

# Apoptosis in rabbit embryos produced by fertilization or nuclear transfer with fibroblasts and cumulus cells

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## Abstract

In this study, we investigated the development, the cell number of the blastocyst, and apoptosis in rabbit nuclear transfer (NT) embryos derived from adult fibroblasts and cumulus cells as compared with embryos derived from *in vivo* fertilization and *in vitro* culture. The developmental rate and the total cell number of the blastocyst were significantly lower in NT embryos than in fertilized embryos (FEs). The type of donor cells did not affect the embryonic developmental rate and the total cell number of blastocysts in NT groups. The present study investigated the onset and the frequency of apoptosis in NT embryos and FEs by using a terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) assay. The earliest positive TUNEL signals were detected at the eight-cell stage in NT embryos and at the morula stage in FEs. The apoptotic index of the total blastocysts, the inner cell mass and the trophoderm was greatly higher in the NT embryos than in FEs. Moreover, the apoptotic index of the blastocyst from fibroblasts was significantly higher than that of the blastocyst from cumulus cells.

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## Introduction

Sheep (Wilmot *et al.* 1997), mice (Wakayama *et al.* 1998), cows (Cibelli *et al.* 1998, Kato *et al.* 1998, Wells *et al.* 1999), goat (Baguisi *et al.* 1999), rabbit (Chesne *et al.* 2002), pig (Polejaeva *et al.* 2000), cat (Shin *et al.* 2002), mule (Woods *et al.* 2003), rat (Zhou *et al.* 2003) and horse (Galli *et al.* 2003) have been cloned from several adult cell types, including mammary gland (Wilmot *et al.* 1997), cumulus (Kato *et al.* 1998, Wakayama *et al.* 1998, Wells *et al.* 1999, Chesne *et al.* 2002, Shin *et al.* 2002), epithelial (Kato *et al.* 1998) and T and B cells (Hochedlinger & Jaenisch 2002). Sheep (Schnieke *et al.* 1997, Wilmot *et al.* 1997), cows (Cibelli *et al.* 1998, Zakhartchenko *et al.* 1999), goats (Baguisi *et al.* 1999), mule (Woods *et al.* 2003), rat (Zhou *et al.* 2003) and horse (Galli *et al.* 2003) have been cloned from fibroblasts. However, only a few cell types have been used for successful cloning, and it is not known which cell types are the most efficient for nuclear transfer (NT). Moreover, the developmental rates are still low and less than 5% of all reconstructed embryos develop to full-term live offspring. The difficulties with cloning may be due to deficiencies in the NT

manipulation, nuclear reprogramming, the type of donor cells and/or the *in vitro* culture systems.

Apoptosis, a morphologically and biochemically distinct physiological process triggered by changes in the levels of specific stimuli, is a highly conserved form of cell death that plays a major role in animal development (Hardy *et al.* 1989) and cellular homeostasis by acting as a quality control mechanism to remove cells that are damaged, non-functional, misplaced or supernumerary (King & Cidlowski 1995, Teraki & Shiohara 1999, Reed 2000). The major characteristics of apoptotic death are nuclear and cytoplasmic condensation, endoplasmic reticulum swelling and cytoplasmic blebbing. In addition, endogenous nucleases digest DNA from apoptotic cells into oligonucleosomal fragments (multiples of 185–200 bp), which give the appearance of a DNA ladder after electrophoresis (Raff 1992, Cory & Adams 1998). The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) reaction (Gavrieli *et al.* 1992) enables *in situ* detection of apoptotic cells by labeling of extensive oligonucleosomal DNA fragmentation generated by endogenous DNase activity during the apoptotic process. Initial application of the TUNEL reaction assay to

preimplantation embryos (Jurisicova *et al.* 1996) has opened a new line of research.

A previous study evaluated the effect of age of donor cells (passages) on NT embryo apoptosis (Jang *et al.* 2004). In order to inhibit apoptosis in NT embryos, treatment of donor somatic cells with putative apoptosis inhibitors (Park *et al.* 2004) has also been investigated. Little is known about the effects of different types of donor cells on apoptosis of NT embryos. The rabbit provides an excellent model system for basic research elucidating NT mechanism (Yang *et al.* 1992, Mitalipov *et al.* 1999, Yin *et al.* 2000, Dinnyes *et al.* 2001). We conducted this study to compare *in vitro* embryo developmental rates and the cell number of blastocysts produced by *in vivo* fertilization (IVF), cumulus cell NT (CNT) and fibroblast NT (FNT), and to evaluate the apoptosis of NT embryos and fertilized embryos (FEs). We identify apoptosis according to two key apoptotic markers: (i) changes in nuclear morphology, such as nuclear and chromatin condensation and nuclear fragmentation, typical of apoptosis, and (ii) DNA degradation detectable by the TUNEL reaction (Gjørret *et al.* 2003).

## Materials and Methods

### Animals

Animal care and handling were in accordance with the policy on the Care and Use of Animals of the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Female Japan Big Eared white rabbits (purchased from Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences) aged 6–8 months had free access to regular rabbit fodder and water.

### Recipient cytoplasm preparation

The procedure of recipient oocyte preparation has been described previously (Yang *et al.* 2003). Briefly, female were superovulated by administering pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) (Institute of Zoology, Chinese Academy of Sciences). Each rabbit was injected with 150 IU PMSG for 4 days, followed by 100 IU hCG injection. Cumulus–oocyte complexes (COCs) were collected 14 h after the hCG injection by flushing the separated oviducts with M2 medium (Sigma Chemical Company) and were treated shortly thereafter with 300 IU/ml hyaluronidase (Sigma) in M2 medium. Cumulus cells were stripped from the oocytes by repeated gentle pipetting. The cumulus-free eggs were transferred to M2 medium containing 7.5 µg/ml cytochalasin B (Sigma), 7.5 µg/ml Hoechst 33342 (Sigma) and 10% fetal bovine serum (FBS) (Gibco BRL) for 10 min and used for micromanipulation. Oocyte enucleation was manipulated according to our previously reported method and only the oocytes

from which the chromosomes were removed were used for NT (Chen *et al.* 1999).

### Donor cell culture, NT, activation and embryo culture

Rabbit cumulus cells and adult skin fibroblasts from primary cultures were used as nuclear donors. For adult fibroblasts, the ear tissue biopsies from an adult rabbit were cut into small pieces. Then the small pieces were digested with 0.25% (w/v) trypsin for 12 h at 4 °C and 40 min at 37 °C and subsequently cultured in DMEM/F12 (Gibco BRL) supplemented with 20% FBS in an incubator at 37 °C in 5% CO<sub>2</sub>. Cumulus cells were derived from COCs aspirated from the same rabbit which the fibroblasts were derived from injected with 150 IU PMSG before 4 days. Briefly, COCs were treated shortly thereafter with 300 IU/ml hyaluronidase in M2 medium and then cumulus cells were stripped from the oocytes by repeated gentle pipetting; after three washings in M2 medium, the cumulus cells were placed in DMEM/F12 supplemented with 20% FBS in an incubator at 37 °C in 5% CO<sub>2</sub>. The two types of cells were passaged at 100% confluence and were used as donor cells at passages four to eight. The primary cultures of the two types cells were repeated three times.

NT was conducted as previously described (Chen *et al.* 1999, 2002). A single donor cell was placed in the perivitelline space. The couplets were transferred to a fusion chamber consisting of two wires, 1 mm apart and overlaid with 100 µl fusion medium (0.25 M sorbitol, 0.5 mM magnesium acetate, 0.1 mM calcium acetate, 0.5 mM HEPES and 100 mg/100 ml BSA). Fusion was induced by double DC pulses of 1.4 kV/cm for 80 µs with an ECM2001 Electrocell Manipulator (BTX Inc., San Diego, CA, USA). Couplets were incubated in the same medium for 30 min at 38 °C in humidified air containing 5% CO<sub>2</sub>. The fused couplets were activated by double DC pulses of 1.2 kV/cm for 40 µs, and cultured overnight in M199 (Gibco BRL) supplemented with 10% FBS.

### In vivo embryo production

Female rabbits were superovulated as described above and mated immediately after hCG injection. Zygotes were collected 20 h after mating. The oviducts were flushed and the fertilized eggs were transferred into 50 µl M199 medium supplemented with 10% FCS, covered with mineral oil and cultured at 38 °C in a humidified atmosphere of 5% CO<sub>2</sub> for 4 days.

### TUNEL and confocal microscopy

The developmental stage of all embryos were assessed and scored by stereomicroscopy. To assess accurately the inner cell mass (ICM) and trophectoderm (TE), the

expanded and the hatched blastocysts from three groups were randomly selected and fixed.

The NT embryos and FEs at different stages were washed three times in PBS supplemented with 0.1% polyvinylpyrrolidone and fixed in 4% (v/v) paraformaldehyde solution for 2 h at room temperature. Membranes were permeabilized in 0.1% Triton X-100 in 0.1% citrate solution for 1 h at room temperature. The TUNEL assay was used to assess the presence of apoptotic cells (*in situ* Cell Death Detection Kit, TMR red; Roche, Mannheim, Germany). Fixed embryos were incubated in TUNEL reaction medium for 1 h at 38.5°C in the dark. After the reaction was stopped, labeled embryos were all incubated in 0.1 mg/ml RNase A (Sigma) and DNA was counterstained with 10 mg/ml propidium iodide (PI) (Sigma) for 5 min. As positive controls for TUNEL, fixed embryos were incubated in RQ1 RNase-Free DNase (Promega) for 40 min in the dark before TUNEL at room temperature. The negative controls were generated by omitting terminal transferase in the reaction. Embryos at two-cell to blastocyst stages were mounted on glass slides in 10–15 µl Fluoroguard antifade (BioRad) under coverslip compression. Slides were stored at –20°C up to 7 days before fluorescence microscopic evaluation. Selected two-cell to blastocyst stage embryos were subsequently subjected to confocal laser scanning microscopy on a Leica TCS4D microscope (Leica Laser Technik, GmbH, Heidelberg, Germany) using an argon/krypton laser at 488 and 543 nm and two-channel scanning for detection of fluorescein isothiocyanate and PI respectively. Complete Z series of 20–25 optical sections at 3- to 4-µm intervals were acquired from each embryo using Leica Scanware software. Image stacks were reconstructed with a Silicon Graphics octane computer (SGI, Mountain View, CA, USA) equipped with an Imaris image analysis software package (Bitplane, Zurich, Switzerland), and reconstructed confocal images were used for scoring of nuclei in the trophoblast and ICM separately in each blastocyst.

### Quantitative analysis of TUNEL labeling and apoptosis

Digitally recombined, composite images were analyzed using the Imaris software. All 20–25 optical sections were divided using a standard grid over each layer to count all nuclei as a measure of the cell number of the embryo. Nuclei were scored for TUNEL labeling, signs of fragmentation, and condensation. Cells were judged to be apoptotic when the nucleus displayed both the biochemical feature (TUNEL labeling) and the morphological feature (fragmentation and/or condensation). Allocation of nuclei to ICM and TE was based on their position in the reconstructed images (Fig. 1, FE3). The nuclei belonging to the polar TE were counted together with the ICM nuclei, leading to an overestimation of the ICM. Differential staining of the ICM to provide a more accurate number of nuclei for the ICM (Cohen *et al.* 1992) was not performed,

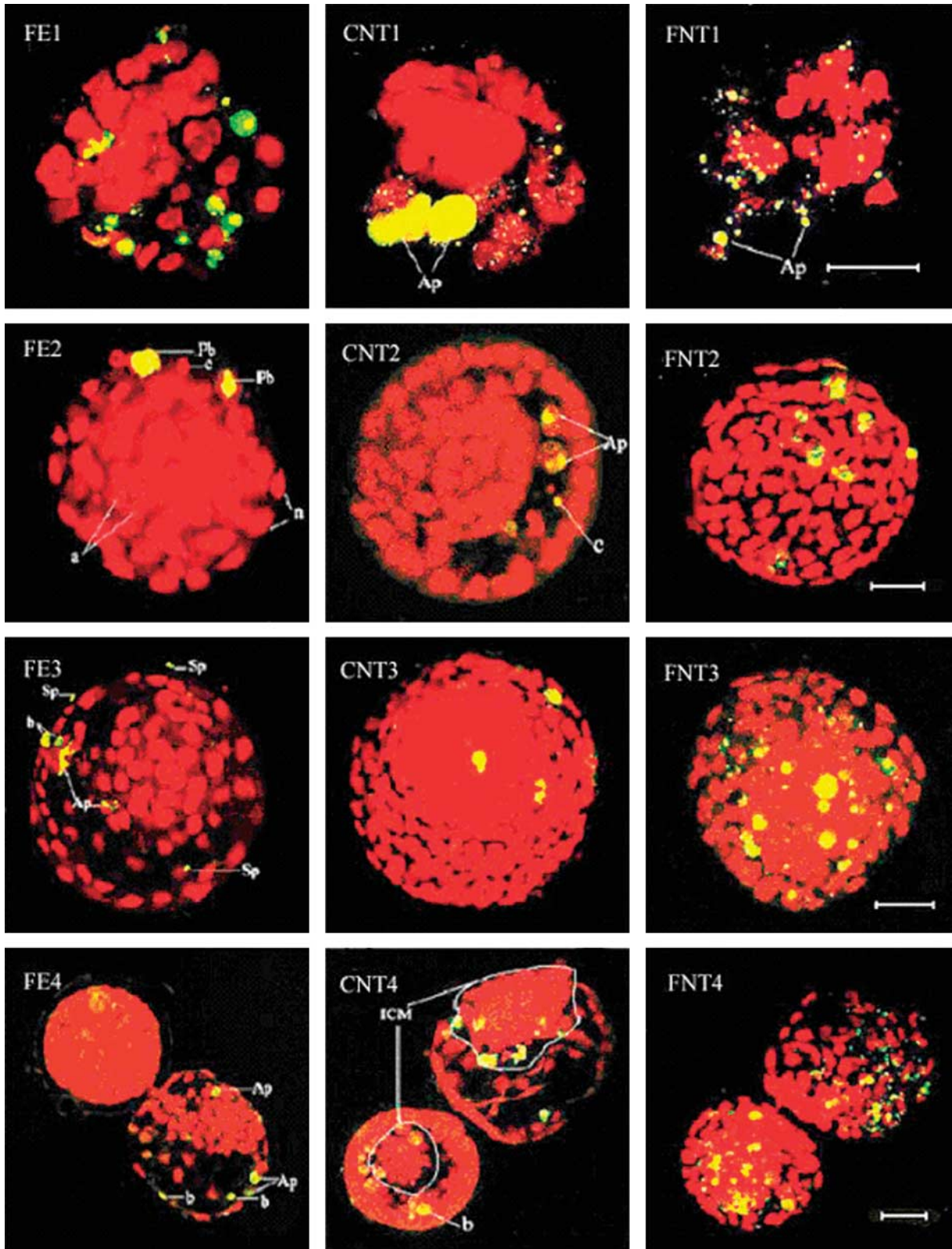
because this technique cannot be combined with TUNEL labeling. After renewed allocation of the nuclei to the ICM and TE, counting was performed a second time.

### Statistical analysis

Data were analyzed using SPSS 11.0 statistical software (SPSS, Inc., Chicago, IL, USA). Random distribution of embryos was made in each experimental group and experiments were replicated at least ten times. Interaction analysis among experimental parameters was first performed. As no interaction was found, the data were subjected to ANOVA and protected least significant different tests using general linear models to determine differences among experimental groups. When a significant model effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was determined when the *P* value was less than 0.05.

### Scoring of nuclei

The total number of nuclei was counted during scoring, and nuclear morphology was assessed on the basis of PI staining and was scored as being normal, condensed (i.e. pyknotic) or fragmented. Normal nuclei displayed loose reticulated chromatin content and sharp delineations, whereas condensed nuclei exhibited stronger PI staining of compacted chromatin in a decreased volume when compared with normal nuclei within the same embryo (Fig. 1, FE2). Condensed nuclei displayed sharp delineations, were often spherical in shape, and contained either a homogeneous chromatin content or chromatin aggregated in marginalized clumps along the nuclear envelope, which sometimes gave the nucleus a lobulated appearance. Fragmented nuclei had two or more condensed chromatin fragments, also with sharp delineations (Fig. 1, FE2). A cluster of nuclear fragments confined in an area comparable with or smaller than the volume of a normal nucleus was regarded as originating from a single nucleus. Conversely, when two fragments were separated by a distance of at least the diameter of an average nucleus, they were regarded as originating from different nuclei (Gjørret *et al.* 2003). Some nuclei displayed a different mode of disintegration; they had increased PI staining intensity but lacked a reduction in volume and had an unclear or fluffy delineation (and were often TUNEL positive; Fig. 1, FE3), and they were sometimes fragmenting into numerous minute elements in an expanded volume. These nuclei were not classified as apoptotic because they could represent necrotic or other types of cell death. Nuclei displaying TUNEL positivity, being separate from the other cells and being at the margin of FEs as assessed by stereomicroscopy, were regarded as polar bodies (Fig. 1, FE2). In this study, nuclei were regarded as apoptotic only if they displayed apoptotic morphology, in addition to being TUNEL positive (Gjørret *et al.* 2003).



## Results

### Developmental capacity and apoptosis of preimplantation embryos

When cultured in M199 medium, the cloned rabbit embryos developed to blastocysts and the embryonic developmental rates of the CNT and FNT groups were not significantly different. There was a significant difference in the rate of development from the two-cell to blastocyst stage between NT embryos and FEs (Table 1).

Embryos subjected to preincubation in DNase (positive controls) displayed the TUNEL reaction in all nuclei, whereas when terminal transferase was omitted (negative controls) no labeling of any nuclei was observed (data not shown). Apoptosis of embryos was not observed in any six-cell embryos, but it was first observed as nuclear condensation and TUNEL labeling in 8- to 16-cell NT embryos (Fig. 1, FNT1, CNT1) and the morula stage in FEs (Fig. 1, FE1). At the 8- to 16-cell stage, 6.5% of CNT embryos and 10.7% of FNT embryos and 8% of FE embryos at the morula stage displayed apoptosis.

### Mean cell number of the ICM and TE

In general, the total cell number of the blastocysts and the cell number of the TE were significantly lower in embryos produced by NT compared with those of embryos produced from fertilization, while the cell number of the ICM was significantly higher in embryos produced from NT compared with that of embryos produced from fertilization (Table 2,  $P < 0.05$ ). Although the mean cell number of CNT embryos ( $145.4 \pm 20.2$ ) tended to increase compared with that of FNT embryos ( $126.1 \pm 21.8$ ), there was no significant difference between the two groups. The CNT and FNT blastocysts had a higher ICM:total cell ratio

( $53.8 \pm 10.3\%$ ;  $59.6 \pm 14.3\%$ ) as compared with IVF ( $38.4 \pm 9.1\%$ ), but no significant difference was observed in the cell ratio between CNT and FNT groups (Table 2).

### Apoptosis in the ICM and TE

As shown in Table 3, the apoptotic indexes of the total blastocyst, ICM and TE in the CNT and FNT group were greatly higher than those of FE ( $P < 0.05$ ). Meanwhile, the apoptotic indexes of the FNT blastocysts in the total blastocyst (8.1%), ICM (9.9%) and TE (7.7%) were significantly higher than those of CNT blastocyst (5.2%, 7.6%, 4.7%).

## Discussion

The number of cells and the amount of apoptosis are important parameters of embryo development and health (Brison & Schultz 1997, 1998). It is thought that embryos with a large number of cells are more likely to implant and give rise to live offspring (Van Soom *et al.* 1997). The total number of cells of NT blastocysts was lower than that of fertilized blastocysts. These findings indicate that NT blastocysts consisting of a small number of cells may relate to a low pregnancy rate and a high embryonic loss after transfer in the rabbit.

In mammals, the functional differentiation of embryonic cells leads to the segregation of TE cells and ICM cells. The ICM cells contribute to all embryonic tissues and to a part of the extra-embryonic membranes. Later in pregnancy, the TE cells combine with the ICM-derived extra-embryonic membranes to form the fetal placenta (Gardner 1989). Both cell lineages are vital and essential for embryonic and fetal survival (Hardy *et al.* 1989, Kang *et al.* 2002). In the present study, the cell number of the

**Table 1** *In vitro* development of rabbit FEs and CNT and FNT embryos.

Group	No. of fused/fertilized	Two-cell (%)	Eight–16-cell (%)	Morula (%)	Blastocyst (%)
FE	160	137 (85.6) <sup>a</sup>	124 (77.5) <sup>a</sup>	117 (73.1) <sup>a</sup>	114 (71.2) <sup>a</sup>
CNT	138	83 (60.1) <sup>b</sup>	72 (52.2) <sup>b</sup>	58 (42.0) <sup>b</sup>	53 (38.4) <sup>b</sup>
FNT	142	99 (69.71) <sup>b</sup>	65 (45.8) <sup>b</sup>	48 (33.6) <sup>b</sup>	45 (31.7) <sup>b</sup>

Fused (%): Fused oocytes/NT units, development rate of embryos at different stages: no. of embryos/no. of fused oocytes or no. of embryos/no. of fertilized embryos.

Values with different superscripts within each column are significantly different ( $P < 0.05$ ).

Figures in parentheses indicate percentage.

**Figure 1** Apoptosis in rabbit FE and CNT and FNT embryos. Confocal laser scanning images of rabbit embryos. The chromatin was stained by PI (red), fragmented nuclei were labeled by TUNEL reaction (green), and colocalization with PI is indicated as yellow. FE1–FE4: fertilized rabbit morula, early blastocyst, expanded blastocyst and hatched blastocyst. CNT1–CNT4: NT rabbit morula, early blastocyst, expanded blastocyst and hatched blastocyst produced by using cumulus cells as donors. FNT1–FNT4: NT rabbit morula, early blastocyst, expanded blastocyst and hatched blastocyst produced by using fibroblast cells as donors. a, nuclear condensation and fragmentation by karyorrhexis without the TUNEL reaction; b, nuclei with the TUNEL reaction but no apparent nuclear condensation; c, a highly condensed but not fragmented nucleus; n, normal-appearing nuclei; Ap, nuclei fulfilling the criteria for apoptosis in this study; Pb, polar body; Sp, sperm; ICM, inner cell mass. Magnifications was  $\times 400$  for all panels except FE4, CNT4 and FNT4 ( $\times 200$ ). Same bar in each line. Bar = 50  $\mu\text{m}$ .

**Table 2** Mean cell number of ICM and TE in rabbit FEs and CNT and FNT blastocysts. Values are means  $\pm$  S.E.M.

Group	No. of blastocysts examined	Cell numbers of blastocysts			ICM: total cells ratio (%)
		Total	ICM	TE	
FE	42	179.6 $\pm$ 18.1 <sup>a</sup>	69.9 $\pm$ 10.2 <sup>a</sup>	109.8 $\pm$ 11.8 <sup>a</sup>	38.4 $\pm$ 9.1 <sup>a</sup>
CNT	38	145.4 $\pm$ 20.2 <sup>b</sup>	76.9 $\pm$ 11.9 <sup>b</sup>	68.8 $\pm$ 14.2 <sup>b</sup>	53.8 $\pm$ 10.3 <sup>b</sup>
FNT	35	126.1 $\pm$ 21.8 <sup>b</sup>	73.2 $\pm$ 13.7 <sup>b</sup>	47.0 $\pm$ 17.9 <sup>b</sup>	59.6 $\pm$ 14.3 <sup>b</sup>

Values with different superscripts within each column are significantly different ( $P < 0.05$ ; ANOVA).

ICM in NT blastocysts was significantly higher than that in FE blastocysts. An oversize ICM could have problems maintaining healthy central cells because of the increased distance over which oxygen and nutrients would have to diffuse. In addition, a disproportionately large ICM could result in an oversize fetus and large-offspring syndrome, as has previously been proposed (Leese *et al.* 1998). In the present cloning system, the primary cause of cloned fetus loss is placental abnormality (Cibelli *et al.* 1998, Baguisi *et al.* 1999, Wells *et al.* 1999, Kubota *et al.* 2000). In our results, aberrant ICM:total cell or a decrease of TE cells in NT blastocysts (Table 2) is similar to bovine NT blastocysts (Koo *et al.* 2002). These results may provide a clue to further clarify the reason why NT-derived embryos frequently make an insufficient placenta after implantation and eventually result in a high rate of fetal abortion at the first trimester of gestation.

Our results demonstrated that apoptosis was first observed at the 8- to 16-cell stage in the rabbit CNT and FNT embryos and at the morula stage in FEs, while it was at the four-cell in bovine NT embryos (Fahrudin *et al.* 2002) and the 8- to 16-cell in bovine IVF embryos (Matwee *et al.* 2000). The onset of apoptosis may correlate with the major burst of embryonic genome activation (Kanka *et al.* 1991, Hardy 1997, Jurisicova *et al.* 1998). At present, there is no related information about why the onset of apoptosis was different in FE and NT embryos. The apoptotic indexes of NT embryos were about 2–3 times greater than that of the FE group (Table 3). This observation was similar to what had been found in bovine NT embryos (Fahrudin *et al.* 2002). Apoptosis may play an important role in eliminating the cells with abnormal chromosomes (Hardy 1997). At the blastocyst stage, apop-

osis probably acts to eliminate cells that are damaged, are in excess, are no longer required, are developmentally incompetent, or have acquired TE potential (Matsuyama & Reed 2000). Apoptosis can be considered to be a normal process in preimplantation embryos to eliminate deviating cells, but a high incidence of apoptotic cells is correlated with abnormal morphology of the embryo (Hardy *et al.* 1989). In the present study, NT embryos with low cell numbers (Table 2) showed a higher apoptotic index (Table 3) than FEs. This may contribute to the low efficiency of rabbit cloning.

The type of donor cell is one of the important factors affecting the efficiency of somatic cell nuclear transfer (SCNT). The efficiency of mouse and bovine cloning using adult somatic cells has been reported as highest when cumulus cells were employed as donors (Wakayama *et al.* 1998, Wakayama & Yanagimachi 2001, Cho *et al.* 2002). In our study, the type of donor cells did not affect the rate of blastocyst formation and the number of ICM and TE cells. However, the apoptotic rates of the ICM and TE in the blastocysts derived from the two types of donor cells were significantly different. The explanation may be that compared with adult fibroblast cells, cumulus cells can potentially communicate with oocytes, and the morphology and function of each microorganelle in cumulus cells are suitable for the synchronization of cellular events after reconstruction. Throughout oogenesis, granulosa (cumulus) cells are coupled to oocytes and to each other by gap junctions (Baird 1984). Thus, follicular cumulus cells are close relatives of oocytes in their cytoplasmic components (Kato *et al.* 1999). In NT protocols employed at this time, donor cell cytoplasm, containing a varying mitochondrial population, is routinely transferred into recipient oocytes. The transferred mitochondria from adult fibroblast may not be easily intercalated into the endogenous mitochondrial network in the recipient oocytes. Many proteins related to apoptosis, such as Bcl-2 (Matsuyama & Reed 2000), localize in mitochondria. Bcl-2 family proteins are involved in the regulation of apoptosis during mouse (Jurisicova *et al.* 1998, Exley *et al.* 1999) and human (Metcalfe *et al.* 2004) preimplantation development. Although what role the mitochondria play in rabbit NT embryos during apoptosis remains unsolved, nucleo-cytoplasmic interactions might be more compatible in CNT embryos than in FNT embryos. The lower apoptotic rate in the NT embryos derived from the cumulus cell may

**Table 3** Apoptosis in rabbit FEs and CNT and FNT blastocysts. The apoptotic index (means  $\pm$  S.E.M.) is the percentage of cells displaying both TUNEL labeling and fragmentation.

Group	No. of blastocysts examined	Apoptotic index		
		Total	ICM	TE
FE	40	2.9 $\pm$ 1.1 <sup>a</sup>	5.0 $\pm$ 0.5 <sup>a</sup>	2.4 $\pm$ 0.3 <sup>a</sup>
CNT	36	5.2 $\pm$ 0.9 <sup>b</sup>	7.6 $\pm$ 0.6 <sup>b</sup>	4.7 $\pm$ 0.5 <sup>b</sup>
FNT	32	8.1 $\pm$ 1.3 <sup>c</sup>	9.9 $\pm$ 1.5 <sup>c</sup>	7.7 $\pm$ 1.0 <sup>c</sup>

Values with different superscripts within a column are significantly different ( $P < 0.05$ , ANOVA).

be one factor resulting in the success of cloning rabbits by using cumulus cells as donor cells (Chesne *et al.* 2002). This remains to be tested.

In conclusion, the earliest positive TUNEL signals were detected at the eight-cell stage in the rabbit NT embryos and at the morula stage in FEs. The types of donor cells did not affect rabbit embryonic developmental rate and the number of ICM and TE cells. The apoptotic indexes of the NT embryos were significantly higher than those of FEs; moreover they were significantly greater in embryos derived from fibroblast donor cells than from cumulus cells.

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