



Effects of Donor Cell Passage, Size and Type on Development of Porcine Embryos Derived from Somatic Cell Nuclear Transfer

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ABSTRACT : The aim of this study was to investigate the effects of donor cell passage, size and type on the development of nuclear transfer embryos. Porcine cumulus cells, fetal fibroblasts and oviductal epithelial cells from 1-2, 3-6 and 7-10 passages were used for the nuclear transfer. In the oocytes with the cumulus donor cells, fusion and cleavage rates of oocytes and cell numbers per blastocyst among the three different passage groups did not show any differences, but the rates of blastocyst formation from 1-2 and 3-6 passage groups were higher than those from 7-10 passage group. The rates of fusion, cleavage and blastocyst formation, and the cell numbers per blastocyst were higher in the embryos with the sizes of <20 and 20 μm cumulus donor cells compared to the >20 μm cumulus donor cell. In the oocytes with the fetal fibroblast donor cells, the rate of blastocyst formation from the 3-6 passage group was higher than from 1-2 and 7-10 passage groups. The embryos with the size of 20 μm fetal fibroblast donor cell showed higher rate of blastocyst formation compared to those with <20 and >20 μm donor cells. In the oocytes with the oviductal epithelial cells, the rates of blastocyst formation from 1-2 and 3-6 passage groups were higher compared to those from 7-10 passage group. The embryos with the sizes of <20 and 20 μm oviductal epithelial donor cells had a higher rate of blastocyst formation compared to those with >20 μm donor cell. Fusion and cleavage rates of oocytes, and cell numbers per blastocyst among the three different donor cell types from the 3-6 passage did not show any differences. However, the rate of blastocyst formation of somatic cell nuclear transfer (SCNT) embryos with the fetal fibroblast donor cell was higher than that of blastocyst formation of SCNT embryos with the cumulus and oviductal epithelial donor cells. (**Key Words :** Donor Cell, Passage, Size, Nuclear Transfer, Porcine)

INTRODUCTION

The birth of cloned piglets by somatic cell nuclear transfer (SCNT) was reported in 2000 (Betthausen et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000). However, in the pig, the viability of SCNT embryos is poor, with an extremely low rate of cloned piglet production. Long-term cultured somatic cells undergo cellular senescence and have numerous mutations or allelic loss of genes accumulated through many rounds of cell divisions, which are known to cause improper genetic reprogramming after SCNT and subsequent abnormal development of the embryos (Aladjem et al., 1998; Cibelli et al., 1998; Kühholzer and Prather, 2000). Therefore, it is suggested that the use of

long-term cultured senescent cells may decrease cloning efficiency, which may be a limiting factor in the application of SCNT in animals for gene targeting. Most studies have used early passage fibroblasts as donor cells for SCNT to produce successful cloned animals (Cibelli et al., 1998; Schinieke et al., 1997; Wells et al., 1999; Onishi et al., 2000; Polejaeva et al., 2000). In contrast, Kubota et al. (2000) demonstrated that use of long-term cultured (up to 30 passage) skin fibroblasts derived from an aged bull did not compromise their cloning competence in terms of full term development, and resulted in higher developmental rates than those derived from use of early passage fibroblast.

The sizes of donor cells varied with different confluence degrees even though in the same culture dish. According to the experiment results of Tao et al. (1999), it was showed that donor cell size could affect the reprogramming of nuclear transfer embryos. The percentage of chromosome condensation and nuclear formation were higher in reconstructed embryos derived from small donor cells (15 μm) compared to large donor cells (20 μm), respectively.

Variety of donor cell types have been used to generate

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Figure 1. Porcine cumulus cells (A), fetal fibroblasts (B) and oviductal epithelial cells (C) at passage 4, respectively.

viable cloned offspring in sheep (Schnieke et al., 1997; Wilmut et al., 1997), cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999), pigs (Onishi et al., 2000; Polejaeva et al., 2000; Lee et al., 2007), rabbits (Chesne et al., 2002; Yoo and Rho, 2007), and cats (Shin et al., 2002). The aim of this study was to investigate the effects of donor cell passage, size and type on the development of nuclear transfer embryos.

MATERIALS AND METHODS

Oocyte collection and *in vitro* maturation (IVM)

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in saline (0.9% (w/v) NaCl) at 30-35°C. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from follicles of 2-6 mm in diameter using an 18-gauge needle fixed to a 10 ml disposable syringe. The follicular contents were pooled into 50 ml tubes and allowed to sediment. The sediment was placed into HEPES buffered Tyrode lactate medium (TL-HEPES-PVA; 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO₃, 0.34 mM NaH₂PO₄, 10 mM sodium lactate, 12 mM sorbitol, 2 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 10 mM HEPES, 0.2 mM sodium pyruvate, 65 µg/ml penicillin G and 25 µg/ml gentamycin sulfate) containing 0.1% (w/v) polyvinyl alcohol (PVA). Oocytes with uniform ooplasm surrounded by a compact cumulus cell mass were selected and washed with TL-HEPES-PVA and then washed twice with the maturation medium. The basic media used for *in vitro* maturation modified tissue culture medium (TCM) 199 supplemented with 26.19 mM sodium bicarbonate, 3.05 mM glucose, 0.91 mM sodium pyruvate, 75 µg/ml sodium penicillin G, 50 µg/ml streptomycin sulfate and 0.1% (w/v) PVA. COCs were cultured in maturation medium containing the 0.5 µg/ml luteinizing hormone (LH), 0.5 µg/ml follicle stimulating hormone (FSH), 10 ng/ml epidermal growth factor (EGF), 10% (v/v) porcine follicular fluids (pFF) and 0.57 mM cysteine. After 22 h of culture, oocytes were cultured without hormones for

22 h at 38.5°C, 5% CO₂ in air.

Preparation of cumulus cell, fetal fibroblast and oviductal epithelial cell as donor cell

COCs were aspirated from follicles of 2-6 mm in diameter using an 18-gauge needle fixed to a 10 ml disposable syringe. Oocytes with uniform ooplasm surrounded by a compact cumulus cell mass were selected and washed with TL-HEPES-PVA. Cumulus cells were isolated from oocytes by vigorous pipetting in TL-HEPES-PVA supplemented with 0.1% (w/v) hyaluronidase. Oocytes were removed, and cumulus cells were washed twice with PBS by centrifugation at 800×g for 3 min. The cell pellet was resuspended and cultured in DMEM medium supplemented with 10% (v/v) FBS, 75 µg/ml penicillin G and 50 µg/ml streptomycin.

A porcine fetus on day 35 of gestation was obtained from a pregnant gilt. After the brain, intestines, and four limbs were removed, tissue was cut into small pieces with fine scissors. Cells were incubated for 30 min at 38.5°C in PBS containing 0.05% (v/v) trypsin and 0.5 mM EDTA, and the suspension was centrifuged at 500×g for 20 min. The cell pellet was resuspended and cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 75 µg/ml penicillin G and 50 µg/ml streptomycin. The cells were frozen using DMEM supplemented with 10% (v/v) dimethyl sulfoxide (DMSO).

Ovary-oviduct-uterus was collected from prepubertal gilts at a local slaughter house and transported to the laboratory in saline (0.9% (w/v) NaCl) at 30-35°C. The oviduct was cut by using sterilized scissors between infundibulum and isthmus. Oviduct cells were collected by flushing with 10 ml PBS, and washed with PBS by centrifugation at 800×g for 3 min. The cell pellet was resuspended and cultured in DMEM medium supplemented with 10% FBS, 75 µg/ml penicillin G and 50 µg/ml streptomycin. Porcine cumulus cells (A), fetal fibroblasts (B) and oviductal epithelial cells (C) at passage 4 were shown in Figure 1.

Table 1. Effect of donor cell passage on development of embryos produced by nuclear transfer of porcine cumulus cell¹

Cell passage	No. of oocytes	% of oocytes fused ¹	% of oocytes cleaved ¹	% of blastocysts ¹	Cell no. per blastocyst ¹
1-2	135	91.4±1.0	75.2±4.4	18.9±1.0 ^a	33.4±2.8
3-6	141	90.6±1.9	77.4±3.2	21.3±1.0 ^a	34.2±1.5
7-10	148	89.7±1.9	75.7±3.1	13.8±0.9 ^b	35.3±2.1

¹ Mean±SE. Experiments were repeated 6 times.

^{a,b} Values in the same column with different superscripts differ significantly ($p < 0.05$).

Table 2. Effect of donor cell size on development of embryos produced by nuclear transfer of porcine cumulus cell¹

Cell size (µm)	No. of oocytes	% of oocytes fused ²	% of oocytes cleaved ²	% of blastocysts ²	Cell no. per blastocyst ²
<20	141	90.9±1.5 ^a	78.0±1.3 ^a	20.6±3.5 ^a	36.2±1.8 ^a
20	143	89.6±2.0 ^{ab}	79.4±1.5 ^a	17.6±1.2 ^{ab}	39.5±1.8 ^a
>20	132	83.2±3.3 ^b	70.4±1.3 ^b	11.6±1.0 ^b	30.5±1.5 ^b

¹ Donor cells were used from 3 to 6 passages. ² Mean±SE. Experiments were repeated 6 times.

^{a,b} Values in the same column with different superscripts differ significantly ($p < 0.05$).

To be used as donor cell in nuclear transfer, cells were thawed and cultured until they reached confluence. Different sizes of donor cells (<20, = 20 and >20 µm) were classified and modified by the experiment results of Tao et al. (1999). Before nuclear transfer, cells were treated with 0.05% (v/v) trypsin and 0.5 mM EDTA for single-cell isolation at 2-5 min in 38.5°C incubator. After washing with PBS, cells were resuspended in manipulation medium (TCM199 supplemented with 0.6 mM NaHCO₃, 3.15 mM HEPES, 30 mM NaCl, 60 µg/ml penicillin, 50 µg/ml streptomycin and 0.3% bovine serum albumin (BSA)).

Procedure of nuclear transfer

After IVM 44 h, the cumulus cells were removed from the oocytes by pipetting in manipulation medium supplemented with 0.1% (w/v) hyaluronidase. For micromanipulation, oocytes and donor cells were placed in a 50 µl drop under oil of manipulation medium supplemented with 7.5 µg/ml cytochalasin B. Oocytes were enucleated by removing the first polar body along with adjacent cytoplasm containing the metaphase plate using a glass micropipette with an inner diameter of 20 µm. Through the same hole in the zona pellucida created during enucleation, a cell was then placed in contact with the cytoplasm of each oocyte to form a couplet. After manipulation, couplets were washed once and equilibrated in TCM199 for 2 h at 38.5°C, 5% CO₂ in air before fusion and activation. Fusion/activation was accomplished with 1 DC pulse of 1.2 kV/cm for 30 µsec provided by a BTX Electro-cell Manipulator 200 (BTX, San Diego, CA, USA). Fusion medium was 0.3 M mannitol supplemented with 1.0 mM CaCl₂·2H₂O, 0.1 mM MgCl₂·6H₂O and 0.5 mM HEPES.

Culture of nuclear transfer embryos

After fusion and activation, the reconstructed embryos were immediately cultured in PZM-3 medium containing 0.3% BSA (Yoshioka et al., 2002) at 38.5°C in a humidified

atmosphere of 5% CO₂. The rates of embryos at the cleavage and blastocyst stages were evaluated at 48 h and 6-7 days after activation, respectively.

Evaluation of embryo and blastocyst produced *in vitro*

Embryos and Blastocysts were fixed with 2% formaldehyde for 40 min at room temperature, washed with PBS three times, permeated with PBS containing 0.1% (v/v) Triton-X for 40 min at room temperature, and stained with 2.5 µg/ml DAPI (Molecular Probes, Eugene, OR, USA) for 40 min. Cell number per blastocyst was counted under epifluorescence microscope (Olympus, Korea).

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS package in a completely randomized design. Duncan's multiple range test was used to compare values of individual treatment, when the F-value was significant ($p < 0.05$).

RESULTS

Effects of donor cell passage and size on development of embryos derived from somatic cell nuclear transfer

The porcine cumulus cells from 1-2, 3-6 and 7-10 passages were used for the nuclear transfer (NT) experiments as shown in Table 1. Fusion and cleavage rates of oocytes, and cell numbers per blastocyst among the three different passage groups did not show any differences, but the rates of blastocyst formation from 1-2 and 3-6 passage groups were higher than those from 7-10 passage group.

As shown in Table 2, the rates of fusion, cleavage and blastocyst formation, and the cell numbers per blastocyst were higher in the embryos with the sizes of <20 and 20 µm donor cells compared to the size of >20 µm donor cells.

Table 3. Effect of donor cell passage on development of embryos produced by nuclear transfer of porcine fetal fibroblast

Cell passage	No. of oocytes	% of oocytes fused ¹	% of oocytes cleaved ¹	% of blastocysts ¹	Cell no. per blastocyst ¹
1-2	137	90.4±2.5	76.3±2.0	16.1±1.7 ^b	32.3±1.4
3-6	148	92.1±0.9	78.3±2.8	25.6±0.7 ^a	34.8±2.5
7-10	141	92.4±1.8	75.3±2.9	12.7±1.9 ^b	28.4±2.0

¹ Mean±SE. Experiments were repeated 6 times.^{a,b} Values in the same column with different superscripts differ significantly (p<0.05).**Table 4.** Effect of donor cell size on development of embryos produced by nuclear transfer of porcine fetal fibroblast¹

Cell size (µm)	No. of oocytes	% of oocytes fused ²	% of oocytes cleaved ²	% of blastocysts ²	Cell no. per blastocyst ²
<20	139	91.6±2.7	76.8±4.2	21.4±3.9 ^{ab}	40.7±3.5
20	148	91.3±5.0	77.3±4.2	24.9±2.9 ^a	36.4±1.4
>20	151	91.6±5.5	71.8±8.1	17.9±2.3 ^b	32.7±3.4

¹ Donor cells were used from 3 to 6 passages. ² Mean±SE. Experiments were repeated 6 times.^{a,b} Values in the same column with different superscripts differ significantly (p<0.05).

The fetal fibroblasts from 1-2, 3-6 and 7-10 passages were used for the NT experiments as shown in Table 3. Fusion and cleavage rates of oocytes, and cell numbers per blastocyst among the three different passage groups did not show any differences, but the rate of blastocyst formation from 3-6 passage group was higher than that from 1-2 and 7-10 passage groups.

As shown in Table 4, there were no significant differences in the rates of fusion and cleavage, and the cell numbers per blastocyst among the three different cell size groups. However, the rates of blastocyst formation were higher in the embryos with the size of 20 µm donor cell compared to the sizes of <20 and >20 µm donor cells.

The porcine oviductal epithelial cells from 1-2, 3-6 and 7-10 passages were used for the NT experiments as shown in Table 5. The rates of fusion and cleavage of oocytes from

3-6 passage group were higher than those from 1-2 and 7-10 passage groups. The rates of blastocyst formation from 1-2 and 3-6 passage groups were higher compared to those from 7-10 passage group. However, the cell numbers per blastocyst did not show any differences among the three different cell passage groups.

As shown in Table 6, there were no significant differences in the rates of fusion and cleavage, and the cell numbers per blastocyst among the three different cell size groups. However, the rates of blastocyst formation were higher in the embryos with sizes of <20 and 20 µm donor cells compared to the size of >20 µm donor cells.

Effects of donor cell type on development of embryos derived from somatic cell nuclear transfer

As shown in Table 7, the effects of donor cell types on

Table 5. Effect of donor cell passage on development of embryos produced by nuclear transfer of porcine oviductal epithelial cell

Cell passage	No. of oocytes	% of oocytes fused ¹	% of oocytes cleaved ¹	% of blastocysts ¹	Cell no. per blastocyst ¹
1-2	163	81.2±3.1 ^b	65.1±3.4 ^b	19.1±0.9 ^a	29.9±1.3
3-6	148	89.1±1.2 ^a	76.9±2.5 ^a	20.9±1.3 ^a	30.4±2.4
7-10	153	81.5±1.3 ^b	62.1±1.8 ^b	12.7±1.3 ^b	29.2±1.5

¹ Mean±SE. Experiments were repeated 6 times.^{a,b} Values in the same column with different superscripts differ significantly (p<0.05).**Table 6.** Effect of donor cell size on development of embryos produced by nuclear transfer of porcine oviductal epithelial cell¹

Cell size (µm)	No. of oocytes	% of oocytes fused ²	% of oocytes cleaved ²	% of blastocysts ²	Cell no. per blastocyst ²
<20	144	85.9±2.0	73.1±2.0	19.3±3.3 ^a	34.6±2.4
20	154	89.0±3.2	73.5±4.8	18.0±1.6 ^{ab}	36.2±2.4
>20	149	86.6±2.1	71.3±5.4	11.6±1.5 ^b	33.4±2.7

¹ Donor cells were used from 3 to 6 passages. ² Mean±SE. Experiments were repeated 6 times.^{a,b} Values in the same column with different superscripts differ significantly (p<0.05).**Table 7.** Effect of various donor cell types on development of embryos produced by nuclear transfer

Type of donor cell ¹	No. of oocytes	% of oocytes fused ²	% of oocytes cleaved ²	% of blastocysts ²	Cell no. per blastocyst ²
Cumulus	141	90.6±1.9	77.4±3.2	21.3±1.0 ^b	34.2±1.5
Fetal fibroblast	148	92.1±0.9	78.3±2.8	25.6±0.7 ^a	34.8±2.5
Oviductal epithelium	148	89.1±1.2	76.9±2.5	20.9±1.3 ^b	30.4±2.4

¹ Donor cells were used from 3 to 6 passages. ² Mean±SE. Experiments were repeated 6 times.^{a,b} Values in the same column with different superscripts differ significantly (p<0.05).

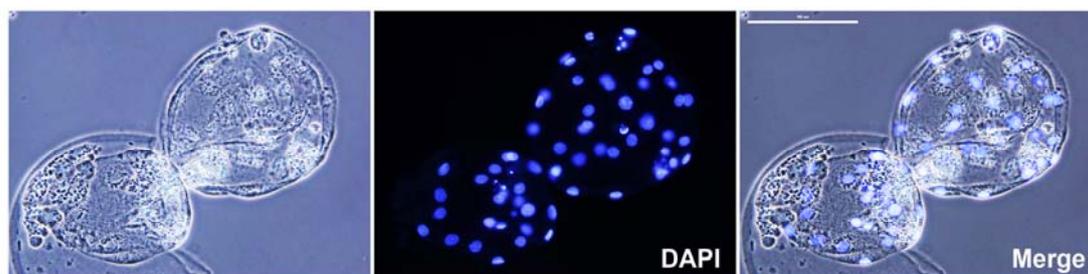


Figure 2. A hatched blastocyst derived from nuclear transfer of a fetal fibroblast.

development of embryos produced by nuclear transfer were investigated. Fusion and cleavage rates of oocytes, and cell numbers per blastocyst among the three different donor cell types at the 3-6 passage did not show any differences. However, the rate of blastocyst formation of SCNT embryos with the donor cell of fetal fibroblast was higher than that of blastocyst formation of SCNT embryos with the donor cells of cumulus and oviductal epithelial cells. A hatched blastocyst derived from nuclear transfer of a fetal fibroblast was shown in Figure 2.

DISCUSSION

The genetic modification of donor cells involves establishment of the cell line, transfection, and then clonal propagation of selected cells that requires long-term culture (Houdebine, 1997; Schnieke et al., 1997; Kato et al., 1998; Wells et al., 1999; Koo et al., 2001; Reik et al., 2001; Rideout et al., 2001). The effects of donor cell passages on embryo development are still controversial. Kubota et al. (2000) reported that long-term culture of donor cells was better than short-term culture, but Rho et al. (2000) reported that early passage donor cells were better than late passage cells. In addition, Wells et al. (1999) reported that the number of donor cell passages did not show any differences on the development of somatic cell nuclear transfer (SCNT) embryos. In this study, we investigated the effects of passage numbers of donor cells (cumulus cell, fetal fibroblast and oviductal epithelial cell) on the development of SCNT embryos. Our results demonstrated that there were no differences in the rates of fusion and cleavage, and the cell numbers per blastocyst, but the rates of blastocyst formation from 3-6 passage group were higher than those from 1-2 and 7-10 passage groups. The above result is suggested that the use of long-term cultured senescent cells may decrease cloning efficiency which may be a limiting factor in the application of SCNT in porcine for gene targeting. When cells are cultured long-term, both genetic and epigenetic alterations would accumulate, resulting in improper nuclear reprogramming of cloned embryos reconstructed with these cells in SCNT (Campbell et al., 1996; Wakayama et al., 1998). Furthermore, possible

disruption to the regulation of imprinted genes could be resulted from the long-term culture of donor cells and lead to perturbations in embryonic and fetal development (Walker et al., 1996; Young et al., 1998). However, further research is necessary to demonstrate the effects of donor cell passages on the development of SCNT embryos.

The small cell size below 20 μm was more efficient than large cell size over 20 μm in the rates of blastocyst formation of SCNT embryos in this study. Boquest et al. (2002) reported that large cells showed relatively low percentages of G0/G1 stage, whereas most small cells were in the G0/G1 stages. Low embryo development rates might be caused by chromosomal damage from aneuploidy of recipient oocytes and donor cells when large cells were used as the donor cells (Barnes et al., 1993; Campbell, 1999).

In this study, three types of donor cells including cumulus cell, fetal fibroblast and oviductal epithelial cell were used for the production of SCNT embryos. The results showed that fetal fibroblast was the most efficient cell type on the developmental rate of blastocyst formation. Fetal cells have been used for transgenic animal production because of their rapid growth and potential for many cell divisions before senescence in culture (Cibelli et al., 1998; Baguisi et al., 1999; Kühholzer and Prather, 2000). Fetal fibroblasts are highly undifferentiated cells compared with other cells retrieved from adult tissue (Mather and Barnes, 1998), which are currently considered much more amenable to reprogramming after reconstruction (Rideout et al., 2001; Yamazaki et al., 2001). Among adult cells used for cloned animal production, only granulosa cells and ear fibroblasts have been utilized in transgenic SCNT (Arat et al., 2001, 2002; Bondioli et al., 2001).

In conclusion, nuclear transfer oocytes receiving porcine fetal fibroblast at 3-6 passage and 20 μm cell size had higher potential to develop into blastocyst.

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