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Construction of Targeting Vector for Expressing Human GDNF in Cattle Mammary Gland

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Abstract Glial cell line-derived neurotrophic factor (GDNF) is a type of neurotrophic factor with significant potential in treatment of Parkinson's disease. Combining gene targeting of animal somatic cells with nuclear transfer technique has provided a powerful method to produce transgenic animal mammary gland bioreactor. The aim of this study was to construct a gene-targeting vector for the human *gdnf* gene knockin at the bovine *beta-casein* gene locus so that human GDNF protein can be produced in the mammary gland of the gene-targeted bovine. The constructed vector contains the 2.2 kb 5′ homologous arm and the 5.7 kb 3′ homologous arm. The human *gdnf* cDNA was located at the downstream of the 5′ homologous arm. The *neo* gene placed between the 5′ and 3′ homologous arms as positive selection marker gene. The HSV-*tk* gene and *DsRed2* gene were located outside the homologous recombinant area as negative selection marker genes, respectively. The recombinant plasmids were identified by restriction fragment analysis and partial DNA sequencing. The results show that the structure of the final constructed vector accords with the designed plasmid map. In order to analyze the bioactivity of the vector, the plasmid DNA was transfected into human mammary tumor cell line Bcap-37 by lipofectamine. Reverse transcription polymerase chain reaction and Western-blotting analysis showed that the transfected cells produced human GDNF mRNA and protein. The results show that the constructed targeting vector pNRTCNbG has bioactivity to efficiently express GDNF in mammary gland cells. At the same time, it is first time to confirm that human mammary tumor cell line Bcap-37 is valid for bioactivity analysis of mammary gland specific expression vector.

Keywords Targeting vector · Human GDNF · Bovine β-casein gene locus · Bcap-37 cell line

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Introduction

Gene targeting is a genetic technique that uses homologous recombination to delete endogenous gene expression or expresses other genes in targeted cells or individual animal [1]. Since the first demonstration that mouse embryonic stem cells could be used to transfer a predetermined genetic modification (*hprt*) to a whole animal in 1987 [2, 3], gene targeting has been a routine tool for modifying the genome of mice precisely. In spite of considerable efforts, ES cells that can contribute to the germ line of any livestock species are still not available, which limits the widespread use of this technology. The breakthrough of cloning animals by somatic cell nuclear transfer, such as "dolly" sheep production [4], has brought about a great innovation in targeted genetic manipulations of livestock species by combining somatic cell gene targeting with animal cloning. The *BLG-AAT* gene has been targeted on sheep fetal fibroblasts by gene knockin into the *COLLAI* locus and displayed a high level expression [5]. Subsequently, gene-targeted sheep, goats, and pigs had been produced in other laboratories [6–9]. This implies that gene targeting in mammary gland is possible.

Glial cell line-derived neurotrophic factor (GDNF), a distant member of the transforming growth factor [beta] (TGF-[beta]) super family, is a type of neurotrophic factor cloned in 1993 [10]. Researches revealed that GDNF has potential in treatment of various diseases of the central nervous system, including stroke [11], spinal cord injury [12], traumatic brain injury [13], and neurodegenerative diseases, such as Parkinson's disease (PD) [14, 15]. GDNF is considered to be one of the strongest neuroprotectants for dopaminergic neurons. Based on the successful results of animal experiments, clinical trials were performed for PD patients using GDNF [16–20].

However, GDNF content in human and other animal is very low that it is impossible to extract it for clinic trials. As a glycoprotein, GDNF produced by transgenetic cells on a commercial scale is very expensive [10, 21]. Mammary gland bioreactor will be an ideal option for producing this kind of proteins. For random integration of mammary gland bioreactor, inserted gene expression level varied and stayed at a low level because of affecting by flanking DNA sequences [1]. In contrast with random integration, site-specific integration of mammary gland bioreactor created by gene targeting at high expressing locus can improve gene expression [22]. Construction of the targeting vector is one of the most important techniques for creating gene targeting mammary gland bioreactors [23–25]. In the present study, we constructed a targeting vector for the human *gdnf* gene knockin at the bovine *beta-casein* gene locus so that human GDNF protein can be produced at high level in the mammary gland of the gene-targeted bovine. The plasmid DNA was transfected into human mammary tumor cell line Bcap-37 by lipofectamine, and mature recombinant GDNF protein was detected in the supernatant of transfected cells. The results show that the constructed targeting vector pNRTCNbG has bioactivity to efficiently express GDNF in mammary gland cells.

Materials and Methods

Construction of the Gene-Targeting Vector pNRTCNbG

To construct the vector pNRTCNbG, pPGK-neoLoxP (Washington University School of Medicine) plasmid DNA containing *neo* gene between two LoxP sites was used as plasmid backbone. The 5′ and 3′ homologous arms of the *beta-casein* gene were amplified from

purified bovine genomic DNA by polymerase chain reaction (PCR). The human *gdnf* cDNA cloned from human glial tumor cell line BT-325 (Cell Bank, Chinese Academy of Sciences, Shanghai) by reverse transcription PCR (RT-PCR) was located at the downstream of the 5′ homologous arm. Moreover, a 260-bp DNA fragment containing SV 40 polyadenylation signals sequence was located at the downstream of the human *gdnf* gene as its transcriptional ending signals. The *neo* gene, positive selection marker gene, was located between the 5′ and 3′ homologous arms. The HSV-*tk* gene subcloned from the plasmid pUMVC1-tk (University of Michigan Vector Core Lab.) by PCR and *DsRed2* gene obtained from the plasmid pCMV-RED (derived from pDsRed2-1, Clontech) by enzyme digestion were located outside the homologous recombinant area as negative selection marker genes, respectively. Flow of the gene-targeting vector construction was described in Fig. 1.

To ensure the validity of the gene-targeting vector construction in each step, every transformant was screened with restriction enzymatic digestion analysis or PCR assay plus restriction enzymatic digestion analysis. Furthermore, the recombinant plasmid was confirmed by partial DNA sequencing. Primer sequences and PCR parameters were described in Table 1. All primers used in this paper were synthesized by Sangon Co., Ltd (China). Partial DNA sequencing was also carried out by Sangon Co., Ltd (China).

Stable Transfection of Bcap-37 Cells

Human mammary tumor cell line Bcap-37 cells (Cell Bank, Chinese Academy of Sciences, Shanghai. China) were cultured for 3 days to subconfluence approximately 80% in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS; Tianjin Hanyang Biologicals Technology Co., Ltd) in a humidified environment with 5% CO_2 before the vector pNRTCNbG transfection. Then, Bcap-37 cells were plated at 3×10^5 cells/well in a 6-well plate for 1 day, and 2.0 μg PmaCIdigested linear vector DNA was added to every well for gene transfection using Lipofectamine (Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. After 48 h, the cells from each well were trypsinized and transferred to a 10-cm dish in selection medium with 250 μg/ml G418 (Gibco-BRL). After 8–10 days selection, healthy cells expressing red fluorescence protein were isolated, cultured, expanded, and cryopreserved by standard procedures.

PCR Assay for Identification of the Transfected Bcap-37 Cells

In order to detect that the human *gdnf* mammary gland expression vector integrated into the chromosome of the transfected Bcap-37 cells, genomic DNA was extracted from the transfected Bcap-37 cells expressed red fluorescence protein. PCR amplification was performed using one set of primers that were designed for identifying the *gdnf* expression cassette (the 5′ homologous arm + *gdnf* cDNA + SV 40 polyadenylation signals sequence) in the chromosome. Primer sequences for *gdnf* expression cassette and PCR parameters were described in Table 1.

Induction Culture of Transfected Bcap-37 Cells for Expression of Recombinant Gene

The positive transgenic Bcap-37 cells were cultured to subconfluence approximately 80% in RPMI-1640 medium supplemented with 20% FBS before induction of expression of recombinant gene. Then, medium was changed to induct medium containing serum-free RPMI-1640 medium with prolactin (Sigma, St. Louis, MO, USA) 4 μg/ml, insulin (Sigma, St. Louis, MO, USA) 10 μg/ml, and hydrocortisone (Sigma, St. Louis, MO, USA) 1 μg/ml.

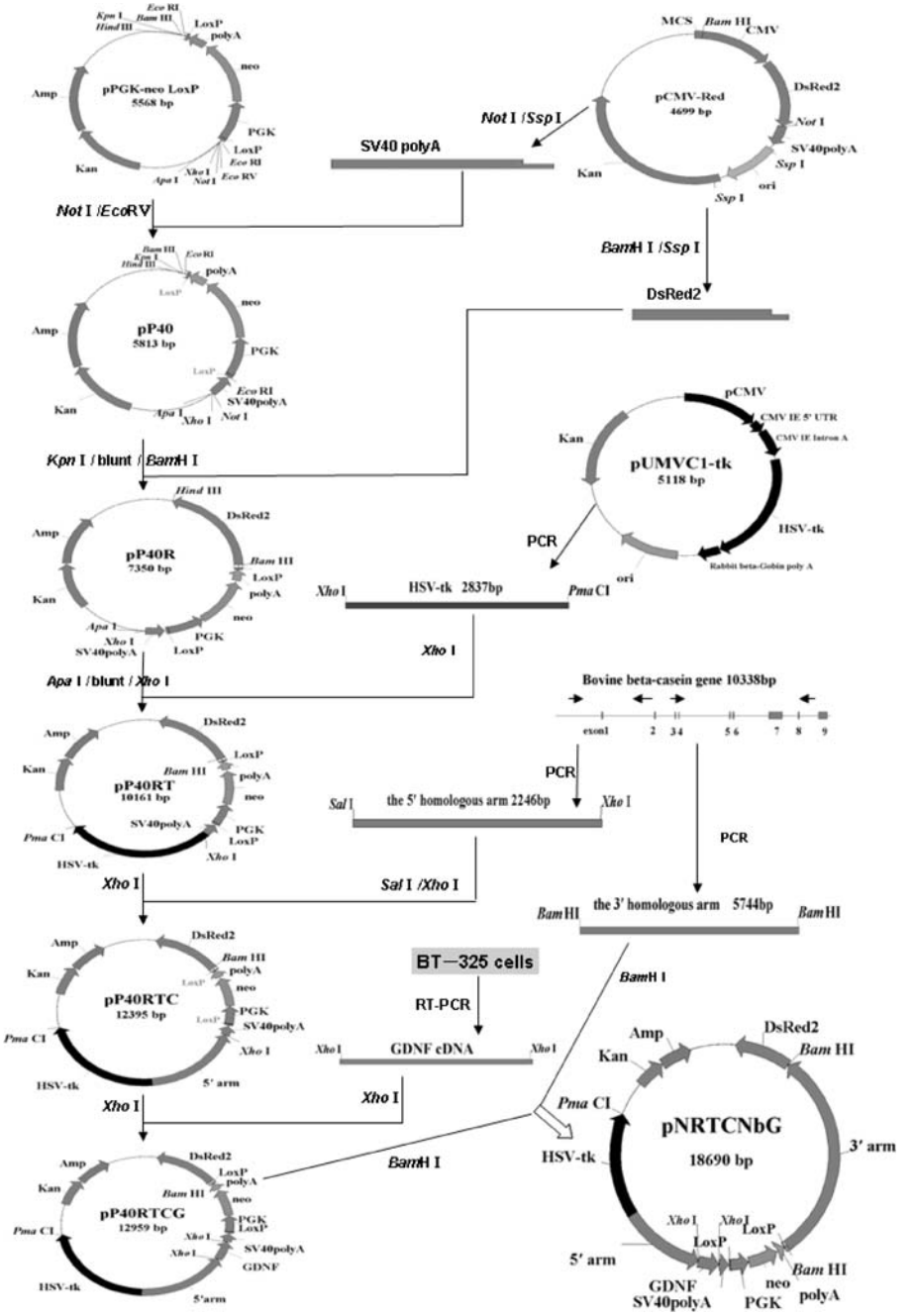

Fig. 1 Flow chart of the vector construction

Table 1 Primer sequences and PCR parameters.

Genes or DNA fragment	Primers	Restriction enzymes	PCR parameters
gdnf	5′GACCTCGAGATGAAGTTATGGGATGTCGTGGCT GTC 3′	XhoI	94 °C for 45 s 60 °C for 45 s
	5′GAGCTCGAGTCAGATACATCCACACCTTTTAG 3′	XhoI	72 °C for 1 min
5′ arm	5′GACGTCGACAAAACTTCCGTGTGTCCCAGC 3′	*Sal*I	94 °C for 45 s 62 °C for 45 s
	5′GGCCTCGAGCTCCTGGGAATGGGAAGATGA 3′	*Xho*I	72 °C for 2.5 min
3′ arm	5′AAAGGATCCAGCAACAGACTAACAAGAAGG 3′	*Bam*HI	94 °C for 45 s 58 °C for 45 s
	5′CTCAGGATCCAAACATCGGCTTACTTG 3′	*Bam*HI	72 °C for 6 min
HSV-*tk*	5′GTTCTCGAGTGTCGGGGCTGGCTTA 3′	*Xho*I	94 °C for 45 s 62 °C for 45 s
	5′CCACACGTGATAGTCCTGTCGGGTTTCGC 3′	*Pma*CI	72 °C for 3 min
gdnf cassette	5′GACGTCGACAAAACTTCCGTGTGTCCCAGC 3′ 5′GGTGGATGTGGAATGTGTGCGA3′		94 °C for 45 s 60 °C for 45 s 72 °C for 3 min
GAPDH	5′AACGGATTTGGTCGTATTGGGCG3′ 5′CAAAGGTGGAGGAGTGGGTGTCG 3′		94 °C for 45 s 60 °C for 45 s 72 °C for 1 min

The cells were harvested for RT-PCR, and the supernatant was collected for western blot analysis after induction for 24, 48, and 72 h, respectively.

RT-PCR Analysis of *gdnf* Transcription in Induced Bcap-37 Cells

Total RNA (DNA-free) was isolated from the transfected Bcap-37 cells and nontransfected Bcap-37 cells after induction culture as described above, using RNAsimple total RNA kit (Tiangen Co., Ltd) following the manufacturer's instructions. First-strand cDNA was synthesized with 1 μg total RNA by using reverse transcriptase in a volume of 20 μl. Subsequent PCR was carried out by using 1 μl cDNA sample as template. Primer sequences for *gdnf* gene and PCR parameters were described in Table 1.

Western Blot Analysis of the GDNF Protein Secretion

Proteins in the supernatant from induction culture of the transfected Bcap-37 cells was concentrated by trichloracetic acid methods [26]. The protein concentration was measured by the method of Lowry et al. [27]. Western blot was carried out following a standard western blotting procedure. Briefly, proteins were separated by SDS-polyacrylamide gel electrophoresis (12% gels). The separated proteins were transferred to PVDF membrane. After blocking the membrane in blocking solution (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% (*v*/*v*) Tween-20, 5% skim milk powder), immune reaction was performed overnight at 4 °C using a rabbit polyclonal GDNF antibody (Santa Cruz) in a 1:100 blocking solution dilution. After incubating the membrane with a goat anti-rabbit second antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA) for 1 h at room temperature, detection for the bound antibodies was carried out using BCIP/NBT kit (Boster, Co., Ltd), according to the manufacturer's instructions.

Results

Construction of the Gene-Targeting Vector pNRTCNbG

To construct the gene-targeting vector pNRTCNbG, two homologous arms (5′ arm and 3′ arm) had been generated by PCR from the purified bovine genomic DNA by using primers that were designed according to reported sequences of the bovine *beta-casein* gene (GenBank accession no. M55158). The 5′ arm was 2.2 kb fragment including promoter, exon 1, intron 1, and part of exon 2 of the bovine *beta-casein* gene, and the 3′ arm was 5.7 kb fragment including 3′ flanking sequence of the bovine *beta-casein* gene. The *gdnf* cDNA was subcloned in the bovine *beta-casein* gene exon 2, and the endogenous start code was replaced by that of *gdnf* gene. The gene-targeted vector pNRTCNbG was identified with restriction enzymatic digestion analysis. The DNA sequences of junctions were analyzed and verified (data not shown). The vector was linearized with *Pma*CIbefore transfection of Bcap-37 cells.

Integration of *gdnf* Cassette into the Genomic DNA of Bcap-37 Cells

Linearized pNRTCNbG was transfected into Bcap-37 cells by Lipofectamine. After 8-10 days G418 selection, cell clones expressing red fluorescence protein were obtained (Fig. 2). These cells were isolated, cultured, expanded, and cryopreserved by standard procedures. To conform that *gdnf* cassette had integrated into the genomic DNA of the cells expressing red fluorescence protein, PCR amplification was carried out by using the purified genomic DNA as template. The expected products were observed on an ethidium bromide-stained gel (Fig. 3). Since the sense primer for the *gdnf* cassette was the sense primer for the 5′ homologous arm and the antisense primer was located outside of the 3′-end of SV 40 polyadenylation signals sequence, the results demonstrated that *gdnf* cassette had integrated into the genomic DNA of the transfected Bcap-37 cells.

Induced Expression of Recombinant Protein GDNF

In order to confirm expression of recombinant protein GDNF, the transgenic Bcap-37 cells were cultured in the induction medium. RT-PCR and Western blot were carried out for analysis of gene transcription and protein secretion for the induced cells.

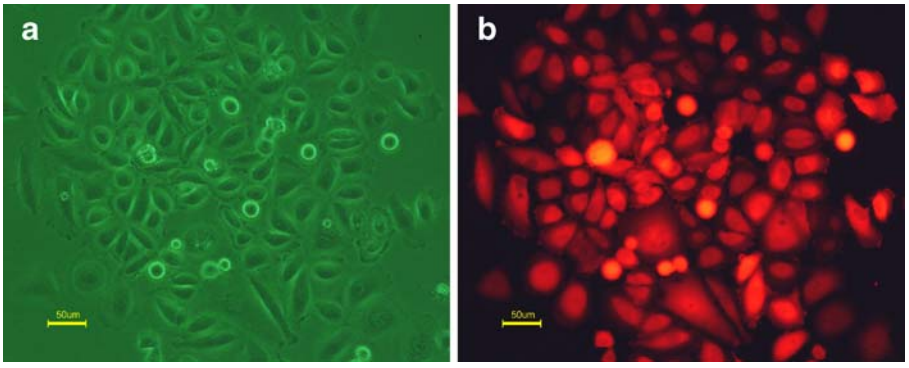

Fig. 2 G418 resistant cell clones transfected with pNRTCNbG. **a** contrast microscope, ×200. **b** fluorescent microscope, ×200. *Bar*=50 μm

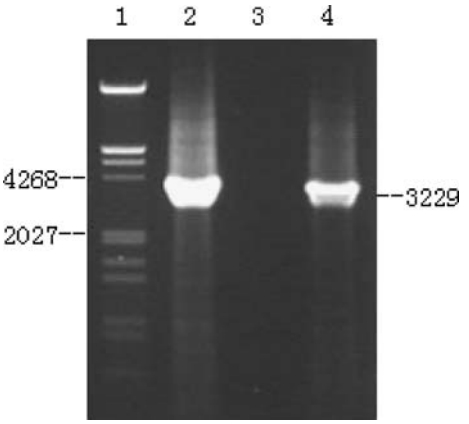

Fig. 3 PCR analysis of the transfected cells. *1*. λDNA/*Hin*dIII+ *Eco*RIMarker. *2*. pNRTCNbG plasmid DNA, positive control. *3*. Normal Bcap-37 cells, negative control. *4*. The transfected Bcap-37 cells

RT-PCR was performed with RNA samples or reverse-transcripted cDNA samples from the induced nontransgenic cells or transgenic cells, respectively (Fig. 4). PCR with RNA samples as template revealed no band, which indicated that the RNA samples did not contaminate with the genomic DNA of the cells. RT-PCR with reverse-transcripted cDNA samples from the induced nontransgenic cells also revealed no band. However, RT-PCR with reverse-transcripted cDNA samples from the induced transgenic cells revealed a 558-bp band. Sequencing result showed that it was identical with human *gdnf* cDNA fragment.

Western blot analysis was carried out to detect the secretion of the expressed GDNA protein from the supernatants of the induction culture (Fig. 5). The protein sample derived from the supernatant of induction culture of the transgenic Bcap-37 cells at 48 h revealed two bands in western blot detection, one band was about 15 kDa and another about 18 kDa. The 18 kDa band, the dominant signal, corresponded to a more glycosylated form of GDNF [10].The results showed that mature GDNF protein was secreted into the supernatant of the induction culture of the transgenic Bcap-37 cells.

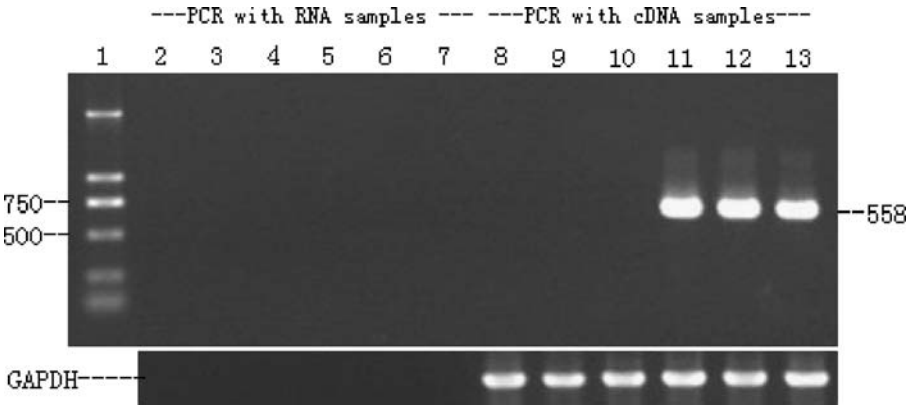

Fig. 4 RT-PCR analysis of recombinant *gdnf* transcription in Bcap-37 cells. *1*: D2000 marker. *2–4*: PCR with RNA samples of the nontransgenic cells on induction at 24, 48, and 72 h. *5–7*: PCR with RNA samples of the transgenic cells on induction at 24, 48, and 72 h. *8–10*: RT-PCR with cDNA samples of the nontransgenic cells on induction at 24, 48, and 72 h. *11–13*: RT-PCR with cDNA samples of the transgenic cells on induction at 24, 48, and 72 h

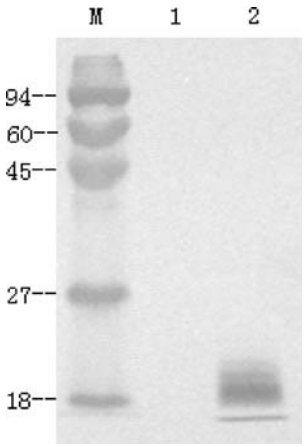

Fig. 5 GDNF protein secretion detected by western blot. *M*: marker. *1*: Supernatant of induction culture of the Bcap-37 cells at 48 h. *2*: Supernatant of induction culture of the transgenic Bcap-37 cells at 48 h

Discussion

Gene targeting has been so difficult in somatic cells because the absolute frequency of homologous recombination in somatic cells is some two orders of magnitude lower than in ES cells [28, 29]. To complicate matters further, frequencies of random integration are typically very high [28]. This means that the targeting vector must provide a very powerful enrichment factor to eliminate a larger proportion of random integration events. With the promoterless gene-targeting vector, the promoter-trap strategy was employed for increasing enrichment of gene-targeting events in the past research [5–9]. However, promoterless gene-targeting vector of the beta-casein locus could not be used in fetal fibroblasts, since the bovine beta-casein locus was the mammary gland organ-specific expressive locus. Positive–negative selection (PNS) is less powerful than promoter-trap selection. However, the enrichment factor for PNS procedure can be increased dramatically by inserting into the targeting vector two identical HSV-*tk* genes, one at each end [30].

In the present study, we used a novel strategy to constructing the gene-targeting vector pNRTCNbG. Instead of two identical HSV-*tk* genes in the gene targeting vector, the red fluorescence protein gene *DsRed2* was used as one of the negative selection marker genes located downstream of the 3′ homologous arm. Only one HSV-*tk* gene was kept in the gene-targeting vector. After stable transfection of somatic cells with the vector, the random integration of the vector DNA into the genome of the host cells resulted in expressing red fluorescent protein in transfected cells (Fig. 2). It is very easy to detect the living cells marked with a red fluorescent protein by microscopy. The homologous recombinant cells were limited within the unmarked cells. The advantages of the *DsRed2* gene used as a negative selection marker gene are not only that a higher enrichment can be obtained via a fluorescent microscope after the enrichment with the HSV-*tk* gene but also that red fluorescent protein can display the efficiency of random integration of the vector DNA into cells.

RT-PCR experiments demonstrated at least three human *gdnf* transcripts of different lengths including the first transcript 636 bp, the second transcript 558 bp, and the third transcript 402 bp [31]. GDNF encoded by the first two transcripts is a secreted protein, and their treatment function for Parkinson's disease is the same, but GDNF encoded by the third transcript is an intracellular protein that could initiated an intracellular autocrine loop [14, 31]. In the present study, the second *gdnf* transcript (558 bp) was cloned by RT-PCR and

located at the downstream of the 5′ homologous arm, followed by a 260-bp SV40 polyadenylation signal sequence as its transcrible ending signal.

Bioactivity is essential for an expression vector. To evaluate the bioactivity of the gene-targeting vector pNRTCNbG, the transgenic Bcap-37 cells expressing red fluorescent protein were used. The negative selection marker gene DsRed2 showed that the stable transfected cells were randomly integrated with the pNRTCNbG vector DNA. Because of the vector containing the *gdnf* cassette, the transfected cells can express and secrete the recombinant GDNG protein. RT-PCR analysis revealed that the transcript of the *gdnf* cDNA inserted in the vector existed in the transgenic cells. Western blot analysis demonstrated that human GDNF protein was secreted in the supernatant of the induction culture of the transgenic Bcap-37 cells, and the majority of the product was in glycosylated form. The results confirm that the constructed targeting vector pNRTCNbG has correct structure and bioactivity to efficiently express and secrete GDNF protein in mammary gland cells. At the same time, it is firstly confirmed that human mammary tumor cell line Bcap-37 is valid for assaying bioactivity of mammary gland specific expression vector.

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