Selection of Donor Nuclei in Somatic Cell-Mediated Gene Transfer Using a Co-Transfection Method

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Abstract. In this study, we introduced a co-transfection method for the selection of donor nuclei in somatic cell-mediated nuclear transfer. Two vectors were constructed in our experiment. One was pMSCV-GFP carrying the neomycin-resistant gene (Neor) and the green fluorescent protein (GFP) reporter gene; the other was pBC1-GFP carrying the mammary gland-specific promoter and target gene GFP. Ovine adult fibroblasts were co-transfected with pMSCV-GFP and pBC1-GFP. The data from this work demonstrated that the GFP genes in both vectors could successfully co-integrate into the genomes of ovine adult fibroblasts in three of the four transgenic cell clones assayed. Furthermore, PCR analysis of transgenic embryos proved that the GFP genes in both vectors could co-integrate into the genomes of the reconstructed embryos. Subsequently, analysis of the developmental rate of the reconstructed embryos after nuclear transfer indicated that the blastocyst rate from the co-transfected donor cells was similar (approximate 8 percent) to that from individual pMSCV-GFP transfected donor cells. The influence of co-transfection resulting in modification of donor nuclei on development of reconstructed embryos was also investigated. The results of flow cytometric analysis indicated that the co-transfected ovine fibroblasts had similar quiescent characteristics in terms of cell cycle (G0+G1 percent: 73.20 ± 4.04) to the individual pMSCV-GFP transfected fibroblasts (G0+G1 percent: 70.77 ± 1.19) after they were treated with serum starvation for five days. Our results suggest that the co-transfection method can be used for selection of donor cell clones in somatic cell-mediated gene transfer experiments. It can be potentially extended to applications related to expression of functional protein in mammary glands and other transgenic research relevant to nuclear transfer.

Key words: Cell cycle, Co-transfection, Mammary gland-specific promoter, Ovine adult fibroblast, Somatic cell-mediated nuclear transfer

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research. Vector construction is the first step in producing transgenic animals using the somatic cell-mediated nuclear transfer technique, which is often a difficult step since it includes multiple procedures related to molecular biology.

Co-transfection is a routine method used in the analysis of protein-protein interaction and DNA-protein interaction in eukaryotes [4–6]. Characteristic of this approach is that cells are co-transfected with two plasmids and the cells that take up the DNA usually take up both plasmids. If a target gene and a reporter gene or a reporter gene and a drug-resistance gene are located in the two separate plasmids, they almost always co-integrate into the same location in the cell [7]. Co-transfection may avoid new vector re-construction under the conditions that the promoter, target gene, reporter gene, and drug-resistance gene are not included in the same vector in the cell transfection procedure. To date, however, no studies have been published in the literature in which a co-transfection procedure has been used for selection of a transgenic donor cell line in somatic cell-mediated nuclear transfer.

In the present study, we introduced a co-transfection method for the selection of donor nuclei in somatic cell-mediated nuclear transfer. Two vectors, which possess different functions in the co-transfection method, were used in this co-transfection procedure. One vector, pMSCV-GFP, carried the drug resistance gene (Neo') and the reporter gene (GFP gene), and the other one, pBC1-GFP, carried the mammary gland-specific promoter and the target gene (GFP gene). Ovine adult fibroblasts were co-transfected with both of the vectors. Our results proved the feasibility of this method through the production of reconstructed embryos after nuclear transfer. We believe that the co-transfection method can be used for selection of donor cell clones in somatic cell-mediated gene transfer experiments. It appears to be particularly convenient since plasmids are large and difficult to reconstruct into a new vector, as was done in our experiment. In addition, the co-transfection method can potentially be extended to applications related to expression of functional protein in mammary glands and transgenic research relevant to nuclear transfer. Gene co-integration, development of reconstructed embryos, and analysis of cell cycle in donor cell lines were used as parameters to determine the success of this study.

Materials and Methods

Construction of pMSCV-GFP and pBC1-GFP and genes expression in pMSCV-GFP

Construction of pMSCV-GFP (Fig.1) was conducted by inserting a 714 bp fragment of the GFP gene, which was isolated from pGFP (Clontech Laboratories, Mountain View, CA, USA) after EcoRI and XhoI digestion, into pMSCV (Clontech) treated with EcoRI and XhoI. Construction of pBC1-GFP (Fig. 1) was conducted by inserting a linked DNA fragment composed of a synthetic secretion signal sequence (5’-TCAGCTCGAGATGAAGGTCCTCATCC TTGCTGTCTGTCGCTGCCCATTGAGAGCTCGAATCGACT-3’) treated with EcoRI and a 714 bp fragment of GFP gene PCR-amplified from pGFP and treated with EcoRI, after XhoI digestion, into pBC1 (Invitrogen, Carlsbad, CA, USA) digested with XhoI.

The pMSCV-GFP and pBC1-GFP were transformed into DH5α Escherichia and TOP10 Escherichia competent cells, respectively. For eukaryotic cell transfection, amplification and purification of the DNA of both plasmids from 100 ml Luria-Bertani (LB) medium were conducted using an EndoFree Plasmid Maxi Kit (Qiagen, Fremont, CA, USA). Identification of the inserted GFP gene fragment in pMSCV-GFP and pBC1-GFP was completed through digestion of the restriction enzymes EcoRI and XhoI and separation using the agarose gel electrophoresis technique. All enzymes for manipulation were purchased from Fermentas (Burlington, ON, Canada).

The GFP gene in pMSCV-GFP was to be expressed under the control of the 5’ long terminal repeat (LTR) sequence of the murine stem cell PCMV virus. The Neo resistance gene in pMSCV-GFP was to be expressed and driven by phosphoglycerate kinase promoter (PGK) (Fig.1).

Establishment of a primary ovine adult fibroblast line

Primary ovine adult fibroblast line AF1 was isolated from the ear skin tissue of a four-year-old female sheep from a local slaughterhouse. Ear skin tissue was minced and digested with 0.25% trypsin, penicillin (200 U/ml), and streptomycin (200 µg / ml) for 18 h at 4°C in several 1.5 ml tubes. Isolated cells were cultured with Dulbecco’s Modified Eagle’s Medium/F12 (DMEM/F12; Gibco BRL, Grand Island, NY, USA) supplemented with 10%
fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), and streptomycin (100 µg/ml). Epithelial-like cells were carefully scraped with a cell scraper after the cells were cultured in 50 ml tissue culture flasks for a week. Scraping was repeated once in the following several days and then pure fibroblasts were collected at around the 20th day. Once confluent, the cells were frozen in 10% dimethyl sulfoxide in DMEM supplemented with 20% FBS.

**Co-transfection of the ovine adult fibroblast line and establishment of transgenic cell lines**

Purified fibroblast line AF1 at passage 2 was co-transfected with pBC1-GFP and pMSCV-GFP. The whole procedure was conducted according to the description provided with Lipofectamine Reagent (Invitrogen). Briefly, $1 \times 10^5$ cells were seeded in each well of a 6-well plate one day before transfection. The cells in each well were transfected with 2 µg mixture of pBC1-GFP/pMSCV-GFP (the ratio of pBC1-GFP to pMSCV-GFP was 10:1). After 72 h to 96 h of transfection, the cells were passaged based on cell density at 1:3 or 1:4 into the appropriate medium and selected using 600 µg/ml G418 (Amresco, Solon, OH, USA) for 8 to 9 days. Green clones that scattered in non-green cells were observed under a fluorescence microscope. The observed green-positive cell clones were marked with circles at the corresponding spot on the bottom of the culture flask or plate. Each marked circle was digested with 0.25% trypsin for 2–3 min and then transferred into a well of a 24-well plate supplemented with DMEM/F12 medium, 10% FBS, and 300 µg/ml G418. Each well of the 24-well was passaged into one well of a 6-well plate, and once it was confluent, it was passaged into a 50 ml flask. Finally, four positive green clones (ATF1, ATF2, ATF8 and ATF9) were generated, which were used for the following analysis of GFP expression rate. The whole procedure of establishment of transgenic cell lines took approximately 45 days.

**Polymerase chain reaction analysis of transgenic cell lines and transgenic embryos**

Genomic DNAs were isolated from the above four GFP-positive cell lines and non-transgenic AF1 with a DNeasy Tissue Kit (Qiagen). The fragments including the GFP genes in the two vectors were analyzed by PCR. The upstream primer (5'-CCCTGAACCTCCTCGTTCGACC-3') used to amplify a 1064 bp fragment including the GFP gene in pMSCV-GFP corresponds to nucleotides 1333–1355 of pMSCV, and the downstream primer (5'-GAGACGTGCTACTT-CCATTTGTC-3') represents the reverse complement of nucleotides 1660–1682 of pMSCV. Amplification was conducted under the conditions of initial denaturation for 3 min at 94 C; 3-step cycling including denaturation for 1 min at 94 C, annealing for 1 min at 58 C, and extension for 1 min at 72 C for a total of 35 cycles; and a final

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**Fig. 1.** Structure of the pMSCV-GFP and pBC1-GFP vectors (drawing is not to scale). LTR: long terminal repeat. ψ: the extended viral packaging signal. PPKG: the murine phosphoglycerate kinase (PKG) promoter. SS: β-casein secretion signal. E: β-casein exon.
extension of 10 min at 72°C. The upstream primer (5’-GATTGACAA GATAACGTGTTTCCCTC-3’) used to amplify an 896 bp fragment including the GFP gene in pBC1-GFP corresponds to nucleotides 8554–8580 of pBC1, and the downstream primer (5’-CATCAGAAGTTAACAGCACAGTTA G-3’) corresponds to the reverse complement nucleotides 8653–8678 of pBC1. Amplification was conducted after initial denaturation for 2 min at 94°C, 3-step cycling (denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 74°C) for a total of 35 cycles, and final extension for 7 min at 74°C. PCR products were applied to 1.5% agarose gel electrophoresis analysis.

PCR analysis for 10 green-positive embryos randomly selected at the blastocyst stage was conducted as follows: each embryo was heated at 94°C for 10 min in a 0.2 ml tube and was then analyzed with the above PCR primers.

Preparation of metaphase II recipient cytoplasm

Ovaries were collected from sheep slaughtered less than 2 h previously at a local abattoir and transported to the laboratory at 39°C. Follicles with diameters of 3–8 mm were punctured with an 18-gauge needle. Cumulus-oocyte complexes (COCs) with several layers of cumulus cells and homogeneous oocyte cytoplasm were washed 3 times in pre-balanced M199 medium supplemented with 10% FBS (Invitrogen), 10 μg/ml FSH, 1 μg/ml estradiol-17β (Sigma), and 1 μg/ml LH. Oocyte maturation was performed in a 4-well plate overlaid with mineral oil at 38.5°C in a humidified incubator with 5% CO2 in air for 18–22 h. After oocyte maturation, the cumulus layers were removed in 0.5% hyaluronidase solution, and the oocytes with a first polar body were selected as candidates for recipient cytoplasm.

Preparation of donor cells and nuclear transfer

The donor cell candidates included co-transfected cell line ATF9, individual pMSCV-GFP transfected cell line AITF1 (preserved in our lab), and non-transfected cell line AF1. Briefly, the donor cell candidates at passage 6 were exposed to DMEM/F12 medium supplemented with 10% FBS (300 μg/ml G418 was also added for ATF9 and AITF1) for a five extra days. On the sixth day, the donor cell candidates were digested with 0.25% trypsin and re-suspended with DMEM/F12 medium supplemented with 10% FBS. They were then centrifuged for 5 min, balanced for 30 min with DMEM/F12 medium supplemented with 10% FBS, and then used as donor cells.

Cumulus-removed oocytes in batches of 10 were placed into a drop of M2 medium containing 7.5 μg/ml cytochalasin B (Sigma) covered with mineral oil. An oocyte was picked up and held at the 2 O’clock position by negative pressure of a hold pipette (75 μm outside diameter). The oocyte was enucleated by aspirating the first polar body and approximately 30% of the adjacent cytoplasm using a soft glass enucleation pipette (20–25 μm outside diameter). Then, a donor cell was introduced through the same notch into the perivitelline space of the enucleated oocyte, and the donor cell membrane was situated in close contact with the inner membrane of the oocyte.

Fusion and activation of the cytoplasm-cell couplet and culture in vitro

After nuclear transfer, the oocyte-cell couplet was fused immediately. Couplets were manually aligned in a slot full of fusion buffer (0.28 M mannitol, 0.5 mM Hepes and 1 mg/ml BSA) between both metal electrodes and kept at the interface of the donor cell and cytoplasm parallel to the electrodes. The distance between the electrodes was 0.5 mm. A direct current pulse (1.4 kV/cm, 20 μs per time, 2 times total) was administered to induce fusion of the cell and cytoplasm. After that, the cell-cytoplasm couplet was washed three times with M199 and 10% FBS and incubated at 38.5°C in 5% CO2 and 95% atmosphere for 0.5 h. The fusion rate was then determined. Couplets that had not fused were administered a second fusion pulse as described previously.

Two to three h after application of the first fusion pulse, the fused couplets were activated using 7% alcohol and 6-dimethylaminopurine (DMAP). Briefly, the couplets were incubated for 7 min in SOF containing 7% ethanol. The activated couplets were cultured for 2.5–4 h in 2 mM DMAP, and then washed in handling medium, and placed into culture drops consisting of SOF medium under an oil overlay. The couplets were observed, and statistical analysis was conducted for cleavage and
development of embryos reconstructed in the following week. Statistical data were obtained from 50 oocytes enucleated per sample. Each entire nuclear transfer experiment was repeated three times. On each occasion, two samples of each treatment were analyzed (n=6).

Cell cycle assay
Three types of ovine adult fibroblast cell line were used for analysis of cell cycles at passpage 6: co-transfected ATF9, individually pMSCV-GFP transfected AITF1, and non-transfected AF1. The cell lines were cultured in DMEM/F12 medium supplemented with 10% FBS (300 µg/ml G418 was extra added for ATF9 and AITF1) until they were 60–80% confluent. Then, the medium was removed, and the donor cell candidates were exposed to DMEM/F12 medium supplemented with 0.5% FBS (300 µg/ml G418 was extra added for ATF9 and AITF1) for an additional five days. On the sixth day, each cell line was digested with 0.25% trypsin, re-suspended in DMEM with 10% FBS, centrifuged at 500 g for 10 min, thoroughly re-suspended in 1 ml of cold PBS, and then 1 ml of ethanol (4 C) was slowly added to each tube while vortexing. After ethanol fixation overnight, the cells were pelleted and washed once with PBS containing 5 mM EDTA. The pelleted cells were stained by adding 1 ml PBS containing 30 µg/ml propidium iodide and 0.3 mg/ml RNase A (Sigma). Staining was achieved at room temperature and in the absence of light for at least 0.5 h. The stained cells were filtered through a 30-µm nylon mesh (Spectrum, Los Angeles, CA, USA), and then the cell cycles were assayed with flow cytometry. Fluorescence data was obtained from 10,000 viable cells per sample. The above-mentioned experiments were repeated three times. Cells from each replicate were analyzed by flow cytometry on separate occasions. On each occasion, two samples of each treatment were analyzed (n=6).

Statistical analysis of cell cycle
Statistical analysis was performed with the SPSS 14 software (SPSS, Chicago, IL, USA). Differences between treatments were determined using the Student’s t-test and were judged to be significant when P<0.05.

Results
Expression of the GFP gene in pMSCV-GFP in ovine adult fibroblast lines
Expression of the GFP gene in pMSCV-GFP was initially observed within 24 h after cells were co-transfected with pMSCV-GFP and pBC1-GFP. After selection with 600 µg/ml G418 for 8 to 9 days, green-positive clones that scattered in non-green cells were observed under a fluorescence microscope. Four green fluorescent cell lines, ATF1, ATF2, ATF8, and ATF9, were generated after the selected green clones continued to be treated with 300 µg/ml G418 for 2 weeks. Expression of the GFP gene in pMSCV-GFP in the above four green fluorescent cell lines is shown in Fig. 2A, respectively. The non-transfected cell line, AF1, was used as a negative control. The green fluorescence rates of the above green-positive clone lines were estimated by flow cytometry (Fig. 2B). The green fluorescence rate of ATF9 was 93.7 percent, the highest of the four green clones. Therefore, ATF9 was selected as the donor cell line in the following nuclear transfer.

The GFP genes in pBC1-GFP and pMSCV-GFP co-integration into the genomes of ovine adult fibroblast lines and reconstructed embryos
Since pBC1-GFP is a mammary gland-specific expression vector, the GFP gene in pBC1-GFP cannot be expressed in ovine adult fibroblasts. To clarify whether the GFP genes in pBC1-GFP and pMSCV-GFP co-integrate into the genomes of green fluorescent cell lines or not, it was necessary to conduct a second selection for the green fluorescent clones. Fragments, including the GFP genes in pMSCV-GFP and pBC1-GFP, were amplified by PCR from the genomic DNAs of green fluorescent cell lines ATF1, ATF2, ATF8, and ATF9 (Fig. 3A), respectively. The amplified fragment for pMSCV-GFP included 350 bp of pMSCV and 714 bp of the GFP gene, a total of 1,064 bp. The results showed that all the green fluorescent cell lines carried the 1064 bp fragment compared with AF1 (Fig. 3B). An 896 bp fragment for pBC1-GFP was also amplified, which consisted of 125 bp of pBC1, 45 bp of the signal peptide sequence, 12 bp of the restriction enzyme SacI and EcoRI fragment, and 714 bp of the GFP gene. The results showed that the 896 bp fragment for pBC1-GFP was included in green fluorescent cell lines ATF1, ATF2, and ATF9, but
not in ATF8 for reasons unknown (Fig. 3C). The above results indicate that the GFP genes in pBC1-GFP and pMSCV-GFP co-integrated into the genomes of transgenic clone lines ATF1, ATF2, and ATF9, but not ATF8.

To further clarify whether the GFP genes in both vectors co-integrate into the genomes of transgenic embryos, the above 896 bp fragment in pBC1-GFP and 1064 bp fragment in pMSCV-GFP were amplified from ten green embryos randomly selected at the blastocyst stage using PCR methods, respectively. The results proved that the GFP genes in pBC1-GFP and pMSCV-GFP co-integrated into the genomes of all ten reconstructed embryos. Fig. 3D shows the results of PCR amplification from a reconstructed embryo.

**Expression of GFP in reconstructed embryos and production analysis of reconstructed embryos**

Expression of GFP was examined under blue light on the sixth day after NT in developing embryos in vitro, whose nuclei were derived from ATF9 expressing the gene. All reconstructed embryos emitted green fluorescence, and there was no evidence of mosaicism in any expressing embryos (Fig. 4). The morulae or blastocyst rates of the reconstructed embryos were assayed for co-transfected ATF9, individual pMSCV-GFP transfected AITF1, and non-transfected AF1. The results (Table 1) indicate that there was a similar (approximate 8%) blastocyst rate for reconstructed embryos derived from ATF9 co-transfected by pBC1-GFP and pMSCV-GFP to those from AITF1 transfected by individual pMSCV-GFP. There was no significant difference (P>0.05) between the blastocyst rates of ATF9 and AITF1. The results also indicate that there was an approximate 10 percent lower blastocyst rate when using ATF9 or AITF1 as the donor cell line in NT than when using AF1. There was a significant difference (P<0.05) between the blastocyst rates of reconstructed embryos derived from transgenic ATF9, AITF1, and non-transgenic AF1.

**Transgenic donor cells could not be synchronized in the G0/G1 phase by serum starvation**

Quiescent status of donor nuclei is an important

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**Fig. 2.** Expression of GFP and analysis of green fluorescence rates in four green fluorescent ovine adult fibroblast lines, ATF1, ATF2, ATF8, and ATF9. A: Expression of the GFP gene in pMSCV-GFP in ATF1, ATF2, ATF8, and ATF9. Purified fibroblast line AF1 at passage 2 co-transfected with pBC1-GFP and pMSCV-GFP at a ratio of 10:1 was selected with G418 for 2–3 weeks. Green fluorescent ovine adult fibroblast lines ATF1, ATF2, ATF8, and ATF9 were generated and examined under a fluorescence microscope. Original magnification: × 400. Non-transfected AF1 was used as a negative control. B: Analysis of green fluorescent rates in ATF1, ATF2, ATF8, and ATF9. Analysis was conducted using flow cytometry after the cell lines were treated with 0.25% trypsin and washed twice in PBS. Non-transfected ovine adult fibroblast line AF1 was used as a negative control.
Fig. 3. Detection of the GFP genes in pMSCV-GFP and pBC1-GFP co-integration into the genomes of ovine adult fibroblast lines and reconstructed embryos. A: The genomic DNA (approximate 50 kb) was isolated from co-transfected ovine fibroblast line ATF1, ATF2, ATF8, and ATF9 and non-transfected AF1, respectively, and electrophoresed on 0.5% agarose gel. B: The 1064 bp fragment including the GFP gene in pMSCV-GFP was amplified from the genomic DNA of ATF1, ATF2, ATF8, ATF9, and AF1 by PCR and electrophoresed on 1.5% agarose gel. C: The 896 bp fragment including the GFP gene in pBC1-GFP was amplified by PCR and electrophoresed on 1.5% agarose gel. D: The 896 bp fragment (ATF9a) in pBC1-GFP and 1064 bp fragment (ATF9b) in pMSCV-GFP were amplified by PCR from the genomic DNA of a reconstructed green embryo at the blastocyst stage from the NT ATF9, respectively. The PCR-amplified products were confirmed by 1.5% agarose gel electrophoresis. The size (bp) of the predicted amplified product is indicated.

Fig. 4. Expression of GFP in NT ovine embryos. Reconstructed embryos were derived from NT ovine matured oocytes nuclear transferred with ATF9 co-transfected by pBC1-GFP and pMSCV-GFP. Reconstructed in vitro cultured embryos were examined on the sixth day after NT by fluorescence microscopy using a standard FITC filter set panel and magnification of ×200. The arrow indicates embryos reconstructed at the morula stage.
factor influencing development of transgenic reconstructed embryos. To clarify why the co-transfected and individual pMSCV-GFP transfected donor cells resulted in lower blastocyst rates than the non-transfected donor cells, the cell cycles of co-transfected cell line ATF9, individual pMSCV-GFP transfected cell line AITF1 and non-transfected cell line AF1 were assayed after treatment with serum starvation for five days. The typical distribution of the cell cycle of the ovine adult fibroblast line in the various phases of the cell cycle as determined by flow cytometry is presented in Fig. 5. The cell cycle histograms showed that almost all the cells in non-transfected adult fibroblast AF1 were in the G0 or G1 phases (Fig. 5A). On the other hand, larger percentages of cells in the G2/M or S phases were included in the co-transfected and individual pMSCV-GFP transfected cell lines (Fig. 5B and Fig. 5C). The distribution of the cell cycles of the three types of cell line is shown in Table 2. The results demonstrate that serum starvation synchronized higher (P<0.05) percentages of G0+G1 (97.3 ± 1.50) cells in AF1 than in ATF9 (G0+G1:73.2 ± 4.04) or

<table>
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<th>Donor cell line</th>
<th>Donor cell name</th>
<th>Percentages (± SD) of oocytes fused</th>
<th>Percentages (± SD) of cleaved embryos reconstructed</th>
<th>Percentages (± SD) of blastocysts of embryos reconstructed</th>
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<tr>
<td>Co-transfected</td>
<td>ATF9</td>
<td>62.1 ± 0.35</td>
<td>45.3 ± 1.72</td>
<td>8.2 ± 1.41b</td>
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<tr>
<td>Individual pMSCV-GFP transfected</td>
<td>AITF1</td>
<td>66.7 ± 1.01</td>
<td>41.7 ± 3.56</td>
<td>8.4 ± 1.79b</td>
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<tr>
<td>Non-transfected</td>
<td>AF1</td>
<td>80.0 ± 1.41</td>
<td>61.9 ± 4.37</td>
<td>18.6 ± 2.41a</td>
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a, b Means with different superscripts within the same column differ (P<0.05).

Table 2. Percentages (± SD) of three types of ovine adult fibroblast line in various phases of the cell cycle after treatment with serum starvation for five days

<table>
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<th>Ovine adult fibroblast line</th>
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<th>G2+M</th>
<th>S</th>
</tr>
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<tbody>
<tr>
<td>Co-transfected</td>
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<td>12.26 ± 0.77</td>
<td>16.97 ± 0.45</td>
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<tr>
<td>Individual pMSCV-GFP transfected</td>
<td>73.2 ± 4.04b</td>
<td>7.13 ± 6.24</td>
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<td>Non-transfected</td>
<td>97.3 ± 1.5a</td>
<td>2.56 ± 3.49</td>
<td>0.1 ± 0.07</td>
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a, b Means with different superscripts within the same column differ (P<0.05).
AITF1 (G0+G1:70.77 ± 1.19); however, the percentages of G0+G1 cells in ATF9 treated with serum starvation for 5 days did not significantly differ (P>0.05) compared with that in AITF1.

Discussion

The main objective of the study was to prove the feasibility of the co-transfection method for selection of donor nuclei cells prior to nuclear transfer. Two vectors were employed in the present study: pBC1-GFP, which carried the mammary gland-specific promoter, partial exons, introns and 3’ genomic DNA of goat β-casein protein and the target gene GFP, and mammalian expression vector pMSCV-GFP, which carried the 5’ and 3’ long LTR of MSCV, Neo’, and the GFP reporter gene. In routine practice, pBC1 is directly used in microinjection. The microinjection method has been used to prove that several types of heterologous proteins are highly expressed in milk using pBC1 [8–10]. However, the constructed pBC1-GFP vector based on pBC1 was used to stably transfec ovine adult fibroblasts in our experiment. Since pBC1-GFP lacked the eukaryotic reporter gene and drug resistance gene, we relied on another mammalian expression vector, pMSCV-GFP, to select the transgenic cell lines in which pBC1-GFP integrated into the genome. In addition, the GFP genes in the two vectors played different roles in our experiments. The GFP gene in pMSCV-GFP conferred ubiquitous expression of GFP, which was used as the reporter gene to trace, select, and purify the transgenic clones. More importantly, it hinted of probable co-integration of pBC1-GFP in the target genome and provided the candidate clones for further selection of cell lines carrying pBC1-GFP. The GFP gene in pBC1-GFP was regarded as the target gene and was only to be expressed in the mammary gland in our present study. This will help us to assay the expression of GFP in the mammary gland of live animals in the future and build a practical protocol for expression of heterogenous protein using ovine adult fibroblast cell-based nuclear transfer.

The results indicated the feasibility of the co-transfection method for selection of donor cell lines prior to nuclear transfer. We initially proved that the GFP genes in pBC1-GFP and pMSCV-GFP co-integrated into the genomes of transgenic fibroblast lines in three of the four pMSCV-positive fibroblast lines by PCR analysis after stable co-transfection. The reason why the GFP gene in pBC1-GFP could not co-integrate into the genome of ATF8 is probably the absence of pBC1 during passaging due to the large size of pBC1-GFP. Moreover, we further identified that the GFP genes in pBC1-GFP and pMSCV-GFP could co-integrate into transgenic embryos through the PCR amplification method. There was no evidence of mosaicism in any of the expressing embryos. The blastocyst rate of reconstructed embryos is an important parameter in judgment of the success of nuclear transfer. Our results showed that there was a similar blastocyst rate (approximately 8%) for reconstructed embryos with ATF9 co-transfected by pBC1-GFP and pMSCV-GFP to that with AITF1 transfected by individual pMSCV-GFP. This indicates that the co-integration of pBC1-GFP and pMSCV-GFP into the genomes of the donor nuclei did not have more apparent deleterious effects on the development of reconstructed embryos than the integration of individual pMSCV-GFP. However, the results also show that the blastocyst rates of the transgenic reconstructed embryos were approximately 10% lower than those of non-transgenic reconstructed embryos. We presume that the reasons for these two aspects might be as follows. 1) Expression of GFP is toxic to living cells [11] and therefore probably detrimental to embryonic development. 2) The genomes of the donor cell nuclei are modified by heterologous genes, which probably results in disruption of regulation of the phases of the cell cycle of donor cell nuclei to some extent, and therefore serum starvation is not a sensitive signal for synchronization of transgenic donor cells in G0/G1 phase.

An important factor governing the developmental rates of reconstructed embryos is the phase of the cell cycle that the donor nuclei are at prior to nuclear transfer. Nuclei transferred to metaphase II recipient cytoplasts during or before activation, when maturation-promoting factor levels are high, must be in the G1 phase to maintain correct ploidy of the reconstructed embryos at the end of the first cell cycle [12]. The results of flow cytometric analysis indicated that the co-transfected fibroblast line contained lower percentages (P<0.05) of G0/G1 cells than the non-transfected fibroblast line (Table 2). On the other hand, the co-transfected fibroblast line contained
similar percentages of (P>0.05) G0/G1 cells to the individual pMSCV-GFP-transfected fibroblast line. These results prove our hypothesis that a lower percentage of G0/G1 cells in the donor cell lines was probably an important factor that resulted in lower blastocyst rates for the reconstructed embryos.

Why were transgenic donor nuclei unable to be synchronized by serum starvation in the G0/G1 phases in our experiment? We believe that the donor cell nuclei modified by the heterologous genes possibly led to disruption of expression of the genes required for proper cell cycle regulation, so that they appeared incapable of performing their scrutiny and regulation duties for S/G1 phase shift. As the results showed, the transgenic cell lines contained lower percentages of G0/G1 cells after they were subjected to serum starvation. This phenomenon is common in transgenic cell lines and is not merely limited to co-transfected cell lines. It appears that research needs to be focused on their mechanisms in order to determine the proper cell cycle for transgenic donor nuclei.

In conclusion, using a co-transfection method, we proved that the GFP genes in pBC1-GFP and pMSCV-GFP co-integrated into the genomes of ovine adult fibroblast lines and reconstructed embryos. Furthermore, we proved its feasibility through the production of reconstructed embryos after nuclear transfer. All our results show that the co-transfection method can be used for selection of donor cells prior to nuclear transfer.

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