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Efficiency of donor cell preparation and recipient oocyte source for production of transgenic cloned dairy goats harboring human lactoferrin

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Abstract

The objective was to investigate the effects of the transgenic donor cell synchronization method, oocyte sources, and other factors, on production of *hLF*-gene nucleus transfer dairy goats. Three transfected cell lines from ear biopsies from three 3-mo-old Saanen dairy goats (designated Number 1, Number 2, and Number 3, respectively) were selected as karyoplast donors for somatic cell nuclear transfer (SCNT) after detailed identification (including PCR and sequencing of PCR products). In donor cell cycle synchronization studies, the apoptosis rate of *hLF* transgenic fibroblasts was not different ($P > 0.05$) after 3 days of serum starvation or 2 days of contact inhibition. Additionally, there was no effect ($P > 0.05$) on developmental capacity of reconstructed embryos; however, the kidding rate of recipients in the serum starvation group was higher than that in the contact inhibition group (18 vs. 0%, respectively). The production efficiency of the transgenic cloned goats using donor cells from the Number 1 dairy goat cell line was higher than those using the Number 2 and the Number 3 cell lines (kidding rates were 18, 2, and 0%, respectively, $P < 0.05$). The oocyte source did not significantly affect the pregnancy rate of *hLF*-transgenic cloned dairy goats, but more fetuses were aborted when using *in vitro* matured oocytes compared to *in vivo* matured oocytes. In summary, utilizing transfected 3-mo-old dairy goat fibroblasts as donor cells, seven live offspring were produced, and the *hLF* gene was successfully integrated. This study provided additional insights into preparation of donor cells and recipient oocytes for producing transgenic cloned goats through SCNT.

Keywords: Human lactoferrin; Somatic cell nuclear transfer; Synchronization; Cell cycle; Oocyte; Dairy goat

1. Introduction

Since Wilmut, et al. [1] used serum starvation treatment of mammary epithelial cells as nuclear donor cells to produce the first cloned sheep "Dolly" in 1996, somatic cell nuclear transfer (SCNT) has been successfully applied in multiple species, including cattle [2], mice [3], and goats [4]. Although SCNT is the preferred approach for producing transgenic large animals, there are still numerous technical challenges, with an overall efficiency of <5% [5], which substantially limits widespread application.

Somatic cell nuclear transfer is a complex technique, involving donor cell culture, gene transfer and cell

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selection, oocyte collection and enucleation, nuclear reconstruction, and embryo transfer. During normal mammalian development, most cells gradually lose their pluripotency and develop into differentiated cells. The critical aspect of SCNT is reprogramming differentiated cells (karyoplast) so the reconstructed donor cells and recipient cytoplasm (cytoplast) can develop into live clones. Therefore, type of donor cell, the synchronization process, and quality of receptor cytoplasm all affect SCNT efficiency.

The objective of the present study was to systematically investigate the effects of the transgenic donor cell synchronization method, oocyte sources, and other factors, on production of *hLF*-gene NT dairy goats, thereby providing insights for improving transgenic cloning efficiency.

2. Materials and methods

Unless otherwise indicated, all chemicals used were obtained from Sigma-Aldrich Company (St. Louis, MO, USA), whereas media was from Gibco (Grand Island, NY, 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. Culture of hLF-transgenic fibroblasts

The *hLF*-gene mammary expression vector was constructed in our laboratory [6] and introduced into dairy goat ear fibroblasts using a liposome transfection method [7]. Positive colonies were identified, expanded, and cryopreserved. Before SCNT, the *hLF*-transgenic dairy goat fibroblasts were taken from liquid nitrogen storage, placed immediately in a 37 °C water bath for thawing, transferred into Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, and cultured in a CO_2 incubator at 37 °C under 5% CO_2 and saturated humidity.

2.2. Synchronization

The *hLF*-transgenic fibroblasts were inoculated at a density of 1×10^5/mL and were divided into three groups, namely; a) normal growing, b) serum starvation, and c) contact inhibition. The cells were cultured in DMEM containing 10% FBS until logarithmic growth phase (80% confluence). The normal growing group was taken to the logarithmic growth phase (80% confluence) and was directly used for the subsequent operations. The serum starvation group were cultured as described above and cells were then washed twice with D–PBS and cultured in DMEM with 0.5% FBS for 3 days. The contact inhibition cells were grown in DMEM containing 10% FBS to 100% confluence and cultured for an additional 2 days.

2.3. Cell cycle analysis

The *hLF*-transgenic fibroblasts in the three groups as defined above were treated with 0.25% trypsin, collected in 1 mL tubes (with each tube containing 2×10^5 to 1×10^6 cells in suspension), centrifuged to obtain the cell pellet, washed once with PBS, centrifuged again, and the supernatant discarded. Then, cells collected were treated with 50 mg/mL RNase for 1 h at 37 °C, and treated with 50 mg/mL PI (propidium iodide) for 1 h at 4 to 8 °C. Samples of cells were analyzed by flow cytometry (Becton Dickinson, Mountain View, CA, USA) with respect to the G1, S, G2, M and G0 phases. Histogram plots were created using the Cell Quest Program (Becton Dickinson). Percentages of cells within the various phases of the cell cycles were calculated using Cell Quest by gating G0+G1, S, and G2+M cell populations visualized, with a scatterplot of green fluorescence against red fluorescence.

2.4. Apoptosis analysis

The *hLF*-transgenic fibroblasts in the three groups as defined above were treated with 0.25% trypsin, collected in 1 mL tubes at a density of 10^6/mL, and stained with Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA). Before use, the PI solution was diluted to 100 μg/mL. Then, 5 μL Alexa Fluor 488 annexin V and 1 μL PI were added to each 100 μL of cell suspension, and were allowed to react for 15 min at room temperature in the dark. Binding buffer (400 μL) was added and mixed gently. Samples were kept on ice for immediate apoptosis analysis by flow cytometry.

2.5. Oocyte collection

To obtain *in vitro* matured oocytes, goat ovaries were collected from an abattoir, transported to the laboratory within 3 to 4 h after death of the donor, and washed with normal saline. Connective tissues and oviducts were removed. The ovaries were transferred into Petri dishes, and 2 to 6 mm follicles were sectioned. Cumulus-oocyte complexes (COCs) appropriate for *in vitro* maturation [8] were sorted under stereomicroscopy, and cultured for 20 to 23 h at 38.5 °C under 5% CO_2 in humidified air. Matured oocytes were then pipetted with 0.3% hyaluronidase to remove granulosa cells; those that had released their polar bodies were selected for use.