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Short Communication

Enhanced development of porcine embryos cloned from bone marrow mesenchymal stem cells

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ABSTRACT In the present study, we have characterized an isolated population of porcine bone marrow mesenchymal stem cells (MSCs) for multilineage commitment and compared the developmental potential of cloned embryos with porcine MSCs and fetal fibroblasts (FFs). MSCs exhibited robust alkaline phosphatase activity and later transformed into mineralized nodules following osteoinduction. Furthermore, MSCs underwent adipogenic and chondrogenic differentiation by producing lipid droplets and proteoglycans, respectively. Primary cultures of FFs from a female fetus at ~30 day of gestation were established. Donor cells at 3-4 passage were employed for nuclear transfer (NT). Cell cycle analysis showed that the majority of MSCs in confluence were in the G0/G1 stage. Cumulus-oocyte complexes were matured and fertilized *in vitro* (IVF) as control. The cleavage rate was significantly ($P<0.05$) higher in IVF than in NT embryos with MSCs and FFs (84.5±4.6% vs. 52.2±5.4% and 50.8±5.2%, respectively). However, blastocyst rates in IVF and NT embryos derived from MSCs (20.6±2.5% and 18.4±3.0%) did not differ, but were significantly ($P<0.05$) higher than NT derived from FFs (9.5±2.1%). Total cell number and the ratio of ICM to total cells among blastocysts cloned from MSCs (34.4±5.2 and 0.38±0.08, respectively) were significantly ($P<0.05$) higher than those from FFs (22.6±5.5 and 0.18±0.12, respectively). Proportions of TUNEL positive cells in NT embryos from FFs (7.3±1.8%) were significantly ($P<0.05$) higher than in MSCs (4.6±1.3%) and IVF (2.5±0.9%). The results clearly demonstrate that multipotent bone marrow MSCs have a greater potential as donor cells than FFs in achieving enhanced production of cloned porcine embryos.

KEY WORDS: *mesenchymal stem cells, nuclear transfer, porcine*

In nuclear transfer (NT), the genome of undifferentiated stem cells may be more easily reprogrammed to resemble the genome of the zygote, which may make stem cells more efficient as donors (Rideout *et al.*, 2001). In mouse NT experiments, the developmental potential of embryonic stem (ES) cell clones was compared with somatic cell clones and despite lower blastocyst developmental rates, higher number of embryos derived from ES cells survived to term (Rideout *et al.*, 2000, 2001; Eggan *et al.*, 2001). However, producing cloned mice from T or B-cell lymphocytes by NT was very inefficient (Hochedlinger and Jaenisch, 2002). Quite recently, cloned mice were produced from the nucleus of natural killer T-cell lymphocytes, but not from peripheral T-cells (Inoue *et al.*, 2005). By contrast, the extremely low cloning outcome was observed using hematopoietic stem cells demonstrating the differences in cloning efficiency, at least for cells within hematopoietic lineage (Inoue *et al.*, 2006).

In domestic animals, technical difficulty in obtaining and maintaining an undifferentiated ES cell line has constrained the comparative studies on the cloning efficiency using ES and somatic cells. Recently, bovine mesenchymal stem cells (MSCs) isolated from bone marrow showed the developmental totipotency successfully undergoing nuclear reprogramming after NT and supported development to term (Kato *et al.*, 2004). Cloned embryos derived from bovine and porcine undifferentiated MSCs and their derivatives along the osteogenic lineage resulted in consistently high preimplantation development compared to adult fibroblasts

Abbreviations used in this paper: ES, embryonic stem cell; FF, fetal fibroblast; ICM, inner cell mass; IVC, in vitro culture; IVF, in vitro fertilization; MSC, mesenchymal stem cell; NT, nuclear transfer.

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(Colleoni *et al.*, 2005). Furthermore, porcine bone marrow MSCs were able to undergo transient and stable genetic modifications with non viral and viral vectors and were found to be an attractive cell type for therapy models and for NT transgenesis (Colleoni *et al.*, 2005; Bosch *et al.*, 2006). Collectively, these results indicate that the characteristics of NT embryos depend on the type of nuclear donors and MSCs may have adequate reprogramming potential for NT.

Attempts have been made to determine porcine MSCs reprogramming and developmental potential of embryos by NT, but information on parameters were limited to cleavage and blastocyst formation. It is important that, the structural composition of NT embryos should also be considered as another factor responsible for the developmental obstacles since the formation of developmentally competent blastocyst involves the processes of cell division, differentiation and cell death. It has been shown that embryonic development *in vitro* is retarded and results in fewer cell numbers compared to those in embryos developed *in vivo* (Machaty *et al.*, 1998). Embryo quality has also been linked with the number of inner cell mass (ICM) and trophectoderm (TE) cells and both cell lineages are vital and essential for embryonic and fetal survival. Apoptotic cell death during the preimplantation development stage may contribute to embryonic loss and hence its evaluation could help to achieve higher quality and survival rates in embryo production.

MSCs of bone marrow origin represent an ideal source because of their easy purification and amplification and their multipotency (Pittenger *et al.*, 1999; Ringe *et al.*, 2002; Colleoni *et al.*, 2005; Bosch *et al.*, 2006). Hence, the present study was conducted to characterize an isolated population of MSCs from porcine bone marrow by assessing their multilineage potential, to compare the developmental potential of embryos cloned with MSCs and fetal fibroblasts (FFs), cell cycle analysis of MSCs and FFs and assess the quality of embryos.

Results

Isolation and multilineage differentiation of porcine MSCs

In Experiment 1, bone marrow MSCs were efficiently isolated based on their characteristic property of attaching to plastic culture dishes and expanded in ADMEM medium. Cells appeared as single, stretched or spindle shaped with long processes leading to large clusters of stellate cells as they multiplied (Fig. 1A), with no obvious reduction in their proliferation potential.

MSCs under osteogenic conditions expressed alkaline phosphatase (AP) activity by day 12 (Fig. 1B) with a steady increase as time progressed. Mineralized matrix was evidenced by staining with von Kossa and Alizarin-red S solution (Fig. 1 C,D). At day 21, almost the whole cell layer was heavily covered with mineralized nodules. Upon induction in adipogenic media, lipid vacuoles or droplets filling the whole cytoplasm of single cells with typical characteristics of adipocytes were observed. Neutral lipid vacuoles were noticeable as early as 1 week and visualized by staining with oil red O (Fig. 1E). In chondrogenic media, accumulation of sulfated proteoglycans was visualized by Alcian blue 8GX staining (Fig. 1F). The presence of transforming growth factor (TGF)-β evolved a compact and homogeneous deposition of mucopolysaccharides, whereas, control MSCs exhibited a more fibrous pellet structure.

Cell cycle analysis

Experiment 2 represents the distribution of FFs and MSCs in confluent existing in the various phases of the cell cycle as determined by flow cytometry. The distribution of cells at different stages of the cell cycle was similar and no significant ($P<0.05$) differences were observed between FFs and MSCs (data not shown). The majority of FFs and MSCs (81.3±1.2% and 78.1±0.9 %, respectively) were in G0/G1 stage of the cell cycle. Similar to the rates of the G0/G1 phase, cells arrested at S or G2/M phases did not differ between FFs and MSCs (5.8±0.3 %, 12.9±0.4% and 6.5± 0.2%, 15.4±0.5 %, respectively).

Cleavage and development

Table 1 shows the cleavage, blastocyst and total cell number of NT embryos cloned with MSCs compared with that of FFs (Experiment 3). Cleavage rate was significantly ($P<0.05$) higher in IVF than in NT embryos with MSCs and FFs (84.5±4.6% vs. 52.2±5.4% and 50.8±5.2%, respectively). However, blastocyst rates in IVF and NT derived from MSCs (20.6±2.5% and 18.4±3.0%)

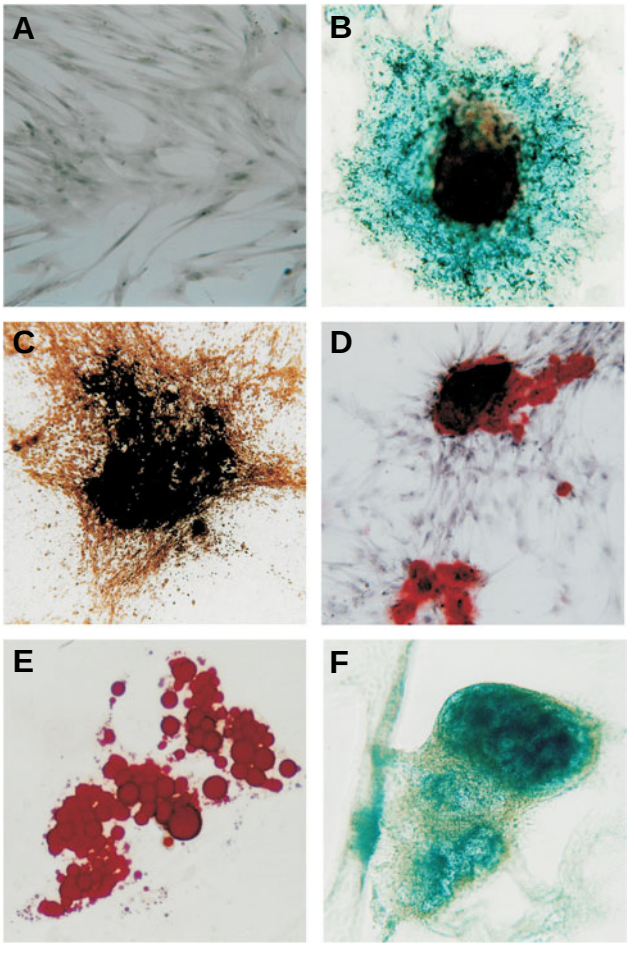

Fig. 1. Morphology and multilineage differentiation of porcine bone marrow mesenchymal stem cells (MSCs). (A) *Morphological characterization of porcine bone marrow MSCs with long flattened cells of fibroblast-like shape.* **(B-F)** *Osteogenic, adipogenic and chondrogenic differentiation from porcine bone marrow MSCs. Osteogenic differentiation was evidenced by the formation of mineralized matrix as shown by visualization of alkaline phosphatase activity* **(B)** *von Kossa staining* **(C)** *and Alizarin red S* **(D)**. *Adipocytic differentiation was evidenced by the formation of lipid vacuoles shown by Oil red O staining* **(E)**. *Chondrogenic differentiation by the presence of mucopolysaccharides was evidenced by Alcian blue 8GX staining* **(F)**; *(A-F), 200 X.*

did not differ but these rates were significantly (P<0.05) higher than NT derived from FFs (9.5±2.1%). The blastocyst cell number was analyzed by labeling the nuclei with DNA stain, bisbenzimide. Total cell number of NT embryos from MSCs (34.4±5.2) were significantly (P<0.05) higher than that of FFs (22.6±5.5).

ICM ratio and apoptosis

The results of Experiment 4 are presented in Table 2. The ratio of ICM to total cells among embryos developed in IVF and NT from MSCs (0.45±0.03 and 0.38±0.08, respectively) were significantly (P<0.05) higher than from FFs (0.18±0.12). ICM: total cells ratio in the embryos between IVF and NT from MSCs did not differ.

Figure 2A and B shows the apoptotic expression of NT embryos from FFs and MSCs. All embryos produced by IVF and NT had apoptotic cells. However, proportional data of TUNEL positive cells in NT embryos from FFs (7.3±1.8%) were significantly (P<0.05) higher than from MSCs (4.6±1.3%) and IVF (2.5±0.9%).

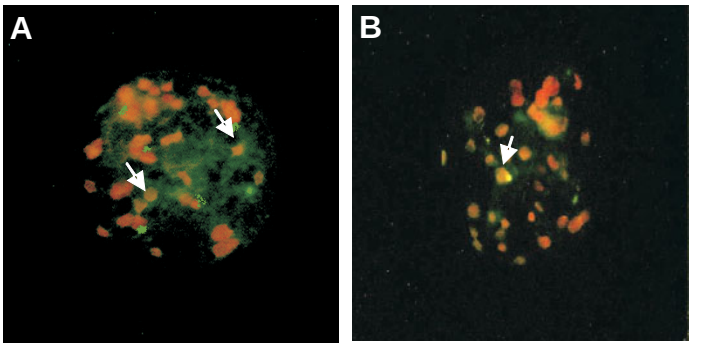

Fig. 2. Fluorescent microscopy images illustrating the apoptotic nuclei in fetal fibroblast (A) and mesenchymal stem cell (B) cloned embryos subjected to TUNEL analysis. *Embryos were labeled with fluorescein isothiocyanate-conjugated dUTP (green) and propidium iodide (red). Apoptotic cells were stained with green and yellow. Arrows indicate apoptotic nuclei. (A-B) 200 X.*

Discussion

Successful NT with a variety of cell types in several species demonstrated the ability of oocyte cytoplasm to reprogram a somatic donor nucleus to a pluripotent state (Rideout *et al.*, 2001). It has been hypothesized that the genome of undifferentiated cells, such as stem cells, may be more easily reprogrammed by the recipient oocyte. The relationship between donor cell differentiation status and NT success has been demonstrated in mice, with NT embryos derived from ES cells (Rideout *et al.*, 2000; Eggan *et al.*, 2001) showing significantly enhanced survival to term compared with those derived from somatic cell nuclei (Wakayama and Yanagimachi, 1999). However, Hochedlinger and Jaenisch (2002) obtained cloned mice from terminally differentiated, mature T and B-cells using a two-step method, but they never succeeded with a simple NT. Cloned mice were successfully obtained from the nucleus of natural killer T-cell lymphocytes, a lymphocyte population in their same hematopoietic lineage, but not from peripheral T cells (Inoue *et al.*, 2005). Contrastingly, *in vitro* development of HSC cloned embryos was very poor and the birth rates per transfer were no better than those of clones from other somatic cell types such as cumulus, immature sertoli and fibroblast cells (Inoue *et al.*, 2006) suggesting that, the genome of HSC may have a lower genomic plasticity, at least in terms of the ability to be reprogrammed in the MII cytoplasm after NT.

In this study, fibroblast-like appearing adherent MSCs isolated from porcine adult bone marrow were successfully induced to multipotential differentiation into osteogenic, adipogenic and chondrogenic lineages under specific culture conditions. The morphological features of MSCs were similar to the earlier observations of Ringe *et al.* (2002). On exposure to osteoinductive medium, MSCs transformed from a fibroblastic to a cuboidal shape, thereby exhibiting osteoblastic morphology. Uncommitted stem cells are recruited by dexamethasone towards the osteogenic lineage, presumably leading to bone cell differentiation at the expense of growth and proliferation (Vacanti *et al.*, 2005). Further, adipogenic differentiation was apparent by the accumulation of lipid vacuoles within cells developed, coalesced and eventually filled the entire cell over a period of 3 weeks. The mechanism of action of stimulants is not completely understood and nothing is known about the mechanisms that contribute to the adipogenesis during this spontaneous regression. However, it has been shown that, the balance between MSCs osteogenesis and adipogenesis is known to be controlled by differentially regulating mechanisms through peroxisome proliferators-activated receptor-γ, Wnt signaling cascade and the notch and RhoA signaling pathways (Vacanti *et al.*, 2005). In this study, treatment with TGF-β in MSCs monolayer induced chondrogenesis by an increased proteoglycan rich extra cellular matrix during culture. The functional abilities of bone marrow derived fibroblasts to differentiate under appropriate stimuli along three principal lineages: osteoblastic, adipocytic and chondrocytic allows their affirmation as MSCs (Pittenger *et al.*, 1999).

The cell cycle analysis showed that majority of MSCs in confluent was in the G0/G1 stage, which is desirable for NT (Wilmut *et al.*, 2002). The lower incidence of nuclear abnormalities and enhanced *in vitro* development of preimplantation NT embryos in pig from fetal skin derived stem cells suggested that

TABLE 1

DEVELOPMENT OF IVF AND CLONED PORCINE EMBRYOS

Groups	Donor cells	Oocytes used	Mean±SEM		
			Cleavage (%)	Blastocyst (%)	Total cell number
IVF		238	201 (84.5±4.6)^{b}	49 (20.6±2.5)^{b}	38.5±4.8^{b}
NT	FFs	242	123 (50.8±5.2)^{a}	23 (9.5±2.1)^{a}	22.6±5.5^{a}
	MSCs	255	133 (52.2±5.4)^{a}	47 (18.4±3.0)^{b}	34.4±5.2^{b}

Different superscipts in the same column denote significant difference (P<0.05). 5 replicates.

TABLE 2

APOPTOSIS AND ICM RATIO IN IVF AND CLONED PORCINE EMBRYOS

Groups	Donor cells	Blastocysts used	Mean±SEM	
			Apoptosis	ICM ratio
IVF		34	2.5±0.9^{a}	0.45±0.03^{b}
NT	FFs	20	7.3±1.8^{c}	0.18±0.12^{a}
	MSCs	31	4.6±1.3^{b}	0.38±0.08^{b}

Different superscipts in the same column denote significant difference (P<0.05). 5 replicates

these embryos were more competent to undergo appropriate remodeling during early development (Zhu *et al.*, 2004). The highest formation of morulae/blastocysts per cleaved porcine embryos reconstructed with olfactory bulb progenitor cells (23.5% vs. 17.0% using skin stem cells and 11.6% using fibroblasts) implied that undifferentiated cells as nuclear donors may increase the efficiency of NT (Lee *et al.*, 2006). Recently, porcine MSCs supported blastocyst development after being transferred to enucleated MII oocytes (Bosch *et al.*, 2006). However, no significant differences were observed in the cleavage and blastocyst rates between MSCs and skin fibroblasts. Similarly, cloned embryos derived from bovine and porcine undifferentiated MSCs and their derivatives along the osteogenic lineage gave rise to consistently high preimplantation development comparable to adult fibroblasts (Colleoni *et al.*, 2005). Further, the development of porcine NT embryos (32.9-44.7% on day 7) using MSCs indicated that consistently high development can be obtained *in vitro*. In the present study, cleavage rate was significantly higher in IVF embryos than in NT embryos derived from MSCs and FFs. However, blastocyst rates in IVF and NT embryos derived from MSCs did not differ but these rates were significantly higher than that for NT embryos derived from FFs. Our observations are well in accordance with the results of above studies. However, further data on post implantation development are needed to shed more light on the effect of the stem cell nature of the donor nucleus.

In this study, total cell numbers and the ratio of ICM to total cells in NT embryos from MSCs were significantly higher than for those from FFs, but did not differ to IVF embryos. Similar were the observations of Zhu *et al.* (2004), suggesting that fetal stem cells derived cloned embryos are of higher quality than fibroblast cloned embryos. It is presumed that the ratio of ICM to total cells in embryos is more important for subsequent normal development after transfer than total cell number, because pig demi-embryos that had only half the cell numbers of normal embryos, but were bisected at the correct ratio of ICM to total cells, could develop to term (Tao *et al.*, 1995).

In the present study, proportions of TUNEL-positive cells in NT embryos from FFs were significantly higher than in those from MSCs and in IVF embryos. This finding supports a relationship between incidence of cell death and developmental potential as indicated by lower developmental rate in NT embryos derived from FFs. It is well known that the postimplantation developmental potential or embryo quality is likely to be affected by apoptotic incidence during preimplantation stages. NT porcine embryos exhibited higher rates of cytoplasmic fragmentation and developmental arrest as well as higher levels of apoptotic cells than IVF embryos (Hao *et al.*, 2003). As shown in our results, it may be possible to improve the developmental potential of NT embryos using MSCs as donors by preventing activation of the apoptotic pathway, especially during preimplantation embryo development.

In conclusion, it is demonstrated in the present study that porcine MSCs exhibit high proliferation rates and multilineage differentiation potential. NT embryos reconstructed with MSCs show enhanced developmental potential compared with those reconstructed with FFs, with a significantly higher proportion of embryos reaching the blastocyst stage. High total cell number and ICM ratio and low apoptotic positive cells were associated with NT embryos derived from MSCs. Cumulatively, this evidence suggests that MSCs have a greater potential as donor cells and are capable of driving efficiently the preimplantation development of cloned pig embryos.

Materials and Methods

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) and media from Gibco (Life Technologies, Burlington, ON, Canada), unless otherwise specified.

Preparation of donor cells

Porcine female fetus was obtained via hysterectomy of pregnant gilt on day ~30 of gestation. After removal of head, limbs and visceral organs, remaining tissues were washed in Dulbecco phosphate buffered saline (DPBS) supplemented with 10% fetal bovine serum (FBS) and transferred into 0.05% (w/v) trypsin-elthylenediamine tetra acetic acid (EDTA) solution for 5 min. Trypsinized cells were washed once by centrifugation in Dulbecco modified Eagle medium (DMEM) by being centrifuged at 300 x*g* for 10min to take cell pellet. Fetal fibroblasts (FFs) at a final concentration of $2x10^5$ cell/mL were then cultured in DMEM medium (high glucose) supplemented with 110 μg/mL Na-pyruvate and 4 μg/mL pyridoxine hydrochloride and 10% FBS at 38.5ºC in a humidified atmosphere of 5% CO_2 in air.

Under sterile conditions, gelatinous bone marrow was extracted from a femur of ~ 6 month old female pig. MSCs were isolated according to the method of Ringe *et al.* (2002) with minor modifications. Briefly, 3-4 g of gelatinous bone marrow was resuspended in phosphate buffered saline (PBS) and dispersed mechanically and centrifuged at 400 x *g* for 10min. Cells were resuspended and layered upon a Ficoll gradient (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 400 x*g* for 30-40 min at 20ºC. The interface buffy layer was collected and washed twice with PBS and twice with advanced-DMEM (ADMEM) supplemented with 10% FBS and cultured at 38.5ºC in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were gently rinsed off 2 days after plating. Attached MSCs reached confluency in 12-14 days after plating (passage 0). Once confluent, cells were dissociated using 0.1% (w/v) trypsin-EDTA solution and pelleted at 300 x*g* for 5 min. Cells were then regrown and passaged for *in vitro* differentiation studies. FFs and MSCs were frozen in DMEM and ADMEM, respectively containing 10% FBS and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. For NT, cells at passage 3 were thawed, cultured to reach confluency to synchronize the majority into G0/G1 stage and subsequently used.

In vitro *differentiation of porcine MSCs*

Multilineage potential of porcine MSCs was evaluated by culturing first- to third- passage cells ($2x10^5$ cells/35 mm dish) in ADMEM containing 10% FBS under conditions conducive for osteogenic, chondrogenic and adipogenic differentiation for 3 weeks adopting previously published protocols with minor modifications (Pittenger *et al.*, 1999; Vacanti *et al.*, 2005). The cells in the control groups were cultured in ADMEM supplemented with 10% FBS. Cells were stained with BCIP/NBT (Promega, Madison, WI, USA) to detect alkaline phosphatase activity. Presence of mineralized matrix was analyzed using von Kossa and Alizarin-red S stains. Cytoplasmic fat globules were identified by Oil red O staining and mucosubstances by Alcian blue 8GX solution

Cell cycle analysis

FFs and MSCs were analyzed in 3 runs for DNA content to evaluate their stages of the cell cycle by flow cytometry (PARTEC. PA-1, Münster, Germany) using a CYSTAIN DNA 2 steps kit (PARTEC). Briefly, the separated cells ($1x10^6$ cells/mL) were fixed in 70% ethanol at 4ºC for 18 h and centrifuged at 200 x *g* for 10 min in order to completely remove the fixative. The cells were resuspended into 21 mg buffer reagent per ml extraction buffer and incubated for 15 min at RT with gentle shaking. Pelleted cells were stained with 2 mL DNA flurochrome 4, 6-diamidino-2-phenylindole for minimum 1 h at RT and relative DNA content of each cell

was measured and classified as G0/G1, S or G2/M phase.

Oocyte collection and* in vitro *maturation (IVM)

Porcine ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in PBS at 35-39ºC. Cumulus-oocyte-complexes (COCs) were collected from follicles of 3-6 mm in diameter with an 18 G needle and a 10 mL syringe. COCs were washed three times with nutrient mixture F-10 (Ham-F10) and two times with *in vitro* maturation medium (IVM) and cultured (50 COCs/500 μl drop) in M-199 containing 5% FBS, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 25 mM HEPES, 2.5 mM Na- pyruvate, 1 mM L-glutamine, 1.0% penicillin-streptomycin (10,000 IU and 10,000 mg/mL, respectively; Pen-Strep, Gibco), 0.5 μg/mL LH, 0.5 μg/mL FSH for 22 h at 39ºC in a humidified atmosphere of 5% CO_2 in air. COCs were further cultured for an additional 20 h in the fresh IVM medium without hormone supplements.

After IVM, oocytes were freed off their cumulus cells by vortexing in DPBS medium supplemented with 0.1% (W/V) hyaluronidase for 1 min. Oocytes with a polar body (PB) and even cytoplasm were selected for production of IVF and NT embryos.

In vitro *fertilization (IVF)*

Each set of 20 cumulus free oocytes were transferred into 50 μl drop of modified tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3.0 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20.0 mM Tris crystallized free base, 11.0 mM glucose and 5.0 mM Na-pyruvate, supplemented with 2 mM caffeine and 0.04g BSA (Fatty acid free, Fraction V). Oocytes were inseminated with frozen-thawed sperm prepared by Percoll (Pharmacia, Uppsala, Sweden) density gradient as described by Rosenkrans *et al.* (1993). The final sperm concentration was adjusted to $1 ¥ 10^5$ sperm/mL. Coincubation was carried out at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 5 h.

Nuclear transfer (NT)

NT was carried out with minor modifications of previously described protocol (Kim *et al.*, 2005). Denuded MII-stage oocytes were enucleated by micromanipulation technique in Hepes-buffered M-199 supplemented with 10% FBS, 7.5 μg/mL cytochalasin B (CCB) and 12 mM sorbitol. Briefly, the first polar body and metaphase plate with a small volume of cytoplasm were removed together using a 15 μm beveled micropipette. Single donor cell (FF or MSC) of approximately 10 μm diameter was used for NT. For fusion, the reconstructed eggs were oriented in BTX Electro chamber (BTX, Inc., San Diego, CA) filled with 0.28 M mannitol solution containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.01% BSA and pulsed twice with 2.0 KV/cm DC for 30 μsec using a BTX Electro Square Porator (ECM 830, BTX, Inc., San Diego, CA). After fusion, eggs were cultured in 50 μL drops of NCSU-23 medium supplemented with 7.5 μg/mL CCB at 38.5ºC in a humidified atmosphere of 5% CO_2 in air for 3 h.

In vitro *culture (IVC)*

IVF and NT embryos were cultured using a protocol as previously described (Ock *et al.*, 2006). Briefly, the presumptive zygotes (20 zygotes/50 μL drop) were cultured in NCSU-23 (IVC-PyrLac) supplemented with 4 mg/mL BSA, 0.17 mM Na-pyruvate, 2.73 mM Na-lactate, 20 μL/mL eagle amino acids in basal medium (BME) and 10 μL/mL nonessential amino acids in minimum essential medium (NEAA) for 2 days and further cultured in the same medium (NCSU-23, IVC-Glu) supplemented with 5.55 mM glucose instead of Na-pyruvate and Na-lactate at 38.5ºC in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 5 days. Cleavage and blastocyst rates were assessed on day 2 and day 7, respectively.

Cytological analysis

To count total cell number, day-7 IVF and NT blastocysts fixed in methanol-acetic acid (3:1) for overnight were stained with 10 μg/mL bisbenzimide (Hoechst 33342) in HEPES-TALP for 10 min and the nuclei were counted under an epifluorescence microscope (Nikon, Tokyo, Japan).

The apoptosis of embryos was detected by the Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) method using the *in situ* cell death detection kit (Roche, Penzberg, Germany) following manufacturer's protocol. Briefly, blastocysts were fixed in 3.7% formaldehyde for 4 h at room temperature (RT), washed in PBS, permeabilized by incubation in 0.5% Triton X-100 for 1 h, washed in PBS, incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase enzyme for 1 h at 37 ºC in dark room and counterstained with 40 μg/mL Propidium iodide (PI) for 1 h at 37 ºC after treatment of 50 μg/mL RNase at RT for 1 h. Samples were examined under an epifluorescence microscope and those stained red were nucleus and those stained green and yellow were apoptotic body.

Differential cell count was performed as described previously by Machaty *et al.*, (1998). Briefly, blastocysts were removed of their zona pellucida using 0.5% (w/v) pronase for 2 min. Zona-free embryos were incubated in rabbit anti-pig whole serum diluted 1:9 (v/v) in IVC-Glu medium for 40 min and finally incubated in guinea pig complement diluted 1:9 (v/v) in IVC-Glu medium supplemented with 10 μg/mL of PI for 1 h. Subsequently fixed in ice-cold ethanol for 5 min and then stained with 10 μg/mL of bisbenzimide in ethanol for 5 min at RT and observed under an epifluorescent microscope (200 X). ICM nuclei labeled with bisbenzimide appeared blue and TE nuclei labeled with both bisbenzimide and PI appeared pink to red.

Experimental design

The present study comprised of four experiments. In Experiment 1, isolation and characterization of adult porcine MSCs for the developmental pluripotency by multilineage differentiation were evaluated. In Experiment 2, cell cycle comparisons of stem cells (MSCs) and somatic cells (FFs) were made using flow cytometric analysis. In Experiment 3, the suitability of MSCs and FFs as nuclear donors was assessed by comparing the developmental rate and total cell number. Finally, experiment 4 examined the apoptosis and ICM ratio in embryos reconstructed with MSCs and FFs. Embryos produced by IVF were used as control.

Statistical analysis

Differences were analyzed among treatments using one-way ANOVA after arc-sine transformation of the proportional data. Data were expressed as mean±SEM. Comparisons of mean values among treatments were performed using Duncan's and Tukey's multiple comparisons test. Differences were considered significant at $P<0.05$.

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