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In vitro development of nuclear transfer embryos derived from porcine embryonic germ cells and their descendent neural precursor cells

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Summary

Undifferentiated stem cells may support a greater development of cloned embryos compared with differentiated cell types due to their ease of reprogramming during the nuclear transfer (NT) process. Hence, stem cells may be more suitable as nuclear donor cells for NT procedures than are somatic cells. Embryonic germ (EG) cells are undifferentiated stem cells that are isolated from cultured primordial germ cells (PGC) and can differentiate into several cell types. In this study, the *in vitro* development of NT embryos using porcine EG cells and their derivative neural precursor (NP) cells was investigated, thus eliminating any variation in genetic differences. The rates of fusion did not differ between NT embryos from EG and NP cells; however, the rate of cleavage in NT embryos derived from EG cells was significantly higher ($p < 0.05$) than that from NP cells (141/247 [57.1%] vs. 105/228 [46.1%]). Similarly, the rate of blastocyst development was significantly higher ($P < 0.05$) in NT using EG cells than the rate using NP cells (43/247 [17.4%] vs. 18/228 [7.9%]). The results obtained from the present study in pigs demonstrate a reduced capability for nuclear donor cells to be reprogrammed following the differentiation of porcine EG cells. Undifferentiated EG cells may be more amenable to reprogramming after reconstruction compared with differentiated somatic cells.

Keywords: Embryonic germ cell, Differentiation, Neural precursor cell, Nuclear transfer, Pig

Introduction

The production of cloned animals by somatic cell nuclear transfer (NT) has been described previously for several mammalian species, including sheep (Wilmut *et al.*, 1997), cattle (Cibelli *et al.*, 1998), goats (Baguisi *et al.*, 1999), and pigs (Polejaeva *et al.*, 2000). However, despite intensive efforts, the efficiency of cloning by somatic cell NT has been low. Most cloned embryos die *in utero*, and the few embryos that develop to term show a high incidence of abnormalities (Yang *et al.*, 2007). Overall, in pigs, only 1–3% of cloned embryos survive to term (Kues & Niemann, 2004).

Although the reasons behind this developmental failure are not yet fully understood, one of the critical factors to produce normal cloned animals might be the appropriate reprogramming of nuclear donor cells. Nuclear reprogramming refers to the disappearance of the donor cell epigenetic pattern after NT and the re-establishment of embryonic epigenetic characteristics and gene expression in the cloned embryo (Yang *et al.*, 2007). This change includes remodelling of the chromatin structure, changes in DNA methylation, transcriptional regulation of imprinted genes, regeneration of telomere length, and inactivation of the X chromosome (Han *et al.*, 2003;

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Westphal, 2005). In this regard, the choice of nuclear donor cells may affect subsequent development of reconstructed embryos. The differentiation status of the donor cells may contribute to the success of cloning as correct epigenetic reprogramming and the resulting changes in transcriptional control are the key processes in the development of somatic cell NT embryos (Jaenisch *et al.*, 2004). It has been shown that the success of nuclear reprogramming decreases as donor cells become more differentiated (Gurdon, 1962; Yang *et al.*, 2007), hence a less differentiated cell type may support a greater development of NT embryos compared with terminally differentiated cell types (Faast *et al.*, 2006). One study showed that the use of murine ES (embryonic stem) cells increased the efficiency of the production of cloned offspring per transferred embryos from 1–3% to 15% (Wakayama *et al.*, 1999). In pigs, mesenchymal stem cells isolated from porcine bone marrow resulted in rates of preimplantation development comparable with (Colleoni *et al.*, 2005; Bosch *et al.*, 2006; Faast *et al.*, 2006) or superior to (Jin *et al.*, 2007) their somatic cell counterparts. In addition, embryos cloned from porcine fetal skin-originated sphere stem cells exhibited enhanced preimplantation development compared with fibroblast-cloned embryos, as evidenced by an increased rate of blastocyst development and a higher total cell number in blastocysts (Zhu *et al.*, 2004).

Primordial germ cells (PGC) are embryonic cells that migrate from the root of the allantois to the genital ridge, where ultimately they give rise to gametes. Murine embryonic germ (EG) cells share several morphological, biochemical, immunological and developmental properties with ES cells, which include pluripotency and the capacity to contribute to the germ line of chimeras (Matsui *et al.*, 1992; Resnick *et al.*, 1992; Onishi *et al.*, 1994). Undifferentiated porcine EG cell lines that can be differentiated into various cell lineages, both *in vitro* and *in vivo*, have also been reported (Shim *et al.*, 1997).

In the current study, the *in vitro* development of NT embryos using porcine EG cells and their descendent neural precursor (NP) cells was investigated. As porcine EG cells and their derivative NP cells possess an identical genetic constitution, the efficiency of NT between undifferentiated and differentiated cells may be compared without attendant bias from genetic variation.

Materials and methods

Animal ethics

All procedures in this study were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes, as approved by the Animal Ethics Committee of Dankook University School of Medicine, Cheonan, Korea.

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Culture of porcine EG cells

Porcine EG cells isolated from PGC of day 23 embryos were maintained, as described previously (Shim *et al.*, 1997). To prepare feeder-free EG cells, a mixture of trypsinized EG and feeder cells were cultured for 15 min on 0.1% gelatin-coated plates until the fibroblasts were attached on the dish, while the majority of EG cells was floating. Then, the cells in the supernatant were collected and washed by centrifugation at 800 *g* for 5 min, and transferred onto 0.1% gelatin-coated plates. The cells were grown continuously in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 15% (v/v) ES-qualified fetal bovine serum (FBS; HyClone), 1 mM L-glutamine, 0.1 M MEM non-essential amino acids (Invitrogen), 10 μM 2-mercaptoethanol (Invitrogen), 100 U/ml of penicillin, 0.5 mg/ml of streptomycin, and 1000 U/ml of leukemia inhibitory factor (LIF; Millipore) in a humidified atmosphere of 5% CO_2 in 95% air.

In vitro differentiation of EG cells into NP cells

To produce embryoid bodies (EBs), dissociated EG cells at a density of 2.0–2.5 × 10^4 cells/cm^2 were cultured in N2B27 medium, which consisted of a 1:1 mixture of DMEM/F12 (Invitrogen) and neurobasal medium (Invitrogen) supplemented with N2 supplement (Invitrogen), B27 supplement (Invitrogen), and 2 mM L-glutamine, in a 35-mm bacteriological culture dish coated with F-127, as previously described by Ying *et al.* (2003). After their formation, EBs were induced to differentiate into neural rosettes by culture on 0.2% gelatinized dishes in N2B27 medium with 10 μM retinoic acid for 7 days. Individual neural rosettes were detached physically from the dish using finely drawn Pasteur pipettes and dissociated by treatment with 0.25% trypsin–EDTA. The resulting single cells were used as nuclear donor cells for subsequent NT experiments. Furthermore, neural rosettes were induced to form floating neurospheres by culture in neurosphere medium (NSM), which consisted of DMEM/F12 supplemented with N2 supplement and 10 ng/ml bFGF on tissue culture plates coated with fibronectin.

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Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from EG and NP cells using an Easy-Spin total RNA extraction kit (Intron Biotechnology). Aliquots of total RNA (1 μg) were used for cDNA synthesis using the SuperScript III first-strand synthesis system with oligo(dT) (Invitrogen), according to the manufacturer's protocol. Synthesized cDNA was amplified by *Taq* DNA polymerase (Takara Korea Biomedical). Thirty cycles of PCR amplification were performed as follows: denaturation at 94°C for 30 s, annealing at 50–65°C for 30 s, and extension at 72°C for 30 s. Products were analysed on 0.8% agarose gels and visualized by ethidium bromide staining. The sequences of the upstream and downstream primer pairs, and annealing temperature (°C), and lengths of PCR products were as follows: Oct4, 5′-ATGTGTAAGCTGCGGCCCCTG-3′, 5′-AGGAGACC-CAGCAGCCTC-3′, 62°C, 324 bp; Nanog, 5′-AAT-ACCCGGGCTTCTATTCC-3′, 5′-GCTGGCTATTCCA-AGTCTGG-3′, 50°C, 198 bp; Nestin, 5′-GGCAG-CGTTGGAACAGAGGTTGGA-3′, 5′-CTCTAAACTG-GAGTGGTCAGGGCT-3′, 65°C, 718 bp; glial fibrillary acidic protein (GFAP), 5′-ACATCGAGATCGCCA-CCTAC-3′, 5′-ACATCACATCCTTGTGCTCC-3′, 64°C, 219 bp; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-TCGGAGTGAACGGATTTG-3′, 5′-CCT-GGAAGATGGTGATGG-3′, 50°C, 219 bp.

In vitro maturation of oocytes

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in a warm box (25–30°C) within 2 h. Follicular fluid and cumulus–oocyte complexes (COC) from follicles (5–6 mm in diameter) were aspirated using an 18-gauge needle attached to a 5-ml disposable syringe. Compact COC were selected and washed five times in HEPES-buffered tissue culture medium (TCM)-199 (Invitrogen). The *in vitro* maturation (IVM) medium used was TCM-199 supplemented with 10 ng/ml of epidermal growth factor, 10 IU/ml equine chorionic gonadotropin (eCG; Intervet), 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°C in a humidified atmosphere of 5% CO_2 and 95% air. After culture for 22 h, the COC were transferred to eCG- and hCG-free IVM medium and cultured for another 20 h. At the end of the maturation stage, oocytes were freed from cumulus cells by repeated pipetting in IVM medium containing 0.5 mg/ml hyaluronidase for 1 min.

Nuclear transfer

Forty-two hours after the onset of IVM, oocytes were enucleated with a 20-μm (internal diameter) glass pipette by aspiration of 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) and 7.5 μg/ml cytochalasin B. After enucleation, oocytes were stained with 5 μg/ml bisbenzimide (Hoechst 33342) for 5 min and observed under a Nikon TE-300 inverted microscope equipped with epifluorescence (Nikon Instrument, Tokyo, Japan). Oocytes that contained DNA materials were excluded from subsequent experiments. As nuclear donor cells, EG or NP cells were trypsinized into single cells, and 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 that contained two electrodes. Then, couplets were fused with a double direct current (DC) pulse (1.5 kV/cm for 40 μs) using a BTX Electro-Cell Manipulator 2001 (Genetronics). Following the electrical stimulation, reconstructed oocytes were cultured in NCSU23 supplemented with 4 mg/ml of fatty acid-free BSA and 7.5 μg/ml cytochalasin B for 3 h to suppress extrusion of the second polar body. Then, oocytes were cultured for 4 days in NCSU23 containing 4 mg/ml of fatty acid-free BSA and transferred to NCSU23 containing 10% FBS and cultured for another 3 days. All NT embryos were cultured at 39°C in a humidified atmosphere containing 5% CO_2 in 95% air.

Statistical analysis

To compare the two different types of donor cells (EG and NP cells), both cell types were tested in each replicate. At least three replicates were used 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 in *p*-values of <0.05 were considered to be significant.

Results

In vitro differentiation of EG cells into NP cells

Porcine EG cells in feeder-free culture were induced to differentiate into neural lineages. Under appropriate culture conditions, as demonstrated in Fig. 1, EG cells were sequentially differentiated into EBs, neural rosettes, neurospheres, and neurons with neurites. In addition to the morphological characteristics of each stage of neural differentiation (Fig. 1*B–E*), RT-PCR analysis revealed a change in gene expression along with differentiation. As shown in Fig. 2, the expression of pluripotency-related genes, including

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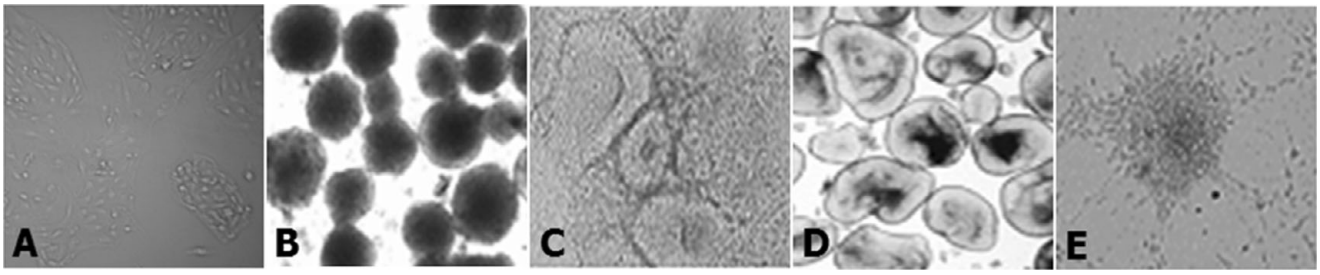

Figure 1 *In vitro* differentiation of porcine embryonic germ (EG) cells into the neural lineage. (*A*) Porcine EG cells, ×200 magnification. (*B*) Embryoid bodies, ×100 magnification. (*C*) Neural rosettes, ×100 magnification. (*D*) Neurospheres ×100 magnification. (*E*) Neurons with neuritis, ×100 magnification.

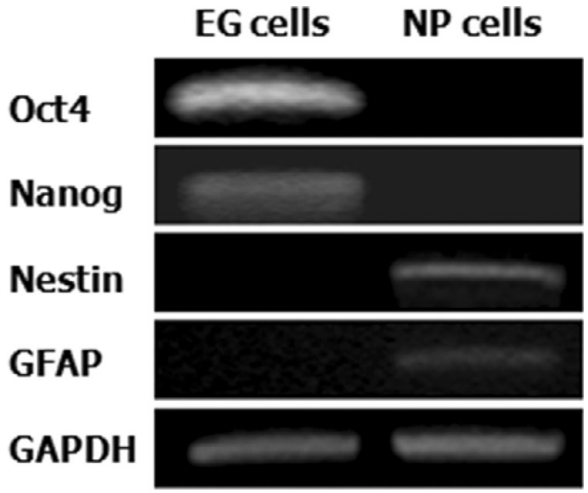

Figure 2 Expression of stem and neural cell markers detected by reverse transcription-polymerase chain reaction (RT-PCR). Note the expression of the stem cell markers Oct4 and Nanog from embryonic germ (EG) cells, compared with the expression of Nestin and glial fibrillary acidic protein (GFAP) from neural precursor (NP) cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Oct4 and Nanog, were detected in EG cells. Upon *in vitro* differentiation into neural lineages, the expression of these genes was down-regulated. In comparison, neuronal lineage-specific genes, including Nestin and GFAP, were up-regulated. Based on the observation that expression of these genes started at the neural rosette stage, these cells were considered to be NP cells and used as donor cells for subsequent NT experiments.

In vitro development of nuclear transfer embryos from EG and NP cells

A summary of the *in vitro* development of NT embryos derived from porcine EG and NP cells is presented in Table 1. No difference was observed in the rate of fusion between the two different donor cells used for NT in this study, however, the rates of cleavage and development to the blastocyst stage from EG cell NT (57.1% and 17.4%, respectively) were significantly greater than those from NP cell NT (46.1% and 7.9%, respectively).

Discussion

Nuclear transfer using various types and levels of differentiated cells has been shown previously to generate cloned offspring in mammals (Galli *et al.*, 1999; Kato *et al.*, 2000; Ogura *et al.*, 2000; Hochedlinger & Jaenisch, 2002; Miyashita *et al.*, 2002). However, the transfer of stem cells with low levels of epigenetic marks may be an advantageous approach because such cells could be more easily reprogrammable and support greater development of NT embryos compared with terminally differentiated cell types (Faast *et al.*, 2006). For instance, oocytes reconstructed from murine 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). It has been previously demonstrated that porcine EG cell NT increased the efficiency of cloned embryo production compared with conventional somatic cell NT using fetal fibroblast cells (Ahn *et al.*, 2007). Hence, porcine EG cells may be more amenable to reprogramming after reconstruction than are differentiated somatic cells. Developmental hindrance due to hypermethylation of DNA in somatic cell NT embryos has been commonly observed (Han *et al.*, 2003). However, the global DNA methylation of EG cells derived from migratory PGC may be lower than differentiated somatic cells, as genome-wide demethylation of DNA occurs during PGC migration, similar to the phenomenon found during preimplantation development of embryos. Hence, NT embryos using EG cells rather than somatic cells may be more easily reprogrammable, as measured by the disappearance of their epigenetic markers, resulting in embryos that have characteristics close to the embryos from normal fertilization in terms of DNA methylation status (Ahn *et al.*, 2007).

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Table 1 *In vitro* development of nuclear transfer embryos

Nuclear donor cells	No. of oocytes	No. (%) of fused oocytes[a]	No. (%) of embryos developed to	
			2-cell[b]	Blastocyst[b]
Embryonic germ cells	319	247 (77.4)	141 (57.1)[c]	43 (17.4)[c]
Neural precursor cells	295	228 (77.3)	105 (46.1)[d]	18 (7.9)[d]

[a]Calculated from the number of oocytes.
[b]Calculated from the number of fused oocytes.
[c,d] $p < 0.05$.

In pigs, studies that involve induced differentiation of EG cells or NT using stem cells and their differentiated descendant cells have not yet been reported to our knowledge. In the present study, porcine EG cells were induced to be differentiated into neural precursor (NP) cells. Under culture conditions, EG cells differentiated successfully into the neural lineage in the correct order of EBs, neural rosettes, neurospheres, and neurons (Fig. 1). These neural cells differentiated from EG cells not only showed morphological characteristics of each stage of neural cell differentiation, but were also verified by expression of neural lineage-specific genes (Fig. 2). Embryoid bodies are structures formed by aggregation of ES cells and can differentiate into derivatives of all three germ layers. The process of aggregation of ES cells to EBs is a good model to study cell differentiation (Zhang *et al.*, 2001). The present study demonstrated that NP cells were differentiated from EG cells by formation of EBs in N2B27 medium supplemented with 10 μM retinoic acid (Fig. 1*A*,*B*). The yield of neural lineage cells generated from EBs can be increased dramatically by the addition of retinoic acid (Guan *et al.*, 2001; Gottlieb, 2002). Then, EBs were dissociated, replated, and differentiated into neural rosettes (Fig. 1*C*). The morphology of the rosettes resembled neuroepithelium in neural development and they retained typical characteristics of NP cells. These cells grown in suspension culture formed neurospheres (Fig. 1*D*). Dissociation and replating of neurospheres gave rise ultimately to terminal differentiation into neuron with neurites projecting from the cells in the periphery of neurospheres (Fig. 1*E*). In addition to the morphological characteristics of NP cells differentiated from EG cells, lineage-specific gene expression was confirmed by RT-PCR (Fig. 2). The results of this study demonstrated that Nestin and GFAP mRNA transcripts were up-regulated in NP cells, whereas the expression of Oct4 and Nanog mRNA was down-regulated. Transcription factors Oct4 and Nanog are involved in the regulation of self-renewal and pluripotency in stem cells and are highly expressed in ES cells of different species, including pigs (Brevini *et al.*, 2007). In contrast, *Nestin* is a gene specifically expressed in neuroepithelial stem cells in neural tube formation (Lendahl *et al.*, 1990), and GFAP expression is associated with astroglia, as well as NPs (Schwartz *et al*, 2005).

The *in vitro* development of NT embryos derived from EG cells before and after differentiation is summarized in Table 1. The rates of cleavage and blastocyst development from EG cell NT were significantly higher than those from NP cell NT when comparing the two different donor cells used for NT in this study. In a previous report (Ahn, *et al.*, 2007), NT embryos using EG rather than fibroblast cells contributed to an increased blastocyst development, perhaps due to the ease of reprogramming of EG cells compared with that of fibroblast cells. However, differences in the genetic background in the two different types of donor cells might affect the reprogramming of donor cells in the NT procedure and, in turn, the developmental competence of NT embryos. In the present study, such differences were eliminated using EG cells and their descendent NP cells for comparison, and the observation that EG cell NT was more efficient than somatic cell NT was extended to superior competence in reprogramming using EG cells to somatic cells of the same genetic constitution.

In conclusion, the results obtained from the present study demonstrate a reduced capability of nuclear donor cells to be reprogrammed following the differentiation of porcine EG cells. Undifferentiated stem cells are more amenable to reprogramming after reconstruction than are differentiated somatic cells. However, the molecular mechanisms underlying the reprogramming of EG and their descendent NP cells that results in different developmental competence requires further study.

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