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Targeting exogenous *GDNF* gene to the bovine somatic cell beta-casein locus for the production of transgenic bovine animals

X.M. Zhang[1], F.H. Luo[2], H.M. Ding[1], B. Li[1], J.J. Zhang[1] and Y.J. Wu[2]

[1]Baotou Medical College, Inner Mongolia University of Science and Technology, Baotou, China
[2]Key Laboratory of National Education Ministry for Research of Mammal Reproductive Biology and Biotechnology, Inner Mongolia University, Hohhot, China

Corresponding author: Y.J. Wu
E-mail: wuyj1211@163.com

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ABSTRACT. Considerable attention is currently being directed toward methods for producing recombinant human proteins in the mammary glands of genetically modified transgenic livestock. However, the expression of inserted genes in transgenic animals is variable and often very low because of the randomness of the site of transgene integration. One possible strategy to avoid the expression problem associated with random integration is to use site-specific integration by targeting integration to a high expression locus and, thereby, to improve expression of the transferred gene. In the present study, we focused on glial cell line-derived neurotrophic factor (GDNF), a novel type of neurotrophic factor first cloned in 1993. Research has shown that GDNF may have potential applications in the treatment of Parkinson's disease and other diseases of the central nervous system since it acts as a protective factor for central dopaminergic neurons. Here, we constructed a gene targeting vector to knock-in the human *GDNF* gene at the bovine beta-casein gene locus as a

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first step to producing transgenic animals with a high level of expression of human GDNF protein in their mammary glands. Bovine fetal fibroblast cells were transfected with linearized *pNRTCNbG* by electroporation. Three cell clones were identified with successful targeting to the beta-casein locus; and were confirmed using both polymerase chain reaction analysis and sequencing. Gene-targeted cells were used as nuclear donors; a total of 161 embryos were reconstructed, 23 of which developed to the blastocyst stage. These blastocysts were transferred to 8 recipient cows, but no offspring were obtained.

Key words: Gene targeting; Somatic cell nuclear transfer; GDNF; Beta-casein locus

INTRODUCTION

Although pronuclear injection is routinely used for producing genetically modified organisms in a variety of species, it is limited by a low success rate and the unpredictable levels of expression of the transferred gene because of random transgene integration. Development of gene targeting techniques using embryonic stem (ES) cells has provided an alternative method for generating transgenic animals. Gene targeting is a technique that uses homologous recombination to delete endogenous genes or express exogenous genes in targeted cells or animals (Jasin et al., 1996). This approach to genome modification is now used routinely in mice because of the reliability of mouse ES cell methods. However, despite considerable effort, ES cell lines that can contribute to the germ line of livestock species are still not available, which limits the application of gene-targeting technology. Another approach has been the development of cloning techniques, such as those used to produce “Dolly” the sheep (Wilmut et al., 1997). Through this method, gene-targeted livestock can be generated by nuclear transfer from transfected fetal fibroblasts that have been cultured *in vitro*. McCreath et al. (2000) targeted the *BLG-AAT* gene in sheep fetal fibroblasts to knock-in the *COL1A1* locus, which generated high-level expression in the gene-targeted sheep. Subsequently, gene-targeted sheep, pigs, goats, and bovine species were produced in other laboratories (Denning et al., 2001; Dai et al., 2002; Lai et al., 2002; Kuroiwa et al., 2004; Yu et al., 2006).

Glial cell line-derived neurotrophic factor (GDNF), a distant member of the transforming growth factor-β superfamily, is a neurotrophic factor that was first cloned in 1993 (Lin et al., 1993). Studies revealed that the GDNF protein has the potential to treat various central nervous system conditions, including stroke, spinal cord injury, and traumatic brain injury, as well as neurodegenerative diseases such as Parkinson’s disease (Yasuhara et al., 2007). GDNF is one of the strongest neuroprotectants for dopaminergic neurons. Based on the successful results of animal experiments, clinical trials with GDNF were performed on Parkinson’s disease patients and the treatment was shown to be safe and to produce an improvement in patient health (Slevin et al., 2005; Mínguez-Castellanos et al., 2007; Yasuhara et al., 2007; Patel et al., 2005, 2013). However, commercial scale production of GDNF as a glycoprotein in transgenic cells is very expensive. The alternative use of gene-targeted mammary gland bioreactors would facilitate the cost-effective production of this protein (Zhang et al., 2009).

The red fluorescence protein gene *DsRed2* is used widely as a positive marker in transgenic mammalian cells. It is likely that *DsRed2* could be used to replace *HSV-tk* as the negative selection gene during positive-negative selection (PNS) for gene targeting in somatic

cells, which might enrich G418-resistant clones. Expression of *DsRed2* can be detected using a fluorescence microscope and its use would not only avoid the cytotoxic effects of GANC on gene-targeted clones, but might also induce efficient transfer of a PNS vector into somatic cells. In the present study, we constructed a gene-targeting vector to knock-in human *GDNF* cDNA into the bovine beta-casein gene locus; the vector contained the *neo* gene as a positive selection marker gene and *DsRed2* and *HSV-tk* as negative selection marker genes. Bovine fetal fibroblast cells were then transfected with the targeting vector, and the utility of *DsRed2* and *HSV-tk* as negative selection marker genes was compared. Subsequently, gene-targeted fibroblast clones were produced using only *DsRed2* as the negative selection marker gene, and gene-targeted blastocysts were produced by nuclear transfer of these cloned cells. Our results provide a basis for the development of methods for the production of recombinant human GDNF protein using gene-targeted bovine mammary gland bioreactors.

MATERIAL AND METHODS

Construction of the gene-targeting vector pNRTCNbG

Plasmid DNA containing a *neo* gene between two LoxP sites was used as a backbone to construct the vector *pPGK-neoLoxP* (Washington University School of Medicine). The 5' homologous arm was a 2.2-kb fragment comprising the promoter, exon 1, intron 1, and part of exon 2 of the bovine beta-casein gene. The 3' homologous arm was a 5.7-kb fragment consisting of exons 3-8, part of intron 2, introns 3-7, and part of intron 8 of the bovine beta-casein gene. Human *GDNF* cDNA was located downstream of the 5' homologous arm (approved by the research ethics board of Baotou Medical College, Inner Mongolia University of Science and Technology). In addition, a 260-bp DNA fragment containing the SV40 polyadenylation sequence was located downstream of the human *GDNF* gene as a transcriptional end signal. The *neo* gene, a positive selection marker, was located between the 5' and 3' homologous arms. The *HSV-tk* and *DsRed2* genes were located outside the homologous recombination area as negative selection marker genes.

Isolation and culture of primary bovine fetal fibroblasts

The animal study reported here was approved by Inner Mongolia University of Science and Technology of Medicine Animal Institute Committee. Bovine fetal fibroblast cell lines were isolated from tissue explants from the ear skin of Chinese Holstein cattle fetuses at 60-90-days of gestation. The explants were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS; Tianjin Haoyang Biologicals Technology Co., Ltd., Tianjin, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich, Shanghai, China) in a humidified environment containing 5% CO_2. The sex of the fetal fibroblast cell lines was determined by polymerase chain reaction (PCR) analysis for the *SRY* gene using the primers SRYf (5'-TTCAGAGGTAGGACTCCACGCTT-3') and SRYr (5'-AAATGACAATCCACCCCAGCACTC-3').

Transfection and selection of bovine fetal fibroblasts

After linearization with *Pma*CI, the targeting vector *pNRTCNbG* was transfected into the third passage fetal fibroblasts by electroporation. Approximately 1.0 x 10^7 cells were harvested at 70-80% confluence, mixed with 20 µg linearized and purified gene-targeting vector *pNRTCNbG*,

and transferred into a 0.4-cm cuvette (Bio-Rad, USA). The cells were subjected to a pulse of 550 V, 50 uF, from a Gene Pulser II (Bio-Rad). Transfected cells were plated on 10-cm dishes in DMEM/F12 without selection. After 48 h, the cells were trypsinized and reseeded in selective cell culture medium containing either 500 mg/mL G418 (Gibco-BRL, Shanghai, China) and 2 μM GANC (Gibco-BRL), or only 500 mg/mL G418. After 8-10 days of selection, healthy and well-separated colonies that did not express red fluorescent protein were isolated using cloning rings and transferred to 48-well cell culture plates. A small number of cells were isolated at sub-confluence and transferred to 48-well plates for analysis using PCR, and the remaining cells were transferred to 24-well plates. They were then cultured to sub-confluence for cryopreservation or nuclear transfer.

Detection and analysis of transfected bovine fetal fibroblasts

$G418^+/DsRed^-$ and $G418^+/GANC^-/DsRed^-$ colonies were screened for successful targeting using PCR and DNA sequencing. The positions of the PCR primers are shown in Figure 1. Cells were grown on 48-well plates, lysed in 40 μL lysis buffer (40 mM Tris-HCl, pH 8.0, 0.9% Triton X-100, 0.9% Nonidet P-40, and 0.4 mg/mL proteinase K) at 65°C for 30 min; they were then heated to 95°C for 10 min to inactivate proteinase K. PCR amplification was performed in a 20-μL reaction volume using the TaKaRa LA Taq system with 2 μL cell lysate as the DNA template. The primer sequences were: P1, 5'-CTCTCTTCTCACCTCCATCTACTCCTTTTTCC-3'; P2, 5'-CTCTCTTCTCA CCTCCATCTACTCCTTTTTCC-3'; P3, 5'-ATGAGATTGTGTTCTTTGTGTGGC-3'; and P 5'-CAG CCACGATAGCCGCGCTGCCTC-3'. The amplification conditions were 4 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 59°C, and 2.5 min (P1/P2) or 6 min (P3/P4) at 72°C, followed by 10 min at 72°C.

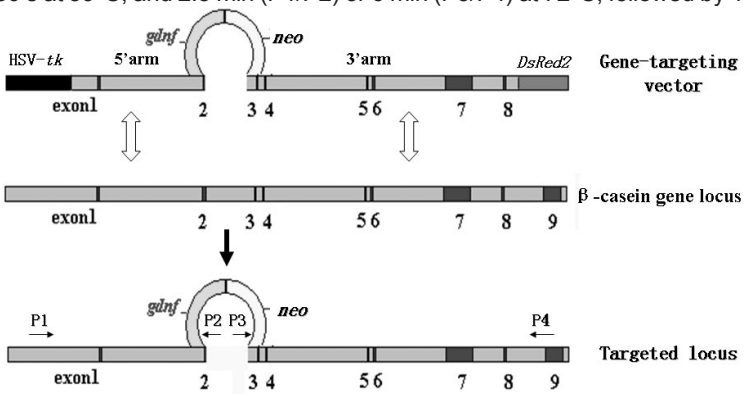

Figure 1. Gene targeting at the beta-casein locus in bovine fetal fibroblasts. The nine exons of the bovine beta-casein locus are presented as black boxes. The corresponding beta-casein genomic sequences were used as the 5' and 3' homologous arms in the gene-targeting vector, and the structure of the targeted locus after homologous recombination.

Nuclear transfer to produce gene-targeted blastocysts

Gene-targeted bovine fetal cells (potential donor cells) were cultured in DMEM/F12 supplemented with 10% FBS for 3 days. Bovine cumulus-oocyte complexes (COCs) were cultured in maturation medium at 38.5°C in an atmosphere containing 5% CO_2. M199 (Gibco-BRL) supplemented with 10 μg/mL FSH, 0.1 μg/mL estradiol-17β, 10 mM HEPES (all from Sigma-Aldrich), 10% FBS, 50 U/mL penicillin, and 60 mg/mL streptomycin was used as the maturation

medium. After 18 h maturation, the COCs were removed, and denuded oocytes with intact cytoplasmic membranes and a clear perivitelline space were selected as recipient cytoplasts. After oocyte enucleation, donor cells were injected into the perivitelline space of the enucleated oocytes using a smooth pipette. The cell-cytoplast couplets were transferred into maturation medium for recovery for 1 h until fusion and activation was performed. Groups of five couplets were placed in a BTX fusion chamber (electrode width 0.5 mm) filled with fusion solution. A DC pulse of 25 V/150 µm was applied for 10 µs, followed by a DC pulse of 100 V/mm for 60 µs. The fused cell-cytoplast couplets were transferred into maturation medium for recovery for 30 min. The reconstructed couplets were then activated using 7% ethanol for 7 min, followed by incubation in SOFaa-BSA medium containing 2 mM 6-DMAP (Sigma-Aldrich) for 4 h. After activation the embryos were cultured in SOFaa-BSA medium for 36 h; embryos undergoing cleavage were co-cultured with cumulus cells in SOFaa medium containing 4% FBS for 5.5 to 7.5 days. On days 7 and 9 after activation, the rate of blastocyst formation was determined under a stereomicroscope. The blastocysts were transferred to synchronized foster-mothers to produce offspring carrying the targeted human *GDNF* gene.

Statistical analyses

Means were compared by a contingency table analysis followed by chi-square testing. A value of $P < 0.05$ was chosen as statistically significant.

RESULTS

Construction of the gene-targeting vector *pNRTCNbG*

To increase the rate of successful gene-targeting events in the somatic cells, we sought to eliminate a larger proportion of random integration events using a positive-negative selection strategy for the *pP40RTCGC* vector. The vector contained a *neo* gene as a positive selection marker gene and an *HSV-tk* gene and red fluorescence protein gene *DsRed2* as negative selection markers. Recombinant plasmids were identified by restriction fragment analysis and partial DNA sequencing. These analyses indicated that the final vector structure was consistent with the designed plasmid map (Figure 1). To confirm the bioactivity of the vector, plasmid DNA was transfected into the human mammary tumor cell line Bcap-37 using lipofectamine. Reverse transcription-PCR and western blotting showed that the transfected cells produced human *GDNF* mRNA and protein. Therefore, the constructed targeting vector *pNRTCNbG* was bioactive and efficiently expressed *GDNF* in mammary gland cells (Zhang et al., 2009).

Comparative efficiencies of *DsRed2* and *HSV-tk* as negative selection marker genes

We compared the enrichment of successful targeting events by *pNRTCNbG* using *DsRed2* and *HSV-tk* as negative selection marker genes. Bovine fetal fibroblast cells (4.0×10^7) were transfected with a linearized targeting vector by electroporation, the cells were divided into two aliquots, and G418 or G418 plus GANC was added to the aliquots. After selection for 8-10 days, we counted resistant clones expressing the red fluorescent protein (Figure 2a and b) and those lacking the protein (Figure 2c and d). The results indicated that the enrichment of the negative selection marker genes *DsRed2* and *HSV-tk* was about four-fold (2026/501 or 1057/267) and two-fold (2026/1057), respectively (Table

1). The resistant clone cells were large, flat, amorphous, and vacuolated after selection. Moreover, the proliferation of the resistant clone cells was remarkably more sensitive to exposure to the double selection drugs (G418 plus GANC) than to the single selection drug (G418 only) (Table 2).

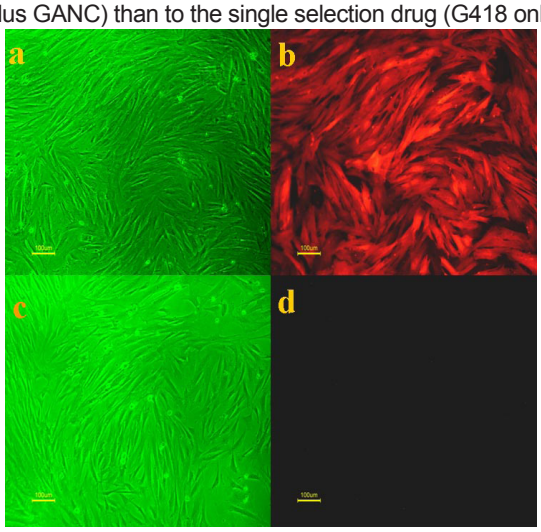

Figure 2. Bovine fetal fibroblasts transfected with the targeting vector and selected using antibiotics. **a. b.** Monoclonal cells expressing red fluorescence protein after selection with G418. **c. d.** Monoclonal cells that did not express red fluorescence protein after selection with G418 (a, c: contrast microscopy images, 200X; b, d: fluorescence microscopy images, 200X). Bar = 100 μm.

Table 1. Efficiency of gene targeting in bovine fetal fibroblasts.

No. of cells transfected with the targeting vector	No. of *neo*$^+$ clones	No. of *neo*$^+$/HSV-*tk*$^-$ clones	No. of *neo*$^+$/*DsRed2*$^-$ clones	No. of *neo*$^+$/HSV-*tk*$^-$/*DsRed2*$^-$ clones	No. of targeted clones	Absolute targeting frequency
2.0 x 10^7	2026	-	501	-	3	1.5 x 10^{-7}*
2.0 x 10^7	-	1057	-	267	1	0.5 x 10^{-7}*

*Significant difference ($P < 0.05$).

Table 2. Clone growth of bovine fetal fibroblasts transfected with the targeting vector and then subjected to drug selection.

Selective marker genes	No. of resistant clones	No. of resistant clones with senescence[a]	Ratio of resistant clones with senescence to resistant clones
neo$^+$/*DsRed2*	501	97	19.4%*
neo$^+$/HSV-*tk*$^+$/*DsRed2*	267	126	47.2%*

*Significant difference ($P < 0.05$). [a]Clones did not proliferate sufficiently after drug selection to allow genomic DNA screening.

Human *GDNF* cDNA knock-in targeted to the beta-casein locus

PCR analysis and sequencing were used to identify clones with successful gene targeting (Figure 3). One clone from the G418$^+$/GANC$^-$/DsRed$^-$ colonies was confirmed as gene-targeted;

however, the cells failed to proliferate sufficiently for use in nuclear transfer. Three other gene-targeted clones were confirmed from G418^{+}/DsRed^{-} colonies; these cells proliferated sufficiently for use in nuclear transfer. Overall, a targeting frequency of 1.5 x 10^{-7} (3/2.0 x 10^{7}) was achieved (Table 1).

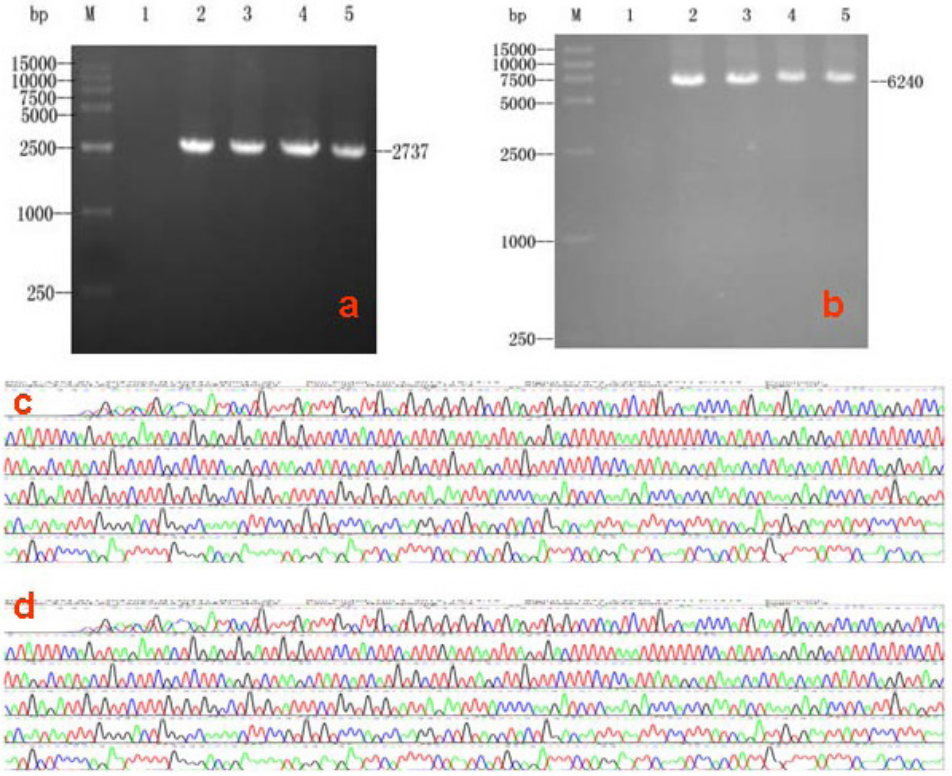

Figure 3. Identification of neo^{+}/HSV-tk^{-}/DsRed2− and neo^{+}/DsRed2^{-} clones. **a.** Separation and identification of amplification products after 5'-end-PCR (P1/P2). **b.** Separation and identification of amplification products after 3'-end-PCR (P3/P4). **c.** Sequencing results for fragments produced from the 5'-flanking regions by 5'-end PCR. **d.** Sequencing results for fragments produced from the 3' flanking regions by 3'-end PCR.

Production of blastocysts carrying targeted human *GDNF* cDNA

Blastocysts carrying the *GDNF* cDNA were produced by somatic cell nuclear transfer using the gene-targeted cells as the nuclear donor cells and enucleated oocytes matured *in vitro* as the recipients. In total, 184 *in vitro* matured oocytes were enucleated and electrofused with donor cells (Table 3). These transfers yielded 161 (87.5%) reconstructed embryos; these were activated and cultured *in vitro* and 23 (14.3%) developed to the blastocyst stage (Figure 4). These blastocysts were transferred to 8 recipient cows, but no offspring were obtained.

Table 3. Development of embryos reconstructed by nuclear transfer of gene-targeted bovine fetal fibroblasts.

No. of NT embryos	No. of fused embryos (%)	No. of cleavage embryos (%)	No. of blastocysts (%)
184	161 (87.5)	128 (79.5)	23 (14.3)

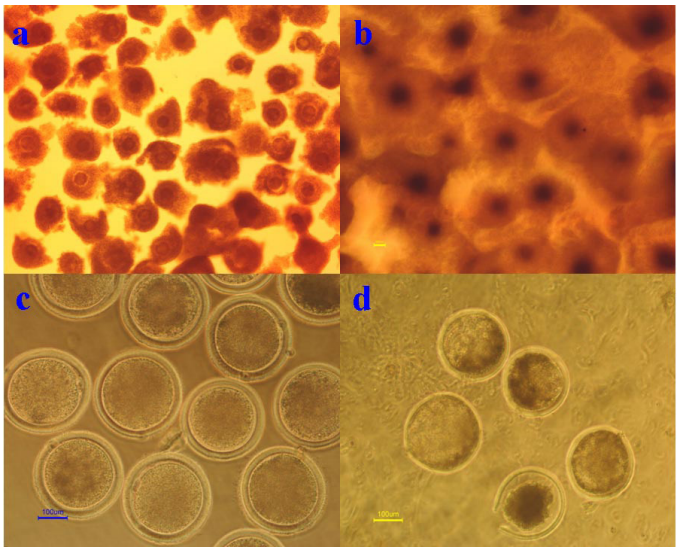

Figure 4. Differential interference microscopic analysis of *in vitro* maturation (IVM) of bovine COCs and *in vitro* development (IVD) of embryos created using gene-targeted clones. **a.** Bovine COCs (40X); **b.** bovine COCs at 18 h of IVM (40X), **c.** Oocytes at 18 h of IVM (100X). **d.** Blastocysts from embryos reconstructed from gene-targeted clones (100X). Bar = 100 µm.

DISCUSSION

The mammary glands of transgenic animals can be used as bioreactors to produce safe and renewable sources of important proteins for medicinal purposes that cannot be produced efficiently in adequate quantities using other methods (Rudolph, 1999). Unfortunately, the level of expression of inserted genes in transgenic animals varies but is generally low because of the randomness of the site of transgene integration (Jasin et al., 1996). It is possible to avoid this difficulty through the use of site-specific integration. For example, Smithies et al. (1985) used homologous recombination to target globin gene sequences to the human beta-globin locus in cell cultures. However, gene targeting in somatic cells is challenging because primary cells have a limited lifespan and the absolute frequency of homologous recombination in somatic cells is some two orders of magnitude lower than in ES cells (Waldman, 1992; Hanson and Sedivy, 1995). To complicate matters further, the frequency of random integration is typically very high (Waldman, 1992; Hanson and Sedivy, 1995). To overcome these obstacles, a promoterless targeting strategy has been developed and applied successfully to generate transgenic domesticated animals carrying sequences inserted at specific target sites (McCreath et al., 2000; Denning et al., 2001; Dai et al., 2002; Lai et al., 2002; Yu et al., 2006). The promoterless strategy uses vectors in which a positively selectable marker gene lacks its own promoter but can be activated by the promoter of the target gene following homologous integration (Hanson and Sedivy, 1995). This strategy is limited to target genes that are actively expressed in fetal fibroblasts, which is currently the only validated cell type with the longevity to allow the selection of gene-targeted cells for nuclear transfer (Denning et al., 2001).

In 1988, Mansour et al. (1988) described a PNS procedure for gene targeting in ES cells.

Their procedure combined the use of a *neo* gene for positive selection and an *HSV-tk* gene for negative selection: first the transfected cells were grown in the presence of G418 for positive selection of those that contained the targeting vector; then the cells were grown in the presence of GANC as a negative selection against cells with random integration of the targeting vector. In contrast to the promoterless selection procedure, PNS can be applied to any gene.

In the present study, a recombinant human *GDNF* cDNA sequence was used to target the beta-casein gene locus. The beta-casein gene is not expressed in primary bovine fetal fibroblasts; therefore, we chose to use a PNS targeting vector in the current study. The targeting vector contained the *neo* gene as the positive selection marker, and *DsRed2* and *HSV-tk* as negative selection genes. We compared the enrichment achieved using *DsRed2* and *HSV-tk* and found that the former gave about four-fold enrichment while the latter achieved two-fold enrichment. Culture of transfected cells in G418 plus GANC allowed the elimination of cell clones expressing the red fluorescent protein. We also confirmed the presence of the vector in the selected cells by PCR and sequencing. When we cultured transfect cells in the presence of only G418 we identified three gene-targeted clones that could be used for nuclear transfer. The PNS procedure using the G418 plus GANC identified fewer successful integration events than using only G418. Possibly, this was because proliferation of resistant cell clones was more sensitive to exposure to the double drug combination than to the single drug. We found that using DsRed2 as a negative selection marker appeared to improve the efficiency of identifying successful gene targeting events in somatic cells and that the cells identified were suitable for use in nuclear transfer-based genetic modification processes.

In conclusion, this study describes an efficient and reproducible gene targeting system for bovine fetal fibroblasts and demonstrated this approach by insertion of an exogenous human *GDNF* cDNA sequence into the beta-casein locus. This approach provides the first step in producing a reliable method for generating transgenic animals that might function for human GDNF production in mammary glands.

Conflicts of interest

The authors declare no conflict of interest.

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