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Development of bovine–ovine interspecies cloned embryos and mitochondria segregation in blastomeres during preimplantation

Song Hua [a], Yong Zhang [a,*], Kai Song [b], Jimei Song [a], Zhipeng Zhang [a], Lin Zhang [a], Chi Zhang [a], Junwei Cao [a], Libing Ma [a]

[a] *Institute of Bioengineering, Northwest A & F University, Yangling, Shaanxi Province 712100, China*
[b] *Xuzhou Higher Vocational School of Bioengineering, Xuzhou, Jiangsu Province 221006, China*

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Abstract

The objective of the study was to investigate interspecies somatic cell nuclear transfer (iSCNT) embryonic potential and mitochondrial DNA (mtDNA) segregation during preimplantation development. We generated bovine–ovine reconstructed embryos via iSCNT using bovine oocytes as recipient cytoplasm and ovine fetal fibroblast as donor cells. Chromosome composition, the total cell number of blastocyst and embryonic morphology were analyzed. In addition, mtDNA copy numbers both from donor cell and recipient cytoplasm were assessed by real-time PCR in individual blastocysts and blastomeres from 1- to 16-cell stage embryos. The results indicated the following: (1) cell nuclei of ovine fetal fibroblasts can dedifferentiate in enucleated bovine ooplasm, and the reconstructed embryos can develop to blastocysts. (2) 66% of iSCNT embryos had the same number of chromosome as that of donor cell, and the total cell number of iSCNT blastocysts was comparable to that of sheep parthenogenetic blastocysts. (3) RT-PCR analysis in individual blastomeres revealed that the ratio of donor cell mtDNA: recipient cytoplasm mtDNA remained constant (1%) from the one- to eight-cell stage. However, the ratio decreased from 0.6% at the 16-cell stage to 0.1% at the blastocyst stage. (4) Both donor cell- and recipient cytoplasm-derived mitochondria distributed unequally in blastomeres with progression of cell mitotic division. Considerable unequal mitochondrial segregation occurred between blastomeres from the same iSCNT embryos.
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Keywords: Bovine; Ovine; Nuclear transfer; Mitochondrion; Real-time PCR

* Corresponding author. Tel.: +86 29 87080085; fax: +86 29 87080085.
E-mail address: hs863@nwsuaf.edu.cn (Y. Zhang).

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1. Introduction

ISCNT has been widely used for researching the mechanism of nuclear–cytoplasm interactions and rescuing highly endangered species (Lanza et al., 2000; Loi et al., 2001). To date, successful iSCNT has been achieved in several species. Chen et al. (2002) reported success in obtaining panda–rabbit cloned blastocysts, and Yang et al. (2003) obtained macaca–rabbit blastocysts. White et al. (1999) established pregnancy of argali–sheep cloned embryos. Furthermore, iSCNT offspring gaur (Lanza et al., 2000) and mouflon (Loi et al., 2001) have been obtained. Dominko et al. (1999) proved that oocyte cytoplasm from bovines can support the introduced differentiated nucleus regardless of donor cell species. Currently, enucleated bovine oocytes have been used as recipient cytoplasm extensively. Many studies (human, Chang et al., 2003; Illmensee et al., 2006, water buffalo, Kitiyanant et al., 2001; Li et al., 2005 and takin, Li et al., 2006) have achieved successes in blastocyst development using enucleated bovine oocytes as recipient cytoplasm. Furthermore, Sansinena et al. (2005) reported that two pregnancies of banteng–bovine cloned embryos were established.

Although, considerable progress has been achieved, a number of problems associated with the somatic cell nuclear transfer (SCNT) technology remain. One obvious problem is low efficiency, with low proportions of embryos developing to be implanted, survive pregnancy and birth live cloned offspring (Spikings et al., 2006; Wilmut et al., 1997). The causes for this extremely high failure rate are unclear. A better understanding of mtDNA distribution with the progression of iSCNT embryo development, holds promise for future improvement of nuclear transfer efficiency. Data concerning the segregation pattern of mtDNA leading to heteroplasmy in nuclear transfer animals is still controversial. There are three possible outcomes in embryos and animals produced by SCNT via whole donor cell electrofusion: (1) homoplasmy of donor cell mtDNA; (2) homoplasmy of recipient cytoplasm mtDNA; (3) heteroplasmy of donor cell and recipient cytoplasm mtDNA. Evans et al. (1999) reported that 10 cloned sheep only contained recipient oocyte-derived mtDNA. Lanza et al. (2000) and Loi et al. (2001) also reported that mtDNA of iSCNT animals were exclusively derived from the recipient oocytes. Conversely, Chen et al. (2002) reported that in panda–rabbit cloned embryos, mtDNA from both panda somatic cells and rabbit oocyte cytoplasm coexisted in early blastocysts, but mtDNA from rabbit oocyte cytoplasm was eliminated after implantation. Heteroplasmy has also been reported, Steinborn et al. (2002), observed coexistence of mtDNA derived from donor cells and recipient oocytes in cloned cattle. mtDNA heteroplasmy has also been reported in other somatic cell cloned embryos, such as ibex–hirus cloned embryos (Jiang et al., 2004), ibex–rabbit cloned embryos (Jiang et al., 2005) and human–bovine cloned embryos (Chang et al., 2003; Illmensee et al., 2006).

Bovine oocytes have been successfully employed in iSCNT using adult sheep fibroblasts as the nuclear donor. The cloned embryos were able to develop to the blastocyst stage. However, no studied on cytogenetic analysis, morphological characteristics and cell numbers of blastocyst have been reported in order to document the iSCNT embryonic quality. In addition, the authors did not present any data on the segregation of mtDNA in cloned embryos. In order to reveal further the interaction of nucleus–cytoplasm at the molecular level, and improve the efficiency of nuclear transfer, we created iSCNT embryos by fusion of ear fibroblasts from ovine fetuses with enucleated bovine oocytes, and investigated the quality of cloned embryos. In addition, we analyzed the distribution of two types of mtDNA by real-time PCR (RT-PCR) in individual blastomeres.

2. Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA). Disposable, sterile plasticware was purchased from Nunclon (Roskilde, Denmark).

2.1. *Preparation of main media*

Phosphate-buffered saline (PBS) was prepared with 0.24 g/L KH_2PO_4, 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L $Na_2HPO_4{\cdot}2H_2O$, 5% fetal bovine serum (FBS, Gibcol), and 50 μg/mL gentamycin sulfate. Bovine oocyte maturation medium (BOM) consisted of bicarbonate-buffered tissue culture medium 199 (TCM199, Life Technologies) supplemented with 10% FBS, 22 μg/L sodium pyruvate, 0.2 IU/mL human menopausal gonadotrophin (HMG), 1 μg/mL estradiol, and 50 μg/mL gentamycin. The sheep oocyte maturation medium (SOM) was composed of TCM199 enriched 10% FBS, 5 μg/mL follicle-stimulating hormone (FSH), 5 μg/mL luteiniting hormone (LH), 1 μg/mL estradiol, 0.3 mg/mL sodium pyruvate, and 50 μg/mL gentamycin.

2.2. *In vitro maturation (IVM) of oocytes*

Holstein cow ovaries and Mongolian sheep ovaries were collected from the local abattoir. After aspirating bovine and ovine cumulus–oocyte complexes (COCs), only oocytes surrounded by at least three layers of granulosa cells were selected for in vitro maturation (IVM). IVM was carried out in a humidified atmosphere of 5% CO_2 at 38.5 °C for 22–24 h. Next, surrounding cumulus cells were removed by pipetting in PBS containing 1 mg/mL hyaluronidase, and denuded oocytes with an extruded first polar body were used for the further experiments.

2.3. *Preparation of donor somatic cells*

Small pieces of sheep fetal (Mongolian sheep, 90 days) ear skin tissue were collected from the abbatoir. The tissue was washed three times in PBS and finely cut into 1 mm^2 pieces, then digested in 0.25% trypsin–EDTA solution for 10–15 min at 38.5 °C. After washing twice in PBS by centrifugation at 1000 rpm for 5 min, dissociated cells were washed three times by centrifugation to obtain a cell pellet. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS, and placed in a plastic culture dish. After the cells had formed a confluent fibroblast monolayer, culture was continued for three to five passages. The serum concentration was decreased to 0.5% (v/v) to starve the donor cells for 3–5 days before nuclear transfer.

2.4. *Nuclear transfer and embryo culture*

The basic NT procedures were described by Campbell et al. (1996). Briefly, bovine oocytes matured in vitro were enucleated by aspirating the first polar body and the MII plate. Successful enucleation was confirmed by staining with 5 μg/mL Hoechst 33342 and visualizing under UV light. At 1–2 h postenucleation, donor cells were placed in a drop of TCM199 supplemented with 5% FBS, and 5 μg/mL cytochalasin B, then the enucleated oocytes (cytoplasts) were placed in the same drop. Individual donor cells were picked up with the injection pipette and injected into the perivitelline space. After injection, the reconstructed embryos were transferred into an electrical

fusion chamber overlaid with fusion solution comprising 0.3 M mannitol, 0.1 mM $MgSO_4$, and 0.05 mM $CaCl_2$. Cell fusion was induced with two direct current pulses (1.0 kV/cm, 60 μs each, 1 s apart). Successful fusion was confirmed under an inverted microscope, and fused reconstructed embryos were activated by 5 μm ionophare in TCM199 for 5 min, followed by a 4 h incubation in 2 mM 6-DMAP. Activated embryos were transferred to modified synthetic oviduct fluid (mSOF, Gardner et al., 1994), supplemented with 2% MEM essential amino acids, 1% MEM nonessential amino acids, 1 mM glutathione and 10 μM EDTA. Nonmanipulated bovine oocytes and ovine oocytes were activated directly under the same conditions applied to the SCNT-reconstructed oocytes. The parthenogenetically activated oocytes were cultured and used as a control of reconstructed embryonic development. Interspecies and parthenogenetic embryos development was monitored daily up to 8 days.

2.5. The evaluation of reconstructed embryos

Embryo quality was determined after 120–144 h of culture according to embryonic morphology and the total cell number of blastocyst. Numbers and size of blastomeres and the presence of anucleate cytoplasmic fragments were recorded in accordance with the following morphological criteria: Grade 1 (excellent quality), embryos were defined as embryos in which blastomeres were of an equal size and without fragments. Grade 2 (good quality), embryos have blastomeres of equal or unequal size and a maximum of 20% of the total volume of the embryo filled with anucleate fragments. Grade 3 (fair quality embryos), anucleate fragments were present in <50% of the total volume embryo. Grade 4 (poor quality), anucleate fragments were present in >50% of the total volume of the embryo. The first three grades of embryos are deemed available embryos and the Grade 4 is unavailable embryo. Cell number of blastocysts was determined at the end of the culture period by staining embryos with the fluorescent bisbenzimidazole stain, described previously by Pursel et al. (1985).

2.6. Chromosomal analysis

Chromosome preparations of interspecies cloned embryos were undertaken according to the improved air-drying technique described by Burgoyne (1993). Briefly, interspecies blastocysts were treated with 0.05 μg/mL colcemid to arrest the cell cycle. They were then placed in 0.075 M hypotonic KCl solution on a grease-free slide and fixed with methanol:acetic acid (3:1). After air-drying for 1 h, the chromosomes were stained with 5% (v/v) Giemsa solution and observed under a microscope. Chromosome preparation of somatic donor cells was carried out based on the routine procedures (Li et al., 2006).

2.7. Preparation of mtDNA from single blastomeres or reconstructed blastocysts

The zona pellucida of reconstructed embryos was removed by a brief exposure to acidified PBS (pH 3.0). Individual blastomeres were isolated by exposing the zona pellucida-free embryos to 1.0% pronase in PBS with 5% FBS for 1–2 min followed by repeated pipetting. Blastomeres derived from 2-, 4-, 8- and 16-cell stage reconstructed embryos were designated as 1/2, 1/4, 1/8 and 1/16 blastomeres, respectively.

Single blastocysts or blastomeres at different developmental stages were collected into separate PCR Eppendorf tubes containing 20 μL lysis solution (1× PCR buffer, 2.5% Triton X-100, proteinase 100 μg/mL). The samples were incubated for 30 min at 55 °C, then 5 min at 100 °C and

followed by centrifugation at 12,000 rpm for 1 min, 20 μL of the supernatant was diluted 25-fold and was then used for template DNA (Wan et al., 2003).

2.8. Generation of DNA standard

The external standards of 225 bp (bovine) and 128 bp (ovine) products were generated using two pairs of specific primers. The nucleotide positions of the bovine primers (GeneBank accession no. NC 001567) were: sense primer (496–515) 5′-CAGTG AGAAT GCCCT CTAGG-3′, and antisense primer (703–720) 5′-TTTAC GCCGT ACTCC TGT-3′, and the sheep primers (GeneBank accession no. NC 001941) were: sense primer (1102–1123) 5′-TAGCC CAAAA TCTCC CACTC TC-3′, and antisense primer (1211–1229) 5′-CATTC CCTTG CGGTA CTTT-3′. PCR products were purified from agarose gel and integrated into a pMD18-T Vector (TaKaRa, Biotech. Co. Ltd.). The identities of the PCR products were confirmed by sequencing using an automated DNA sequencer (ABI PRTSM 377DNA sequencer-C Sangon). Standard curves covering 10^5 to 10^0 copies for sheep mtDNA and 10^6 to 10^0 copies for bovine mtDNA were set up for PCR quantification.

2.9. Real-time PCR quantification

A SmartCycler (Cepheid. USA) was used to determine mtDNA copy number. PCR reactions were set up in 25 μL reaction mixtures containing 12.5 μL 1× SYBR@ Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 μL 0.2 μM sense primer, 0.5 μL 0.2 μM antisense primer, 1.5 μL distilled water, and 10 μL of template. The reaction conditions were as follows: 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s, and 60 °C for 20 s. Each experiment included a lysis solution control and a distilled water control. All raw data were multiplied 50-fold to determine total mtDNA copy number in each embryo or blastomere.

2.10. Statistical analysis

Each replicate was carried out using oocytes harvested and cultured on the same day thus removing any effect of different batches. All experiments were repeated at least three times. The software package SPSS 9.0 was used to analyze the data. A P-value <0.05 was considered statistically significant.

3. Results

3.1. In vitro development of iSCNT embryos

A total of 219 bovine oocytes were successfully enucleated and fused with ovine skin fibroblasts. First cleavage division occurred at 18 h post-culture, 73.4% (161) of iSCNT embryos cleaved within 36 h (Table 1). The second mitosis was observed between 36 and 60 h after culture. Timing of the first two cleavage divisions corresponded to the timing of cleavage division that occurred in the bovine and the ovine parthenogenetic embryos. However, with progression of embryo culture, the iSCNT embryos gradually approached the timing of sheep parthenogenetic embryo development. iSCNT (24.6%) and ovine parthenogenetic blastocysts (46.8%) were observed between 108 and 144 h, but the rate of bovine parthenogenetic blastocysts was 1.1% during this time.

Table 1
Developmental process of iSCNT embryos, bovine and ovine parthenogenetic embryos

Embryo	N	Two cell (%) (18–36 h)	Four cell (%) (−60 h)	8–16 cell (%) (−84 h)	Morula (%) (−108 h)	Blastocyst (%) (−144 h)
Sheep PE[a]	292	249 (85.3) a	237 (81.2) a	204 (69.9) a	159 (54.5) a	136 (46.8) a
iSCNT[b]	219	161 (73.4) b	137 (62.6) b	93 (42.6) b	69 (31.5) b	54 (24.6) b
Bovine PE	271	247 (91.1) c	241 (88.9) c	39 (14.4) c	4 (1.5) c	3 (1.1) c

Values within each column with different letters differ significantly ($P < 0.05$).
[a] PE refers to parthenogenetic embryos.
[b] iSCNT refers to interspecies somatic cell nuclear transfer.

Table 2
Morphological assessment of iSCNT blastocysts, bovine and ovine parthenogenetic balstocysts

Embryo	n	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)
Sheep PE[a]	64	38 (59.4) a	14 (21.8) a	8 (12.5) a	4 (6.3) a
iSCNT[b]	49	18 (36.6) b	11 (22.5) a	9 (18.4) a	11 (22.5) b
Bovine PE	72	40 (55.6) a	19 (26.4) a	7 (9.7) a	6 (8.3) a

Values within each column with different letters differ significantly ($P < 0.05$).
[a] PE refers to parthenogenetic embryos.
[b] iSCNT refers to interspecies somatic cell nuclear transfer.

3.2. iSCNT embryonic morphology

Similar proportions of good (22.5% versus 26.4%, 21.8%, $P > 0.05$) and fair quality embryos (18.4% versus 12.5%, 9.7%, $P > 0.05$) were obtained, although the proportion of excellent quality embryos was lower in the iSCNT group than in the bovine and ovine parthenogenetic activation groups. For available embryos (except for excellent quality embryos), iSCNT and parthenogenetic embryos showed comparable cellular and morphologic features. The rate of poor quality embryos (22.5%) was significantly higher in the iSCNT group than in the bovine and ovine parthenogenetic activation groups, but these poor quality embryos are designated unavailable embryos. There was no significant difference between ovine and bovine parthenogenetic activation groups in all embryo grades (shown in Table 2).

3.3. Chromosomal composition of embryos

The chromosome constitution of donor cells and blastocysts derived from iSCNT is presented in Table 3. Of the 63 iSCNT embryos evaluated, 50 (79.4%) embryos were successfully analyzed for chromosome composition, yet 13 (20.6%) embryos were not assessed due to technical problems.

Table 3
Karyotype analysis of donor cells and blastocysts from interspecies nuclear transfer between bovines and ovines

Source	n	Diploid (%)	Tetraploid (%)	Aneuploid (%)
Donor cells (control)	45	36 (80.0)	5 (11.1)	4 (8.9)
iSCNT[a] embryos	50	33 (66.0)	7 (14.0)	10 (20.0)

Values within the same column are not statistically different at the 0.05 levels; values based on three replicates.
[a] iSCNT refers to interspecies somatic cell nuclear transfer.

Table 4
Comparison of the cell number of iSCNT embryos (day 6), bovine parthenogenetic activation embryos (day 8), and ovine parthenogenetic embryos (day 6)

Embryo	n[a]	No. of blastomeres
Ovine PE[b]	15	122 ± 9 a
iSCNT[c] embryo	12	117 ± 13 a
Bovine PE	18	131 ± 11 b

[a] Experiments were repeated three times. The letters (a, b) indicate that the mean values are different ($P < 0.05$).
[b] PE refers to parthenogenetic embryos.
[c] iSCNT refers to interspecies somatic cell nuclear transfer.

The results indicated that 33 (66.0%) iSCNT embryos had the same number of chromosomes as that of the donor cells ($2n = 54$), 7 out of 50 iSCNT embryos analyzed were tetraploid and the rest (10) of the iSCNT embryos had aberrant numbers of ovine chromosomes with or without sex chromosomes. There was no significant difference in the proportion of diploid, tetraploid or aneuploid karyotypes between donor cells and iSCNT embryos.

3.4. The cell number of blastocyst

The cell numbers of blastocysts derived from iSCNT and sheep parthenogenetic blastocysts by day 6 and bovine parthenogenetic blastocysts by day 8 are summarized in Table 4. The total cell number of blastocysts in the iSCNT group was comparable with that in the ovine parthenogenesis embryos, though both of them were lower than that in bovine parthenogenetic activation embryos.

3.5. Species-specific mtDNA primers and standard curves

To confirm the specificity of primers, single donor cell and recipient oocyte mtDNA were amplified using two sets of primers and cross PCR amplification was also carried out. PCR products were verified by sequencing. The specific primers designed in this study were sufficient to allow detection of mtDNA in very small quantities. The sequences of 128 and 255 bp PCR products were identical with those of the counterparts in the donor cells and recipient cytoplasm. In cross-species tests, the ovine mtDNA-species primers detected PCR product only in ovine mtDNA but not in bovine mtDNA and *vice versa*; the bovine mtDNA-species primers detected PCR product only in bovine mtDNA but not in ovine mtDNA. The results also indicated that the lysis buffer and distilled water controls were negative (data not shown). The DNA standards were well proportioned and the standard curves were generated from six or seven points spanning the expected unknown values (Fig. 1).

3.6. Quantitation of mtDNA in iSCNT embryos

In order to reveal and document accurately the distribution of mtDNA in iSCNT embryos, we detected the levels of mtDNA originating from the donor cell and recipient cytoplasm in single one-cell embryo, 1/2, 1/4, 1/8, 1/16 blastomeres and blastocysts using a RT-PCR method (Table 5). At the one-cell stage, the mean copy number of mtDNA from the donor cell was 4050, while there were 399,600 mtDNA copies from the recipient oocyte. At the 16-cell stage, the mean copy number of mtDNA in individual blastomeres from donor cells and recipient cytoplasm were 200 and 31,500, respectively.

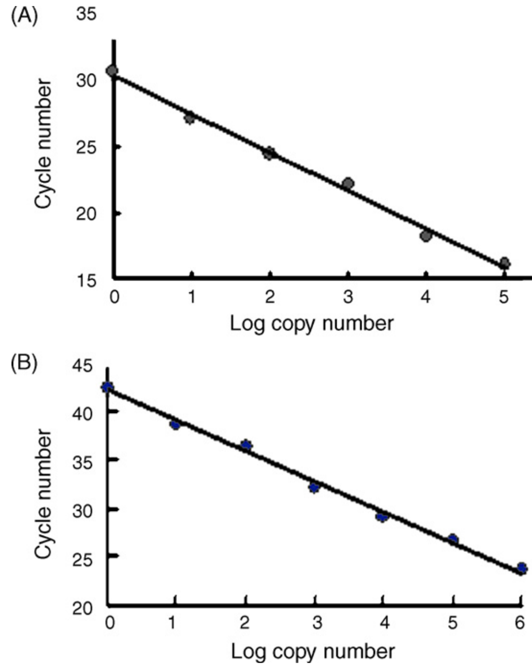

Fig. 1. Regression lines of standards with log copy numbers plotted on the *X*-axis and the threshold cycles on the *Y*-axis. (A) Ovine DNA standards; (B) bovine DNA standards.

During the first three cleavage divisions, there were slight changes in ratios of mtDNA from donor cells to from recipient cytoplasm, showing a similar tendency both for donor cells and recipient cytoplasm mtDNA with progression of cleavage division. At the 16-cell stage, the ratio of donor cell mtDNA copy number to recipient cytoplasm mtDNA copy number decreased significantly. At the blastocyst stage, the total copy numbers of donor cell- and recipient cytoplasm-derived mtDNA were 1050 and 855,700, respectively, and the ratio of donor cell mtDNA to recipient cytoplasm mtDNA copy numbers was 0.11%. The mean copy numbers of mtDNA from either donor cell or recipient cytoplasm in blastocysts changed greatly compared with those in one-cell embryos. Since there was a great variation between the ratios of donor cell mtDNA copy number: recipient cytoplasm mtDNA copy number and the number of embryonic mitotic divisions, we were unable to establish any correlation between the embryo cleavage division and the segregation of mtDNA.

Table 5
The mean copy numbers of donor mtDNA and recipient cytoplasm mtDNA in single blastomeres or blastocysts

Species	One-cell embryo	1/2 blastomere	1/4 blastomere	1/8 blastomere	1/16 blastomere	Blastocyst
Sheep	4050 ± 410	2350 ± 420	1450 ± 390	600 ± 190	200 ± 90	1050 ± 120
CV (%)	10	18	27	31	45	11
Bovine ($\times 10^2$)	3996 ± 439	2132 ± 280	1291 ± 170	562 ± 140	315 ± 110	$8557 \pm 1\,880$
CV (%)	11	13	14	25	34	22
Ratios (%)	1.01	1.10	1.12	1.06	0.66	0.11

CV: coefficient of variation.

Table 6
Variation on mtDNA copy number within individual blastomeres from the same embryos

Blastomere	No. of bovine mtDNA copy	No. of sheep mtDNA copy	Total mtDNA copy number in individual blastomeres
Two-cell embryo	257,100	3100	260,200
	304,000	1800	305,800
Two-cell embryo	178,300	2700	181,000
	246,400	2900	249,300
Two-cell embryo	191,700	1300	193,000
	315,700	2700	318,400
Four-cell embryo	193,500	900	194,400
	101,600	1100	102,700
	171,300	1800	173,100
	97,300	1000	98,300
Four-cell embryo	87,400	800	88,200
	172,100	1300	173,400
	111,300	600	111,900
	206,400	1000	207,400
Four-cell embryo	217,300	1100	218,400
	95,700	1800	97,500
	196,300	1000	197,300
	87,200	600	87,800

In addition, there were considerable variations in copy numbers of mtDNA either from donor cells or recipient cytoplasm among separate blastomeres within the same embryo (Table 6). Total copy numbers in single blastomeres were also different within the same embryo. Furthermore, in single embryos, the total copy numbers were also different between embryos at the same development stage (Table 6).

4. Discussion

ISCNT may offer an opportunity to support the study of nucleus cytoplasm interactions and save critically endangered animals, especially improve nuclear transfer efficiency. In the present study, we have shown that bovine–ovine cloned embryos could develop to the blastocyst stage in vitro, suggesting that sheep fetal fibroblast nuclei can dedifferentiate in enucleated metaphase bovine oocytes, and confirmed again the report by Dominko et al. (1999). The authors reported that the developmental chronology is determined by the donor cell nucleus (Dominko et al., 1999). Following in vitro culture, we found that timing of the first two cleavage divisions were very close between iSCNT embryos, bovine and ovine parthenogenetic embryos. Conversely, in subsequent development, the iSCNT embryos started approaching the donor species-specific timing of development. Those factors regulating the timing of early cleavage division may exist in oocyte cytoplasm, but the time to reach the blastocyst stage for iSCNT embryos is donor-nucleus-specific (Wen et al., 2003).

Following nuclear transfer, we obtained a relatively high proportion of blastocysts. In comparison with the results reported by Dominko et al. (1999), the blastocyst yield in this study was extremely high (24.6% versus 13.9%). One factor contributing to the different results may be the

difference in donor animal ages (fetal fibroblasts versus adult fibroblasts). Saikhun et al. (2002) reported that more embryos were derived from fetal fibroblasts after fusion of adult cumulus cells and fetal fibroblasts with enucleated bovine oocytes. As such, it seems more likely that ovine fetal fibroblast nuclei can be easily reprogrammed in enucleated bovine oocytes when compared with adult ovine fibroblast nuclei. Another factor leading to the discrepancy may be related to nuclear transfer protocol. Though the rate of cleavage division obtained following fusion and activation was relatively high, a considerable number of fused embryos did not show signs of cleavage division. This initial failure may possibly be attributed to an incomplete reprogramming of the differentiated nucleus. Considerable efforts are now being focused on understanding the mechanisms underlying reprogramming, and it is possible that in the near future somatic cells will be reprogrammed in vitro before nuclear transfer (Kikyo et al., 2000; Loi et al., 2001).

Cytogenetic analysis showed that 66.0% of iSCNT embryos had the same number of chromosomes as that of donor cells. There was no significant difference in the proportion of diploid, tetraploid or aneuploid karyotypes between donor cells and iSCNT embryos. It was further revealed that the protocol of nuclear transfer employed in the study is feasible and reliable. In spite of the promising results of chromosome numbers, it is necessary to analyze the causes of chromosome anomalies that were observed in the rest of the embryos (34.0%). Probably, this was simply due to chromosome loss or splitting during preparation, alternatively it might result from specific chromosomal damage during iSCNT or heteroplasmic epigenetic reprogramming (Chang et al., 2003).

In this study, the cell number of blastocysts derived from iSCNT embryos was lower than that derived from bovine parthenogenetic embryos, but similar to that derived from ovine parthenogenetic embryos. Previous studies have shown that if the blastocysts have more blastomeres, the capacity of these blastocysts to develop further is elevated. In general, the total cell number is 50–317 (De La Fuente and King, 1997) per bovine blastocyst, and 52–173 per ovine blastocyst (Gardner et al., 1994) produced, respectively, in vitro. Moreover, the total cell number per nuclear transfer blastocyst usually was less than that of in vivo counterparts (Watson et al., 2000). Therefore, in this study, the proportions of good and fair quality iSCNT embryos were comparable to those in parthenogenetically activated bovine and ovine embryos, although the proportion of excellent quality embryos was lower in iSCNT embryos than in parthenogenetcially activated bovine and ovine embryos.

The heteroplasmy of mtDNA caused by whole cell electrofusion has been explored in various species by many investigators. The first investigation on mtDNA inheritance in somatic cloned sheep showed that tissue only contained recipient cytoplasm-derived mtDNA (Evans et al., 1999). However, several reports indicated that somatic cell mitochondria microinjected into zygotes escaped destruction and persisted in embryos (Pinkert et al., 1997; Shitara et al., 2000) and offspring (Irwin et al., 1999; Shitara et al., 2000). Similarly, another study on mtDNA inheritance in cloned bovines revealed low proportions of nuclear donor-derived mtDNA (Steinborn et al., 2000). Different species or experimental parameters, such as nuclear donor cell type or passage number of donor cells, might be responsible for the differences between the latter study and the Evans' findings (Steinborn et al., 2000). Following quantitative analysis, we calculated the average copy number of mtDNA per blastomere in each developmental stage (Table 5). Values derived from three separate reactions with the same samples were very similar. However, the degree of variation was remarkable between different samples.

Real-time quantitative PCR analysis in individual blastomeres revealed that mtDNA copy numbers from donor cell and recipient cytoplasm decreased disproportionally between blastomeres, as observed in human embryos (Brenner et al., 2000; Van Blerkom, 2000), in pig embryos (El

Shourbagy et al., 2006) and the ratio of donor cell mtDNA to recipient oocyte mtDNA is about 1%, remaining constant from the one- to eight-cell stage development (Table 5). It could be inferred that in individual blastomeres, there was a similar tendency toward decreased mtDNA from donor cells and recipient oocytes with progression of embryonic cleavage divisions prior to eight-cell stage. In subsequent development, different changes occurred, although the amount of both donor cell and recipient cytoplasm mtDNA in single blastomeres decreased further. The changes may be due to two events: (1) transcriptional activation: transcriptional activation of embryonic nuclei occurs at the 8–16-cell stages in sheep (Crosby et al., 1988) and cow (Jones and First, 1995). Nonetheless, it remains to be determined whether there is a detailed relationship between transcriptional activation and mtDNA level change; (2) morphological transformation of mitochondria: mitochondrial morphology varies during oogenesis and throughout the early cleavage stages. In bovine embryos, mitochondrial maturation begins at the eight-cell stage and is completed by the blastocyst stage. Therefore, it seemed that a number of the mature mitochondria cotransferred with the donor cell nucleus might be destroyed during morphological transformation of mitochondria after the eight-cell stage (Takeda et al., 1999).

Coefficient of variations (CVs) are directly related to embryo cleavage divisions. CVs either mean numbers of donor cell mtDNA or recipient cytoplasm mtDNA became increasingly greater, indicating a considerable variation of mtDNA copy numbers occurring in individual blastomeres derived from different embryos at the same developmental stage. The degree of variation in mtDNA amount derived from the donor cell, the amount derived from the recipient cytoplasm and the total amount in individual blastomeres within the same embryos was very high (Table 6). It could be inferred that mitochondria, both those from the donor cell and those from the recipient cytoplasm distributed unequally between blastomeres with progression of embryonic mitotic division, so did the total mitochondria (donor cell-derived + recipient cytoplasm-derived). Therefore, more blastomeres will be distributed less mitochondria with further development. Those blastomeres with insufficient mitochondria mtDNA, and therefore the incapacity to generate ATP, remained undivided and lysed or fragmented during subsequent culture (Van Blerkom et al., 1997). Mitochondria are distributed in each cell of the early embryo, and they do not replicate, because each blastomere relies on the energy provided by mitochondria until after the blastocyst stage when mitochondrial replication initiates (Smith and Alcivar, 1993; Cummins, 1998). This may explain why a considerable proportion of poor quality embryos were obtained after iSCNT.

In summary, our findings reconfirmed again the ability of enucleated bovine oocytes to support normal karyotypic and phenotypic development to the blastocyst stage under the direction of differentiated ovine fibroblast cell nuclei. No changes were observed in the ratios of donor cell mtDNA to recipient cytoplasm mtDNA prior to eight-cell stage in individual blastomeres, but the ratio changed remarkably at the 16-cell stage. Yet, at the blastocyst stage, the ratio of donor cell mtDNA to recipient cytoplasm mtDNA was only 0.11%. Furthermore, unequal distribution of mitochondria occurred in individual blastomeres within the same embryo. The variability of mitochondrial segregation in iSCNT embryos after implantation needs further investigations.

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