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Production of *myostatin*-targeted goat by nuclear transfer from cultured adult somatic cells

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ABSTRACT

Myostatin, a member of the transforming growth factor-β family, acts as a negative regulator of skeletal muscle mass. In this study, *myostatin*-targeted caprine fibroblasts were obtained and subjected to SCNT to determine whether *myostatin*-knockout goats could be created. Fibroblasts from a 2-mo-old goat were transfected with a *myostatin*-targeted vector to prepare transgenic donor cells for nuclear transfer. After serum-starvation (for synchronization of the cell cycle), the percentage of transgenic fibroblasts in the G_0/G_1 phase increased (66.2% vs. 82.9%; $P < 0.05$) compared with that in the control group, whereas the apoptosis rate and mitochondrial membrane potential were unaffected ($P > 0.05$). There were no significant differences between *in vivo*- and *in vitro*-matured oocytes as recipient cytoplasts for rates of fusion (86.5% vs. 78.4%), pregnancy (21.6% vs. 16.7%), or kidding (2.7% vs. 0%). One female kid from an *in vivo*-matured oocyte was born, but died a few hours later. Microsatellite analysis and polymerase chain reaction identification confirmed that this kid was genetically identical to the donor cells. Based on Western blot analysis, myostatin of the cloned kid was not expressed compared with that of nontransgenic kids. In conclusion, SCNT using *myostatin*-targeted 2-mo-old goat fibroblasts as donors has potential as a method for producing *myostatin*-targeted goats.

1. Introduction

Transgenic livestock have wide potential applications in biomedical and agricultural fields, e.g., pharmaceutical protein production using mammary gland bioreactors and in improving economic characteristics through gene targeting [1–3]. However, the efficiency of transgenic animal generation remains low [4,5]. Nuclear transfer (NT) using transgenic donor cells provides an alternative technique for production of transgenic animals [6,7]. Donor cells can be selected for genetic modification to incorporate or knockout a transgene of interest; therefore, all cloned animals should be transgenic [8–12]. However, several foreign genes are randomly integrated and subsequently expressed at low levels. Gene targeting generally improves the repeatability of transgene expression and provides an efficient method of introducing single-copy transgenes via homologous recombination [13,14]. Thus, animal somatic cell gene targeting combined with NT is a powerful tool for producing transgenic animals. Although several gene-targeted animals were successfully produced using this method [11,15,16], production of *myostatin*-targeted goats has apparently not been reported.

Goat meat is an important food worldwide [17]; it has high-quality protein and low cholesterol, thus providing health benefits to humans [18]. *Myostatin* is a negative regulator of skeletal muscle growth [19,20] and has been identified as a direct mediator of skeletal muscle atrophy associated with heart failure. *Myostatin* deficiency may confer metabolic benefits [21,22] and is a promising strategy for treating cardiac cachexia and other metabolic

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diseases [23]. In addition, *myostatin* in mice and cattle has been intensively studied and reviewed. Several research institutions have attempted to produce *myostatin*-knockout goats or sheep. However, because *myostatin* affects organism development, the targeting technique is more difficult than with other genetic modifications, and reports on goat *myostatin* remain limited [24,25].

The objective of the present study was to establish an effective system for producing *myostatin*-targeted goats via SCNT. The system involved preparation of competent transgenic donor cells for NT, followed by a comparison of the effects of serum starvation on donor cell characteristics and evaluation of the production efficiency of transgenic goats derived from various oocyte sources.

2. Materials and methods

Unless otherwise indicated, all chemicals used in this study were obtained from the Sigma-Aldrich Company (St. Louis, MO, USA). Media were obtained from Gibco Invitrogen Corporation (Grand Island, NY, USA). Antibodies for myostatin (Cat. No. ab98337), β-actin (Cat. No. ab8229), goat polyclonal secondary antibody for rabbit IgG (Cat. No. ab97080), and rabbit polyclonal secondary antibody for goat IgG (Cat. No. ab6741) were purchased from the AbCam Company (AbCam, Inc., Cambridge, MA, USA). All trials were conducted in accordance with the Guidelines for the Care and Use of College of Animal Science and Technology, Nanjing Agricultural University.

2.1. Construction of the gene-targeted vector pLoxp-myostatin

To construct the gene-targeted vector pLoxp-myostatin, genomic DNA was extracted from the ear of a Huanghuai goat using the TIANamp Genomic DNA Kit (Tiangen, Beijing, China). The pLoxp-myostatin vector was constructed via a ligation-anchored polymerase chain reaction from purified isogenic DNA. The 4.9 kilobase (kb) homologous long arm contained whole exon1, intron1, and exon2, as well as part of the promoter and part of intron2. Meanwhile, the 2.5 kb homologous short arm contained a partial exon3 sequence and a 3′ untranslated regions (UTR) sequence. The two arms were cloned into the knockout plasmid pLoxp (donated by Wei Shen) [26]. The thymidine kinase (TK) fragment and the bacterial neomycin gene (the positive selection marker gene) were then placed between the two homologous arms. Thymidine kinase was positioned just outside the short arm as a negative selection marker gene. A schematic representation of the vectors is shown (Fig. 1).

2.2. Myostatin-targeted fibroblast (MTFF) culture

The resulting 13.71 kb gene-targeted vector was linearized with *Sal* I for somatic cell transfection. In brief, fibroblasts from a 2-mo-old goat were seeded in a six-well dish at 3×10^3 cells per well and then incubated for 48 h in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Cells were transfected using Lipofectamine2000 (Invitrogen, Inc.), in accordance with the

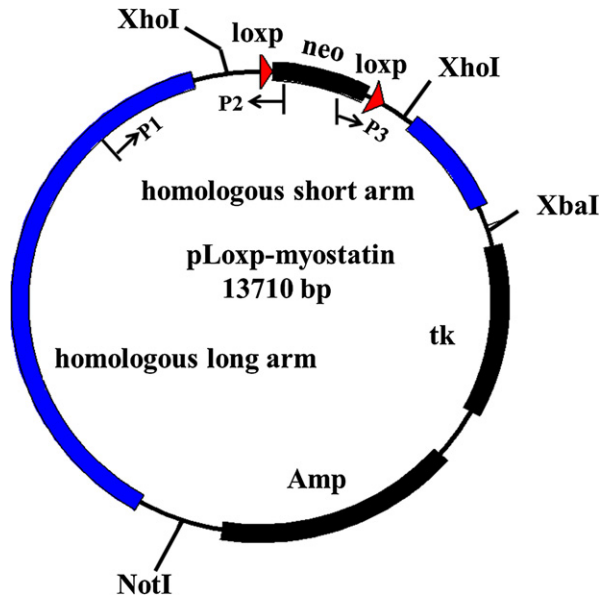

Fig. 1. Diagram of the goat pLoxp-*myostatin*-targeted vector (13.71 kilobase).

manufacturer's guidelines. Transfected cells were then placed in 100 mm plates and cultured. After 48 h, colonies were selected using 500 mg/mL G418 (Invitrogen, Inc.,) for 14 days. Well-proliferated colonies were then isolated and screened for the presence of the transgene. Genomic DNA was isolated from G418-positive cells, and the gene-targeted cell lines were then identified using polymerase chain reaction (PCR) and DNA sequencing. The PCR primers were as follows: P1, 5′-CTCTCTCGGAGTTGGTC-3′; P2, 5′-CTTCAGTGACAACGTCG-3′; P3, 5′-CTCCTGCCGAGAAAGT-3′; and P4, 5′-ACAGCCATCACGAACC-3′. In that regard, P1 and P2 were used to amplify 0.9 kb of the partial neo fragment and partial homologous long arm, whereas P3 and P4 were used to amplify 2.5 kb of the partial neo fragment, the entire homologous short arm, and a portion of the downstream area of the homologous short arm. The PCR products of positive colonies were sequenced to further confirm the targeting events. After PCR identification, targeted cells were collected for Western blot analysis using a standard protocol. Rabbit anti-GDF8/myostatin polyclonal antibodies were used as the primary antibody, whereas the secondary antibody was goat polyclonal secondary antibody to rabbit IgG. Nontransfected goat cells were used as negative controls. Colonies were then frozen until use for nuclear transfer.

2.3. Cell synchronization

The MTFFs were seeded at a density of 1×10^5 cells per mL and divided into control and serum starvation groups. For the various treatments, cells were cultured in DMEM containing 10% FBS until the logarithmic growth phase (80% confluence) as follows: (1) for the control group, cells were grown until the logarithmic growth phase (80% confluence) and were directly used for subsequent operations; and (2) for the serum starvation group, the original