**INTRODUCTION**

FLICE inhibitory protein (FLIP) is one of the important anti-apoptotic 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).

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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\textsubscript{260}/OD\textsubscript{280}>1.8) was chosen and then reverse-transcribed using the cDNA synthesis reverse transcription 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'-TTTCTTGGAATGACACTGTA-3' and reverse 5'-CTTTTTATTTGTGAGAGAGGAAGA-3'. PCR amplification cycles was 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 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 the AcGFP-N1 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 colonies using TIANprep Mini Plasmid Kit (Tiangen, Beijing, China) and digested with BgII and EcoRI enzymes (Takara). A cDNA fragment of 1,477 bp was recovered and directly ligated to the AcGFP-N1 eukaryotic expression vector that digested with BgII and EcoRI 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 colonies and digested with BgII and EcoRI 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, Company (Beijing, China).
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 colonies 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 CO2 incubator (Thermo, Marietta, Ohio, USA) at 37°C for 24 h, 5% CO2 in 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) 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% CO2 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 0.5-2×10^5 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 μl was added to each well containing the cells and medium. The cells were incubated at 37°C in a CO2 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'- ACTAGATCTGCCACCATGTGC TGCTGAAAGTCAT-3' and reverse 5'-ACTGAATTCCT TTGGAGAGAGAAGA-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 rabbit anti-bovine c-FLIP(L) polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at 4°C for 1 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...
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).

**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 Simple vector, then transformed into DH5α, the plasmids were extracted from positive clones and digested with BgIII and EcoRI enzyme (Takara) for 6 h at 37°C following the supplier’s direction. A: Result of bovin c-FLIP(L) gene with BgIII, EcoRI 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 BgIII/EcoRI).

The target gene fragment was successfully connected to the 5’ end of the AcGFP cDNA, which had guaranteed that c-FLIP(L) coding 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 BgIII and EcoRI (Figure 4).

The sequence analysis showed that bovine c-FLIP(L) gene was successfully cloned into BgIII/EcoRI 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).
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 lost its balance, 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 4. Identification of recombinant plasmid pAcGFP-bFLIP(L) by restriction enzyme digestion. The restriction fragments of BglII/EcoRI was cloned into the pAcGFP-N1 vector then transformed into DH5a, the plasmids were extracted from positive clones and digested with BglII and EcoRI 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 BglII/EcoRI; 5. pAcGFP-bFLIP(L) recombinant plasmid.](image)

![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 BglII and EcoRI, 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).](image)
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 nucleus 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).

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

<table>
<thead>
<tr>
<th>G418 concentration (μg/ml)</th>
<th>100</th>
<th>200</th>
<th>300</th>
<th>400</th>
<th>500</th>
<th>600</th>
<th>700</th>
<th>800</th>
<th>900</th>
<th>1,000</th>
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<tr>
<td>Survival rate (%)</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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+++ Survival rate of 80%; ++ Survival rate of 50%; + Survival rate of 30%; - Survival rate of 0%.

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 Lipoctectamine 2000. After transfection, green fluorescent was observed by fluorescent microscopy. The expression rates of green fluorescent 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 nucleus 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.
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).
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 (FLIPL) and short (FLIPS) splice variants in human cells. Although FLIP has apoptotic 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 oligozoospermous 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 BglII (AGATCT) and EcoRI (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 BglII 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, resellected 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 LipofectamiTM 2000 with transfection efficiency reaching 65%. After screening 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 function 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 in vivo for oocyte gene expression was also beneficial for regulation environment simulation and study regulation of c-FLIP in modulation of CD95-induced apoptosis. J. Biol. Chem. 274:1541-1548.


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