

Expression of Recombinant Human Nerve Growth Factor Beta in Milk of Goats by Recombinant Replication-Defective Adenovirus

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Abstract For producing a large quantity of human nerve growth factor beta (hNGF- β), the recombinant adenovirus containing an expression cassette of hNGF- β complementary DNA gene was constructed and then instilled directly into goats' mammary glands. The recombinant hNGF- β was detected in the milk. The maximal expression level of recombinant hNGF- β in the milk reached 196.8 mg/l. The biological activity of the recombinant hNGF- β , compared with the commercial hNGF- β , was tested using PC12 cells. The results demonstrate that instilling recombinant adenovirus directly into the mammary gland of goat is an efficient approach to producing a large quantity of hNGF- β .

Keywords Adenovirus · Goat · Human nerve growth factor beta · Mammary gland · Milk

Introduction

As one of the earliest revealed cellular growth regulators, the nerve growth factor (NGF) is essential to the survival, growth, and differentiation of central and peripheral nerves [1, 2]. Some studies have suggested that NGF could improve cognitive function in impaired, aged, or cholinergic lesion models [3–7]. NGF clinical use has a potential to repair or regrow damaged nerves, to prevent and treat retrograde neurological diseases such as Alzheimer's

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disease, and to promote differentiation of neuroblast [8–11]. Furthermore, NGF could also accelerate wound healing, restrain tumor cells, and has been used in ulcer treatment [12, 13]. Therefore, NGF is required to be possibly produced at a reasonable cost and in sufficient amounts for clinical trials and therapeutic purposes.

For more than a decade, several attempts have been made to produce large amounts of recombinant human nerve growth factor beta (rhNGF- β) (the functional subunit of human NGF) by genetic approaches from *Escherichia coli*, yeast, insect, and mammalian cells. However, the quantity of rhNGF- β produced by these approaches could not suffice for the clinical requirement. Although the use of transgenic animals is considered as another method for the large-scale production of rhNGF- β in terms of overall yield, the long time and high expense for constructing a transgenic animal make the large-scale production of rhNGF- β uneconomical and inefficient. Therefore, large-scale production of rhNGF- β still remains challenged.

In recent years, a new approach for expressing exogenous genes in secretory mammary epithelial cells has been proven to be the most effective versatile alternative [14–18]. A target protein could be obtained from milk by employing the recombinant adenovirus directly transfected into the mammary gland. However, as suggested in previous reports [15, 16], the successful expression of a recombinant protein using this approach relies on many factors, including the physiological and biological properties of the recombinant protein itself, the promoter driving the recombinant protein expression *in vivo*, and the animals used to express the recombinant protein.

In this study, we used goats as animal models to produce rhNGF- β in the mammary gland. The replication-defective recombinant adenovirus encoding hNGF- β complementary DNA (cDNA) gene was constructed and transfected into goats' mammary glands in lactation. The efficiency of expressing rhNGF- β was investigated by detecting the rhNGF- β in the milk of goats.

Materials and Methods

Preparation of Bacteria, Cells and Animals

The *E. coli* DH5 α and BJ5183 were purchased from Invitrogen Ltd. (Shanghai, China) and preserved in our institution.

The human embryonic kidney 293 (HEK-293) cells and the rat pheochromocytoma cells (PC12 cells) were purchased from the center of cells of Peking Union Medical College (Beijing, China). The HEK-293 cells were seeded in DMEM supplemented with 10% fetal bovine serum. The PC12 cells were seeded in RPMI 1640 supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum.

Six adult lactating goats (45 \pm 2.5 kg) were bred from a local goat farm and kept in the animal facility at the Department of Biological Engineering, the University of Yanshan. All animal procedures were approved by the Animal Care and Use Committee of the University of Yanshan.

Construction of Recombinant Adenoviral Vectors

Total RNA was extracted from a legally obtained human embryo heart with Trizol reagent according to the protocol described by the supplier (Promega, Beijing, China). Then the

cDNA of hNGF- β was obtained from the total RNA by reverse transcriptase polymerase chain reaction (RT-PCR). The primer pair was as follows: P1: 5'-GTggtaccGCATAGCGTAATGTCCAT-3' (corresponding to positions 152–177 of the NGF- β mRNA [GenBank accession no. NM-002506]) with an introduced *KpnI* recognition site (underlined in the primer); P2: 5'-GTtctcgagTCGGCAGGTCAGGC-3' (positions 890–911) with an introduced *XhoI* recognition site (underlined in the primer). The procedure of RT-PCR was as follows: Ten microliters of reverse transcription reaction mixture containing 5 μ g of total RNA, 500 μ M deoxyribonucleotide triphosphates (dNTPs), 0.2 μ M each of primers and DEPC-treated water, was incubated at 65°C for 5 min, and then placed on ice for 1 min. After that, the reverse transcription was carried out in the presence of 1 μ l of SuperscriptTMIII reverse transcriptase (200 U/ μ l), 1 μ l of RNase inhibitor (40 U/ μ l), 2 μ l of 10 \times buffer, 4 μ l of MgCl₂ (25 mM) and 2 μ l of DTT (0.1 M) at 50°C for 50 min. Subsequently, the reaction was terminated at 85°C for 5 min. The reverse transcription product was kept at -20°C until use. PCR was performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Munich, Germany). One microliter of reverse transcription product was subjected to amplification in 50 μ l of mixture containing 1.5 units of KOD Plus DNA polymerase, 5 μ l of 10 \times PCR buffer, 100 μ M dNTP, and 25 pM primer P1 and P2. The PCR cycle was as follows: initial denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 90 s; and a final extension at 72°C for 7 min. The RT-PCR product was stored at -70°C until use.

All standard recombinant DNA protocols were followed as described [19, 20]. The RT-PCR product was digested with *KpnI/XhoI*, purified and inserted into *KpnI/XhoI*-digested pShuttle-cytomegalovirus (CMV) vector. The resulted plasmid was designated pSh-NGF (8125 bp). After being cloned in *E. coli* DH5 α , the pSh-NGF containing the cDNA sequence of hNGF- β was sent out for sequencing.

A 1,308-bp *XhoI/EcoRV* DNA fragment including internal ribosome entry site (IRES) and green fluorescent protein (GFP) gene was excised from the pIR-G vector (constructed by our institution). After gel purification, the 1,308-bp DNA fragment was cloned into the pSh-NGF. The resulting vector was designated pSh-hNGF-GFP and linearized by *PmeI*. The linearized pSh-hNGF-GFP and an Ad-easy vector (Stratagene, La Jolla, CA, USA) containing the adenovirus type 5 genome deleted E1 and E3 regions were simultaneously transformed in ultracompetent BJ5183 bacterial cells to yield an Ad-hNGF recombinant adenoviral vector. At the same time, a recombinant adenoviral vector containing GFP gene was also constructed using the similar ways as above and designated Ad-GFP. The Ad-hNGF and Ad-GFP were linearized with *PacI*, and then purified by the commercial purification kits (BioDev-Tech, Beijing, China) according to the manufacturer's instructions. The final linearized Ad-hNGF and Ad-GFP were stored at -70°C until use.

Preparation of Recombinant Viruses

Intermediated by Lipofectamine reagent, the linearized Ad-hNGF or Ad-GFP was transfected into host HEK-293 cells and then the primary adenoviral stock was obtained. After further amplification in HEK-293 cells, the recombinant adenoviral stocks were collected and purified using an Adeno-XTM Virus Purification Kit (Takara, Dalian, China). The titer of each viral stock was determined from the GFP expression in semiconfluent HEK-293 cells and expressed in gene transfer units (GTU). The concentrated viral stock, containing Ad-hNGF or Ad-GFP, was designated Ad-N or Ad-G, and stored at -70°C until use.

Adenovirus Infusion and Milk Processing

Six female goats were divided randomly into two equal groups: the experimental group and the control group. The adenoviral infusion was consulted as described elsewhere [18]. For all mammary infusions and sample collections, the teats were routinely sterilized with 70% alcohol and iodine to prevent mammary gland infection. A 200-ml phosphate-buffered saline (PBS) solution containing 10^9 GTU/ml of Ad-N was infused into the right mammary glands of the experimental group goats and the same viral dose of Ad-N in a solution of PBS supplemented with 40 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was infused into the left glands. At the same time, the same volume of PBS solution was infused into the right mammary glands of the control group goats for vacant control group and the same viral dose of Ad-G in a solution of PBS was infused into the left glands of the control group goats for negative control group.

All the mammary glands were milked on the indicated days. The collected milk was immediately centrifuged at $15,000\times g$ for 20 min at 4°C . The supernatant of milk was separated from the layer as completely as possible, and then diluted with equal volume of PBS. The diluted supernatant of milk was adjusted to pH 4.5 by adding 10% acetic acid. After 3 h of incubation at room temperature, the precipitated protein was removed by centrifugation at $17,000\times g$ for 30 min. The supernatant was immediately neutralized with 1 M NaOH and clarified by sterile filtration ($0.22\ \mu\text{m}$). The clear filtrate was named milk serum and stored at -70°C for later assay.

rhNGF- β Detection

The supernatant of the cell culture and the milk serum of the goats were fractionated on SDS-polyacrylamide gel electrophoresis (PAGE) in 12% gels. After that, the transfer of proteins to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) was performed according to standard protocols on a Trans-blot and Semi-dry Electrophoretic Transfer Cell (Biometra, Göttingen, Germany). Detection of rhNGF- β was carried out by Western blot analysis with mouse anti-hNGF monoclonal antibody (R&D Systems, Minneapolis, MN, USA) as the primary antibody, and goat anti-mouse IgG-conjugated horseradish peroxidase (Dingguo, Beijing, China) as secondary antibody. The recombinant hNGF- β concentration was measured with DuoSet[®] ELISA Development kit (R&D Systems) according to the manufacturer's protocol.

Biological Activity Assay

The biological activity of the recombinant hNGF- β in the milk was determined using PC12 cells. PC12 cells were plated at a density of about 2×10^5 cells/cm² in 12-well plastic culture dishes. After incubation for 3 h at 37°C in 800 μl RPMI 1640 supplemented with 10% FBS in 5% CO₂, the medium overlaid on the PC12 cells was replaced with fresh RPMI 1640 supplemented with 2% FBS and 1% glutamine. The milk serum from the goats infused with recombinant adenoviruses were added to the plates in final doses of 1, 2, 5, 10, 25, and 50 ng/ml rhNGF- β and compared to the same concentrations of commercial recombinant hNGF- β (R&D Systems) likewise diluted with RPMI 1640. Untreated PC12 cells and the PC12 cells treated with milk serum from control group goats diluted with RPMI 1640 were used as controls. Fresh medium with or without additive was added on day 3. On day 6 (dose-dependent neurite outgrowth) and days 2, 4, and 6 (time-dependent neurite outgrowth) the cells were examined.

Results

Construction of Recombinant Adenoviral Vectors and Preparation of Viral Stock

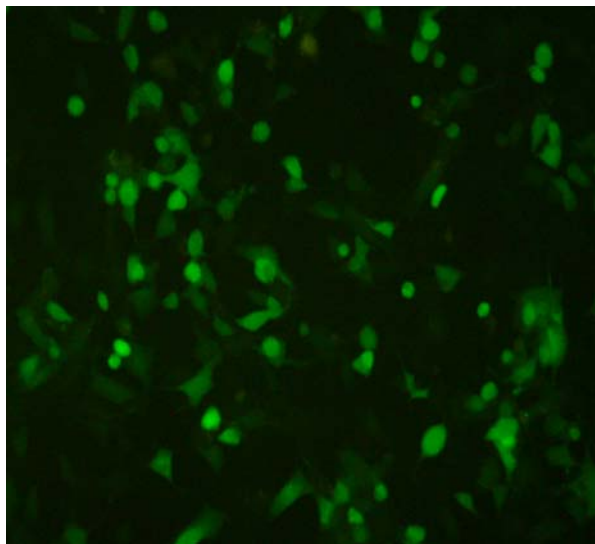
As HEK-293 cells could supply the material for replication-defective adenovirus propagation, after the linearized Ad-hNGF was transfected into HEK-293 cells for 48 h, the expression of GFP gene was detected in 100% of infected HEK-293 cells (Fig. 1) along with some cells floating in the media. The expression of GFP implied that the recombinant adenoviral vector was correctly constructed and the heterologous genes in the vector could be expressed in infected HEK-293 cells. After 7 to 10 days, the forming of the pathologic HEK-293 cells implied that the recombinant adenoviruses were successfully packaged. When up to 90% of the pathologic HEK-293 cells were formed, the recombinant adenoviruses were harvested.

After further amplified in HEK-293 cells, the recombinant adenoviruses were harvested and purified. The titer of the purified stock was determined by the GFP expression on semi-confluent HEK-293 cells and expressed in GTU. The titer of the purified stock reached 2×10^{10} GTU/ml.

Adenoviral Transduction and Detection of rhNGF- β

Previous studies have shown that the efficiency of viral transduction is diminished owing to the low permeability of the secretory epithelium during active lactation [15]. Some reports [17, 21] have shown that a temporal disruption of tight junctions through an EGTA treatment could be a potential solution to this problem. We infused a PBS solution containing 10^9 GTU/ml of the Ad-N recombinant adenovirus supplemented with 40 mM of EGTA into the left mammary glands of experimental group goats and compared to the counterpart. During the first 2 days of milking, the milk yield from the mammary glands of goats infused with viruses was particularly low but increased on the subsequent days. No appreciable differences in milk volumes were found between both halves of the udders in

Fig. 1 The expression of GFP in HEK-293 cells (magnification is $\times 400$). The expression of GFP implied the viral genes were also expressed in HEK-293 cells



experimental group ($P>0.05$) (Fig. 2). The cutdown and subsequent increase of the milk yield indicated that the adenoviruses affected the lactation of the lactating cells in the early stage of infection, and after the viruses transfected into the epithelia, the lactation of lactating cells were restored to the normal level. Meanwhile, the inconspicuous change of the milkability from the mammary glands of goats only infused with PBS suggested that the infusion of PBS did not affect the milk yield. The similarity of change of the milkability in the experimental group suggested that the temporary disruption of tight junctions mediated by EGTA did not affect the milk yield either.

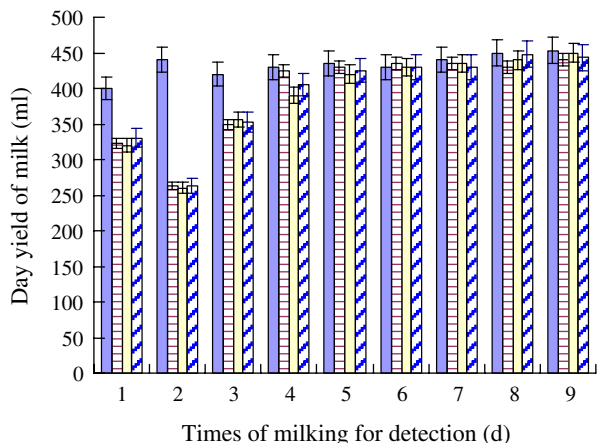
On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or Western blot, milk-derived rhNGF- β from experimental groups migrated as a narrow compact band with a similar size to the commercial counterpart. Meanwhile, no specific band with the similar size as commercial hNGF- β was found in the vacant control or negative control groups (Fig. 3). According to the enzyme-linked immunosorbent assay (ELISA) results, the expression level of rhNGF- β averages in the range from 165 to 11.5 mg/l on the indicated milking days. The maximal quantity of rhNGF- β expressed in the milk achieved 196.8 mg/l.

The mammary gland infused with viral inoculants containing EGTA showed an expression of rhNGF- β of about one fold and a half higher than the counterpart infused with a solution devoid of EGTA (Fig. 4). During experiments, the EGTA-inocula treated udder half yielded rhNGF- β at an average of 118.6 mg/l, whereas the non-EGTA treated udder half yielded hNGF- β at an average of only about 77.2 mg/l. Regardless of the treatment used in both experimental groups, the expression level of rhNGF- β dropped daily, and it was below 15 mg/l by day 9 of milking (Fig. 4).

Biological Activity Assay

The PC12 cells were excited using the milk serum from adenovirus-transfected mammary glands, the milk serum from control mammary glands and the commercial hNGF- β , respectively. After 48 h, the difference between these groups of cells was examined under an inverted microscope. On day 6, cells treated with milk serum containing 50 ng/ml hNGF- β exhibited substantial neurite outgrowth, whereas most cells treated with milk serum from the control and untreated groups died. The increased neurite outgrowth in both

Fig. 2 The comparison of milking yields in the indicated days among various groups. The volume of milk presents one gland from vacant control group (solid rectangle), negative control group (empty rectangle), experimental group infused with PBS containing EGTA (*horizontal line* in empty rectangle) and experimental group infused with PBS devoid of EGTA (*slant line* in empty rectangle). As each group has three glands, the *error bars* represent S.E.M. ($n=3$)



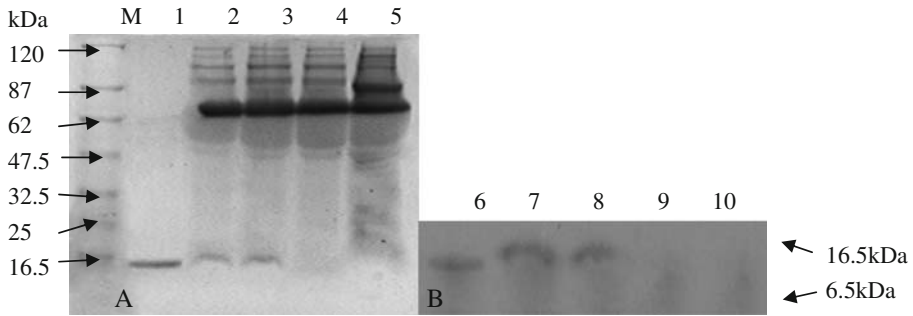


Fig. 3 SDS-PAGE and Western Blot detected the expression of hNGF- β in milk. Twenty microliters of each of samples was loaded in each well on the gels. *A.* Detection by SDS-PAGE. Lane *M*, protein molecular weight markers; lane *1*, standard protein of hNGF- β from R & D; lane *2*, milk serum from glands infused with PBS containing recombinant adenoviruses and EGTA; lane *3*, milk serum from glands infused with PBS containing recombinant adenoviruses devoid of EGTA; lane *4*, milk serum from glands infused with PBS; lane *5*, milk serum from glands infused with PBS containing vacant adenoviruses. *B.* Detection by Western blotting. Lane *6*, standard protein of hNGF- β from R & D; lane *7*, milk serum from glands infused with PBS containing recombinant adenoviruses and EGTA; lane *8*, milk serum from glands infused with PBS containing recombinant adenoviruses devoid of EGTA; lane *9*, milk serum from glands infused with PBS; lane *10*, milk serum from glands infused with PBS containing vacant adenoviruses

experiments for rhNGF- β and commercial hNGF- β were shown as Fig. 5. These results indicate that our rhNGF- β had full bioactivity compared with the commercial hNGF- β .

Discussion

The Advantage of GFP

In construction of the adenoviral vector, we have employed a dual-expression system with IRES sequence and included a GFP gene as a live marker for the recombinant protein expression. As the GFP gene in the downstream of the IRES is controlled by the same CMV promoter to the hNGF- β in the upstream of the IRES, the expression of the GFP gene implies the certainly expression of the recombinant hNGF- β . Besides simplifying the adenovirus titration, the inclusion of GFP gene in the downstream of the target gene under

Fig. 4 The change of quantity of hNGF- β obtained in milk from goats on indicated days infused with viruses. The error bars represent minus warp in mensuration

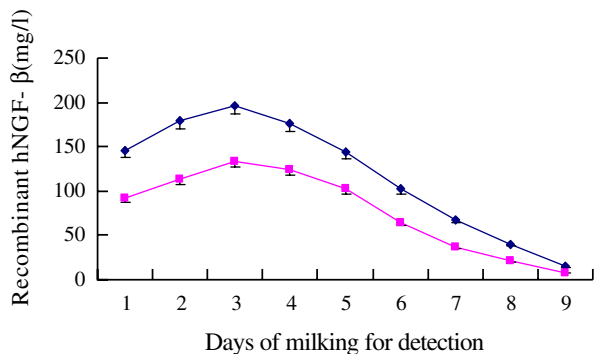
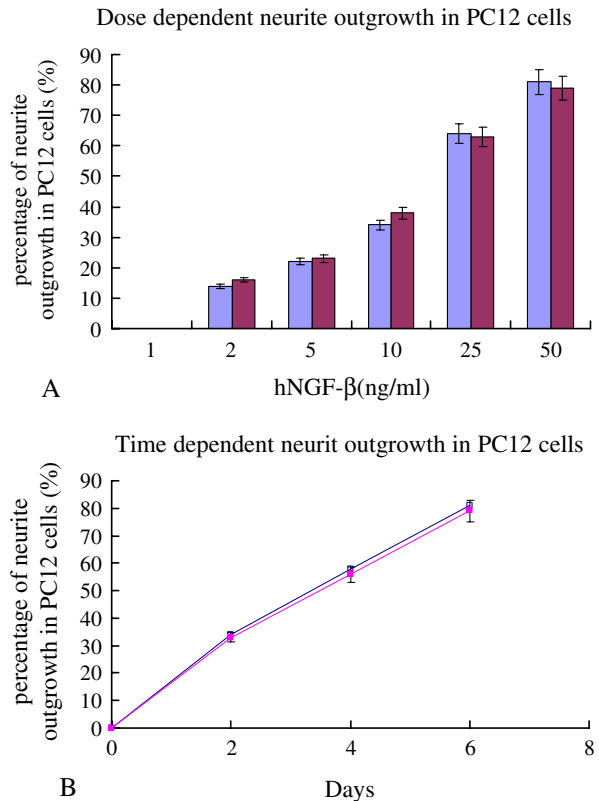


Fig. 5 Dose- and time-dependent neurite outgrowth in PC12 cells.

A. On day 6, for dose-dependent neurite outgrowth in response to diluted milk containing 1, 2, 5, 10, 25, and 50 ng/ml of rhNGF- β (*empty square*) and commercial rhNGF- β (*solid square*) diluted with RPMI 1640. **B.** Time-dependent neurite outgrowth in response to diluted milk containing 50 ng/ml of rhNGF- β (*empty square*) or commercial rhNGF- β (*solid square*) on days 2, 4 and 6. Five random microscopic fields (magnification is $\times 100$) of neurite-bearing cells were counted under the converted microscope. Neurite-bearing cells were scored as cells with neurites longer than the cell body. Percent neurite outgrowth was calculated as the number of cells with neuritis $\times 100$ /total number of cells counted. Experiments were done in triplicate. *Error bars* represent S.E.M. ($n=3$)



controlling by the same CMV promoter in this vector could more clearly indicate the expression of target gene than the inclusion of GFP gene under controlling by another CMV promoter in other vectors.

The Efficiency of Viral Transduction

Toledo et al. [17] reported that a temporary disruption of tight junctions increased the accessibility of the adenoviral vector to the basolateral surface of the mammary epithelium where the Coxsackie adenovirus receptor (CAR) was supposed to be located. In our results, the higher levels of rhNGF- β in the EGTA-permeated mammary glands may be a consequence of both a greater number of transduced cells and a greater number of viral copies per cell. The temporary disruption of tight junctions mediated by EGTA could give more adenoviruses the chance to infect the mammary epithelial cells. At the same time, each of the epithelial cells has a chance to be infected by lots of adenoviruses. Therefore, the yield of rhNGF- β from the mammary glands infused with PBS-containing EGTA is higher than the counterpart from the mammary glands infused with PBS devoid of EGTA.

The Relative Merits of Using Adenovirus

Recombinant adenoviruses provide a versatile system for gene expression studies and therapeutic applications. One of the main advantages of adenoviral vector is their ability to

replicate at high titers in complementing cell lines. As stated above, the titer of recombinant adenoviruses is related to the efficiency of infection. A moderated titer of recombinant adenoviruses in high level would improve the efficiency of infection. Another advantage of adenoviral vector is that the transgene expression is not controlled by the host genome for their nonintegrative nature. Thus, the expression of the heterologous gene could be carried out once the chimeric genome (heterologous gene sequences and vector host genome sequences) is present in host cells. Therefore, the adenovirus was used as the vector to transduce hNGF- β gene in our experiments.

In the course of our experiments, the level of rhNGF- β was dropped gradually. This drop of the expression level was also observed in other reports [15–17]. One of the reasons could be that this kind of recombinant adenovirus only has a one-time ability of infection in normal cells and it could not produce progeny viruses for another infection; with the metabolism of epithelial cells, the infected cells are less and less, and then the expression level of rhNGF- β is correspondingly dropped. Another reason could be that the immune system of the goats precludes the reinfection of adenoviruses. With the immune reaction of host, the quantity of the infected mammary epithelial cells is reduced. Thus, the expression level of rhNGF- β is correspondingly dropped. Toledo et al. [17] did the viral re-administration experiments, but no target protein was obtained; this might be because the immune system of the goats precludes the infection of adenovirus. Therefore, further studies should be required to elucidate the exact mechanisms through which the immune system precludes a successful viral re-administration in the mammary gland. This knowledge is very important to design new strategies aimed to improve viral re-inoculation allowing for the repeated use of the same animals.

All in all, we successfully expressed rhNGF- β with the properties of native hNGF- β in the milk of goats. Although the maximal level of rhNGF- β expressed in the milk only achieved 196.8 mg/l which is lower than that expressed in the milk from transgenic rabbits [22], the yield of rhNGF- β from the milk of goats are much higher than that from rabbits. Furthermore, the rhNGF- β could be obtained simultaneously from many goats infused with recombinant adenoviruses. These results demonstrate that the mammary gland of goats is a potential source of producing considerable rhNGF- β with the properties of native hNGF- β . The expression of rhNGF- β in the milk of goats confirmed that direct transduction of recombinant adenovirus into the mammary epithelial cells could be a suitable alternative for producing recombinant proteins of biopharmaceutical interest.

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