African Journal of Biotechnology Vol. 11(27), pp. 6946-6955, 3 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4069
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

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.

Accepted 16 February, 2012

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, pIN, 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* JM 109 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 (Gene Bank 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, 50 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* JM 109 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 pI. Then we designed a primer to check plasmid pI. Furthermore, to produce *neo* fragment, recombinant plasmid pMD19-T-*neo* was digested with restriction enzyme *Not* I. Plasmid pI was also digested with *Not* I. Then we used T4 DNA ligase to link digested plasmid pI 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% CO_2.

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 radioimmunoassay (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 pIN only) and transfection with induction group (transfected with pIN and induced with insulin, PRL and Hydrocortisone). 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 added with insulin (10 µg/ml), PRL (1 µg/ml) and hydrocortisone (20 µg/ml). Cells and medium were collected at 12, 24, 36 and 48 h for subsequent test.

For Western-blot analysis, the cells were collected and dissolved in radioimmunoprecipitation assay (RIPA) buffer (1 mm phenylmethylsulfonyl fluoride (PMSF)). Supernatants were loaded on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Then gels were wet transferred onto nitrocellulose (NC) membranes. Membranes were blocked with Tris-buffer saline Tween20 (TBST) buffer containing 5% non-fat milk at room temperature for 2 h, then incubated with mouse anti-human IGF-1 monoclonal antibody at 4°C for 18 h and subsequently incubated with secondary HRP-IgG (1:10000) antibody at room temperature for 2 h. Then IGF-1 proteins were

Table 1. PCR primers.

Gene	Sense primer	Anti-sense primer	Product length (bp)
IGF-1	ACAGGTACCATGGGAAAAATCAGCAGTCT	CGCAAGCTTCTACATTCTGTAGTTCTTGT	501
infusion IGF-1	ATCGCGGATCCTCGAGATGGGAAAAATCAG	ATCGCGGATCCTCGAGATGGGAAAAATCAG	513
neo	GCGGCCGCTGTGGAATGTGTGTCAGTTA	GCGGCCGCACAGACATGATAAGATACAT	1521
pBC1- IGF-1	TTTGCAGGATCTTGGTTC		
pBC1354	GTGAATGAGATGAAAAAGAGT	TCAAAAACAAGATGTGAAATG	1354
pIN799	ACATCCTCCTCGCATCTCTTC	TTAGGTTTGTTATTCTTAGCC	799

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 aspired for testing.

One hundred microliters each of cells lysate and cells culture medium extracts (or standards), IGF-1 anti-serum, and 125I-IGF-1 were pipetted into duplicate RIA tubes. Tubes were vortexed and incubated with rotation at 4°C for 48 h. At the end of the incubation, 500 µL of the separation reagents was added to each tube followed by 30 min incubation at room temperature. The tubes were then centrifuged at 3600 rpm, 4°C for 20 min, to separate bound 125I-IGF-1 from unbound. Immediately after centrifugation, the supernatant was removed by aspiration. All RIA tubes were then placed in a Beckman Gamma 4000 counter, and the bound 125I-IGF-1 fraction radioactivity was counted for 2 min per tube.

The expression of recombinant plasmid pIN in goat mammary

Plasmid injection

10 healthy goats were divided into two groups: control group (lactating goat primed with saline) and experimental group (lactating goat injected with plasmid pIN). For the experimental group, 400 ng plasmid pIN was dissolved into saline and injected into right side mammary gland of each goat in day three and seven. Then mammary gland tissues of each group were collected to conduct subsequent testing in day 10.

Detecting the expression of IGF-1 protein in goat mammary

The concentration of growth hormone (GH) protein in goat mammary of different groups was measured by human IGF-I Quantikine ELISA Kit (R&D, USA). ELISA assays were performed in accordance with protocol.

Transfection of the goat ear fibroblast lines and establishment of transgenic cell lines

Purified goat ear fibroblast cells (donated by shanghai transgenic research) were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). The whole procedure was conducted according to the description provided with liposome 2000 regent (Invitrogen). Briefly, 1×10^5 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 group 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 ig*f-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.

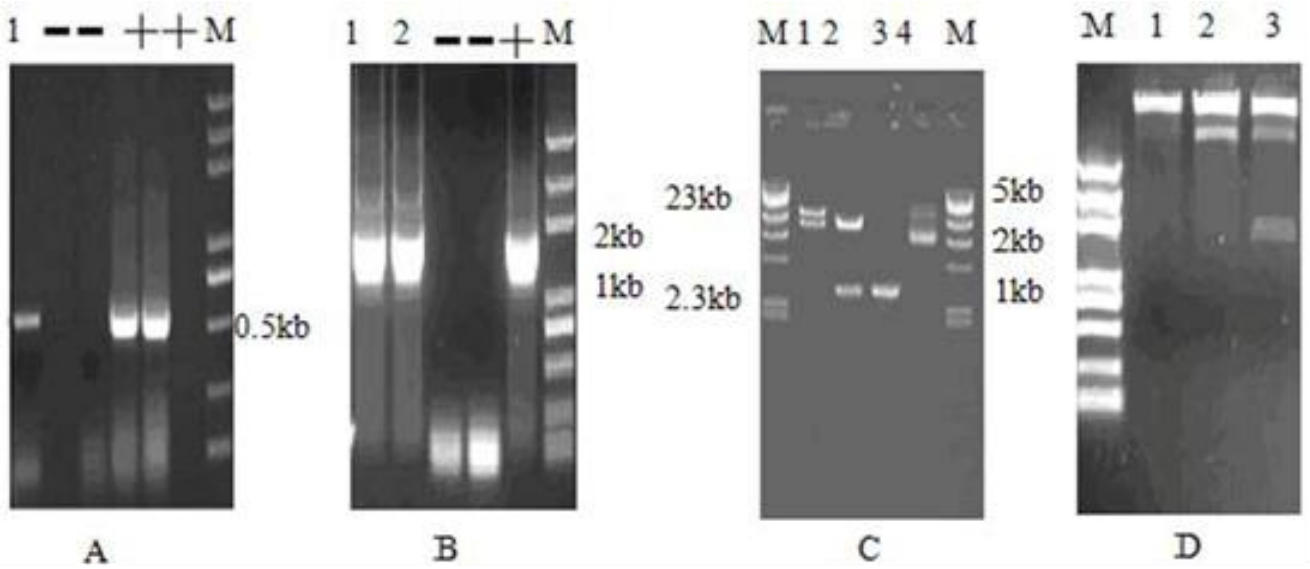

Figure 1. Construction and verification of vector pIN. (A) IGF-1 electrophoresis of PCR product (1, IGF-1; -, negative clone; +, positive clone; M, DNA marker / Trans2K Plus). (B) Neo electrophoresis of PCR product (1, 2, neo; -, negative clone; +, positive clone; M, Marker DNA /Trans2K Plus). (C) Identification of vector PI digested with restriction enzymes (Kpn I/Cla I) (1, vector pBI-Kpn I/Cla; 2, vector pI-Kpn I/Cla I; 3, pBC I-Kpn I/Cla I; 4, vector pI; M, marker DNA /λ-Hind III digest). (D) Identification of vector pIN digested with restriction enzyme (Not I) (1, 2, vector pIN; 3, vector pI-Not I; M, marker DNA /λ-Hind III digest).

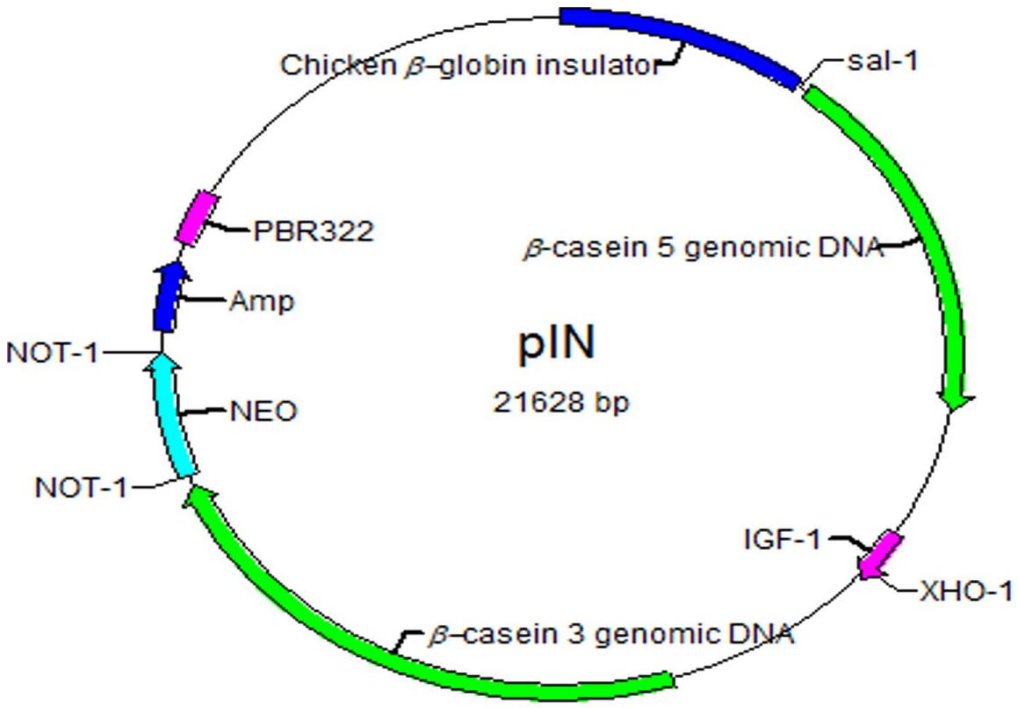

Figure 2. The map of vector pIN.

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

Table 2. Quantitative PCR primers.

Gene	Sense primer	Anti-sense primer	Product length (bp)
IGF-1	cgtctgtgaacccggagtat	gcctcgttcaccgtcttaat	193
Bcap-37 beta-actin	gatcattgctcctcctgagc	tgtggacttgggagaggact	385

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

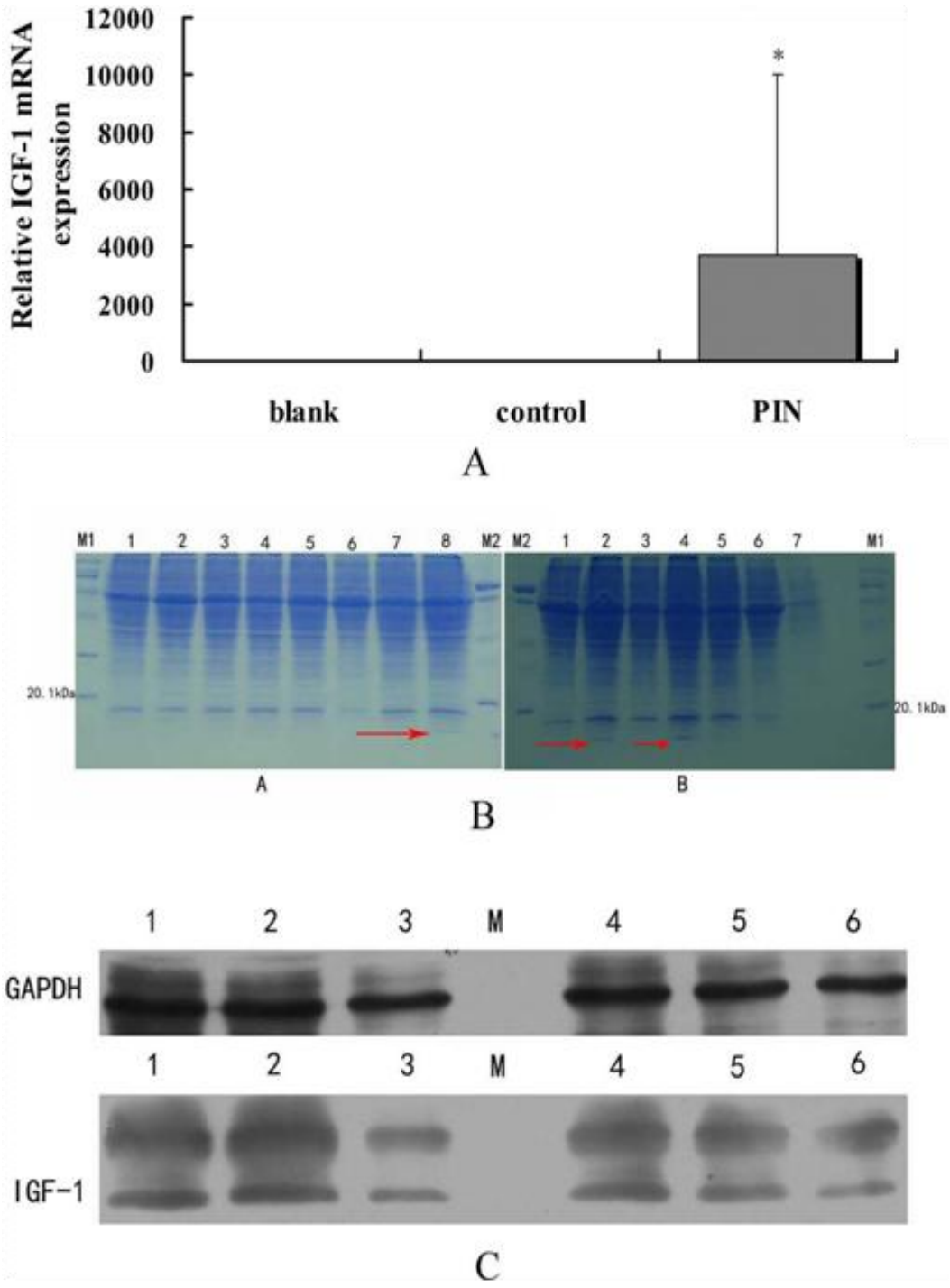

Figure 3. The expression of IGF-1 in Bcap-37 cell line. (A) The relative IGF-1 mRNA expression in Bcap-37 cell line. (B) SDS-PAGE result of IGF-1 protein in Bcap-37 cell line (A: M1, PageRuler™Prestained Protein Ladder; 1, control group/12 h; 2, transfection group/12 h; 3, transfection with induction 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.

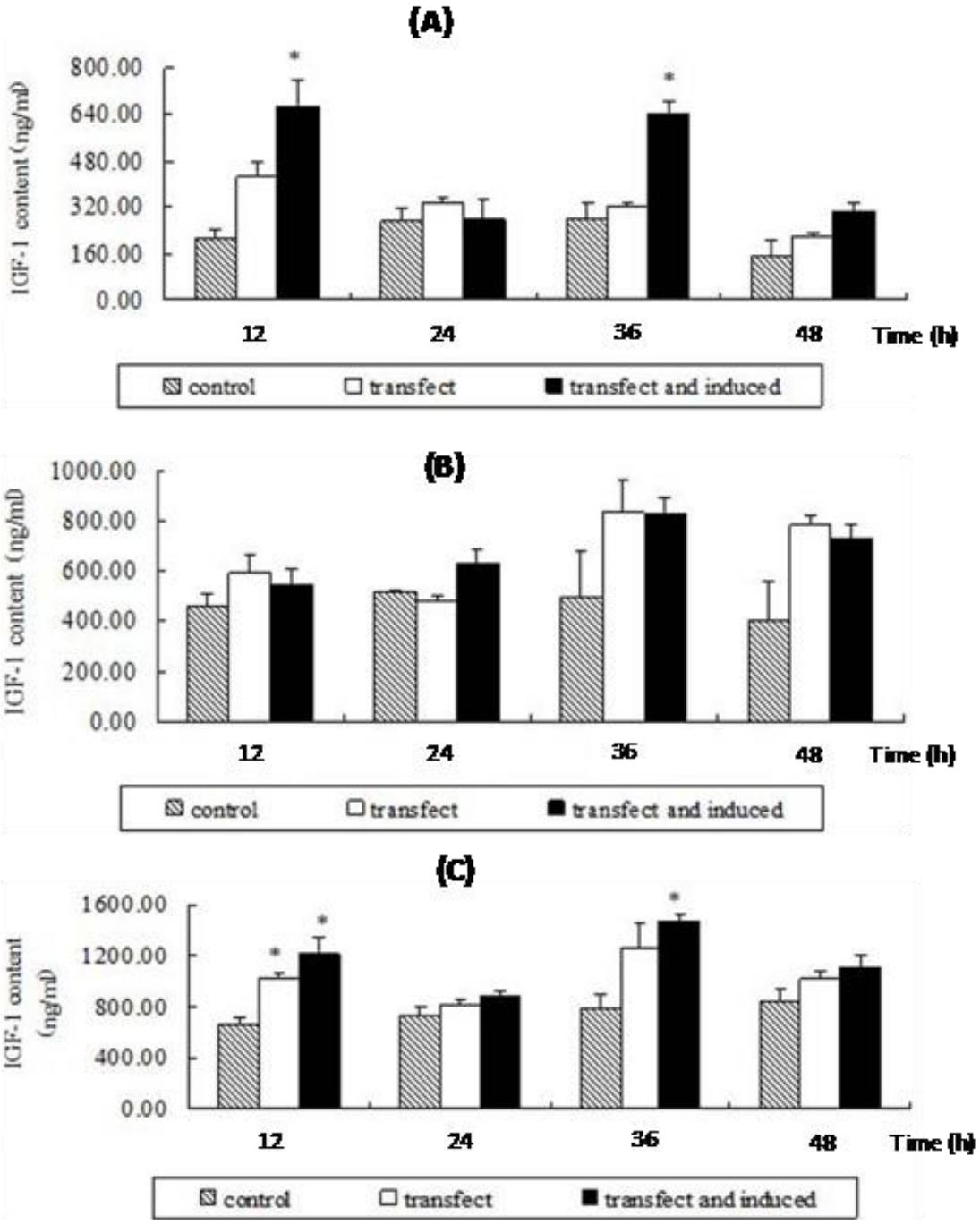

Figure 4. RIA result of IGF-1 content in Bcap-37 cell line. (A) The IGF-1 content in cell lysate in different group at the same time (*significant difference $P < 0.05$). (B) The IGF-1 content in cell culture medium in different group at the same time. (C) The IGF-1content in cell culture medium and cell lysate of different group at the same time (*significant difference $P < 0.05$). RIA, Radioimmunoassay; IGF-1, insulin-like growth factor 1.

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

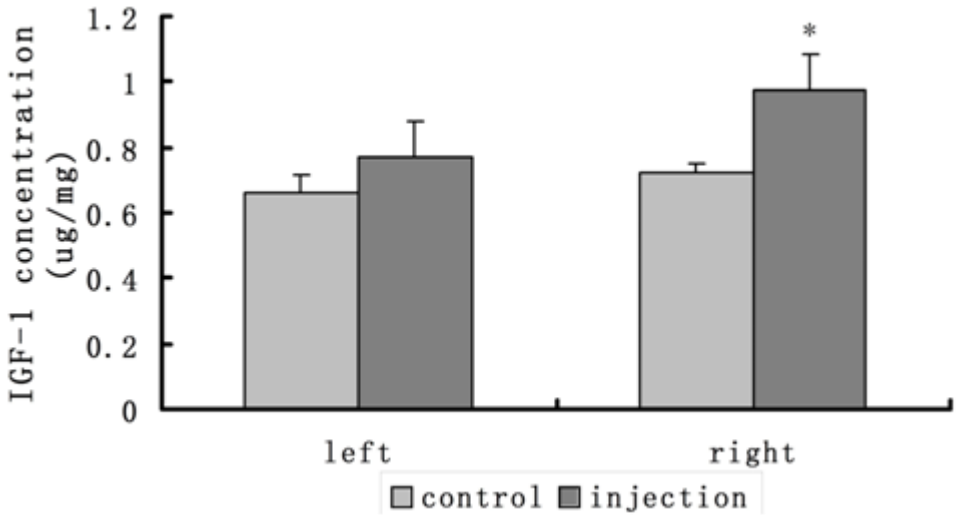

Figure 5. The IGF-1 concentration in goat mammary gland. IGF-1, Insulin-like growth factor 1.

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, over-expressed 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 transfected group not only proved that β-casein 5′ regulatory element highly initialed 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 inducted 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

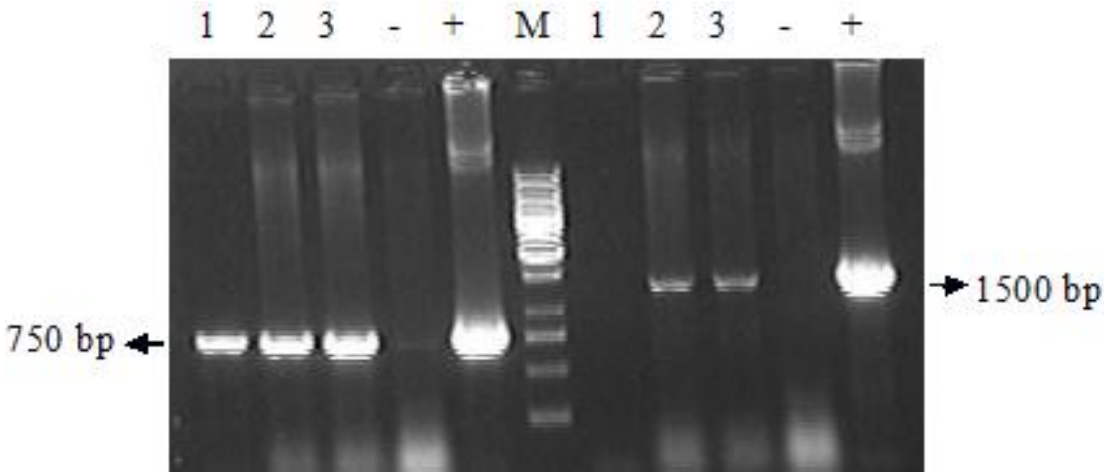

Figure 6. The detection of established transgenic cell lines by PCR. Lanes 1, 2 and 3, Positive transgenic cell lines; -, negative clone; +, plasmid pIN; M, marker DNA.

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. This overall rising, whether direct, indirect, or both, would require a coordinated response within the mammary gland that involved in many different pathways.

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 plasmidpIN could exhibit favorable bioactivity in efficiently expressing IGF-1, which would be a promising way in increasing the milk production.

REFERENCES

Bevilacqua C, Helbling JC, Miranda G, Martin P (2006). Translational efficiency of casein transcripts in the mammary tissue of lactating ruminants. Reprod. Nutr. Dev. 46: 567-578.

Burgos SA, Cant JP (2010). IGF-1 stimulates protein synthesis by enhanced signaling through mTORC1 in bovine mammary epithelial cells. Domest. Anim. Endocrinol. 38: 211-221.

Capuco AV, Wood DL, Baldwin R, McLeod K, Paape MJ (2001). Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of bST. J. Dairy. Sci. 84: 2177-2187.

Hadsell DL, Bonnette SG, Lee AV (2002). Genetic manipulation of the IGF-I axis to regulate mammary gland development and function. J. Dairy. Sci. 85: 365-377.

Hadsell DL, Greenberg NM, Fligger JM, Baumrucker CR, Rosen JM (1996). Targeted expression of des (1-3) human insulin-like growth

factor I in transgenic mice influences mammary gland development and IGF-binding protein expression. Endocrinology, 137: 321-330.
Hadsell DL, Parlow AF, Torres D, George J, Olea W (2008). Enhancement of maternal lactation performance during prolonged lactation in the mouse by mouse GH and long-R3-IGF-I is linked to changes in mammary signaling and gene expression. J. Endocrinol. 198: 61-70.
Kang YK, Lee CS, Chung AS, Lee KK (1998). Prolactin-inducible enhancer activity of the first intron of the bovine beta-casein gene. Mol. Cells, 8: 259-265.
Kaskous S, Grun E, Gottschalk J, Hippel T (2003). The behavior of lactogenic and steroid hormones in the blood of Awassi ewes in Syria during lactation. Berl. Munch. Tierarztl. Wochenschr. 116: 117-123.
Lee CS, Kim K, Yu DY, Lee KK (1996). An efficient expression of human growth hormone (hGH) in the milk of transgenic mice using rat beta-casein/hGH fusion genes. Appl. Biochem. Biotechnol. 56: 211-222.
Loladze AV, Stull MA, Rowzee AM, Demarco J, Lantry JH, Rosen CJ, Leroith D, Wagner KU, Hennighausen L, Wood TL (2006). Epithelial-specific and stage-specific functions of insulin-like growth factor-I during postnatal mammary development. Endocrinology, 147: 5412-5423.
Monaco MH, Gronlund DE, Bleck GT, Hurley WL, Wheeler MB, Donovan SM (2005). Mammary specific transgenic over-expression of insulin-like growth factor-I (IGF-I) increases pig milk IGF-I and IGF binding proteins, with no effect on milk composition or yield. Transgenic. Res. 14: 761-773.
Noble MS, Rodriguez-Zas S, Cook JB, Bleck GT, Hurley WL, Wheeler MB (2002). Lactational performance of first-parity transgenic gilts expressing bovine alpha-lactalbumin in their milk. J. Anim. Sci. 80: 1090-1096.
Parker MH, Birck-Wilson E, Allard G, Masiello N, Day M, Murphy KP, Paragas V, Silver S, Moody MD (2004). Purification and characterization of a recombinant version of human alpha-fetoprotein expressed in the milk of transgenic goats. Protein Expr. Purif. 38: 177-183.
Plante I, Stewart MK, Laird DW (2011). Evaluation of mammary gland development and function in mouse models. J. Vis. Exp. 53: 2828-2836.
Prosser CG, Davis SR, Farr VC, Moore LG, Gluckman PD (1994). Effects of close-arterial (external pudic) infusion of insulin-like growth factor-II on milk yield and mammary blood flow in lactating goats. J. Endocrinol. 142: 93-99.
Ruan W, Kleinberg DL (1999). Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. Endocrinology, 140: 5075-5081.
Su HY, Cheng WT (2004). Increased milk yield in transgenic mice expressing insulin-like growth f actor 1. Anim. Biotechnol. 15: 9-19.
Tokunou T, Miller R, Patwari P, Davis ME, Segers VF, Grodzinsky AJ, Lee RT (2008). Engineering insulin-like growth factor-1 for local delivery. Faseb. J. 22: 1886-1893.
Van Berkel PH, Welling MM, Geerts M, Van Veen HA, Ravensbergen B, Salaheddine M, Pauwels EK, Pieper F, Nuijens JH, Nibbering PH (2002). Large scale production of recombinant human lactoferrin in the milk of transgenic cows. Nat. Biotechnol. 20: 484-487.
Wright G, Carver A, Cottom D, Reeves D, Scott A, Simons P, Wilmut I, Garner I, Colman A (1991). High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Biotechnology (N. Y.). 9: 830-834.
Yee D, Wood TL (2008). The IGF system in mammary development and breast cancer. Preface. J. Mammary. Gland. Biol. Neoplasia, 13: 351-352.
Zhang X, Wu Y, Luo F, Su H, Bai Y, Hou Y, Yu B (2009). Construction of targeting vector for expressing human GDNF in cattle mammary gland. Appl. Biochem. Biotechnol. 159: 718-727.
Zinovieva N, Lassnig C, Schams D, Besenfelder U, Wolf E, Muller S, Frenyo L, Seregi J, Muller M, Brem G (1998). Stable production of human insulin-like growth factor 1(IGF-1) in the milk of hemi- and homozygous transgenic rabbits over several generations. Transgenic Res. 7: 437-447.