Comparison of *In Vitro* Development of Porcine Embryos Derived from Transfer of Embryonic Germ Cell Nuclei into Oocytes by Electrofusion and Piezo-Driven Microinjection

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ABSTRACT

Embryonic germ (EG) cells are undifferentiated stem cells isolated from cultured primordial germ cells (PGC). These cells share many characteristics with embryonic stem cells including morphology and pluripotency. Undifferentiated porcine EG cell lines demonstrating capacities of differentiation both *in vitro* and *in vivo* have been established. Since EG cells can be cultured indefinitely in an undifferentiated state, whereas somatic cells in primary culture are often unstable and have limited lifespan, EG cells may provide inexhaustible source of karyoplasts in nuclear transfer (NT). In this study the efficiencies of NT using porcine EG and fetal fibroblast cells were compared. Two different techniques were used to perform NT. With conventional NT procedure (Roslin method) involving fusion of donor cells with enucleated oocytes, the rates of development to the blastocyst stage in EG and somatic cell NT were 16.8% (59/351) and 14.5% (98/677), respectively. In piezo-driven microinjection (Honolulu method) of donor nuclei into enucleated oocytes, the rates of blastocyst formation in EG and somatic cell NT were 11.9% (15/126) and 9.4% (9/96), respectively. Regardless of NT methods used in this study, EG cell NT gave rise to comparable rate of blastocyst development to somatic cell NT. Overall, EG cells can be used as karyoplast donor in NT procedure, and embryos can be produced by EG cell NT that may be used as an alternative to conventional somatic cell NT.

(Key words : Pigs, Embryonic germ cells, Nuclear transfer)

INTRODUCTION

Since a cloning by somatic cell nuclear transfer (NT) has been first achieved in sheep (Wilmut *et al.*, 1997), a large number of clones have been produced in various species including pigs (Polejaeva *et al.*, 2000). However, overall efficiencies of generating live offspring from nuclear-transferred oocytes remain disappointingly low. The reasons behind the failures maintaining pregnancy are not yet fully understood. They may originate from the recipient cytoplast (e.g. insufficient activation, lack of reprogramming factors, reduced viability owing to micromanipulation), or from the DNA of the donor cell (e.g., inappropriate nuclear status, incomplete preprogramming, chromosomal abnormalities, damaged DNA), or both. One of the factors to determine the efficacy of NT might be the type of donor cells. A comparison of efficacy among donor cells of different types including undifferentiated stem cells would be of interest because a less differentiated cell type may support greater development of NT embryos compared with terminally differentiated cell types (Faast *et al.*, 2006).

Primordial germ cells (PGC) are embryonic cells that migrate from the root of the allantois to the genital ridge, where they ultimately give rise to gametes. Cells having morphological, biochemical, immunological and developmental properties in common with embryonic stem (ES) cells, including pluripotency and the capacity to contribute to the germ line of chimeras, have been isolated from murine PGC (Matsui *et al.*, 1992; Resnick *et al.*, 1992; Labosky *et al.*, 1994). These PGC-derived stem cells have been referred to as embryonic germ (EG) cells to be distinguished from ES cells. Undifferentiated porcine EG cell lines with capacities of differentiation both *in vitro* and *in vivo* have also been established (Shim *et al.*, 1997). Unlike somatic cells that have limited lifespan, EG cells can be cultured indefinitely in an undifferentiated state. Providing an abundance of pluripotent stem cells that can be genetically manipulated by conventional recombinant DNA techniques may enable stable genetic mutations to be established and maintained. If these cells are used for a source of karyoplasts in NT, it would be particularly advantageous in producing transgenic animals. Hence in the present study porcine EG cells were tested for

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nuclear donor cells to improve the efficiency in the production of NT embryos.

Two procedures have traditionally been used for NT. Nuclear donor cells may be fused with enucleated oocytes using electric pulses (Roslin method; Wilmut *et al.*, 1997). Alternatively, direct microinjection of donor cell nuclei into enucleated oocytes may be performed using piezo-driven micromanipulator (Honolulu method; Wakayama *et al.*, 1998). A few studies have compared the efficiency of somatic cell NT using both methods (Roh *et al.*, 2001; Kawano *et al.*, 2004). The rates of blastocyst development from NT embryos using Roslin method have been reported to be higher than (Roh *et al.*, 2001) or comparable to (Kawano *et al.*, 2004) those using Honolulu method in pigs. These studies were conducted only with the use of fetal fibroblast cells as nuclear donor cells. Therefore, the test of other cell types such as undifferentiated stem cells may be necessary not to limit but to extend the results obtained from such studies.

In the present study *in vitro* development of porcine oocytes following two different procedures of NT (electrofusion vs. piezo-driven microinjection) using two different cell types (EG cells vs. fetal fibroblast cells) was examined.

MATERIALS AND METHODS

In Vitro Maturation of Porcine Oocytes

Porcine oocytes were matured *in vitro* by the method modified from Hyun *et al.* (2003). Briefly, ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to laboratory in a warm box (25 to 30℃) within 2 hr. Follicular fluid and cumulus-oocytes complexes (COC) from follicles of 5 to 6 mm in diameter were aspirated using an 18-gauge needle attached to 5-ml disposable syringe. Compact COC were selected and washed six times in HEPES-buffered tissue culture medium (TCM)-199 (Gibco BRL, Gaithersburg, MD). The *in vitro* maturation (IVM) medium was modified TCM-199 (Gibco BRL) supplemented with 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO), 10 IU/ml pregnant mare serum gonadotropin (PMSG; Intervet, Boxmeer, The Netherlands), 10 IU/ml human chorionic gonadotropin (hCG; Intervet) and 10% (v/v) porcine follicular fluid. A group of 50 COC was cultured in 500 μl of IVM medium at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 hr, COC were transferred to PMSG- and hCG-free IVM medium and cultured for additional 20 hr. At the end of the maturation, oocytes were freed from cumulus cells by repeated pipetting in the IVM medium containing 0.5 mg/ml hyaluronidase (Sigma) for 1 min.

Preparation of Porcine Fetal Fibroblasts

Fibroblasts were isolated from pig fetuses on day 23 of gestation. Briefly, fetuses were washed three times with $Ca^{2+}-$ and $Mg^{2+}-$ free phosphate-buffered saline (PBS; Gibco BRL). The heads and internal organs were removed using iris scissors and forceps. The remnants were washed twice in Dulbecco's PBS (DPBS), minced with a surgical blade on a 100-mm petridish. Cells were dissociated from the tissues in 0.25% (v/v) trypsin-EDTA (Gibco BRL) for 5 min at 39℃. After centrifuging cell suspension three times at 800 × g for 10 min, pellets were subsequently seeded onto 100-mm plastic culture dishes (Falcon, Franklin Lakes, NJ) and cultured for 6 to 8 days in Dulbecco's modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 1 mM L-glutamine (Gibco BRL), 100 units/ml penicillin (Gibco BRL) and 0.5 mg/ml streptomycin (Gibco BRL) in a humidified atmosphere of 5% CO_2 in 95% air. After removal of unattached clumps of cells, attached cells were further cultured until confluent, subcultured at intervals of 5 to 7 days by trypsinization for 5 min using 0.25% trypsin-EDTA and stored after two passages in freezing medium in liquid nitrogen at −196℃. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) dimethyl sulfoxide (Sigma) and 10% (v/v) FBS. After thawing, cells were cultured in DMEM supplemented with 10% FBS until approximately 80% confluency and used for NT experiment.

Isolation and Culture of Porcine EG Cells

Porcine EG cells were isolated from PGC of day 23 embryos collected from Hampshire × Yorkshire crossbred gilts as described previously (Shim *et al.*, 1997). Briefly, the dorsal mesentery was removed from the embryos and placed in 0.02% EDTA solution for 20 min. Primordial germ cells were released from the dorsal mesentery by gentle pressing and pricking the tissue using fine forceps and collected by centrifugation at 800 × g for 5 min. Harvested PGC were cultured in DMEM containing 15% ES-qualified FBS (Gibco BRL), 1 mM L-glutamine, 0.1 M MEM nonessential amino acids, 10 μM 2-mercaptoethanol, 100 units/ml penicillin, 0.5 mg/ml streptomycin and 1,000 units/ml murine leukaemia inhibitory factor (Chemicon, Temecula, CA) on inactivated STO feeder cells prepared by the treatment of 10 μg/ml mitomycin C (Sigma) for 2 hr. Approximately 30,000 PGC were seeded per well of a 96-well plate (Falcon) containing feeder cells. Resulting EG cell colonies from PGC culture were disaggregated by incubation in 0.25% trypsin-EDTA for 10 to 15 min and subcultured onto fresh feeder cells in 4-well multidish (Nunclon, Roskilde, Denmark) approximately every 5 to 7 days. All cultures were maintained at 39℃ in 5% CO_2, 95% air with culture medium changed every other day.

Nuclear Transfer

At 42 hr after the onset of IVM, oocytes were enucleated with a 20-μm (internal diameter) glass pipette by aspirating the first polar body and the second metaphase plate with a small volume of surrounding cytoplasm in HEPES-buffered TCM-199 supplemented with 0.4% bovine serum albumin (BSA; Sigma) and 7.5 mg/ml cytochalasin B (Sigma). After the enucleation, oocytes were stained with 5 mg/ml bisbenzimide (Hoechst 33342; Sigma) for 5 min and observed under an inverted microscope equipped with epifluorescence. Oocytes containing DNA materials were excluded from the subsequent experiments. Nuclear donor cells including porcine EG and fibroblast cells were trypsinized into single cells and placed in HEPES-buffered TCM-199 supplemented with 0.4% BSA and 2% polyvinyl alcohol (PVA; Sigma).

For NT using Roslin method, nuclear donor cells were transferred into the perivitelline space of enucleated oocytes. The resulting couplets were equilibrated for 1 min in 0.3 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. Then, couplets were fused with double DC pulse of 1.5 kV/cm for 40 μsec using BTX Electro-Cell Manipulator 2001 (Gentronics, San Diego, CA). Following the electrical stimulation, reconstructed oocytes were washed three times with NCSU 23 supplemented with 4 mg/ml fatty acid-free BSA (Sigma) and cultured in the same medium containing 7.5 mg/ml cytochalasin B for 3 hr to suppress extrusion of the second polar body.

For NT using Honolulu method, a piezo-driven micromanipulator (PrimeTech, Ibaraki, Japan) was used as previously reported (Wakayama *et al.*, 1998). An injection pipette with blunt 10-μM tip was attached to the micromanipulator, and a small amount of mercury was loaded into the pipette 10 mm from the tip. The plasma membrane of the nuclear donor cells was broken and visible cytoplasmic material was removed by gently aspirating the cells in and out of the pipette. Isolated nuclei with associated cytoplasm were individually injected into the cytoplasm of enucleated oocytes using the slit in the zona pellucida made during the enucleation. Care was taken to inject a cell into cytoplasm with as little medium as possible. The injected oocytes were placed in NCSU 23 containing 4 mg/ml fatty acid-free BSA for 1 hr. Electrical stimulation of reconstructed oocytes was performed as the Roslin method described above.

Next, reconstructed oocytes prepared from two different methods were cultured for 4 days in NCSU 23 containing 4 mg/ml fatty acid-free BSA and cultured for additional 4 days in NCSU 23 containing 10% FBS. All NT embryos were cultured at 39℃ in a humidified atmosphere containing 5% CO_2 in 95% air, and the rate of *in vitro* development was monitored.

Statistical Analysis

In comparison of two different types of donor cells (fibroblasts vs. EG cells) using two different methods, both cell types were tested in each replicate. At least three replicates were conducted for each experiment. Data on the rates of fusion, cleavage and subsequent development to the blastocyst stage were subjected to Student's t-test. Differences of $p<0.05$ were considered to be significant.

RESULTS

In vitro development of embryos from NT of fibroblast and EG cells was assessed. The result from NT using electrofusion (Roslin method) was summarized in Table 1. The rates of fusion and cleavage from EG cell NT were comparable to those from somatic cell NT. Similarly, the rates of development to the blastocyst stage in EG and somatic cell NT were not different (16.8%, 59/351 vs. 14.5%, 98/677).

The result from NT using piezo-driven microinjection (Honolulu method) was shown in Table 2. Similar to the result demonstrated in NT using electrofusion, not only the rates of cleavage were not different between EG and somatic cell NT, but the rate of blastocyst development from EG cell NT (11.9%, 15/126) was comparable to that from somatic cell NT (9.4%, 9/96).

DISCUSSION

Table 1. *In vitro* development of nuclear transfer embryos from electrofusion

Nuclear donor cells	No. of used oocytes	No. (%) of fused oocytes*	No. (%) of embryos developed to**	
			2-Cell	Blastocyst
EG cells	583	351(60.2)	199(56.7)	59(16.8)
Fibroblasts	1,141	677(59.3)	406(60.0)	98(14.5)

* Calculated from the number of used oocytes.

** Calculated from the number of fused oocytes.

Table 2. *In vitro* development of nuclear transfer embryos from piezo-driven microinjection

Nuclear donor cells	No. of injected oocytes	No. (%) of embryos developed to*	
		2-Cell	Blastocyst
EG cells	126	97(77.0)	15(11.9)
Fibroblasts	96	64(66.7)	9(9.4)

* Calculated from the number of injected oocytes.

Although somatic cell NT has been a widely used to generate cloned offspring in mammals, use of undifferentiated stem cells for nuclear donor may be advantageous because such cells could be more easily reprogrammable. In mice for instance, oocytes reconstructed from ES cells gave rise to an increase in the number of viable offspring compared with those from somatic cells (Wakayama *et al.*, 1999; Rideout *et al.*, 2000). However, similar studies have not been performed in domestic animals perhaps due to limited availability of ES cells. Instead, adult stem cells such as mesenchymal stem cells isolated from porcine bone marrow resulted in the rates of preimplantation development comparable to (Colleoni *et al.*, 2005; Bosch *et al.*, 2006; Faast *et al.*, 2006) or better than (Jin *et al.*, 2007) their somatic cell counterpart. In addition, embryos cloned from porcine skin-originated sphere stem cells from fetal skin showed enhanced preimplantation development compared with fibroblast cloned embryos, which is indicated by an increased rate of blastocyst development and a higher total cell number in Day 7 blastocysts (Zhu *et al.*, 2004). These results imply that stem cell population may be used for alternative source of nuclear donor cells for NT.

As represented in Table 1 and 2 from this study, the rates of *in vitro* development of NT embryos from EG and fibroblasts were not different regardless of the NT procedures used (electrofusion and piezo-driven microinjection). Although statistically not significant, EG cell NT embryos from both electrofusion and piezo-driven microinjection tended toward slightly greater development than somatic cell NT embryos (Table 1 and 2).

Direct comparison between two different NT procedures (Roslin vs. Honolulu method) was not made in this study. However, NT using electrofusion may seem to support better embryonic development, but such tendency may be biased by that selection for embryos with normal development was feasible after fusion of donor cells with enucleated oocytes when the Roslin method was applied.

Overall, NT of EG cells using electrofusion resulted in the production of blastocysts with the best efficiency, and this may extend the reports from others demonstrated that the rates of blastocyst development from NT embryos using Roslin method have been higher than (Roh *et al.*, 2001) or comparable to (Kawano *et al.*, 2004) those using Honolulu method in pigs.

Compared with other cell types retrieved from adult tissue, EG cells may have greater potential as donor cells than fetal fibroblasts in achieving production of transgenic clone embryos. Since undifferentiated stem cells such as ES or EG cells can be maintained indefinitely in culture, use of such cells for transgenesis may facilitate ease of gene transfer and subsequent selection of transgenic cells. The current problem of using terminally differentiated somatic cells is that they tend to become senescent before sufficient rounds of gene transfer and/or gene targeting followed by antibiotic selection. This may be overcome by isolation and use of cell lines that are capable of transfection and long-term culture. Porcine EG cells may possess such characteristics, and use of these cells would be particularly advantageous in maintaining nuclear donor cells carrying a transgene. If combined with NT technique, EG cells may potentially be useful for genetic manipulation in pigs.

Other advantages in chromosomal stability and DNA methylation may exist in the use of EG cells as nuclear donor cells. Chromosomal stability of porcine EG cells as previously reported (Shim *et al.*, 1997) may yield consistent results in NT compared with somatic cells such as fetal fibroblasts that often exhibit chromosomal abnormality in long-term culture (Mir *et al.*, 2003). In addition, PGC prior to their erasure of DNA methylation have been used as a source of nuclear donor cells to successfully produce clone mice (Yamazaki *et al.*, 2003; Miki *et al.*, 2005). Genome-wide demethylation of DNA occurs during PGC migration similar to the phenomenon during preimplantation development of embryos. Hence, NT embryos using EG cells rather than somatic cells may be close to embryos from normal fertilization in their DNA methylation status, and this may contribute an increased blastocyst development of NT embryos derived from EG cells.

In this study, EG cell NT resulted in comparable rate of *in vitro* development to somatic cell NT regardless of the NT procedures used. Such results suggest that EG cell NT may be used as an alternative to conventional somatic cell NT, and long-term advantages of EG cell NT are still need to be proven in further studies.

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