Construction of mammary gland specific expression plasmid pIN and its expression in vitro and in vivo

Jian Lin, Qinghua Yu, Qiang Zhang and Qian Yang*

Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture, Nanjing Agricultural University, Weigang 1, Nanjing, Jiangsu 210095, People’s Republic of China.

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The aim of this study was to construct a mammary gland specific expression plasmid pIN and validate its function in expressing goat insulin-like growth factor 1 (IGF-1). The backbone plasmid pBC1 contained goat β-casein 5’ arm and β-casein 3’ arm, to target mammary gland-specific gene expression. First, the igf-1 gene was cloned from liver tissue harvested from a Saanen dairy goat and inserted downstream of the β-casein 5’ arm. Then the neo gene was amplified from plasmid pCDNA3.1 and placed downstream of the β-casein 3’ arm as a positive selection marker. In order to analyze the bioactivity of plasmid pIN, it was transfected into the Bcap-37 cell line and injected into goat mammary gland. Western-blotting and quantitative polymerase chain reaction (PCR) results confirmed the expression of IGF-1 protein and mRNA in transfected Bcap-37 cells. Further studies (RIA) demonstrated that the expression of IGF-1 protein in transfected group was much higher than that in control group (p < 0.05). In vivo results showed that the expression of IGF-1 in injected group was significantly higher than that in control group. All our results provide evidence that pIN is a mammary gland specific expression plasmids that can target expression of IGF-1 to mammary tissue, with the goal of increasing milk production.

Key words: IGF-1, plIN, Bcap-37 cell line, goat mammary, milk production.

INTRODUCTION

Improving milk production of goat by transgenic animal technology is a promising strategy, but requires selecting an appropriate gene for increased expression. Mammary gland development and milk production are affected by many hormone genes (Plante et al., 2011), such as growth hormone, prolactin (PRL), progesterone, estrogen and insulin-like growth factor 1 (IGF-1) (Kaskous et al., 2003). IGF-1 is a hormone similar to insulin in molecular structure, which can bind to two receptors: IGF-1 receptor and insulin receptor. In addition to insulin-like effect, IGF-1 can regulate cell growth and development. After binding to IGF-1R, it initiates intracellular signaling: activating AKT signal pathway, a stimulator of cell growth and proliferation. Also IGF-1 has growth-promoting effects on almost every cell. The deficiency of IGF-1 will directly lead to non-development of mammary gland (Ruan et al.,1999).

IGF-1 plays a role in lactation mainly in two aspects: on one hand, IGF-1 increases the milk production of animals in unit time. Prosser et al. (1994) proved that arterial infusion of IGF-1 into mammary gland increased milk yield by 9% in goat. On the other hand, IGF-1 enhances sustainability of lactation in lactating animals. Hadsell et al. (2002) demonstrated that IGF-1 slowed the apoptotic loss of mammary epithelial cells during the recession phase of lactation, which implied that IGF-1 possessed a potent inhibitor of programmed cell death. The ability of modifying mammary gland function through transgenic technology provides an opportunity to enhance the

*Corresponding author. E-mail: zxbyq@njau.edu.cn. Tel: +8602584395817. Fax: +8602584398669.

Abbreviations: IGF-1, Insulin-like growth factor 1; Bcap-37, human mammary tumor cell line; RIA, radioimmunoassay; PRL, prolactin; IGF-1R, insulin-like growth factor 1 receptor; IGF-BP, insulin-like growth factor binding protein; DMEM, Dulbecco’s modified Eagle medium.
production of milk. Previous research had suggested strategies for changing milk composition to reduce the energetic cost of milk production and to reduce the microbial load in milk. The perceived utility of mammary gland may, in part, account for the considerable attention that had been focused on investigating transgene expression in mammary glands. Most studies are designed to address both basic and applied aspects of expressing additional protein in mammary gland, while the bulk of research literature are focused on biomedical rather than agricultural applications.

The aim of our research was to construct a mammary gland specific expression plasmid pIN and validate its function in Bcap-37 cell line and goat mammary gland. Mammary-specific promoter such as casein (Lee et al., 1996; Zinovieva et al., 1998), whey protein (Hadsell et al., 1996) and lactalbumin (Su and Cheng, 2004) were wildly used in transgene animal. We used β-casein to initiate the expression of IGF-1. Our research laid a solid foundation for producing IGF-1 genetically modified goat with increasing milk production.

**MATERIALS AND METHODS**

Materials and reagents

Liver and mammary gland were harvested from Saanen dairy goats. Plasmid pBC1 was generously donated by Professor Cheng of Yangzhou University. Plasmid pCDNA3.1 and Bcap-37 cell line were provided by Professor Wang of Nanjing Agricultural University. Plasmid pBluescript II SK (+) was purchased from Takara (Japan). *Escherichia coli* JM109 strain was kept in our laboratory. Mouse monoclonal antibody to IGF-1 was purchased from Abcam Biotechnology (USA).

**Construction of vector pIN**

**The amplification of igf-1 and neo gene**

The full-length of goat igf-1 gene was 960 bp (GenBank ID: D11378), while the open reading frame (ORF) region was 483 bp. Total RNA was extracted from liver of Saanen dairy goat with Trizol reagent. Reverse Transcriptase M-MLV was used to synthesize cDNA. Parameters for PCR were as follows: 94°C for 5 min; 94°C, 30 s; 57°C, 30 s; 72°C, 30 s; 30 cycles; 72°C, 10 min; 10°C. After amplification, 25 µL PCR products were run on a 1.5% agarose gel with ethidium bromide staining. The PCR primers are listed in Table 1.

**The amplification of neo gene**

The full-length of the neo gene is 1521 bp and contains promoter and poly (a) tail. Using the plasmid pcDNA3.1 as a template, PCR amplification was performed with PrimeSTAR® HS DNA polymerase. PCR primers used for this study were listed in Table 1. The PCR products of IGF-1 and neo gene were isolated and purified. Purified DNA fragments were linked to plasmid pMD19-T. Recombinant plasmid was transformed to *E. coli* JM109 competent cells by heat-stress and extracted from bacterial colonies by colony PCR. The identified recombinant plasmid was then finally sequenced.

**The construction of vector pIN**

As shown in Figure 2, to produce IGF-1 insert fragment, recombinant plasmid pMD19-T-IGF-1 was amplified with infusion primers. Infusion Primers were listed in Table 1. PCR amplification was performed with PrimeSTAR® HS DNA polymerase. Infusion-IGF-1 product was isolated and purified. Then we used In-Fusion™ Advantage PCR cloning Kit to insert infusion-IGF-1 into plasmid pBlue-pBC. Recombinant plasmid was named pBI. Finally, the digested plasmid pBI (Kpn I; Cla I) was linked with plasmid DNA3. Recombinant plasmid was named pl. Then we designed a primer to check plasmid pl. Furthermore, to produce neo fragment, recombinant plasmid pMD19-T-neo was digested with restriction enzyme Not I. Plasmid pl was also digested with Not I. Then we used T4 DNA ligase to link digested plasmid pl with neo fragment. Recombinant plasmid was named pIN. PCR and restriction enzyme digestion methods were used to verify recombinant plasmid pIN.

**Expression of recombinant plasmid pIN in Bcap-37 cell line**

**Cell culture**

Bcap-37 cell line was grown in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) at 37°C in a humidified atmosphere of 5% CO2.

**Detection of the expression of IGF-1 mRNA by quantitative PCR**

Quantitative PCR assays were run to confirm whether the plasmid pIN could be expressed successfully in Bcap-37 cell line. Cells were divided into three groups 6 h after transfection: the blank group (normal cells), the transfection group (transfected with plasmid pIN) and the control group (liposome 2000). Assays were performed with RNA preparation from three groups. As to each group, at least three technical (quantitative PCR) replicates were needed. Gene-specific primers were designed using Primer Express software. The primer sequences are listed in Table 2.

**Detection of the expression of IGF-1 by Western blot and radioimmunooassay (RIA)**

Bcap-37 cell line was transfected with plasmid pIN by liposome2000. Cells were divided into three groups 6 h after transfection: control group (normal cells), transfection group (transfected with plasmid pIN) and the control group (liposome 2000). The medium of control group and transfection group were changed into DMEM containing 10% fetal bovine serum. At the same time, transfection with induction group was performed with DMEM containing 10% fetal bovine serum. At the same time, transfection with induction group was performed with DMEM containing 10% fetal bovine serum. At the same time, transfection with induction group was performed with DMEM containing 10% fetal bovine serum. At the same time, transfection with induction group was performed with DMEM containing 10% fetal bovine serum. At the same time, transfection with induction group was performed with DMEM containing 10% fetal bovine serum.
visualized by enhanced chemiluminescence.

For IGF-1 RIA, the concentration of IGF-1 in Bcap-37 cells lysate and cells culture medium were measured separately after 12, 24, 36 and 48 h by a radioimmunoassay protocol. 125I-IGF-1 kit was purchased from Beijing Sinoukbio Company. Cells lysate and cells culture medium samples 0.1 ml, mixed with acid/ethanol (95% ethanol: 2 M HCL = 87.5:12.5) 0.4 ml, set at room temperature for 30 min and then centrifuged at 3000 rpm / min for 30 min at 4°C. After centrifugation, 0.2 ml of the supernatant mixed with 0.2 ml 0.855 M Tris solution for 30 min at 4°C, then centrifuged 3000 rpm/min for 20 min at 4°C. Subsequently, 100 µl supernatant was aspirated for testing. One hundred microliters each of cells lysate and cells culture medium samples 0.1 ml, mixed with acid/ethanol (95% ethanol: 2 M HCL = 87.5:12.5) 0.4 ml, set at room temperature for 30 min and then centrifuged at 3000 rpm / min for 30 min at 4°C. After centrifugation, 0.2 ml of the supernatant mixed with 0.2 ml 0.855 M Tris solution for 30 min at 4°C, then centrifuged 3000 rpm/min for 20 min at 4°C. Subsequently, 100 µl supernatant was aspirated for testing.

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The whole procedure was conducted according to the description provided with liposome 2000 reagent (Invitrogen). Briefly, 1×105 cells were seeded in each well of 6-well plate one day before transfection. The cells in 5 wells were transfected with 2 µg plasmid pIN, while one well served as control. After 48 h of transfection, cells were passage based on cell density at 1:3 or 1:4 into the appropriate medium and selected using 600 µg/ml G418 (Amresco, USA) for 10 to 14 days. The observed positive cell clones were marked with circles at the corresponding spot on the bottom of the culture flask or plate. Each marked circle was digested with 0.25% trypsin for 2 min and then transferred into 48 well plate supplemented with DMEM, 10% fetal bovine serum (FBS) and 300 µg/ml G418 until each cells in 48 wells passage into six-well plate, three positive clones (1, 2 and 3) were used for following analysis. Genomic DNA was isolated from the above three positive cell lines and non-transgenic cell lines with TIANamp genomic DNA Kit (TIANgen). Two specific primers were designed to detect the positive clones. The primers used are listed in Table 1.

**Statistical analysis**

The statistical analyses of the concentration of IGF-1 in different groups were carried out with SPSS 17 software. Comparison between two groups was carried out using t-test. A one-way analysis of variance (ANOVA) with Turkey's post hoc analysis was performed when comparing more than two groups. Significant differences (P < 0.05) are noted with symbols in the Figures. All data are expressed as mean ± standard error of the mean.

**RESULTS**

**Cloning of igf-1 gene and neo gene**

Result shows that igf-1 gene was amplified and cloned into plasmid pMD19-T (Figure 1A). Subsequently, plasmid pMD19-T- igf-1 was digested with restriction enzyme Xho I and there were two fragments: 501 and 2690 bp as we wished. Nucleotide sequencing showed 100% homology to goat IGF-1 gene sequences. Results also show that neo gene was amplified and cloned into plasmid pMD19-T (Figure 1B). Then plasmid pMD19-T-neo was digested with restriction enzyme Not I and two fragments, 1521 and 2690 bp, were obtained. Nucleotide sequencing showed 100% homology to neo gene.
Identification of recombinant plasmid PI and pIN with restriction enzymes

Recombinant plasmid PI was cleaved with restriction enzymes Kpn I and Cla I (Figure 1C). Compared with digested plasmid pBC1 (Kpn I/ Cla I), plasmid pIN was identified. Furthermore, the recombinant plasmid pIN was cleaved with restriction enzyme Not I. Results show two fragments 1521 and 22108 bp as we supposed (Figure 1D). These results demonstrate that plasmid pIN was
constructed successfully.

**Detecting of the expression of plasmid pIN in Bcap-37 cell line**

**Detection of expression of IGF-1 mRNA by quantitative PCR**

Plasmid pIN was transfected into Bcap-37 cell line to evaluate its biological activity. IGF-1 mRNA was evaluated by quantitative PCR after 36 h. As shown in Figure 3A, the expression of IGF-1 in transfected group was greatly increased by 3000 times than that in control group. Results certified that plasmid pIN could be successfully transcribed in Bcap-37 cell line (Figure 3A).

**SDS-PAGE**

From Figure 3B, we observed a 7.5-kDa major band appearing in only a few transfected group (A8, B2 and B4), while most of them could not spot any band in 7.5-kDa place.

**Western-blotting**

Western blot analysis showed that the molecular weight of the expressed IGF-1 was 7.5-kDa, which was in agreement with the molecular weight of IGF-1 reported previously (Tokunou et al., 2008). Interestingly, we saw a 17-kDa band, which was always associated with a smaller 7.5-kDa IGF-1 band, in the colonic specimens with IGF-1 mouse antibody (Figure 3C).

**RIA**

To be sure whether the expression of IGF-1 was up regulated in transfected cells, cells lysate and cells culture medium of different group were collected to perform RIA analysis. Results confirm that the expression of IGF-1 in transfection group and transfection with induction group was much more than that in control group (Figure 4). In general, the content of total IGF-1 (the addition of IGF-1 in cells lysate and cells culture medium) was stably grown by time from 12 to 36 h, while the content of IGF-1 had a little drop down in 48 h. Compared with control group at 12, 24, 36 and 48 h (666.11 ± 47.78; 730.14 ± 64.31; 776.86 ± 126.14 and 842.69 ± 89.40 µg/L, respectively), the content of total IGF-1 in transfection group (1017.40 ± 51.16; 817.90 ± 37.38; 1267.10 ± 190.51 and 1015.70 ± 61.84 µg/L) were increased significant in 12 and 36 h. However the expression of IGF-1 in transfection and induction group reached to a peak in 36 h (1209.60 ± 130.88, 887.65 ± 39.01, 1472.10 ± 56.47 and 1111.00 ± 86.15 µg/L) (Figure 4C). In cell lysate, the expression of IGF-1 in control group at 36 h (277.30 ± 56.19 µg/L) were precisely lower than that in transfection with induction group (641.13 ± 42.81 µg/L) (Figure 4A). Also in cell culture medium, the content of IGF-1 increased significant from 499.56 ± 182.28 µg/L (36 h, control group) to 837.33 ± 122.50 µg/L (36 h, transfection group) and 830.98 ± 58.32 µg/L (36 h, transfection with induction group) (Figure 4B). Statistical analysis showed that the content of IGF-1 in transfection and induction group was higher than that in control group at 24 and 48 h. The content of IGF-1 in induction group was significantly higher than that in control group (P < 0.05) at 12 and 36 h.

**Detect the expression of plasmid pIN on goat mammary**

The ELISA results of goat mammary gland changed dramatically in different groups. In the right side (injection side), the IGF-1 content in injection group was 0.98 ± 0.11 ng/mg, which was greatly higher than that in control group (0.72 ± 0.03 ng/mg). As to the left sides (non-injection side), the expression of IGF-1 content dropped from 0.77 ± 0.11 ng/mg (injection group) to 0.66 ± 0.05 ng/mg (control group). In general, the total IGF-1 content (two sides) in injection group (0.87 ± 0.07 ng/mg) was higher than that in control group (0.69 ± 0.06 ng/mg) (Figure 5).

**Detection of the established transgenic cell lines by PCR**

To clarify whether plasmid pIN was integrated into the genomes of transgenic cell lines, two specific primers were design to detect the positive clones. An amplified fragment for pIN included 384 bp of IGF-1 and 415 bp β-casein 3’ region, a total of 799 bp. Result shows that three positive clones carried the 799 bp fragment (Figure 6). Another amplified fragments (1354 bp) for pIN contained 389 bp β-casein 5’ region, 465 bp of IGF-1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
<th>Product length (bp)</th>
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<tr>
<td>IGF-1</td>
<td>gctctgtgaaccggagtat</td>
<td>gctctgttaaccgtcttaat</td>
<td>193</td>
</tr>
<tr>
<td>Bcap-37 beta-actin</td>
<td>gtcattgcctcctgagc</td>
<td>tgtgactgaggagagact</td>
<td>385</td>
</tr>
</tbody>
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Table 2. Quantitative PCR primers.
Figure 3. The expression of IGF-1 in Bcap-37 cell line. (A) The relative IGF-1 mRNA expression in Bcap-37 cell line. (B) SDS-PAGE result of IGF-1 protein in Bcap-37 cell line (A: M1, PageRuler™ Prestained Protein Ladder; 1, control group/12 h; 2, transfection group/12 h; 3, transfection with induction group/12 h; 4, control group/24 h; 5, transfection group/24 h; 6, transfection with induction group/24 h; 7, control group/36 h; 8, transfection group/36 h; M2, protein MW marker (low)). B: M2, Protein MW marker (low); 1, transfection with induction group/48 h; 2, transfection group/48 h; 3, control group/48 h; 4, transfection with induction group/36 h; 5, transfection group/36 h; 6, control group/36 h; M1, PageRuler™ Prestained Protein Ladder). (C) Western blot result of IGF-1 protein in Bcap-37 cell line (1, control group /24 h; 2, transfection group /24 h; 3, transfection with induction group/24 h; M, PageRuler™ Prestained Protein Ladder; 4, control group/36 h; 5, transfection group/36 h; 6, transfection with induction group/36 h). IGF-1, Insulin-like growth factor 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
gene and 500 bp β-casein 3' region. Results show that 1354 bp fragment appeared in clone lines 2 and 3, but not in clone line 1 for reason unknown (Figure 6). All these results indicate that plasmid pIN was integrated into the genomes of transgenic clone lines 2 and 3, but not clone lines 1.

DISCUSSION

IGF-1 plays an important role in mammary development (Loladze et al., 2006) and lactation (Hadsell et al., 2008). The mechanism may be that IGF-1 regulates signaling molecules to promote AKT activity in mammary cells.
which increases the persistence of lactation (Burgos and Cant, 2010) or that IGF-1 could reduce the oxidative damage to mammary epithelial cells which in turn could maintain lactation. IGF-1 also blocks the apoptotic process of mammary epithelial cells, thus prolonging the lactation period and improving milk ability (Hadsell et al., 2002). From the important role of IGF-1 in mammary development and lactation, what would be happened if IGF-1 was overexpressed? Su demonstrated that local over expression of IGF-1 would stimulate milk yield during the first lactation in transgenic mice (Su and Cheng, 2004). However, in transgenic swine, overexpressed IGF-1 notably increased IGF-1 and IGFBP content in milk, while it had no impact on lactation performance (Monaco et al., 2005). Interestingly, Noble found an apparent discontinuity between the enhanced milk production in transgenic sows and the increased growth rate of piglets (Noble et al., 2002). Sometimes IGF-1 may not increase milk production, but it really exerted effect in another form (increasing the piglet growth speed). So the over expression of IGF-1 in goat stand a good chance for increasing milk production.

Milk protein regulatory elements were wildly used in regulating lactation gene expression. Recent studies showed that β-lactoglobulin and β-casein obtained high level expression in mammary gland of rat (Lee et al., 1996), sheep (Wright et al., 1991), goats (Parker et al., 2004) and cows (van Berkel et al., 2002). Plasmid pBC1 used in this experiment contained a goat β-casein 5' regulatory element which could regulate the downstream gene expression (Kang et al., 1998). Research on lactating mammary of goat, sheep and cattle found that αs1- and β-casein transcripts were translated three to four fold more efficiently than αs2- and κ-casein transcripts. The percentage of αs1-Cas mRNA dramatically dropped from 30% (αs1-CasA/A) to 3% (αs1-CasE/F), with an intermediate value (17%) (αs1-CasA/E) (Bevilacqua et al., 2006). In order to verify plasmid bioactivity, plasmid pIN was transiently transfected into human breast cancer cell line Bcap-37 (Zhang et al., 2009). Quantitative PCR results showed that pIN could be transcribed successfully in Bcap-37 cell line. The high level expression of IGF-1 mRNA in transected group not only proved that β-casein 5' regulatory element highly initiated the transcription, but also verified that Bcap-37 cell line was suitable for detecting gene expression.

In this study, Western blotting result showed that IGF-1 were expressed at different times (12, 24, 36 and 48 h) and different treatments (control group, transfect group, transfect and induced group), while the concentration of IGF-1 had no significant difference. Taking into account the detection limit of Western blot, we further detected the content of IGF-1 by RIA. RIA results showed that the contents of IGF-1 in cell lysate and cell culture medium were increased with time lasting in control group. However, the increment of IGF-1 was slow and low, which implied that Bcap-37 cells could express IGF-1 stably at a low level. In general, the content of IGF-1 changed greatly in other two groups at different time. At the beginning (12 h), transfection group and transfection with induction group conveyed much more IGF-1 than that in control group. The significant difference (P<0.05) between control group and transfection group convinced us that plasmid pIN played its role in expressing IGF-1. The IGF-1's increment at 12 h was primarily attributed to the increment in cell lysate (Figures 4A and B), which meant that IGF-1 was produced in cells while it did not secrete into cell culture medium at 12 h. As times went on, the IGF-1 contents in transfection and transfection with induction group were only slightly higher than that in control group at 24 h. We supposed that all plasmid pIN were expressed at 12 h. A part of plasmid did not integrate into cells, so they lost the capacity to express IGF-1 which resulted in the drop down of IGF-1 content in
24 h.

The other hypothesis was that the transfection greatly harmed cells which consumed some IGF-1 to repair. Yee proved that IGF-1 was a potent survival factors for mammary epithelial cells (Yee and Wood, 2008). When the detection journey paused at 36 h, the content of IGF-1 in transfection and transfection with induction group had a huge increase, which was significantly higher than that in control group at 36 h. Results in 36 h hinted us that plasmid pIN was stably enrolled into cell chromosome or cells were fully recovered from the damage caused by transfection. Interestingly, the expression of IGF-1 in 36 h reached to a peak. It was also very important to stress that based on our research, the increment of IGF-1 was primarily due to the increasing IGF-1 in cell culture medium which hinted that IGF-1 was synthesized and secreted into cell cultural medium in 36 h. The expression of total IGF-1 showed a downward trend at 48 h. Surprisingly, the content of IGF-1 in cells cultural medium did not decrease notably, especially for transfection group and transfection with induction group which kept a high level of IGF-1 expression in 48 h. However in cells lysate, the content of IGF-1 declined notably in 48 h. This result confirms that the expressed IGF-1 was continuously secreted into cells cultural medium at 48 h. As for the reason, the depletion of nutrient might have led to lack of raw materials to synthesize IGF-1, which would result in the reduction of IGF-1 in cell lysate. Meanwhile, it was important to note that based on our analysis, obvious increase in IGF-1 protein expression in injection side cannot be attributed specifically to plasmid pIN. Observations in vivo also found that the expression level of IGF-1 protein increased not only in injection sides but also in non-injection sides.

All RIA statistics showed that transfected cell could express goat IGF-1 protein and had potential to stimulate the increase of IGF-1 in cell level. Results also prove that plasmid pIN had bioactivity in efficiently expressing IGF-1 in mammary gland cells. Previous studies had found that increased activity of mammary epithelial cells would lead to the intensifying of early lactation (Capuco et al., 2001). IGF-1 could not only increase the number of mammary epithelial cells, but also activate the secretion of mammary epithelial cells. Our data suggests that plasmid pIN had the potential to stimulate the expression of IGF-1 in human breast cancer cell line Bcap-37, which probably suggested that plasmid would be effective in transgenic goat. Hadsell et al. (2002) proved that the sustained expression of IGF-1 not only blocked the apoptosis of mammary epithelial cells, but also slowed the aging of mammary cells. Results also confirm that IGF-1 could prolong the time of lactation and increase the milk secretion capacity. Our research demonstrates that plasmid pIN could exhibit favorable bioactivity in efficiently expressing IGF-1, which would be a promising way in increasing the milk production.

REFERENCES


