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Generation of human lactoferrin transgenic cloned goats using donor cells with dual markers and a modified selection procedure

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Abstract

The objective was to use dual markers to accurately select genetically modified donor cells and ensure that the resulting somatic cell nuclear transfer kids born were transgenic. Fetal fibroblast cells were transfected with dual marking gene vector (pCNLF-ng) that contained the red-shifted variant of the jellyfish green fluorescent protein (LGFP) and neomycin resistance (Neo) markers. Cell clones that were G418-resistant and polymerase chain reaction-positive were subcultured for several passages; individual cells of the clones were examined with fluorescence microscopy to confirm transgenic integration. Clones in which every cell had bright green fluorescence were used as donor cells for nuclear transfer. In total, 86.7% (26/30) cell clones were confirmed to have transgenic integration of the markers by polymerase chain reaction, 76.7% (23/30) exhibited fluorescence, but only 40% (12/30) of these fluorescent cell clones had fluorescence in all cell populations. Moreover, through several cell passages, only 20% (6/30) of the cell clones exhibited stable LGFP expression. Seven transgenic cloned offspring were produced from these cells by nuclear transfer. Overall, the reconstructed embryo fusion rate was 76.6%, pregnancy rates at 35 and 60 days were 39.1% and 21.7%, respectively, and the offspring birth rate was 1.4%. There were no significant differences between nuclear transfer with dual versus a single (Neo) marker (overall, 73.8% embryo fusion rate, 53.8% and 26.9% pregnancy rates, and 1.9% birth rate with five offspring). In conclusion, the use of LGFP/Neo dual markers and an optimized selection procedure reliably screened genetically modified donor cells, excluded pseudotransgenic cells, and led to production of human lactoferrin transgenic goats. Furthermore, the LGFP/Neo markers had no adverse effects on the efficiency of somatic cell nuclear transfer.

Keywords: Transgenic animals; Nuclear transfer; Human lactoferrin; Dual markers; Donor cells; Goat

1. Introduction

Somatic cell nuclear transfer has been widely used for production of human recombinant proteins, including factor IX [1], catalase [2], CD59 [3], α-lactalbumin [4], lysozyme [5], and albumin [6]. The success rate of transgenic nuclear transfer (NT) remains low, as this process is under the influence of various factors, including the transgenic vector, donor cells, source of recipient oocyte, the process of somatic cell nuclear transfer (SCNT), and the effects of exogenous genes on embryo development [7–10]. The initial step of producing a transgenic cloned animal is preparation of genetically modified donor cells. According to previous reports by Echelard et al. [6], and Chen et al. [11], after NT using transfected donor cells from antibiotic drug selection, the animals generated were not transgenic. In that regard, G418-selected cells contained a mixture of transgenic and nontransgenic cells, because of a by-

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stander effect, whereby transgenic cells that expressed the antibiotic resistance product or transgenic cells that were in direct cell contact protected nearby nontransgenic cells [11,12]. In addition, obtaining cell clones either by cloning rings or by limiting dilution did not ensure isolation of certifiable clonal parental cells [13]. Thus, preparation of genetically modified donor cells is crucial for successful production of transgenic animals by NT. To facilitate this process, visual indication of green fluorescent protein (GFP) protein expression was developed to screen transfected cells, because GFP fluorescence under blue light indicates transgene integration [14,15]. As a visual selection marker, GFP has been widely used to generate transgenic offspring by NT in goats [16], pigs [17], cattle [18], mice [19], and rabbits [20]. However, use of GFP as a selection marker to prepare transgenic donor cells for production of NT animals has resulted in nontransgenic animals [21]. Limitations of GFP as a cell lineage marker have been reported by Swenson et al. [22]. Furthermore, during long-term cell culture, GFP expression could be silenced by epigenetic regulatory mechanisms [16]. However, long-term culture of the cell clones is essential to ensure stable integration of the transgene [10]. To overcome these obstacles, a modified strategy for genetic selection to prepare the genetically modified donor cells is required.

The purpose of the present study was to effectively select genetically modified donor cells for NT using jellyfish green fluorescent protein (LGFP)/neomycin resistance (Neo) dual markers, and investigate whether LGFP/Neo dual markers affected the efficiency of NT compared with a single marker.

2. Materials and methods

2.1. Animals

Sannen and Yangtze River delta white goats were kept in the Research Farm of Yangzhou University (Yangzhou, Jiangsu, China), and fed alfalfa hay, with free access to salt, mineral mix, and water. All surgical procedures were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Yangzhou University.

2.2. Construction of the mammary gland-specific expression vector

The plasmid vector pCNLF-ng containing the LGFP/Neo dual markers was based on the vector PbCNCS/LF36, which contains the human lactoferrin (hLF) gene under the control of the bovine αs1-casein promoter and the cytomegalovirus (CMV) enhancer for mammary gland-specific expression. The fragment (Ferh-mEF1/prom-GFP-IRES-Neo) carrying the LGFP gene and the Neo gene driven by a composite ferritin promoter was recovered from pMONO-Neo-GFP (InvivoGen Co., San Diego, CA, USA). The fragment was flanked by loxP sequence and then ligated to the 3′-end of PbCNCS/LF36 to form the dual markers plasmid pCNLF-ng.

The plasmid vector pBLC14 containing the single marker Neo and hLF gene, was promoted by β-lactoglobulin regulatory sequences and the CMV enhancer for mammary gland-specific expression, and neomycin resistance under control of the SV40 promoter.

The pCNLF-ng and pBLC14 vectors were linearized with *Sal*I and *Not*I. The DNA fragments were purified by QIAquick Gel Extraction Kit (Qiagen Co., Hilden, Germany), diluted with dual-distilled water, and quantified.

2.3. Preparation of goat fetal fibroblasts

Fetal fibroblasts were isolated from a 35-day fetus that was recovered surgically from a Sannen goat. After removal of the head and internal organs, the remaining tissues were dissociated into small pieces using scissors and digested with 0.25% trypsin. The supernatant was centrifuged to recover the cells. The cells were then cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12; Life Technologies Co., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies Co.), 100 IU/mL penicillin and 100 μg/mL streptomycin (Thermo, Fisher Scientific Co., Waltham, MA, USA) at 38 °C in a humidified atmosphere of 5% CO_2. Upon reaching 80% confluence, cells were passaged. At passage 2, cells were frozen into aliquots in DMEM/F12 with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO, USA) and 20% FBS.

2.4. Transfection and G418 selection of fetal fibroblasts

Frozen-thawed fetal fibroblasts that had reached 80% confluency were transfected with the pCNLF-ng and pBLC14 vectors by electroporation. Cells (2×10^6 cells/mL) were mixed with 20 μg/mL DNA (pCNLF-ng or pBLC14) fragments in hypo-osmolar buffer (Eppendorf AG., Hamburg, Germany) and transferred into 2-mm gap electroporation cuvettes (Eppendorf AG.). Electroporation was completed using a multiporator (Eppendorf AG.) with the following conditions: 270 V for 100