Comparisons of Developmental Potential and Gene Expression Level in Porcine Nuclear Transfer, Parthenogenetic and Fertilized Embryos

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ABSTRACT

This study was conducted to detect the apoptosis incidence in blastocysts and to compare the abundance of *Bax*, *Bcl2L1*, *VEGF* and *FGFR2* in *in vitro* fertilized (IVF), parthenogenetic (PAT) and nuclear transfer (NT) embryos. Oocytes matured for 40 hr were enucleated and reconstructed with confluenced fetal fibroblasts (FFs) derived from a ~45 day fetus. Reconstructed eggs were then fused with 2 DC pulses (2.0 kV/cm, 30 μsec) and cultured with 7.5 μg/ml cytochalasin B for 3 hr. Parthenotes (PAT) were produced with the same electric strength and culture for NT eggs. The embryos were cultured in NCSU-23 medium at 39℃, 5% CO_2, 5% O_2 in air. In 3 runs, set of 10 embryos at the 4-cell to blastocyst stages were used to extract total RNA for analyzing the gene expression patterns of pro-apoptotic (*Bax*), anti-apoptotic (*Bcl2L1*), vasculogenesis (*VEGF*), implantation (*FGFR2III*) using real-time quantitative PCR. Cleavage and blastocyst rates were significantly higher ($P<0.05$) in IVF and PAT (79.3±8.5 and 25.5±6.1, and 85.0±6.4 and 38.6±5.5, respectively) than NT counterparts (65.1±5.2 and 15.6±3.0, respectively). Significantly higher ($P<0.05$) total cells were observed in IVF controls and PAT (34.7±5.8 and 38.1±4.1) than NT embryos (24.8±3.2). Apoptosis index was significantly lower ($P<0.05$) in IVF than NT embryos. The Relative abundances (RA) of *Bax* and *VEGF* were significantly higher ($P<0.05$) at blastocyst stage in NT than IVF control. The RA of *Bcl2L1* and *FGFR2III* were significantly higher ($P<0.05$) at blastocyst stage in IVF than NT. The present study observed the abnormal gene expressions in NT embryos at various developmental stages, suggesting certain clues to find out the cause of the low efficiency of NT to term.

(Key words : Porcine, Apoptosis, Nuclear transfer, Gene expression)

INTRODUCTION

Since the production of a cloned sheep with a somatic cell (Wilmut *et al.*, 1997), somatic cell nuclear transfer (SCNT) technique has been applied to achieve the successful offspring across a range of species (Kato *et al.*, 1998; Wakayama *et al.*, 1998; Polejaeva *et al.*, 2000; Shin *et al.*, 2002; Galli *et al.*, 2003). Among domestic animals, pig has drawn attentions for generating animal models, human disease, designing animal bioreactors to harvest secreted proteins and producing tissues or organs in order to use in xenotransplantation (Lai *et al.*, 2002; Park *et al.*, 2002). However, the potential of SCNT embryos to develop to term remains still low, due to the high mortality of embryo and fetus in early developmental stage, stillbirth and neonatal death immediately after birth (Wilmut *et al.*, 2002). The failure to get the healthy offspring could result from several reasons, such as deficiencies in DNA fragmentation, nuclear reprogramming and globalgene expression.

Hardy (1999) reported that apoptosis may have an important role in the degeneration of pre-implantation embryos during *in vitro* culture. No cell death can be found during normal development of early embryos, and apoptosis can only be detected under abnormal conditions to eliminate defective embryonic cells (Raff *et al.*, 1993). Apoptosis levels are important parameters that are emerging as useful indicators of embryo development and quality (Watson *et al.*, 1994; Brison *et al.*, 1997, 1998). Consequently, low offspring or high embryonic loss of cloned embryos could be attributed to the decline of normally functional cells along with

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increased number of apoptotic cells.

Incomplete or incorrect nuclear reprogramming of the donor cell transferred into the oocyte is considered to be the primary cause to deteriorate the developmental competence of cloned embryos, resulting in genetic and epigenetic modifications that show throughout embryonic development (Latham, 2004; Santos and Dean, 2004; Smith and Murphy, 2004; Tamada and Kikyo, 2004). In other words, successful cloning is likely to need the epigenetic information of the donor nucleus to be reprogrammed to an embryonic state (Sebastiano *et al.*, 2005). Most of the cloned embryos show a severely deficient and aberrant epigenetic reprogramming, such as DNA methylation (Dean *et al.*, 2001; Kang *et al.*, 2001; Cezar, 2003; Chung *et al.*, 2003; Mann *et al.*, 2003; Enright *et al.*, 2005), histone acetylation (Enright *et al.*, 2003; Santos *et al.*, 2003) and chromatin organization (Kim *et al.*, 2002; Vignon *et al.*, 2002). This may lead to alteration in the transcriptional status of bulky genes. Many recent studies on NT embryos have demonstrated that gene expression patterns in the embryo, fetus and placenta are abnormal (Zhang *et al.*, 2004; Zhu *et al.*, 2004; Hall *et al.*, 2005; Li *et al.*, 2005). Sinceembryonic development requires the correct temporal and spatial expression of many genes, the functions of which are essential for survival (Zhu *et al.*, 2004). Hence, it is essential to analyze the genes related to early embryonic development, so that it would be possible to provide useful information for improvement of cloned pig production.

However, the amount of information gathered on the profile of gene expression in NT pre-implantation embryos is still scarce and limited to a handful of genes described in two species, bovine and mouse (Campbell *et al.*, 2005). Therefore, this study was conducted to detect the apoptosis incidence in blastocysts and to compare the abundance of *Bax*, *Bcl2L1*, *VEGF* and *FGFR2 in vitro* fertilized (IVF), parthenogenetic (PAT) and NT-derived 4-cell, 8-cell, morula and blastocyst stage embryos.

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

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's Phosphate buffered saline (DPBS) supplemented with 10% fetal bovine serum (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 Dulbecco's modified eagle medium (DMEM) to take cell pellet. Fetal fibroblasts 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 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 in order to synchronize the majority of cells at 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 Advanced DMEM with 10% FBS.

Oocyte Collection and *In Vitro* Maturation

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in Phosphate buffered saline (PBS)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 nutrient mixture F-10 (Ham-F10) and two times with *in vitro* maturation medium (IVM) 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 Luteinizing hormone, 0.5 μg/ml Follicle stimulating hormone. Sets of 50 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, parthenote (PAT) 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). Briefly, denuded MII-stage oocytes were enucleated by micromanipulation technique in Hepes-buffered M-199 supplemented with 10% FBS, and 12 mM sorbitol. 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 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.

Activation

Cumulus-free oocytes were transferred into BTX Electro chamber filled with 0.28 M mannitol solution containing 0.01% (W/V) BSA, 0.05 μM $CaCl_2$ and 0.01 μM $MgSO_4$ and pulsed twice with 2.0 kV/cm DC for 30 μsec using a BTX Electro Square Porator (ECM 830, BTX, Inc.) and were cultured in 5.5 mM glucose free NCSU23 medium containing 7.5 μg/ml CCB at 38.5 ℃ in a humidified atmosphere of 5% CO_2 in air for 3 hr.

In Vitro Culture (IVC)

IVF, PATand 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 4 days. Cleavage and blastocyst rates were assessed on Days 2 and 6, respectively.

Each ten embryos were pooled at 48, 72, 120 and 144 hrs after insemination or fusion/activation for obtaining 4-cell, 8-cell, morula and blastocyst stage embryos, respectively, and snapped in liquid nitrogen until the analysis of transcript abundance.

Cytological Analysis

To count total cell number, day-6 blastocysts fixed in methanol-acetic acid (3:1) for overnight were stained with 10 μg/ml bisbenzimide 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 room temperature (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/ml RNase 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.

Total apoptotic indices were calculated for each embryo as follows: Apoptotic index = (number of TUNEL - positive nuclei / total number of nuclei blastocyst) × 100.

RNA Extraction and Reverse Transcription

Total RNA was extracted from pools (triplicates) of 10 embryos at the 4-cell, 8-cell, morula and blastocyst stages from IVF, PAT and NT using Dynabeads® mRNA DIRECT™ kit (Dynal, Oslo, Norway). Briefly, samples were lysed in 50 μl lysis/binding buffer (Dynal). After vortexing and brief centrifugation, the samples were incubated at RT for 10 min. A total of 100 μl prewashed Dynabeads oligo $(dT)_{20}$ were added to each sample. After 5 min of hybridization, the beads were separated from the binding buffer using the Dynal magnetic separator. Then the beads were washed in buffer A and B (Dynal) and the poly (A) RNA was eluted from the beads by adding 11 μl of tris-HCl. A total of 1.0 μg RNA was converted to cDNA using oligo $(dT)_{20}$ primers and superscript™ III reverse transcriptase (Invitrogen Corporation, CA, USA) in a 20 μl reaction for 50 min at 50℃. PCR was performed with 1~2 μl of cDNA in 100 μl using hotstar taq DNA polymerase (Qiagen GmbH, Germany) to confirm product size. Amplification consisted of 25 or 30 cycles of 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 60 sec performed in a PTC-200 peltier thermal cycler (MJ research, Inc, MT, USA).

Real-Time PCR

Real-time PCR was performed on a LightCycler® using FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) containing $MgCl_2$,

Table 1. Sequence specific primers used for Real Time RT-PCR

Transcript	Primer sequence (5'-3')	GenBank Accession no.
Bax	For - AAGCGCATTGGAGATGAACT Rev CGATCTCGAAGGAAGTCCAG	AJ606301
Bcl2L1	For - GAAACCCCTAGTGCCATCAA Rev GGGACGTCAGGTCACTGAAT	AJ578821
VEGF	For - CTACCTCCACCATGCCAAGT Rev ACACTCCAGACCTTCGTCGT	AF318502
FGFR2III	For ATTCTGTGCCGGATGAAGAC Rev GGTGTTGGAGTTCATGGAGG	BE233277
Histone (H2A)	For - GTGGCAAACAAGGAGGAAAG Rev ATGCGGGTCTTCTTGTTGTC	BP459633

dNTP, and FastStart Taq DNA polymerase. Primer sequences, the size of amplified products and the GenBank accession numbers are shown in Table 1. DNase I treated cDNA (2 μl) from 10 embryos at the 4-cell, 8-cell, morula and blastocyst stage was added to 18 μl of master mix containing 11.6 μl of H_2O PCR grade, 2.4 μl $MgCl_2$ stock, 2 μl SYBR Green mix and 1 μl each of forward and reverse bovine specific primers. The program used for all genes consisted of a denaturing cycle of 10 min at 95℃; 45~50 cycles of PCR (95℃ for 10 sec, 57℃ for 5 sec, and 72℃ for 10 sec); a melting cycle consisting of 95℃ for 10 sec, 70℃ for 15 sec, and a step cycle starting at 70℃ until 95℃ with a 0.1 ℃/sec transition rate; and finally, a cooling cycle of 40℃ for 30 sec. The quantification of gene transcripts such as *Bax*, *Bcl2L1*, *VEGF* and *FGFR2* was carried out in 3 replicates. Histone (H2A) was amplified for every sample to confirm the presence of RNA as a house keeping gene for normalization. Melt curve analysis was conducted to confirm the specificity of each product, and products were electrophoresed on agarose gel to confirm product size. As negative controls, tubes were always prepared in which RNA or reverse transcriptase was omitted during the RT-reaction.

Statistical Analysis

Differences among groups were analyzed using one-way analysis of variance (ANOVA) by SPSS after arcsine transformation of 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 to be significant when $P<0.05$.

RESULTS

Development of Embryos Produced *In Vitro*

The rate of cleavage, development and cell number of blastocysts from different origins are shownin Table 2. In IVF and PAT embryos, 79.3% and 85.0% of oocytes were cleaved, respectively, but did not differ statistically. However, the cleavage rate was significantly ($P<0.05$) lower (65.1%) in NT embryos. The develop-

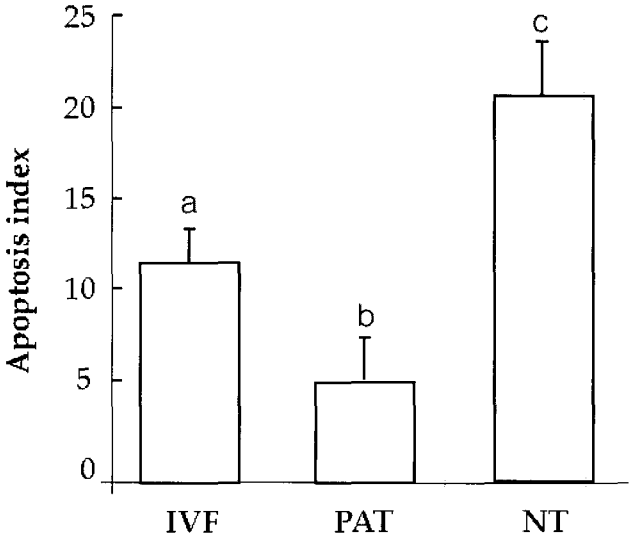

Fig. 1. Apoptosis index in Day-6 blastocyst produced *in vitro*.
[a,b,c] Significantly different ($P<0.05$).

Table 2. Development rate and cell number of embryos produced *in vitro*

Groups*	No. of eggs used	Development to (% mean±SEM)		Total cells (mean±SEM)
		Cleavage	Blastocyst	
IVF	370	293(79.3±8.5)[a]	76(25.1±6.1)[ab]	34.7±5.8[ab]
PAT	567	482(85.0±6.4)[a]	219(36.6±5.5)[a]	38.1±4.1[a]
NT	244	159(65.1±5.2)[b]	25(15.6±4.0)[b]	24.8±3.2[b]

[a,b] Within a column, percentages and mean number with different superscripts differ significantly ($P<0.05$).
* IVF, 5 replicates; PAT, 5 replicates; NT, 9 replicates.
IVF; *in vitro* fertilized, PAT; parthenogenetic, NT; nuclear transferred.

mental rates of PAT to the blastocyst stage were significantly ($P<0.05$) higher than those of IVF and NT (36.6±5.5 vs. 25.1±6.1 and 15.6±4.0%, respectively). In addition, total cell number of PAT blastocyst were significantly ($P<0.05$) higher than those of IVF and NT (38.1±4.1 vs. 34.7±5.8 and 24.8±3.2%, respectively).

Detection of Apoptosis

Fig. 1 shows that the expression of apoptosis in the NT blastocysts was significantly ($P<0.05$) higher than that of IVF and PAT blastocysts (22.8% vs. 12.6% and 4.0%, respectively).

Relative Abundance of Selected Genes at Various Stages of Embryo Development

The relative abundance of specific transcripts in embryos is shown in Fig. 2. *Bax* transcription levels were low in all groups up to the blastocyst stage except at the 8-cell stage, which showed a slight increase in expression. At the blastocyst stage, *Bax* gene showed a significantly ($P<0.05$) higher expression in NT embryos than IVF counterparts.

For the *Bcl2L1* gene, the 4-cell and 8-cell stages in NT embryos showed significantly ($P<0.05$) higher expression levels than IVF counterparts. However, the expression level of NT morula decreased, showing no significant difference compared to IVF embryos whereas, IVF embryos showed significantly ($P<0.05$) higher expression level than NT counterparts at the blastocyst stage.

VEGF transcription levels of NT embryos were sig-

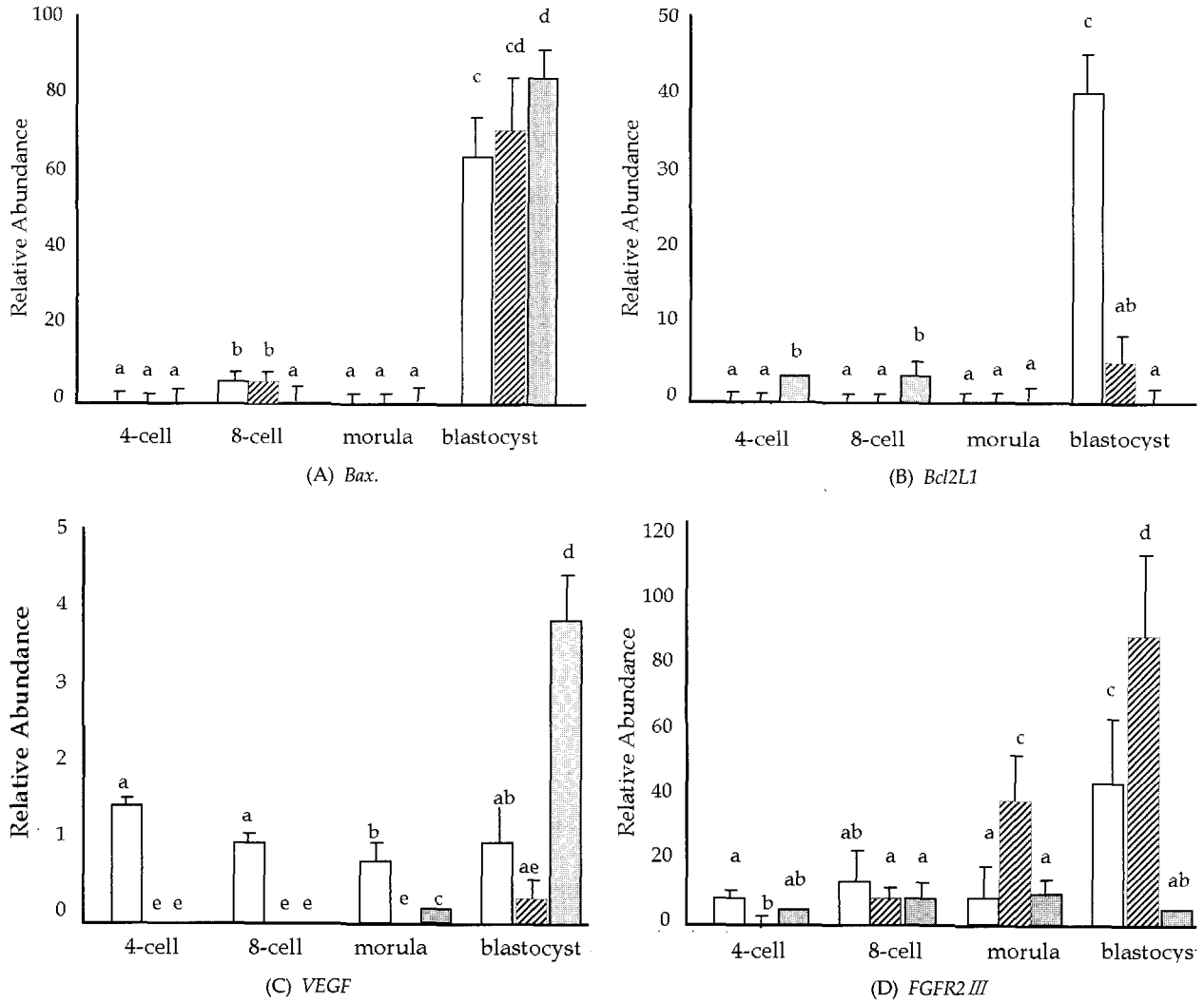

Fig. 2. Relative abundance of six developmentally important genes at different development stages of embryos produced *in vitro*. (A) *Bax*, (B) *Bcl2L1*, (C) *VEGF*, (D) *FGFR2III*. H2A was used as a housekeeping gene for normalization. Open bars indicate IVF, hatched bar PAT and solid bars NT embryos. Data is the mean±SEM. $^{a\sim e}$ Percentages with different superscripts within columns indicate significant differences ($P<0.05$).

nificantly ($P<0.05$) lower than IVF counterparts up to the morula stage, then showed a significantly ($P<0.05$) higher expression at the blastocyst stage.

*FGFR2II I*transcripts were detected in all groups through all stages. The expression level of IVF and NT embryos from the 4-cell to morula stage was not significantly different, but IVF blastocysts showed significantly ($P<0.05$) higher expression level than NT counterparts.

DISCUSSION

Recently porcine have been given attention to several advantages, like xenotransplantation and animal model for human diseases, thereby satisfying several purposes required for the production of SCNT embryos. However, NT embryos compared to IVF or *in vivo* derived embryos showed generally poor developmental ability and incompetent survivability after transfer to the recipients, mainly due to a few total cell number, shortened telomere length, high apoptosis incidence, incomplete epigenetic reprogramming and abnormal gene expressions in many animals (Zhu *et al.*, 2004; Betts *et al.*, 2005; Booth *et al.*, 2005; Gjorret *et al.*, 2005; Pomar *et al.*, 2005). Among those causes, the high apoptosis incidence and aberrant (or even lack of) expression of certain developmentally important genes have been implicated as primary reasons for the low efficiency of SCNT (Hao *et al.*, 2004; Gjorret *et al.*, 2005). Therefore, this study was conducted to investigate the difference of apoptosis incidence and gene expression among embryos produced *in vitro.*

Apoptosis plays an important role in embryo development. The process of apoptotic cell death in preimplantation mammalian embryos has been well described (Levy *et al.*, 2001; Feugang *et al.*, 2002; Gjorret *et al.*, 2002). Apoptosis occurs during the preimplantation development stage in both *in vivo* and *in vitro* produced embryos, and it may contribute to embryonic loss. The incidence of apoptosis is higher in NT derived bovine blastocysts than in embryos produced *in vivo* (Feugang *et al.*, 2002; Gjorret *et al.*, 2002). Human preimplantation embryos exhibit high levels of apoptosis and high rates of developmental arrest during the first week *in vitro* (Hardy, 1999). Thus, apoptosis may contribute to the progressive loss of embryos during the *in vitro* production procedure. Hao *et al.* (2003) have reported that NT embryos exhibited a very high degree of apoptosis as compared to IVF embryos, consistent with results reported by Gjorret *et al.* (2002). In the present study, the higher incidence of apoptosis was observed in NT blastocysts compared to IVF counterparts. However, in contrast to the result by Hao *et al.* (2004), it is demonstrated that IVF blastocysts showed significantly ($P<0.05$) higher apoptosis incidence than PAT counterparts. This difference might be due to the activation protocol and haploid rate. Hao *et al.* (2004) used electric pulse to activate oocytes and the haploid rate in PAT was significantly ($P<0.05$) higher than IVF embryos. Kim *et al.* (2005) have shown that there was significantly ($P<0.05$) higher proportion of haploid in Day-7 NT blastocysts activated with electric pulse than electric pulse and chemicals. In the present study, cytochalasin B, protein synthesis inhibitor, was used to suppress the extrusion of the first polar body. As described by Liu *et al.* (2002), haploid leads to an increased incidence of apoptosis. Therefore, the use ofcytochalasin B might influence the outcome of apoptosis, so that it is interesting to check the effect of different activation protocols on the incidence of apoptosis.

Embryonic development requires the correct temporal and spatial expression of many genes, of which the functions are essential for survival. For example, disrupted expression of *VEGF* or *FGFR2* results in embryonic lethality during early development (Carmeliet *et al.*, 1996; Arman *et al.*, 1998). Abnormal gene expression is frequently observed in NT embryos and is one of the suggested causes of the low success rates of this approach. The present study demonstrated that the transcription timing and levels of the genes examined in NT embryos were significantly different from IVF counterparts.

Apoptosis, a type of programmed cell death, is a common feature of mammalian development (Jacobson *et al.*, 1997). Environmental stresses such as those imposed by *in vitro* culturing can induce unscheduled apoptosis in cultured embryos, which may lead to arrest or abnormal development and lower viability of embryos. There are at least two major protein families involved in the regulation of apoptosis, namely, *Bcl-2* and *Bax*. The *Bcl-2* family members include *Bcl-2, Bcl-xL, Bcl-x,* and *Bcl-w* has been shown to protect cells from apoptosis whereas the *Bax* family members *Bad, Bax,* and *Bak* induce apoptosis in somatic cells. Therefore, the progression in the apoptotic pathway seems to be achieved by the balance of the expression of several conserved genes that have either a pro- or anti-apoptotic effect. Juriscova *et al.* (1998) has shown that the *Bcl-2* apoptotic gene family was expressed in the preimplantation stage of embryo development. *Bcl-2-* related gene products are known to act as a distal step in the apoptotic pathway and may either suppress (*Bcl-2, Bcl-xL*) or promote (*Bax, Bad*) the induction of apoptosis. In the present study, significantly higher expression of *Bax* mRNA and lower expression of *Bcl2L1* mRNA was observed at the blastocyst stage. It could be the counterbalance to overcome the possible defect induced by the *in vitro* culture condition or micromanipulation procedure. Interestingly, the expression of

Bax mRNA in NT embryos was not detected from the 4 cell up to the morula stage in this study. In morphological and biological studies of apoptosis incidence by Hao *et al.* (2003), they detected the onset of apoptosis signal from Day 5 embryos. It is not clear why no expression was observed earlier. It needs to be elucidated by comparing the morphological and biological parameters with geneexpression levels at various developmental stages, so that it might be possible to see the related factors between those results. In addition, *Bcl2L1* mRNA was not expressed at the morula and blastocyst stage in NT embryos. It might be due to the improper reprogramming of donor nucleus injected into the oocytes.

Vascular endothelial growth factor (*VEGF*), also known as vascular permeability factor, has been implicated in the regulation of blood vessel formation (i.e., vasculogenesis and angiogenesis). Development of the cardiovascular system depends on the generation of precise *VEGF* concentration gradients. A decrease in the amount of *VEGF* produced during development of the embryo may lead to decreased angiogenesis, with fatal consequences (Ferrara *et al.*, 1996; Carmeliet *et al.*, 1996). *VEGF* modulates early heart valve formation (Dor *et al.*, 2003). Significantly higher expression of *VEGF* was seen in cloned animal heart, spleen and brain compared to controls. Coincidentally aberrant valvulogenesis was seen in cloned heart, and congestion and hemorrhage were seen in some cloned heart, brain, and spleen. The aberrant expression of *VEGF* may contribute to these defects as reported in the clones that died soon after birth (Li *et al.*, 2005). The present study observed the *VEGF* expression in IVF embryos from the 4-cell stage up to the morula stage, which is significantly higher than NT counterparts. However, NT embryos at the blastocyst stage showed significantly higher expression level than IVF controls. This aberrant expression level of *VEGF* might cause the abnormal vascular formation in post-implantation period, failing to maintain the pregnancy.

The *FGF* receptor family is known to be strongly expressed at the blastocyst stage and play critical roles in post-blastocyst development and implantation (Rappolee *et al.*, 1998; Taniguchi *et al.*, 1998). Knockout of *FGFR2*, a member of the *FGF* receptor family, in mice results in developmental arrest in the blastocyst stage (Arman *et al.*, 1998). Previously, both normal and abnormal expressions of *FGFR2* have been reported. In bovine granulosa cell NT embryos, *FGFR2* expression was found to be down regulated. Furthermore, normal *FGFR2* expression has also been reported and, in this case, is suggested to be related to the donor cell line used in the NT experiments (Daniels *et al.*, 2000). This study observed the significantly lower expression of *FGFR2* mRNA in NT blastocysts than IVF counterparts. Like the up-regulated *VEGF* expression of NT blastocysts, it might be one of the causes to yield the unsuccessful pregnancy.

In conclusions, gene expressions were different in embryos at various developmental stagesderived from different origin. This could provide some clues to elucidatethe cause of the low efficiency of SCNT to term. Many other genes are involved in mammalian embryonic development and organogenesis, and hence further research on suchgenes will increase our understanding of nuclear reprogramming events following SCNT, embryonic development, and organogenesis. Furthermore, it is noteworthy to investigate the gene expression patterns during post-implantation (early, mid and late) stages, following transfers of IVF, PTA and SCNT embryos.

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