be expressed in many species such as virus, eucaryote, mammal (Carsten et al., 1999; Zhang et al., 2004). The bovine c-FLIP(L) gene is located on chromosome 2, and contains 9 exons,which encode 585 amino acids. c-FLIP has two death-effector domains in tandem result from alternative mRNA splicing: a short, 26-kD protein ($FLIP_S$) and a long, 55-kD form ($FLIP_L$). The short form of c-FLIP contains two death effector domains (DEDs) found on the death receptor adaptor protein FADD and the domain of procaspase-8. The long form of c-FLIP shares significant homology with caspase-8 (FLICE), contains an additional death effector domain, but lacks the catalytic active site of the caspases and does not have protease activity (Szperka et al., 2006).

c-FLIP has been identified as a downstream key inhibitor of Fas-mediated apoptosis by virtue of its structural, albeit not functional, similarity to caspase-8, since it lacks a caspase activating unit. Thus, c-FLIP prevents recruitment of procaspase-8 to the death-inducing signalling complex, with consequent abrogation of procaspase-8 autoproteolytic cleavage culminating in activation of the caspase cascade and cell death (Zhang et al., 2005; Zou et al., 2007).

During the development process of the bovine oocytes, Fas/FasL pathway induce apoptosis of ovarian granulosa

* The work was supported by the National High Technology Research and Development Program (863 Program, no. 2008 AA101010) and National Technology Supporting Scheme "Breeding Project of Animals and Plants" during the 11th Five Year Plan Period (no.2006BAD01A10).

** Corresponding Author: Shangzhong Xu. Tel: +86-10-6289 0940, Fax: +86-10-62817806, E-mail: simmenta@vip.sina.com

[2] College of Animal Science and Veterinary Medicine,Jilin University,Jilin Changchun 130062, China.

[3] College of Life Science, Shanxi Agricultural University, Taigu, Shanxi 030801, China.

Received March 21, 2009; Accepted October 8, 2009

cells by apoptosis signal,and then make the follicular atresia.While,c-FLIP completely block Fas-mediated apoptosis signal of ovarian granulosa cells through inhibition of caspase-8 processing at the DISC. So it can induce proliferation of ovarian granulosa cells and promote the development of oocytes, to maintain the equilibrium state of follicular development (Giampietri et al., 2006). It reveals that c-FLIP plays an important role in the regulation of oogenesis.

In this study, the authors inserted the cloned c-FLIP(L) gene into the eukaryotic expression vector pAcGFP-N1, and successfully constructed fusion protein recombinant plasmid pAcGFP-bFLIP(L), and then transfected it into the follicular granulosa cell. It could provide technical support for the basic research on regulation of c-FLIP(L) on the bovine oogonium development, and be important for further research.

MATERIALS AND METHODS

Collection of bovine ovaries

Bovine ovaries were collected at a local abattoir and frozen rapidly in Liquid Nitrogen and then brought back to laboratory.

Extraction of total RNA and cDNA synthesis

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

Gene cloning and sequence analysis

According to the c-FLIP(L) gene total length sequence(GenBank accession number: NM_001012281) a pair of primers was designed, forward 5'-TTCCTTGGAATGACACTGTA-3' and reverse 5'-CTTTTTATTTGTGAGAGAGG-3'. PCR amplification cycles was performed as follows: 94°C for 90 s; 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final extension period at 72°C for 10 min. PCR products were electrophoresised 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 c-FLIP(L) genes were ligated with pMD19-T vector (Takara, Dalian, China) and then were tranformed into the competent cell of DH5α. The positive clones were picked out and shaken overnight at 37°C, and then a random analysis of 10 clones with PCR and sequencing analysis was conducted at Sinogenomax Company (Beijing, China).

Construction of mammalian cell expression vector for pAcGFP-bFLIP(L) fusion protein

According to the Restriction Enzyme Mapping of ORF fragments of bovine c-FLIP(L) and multiple cloning sites of pAcGFP-N1 vector (Clontech, Mountain View, CA, USA), *Bgl*II, *Eco*RI were chosen as clone sites. The authors designed primers at two ends of the c-FLIP(L) open reading frame, and inserted BglII Restriction Enzyme site in the upstream prime and four protective bases before ATG, meanwhile they inserted a Kozak sequence, which could increase inserted gene expression level, in the eukaryotic cell. Forward primer was designed as follows: 5'-ACTAGATCTGCCACCATGTCTGCTGAAGTCAT-3'(AG ATCT is B*gl*II Enzyme site, GCCACCATG is Kozak sequence). When the authors designed the reverse primer, the stop codon TGA was deleted, meanwhile behind it the C base and EcoRI Restriction Enzyme site were inserted. c-FLIP(L) open reading frame should be consistent with the downstream AcGFP gene sequence to ensure coexpression with the fusion protein.The reverse primer was designed as follows: 5'-ACTGAATTCCTTTGTGAGAGAGGAAGA-3' (GAATTC is *Eco*RI Enzyme site).

In order to improve the amplification efficiency, the full-length encoding region of the bovine c-FLIP(L) gene was amplified by TD-PCR from the plasmid template, PCR cycles were performed as follows: 94°C for 90 s; five cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 1 min; five cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min; 28 cycles of 94°C for 30 s, 61°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 the competent cell of DH5α. The positive clones were picked out and shaken overnight at 37°C. Plasmids were extracted from sense colones using TIANprep Mini Plasmid Kit (Tiangen, Beijing, China) and digested with *Bgl*II and *Eco*RI enzymes (Takara). A cDNA fragment of 1,477 bp was recovered and directly ligated to the AcGFP-N1 eukaryotic expression vector that digested with *Bgl*II and *Eco*RI enzymes, and transformed to competent cell DH5α. The positive clones were picked out and shaken overnight at 37°C.

Identification of recombinant plasmid pAcGFP-bFLIP(L)

After random analysis of 20 clones with PCR, plasmids were extracted from sense colones and digested with *Bgl*II and *Eco*RI enzymes to confirm the expression of the bovine c-FLIP(L). The DNA sequence of the ORF was determined using an automatic DNA sequencer (ABI Prism 310, Foster,

CA, USA). All these procedures were performed according to the manufacturer's instructions. The recombinant plasmid pAcGFP-bFLIP(L) was amplified in DH5α, and then the EndoFree Plasmid was extracted from the sense colones using the EndoFree Plasmid Kit (Tiangen). It was 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 plated on 24-well culture plates (Falcon, Franklin Lakes, NJ, USA) were incubated in a CO_2 incubator (Thermo, Marietta, Ohio, USA) at 37°C for 24 h, 5% CO_2 in air. After 24 h of culture, the DMEM medium (GIBCO, Iinvitrogen, Carlsbad, California, USA) supplemented with 10% (V/V) Fetal Bovine Serum (GIBCO) and 1% (V/V) L-Glutamine (GIBCO) were replaced by DMEM medium containing different concentrations of G418 (100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 μg/ml) (Sigma, St. Louis, MO, USA). Cells were incubated at 37°C, 5% CO_2 condition, then replaced every 72 h for two weeks of observation. The optimum concentration of G418, as a selection agent for follicular granulosa cell was of the lowest concentration, under which all the cells were killed 10-14 d after culture in DMEM with G418.

Transfection and fluorescence observation of fusion protein

One day before transfection, plated $0.5\text{-}2\times10^5$ follicular granulosa cells 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, incubated for 5 minutes at room temperature. After 5 minutes of incubation, the diluted DNA was combined with diluted Lipofectamine™ 2000 (total volume = 100 μl). It was mixed gently and incubated for 20 minutes at room temperature. DNA-Lipofectamine 2,000 mixture of 100 μ was added to each well containing the cells and medium. The cells were incubated at 37°C in a CO_2 incubator for 4-6 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 by G418. Twelve hours later, the expression of AcGFP in the cells was observed under fluorescence microscope (NikonTE2000, Japan), and the number of positive expression cells in every 24 h, under high power field, were counted.

Analysis of bovine c-FLIP(L) by RT-PCR and western-blotting

To confirm the insertion of a bovine c-FLIP(L) open reading frame, after stable transfection screening with G418, the cells were harvested. mRNA was extracted from one part of the cells using Quickprep Micro mRNA Purification Kit (Invitrogen), and then it was reverse-transcribed to synthesize the cDNA. The primer for amplification of partial cDNA sequence of bovine c-FLIP(L) was designed as follows: forward 5'- ACTAGATCTGCCACCATGTC TGCTGAAGTCAT-3' and reverse 5'-ACTGAATTCCT TTGTGAGAGAGGAAGA-3'. PCR cycles were performed as follows: 94°C for 90 s; five cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 1 min; five cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min; 28 cycles of 94°C for 30 s, 61°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 dodecylsulfate-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 c-FLIP(L) 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.

RESULTS

Bovine c-FLIP(L) gene cloning and sequence analysis

The optical density ratio of total RNA in bovin ovary

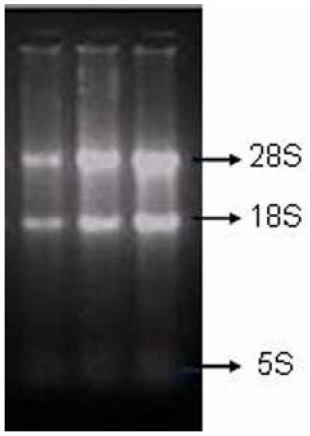

Figure 1. The result of total RNA from bovin ovary.

was 1.96. The result of gel electrophoresis detection showed that: 5S RNA was small and run up to the gelatin boundary, its banding was visible, but weak. The banding of 18S and 28S RNA were bright, and 28S RNA bandings were approximately two times larger than that of the 18S RNA, which indicated that the total RNA was not broken down and the purity was good (Figure 1).

The experimental results showed that a gene fragment with molecular size of about 1,483 bp was obtained by RT-PCR amplification (Figure 2), which was consistent with the expected and it contained 1,455 bp coding region sequences.

Using T/A cloning and choosing positive clones randomly, the double strands cDNAs were sequenced. The length of one sequence was 1,483 bp, which contained ORF of 1,455 bp (485 amino acids: aa). The aligned results showed the sequence for bovine c-FLIP(L) had 100% homology with Gene Bank (NCBI).

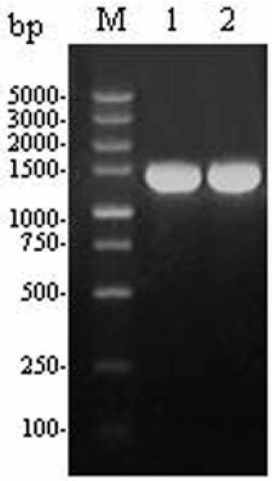

Figure 2. The product of bovine c-FLIP(L) gene. The cDNA from bovine ovary acted as templet, c-FLIP(L) forward primer and c-FLIP(L) reverse primer were used to amplify the c-FLIP(L) fragment. Total volume of the reaction was 20 μl. A 1,483 bp fragment was detected by electrophoresis on 1.2% agarose gel. M:DNA Marker DL 5,000; 1,2:cDNA of bovine c-FLIP(L).

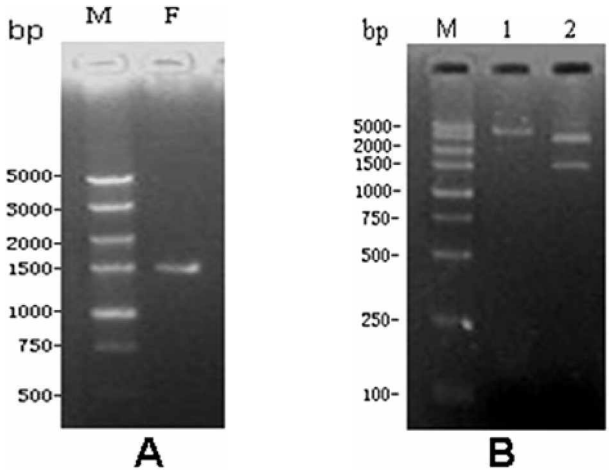

Figure 3. Construction and identification of pMD19T-bFLIP(L). The pT-bFLIP(L) plasmid acted as templet, specificity primers were used to amplify the c-FLIP(L) coding region with *Bgl*II/*Eco*RI site. A 1,477 bp fragment was detected by electrophoresis. The c-FLIP(L) coding region was cloned into the pMD19-T Simple vector, then transformed into DH5a, the plasmids were extracted from positive clones and digested with *Bgl*II and *Eco*RI enzyme (Takara) for 6 h at 37°C following the supplier's direction. A: Result of bovin c-FLIP(L) gene with *Bgl*II, *Eco*RI cloning sites by PCR [M.DNA Marker DL 5000; F.cattle c-FLIP(L)]; B: Identification of pT-bFLIP(L) (M.DNA Marker DL 5000; 1. pT-bFLIP(L) plasmid; 2. pT- bFLIP(L) plasmid digestion by restrictive enzyme *Bgl*II/*Eco*RI).

Construction and identification of the recombinant plasmid pAcGFP-bFLIP(L)

The 1,477 bp encoding region of c-FLIP(L) gene was amplified from pT-bFLIP(L) plasmid with specific primers by touchdown PCR (TD-PCR) (Figure 3A, lane F).

The expected fragments were obtained by complete digestion of PMD19-T- FLIP(L) plasmid, which was extracted from the transformed positive clones with B*gl*II and EcoRI (Figure 3B, lane 2).

The target gene fragment was successfully connected to the 5' end of the AcGFP cDNA, which had guaranteed that the c-FLIP(L) reading frame was consistent with AcGFP. The 1,477 bp fragments were obtained by complete digestion of the recombinant plasmid pAcGFP-bFLIP(L), which was extracted from the transformed positive clones with *Bgl*II and *Eco*RI (Figure 4).

The sequence analysis showed that bovine c-FLIP(L) gene was successfully cloned into *Bgl*II/*Eco*RI site of pAcGFP-N1 vector. The authors made sure that c-FLIP(L) coding region sequence and AcGFP gene sequence had the same reading frame through deleting the stop codon TGA and inserting the C base, so the target gene and fusion protein gene could express at the same time. The reconstructed plasmid was named as the pAcGFP-bFLIP(L)

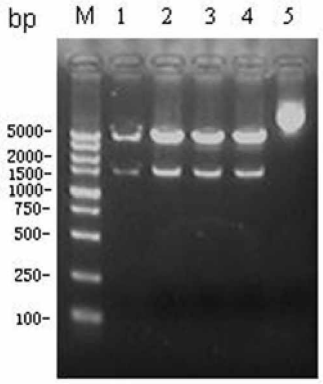

Figure 4. Identification of recombinant plasmid pAcGFP-bFLIP(L) by restriction enzyme digestion. The restriction fragments of *Bgl*II/*Eco*RI was cloned into the pAcGFP-N1 vector then transformed into DH5a, the plasmids were extracted from positive clones and digested with *Bgl*II and *Eco*RI enzyme (Takara) for 6 hours at 37°C following the supplier's direction. M.DNA Marker DL 5000; 1-4. pAcGFP-bFLIP(L) digestion by restrictive enzyme *Bgl*II/*Eco*RI; 5. pAcGFP-bFLIP(L) recombinant plasmid.

vector (Figure 5).

Determine the minimum dose of G418 for follicular granulosa cells

After three days' selection with different concentrations of G418, the cells were in different degrees of death, and the number of suspending and breaking of cells was increasing in the treatments supplemented with higher than 600 μg/ml. Its peak mortality was in the eighth to tenth day duration, and the cells of treatments supplemented with 600 μg/ml and over 600 μg/ml were dead on the tenth day. The concentration of 600 μg/ml was considered as the minimum dose of G418 for follicular granulosa cells (Table 1).

Transfection of follicular granulosa cells with pAcGFP-bFLIP(L) plasmid and G418 selection of resistant cell strain

The cationic liposomes surface's positive charge and the phosphate backbone of pAcGFP-bFLIP(L) plasmid DNA are stably combined by electrostatic interaction forming 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.

DNA-liposome complex transfers into cells, anionic lipid of membrane diffuse into the complex because the membrane losed its banlance, and then anionic lipid of membrane is combined with positive ion of cationic liposomes, forming the neutral ion pair, so that pAcGFP-bFLIP(L) plasmid DNA break away from the DNA-liposome complex, enter the cytoplasm, and then enter the nucleus through the nuclear pore. Finally, the bFLIP(L) gene encoding protein is produced by transcribing and expressing in the nucleus.

Cells transfected with the pAcGFP-bFLIP(L) plasmid by Lipofectamine 2000 were screened with G418 up to the fourteenth day. The negative control cells were all dead. There were cell clones formed in other dishes. Subsequently the maintaining dose of G418 was used to the 18th day when all cell degeneration and necrosis disappeared and the

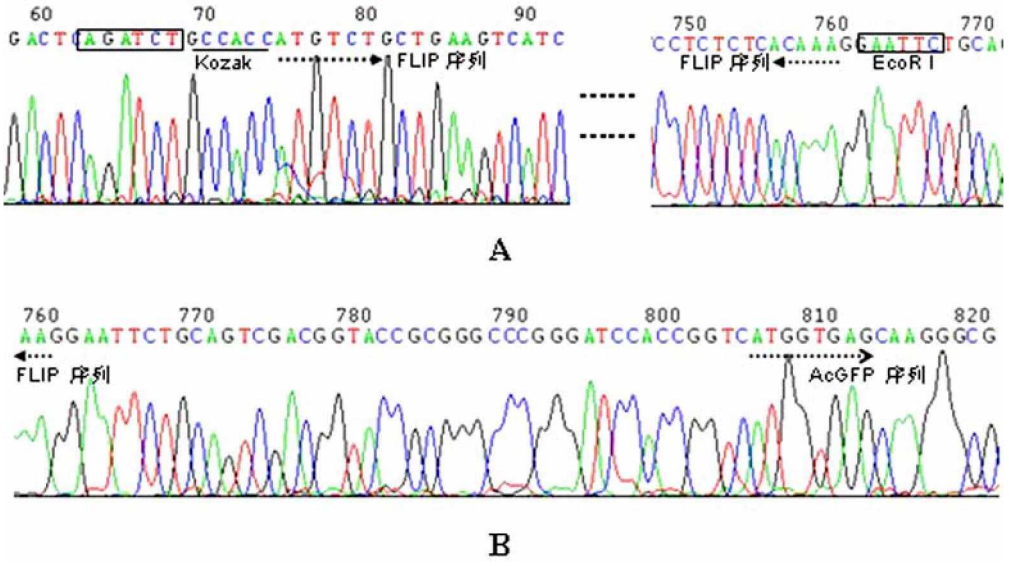

Figure 5. Sequence of recombinant expression vector pAcGFP-bFLIP(L). The pAcGFP-bFLIP(L) plasmids were extracted from positive clones and sequenced by Sinogenomax Company. A: c-FLIP(L) ORF sequence of pAcGFP-bFLIP(L), digestion sites with *Bgl*II and *Eco*RI, Kozak sequence (digestion sites are in the box, underline part is Kozak Sequence, dotted line arrow direction is c-FLIP(L) ORF); B: AcGFP sequence of pAcGFP-bFLIP(L) recombinant (dotted line arrow direction on the right).

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

G418 concentration (μg/ml)	100	200	300	400	500	600	700	800	900	1,000
Survival rate (%)	+++	++	++	+	+	-	-	-	-	-

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

resistant cells formed positive clones and gradually grew up. The expression of AcGFP located in the plasma and nucleus under the inverted fluorescent microscopy (Figure 6).

The observation result of green fluorescence in the cells showed that the untransfected cells were not observed under microscope fluorescent, and AcGFP could be observed in the nucleu and its lateral region in follicular granulosa cells transfected with pAcGFP-bFLIP(L), and uniform distribution throughout on the whole cell in the pAcGFP-N1 transfection group (Figure 7).

RT-PCR analysis of monoclonal cell strain after being selected by G418

The RNA of the monoclonal cells screened by G418

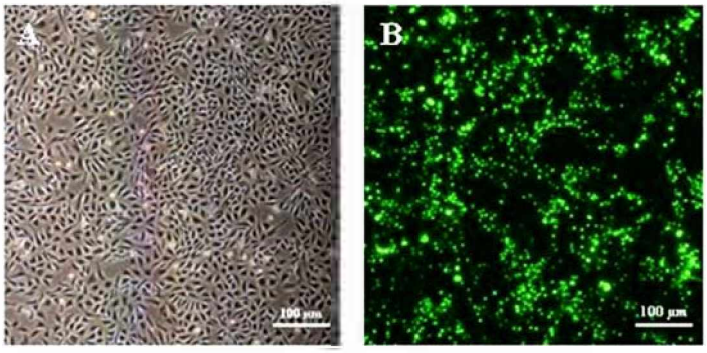

Figure 6. The green fluorescence positive cells after transfected with pAcGFP-bFLIP(L) plasmid. The pAcGFP-bFLIP(L) plasmid was transfected into follicular granulosa cells mediated by Lipofectamine 2000. After transfection, green fluorescent was observed by fluorescent microscopy. The expression rates of green fluorescence in follicular granulosa cells was 65% at 24 h after transfection. A: transfected follicular granulosa cells by pAcGFP-bFLIP(L) under visible light; B: transfected follicular granulosa cells by pAcGFP-bFLIP(L) under fluorescent microscope. Scale bar 100 μm.

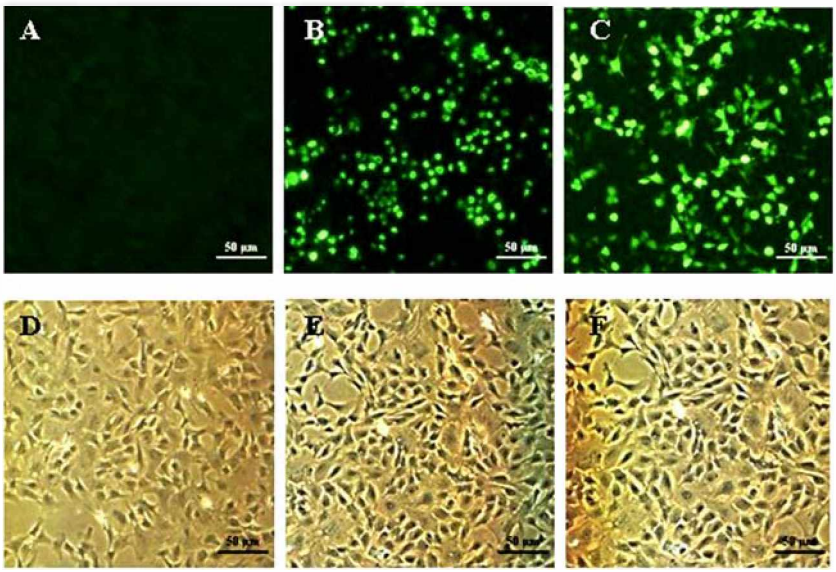

Figure 7. The expression of AcGFP-bFLIP(L) fusion protein and AcGFP protein in follicular granulosa cells after transfection. After transfection, the green fluorescence could be detected in follicular granulosa cells transfected by pAcGFP-bFLIP(L) and pAcGFP-N1 plasmid, while there was no AcGFP expression in follicular granulosa cells untransfected by any plasmid. AcGFP could be observed in the nucleu and its lateral region in pAcGFP-bFLIP(L) transfection group and uniform distribution throughout on whole cell in pAcGFP-N1 transfection group. A, B, C: transfected follicular granulosa cells under fluorescent microscope; D, E, F: transfected Follicular granulosa cells under visible light. A, D: control group; B, E: pAcGFP-bFLIP(L) transfection group; C, F: pAcGFP-N1 transfection group. Scale bar 50 μm.

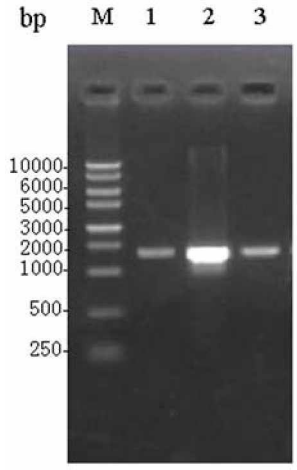

Figure 8. The expression of bovine c-FLIP(L) Mrna on follicular granulosa cells determined RT-PCR. Total RNA was extracted from follicular granulosa cells and cDNA was prepared using universal primer. Specificity primers were used to amplify the c-FLIP(L) sequence,a bright 1,477 bp fragment was detected by electrophoresis on 1.2% agarose gel in pAcGFP-bFLIP(L) transfection group. M.DNA Marker DL 10000; 1. Control group; 2. pAcGFP-bFLIP(L) transfection group; 3 pAcGFP-N1 transfection group.

was extracted. A bright 1,477 bp strap was amplified in the pAcGFP-bFLIP(L) transfected follicular granulosa cells by RT-PCR,but the 1,477 bp strap was weak in the pAcGFP-N1 transfected cells and the negative control cells (Figure 8). The result showed that there was effective expression of c-FLIP(L) in the pAcGFP-bFLIP(L) transfected follicular granulosa cells. It could be considered that the pAcGFP-bFLIP(L) had transfected the follicular granulosa cell successfully.

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

SDS-PAGE analysis indicated that the fusion protein of AcGFP-bFLIP(L) was expressed in pAcGFP-bFLIP(L) transfected cells and its molecular weight was about 83 kD (Figure 9A, lane 3,4), but there was no expression for fusion protein of AcGFP-bFLIP(L) in the pAcGFP-N1 transfected cells and the negative control cells (Figure 9A, lane 1,2). It was preliminarily confirmed that follicular granulosa cells transfected with AcGFP expression vectors of the bovine c-FLIP(L) gene expressed fusion target proteins. The expressed fusion protein showed specificities of c-FLIP(L) polyclonal antibody as proved by Western blot and further proved to be an immunocompetence protein (Figure 9B).

c-FLIP(L) is known to inhibit caspase-8 recruitment to Fas death receptor by direct competition. During the development process of the bovine oocytes, Fas/FasL pathway induced apoptosis of ovarian granulosa cells by apoptosis signal, and then made the follicular atresia. While, c-FLIP(L) completely blocked Fas-mediated apoptosis signal of ovarian granulosa cells through inhibition of caspase-8 processing at the DISC. So it could induce proliferation of follicular granulosa cells and promote the development of oocytes, to maintain the equilibrium state of follicular development.

DISCUSSION

Apoptosis is an important phenomenon involved in cell survival and death during differentiation and development. The death ligand and receptor systems are considered to be apoptosis-inducing factors (Hengartner, 2000).

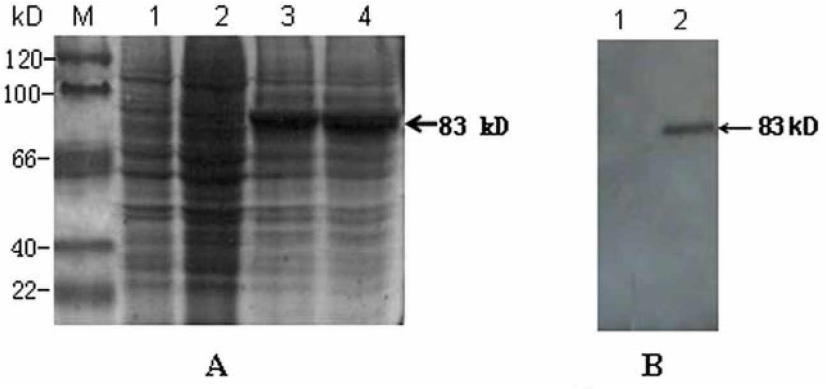

Figure 9. The SDS-PAGE analysis and western blot identification of expression in follicular granulosa cells of bovin c-FLIP(L) fusion protein. Protein sample were loaded onto 15% SDS-PAGE to separate protein and transferred to nylon cellulose membrane. The membrane was probed with anti bovine c-FLIP(L) 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 9A: 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-bFLIP(L) transfection group; Figure 9B: 1. Western blot analysis of pAcGFP-N1 transfection group; 2. Western blot analysis of pAcGFP-bFLIP(L) transfection group.

Apoptosis can be mediated by caspase 8 activation via the extrinsic or death receptor- mediated pathway resulting in formation of the death-inducing signalling complex (DISC) containing the adapter molecule FADD and procaspase 8 (Ferguson et al., 2007). An important regulator of the caspase-8 mediated pathway is Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (FLIP). FLIP is a family of alternatively spliced variants, and primarily exists as long ($FLIP_L$) and short ($FLIP_S$) splice variants in human cells. Although FLIP has apoptogenic activity in some cell contexts, which is currently attributed to heterodimerization with caspase-8 at the DISC, accumulating evidence indicates an anti-apoptotic role for FLIP in various types of human cancers (Moriyama and Yonehara, 2007; Park et al., 2008).

A previous study about an analyzing expression map in our laboratory suggested mRNA of the bovine c-FLIP(L), highly expressed in lymphoid tissue, ovary and testis, whereas less expressed in other tissues. This indicated that c-FLIP(L) in the lymphoid tissue played an important role in keeping the bovine immune environment stable. During the development process of the bovine oocytes, Fas/FasL pathway induced apoptosis of ovarian granulosa cells by apoptosis signal, and then made the follicular atresia. While, c-FLIP(L) completely blocked Fas-mediated apoptosis signal of ovarian granulosa cells through inhibition of caspase-8 processing at the DISC. So it could induce proliferation of follicular granulosa cells and promote the development of oocytes, to maintain the equilibrium state of follicular development. (Margalit et al., 2005; Skarzynski et al.,2007). Gene mutation or abnormal expression of c-FLIP(L) in reproductive system, leading to internal environment disorder and abnormal spermatogenesis and oogenesis could cause bull's oligzoospermous or aspermia, reduce a cow's ovulation rate and conception rate.

When the authors constructed the eukaryotic expression vector for the pAcGFP-bFLIP(L) fusion protein, the authors took advantage of directional cloning, introduced *Bgl*II (AGATCT) and *Eco*RI (GAATTC), wo sites in upstream primer and downstream primer, respectively. These two restriction enzymes produced different 3'cohesive ends, which could realize that target gene was directionally connected to vector. The following were virtues of this method: i) The vector fragment couldn't be cyclized, so there was few false positive recombinant clones, because the vector's two cohesive ends didn't complement each other. ii) Because the foreign bovine c-FLIP(L) gene was inserted into recombinant plasmid in one direction, it was not necessary to screen for right connection. iii) Restriction enzyme sites were preserved, which was beneficial to further identification.

In 2003, Kozak (Marilyn, 1980) analyzed the relationship between sequence of mRNA 5' end and translation efficiency in the eucaryotic expression gene and found that 5' G/N-C/N-C/N-ANNATGG 3'sequence could improve transcription and translation efficiency.especially A in -3 site and G in +4 site were important to improve the translation efficiency. Therefore the Kozak sequence was introduced after upstream primer's *Bgl*II site, to make sure that c-FLIP(L) gene was highly expressed in recombinant plasmid.

In addition, c-FLIP(L) and pAcGFP-N1 were mixed in the proportion of 8:1 (mole number) and connected under 16°C, which could not only improve efficiency but also further reduce the probability of vector cyclization itself.

G418 is one of the aminoglycoside antibiotics, which is toxic to both prokaryotic cells and eukaryotic cells. It is usually utilized to resist screening of transfection (Magin et al., 2003). When a neo gene was inserted into the genome of eukaryotic cells, a sequence coded by neo gene started to transcribe into mRNA, and then amino glycoside phosphotransferase was highly expressed, a resistant production, which made cells grew up in a selective medium, including G418. First, the authors should selected the correct G418 screening concentration because sensitivities of different cells to G418 were different and the activities of G418 from different factories were different although they were the same concentration. In this experiment, all the cells died in the 600 μg/ml concentration group on the twelfth day, so the authors chose the 600 μg/ml as the best screening concentration. During the screening, the authors first selected 600 μg/ml of G418, when clones appeared. Then the authors selected 200 μg/ml of G418 instead of 600 μg/ml of G418. In this condition, the cells grew up rapidly,when the cells spread out full, reselected positive clones again by 600 g/ml of G418. Finally, the authors acquired cell clones which could stably express bovine c-FLIP(L) gene.

pAcGFP-bFLIP(L) was transfected into follicular granulosa cell mediated by Lipofectami™ 2000 with transfection efficiency reaching 65%. After sreening for two weeks by 600 μg/ml of G418, positive clones could emit fluorescence. This indicated that the bovine c-FLIP(L) gene was completely inserted into the follicular granulosa cell genome and the fusion protein was stably expressed. Molecular weight of green fluorescent protein was 28 kD, bovine c-FLIP(L)'s molecular weight was 55 kD, so the fusion protein's molecular weight was about 83 kD, which was consistent with the detection result by SDS-PAGE electrophoresis and Western blotting, and c-FLIP(L)'s antibody binding to the NC membrane showed a specific reaction with the fusion protein. It indicated that transfected follicular granulosa cells by pAcGFP-bFLIP(L), greatly expressed immunocompetent c-FLIP(L) protein. In addition, the background color of the protein immunoblotting ECL was dark because the concentration of horseradish

peroxidase labeled second antibody was a bit high, it was rinsed insufficiently, and exposure time was longer. Decreasing concentration, raising time to rinse, increasing buffer volume, and shortening exposure time could improve the development effect.

This research was prepared for the study on mechanism of bovine oogonium's proliferation and differentiation. c-FLIP(L) was inserted into pAcGFP-N1's N end and fusion protein was expressed driven by pAcGFP-N1 CMV promoter, which could improve c-FLIP(L)'expression level in eukaryotic cells and keep its structure and founction unchanged.

On the other hand, the AcGFP reporter gene, instead of EGFP was extracted from aequorea coerulescens. Compared to EGFP, ACGFP had an opening frame with enhanced codon, so it could improve both transformation efficiency of AcGFP mRNA and its expression level in mammalian cells and it could be detected only 8-12 h after transfection, and fluorescence detection could be carried out for a long time (Jakobs et al., 2000; Hideki et al., 2003). AcGFP as pAcGFP-bFLIP(L)'s reporter gene could improve transfection efficiency and reduce harm for cells. It was also beneficial for regulation environment simulation for oocyte gene expression *in vivo* and study regulation of c-FLIP(L) on differentiation and proliferation of oogonium at the gene level.

Through bovine c-FLIP(L) gene binding to the AcGFP gene, the mammalian expression vector of the pAcGFP-bFLIP(L) fusion protein was constructed and highly expressed in follicular granulosa cells. It could provide technical support for basic research on regulation of c-FLIP(L) on bovine oogonium development and become important for further research.

REFERENCES

Carsten, S., S. Ingo, H. K. Peter and E. P. Marcus. 1999. The role of c-FLIP in modulation of CD95-induced apoptosis. J. Biol. Chem. 274:1541-1548.

Ferguson, B. J., D. Esposito, J. Jovanović, A. Sankar, P. C. Driscoll and H. Mehmet. 2007. Biophysical and cell-based evidence for differential interactions between the death domains of CD95/Fas and FADD. J. Cell Death. Differ. 14:1717-1719.

Giampietri, C., S. Petrungaro, F. G. Klinger, P. Coluccia, A. Paone, E. Vivarelli, A. Filippini, P. De Cesaris, M. De Felici and E. Ziparo. 2006. c-Flip expression and function in fetal mouse gonocytes. FASEB. J. 20:124-126.

Giampietri, C., S. Petrungaro, P. Coluccia, F. Antonangeli, A. Paone, F. Padula, P. De Cesaris, E. Ziparo and A. Filippini. 2006. c-Flip(L) is expressed in undifferentiated mouse male germ cells. FEBS. Lett. 580:6109-6114.

Hengartner, M. 2000. The biochemistry of apoptosis. Nature 407:770-776.

Hideki, T., K. L. Chang, C. Y. Chan, O. Masayoshi, N. Masami, N. Yoshitake, O. Nobuo, K. Yoshinori, W. Rie, M. Rika, M. Kohji and M. Yoshiharu. 2003. *In vitro* and *in vivo* gene delivery by recombinant baculoviruses. J. Virol. 77:9799-9808.

Jakobs, S., V. Subramaniam, A. Schönle, T. M. Jovin and S. W. Hell. 2000. EFGP and DsRed expressing cultures of *Escherichia coli* imaged by confocal, two-photon and fluorescence lifetime microscopy. FEBS. Lett. 479:131-135 .

Magin, L. C., G. Kotzamanis, L. D'Aiuto, E. Wagner and C. Huxley. 2003. Retrofitting BACs with G418 resistance, luciferase, and oriP and EBNA-1- new vectors for *in vitro* and *in vivo* delivery. BMC. Biotechnol. 3:2-10.

Margalit, K. A., R. G. Cowan, R. M. Harman and S. M. Quirk. 2005. Apoptosis of bovine ovarian surface epithelial cells by Fas antigen/Fas ligand signaling. Reproduction 130:751-758.

Marilyn, K. 1980. Binding of wheat germ ribosomes to bisulfite-modified reovirus messenger RNA:Evidence for a scanning mechanism. J. Mol. Biol. 144:291-304.

Moriyama, H. and S. Yonehara. 2007. Rapid up-regulation of c-FLIP expression by BCR signaling through the PI3K/Akt pathway inhibits simultaneously induced Fas-mediated apoptosis in murine Blymphocytes. Immunol. Lett. 109:36-46.

Park, D., E. Shim, Y. Kim, Y. M. Kim, H. Lee, J. Choe, D. Kang, Y. S. Lee and D. Jeoung. 2008. C-FLIP promotes the motility of cancer cells by activating FAK and ERK, and increasing MMP-9 expression. Mol. Cells 25:184-195.

Skarzynski, D. J., M. Shibaya, Y. Tasaki, A. Korzekwa, S. Murakami, Woclawek, I. Potocka, M. Majewska and K. Okuda. 2007. Fas-mediated apoptosis is suppressed by calf serum in cultured bovine luteal cells. Reprod. Biol. 7:3-15.

Szperka, M. E., E. E. Connor, M. J. Paape, J. L.Williams and D. D. Bannerman. 2006. Sequencing, chromosomal mapping, and functional characterization of bovine FLICE-like inhibitory protein (FLIP). Cytogenet. Genome. Res. 112:90-97.

Zhang, S., H. M. Shen and C. N. Ong. 2005. Down-regulation of c-FLIP contributes to the sensitization effect of 3,3'-diindolylmethane on TRAIL-induced apoptosis in cancer cells. J. Mol, Cancer, Ther. 4:972-981.

Zhang, X., T. G. Jin, H. Yang, W. C. DeWolf, F. R. Khosravi and A. F. Olumi. 2004. Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. J. Cancer Res. 64:7086-7091.

Zou, W., X. Liu, P. Yue, F. R. Khuri and S. Y. Sun. 2007. PPAR gamma ligands enhance TRAIL-induced apoptosis through DR5 upregulation and c-FLIP downregulation in human lung cancer cells. Cancer Biol. Ther. 6:99-106.