Donor Cell Source (Miniature Pig and Landrace Pig) Affects Apoptosis and Imprinting Gene Expression in Porcine Nuclear Transfer Embryos

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ABSTRACT

This study investigated the developmental ability and gene expression of somatic cell nuclear transfer embryos using ear skin fibroblast cells derived from miniature pig. When miniature pig (m) and landrace pig (p) were used as donor cells, there were no differences in cleavage (79.2 vs. 78.2%) and blastocyst rates (27.4 vs. 29.7%). However, mNT blastocysts showed significantly higher apoptosis rate than that of pNT blastocysts (6.1 vs. 1.7%) ($p<0.05$). The number of nuclei in pNT blastosysts was significantly higher than that of mNT (35.8 vs. 29.3) ($p<0.05$). Blastocysts were analyzed using Realtime RT-PCR to determine the expression of Bax-α, Bcl-xl, H19, IGF2, IGF2r and Xist. Bax-α was higher in mNT blastocyst than pNT blastocyst ($p<0.05$). There was no difference in Bcl-xl between two NT groups. Bax-α/Bcl-xl was, however, significantly higher in mNT blastocyst compared to pNT. The expression of imprinting genes were aberrant in blastocysts derived from NT compared to *in vivo* blastocysts. H19 and IGF2r were significantly lower in mNT blastocysts ($p<0.05$). The expression of IGF2 and Xist was similar in two NT groups. However, imprinting genes were expressed aberrantly in mNT compared to pNT blastocysts. The present results suggest that the NT between donor cells derived from miniature pig and recipient oocytes derived from crossbred pig might affect reprogramming of donor cell, resulting in high apoptosis and aberrant expression patterns of imprinting genes.

(Key words : miniature pig, nuclear transfer (NT) embryo, apoptosis, imprinting gene)

INTRODUCTION

Since the birth of the first cloned mammals derived from somatic cell nuclear·transfer (Wilmut *et al.,* 1997), nuclear transfer (NT) techniques have advanced remarkably in the last few years, resulting in production of cloned animals in many species (Campbell *et al.*, 1996; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Wells *et al.*, 1999; Onishi *et al.*, 2000; Polejaeva *et al.,* 2000; Shin *et al.,* 2002). Cloned miniature pigs have a potential application in the production of pig for xenotransplantation and human disease model (Lai *et al.*, 2002). Live birth of cloned pigs has been achieved by nuclear transfer of somatic cell nuclei (Onishi *et al.*, 2000; Polejaeva *et al.*, 2000; Dai *et al.,* 2002; Lai *et al.,* 2002; Ramsoondar *et al.*, 2003; Yin *et al.*, 2003). Although healthy offspring was generated, current cloning efficiency is still low (Cibelli *et al.*, 2002; Dinnyes *et al.*, 2002; Wilmut *et al.*, 2002). Cloning creates phenotypic variations that are independent of genetic background (Dean *et al.*, 1998; Humpherys *et al.*, 2002; Ogura *et al.*, 2002; Wilmut *et al.*, 2002; Archer *et al.*, 2003; Shi *et al.*, 2003). Moreover, abnormal development of preimplantation embryo and neonatal death occur frequently in somatic cell cloning due to a variety of afflictions, including fetal overgrowth, placental malformations, deficient immune system and Leydig cell hypoplasia (Wakayama *et al.*, 1998; Renard *et al.*, 1999; McCreath *et al.*, 2000; Park *et al.*, 2004, 2005).

Apoptosis plays an important role in embryo development. The percentage of cells undergoing apoptosis in SCNT porcine embryos was higher than that of IVF embryos (Hao *et al.*, 2003). Apoptosis may contribute to the progressive loss of embryos during the *in vitro* production procedure. However, to our knowledge, miniature pig embryonic apoptosis and its relation to developmental competence, especially in embryos derived by NT, have not been reported previously.

Many imprinted genes are essential for fetal development in both growth regulation and brain development (Tilghman, 1999). *In vitro* culture and embryo manipulation at preimplantation stages may affect phenotype and growth during fetal and peri-

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natal development (Doherty *et al.*, 2000; Khosla *et al.*, 2001; Young *et al.*, 2001). The abnormal phenotype is caused by epigenetic deregulation of genes, and such epigenetic abnormalities would affect in particular the expression of genes that are subject to imprinting. Therefore, NT embryos exhibiting abnormal expression of embryonic genes may be an early indication of incomplete reprogramming that could result in lower survival rates (Dean *et al.*, 1998; Feil, 2001; Moore & Reik, 1996). In addition, the individual effect of the miniature pig and the pig cytoplasm on imprinting gene expression has not been studied.

The present study investigated the development, apoptosis and imprinting gene expression of NT embryos produced using donor cells of miniature pig and recipient oocytes of crossbred pig.

MATERIALS AND METHODS

1. Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

2. Collection of Oocytes and *In Vitro* Maturation (IVM)

Ovaries were obtained from prepubertal crossbred gilts at a local slaughterhouse and transported to the laboratory at 30～35℃. Cumulus-oocyte complexes (COCs) were collected by the aspiration of ovary antral follicles (3～6 mm diameter) with 18 gauge needle fixed to a 10 ml disposable syringe. The COCs with several layers of cumulus cells were selected and washed three times in maturation medium. 50～100 COCs were transferred into 500 μl of maturation medium (TCM-199 Gibco-BRL, Grand Island, NY, USA) covered with mineral oil in a four-well dish (Nunc, Roskilde, Denmark). Oocytes were matured for 40 to 44 h at 38.5℃ under 5% CO_2 in air. The maturation medium supplemented with 0.1% PVA (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml LH, 0.5 μg/ml FSH, 75 μg/ml penicillin G and 50 μg/ml streptomycin.

3. Cell Culture and Preparation of Donor Cells

Fibroblast cells were cultured from ear skin tissue of 8-month-old MHC inbred miniature pig, which was imported from Immerge Bio Therapeutics (Cambridge, MA, USA), and Landrace pig. The cell was cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 15% fetal bovine serum and 75 μg/ml antibiotics. The cells were passaged twice, and then frozen by using DMEM supplemented with 10% dimethylsulfoxide (DMSO). Fibroblast cells were cultured, passaged (2 to 8 passages) and used as donor cells for NT.

4. Production of Nuclear Transfer (NT) Embryos

After maturation, cumulus cells were removed from oocytes by vortexing the COCs in PBS supplemented with 0.1% PVA and 0.1% hyaluronidase for 4 min. Oocytes were enucleated by the aspiration of the first polar body and metaphase-Ⅱ (MⅡ) plate in a small amount of surrounding cytoplasm with a glass pipette. All micromanipulation procedures were performed in TCM-199 supplemented with 3 mg/ml BSA and 5 μg/ml cytochalasin B (CB). Enucleation was confirmed by staining the oocytes with 10 μg/ml Hoechst 33342 for 15～20 min at 39℃. After enucleation, oocytes were held in TCM-199 supplemented with 3 mg/ml BSA until injection of donor cells. A single cell with a smooth surface was transferred into the perivitelline space of an enucleated oocyte. Reconstructed oocytes were then placed between 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber overlaid with 0.3 M mannitol solution supplemented with 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$ and 0.5 mM Hepes. For the fusion, two DC pulse (1 sec interval) of 1.2 kV/cm were applied for 30 μs using a BTX Electro Cell Manipulator 2001 (BTX, San Diago, CA, USA). After fusion, the reconstructed oocytes were cultured in TCM-199 supplemented with 3 mg/ml BSA for 1 h and the fusion was determined. Embryos were washed and transferred into each culture medium covered with mineral oil in a four-well dish. Basic culture medium was porcine zygote medium (PZM-3, Yoshioka *et al.* 2002). The cleavage and development to the blastocyst stage were examined on day 6, respectively.

5. Apoptosis Assays

Day 6 NT blastocysts were washed twice in PBS/PVP (PBS supplemented with 0.1% polyvinylpyrolidone) and fixed in 4% (v/v) paraformaldehyde solution for 24 h at 4℃. Membranes were permeabilized in 0.5% Triton X-100 for 30 min at room temperature. A TUNEL assay was used to assess the presence of apoptotic cells (in situ cell death detection kit, Roche Mannheim, Germany) in the dark for 1 h at 38.5℃. The broken DNA ends of the embryonic cells were labeled with TDT and fluorescein-dUTP. After the reaction stopped, the embryos were washed and transferred into 10 μg/ml Hoechest 33342 for 30 min at room temperature in the dark. The embryos were washed three times and mounted on slides with Prolong antifade Kit

(cat. P-748, Molecular Probes, Eugene, OR, USA). The slides were stored at −20℃. The numbers of apoptotic nuclei and total numbers of nuclei were determined from optical images of whole-mount embryos under an epifluorescent microscope (Nikon, Tokyo, Japan).

6. Collection of *In Vivo* Blastocysts

Embryos were collected from synchronized 7 to 11-month-old Landrace gilts. The gilts were artificially inseminated 24 and 36 h after hCG injection. Immediately after stunning and bleeding of the animals, approximately 168 h after hCG (120 h after estimated ovulation), the genital tract was removed and flushed with DPBS for embryo collection. Embryos were transported to the laboratory in PVA-TL-Hepes buffered at 37℃ within 30 min after collection.

7. Realtime RT-PCR Quantification

The day 6 blastocysts were washed in DEPC-treated water and stored at −70℃. In all experiments, β-actin mRNA was used as an internal standard. First standard cDNA synthesis was achieved by reverse transcription of the RNA by using the Oligo (dT)15 primer and the 1st strand cDNA synthesis kit (Roche). The mRNAs of Bax-α, Bcl-xl, H19, IGF2, IGF2r, Xist and β-actin were then detected by Realtime RT-PCR with specific primer pairs (Table 1). PCR reactions were performed according to the instructions of the Realtime PCR machine manufacturer (LightCycler, Roche) and detected with SYBR Green, a double-stranded DNA-specific fluorescent dye included in the SYBR Green PCR premix. Each PCR run was performed in 20 μl reaction buffer containing 10 μl 2 × SYBR Green premix, 1 μl of forward, reverse primers (100 pmol/μl), 1 μl embryonic cDNA (0.1 blastocyst/μl equivalent). All samples were measured in triplicate. The following amplification program was employed: preincubation for HotStart polymerase activation at 95℃ for 15 min, followed by 40∼55 amplification cycles of denaturation at 95℃ for 1 min (2℃/sec), annealing at 60∼63℃ for 1 min (2℃/sec), elongation at 72℃ for 1 min (2℃/sec), and acquisition of fluorescence at 72 or 80℃ for 1 sec. After the end of the last cycle, the melting curve was generated by starting fluorescence acquisition at 65℃, and taking measurements every 0.2℃ until a temperature of 95℃. Product sizes were confirmed by electrophoresis on a standard 2% agarose gel stained with ethidium bromide, and visualized by exposure to ultraviolet light.

8. Experimental Design

In Expreriment 1, Effect of different sources of donor cells on the developmental rate of nuclear transfer embryos. In Experiment 2, the apoptosis and total nuclei of mNT and pNT blastocysts were detected. In Experiment 3, the apoptosis and imprinting gene expression in mNT and pNT blastocysts were determined.

Table 1. Primer sequences and cycling condition used in realtime RT-PCR

Gene	Primer sequence (5'-3')	Size (bp)	Realtime RT-PCR
β-Actin	5'-CAC TGG CAT TGT CAT GGA CT-3' 3'-GAG AAG AGC TAC GAG CTG CC-5'	285	60℃, 12 sec, 45 cycle
pBax-α	5'-ACT GGA CAG TAA CAT GGA GC-3' 3'-GTC CCA AAG TAG GAG AGG AG-5	294	63℃, 13 sec, 50 cycle
pBcl-xl	5'-GTT GAC TTT CTC TCC TAC AAG C-3' 5'-GGT ACC TCA GTT CAA ACT CAT C-3'	277	62℃, 13 sec, 50 cycle
pH19	5'-AAA GAG CAT CTC AAG CGA GTC T-3' 3'-GCT CCT GTA CCT GCT ACT AAA TGA A-5	180	60℃, 12 sec, 50 cycle
pIGF2	5'-CTC GTG CTG CTA TGC TGC TTA C-3' 5'-CAG GTG TCA TAG CGG AAG AAC T-3'	300	62℃, 12 sec, 55 cycle
pIGF2r	5'-ATA AAC ACC AAT ATA ACA CT-3' 5'-GCA CAC GTT AAT ATA AAA CT-3'	261	62℃, 12 sec, 45 cycle
pXist	5'-ACT AGT GAT GGT TAT GAA AA-3' 5'-GTA AGA GGA AAG AAA TGA AG-3'	217	62℃, 12 sec, 45 cycle

9. Statistical Analysis

To determine the statistical significance between treatments, the rates of cleavage and blastocyst formation, total cell number and percentage of apoptosis were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using the Duncan's multiple range-test. All data were expressed as Least Square (LS) mean±SEM (Standard Error of the sample Mean). Difference were considered significant at $p<0.05$.

RESULTS

1. *In Vitro* Development of Nuclear Transfer Embryos

Table 2 shows developmental rates of pNT (Landrace pig ear fibroblast nuclear transfer) and mNT (Miniature pig ear fibroblast nuclear transfer) embryos. There was no significant difference in fusion and developmental rates to the blastocyst stage between pNT and mNT. Blastocyst formation rates were 29.7 and 29.4%, respectively.

2. Apoptosis and Total Cells in pNT and mNT Blastocysts

Although blastocyst formation rate was not different between two NT groups, the apoptosis rate was significantly lower ($p<0.05$) in pNT blastocysts than in mNT blastocyst (1.7 vs. 6.1%, respectively, Table 3). Also the number of nuclei in blastocysts was significantly higher in the pNT than the mNT (35.8 vs. 29.3; $p<0.05$, Table 3).

3. Analysis of Apoptosis Gene Expression in Blastocysts

In mNT blastocysts, the relative abundance of Bax-α mRNA expression was higher than in pNT blastocyst (Fig. 1, $p<0.05$).

There was no difference in the relative Bcl-xl mRNA expression between mNT and pNT. The relative abundance of Bax-α/Bcl-xl was significantly higher in mNT blastocysts than in pNT blastocyst ($p<0.05$).

Table 3. Apoptosis in porcine blastocysts derived from pNT or mNT

Source of donor cell	No. of blastocyst	No. of total cells (Mean±SE)	Apoptotic cell	% TUNEL (Mean±SE)
Landrace (pNT)	56	2,002 (35.8±1.64)^{a}	34	1.7±0.35^{b}
Mini pig (mNT)	49	1,434 (29.3±1.34)^{b}	88	6.1±1.16^{a}

[a,b] Values with different superscripts differ significantly ($p<0.05$).

4. Imprinting Gene Expression Pattern in pNT, mNT and *In Vivo* Blastocysts

As shown in Fig. 2, the relative gene expression of H19 and IGF2r was significantly higher in *in vivo* blastocysts than in NT blastocysts. Relative IGF2 mRNA expression was significantly higher in both mNT and pNT blastocysts than in *in vivo* blastocysts ($p<0.05$). In case of Xist, *in vivo* blastocysts showed significantly higher expression level than that of mNT or pNT blastocysts ($p<0.05$), but there was no difference between mNT and pNT. The majority of imprinting genes were expressed aberrantly in cloned blastocysts compared to *in vivo* blastosysts. Although imprinting genes were expressed aberrantly in mNT and pNT compared to *in vivo* blastocyst, mNT showed more aberrant expression patterns than pNT (Fig. 2).

DISCUSSION

This study was conducted to investigate the development and gene expression of somatic cell NT embryos generated using miniature pig and Landrace pig cells. In this study, gene expression of apotosis and imprinting genes showed significant differences, while there was no significant difference in fusion and developmental rates between mNT and pNT embryos.

Table 2. *In vitro* development of porcine cloned embryos derived from pNT or mNT

Source of donor cell	No. of oocytes manipulated*	No. (%) of oocytes fused	No. (%) of oocytes cleaved	No. (Mean±SE) of blastocysts
Landrace (pNT)	372	303 (81.5±0.004)	237 (78.2±0.01)	90 (29.7±0.03)
Mini pig (mNT)	299	245 (81.9±0.012)	194 (79.2±0.03)	67 (27.4±0.01)

* Total number of oocytes from three replicates.

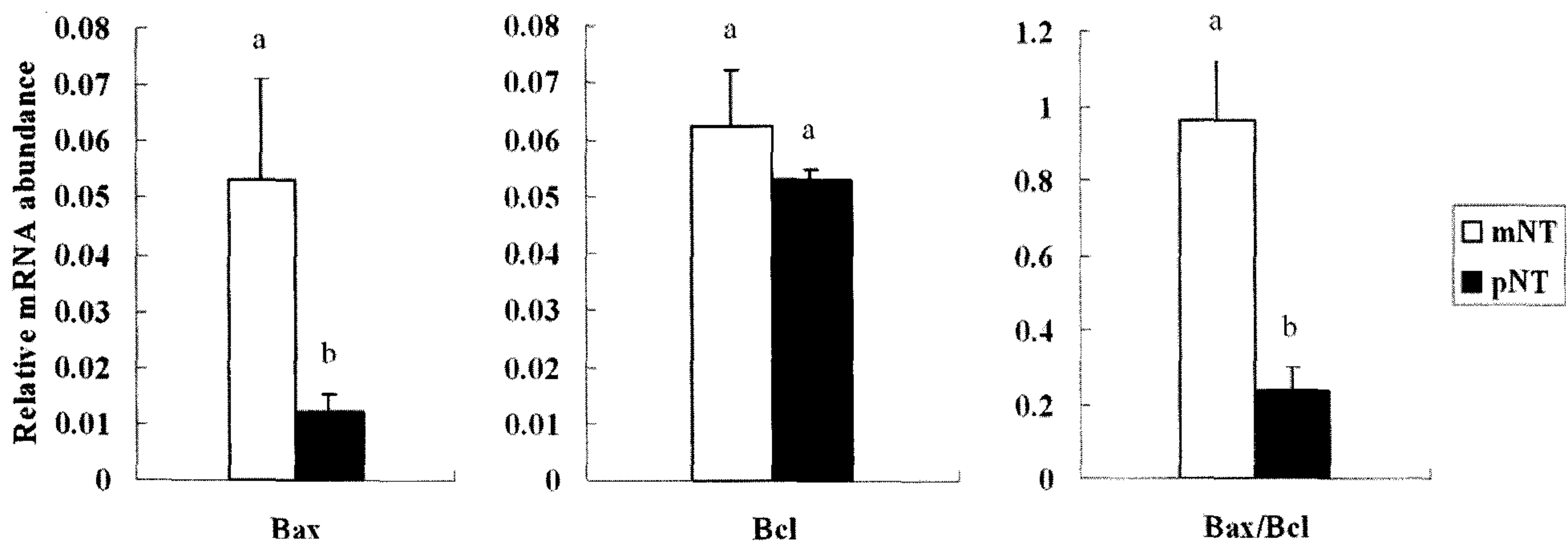

Fig. 1. Relative levels of Bax-α, Bcl-xl and Bax-α/Bcl-xl gene expression in mNT and pNT blastocysts. mRNA from pools of blastocyst was reverse transcribed, and subjected to Realtime RT-PCR using transcript-specific primers (Table 1). Gene expression was normalized for β-actin mRNA expression. Bars with different superscripts are significantly different between the blastocysts (p<0.05).

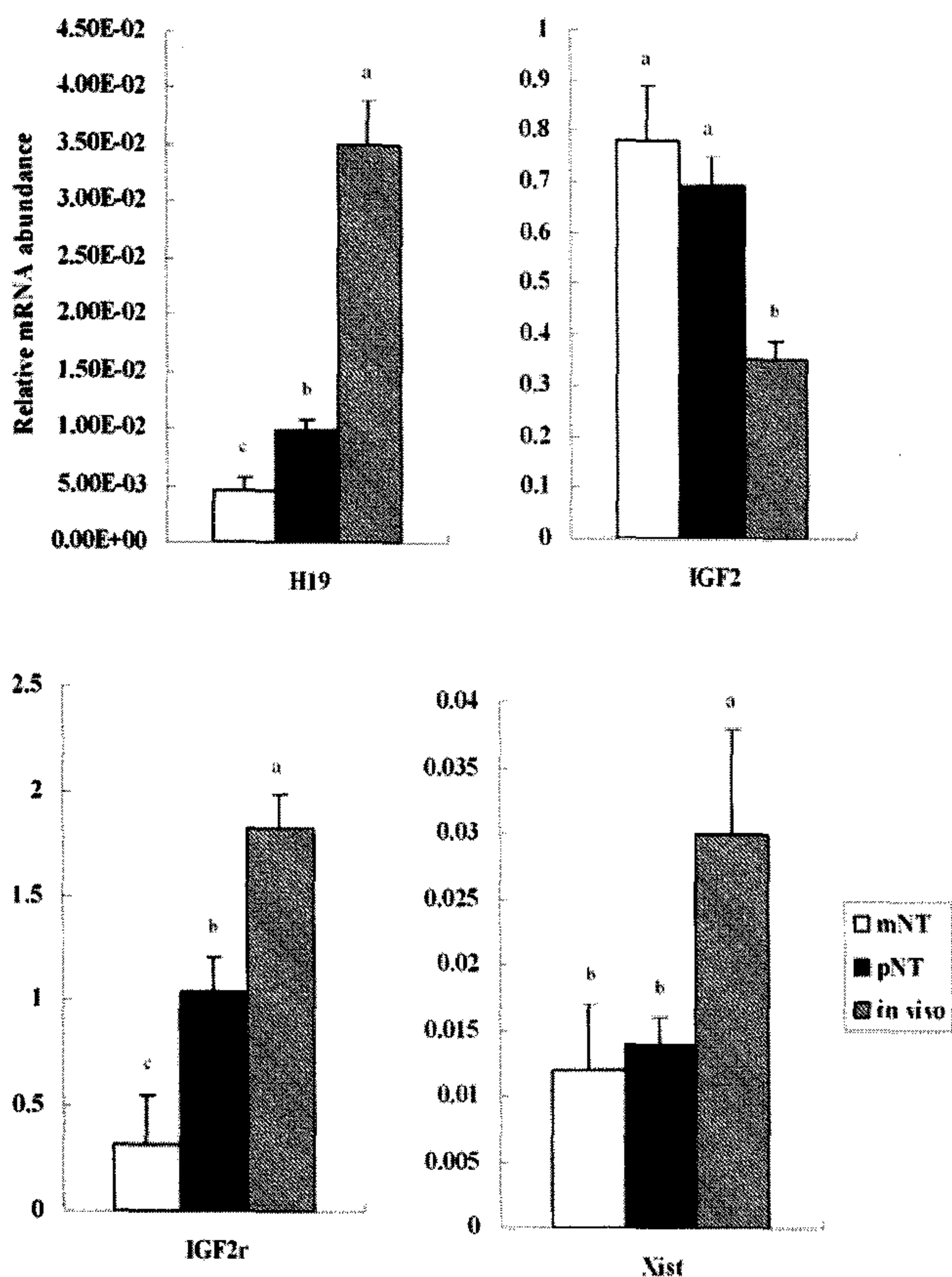

Fig. 2. Relative levels of H19, IGF2, IGF2r and Xist gene expression of mNT, pNT and *in vivo* blastocysts. mRNA from pools of blastocyst was reverse transcribed, and subjected to Realtime RT-PCR using transcript-specific primers (Table 1). Gene expression was normalized for β-actin mRNA expression. Bars with different superscripts are significantly different among the blastocysts (p<0.05).

These results are similar to the report that the more close the species of the donor cell is to the recipient oocyte (yak versus takin), the greater the blastocyst development *in vitro* (Li *et al.*, 2006).

Analysis of gene expression during NT embryogenesis is a valuable tool to investigate nucleo-cytoplasmic interactions, basic cellular and molecular mechanisms of nuclear reprogramming and remodeling. In this study, although blastocyst formation rate was not different between mNT and pNT, the relative abundance of Bax-α/Bcl- xl mRNA expression in mNT blastocysts was significantly higher than in pNT blastocysts. The process of apoptotic cell death in preimplantation mammalian embryos has been well described. Apoptosis plays an important role in embryo development (Levy *et al.*, 2001; Feugang *et al.*, 2003; Gjorret *et al.*, 2003). Apoptosis occurs during the preimplantation stage in both *in vivo*-and *in vitro*- produced embryos, and it can contribute to low developmental rate and embryonic loss. The incidence of apoptosis is higher in bovine blastocysts produced by NT than in blastocysts produced *in vivo* (Feugang *et al.*, 2003; Gjorret *et al.*, 2003; Lee *et al.*, 2007). The percentage of cells undergoing apoptosis in porcine NT embryos was higher than that of IVF embryos and increased with time *in vitro* (Hao *et al.*, 2003). Key regulators of apoptosis in development, autoimmunity and disease include proteins of the Bcl-2 family (Adams & Cory, 1998; Korsmeyer, 1999) that contain both anti-apoptotic (e.g., Bcl-2 and Bcl-xl) and pro-apoptotic (e.g., Bax) members.

In the present study, although imprinting genes were expressed aberrantly in cloned blastocysts compared to *in vivo* blasto-

systs, mNT showed more aberrant expression patterns than pNT (Fig. 2). The role of genomic imprinting in governing the level of allelic activity is an absolute requirement for normal mammalian embryogenesis (Sasaki *et al.*, 1993; Surani *et al.*, 1990). Fundamental disturbances in the imprinting process, which preclude the balanced inheritance of differentially marked maternal and paternal alleles, frequently result in abnormal embryonic development (McGrath & Solter, 1984; Surani *et al.*, 1984). Of the maternally imprinted genes, H19 mRNA is expressed abundantly in the human placenta and in several embryonic tissues. Although the function of the H19 transcript is unclear, it is closely linked to IGF2 gene in both the mouse and human and regulates imprinting of IGF2 gene by sharing an imprinting control element (Bartolomei *et al.*, 1991). In deceased animals generated by somatic cell nuclear transfer, biallelic expression of the H19 gene was found, suggesting the disruption of imprinting, which may have contributed to their abnormal development. Biallelic expression of the H19 gene has been reported in cloned mouse embryos, along with imprinting disruptions of other genes as well (Mann *et al.*, 2003). H19 gene is imprinted in domestic cattle and imprinting disruption of H19 can be present in developmentally abnormal animals produced by nuclear transfer (Zhang *et al.*, 2004). However, although it is increased in tissue, IGF2 concentration is not elevated in serum in H19-null mice in which elevated expression of IGF2 is responsible for the fetal overgrowth (Eggenschwiler *et al.*, 1997). Paternally imprinted genes included Xist and IGF2. Xist is required for X inactivation (Norris *et al.*, 1994) and is also expressed strongly in the blastocyst stage (Hartshorn *et al.*, 2002; De La Fuente *et al.*, 1999; Peippo *et al.*, 2002). In addition, transcriptional defect of Xist is lethal in the early developmental stage (Takagi & Abe, 1990; Migeon *et al.*, 1993). Expression of IGF2 gene has major dosage-sensitive effects on fetal growth. Over-expression results in fetal overgrowth, fetal lethality and skeletal abnormalities, while deletion results in growth deficiency at birth. Given its importance for fetal and neonatal growth, it has been proposed that factors regulating the expression of IGF2 may also be imprinted (Moor & Haig, 1991; Haig, 1992). IGF2r gene is expressed exclusively from the maternally inherited allele whereas the paternally-derived allele remains silent (Stoger, 1993; Barlow *et al.*, 1991). It is also known that IGF2r is involved in embryonic growth control (Wang *et al.*, 1994).

In conclusion, the present results suggest that the NT between donor cells derived from miniature pig and recipient oocytes derived from crossbred pig might affect reprogramming of donor cell, resulting in high apoptosis and aberrant expression patterns of imprinting genes.

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(접수일: 2008. 6. 16 / 채택일: 2008. 6. 20)