Comparison of Developmental Competency of Porcine Embryos Cloned with Mesenchymal Stem Cells and Somatic Cells

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ABSTRACT

The present study compared the developmental potential of cloned porcine embryos with mesenchymal stem cells (MSCs), fetal fibroblasts (FFs) and cumulus cells (CCs) by assessing the cleavage and blastocyst rate, total cell number, inner cell mass (ICM) ratio and apoptosis. MSCs were isolated by ficoll gradients from femur of ~6 month old female pig, and maintained for primary cultures. FFs from a female fetus at ~30 day of gestation were established, and CCs were obtained from cumulus oocyte complexes (COCs) aspirated from 3~6 mm follicles in diameter. Donor cells at 3~4 passage were employed for nuclear transfer (NT). COCs were matured and fertilized *in vitro*(IVF) as control. Cleavage rate was significantly ($P<0.05$) higher in IVF than in NT embryos with MSCs, FFs and CCs (82.7±3.9% vs 70.6±5.4, 68.7±5.1 and 63.4±5.6%,respectively). However, blastocyst rates in IVF and NT embryos derived from MSCs (24.5±2.8 and 20.4±3.3%) did not differ, but were significantly ($P<0.05$) higher than NT derived from FFs and CCs (10.6±2.7 and 9.8±2.1%). Total cell number and the ratio of ICM to total cells among blastocysts cloned from MSCs (35.4±5.2 and 0.40±0.09, respectively) were significantly ($P<0.05$) higher than those from FFs and CCs (24.9±6.2 and 0.19±0.16, 23.6±5.5 and 0.17±0.16, respectively). Proportions of TUNEL positive cells in NT embryos from FFs and CCs (6.9±1.5 and 7.4±1.7%, respectively) were significantly ($P<0.05$) higher than in MSCs (4.8±1.4%) and IVF (2.3±0.9%). The results demonstrate that MSCs have a greater potential as donor cells than FFs and CCs in achieving enhanced production of cloned porcine embryos.

(Key words : Mesenchymal stem cells, Development, Nuclear transfer, Porcine)

INTRODUCTION

Although cloned piglets were obtained by somatic cell nuclear transfer (SCNT) technique (Betthauser *et al.*, 2000; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000), the success of SCNT depends upon many factors, including donor cell types, passages, oocyte maturation, activation, nuclear reprogramming, embryo culture and transfer. With current technology, the rate of SCNT embryos progressing to live young in pig is hovering between 1~4% or less (Wilmut *et al.*, 2002).

Fetal fibroblasts (FFs) have been most commonly used as nuclear donors in porcine SCNT, primarily because embryos derived from these cells showed higher developmental competence than those developed from adult somatic cells (De Sousa *et al.*, 2002). Establishment of FFs, however, inevitably poses an ethical concern, since it requires surgical intervention of the dam and sacrifice of the fetus. A previous study showed that porcine nuclear transfer (NT) embryos regardless of donor cell types (cumulus or fibroblast cells) have developmental potential to the blastocyst stage (Koo *et al.*, 2001). However, *in vitro* development of porcine NT embryos with somatic cells still remains relatively low when compared to *in vitro* fertilized (IVF) embryos. The choice of a donorcell is the key to successful production of SCNT clones, because the type of donor cell determines the developmental potency of cloned embryos (Wakayama *et al.*, 2001). DNA methylation and gene expression patterns required for early development of cloned embryos were affected by the donor cell type (Bortvin *et al.*, 2003). Presumably, this cloning inefficiency may be explained by inadequate reprogramming of differentiated somatic nuclei (Rideout *et al.*, 2001). It has been suggested that the genome of undifferentiated stem cells may be more easily reprogrammed to resemble the genome of the

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zygote, which may make stem cells more efficient donors for NT (Rideout *et al.*, 2001; Oback and Wells, 2002). Quite recently, 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 (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.

The aims of this study therefore were to compare the developmental potential of embryos cloned with MSCs, FFs and CCs. The cell cycle distribution of MSCs, FFs and CCswas analyzed to confirm their feasibility as nuclear donors. The quality of embryos was assessed *in vitro* by the cleavage and blastocyst rates. Until now, no information is available on the structural composition of NT porcine embryos from MSCs. Thus, we examined total cell number, relative proportions of the inner cell mass (ICM) and trophoblast (TE) cells and incidence of apoptosis in NT blastocysts derived from MSCs by comparing with those from FFs, CCs and IVF counterparts.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) and media from Gibco (Invitrogen Corporation, Grand Island, N.Y., USA), unless otherwise specified.

Preparation of Donor Cells

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 Dulbecco's phosphate buffered saline (DPBS) and dispersed mechanically. After being centrifuged at 400 ×g for 10 min, cells were resuspended and layered upon a Ficoll gradient (Amersham Biosciences, Uppsala, Sweden), and centrifuged at 400 ×g for 30~40 min at 20℃. The interface buffy layer was collected and washed twice with DPBS and twice with advanced- Dulbecco's modified eagle medium (ADMEM). Cells at 2×10^5 cell /ml were cultured in ADMEM supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (Pen-strep, Gibco).

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 DPBS supplemented with 10% FBS and transferred into 0.05% (w/v) trypsin-ethylenediamine tetra acetic acid (EDTA) solution for 5 min. Trypsinized cells were washed once by centrifugation at 300 ×g for 10 min in DMEM to take cell pellet. Fetal fibroblasts (FFs) at a final concentration of 2×10^5 cell/ml were then cultured in DMEM (high glucose) supplemented with 110 μg/ml Na-pyruvate and 4 μg/ml pyridoxine hydrochloride, 1% (v/v) pen-strep and 10% FBS.

CCs were collected from cumulus oocyte complexes (COCs) aspirated from 3~6 mm follicles in diameter, and were cultured in DMEM supplemented with 10% FBS and 1% (v/v) pen-strep. All cultures were maintained at 38.5℃ in a humidified atmosphere of 5% CO_2 in air.

Twelve days before NT, cells were passaged, seeded into 35 mm tissue culture dishes and allowed to reach confluency to synchronize the majority into G0/G1 stage. Approximately half an hour before manipulation, cells were dissociated by incubation for 5 min in 0.1% (w/v) trypsin-EDTA solution, pelleted, and resuspended in DMEM or ADMEM with 10% FBS.

Cell Cycle Analysis

MSCs, FFs and CCs were analyzed in triplicates 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 (1×10^6 cells/ml) were fixed in 70% ethanol at 4℃ for 18 hr and centrifuged at 200 ×g for 10 min in order to completely remove the fixative. Cells were resuspended into 21 mg buffer reagent per ml extraction buffer and incubated for 15 min at room temperature (RT) with gentle shaking, stained with 2 ml DNA flurochrome 4,6-diamidino-2-phenylindole (DAPI) for minimum 1 hr at RT, and analyzed for their different phases of the cell cycle.

Oocyte Collection and *In Vitro* Maturation (IVM)

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in Phosphate buffered saline at 35~39℃. 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 nutrientmixture F-10 (Ham-F10) and two times with *in vitro* maturation (IVM) medium which composed of 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% Pen-strep, 0.5 μg/ml LH, 0.5 μg/ml FSH. Sets of so COCs were cultured in 500 μl drop of IVM medium for 22 hr at

38.5℃ in a humidified atmosphere of 5% CO_2 in air. COCs were further cultured for an additional 20 hr 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 (m-TBM) supplemented with 2 mM caffeine and 0.04 g/ml BSA (Fatty acid free, Fraction V). Oocytes were inseminated with frozen-thawed sperm prepared by Percoll (Pharmacia, Uppsala, Sweden) density gradient as previously described (Ock *et al.*, 2006). The final sperm concentration was adjusted to 1×10^5 sperm/ml. Coincubation was carried out at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 5 hr.

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, 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. To validate enucleation process, the enucleated oocytes were stained with 10 μg/ml bisbenzimide (Hoechst 33342) for 2 min and observed under an epifluorescent microscope (Nikon, Tokyo, Japan).

Single donor cell (MSC, FF and CC) 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 cytochalasin B at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 3 hr.

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 38.5℃ 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. After being mounted onto a precleaned microscope slide, the nuclei were counted under an epifluorescence microscope (Nikon, Tokyo, Japan).

Analysis of apoptosis rate was performed following Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) assay. Briefly blastocysts fixed in 3.7% formaldehyde for 4 hr at RT, were permeabilized by incubation in 0.5% Triton X-100 for 1 hr. After being washed in PBS, embryos were incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase enzyme (Roche, Mannheim, Germany) for 1 hr at 38.5℃ in dark room, and counterstained with 40 μg/ml propidium iodide (PI) for 1 hr at 38.5℃ after treatment of 50 μg/mlRNase at RT for 1 hr. Samples were examined under an epifluorescence microscope and those stained red were considered as nucleus and those stained green and yellow were apoptotic body.

Differential cell count was performed as described previously by Machaty *et al.* (1998) with minor modifications. Briefly, blastocysts were removed of their zona pellucida by treatment with 0.5% (w/v) pronase for 2 min. After being cultured individually in drops of IVC-Glu medium for 1~2 hr, 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 hr at 38.5℃ in a humidified atmosphere of 5% CO_2 in air, respectively. After complement-mediated cell lysis and staining of lysed cells by PI, embryos were fixed in ice-cold ethanol for 5 min and then stained with 10 μg/ml of bisbenzimide in ethanol for 5 min at RT. The stained embryos were transferred in to 100% glycerol drop on a microscopic slide and examined in whole mount under an epifluorescent microscope. ICM nuclei labeled with bisbenzimide appeared blue and trophoblast (TE) nuclei labeled with both bisbenzimide and PI appeared pink to red. Numbers of ICM and TE nuclei were counted directly under the microscope (200×).

Experimental Design

The present study comprised of two experiments. Experiment 1 evaluated the suitability of MSCs, FFs and CCs as nuclear donors by comparing the developmental rate and total cell number. Experiment 2 examined the apoptosis and ICM ratio in day 7 blastocysts reconstructed with MSCs, FFs and CCs.

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 $P<0.05$.

RESULTS

Isolation and Identification of MSCs

MSCs from porcine bone marrow were efficiently isolated based on their characteristic property of attaching to plastic culture flasks or dishes and expanded in ADMEM medium. The isolated cells appeared as single, stretched cells or spindle shaped with long processes leading to large clusters of satellitecells as they multiplied and demonstrated no obvious reduction in their proliferation potential during culture. Cytochemical analysis revealed the appropriate phenotype of differentiated cells including osteocytes (alkaline phosphatase activity, von Kossa and alizarin red S), adipocytes (oil red O) and chondrocytes (alcian blue) (data not shown).

Experiment 1: Developmental Rate and Total Cell Number

The *in vitro* preimplantation development of embryos cloned with MSCs was compared with that of embryos cloned with FFs and CCs. Embryos produced by IVF were used as control. Table 1 shows the development rate and total nuclei in IVF and NT embryos (Experiment 1). Cleavage rate was significantly ($P<0.05$) higher in IVF than in NT embryos with MSCs, FFs and CCs (82.7±3.9% vs. 70.6±5.4, 68.7±5.1 and 63.4±5.6%, respectively). Although, blastocyst rates in IVF and NT derived from MSCs (24.5±2.8 and 20.4±3.3%, respectively) did not differ, these rates were significantly ($P<0.05$) higher than NT derived from FFs and CCs (10.6±2.7 and 9.8±2.1%, respectively).

Total cell number among day 7 blastocysts cloned with MSCs (35.4±5.2) were significantly ($P<0.05$) higher than those with FFs and CCs (24.9±6.2 and 23.6±5.5, respectively).

Experiment 2: ICM Ratio and Apoptosis

The results of Experiment 2 are presented in Table 2. The ratio of ICM to total cells among day 7 blastocysts derived from IVF and NT with MSCs (0.47±0.04 and 0.40±0.09, respectively) were significantly ($P<0.05$) higher than those with FFs and CCs (0.19±0.16 and 0.17±0.16, respectively). However there was no difference in the ratio of ICM to total cells in the embryos between IVF and NT from MSCs.

All embryos produced by IVF and NT had apoptotic cells. However, proportional data (mean±SEM) of TUNEL positive cells in NT embryos from FFs and CCs (6.9±1.5 and 7.4±1.7%, respectively) were significantly ($P<0.05$) higher than those from MSCs (4.8±1.4%) and IVF (2.3±0.9%).

DISCUSSION

In the present study, MSCs derived from adult porcine bone marrows were demonstrated to be used as nuclear donors successfully for NT procedure. Although blastocyst rates in IVF and NT embryos derived from MSCs did not differ, these rates were significantly higher than NT derived from FFs and CCs.

Table 1. Development and total cell number of cloned porcine embryos with different donor cells

Groups	Donor cells	Oocytes used	Mean±SEM		
			Cleavage (%)	Blastocyst (%)	Total cell number
IVF		526	435(82.7±3.9)^{b}	129(24.5±2.8)^{b}	39.5±4.7^{b}
	MSCs	470	332(70.6±5.4)^{a}	96(20.4±3.3)^{b}	35.4±5.2^{b}
NT	FFs	451	310(68.7±5.1)^{a}	48(10.6±2.7)^{a}	24.9±6.2^{a}
	CCs	448	284(63.4±5.6)^{a}	44(9.8±2.1)^{a}	23.6±5.5^{a}

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

Table 2. Apoptosis and ICM ratio of cloned porcine embryos with different donor cells

Groups	Donor cells	Blastocysts used	Mean±SEM	
			Apoptosis	ICM ratio
IVF		87	2.3±0.9[a]	0.47±0.04[b]
NT	MSCs	75	4.8±1.4[b]	0.40±0.09[b]
	FFs	41	6.9±1.5[c]	0.19±0.16[a]
	CCs	36	7.4±1.7[c]	0.17±0.16[a]

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

Faulty or incomplete epigenetic reprogramming of the donor genome is still considered a major problem in nuclear cloning despite recent improvements. Little is known about the initial molecular reprogramming events occurring upon transfer of the donor nuclei to the enucleated oocyte, but it is generally believed that this reprogramming must be complete before zygotic transcription commences and that the ease of reprogramming is related to the differentiation status of donor-cell type (Rideout *et al.*, 2001). That reconstructed pig embryos producedby NT of adult somatic cells show a lower rate of blastocyst formation in comparison with the use of fetal fibroblasts (De Sousa *et al.*, 2002). In addition porcine NT embryos with regardless of donor cell types (cumulus or fibroblast cells) have similar developmental potential to the blastocyst stage (Koo *et al.*, 2001). However, *in vitro* development of porcine NT embryos with somatic cells still remains relatively low when compared to IVF derived embryos. In mice, Oback and Wells (2002) reported that NT embryos derived from ES cells show significantly enhanced survival to term compared with those derived from somatic cell nuclei, and up to one third of blastocysts cloned using ES cells develop to term (Rideout *et al.*, 2000). The above reports therefore support the hypothesis that undifferentiated stem cells may be more easily reprogrammed to an embryonic state. Enhanced *in vitro* development of preimplantation pig embryos reconstructed with fetal-skin derived stem cells has been reported with a conclusion that fetal skin derived stem cells may be better donor cells for NT in the pig (Zhu *et al.*, 2004). The lower incidence of nuclear abnormalities in NT embryos derived from fetal skin derived stem cells suggested that these embryos are more competent to undergo correct remodeling during early embryo development.

Although various evaluating parametersincluding cell number, ICM ratio, apoptosis, chromosome number, and gene expression patterns are important, it is well known that ICM ratio and total cells in blastocysts form fundamental parameters for subsequent development after transfer. In the present study, total cell numbers and the ratio of ICM to total cells in NT embryos from MSCs were significantly ($P<0.05$) higher than for those from FFs and CCs, but did not differ to IVF embryos. In accordance with our results, total cell numbers of NT derived from FFs blastocysts were lower than IVF derived blastocysts (Koo *et al.*, 2004), and higher total cell number in the porcine fetal stem cells-cloned embryos observedthan in those of the fibroblast-cloned embryos (Zhu *et al.*, 2004), suggesting that embryos cloned with fetal stem cells are of higher quality than those with fibroblasts.

Apoptosis plays an important role in embryo development, which has received increasing attention mostly because of its potential role in cellular response to suboptimal developmental conditions and stress (Fabian *et al.*, 2005). In the present study, the proportions of TUNEL-positive cells in NT embryos from FFsand CCs were significantly ($P<0.05$) higher than in those from MSCs and in IVF embryos. If has been suggested that a major cause for the level of cell death can be reconciled with the high level of embryo arrest. During *in vitro* culture, the NT embryos exhibited higher rates of cytoplasmic fragmentation and developmental arrest as well as higher levels of apoptotic cells than IVF embryos in pig (Hao *et al.*, 2003). In addition, other factors such as micromanipulation, donor cell types, cell passage, cell cycle and proper remodeling of donor cell into oocyte affect the ontogenesis of cloned animals (Kato *et al.*, 1998; Li *et al.*, 2003). The propensity to apoptosis is continuously counterbalanced in the cell by genes stimulating cell survival and proliferation. Since it is essential that the NT embryos are fully reprogrammed and synchronous with the cytoplasm of the activated oocyte as shown in our results, it may be possible to improve the developmental potential of NT embryos using adult MSCs as donors by preventing activation of the apoptotic pathway, especially during preimplantation embryo development in porcine.

In conclusion, NT embryos reconstructed with MSCs show enhanced developmental potential compared with those reconstructed with FFs and CCs, with a significantly higher proportion of embryos reaching the blas-

tocyst 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.

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