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Human gdnf knock-in at the bovine *β-casein* locus by CRISPR/Cas9-mediated homologous recombination*

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Parkinson's disease (PD) is a common and debilitating neurodegenerative disorder that stems from the loss of dopaminergic neurons and is associated with progressive motor dysfunction. Glial cell-derived neurotrophic factor (GDNF) is very promising for the treatment of PD and other neuropathic diseases. In this study we applied the CRISPR/Cas9 technique to develop a gene-targeting knock-in system for the expression of the human *gdnf* gene at the bovine *β-casein* gene locus. A CRISPR/Cas9 expression plasmid and a pP40-GN vector were constructed. Bovine fetal fibroblasts were cultured and collected using the tissue explant method. Then the pP40-GN and CRISPR/Cas9 vectors were electrotransfected into bovine fetal fibroblasts. Resistant clones were selected using G418, while target clones were identified via PCR analyses and PCR product sequencing. Bovine fetal fibroblasts were successfully isolated and cultured using the ear tissue blocking method. After co-transfecting the bovine fetal fibroblasts with the pP40-GN target vector and the CRISPR/Cas9 expression vector in 7 days of selection with G418 a total of 12 healthy and well-separated cell clones were obtained, of which 5 were involved in gene targeting events. This study lays a foundation for the production of human GDNF protein using a gene-targeted bovine mammary gland bioreactor and provides a new strategy for the targeted therapy of PD.

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Parkinson's disease (PD) is a type of movement disorder resulting from a shortage of dopamine production in nerve cells in the brain. Although there appears to be a genetic component in some cases, the disease does not generally seem to run in families. Exposure to environmental chemicals might play a role. The symptoms begin gradually, often only on one side of the body. Later, they begin to affect both sides. As the disease worsens, sufferers may have trouble walking, talking or performing simple tasks. They may struggle with depression, experience sleep problems, or have trouble chewing, swallowing or speaking. At present the chemical treatment for PD produces major side effects [Ascherio and Schwarzschild 2017, Liu *et al.* 2019].

GDNF is a secreted glycoprotein in the TGF-β family [Popova *et al*. 2017]. It can effectively protect the DA neurons of the substantia nigra striatum from damage and provide them with nutrients to promote the growth of dopaminergic neurons (DNs). This is a potent and relatively specific protective factor for DNs [Duarte *et al*. 2020]. Nevertheless, it is difficult to mass produce safe and bioactive glycosylated GDNF due to high production costs. Animal mammary bioreactors have many advantages in the production of active pharmaceutical proteins, including their low production cost, high quality, easy purification, high activity, as well as stable inheritance and expression of exogenous genes *in vivo* [Jinek *et al*. 2014]. However, when producing transgenic animals we face the challenge of random transgene integration.

Gene targeting is a powerful technique, which replaces an endogenous gene locus with an exogenous gene through homologous recombination [Li *et al.* 2020]. Studies have shown that the gene-targeting technology used to produce transgenic animals can integrate exogenous DNA into the target site of the genome, which avoids position effects due to random transgene insertion and significantly improves the expression of exogenous genes [Zhang *et al*. 2013]. However, the efficiency of gene targeting in somatic cells is lower than that in embryonic stem cells [Li *et al.* 2013, Wang *et al.* 2013].

Artificial nucleases can cleave the targeting gene, which greatly increases homologous recombination repair (HDR) and the efficiency of gene targeting [Ni *et al.* 2014, Salsman and Dellaire 2017, Li *et al*. 2018]. Such nucleases, including ZFN, TALEN, and CRISPR/Cas9, can also cleave target sites to a double strand break (DSB) [Fu *et al.* 2021]. DSB can activate cellular repair through non-homologous end joining (NHEJ), which causes knockout of targeting genes. The other mechanism used is HDR, which provides the ability to precisely insert exogenous genes [Jackson *et al*. 2014, Liang *et al.* 2017, Hu *et al*. 2019]. In recent years innovative gene-editing tools, such as the CRISPR/Cas9 system, have improved the model of gene editing and offer several advantageous characteristics, such as cost effectiveness, flexibility and ease of use.

This study exploited a gene-targeting vector containing the DNA donor with long homologous arms and a recombinant CRISPR/Cas9 plasmid to target cleavage in bovine fetal fibroblasts and integrate the human GDNF exogenous gene into the

bovine β-casein gene locus. This approach could be used for gene targeting in somatic cells, which may contribute to the production of a GDNF mammary gland bioreactor.

Material and methods

Construction of plasmids

CRISPR/Cas9 mediation was used to increase the rate of success for gene targeting in somatic cells. A pP40-GN plasmid vector was built on the basis of the pgk-SV40 skeleton carrier (Inner Mongolia University, Hohhot, China) containing a *neo* gene between two LoxP sites. The 5' homologous arm was a 1052 bp fragment comprising parts of intron 1 and exon 2 of the bovine *β-casein* gene. The 3' homologous arm was a 1080 bp fragment consisting of a part of exon 2 and intron 2 of the bovine *β-casein*. The list of the primer pairs is given in Table 1. Human *gdnf* cDNA was located downstream of the 5' homologous arm (use of human DNA was approved by the research ethics board of the Baotou Medical College, the Inner Mongolia University of Science and Technology). In addition, a 260 bp fragment containing the SV40 polyadenylation sequence was located downstream of the human *gdnf* as a transcriptional end signal. The *neo* gene, a positive selection marker, was located between the 5' homologous arm and the 3' homologous arm. The CRISPR/Cas9 expression vector was constructed by Shanghai Biomodel Organism Science & Technology Development Co. Ltd. (China).

Isolation and culture of primary bovine fetal fibroblasts

The tissue block adhesion method was used to isolate bovine fetal fibroblasts from explants of fetal ear skin tissues at 2-3 months of pregnancy. The explants contained 10% fetal bovine serum (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% CO2.

Assay for validation of the CRISPR/Cas9 system by T7EI

To verify whether the CRISPR/Cas9 system could cleave the endogenous gene in bovine cells we analyzed genomic DNA in bovine fetal fibroblasts cells transfected with the CRISPR/Cas9 vector by T7 endonuclease I (T7EI) (300 U, View Soid Biotech, Beijing), an endonuclease that cleaves heteroduplexes formed by the hybridization of wild-type and mutant DNA sequences in particular. Approximately 1.0×106 cells (at the concentration of about 1.0×107 cells/mL) were harvested at 70-80% confluence, mixed with a 10 μg CRISPR/Cas9 vector transferred into a 0.2-cm cuvette (Bio-Rad, USA). The cells were pulsed at 225 V twice with an electroporation transfection instrument (Bio-Rad). Each pulse was 5 ms and the interval was 0.1 ms. The transfected cells were cultured in DMEM/F12 medium without antibiotics in 10-cm dishes. After 48 h we extracted genomic DNA for PCR amplification using the primer pair (Tab. 1).

Table 1. PCR primer sequences

5' arm	F 5'- ATTGGGCC CGTGTGTCAAGAGATTGTGATGG-3' R 5'- CATCTCG'AGCAAGTCCTGGGAATGGGAAGATG-3'
3' arm	F 5'- ATTGGA TCCGGTCCTCATCCTTGCCTGG-3' R 5'- GCTGGATCCGCTCCTCCT CTATGGGATTTTCG-3'
T7EI	F 5'- ATCTGGATGGCTGGCAGTGAAACA-3' R 5'- GACTCAGACTTGTGGTCCCATAGG-3'
D-5' arm	F 5'- GCTTCCTCGTGCTTTACGGTATCGC-3' R 5'- AATGGGTAGCCTATCCCTTCTCCTG-3'
D-3' arm	F 5'- GCTTCCTCGTGCTTTACGGTATCGC-3' R 5'- AATGGGTAGCCTATCCCTTCTCCTG-3'

The amplification conditions were 95°C for 4 min, followed by 95°C for 30 s, 57°C for 30 s, 72°C for 45 s for 35 cycles and 72°C for 10 min. The PCR product was digested using T7EI. The digestion procedure was as follows: denaturation with heating followed by annealing in a hybridization buffer (10 mM Tris-HCl [pH 8.5], 75 mM KCl, and 1.5 mM MgCl2) to form heteroduplex DNA. After treatment with T7EI at 37°C for 30 min the resulting fragments were subjected to electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide.

Transfection and selection of bovine fetal fibroblasts

Approximately 1.0×106 cells of bovine fetal fibroblasts were collected at 70-80% confluency in the cell culture flask, taking 100 μL cell suspension mixed with 1.5 μg linear pP40-GN vector and 6 μg Cas9 nuclease expression vector to transfer into a 0.2-cm cuvette (Bio-Rad). The cells were subjected twice to pulses of 175 V using a Gene Pulser (Bio-Rad; each pulse lasted for 5 ms and came at an interval of 0.1 ms). The transfected cells were cultured in a DMEM/F12 medium without antibiotics in 10 cm dishes for 48 h. Then they were cultured in DMEM/F12 medium with G418 for 6-7 days. Afterwards healthy and well-separated colonies of cells were transferred to 48-well cell culture plates. The healthier and better-separated colonies in the plates were transferred to 6-well cell culture plates for embryo preparation and a quarter of the colonies were further cultured for PCR analyses.

Detection and analysis of transfected bovine fetal fibroblasts

G418+ colonies were screened with PCR and DNA sequencing for successful targeting. The positions of the PCR primers are shown in Figure 1. The cells were grown on 48-well plates and lysed in 40 μL lysis buffer (100 mM Tris, 200 mM NaCl, 0.2% SDS, 5 mM EDTA, and 0.4 mg/mL proteinase K) at 65°C for 30 min or 37°C overnight. Then they were heated to 95°C for 10 min to inactivate proteinase K. PCR amplification was performed in a 20 μL reaction volume using a TaKaRa LA Taq system (Bao Bioengineering Co., Ltd. Dalian, China). The PCR primer sequences are shown in Table 1.

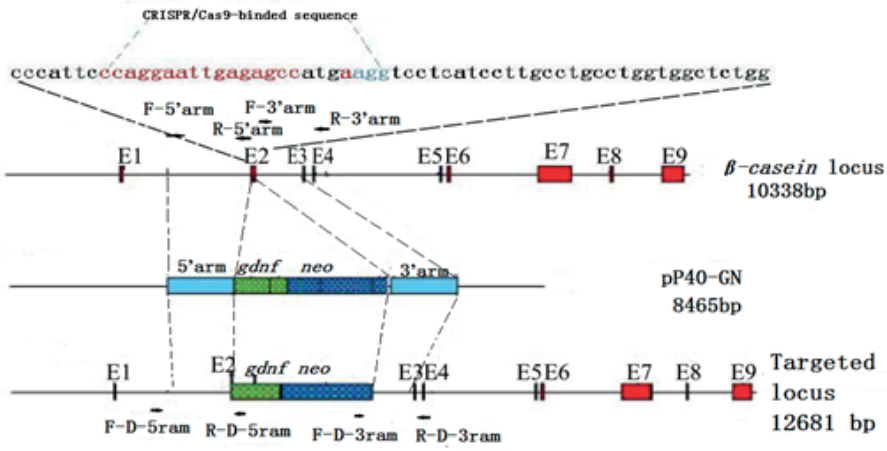

Fig. 1. Knock-in strategy of gdnf at the bovine β-casein locus by CRISPR/Cas9-mediated homologous recombination. The neo gene was used as a positive selection marker. The PCR primer pairs used to detect homologous recombination events are denoted with arrows.

The amplification conditions were 94°C for 4 min, 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1.5 min (5'arm) or 2 min (3'arm), followed by 72°C for 10 min.

Results and discussion

Construction of the pP40-GN gene-targeting vector

The pP40-GN gene-targeting vector was constructed as shown in Figure 2. Two homology arms were inserted into a pP40 skeleton vector, including a phosphoglycerate kinase promoter (PGK) and the neo positive screening gene, so that gene-targeting

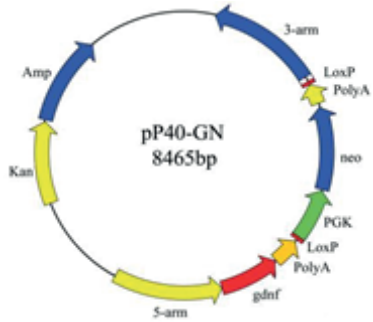

Fig. 2. The pP40-GN targeting vector including gndf, neo, 5'arm, 3'arm and the Cre-LoxP system [Utomo *et al*. 1999].

events were detectable by neo expression and G418 selection. gdnf was located downstream of the five homology arms that provide the driving gene sequence for subsequent gdnf gene expression.

Rapidly proliferating and well-shaped bovine fetal fibroblasts were obtained for gene targeting through the isolated culture of bovine embryos (as shown in Fig. 3). At a concentration of 200 μg/mL, G418 killed all normal cells within 7 days (Fig. 4).

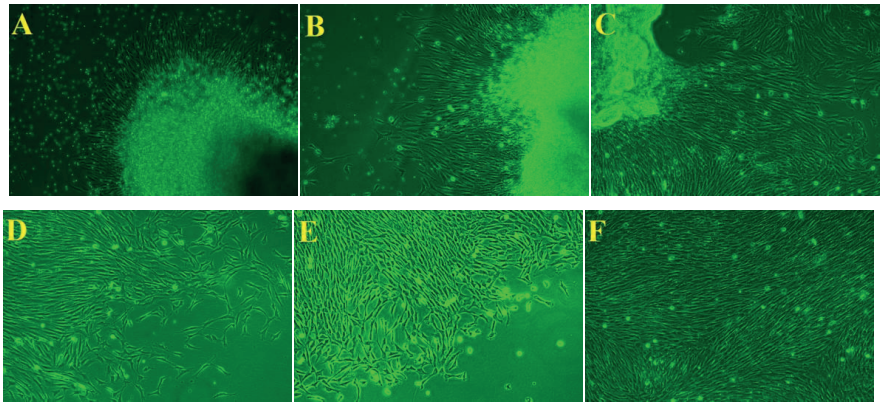

Fig. 3. Isolation and culture of bovine fetal fibroblasts (contrast microscopy images, 100×).

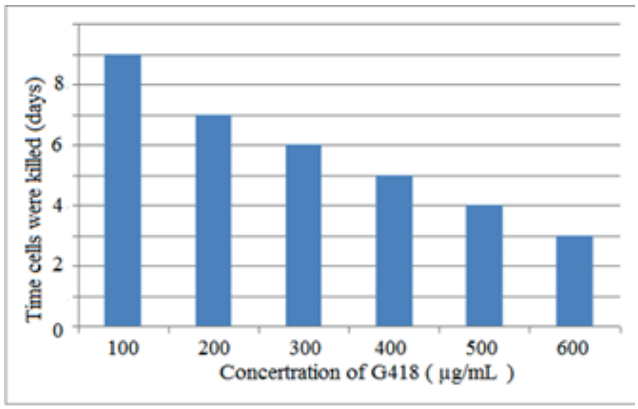

Fig. 4. Analysis of normal cell tolerance of G418. At a concentration of 200 μg/mL G418 is guaranteed to kill all normal cells in 7 days.

CRISPR/Cas9 system function for the beta-casein locus with T7EI

We found that a mutation was produced after the CRISPR/Cas9 plasmid was transfected into the bovine fetal fibroblasts for 3 days. Genomic DNA was extracted for PCR and its fragments were cleaved by T7EI, indicating that CRISPR/Cas9 was effective for target-gene editing (Fig. 5).

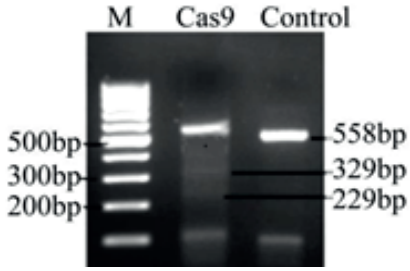

Fig. 5. Assay for CRISPR/Cas9-mediated cleavage at a targeting site at the bovine β-casein locus. (M = 100 bp DNA marker.).

Human gdnf cDNA targeted into the β-casein locus

After transfection for about 48 h, the cells showed green fluorescence (Fig. 6A and B). After 7-day cultivation we obtained monoclonal cells that were healthy and well-separated (Fig. 6C and D). Monoclonal cells were frozen when the convergence of the cells approached 80%. Well-growing cells are beneficial for recombinant embryo production.

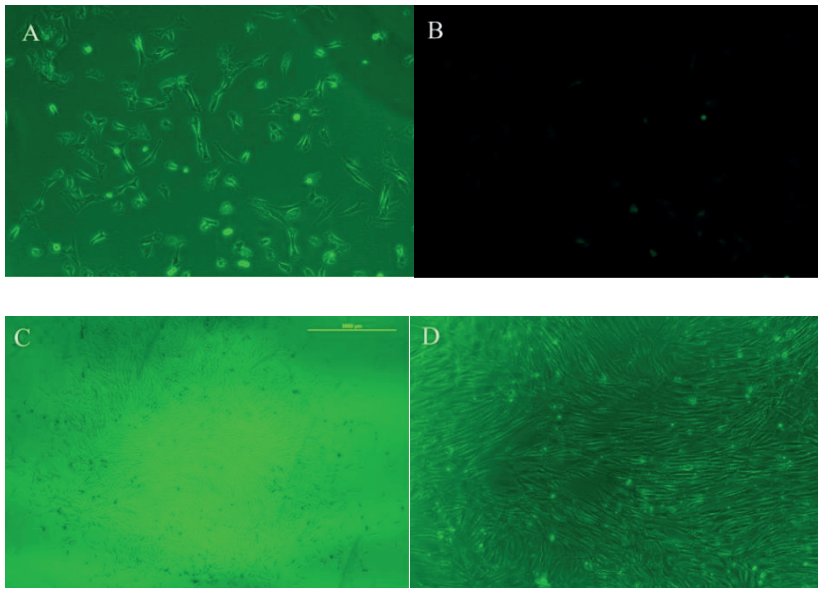

Fig. 6. Bovine fetal fibroblasts transfected with the pP40-GN targeting vector and CRISPR/Cas9 selected by G418. A and B, Monoclonal cells expressing green fluorescent protein after transfection for 48 h. C and D, Monoclonal cells that formed a cell mass after selection by G418 (B, fluorescence microscopy images, 400×; A and D, contrast microscopy images, 400×; C, contrast microscopy image, 100×).

The genome of the monoclonal cells was extracted before freezing. The results of PCR (the primer sites for the F-D-5' arm, R-D-5' arm, F-D-3' arm and R-D-3' arm, as shown in Fig. 1) and gene sequencing showed that human *gdnf* was integrated at the bovine *β-casein* locus (Fig. 7). The efficiency of CRISPR/Cas9 mediated targeting was about 41.7%.

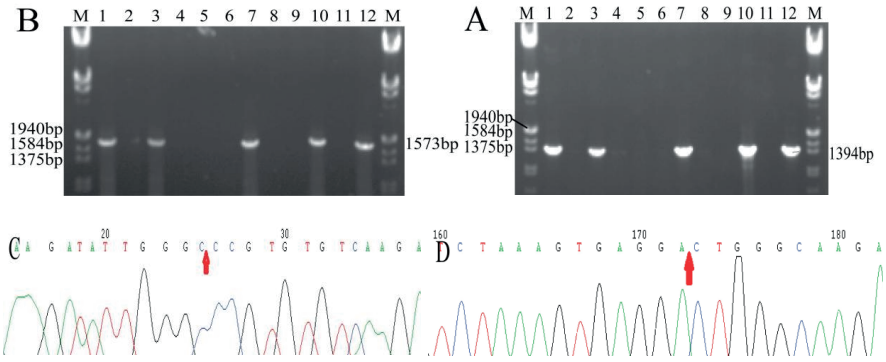

Fig. 7. Identification of *gdnf* insertion at the bovine *β-casein* locus. A and B, Electrophoretic result of amplification products after 5'-end-PCR (F-D-5' arm/R-D-5' arm) and 3'-end-PCR (F-D-3' arm/R-D-3' arm). Positive bands were 1394 bp and 1573 bp, respectively. C and D, Sequencing results for amplified fragments by 5'-end-PCR and 3'-end-PCR, respectively. Arrows indicate integration sites.

CRISPR/Cas9 has been widely used in gene editing since its first application in 2013. CRISPR/Cas9 efficiently cleaves a target site to generate DNA double-strand breaks. It can introduce a change at the target site with HDR repair. The technique is efficient, fast and simple, and it can modify simultaneously multiple gene sites. Furthermore, it has fewer off-target effects than ZFN and TALEN [Wang 2015]. It has been successfully used for gene targeting in several species [Thomas *et al.* 1992, Chen *et al*. 2015, Crispo *et al*. 2015]. In this study CRISPR/Cas9 was used to mediate the insertion of human *gdnf* into the bovine fetal fibroblast *β-casein* locus and positive cell clones were successfully obtained.

The pP40-GN targeting vector includes carrier skeleton sequences, homologous sequences of targeting genes and exogenous targeting gene sequences. The base mutation of homologous sequences affects HDR, significantly reducing the efficiency of gene targeting [Zhao *et al.* 2015]. In addition, HDR is positively correlated with the total length of homologous arms within a certain range. In our study the lengths of homologous sequences were 1050 bp and 1080 bp, respectively. Additionally, the mutation rate was strictly controlled. Thus the efficiency of gene targeting was greatly improved. Studies have shown high targeting efficiencies, reaching 40-69.9% in CRISPR/Cas9-mediated somatic gene targeting research [Zhang *et al*. 2021]. Jeong *et*

al. [2016] first fused the F2A sequence to the small human fibroblast growing factor 2 (FGF2) gene and then integrated it into the third exon of the *β-casein* locus of bovine fetal fibroblasts through CRISPR/Cas9. As a result, the relative targeting efficiency of male bovine fetal fibroblasts was 31.3%, while the relative targeting efficiency in female bovine fetal fibroblasts was 78.45%.

The aging problem for bovine fetal fibroblasts, however, remains a major problem in recombinant embryo production. In the case of electroporation cells can be damaged to a certain extent, which can accelerate their senescence and shorten their life [Chang *et al.* 2016, Polajzer *et al*. 2020]. This can greatly reduce the production of monoclonal cells. In our study the process of electrotransfection was the most important factor leading to the aging of bovine fetal fibroblasts. The electric current, the voltage and ionic concentration of electrotransfection need to be appropriate; otherwise, the efficiency of gene targeting will decrease. Excessive values in the process of electroporation can lead to fibroblast damage, accelerate cell aging and attenuate the subculture and identification of cells. If the current and voltage are too low, foreign plasmids cannot be effectively transfected into fibroblasts, which reduces the efficiency of gene targeting. In addition, the ionic concentration plays an important role in transfection. Therefore, we focused on optimizing the electric current, voltage and the number of plasmids. Finally, we established electrotransfection conditions to be as follows: voltage of 175 V, two pulses, interval of 0.1 ms, 5 ms per pulse, cell concentration of 1×107 cells/mL, volume 100 μL, 6 μg CRISPR/Cas9 plasmid and 1.5 μg pP40-GN plasmid.

In addition to the transfection conditions, high concentrations of G418 can also damage fibroblasts. Therefore, a concentration of 200 μg/mL was chosen for G418 when selecting transfected fibroblasts. To obtain active positive cell clones 3×106 fibroblasts were transfected under optimized conditions. After selection with G418 a total of 12 healthy and well-separated clones were obtained, in which five cell clones involved in gene targeting events were confirmed by PCR analyses and PCR product-sequencing identification.

In conclusion, gene targeting was successfully achieved using the CRISPR/Cas9 technique in bovine fetal fibroblasts. This approach produced reliable materials for subsequent embryo preparation in vitro and the preparation of animal mammary bioreactors.

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