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Effect of Donor Cell Types and Passages on Preimplantation Development and Apoptosis in Porcine Cloned Embryos

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ABSTRACT : In this study, two cell types from porcine females, namely fetal fibroblasts (pFFs) and adult ear fibroblasts (pAEFs) and two passages (3-4 and 7-8) were investigated by evaluating the development rate, blastocyst cell number and the incidence of apoptosis. No significant differences were observed in the cleavage rates of cloned and IVF embryos. The blastocyst rates between the embryos cloned with pFFs (15.1±3.2) and pAEFs (10.4±2.6) did not differ significantly but was significantly ($p<0.05$) lower in pAEFs than that in IVF (22.5±4.5) embryos. Total cell number in pFFs (28.4±4.3) and pAEFs cloned blastocysts (24.2±5.1) was significantly ($p<0.05$) lesser than IVF control (35.4±3.2). Apoptosis rates between cloned blastocysts differed significantly ($p<0.05$) and were significantly ($p<0.05$) higher than IVF embryos. The blastocyst rates between the cloned embryos cloned with different cell passages did not differ significantly but in embryos cloned with 7-8 cell passage was significantly ($p<0.05$) lower than the IVF control. Apoptosis signals were detected in IVF and cloned embryos as early as day 3 and the rates of apoptosis increased concurrently with the embryo development. In conclusion, high apoptosis during *in vitro* preimplantation development resulted in low development rate and total cell number of cloned embryos. Moreover, based on the apoptotic incidence in cloned blastocysts, fetal fibroblasts are more suitable for production of cloned embryos in porcine. (**Key Words :** Donor Cells, Passage, Apoptosis, Development, Cloned Embryos, Porcine)

INTRODUCTION

Since the birth of a cloned sheep (Wilmut et al., 1997), somatic cell nuclear transfer (SCNT) in mammalian species has been developed and advanced in the last 8 years. Successful cloning has now been achieved in several mammalian species as reported by the birth of offspring in cattle, goats, pigs, mice, and rat (Cibelli et al., 1998; Kato et al., 1998; Wakayama and Yanagimachi 1999; Polejaeva et al., 2000). However, there still remains a significant problem associated with low efficiency of the procedure resulting in the high rate of the loss of embryos and fetuses throughout gestation.

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Among many factors affecting the success of cloning efficiency, apoptotic occurrence plays a pivotal role (Hao et al., 2004; Gjorret et al., 2005). The process of apoptotic cell death in preimplantation mammalian embryos has been well described (Levy et al., 2001). Two forms of cell death are known and can be distinguished on the basis of morphological and molecular criteria (Savill, 1994). Necrosis is characterized by nuclear disintegration, cellular swelling and rupture of internal and external membranes with release of lytic enzymes and damage to surrounding cell. In contrast, apoptosis characteristically affects single cell rather than group of cells. Apoptosis-inducing events include various environmental stressors in addition to gross chromosome abnormalities. Morphological features of this cell death include cytoplasm and chromatin condensation, DNA fragmentation, with the separation of the nucleus into discrete masses (Otti et al., 1999; Fahrudin et al., 2002; Neuber et al., 2002; Pomar et al., 2004).

Research has suggested that a major cause for the level of cell death can be reconciled with the high level of embryo arrest (Hao et al., 2004). Since the developmental competence of embryos is already established at the zygote stage, the generation of a healthy zygote is important in

understanding the mechanism that causes chromosomal abnormalities during early cleavage stages (Hardy et al., 2001). Apoptosis has been observed in bovine embryos after the 8-cell stage (Neuber et al., 2002). More than 80% of *in vivo* mouse blastocysts on day 4 or 5 had one or more apoptotic cells (Lui et al., 2001). During *in vitro* culture in pigs, the SCNT embryos exhibited higher rates of cytoplasmic fragmentation and developmental arrest as well as high levels of apoptotic cells than IVF embryos (Hao et al., 2003). However, Fahrudin et al. (2002) demonstrated that SCNT embyros before day 5 of culture did not reveal apoptotic cells. Therefore, apoptosis levels are important parameter and emerging as a useful indicator of early embryonic development and quality.

With this in view, two cell types, namely fetal fibroblasts (pFFs) and adult ear fibroblasts (pAEFs) and two passages (3-4 and 7-8) were investigated by evaluating the development rate, blastocyst cell number and the incidence of apoptosis in cloned embryos compared to those of *in vitro* fertilization (IVF) counterparts in porcine.

MATERIALS AND METHODS

Chemicals and media

All chemicals used in this study were purchased from the Sigma Chemical Company (St. Louis, MO, USA) and media from GIBCO (Life Technologies, Rockville, MD, USA), unless otherwise stated. The medium used for *in vitro* maturation (IVM) was Tissue culture medium 199 (TCM-199) containing Earle's salts, 0.1% polyvinylalcohol (PVA), 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 25 mM HEPES, 2.5 mM Na pyruvate, 1 mM L-glutamine, and 1% penicillin-streptomycin (10,000 IU and 10,000 μg/ml, respectively) with 0.5 μg/ml luteinizing hormone (LH) and 0.5 μg/ml follicle stimulating hormone (FSH) (IVM+H) and without hormones (IVM–H) (Abeydeera et al., 2000). The medium used for IVF was antibiotic free modified Tris-buffered medium (mTBM), consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (crystallized free base; Fisher Scientific, Fair Lawn, NJ, USA), 11 mM glucose, 5 mM Na pyruvate, 2.5 mM caffeine and 4 mg/ml fatty acid free bovine serum albumin (BSA, Fraction V). The medium used for culture of fibroblasts was Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) fetal bovine serum (FBS; Life Technologies, Canada), 0.1 mM non-essential amino acids (NEAA) and 0.5 μl/ml gentamycin (Biowhittaker, Walkesville, MD, USA). Embryo culture medium was North Carolina State University 23 (NCSU 23) supplemented with 4 mg/ml BSA (Petters and Wells, 1993). Tyrode's albumin lactate pyruvate medium containing 2 mg/ml BSA and 10 mM Hepes (HEPES-TALP) was used for oocytes micromanipulation. For all the media, the pH was adjusted to 7.4 and the osmolality to 280 mOsm/kg.

Preparation of donor cells

Two cell types from porcine females, namely pFFs and pAESFs were used in this study. pFFs were prepared from a female fetus obtained via hysterectomy of pregnant gilt on day ~30 of gestation. pAESFs were prepared from cells taken from the ear of a 2-year old sow. pFFs and pAEFs were prepared by digestion in 0.05% Trypsin-elthylenediamine tetra acetic acid (EDTA) solution at 39°C for 15 min, followed by dispersal in DMEM containing 7% (v/v) fetal calf serum (FCS) by pipetting to make a single cell suspension. After being washed by centrifugation at 300×g for 10 min, two types of isolated cells at a final concentration of 2×10^5 cell/ml were seeded on to 35 mm plastic culture dishes (Nunc, Roskilde, Denmark), and subsequently cultured to confluence for 6-8 days at 39°C in a humidified atmosphere of 5% CO_2 in air. For nuclear donor, cells at 3-4 and 7-8 passages were grown to confluence, trypsinized and resuspended in HEPES-TALP medium.

Oocyte collection and *in vitro* maturation

Oocytes were obtained from slaughterhouse ovaries and subjected to IVM based on the protocol of Abeydeera et al. (1998, 2000) with minor modifications. Briefly, COCs were aspirated from antral follicles of 3-6 mm in diameter with an 18 G needle fitted with a 10 ml syringe. COCs were washed twice with IVM-H medium and once in IVM+H medium. COCs with uniform cytoplasm and multilayered cumulus cell mass were selected for the experiments. Sets of 50 COCs were matured in 500 μl of IVM+H medium in a 4-well multidish (Nunc) under paraffin oil (Yakuri, Tokyo, Japan) for 22 h at 39°C in a humidified atmosphere of 5% CO_2. COCs were further cultured for an additional 22 h in IVM-H medium. At the end of oocyte maturation, COCs were removed off their cumulus cells by vortexing in HEPES-TALP medium supplemented with 0.1% (w/v) hyaluronidase for 2 min. Oocytes with a polar body (PB) and even cytoplasm were selected under a microscope ×200 magnification for production of IVF and SCNT embryos.

In vitro production of embryos

After incubation, the oocytes were transferred to HEPES-TALP containing 7.5 μg/ml cytochalasin B for 10 min, and then into a HEPES-TALP drop for the process of nuclear transfer. The first polar body and metaphase II plate with small volume of surrounding cytoplasm were removed by aspiration with a 20 μm internal diameter enucleation pipette. The enucleated oocytes were labeled with 0.5 μg/ml bisbenzimide (Hoechst-33342) for 2 min at room temperature (RT) in HEPES-TALP medium and ensured

that the nucleus was completely removed. After being trypsinized, a single intact cell (either fetal or adult fibroblast) was transferred by micropipette into the perivitelline space of each enucleated oocyte. For fusion of the cytoplast and donor cell, the egg was exactly oriented in BTX Electro-chamber (BTX, Inc., San Diego, CA, USA) filled with 0.28 M mannitol solution containing 0.01 μM $CaCl_2$ and 0.01 μM $MgCl_2$ with the help of holding and injection pipettes under micromanipulator, and pulsed twice with 1.4 KV/cm DC for 20 μsec using a BTX Electro-Cell Manipulator 200. The reconstructed eggs were then cultured in NCSU 23 supplemented with 1.9 mM 6-dimethylaminopurine (6-DMAP) for 3 h in order to maintain the maturation promoting factors (MPF) at a low level (Kim et al., 2005).

As IVF controls, embryos were produced *in vitro* using a modified protocol as previously described (Kikuchi et al., 2002). Briefly, denuded oocytes were washed and sets of 20 cumulus free oocytes were transferred into 50 μl drop of IVF medium. Oocytes were then inseminated with frozen-thawed sperm prepared by Percoll (Pharmacia, Uppsala, Sweden) density gradient as described by Rosenkrans et al. (1993). The final sperm concentration was adjusted to 1×10^5 sperm/ml. Coincubation was carried out for 5 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

All eggs were then cultured in IVC medium (30 eggs/60 μl drops) for 3 days. The cultures were further maintained in IVC medium supplemented with 5.55 mM glucose for additional 4 days at 39°C in a humidified atmosphere of 5% CO_2 in air (Abeydeera et al., 2000). The rates of cleavage and blastocyst formation were assessed on day 2 and day 6, respectively.

Cell number and apoptosis

Blastocysts collected on day 6 were fixed in methanol:acetic acid (3:1) for overnight and stained with 5 μg/ml bisbenzimide (Hoechst 33342) in HEPES-TALP for 10-15 min. The stained embryos were mounted onto pre-cleaned slides and their nuclei were counted under a fluorescent microscope (Nikon, Tokyo, Japan) fitted with a 475 nm emission filter.

Apoptosis was detected in embryos as per the procedure previously described (Hao et al., 2004). Both SCNT and IVF derived embryos at days 2, 3, 4, 5 and 6 were washed three times in PBS supplemented with 0.1% polyvinyl-pyrrolidone and fixed in 4% (v/v) paraformaldehyde/PBS solution overnight at RT. For membrane permeabilization, the embryos were incubated in 0.1% Triton X-100 in 0.1% citrate solution for 1 h at RT. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was used to assess the presence of apoptotic cells (*in situ* Cell Death Detection Kit, TMR red; Roche Diagnostics, Mannheim, Germany). Fixed embryos were incubated in TUNEL reaction medium for 1 h at 38.5°C in the dark. After being washed, the embryos were stained with 2 μg/ml 4', 6'dianidino-2-phenylindole dihydrochloride (DAPI, Roche) for 30 min at 38.5°C in the dark. Embryos were washed three times and mounted on slides with ProLong Antifade Kit (Molecular Probes, Eugene, OR, USA) and stored at -20°C until assessed. As positive controls, fixed embryos were incubated in RQ1 RNase-Free DNase (5 μl/50 μl of PBS; Promega, Madison, WI, USA) for 30 min at 38.5°C in the dark before the TUNEL reacrion. Whole-mount embryos were evaluated under an epifluorescent microscope (Nikon) by detecting the positive staining. The apoptotic positive and total cell number was counted simultaneously.

Experimental design

The present study comprised of 4 experiments for evaluating the developmental rate, cell number and apoptosis of porcine cloned and IVF embryos. In Experiment 1, the rates of cleavage and blastocyst development of cloned embryos produced with different sources of donor cells, such as pFF and pAEF, were compared to those of IVF counterparts. In total, 396 and 370 oocytes were used in 5 replicates for SCNT and IVF, respectively. In Experiment 2, 26 IVF and 39 SCNT blastocysts produced on day 6 were assessed for their total cell number and apoptosis index. In Experiment 3, the rates of cleavage and blastocyst development of SCNT embryos produced with pFF at different cell passages, such as 3-4 and 7-8 passages, were compared to those of IVF counterparts. In Experiment 4, the apoptosis of SCNT and IVF embryos at different developmental stages was detected by the TUNEL method.

Statistical analysis

Differences were analyzed by multiple comparisons among treatments using one-way ANOVA after arc-sine transformation of the proportional data. Comparisons of means were performed using the Duncan's and Tukey multiple comparisons test. Differences were considered significant at $p<0.05$.

RESULTS

Development of SCNT embryos produced from different donor cell types

The cleavage and blastocysts rates of embryos cloned with fibroblasts originated from fetal and adult ear skin were compared to IVF counterparts, as shown in Table 1. No significant differences were observed in the cleavage rates of cloned (fetal and adult) and IVF embryos. The blastocyst rates between the embryos cloned with fetal fibroblasts and adult ear skin did not differ significantly but

Table 1. Development of porcine embryos cloned with fetal and adult ear skin fibroblasts*

Embryo source		Eggs used	% of development (mean±SEM)	
			Cleavage	Blastocyst
SCNT	pFF	185	72.4±6.9	15.1±3.2[ab]
	pAEF	211	68.7±5.6	10.4±2.6[b]
IVF		370	75.3±5.8	22.5±4.5[ac]

5 replicates.
* SCNT, cloned embryos; Pff, porcine female fetal fibroblasts.
pAEF, porcine female adult ear-derived fibroblasts at 3-4 cell passage; IVF, *in vitro* fertilization.
[a, b, c] Percentage with different superscripts within columns indicate significant differences ($p<0.05$).

Table 2. Comparison of cell number and apoptotic incidence of porcine day-6 blastocysts cloned with fetal and adult ear skin fibroblasts*

Embryo source		Blastocysts used	Mean±SEM	
			Total cell number	% apoptosis
SCNT	pFF	22	28.4±4.3[a]	9.2±2.5[a]
	pAEF	17	24.2±5.1[a]	16.6±4.3[b]
IVF		26	35.4±3.2[b]	3.6±1.4[c]

* SCNT, cloned embryos; pFF, porcine female fetal fibroblasts.
pAEF, porcine female adult ear-derived fibroblasts at 3-4 passage; IVF, *in vitro* fertilization.
[a, b, c] Percentage with different superscripts within columns indicate significant differences ($p<0.05$).

Table 3. Development of porcine embryos cloned with fetal fibroblasts at different cell passages*

Embryo source	Cell passage	Eggs used	% of development (mean±SEM)	
			Cleavage	Blastocyst
SCNT	3-4	138	76.1±5.6	18.2±4.1[ab]
	7-8	133	73.6±6.1	13.5±3.6[b]
IVF		142	76.8±6.2	20.4±4.4[ac]

5 replicates.
* SCNT, cloned embryos with porcine female fetal fibroblasts; 3-4 and 7-8 cell passages; IVF, *in vitro* fertilization.
[a, b, c] Percentage with different superscripts within columns indicate significant differences ($p<0.05$).

SCNT embryos cloned with adult ear skin cells was significantly ($p<0.05$) lower than the IVF control.

Comparison of cell number and apoptotic incidence of embryos cloned with fibroblasts originated from fetus and adult ear skin

The total cell number and apoptosis in blastocysts cloned with fetal and adult ear skin fibroblasts were compared to IVF embryos, as presented in Table 2. Total cell number in cloned blastocysts was significantly ($p<0.05$) lesser than IVF control. However, no significant difference was observed between the embryos cloned with fetal and adult ear skin cells. Apoptosis rates between cloned blastocysts differed significantly ($p<0.05$) and were significantly higher than IVF embryos.

Development of embryos cloned with fetal fibroblasts by different passages

Table 3 shows the results of development rates of embryos cloned with fetal fibroblasts at different cell passages, 3-4 and 7-8. No significant differences were observed in the cleavage rates of embryos cloned with different cell passages and IVF embryos. The blastocyst rates between the embryos cloned with different cell passages did not differ significantly but was lower than that in IVF embryos. However, the blastocyst rate in embryos cloned with 7-8 cell passage was significantly ($p<0.05$) lower than the IVF control.

Apoptotic incidence in cloned embryos at different developmental stages

Figure 1 shows the apoptotic incidence as detected by TUNEL in SCNT and IVF embryos at different developmental stages. The earliest apoptotic signals were detected in IVF and cloned embryos on day 3. Concurrent with the embryo development, the rates of apoptosis also increased. On day 6, the rates of apoptosis in IVF were significantly ($p<0.05$) lower than those in embryos cloned with fetal and adult ear skin cells. However, no significant differences were observed within SCNT embryos.

DISCUSSION

To date, several reports are available using different cell types as nuclear donors with varying efficiencies (Wakayama et al., 1998; Zachartchenko et al., 1999). In this study, although there were no significant difference in the rates of cleavage and blastocyst development within donor cell types and cell passages, the blastocysts rates were comparatively higher in SCNT embryos cloned with pFFs

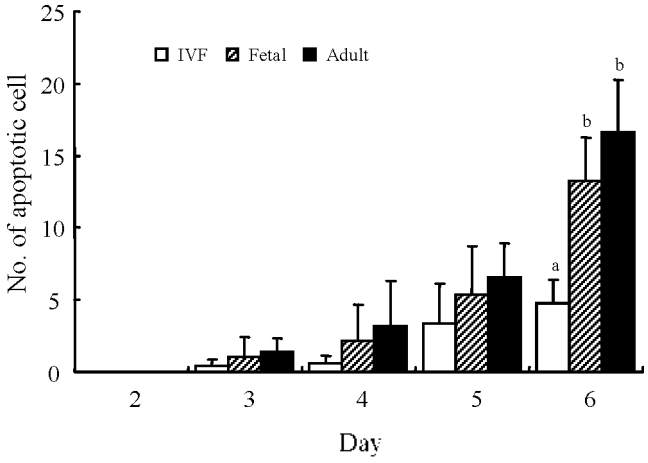

Figure 1. Apoptosis at different developmental stage of embryos produced by IVF and SCNT. Open bars indicate IVF embryos, hatched bars SCNT embryos with pFF and solid bars SCNT embryos with pAEF. [a, b] Different superscripts in bars within each stage indicate significant differences ($p<0.05$).

with 3-4 cell passage. Based on these observations, in general, early passages of donor cells have been used for SCNT as a nuclear donor (Cibelli et al., 1998; Baguisi et al., 1999).

Reported that Dolly not only inherited her mother's short telomeres, but her telomere lengths were further shortened during the brief *in vitro* culture (Shiels et al., 1999). This indicated that the reduced length of telomeres in the young ones produced by cloning was a result of senescence. The explanation to this observation is nuclear status and it is not clear how culture improves the nuclear totipotency of donor cells. In general, most cells face an apoptotic pathway. Especially, in a condition of *in vitro* culture, cells showed higher incidence of apoptosis (Feugang et al., 2003). The propensity to apoptosis is continuously counterbalanced in the cell by genes stimulating cell survival and proliferation. On induction by an appropriate trigger, the cell activates or stops the repression of gene products responsible for control of the suicidal mechanism (Raff, 1992; Matwee et al., 2000).

In the present study, total cell number of IVF embryos (35.4±3.2) were significantly ($p<0.05$) higher than blastocysts cloned with fetal and adult ear skin cells (28.4±4.3 and 24.2±5.1, respectively). Apoptosis detected by the TUNEL method was observed at different developmental stages of IVF and SCNT embryos. The earliest apoptotic signals were detected on day 3 post insemination and fusion in IVF and cloned embryos. Further, as the embryos developed, the rates of apoptosis increased to 4.8% in IVF and 13-17% in cloned embryos on day 6 blastocysts. The rates of apoptosis in IVF embryos on day 6 were significantly ($p<0.05$) lower than those in SCNT embryos cloned with pFFs and pAEFs. However, no difference was observed within SCNT embryos. The reasons for the occurrence of apoptosis in IVF embryos may be due to suboptimal IVP conditions (Feugang et al., 2003), indicating that high apoptosis in cloned embryos might also be related to the *in vitro* culture system that could have probably induced DNA fragmentation of the donor nuclei. In addition, it has been suggested that other factors such as micromanipulation, donor cell types, cell passages, cell cycle, and proper remodeling of donor cells into oocyte affect the ontogenesis of cloned animals (Kato et al., 1998; Renard et al., 1999; Hill et al., 2001; Li et al., 2003).

In the present study, initiation of apoptotic signals was observed on day 3 in both IVF and cloned embryos with normal morphology and development. This was an unexpected observation since in mouse embryos apoptosis did not occur at 8-cell or morula stages derived from either *in vivo* or *in vitro* (Handyside and Hunter, 1986). Similarly, human embryos from the 2-cell to the uncompacted morula stage, were negative with regard to necrosis or apoptosis (Jurisicova et al., 1996). In contrast to these observations, it has also been reported that fragmentation was associated with apoptosis at the 1-cell stage during mouse embryonic development (Jurisicova et al., 1998). In this case, apoptosis may be related to the activation of embryonic genome. In mice and human, the major events take place at the 2- and/or 4-cell stages. However, in mice, activation of the embryonic genome is initiated at late stage of first cell cycle coincident with the first observation of apoptosis in this species.

In bovine IVF and cloned embryos, the earliest

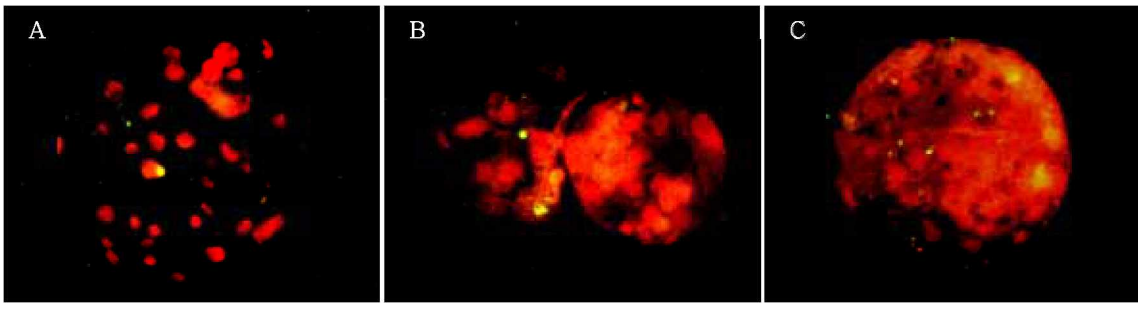

Figure 2. Apoptosis occurrence in day-6 porcine blastocysts produced by IVF (a) and SCNT with pFF (b) and pAEF (×400).

apoptotic signals were detected on day 3 after IVF and activation (Neuber et al., 2002) and as the embryos developed, the rates of apoptosis also increased (Hao et al., 2003). Particularly, Lee et al. (2006) reported that the expression of apoptosis by TUNEL is first detected at 6- to 8-cell stages in all embryos of IVF, SCNT and transgenic (TG) embryos, but the expression rate at each developmental stages was significantly higher ($p<0.05$) in SCNT and TG embryos than in IVF counterparts. Apoptosis may occur due to damaged embryos and that do not undergo appropriate activation of the embryonic genome. However, this does not explain the early expression of apoptotic genes in porcine, as the embryos were neither fragmented nor abnormal. This hypothesis is supported by the high variation in the extent of apoptosis with transcription of early markers representing the activation of the embryonic genome in individual blastomeres (Byrne et al., 1999).

In conclusion, high apoptosis during *in vitro* preimplantation development resulted in low development rate and total cell number of SCNT embryos. Further, the earliest positive TUNEL signals were detected in the SCNT and IVF embryos on day 3, and the rates of apoptosis increased concurrently with the *in vitro* embryo development. Based on the apoptotic incidence in cloned blastocysts, fetal fibroblasts are more suitable for production of SCNT embryos in porcine. However, preventing the activation of apoptotic pathway, especially during preimplantation embryo development would help to enhance the developmental potential of SCNT embryos.

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