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Different fates of donor mitochondrial DNA in bovine-rabbit and cloned bovine-rabbit reconstructed embryos during preimplantation development

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1. ABSTRACT

The functions of mitochondria depend on precise interaction between nuclear and cytoplasmic genomes. Non-balance of mtDNA has been reported in most nuclear transfer embryos and offspring. The reason of the degradation of donor mtDNA is still not clear. To further investigate the mechanism, in this study, we designed an experiment as follows. Two fibroblast cell lines sharing same nuclear genome but different mitochondria genome backgrounds, namely cells from ear tissues of cloned bovine and its donor, were choose as donor cells and introduced into enucleated rabbit oocytes. Similar developmental potential was observed in cloned bovine-rabbit (clone group) and bovine-rabbit (non-clone group) embryos. Real-time PCR assay showed that, in non-clone group**,** bovine mtDNA decreased during the development of reconstructed embryo, and that a sharp decrease was detected at the blastocyst stage. In clone group, bovine mtDNA decreased slightly, and the abrupt reduction of donor mtDNA did not occur during preimplantation development. In addition, an obvious increase in rabbit mtDNA was observed in both groups at the blastocyst stage. Our results demonstrate that: 1) the fates of donor mtDNAs in bovine-rabbit and cloned bovine-rabbit reconstructed embryos were different; and 2) recipient mtDNAs replicate at blastocyst regardless of the difference of donor cells.

2. INTRODUCTION

Mitochondria are the most abundant organelles in mammalian oocytes and early embryos. While their essential role in ATP production has long been recognized, only recently has their contribution to oocyte and embryo competence been investigated. A variety of physiological processes, including normality of spindle organization and chromosomal segregation, timing of the cell cycle, and morphodynamic processes such as compaction, cavitations and blastocyst hatching, were affected by the capacity of mitochondria to produce ATP (1). These functions are controlled by a dual genome system, with precise cross-talk between the mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) (2). In the process of nuclear transfer, heteroplasmic sources of mitochondrial DNA from a donor cell and a recipient oocyte are mixed in the cytoplasm of the reconstituted embryos. Strictly speaking, these animals are not true clones, but are better described as “genomic copies” as they are mosaics of cytoplasmic and nuclear elements from different individuals (3). It is worth reiterating that NT processes bypass some development event and introduce new cross-talk between the cytoplasmic and nucleus genomes derived from two individuals.

In intra-species example “Dolly”, there was no evidence that transferred mtDNA survived (4). And this is

corroborated by the fact that other studies on mtDNAs of cloned calves showed complete replacement of maternal mtDNAs (5-7). By contrast, only one report gave evidence of a significant replicative advantage of donor mtDNA (8). Mitochondria heteroplasmy with varying levels of donor-derived mtDNAs was observed in cloned bovine embryos and offspring by others and us (9-14).

In interspecies somatic cell nuclear transfer (iSCNT) animals where the donor nucleus and recipient oocyte are from closely related species, mtDNAs are primarily oocyte-derived with few divergence (15, 16). As for the iSCNT embryos reconstructed from two distantly related species, our previous studies in monkey-rabbit (17) and human-rabbit (18) embryos demonstrated that donor-derived mtDNA were degraded as the development went further. All together, in cloned animals, the donor-derived mtDNA was replaced by oocyte mtDNA partly or completely.

We presume that in preimplantation embryos, transferred donor nuclei can recognize its own mitochondria and lead to the destruction of the latter. In cloned animals, somatic cells contain mitochondria from recipient oocyte as mentioned above, so whether the nuclei can still recognize them and induce their destruction in NT embryos is an interesting question. In the present study, we choose two fibroblast cell lines sharing the same nuclear genome, but different mitochondrial genome backgrounds, namely cells from ear tissues of normal cloned bovine and its donor. And these cells were transferred into enucleated rabbit oocyte respectively. Real time PCR was used to analyze the fate of mitochondrial DNA in reconstituted embryos during early development.

3. METHODS AND MATERIALS

3.1. Animals

All chemicals used in this study were purchased from Sigma (Sigma Chemical Company, St. Louis, MO) unless otherwise noted.

All animals' experiments were approved and performed under the guidelines of the ethical committee of Institute of Zoology, Chinese Academy of Sciences. Mature female Japan white rabbits (purchased from the Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences) were caged at 20-26 ^{o}C, and were fed regular rabbit fodder and water ad libitum.

3.2. Preparations of oocyte and fibroblast

Each rabbit was super-ovulated by sequential injection of 100IU PMSG and 100IU hCG 72h apart. Both hormones were supplied by Institute of Zoology, Chinese Academy of Sciences, as a lyophilized powder and reconstituted in PBS. At 13.5 h after the hCG injection, the animals were laparotomized, and ovulated oocytes were flushed from the oviducts with Dulbecco's modified PBS (DPBS) supplemented with 1% FBS (Gibco). Cumulus cells were removed by short exposure to 300IU of hyaluronidase in DPBS solution and subsequent pipetting with a small-bore glass pipette. The ear tissue fibroblast cells were obtained from donor cattle and cloned cattle, respectively. Primary cell culture was performed with the same method as that described previously (19). Middle-sized donor cells passed for 4-8 generations were used for NT.

3.3. Reconstruction and culture of NT embryos

NT was performed according to the methods described previously with some modifications (20). Briefly, the eggs were incubated in M_2 medium containing 7.5 μg /ml cytochalasin B, 7.5 μg/ml Hoechst 33342 and 10% FBS for 10 min. The metaphase chromosomes were removed and visualized under a fluorescence microscope to confirm the complete nuclear removal. A singe cell (15 μm in diameter) was chosen and transferred into the perivitelline space. Then the couplets were exposed to a double electric pulse of 1.4 kv/cm for 80 μsec using an ECM2001 Electrocell Manipulator (BTX Inc, San Diego, CA). Fused couplets were assigned to 7% ethanol for 5 min, then 2.5 mM DMAP in TCM 199 for 1.5 h. Embryos were cultured in 100-μl drops of TCM 199 +15% FBS under mineral oil at 38.5 ^{o}C in a humidified atmosphere of 5% CO_2 for further development.

3.4. Embryo collection and DNA extraction

Single embryo at 1-cell, 2-cell, 4/8-cell, morula or blastocyst stage was collected. These samples were washed 3 times in DPBS and then collected into PCR tube directly. The manipulation was performed under a dissection microscope to assure that each embryo was transferred. The genomic DNA was extracted from the collected embryos with ReadyAmp^{TM} GenomicDNA Purification System (Promega, Corporation, Madoson, WI, USA). Single NT embryo was heated at 55 ^{o}C for 30 min, and 99 ^{o}C for 10 min in 20 μl lyses system. The samples were centrifuged at 1.2×10^{3} g for 2 min and then used as PCR templates directly.

3.5. Verification of NT embryos by microsatellite DNA analysis

Semi-nested PCR was performed to identify the iSCNT embryos. The specific primers of the bovine microsatellite DNA were: B_1 5'-CCC CTT TCC CTC TGA TAA CCA C-3'; B_2 5'-CAG GGC TTG TCT TTC TCC ATC TGA-3' and B_3 5'-ACT TCA CAG CCC ATC AAC AGA CGA-3'. The first round amplification was performed at 95 ^{o}C for 5 min, 94 ^{o}C for 45 sec; 55 ^{o}C for 40 sec; 72 ^{o}C for 1 min for 30 cycles, and finally 72 ^{o}C for 5 min using primer B_1 and B_3 in a temperature cycler (PTC-100,MJ Research. Inc.USA). Aliquots of 1 μl PCR production from first round amplification were subjected to second round amplification performed at 95 ^{o}C for 5 min, 94 ^{o}C for 30 sec; 60 ^{o}C for 40 sec; 72 ^{o}C for 50 sec for 30 cycles, and finally 72 ^{o}C for 5 min using primer B_2 and B_3. Cross contamination tests were performed and negative controls were designated to rule out the possibility of contamination during PCR process. The final amplification products were separated via agarose gel electrophoresis and the bands were verified by sequencing for both DNA strains using an automatic DNA Sequencer (ABI377, Perkin Elmer).

Table 1. Developmental potential of reconstructed embryos with different donor sources

Donor cell	Total NT	Fusion (%)	1-cell (%)	2-cell (%)	4/8-cell (%)	Morula (%)	Blastocyst (%)
Bovine	229	170(74.2)	138(81.2)	110(79.7)	92(66.7)	46(33.3)	24(17.4)
Cloned bovine	352	215(61.0)	168(78.1)	144(85.7)	124(73.8)	62(36.9)	28(16.7)
Total	581	385(66.3)	306(79.5)	254(83.0)	216(70.6)	108(35.3)	52(17.0)

Five replicates for each experiment. There was no difference in development potential between the bovine-rabbit and cloned bovine-rabbit groups ($p>0.05$).

3.6. Standard curves of mtDNAs

The primers for bovine mtDNAs were: B_{m1} 5'-ATC ATA GCA ATT GCC ATA GTC-3' and B_{m2} 5'-CGC GAA TAG TAC TAG TAG TAT TAG AG-3'. The primers for rabbit mtDNAs were: R_{m1} 5'-ATT TAT CAT TGC AAC TTT AGT CTT A-3' and R_{m2} 5'-AAG GAG GAG AAG AAT GGC TAC A-3'. Primers sets were tested and optimized to verify the amplification of a single band of the correct predicted size without primer dimmer. PCR products were purified from agarose gel and inserted into pGEM-T-easy vector (Promega). Both strands of two clones, each derived from independent polymerase chain reactions (PCRs) of a single individual, were sequenced by standard procedures on ABI377 automatic DNA Sequencer.

The copy number of the standards was determined by measuring absorbance at 260nm (Eppendolf France). The quality of purification was accepted only with an absorbance ratio (A260-A280) between 1.7 and 1.9. And these plasmids were diluted serially covering from 10^5 to 10^1 copies (10-fold dilutions) for bovine mtDNA and from 10^6 to 10^2 copies for rabbit mtDNA.

3.7. Real-time PCR of mtDNAs

Single embryos were analyzed, and at least six embryos were tested at each developmental stage, independently. The samples and the standards were co-amplified under the same conditions. A standard scale prepared from plasmid was used to convert the "threshold cycle" value at which each fluorescent signal was first detected above background into template copy numbers. The amplification cycling profile was 95 ^oC for 8 sec, 40 cycles consisting of the following two steps: 95 ^oC (4 sec), 61 ^oC (40 sec) using a ABI PRISM 7000 (Applied Biosystems, USA). The reaction mixture consisted of 1 µl NT embryos lysis template (equivalent 0.05 embryos), 50 nM each primer, 2×SYBR Green Master Mix, 0.5µl ROX (Takara, Japan) and distilled water to make a final total volume of 25 µl. Negative control (NTC) was included in each experiment. A melting curve (loss of fluorescence at a given temperature between 60 ^oC and 95 ^oC) was analyzed to check the absence of mispriming and the quality of amplification.

3.8. Statistical analysis

All data were analyzed using the SPSS statistical program, LSD-test (SPSS Inc, Chicago, IL). Data are presented as the mean percentage ± SEM. Differences were considered significant when $p<0.05$. All experiments were repeated at least 3 times.

4. RESULTS

4.1. Developmental potential of the NT embryos

A total of 581 rabbit oocytes were enucleated and 385(66.3%) fibroblast-oocyte pairs were constructed successfully. Six hours after activation, pronucleus (PN)-like structure was observed among most of the reconstructed eggs (79.5%). The overall developmental rates were 83.0% to the 2-cell at 18-24 hours, 70.6% to the 4/8-cell at 48-60 hours, 35.3% to morula stage and 17.0% to the blastocyst stage at 120-144 hours of culture (Table 1). There was no significant difference between the two groups using different donor cells ($P>0.05$).

4.2. Specificity of the mtDNAs assay

The standard curves were generated from a minimum of 5 points spanning the anticipated unknown values. Analyzing the amplification profiles and the corresponding dissociation curves of each amplicon monitored the specificities of the products (Figure 1 and Figure 2). When amplification was performed using bovine-specific primers and bovine genome, a significant increase in SYBR Green fluorescence was recorded. When the same bovine primers were used with rabbit genome, the fluorescence signal was under the threshold, indicating no cross-specific product; vice versa, the rabbit mtDNA-specific primers detected PCR products only in rabbit mtDNA but not in bovine. Single melting peak was observed in Figure1A (Tm=81.8^oC) and Figure2A (Tm=80.8^oC), indicating the absence of nonspecific amplification.

4.3. Analytical sensitivity of SYBR Green PCR using plasmid DNA as the template

A linear relationship was observed between the input copy number of the plasmid template and the CT values for the species-specific product over $\log_{10}$ dilutions, ranging from10^6 copies to 10^2 copies for rabbit and from 10^5 copies to 10^1 copies for bovine. The mtDNA standards were well proportioned (Figure3). A174-bp mtDNA was amplified using bovine primers, and a 163-bp DNA was amplified using rabbit primers (data not shown).

4.4. Dynamic changes in mtDNA copies number of two NT groups

In each group, we choose 6 embryos at each stage. The nuclear genomic background of embryos was confirmed to be identical to their donor cells by microsatellite examination (data not shown). The confirmed embryos were analyzed for the mtDNA dynamic changes during early development.

As for the rabbit mtDNA, in the 1-cell stage embryos, the copy numbers were 5.64×10^5 for cloned bovine-rabbit group (clone group) and 5.06×10^5 for bovine -rabbit group (non-clone group). In subsequent *in vitro* development, both groups showed similar dynamic changes in copy numbers. No significant difference was observed from the 2-cell stage until to morula stage between two groups ($P>0.05$) (Figure 4A). However, at blastocyst stage,

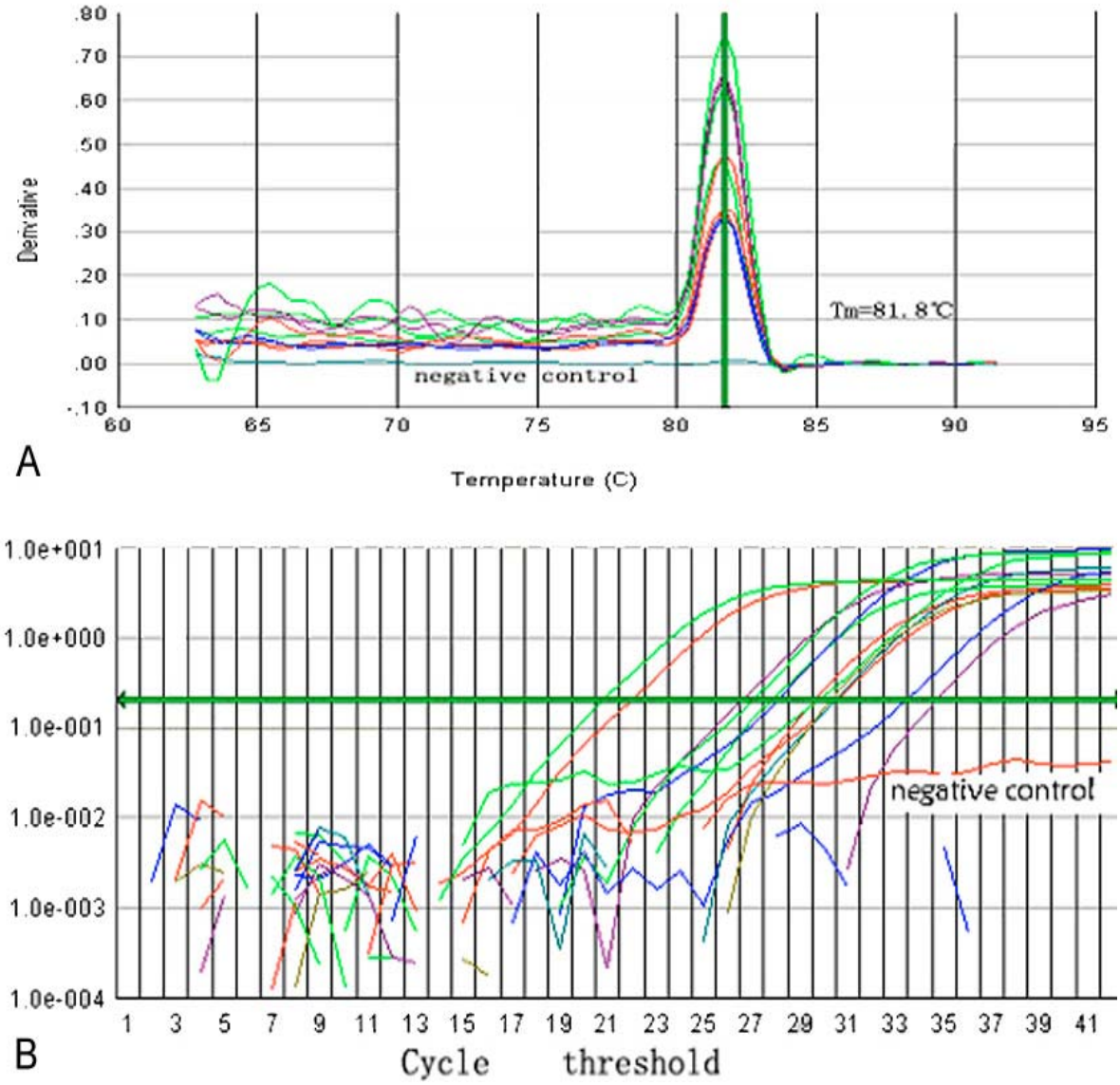

Figure 1. The mtDNA amplification dissociation curves (A) and NT embryos amplification profiles (B) showing log linear phases of real-time PCR reactions using of bovine specific mtDNAs primers. The Tm values and negative control are indicated alongside the curves.

a sharp increase in rabbit mtDNA ($P<0.05$) was observed and the copy number was increased to 5.10×10^6 for clone group and 6.32×10^6 for non-clone group.

Contrary to the similar trends in rabbit mtDNA content, bovine mtDNA showed different fates in the two groups (Figure 4B). After the normal bovine fibroblasts were introduced into rabbit ooplasm, the donor-derived mtDNA gradually decreased as the reconstructed embryos developed further (from $2.74 \pm 1.86 \times 10^3$ at 1-cell stage to $1.60 \pm 0.58 \times 10^3$ at morula stage). This kind of reduction was gradual until morula stage, while a sharp decrease was observed at blastocyst stage, bringing the number of bovine mtDNA copies down to $0.16 \pm 0.07 \times 10^2$ ($P<0.05$). In the other group, where the cloned bovine fibroblast cells were used as donors for NT, the bovine mtDNA copies were decreased slightly (from $2.67 \pm 1.03 \times 10^3$ at 1-cell stage to $1.69 \pm 0.71 \times 10^3$ at blastocyst stage). In contrast to non-clone group, the abrupt reduction in mtDNA did not occur at blastocyst stage in clone group ($P>0.05$).

5. DISCUSSION

Previous studies have shown that bovine and sheep oocyte cytoplasm can support early development of embryos produced by NT of somatic cell nuclei from other mammalian species (21, 22). In the present study, our results verified that the bovine fibroblast and regenerated fibroblast derived from its cloned offspring were dedifferentiated and developed to blastocyst in rabbit

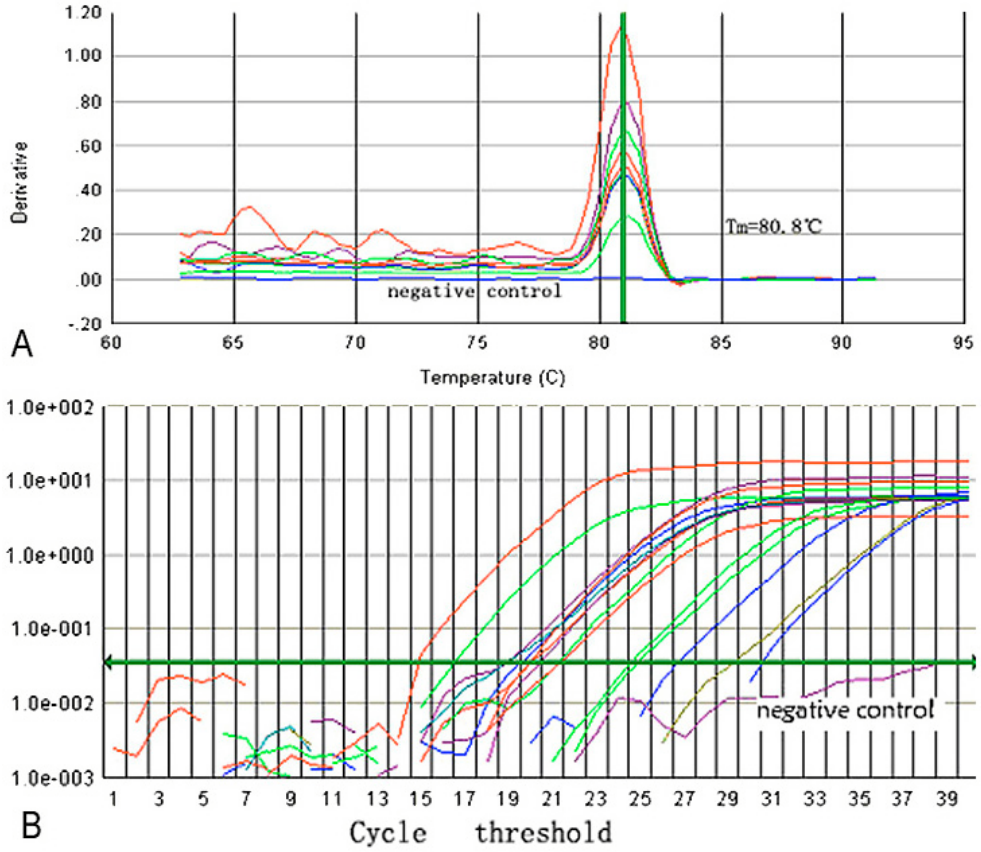

Figure 2. The mtDNA amplification dissociation curves (A) and NT embryos amplification profiles (B) showing log linear phases of real-time PCR reactions using of rabbit specific mtDNAs primers. The Tm values and negative control are indicated alongside the curves.

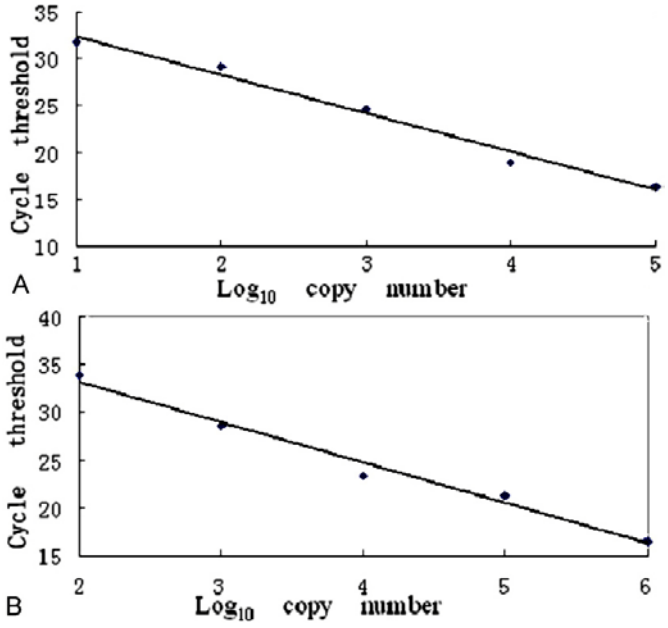

Figure 3. Linear relationship between CT values and the dilutions of the plasmid DNAs of Cytb fragment. (A) Bovine mtDNA standards from 10^5 copies/µl to 10^1 copies/µl, and (B) Rabbit mtDNA standards from 10^6 copies/µl to 10^2 copies/µl.

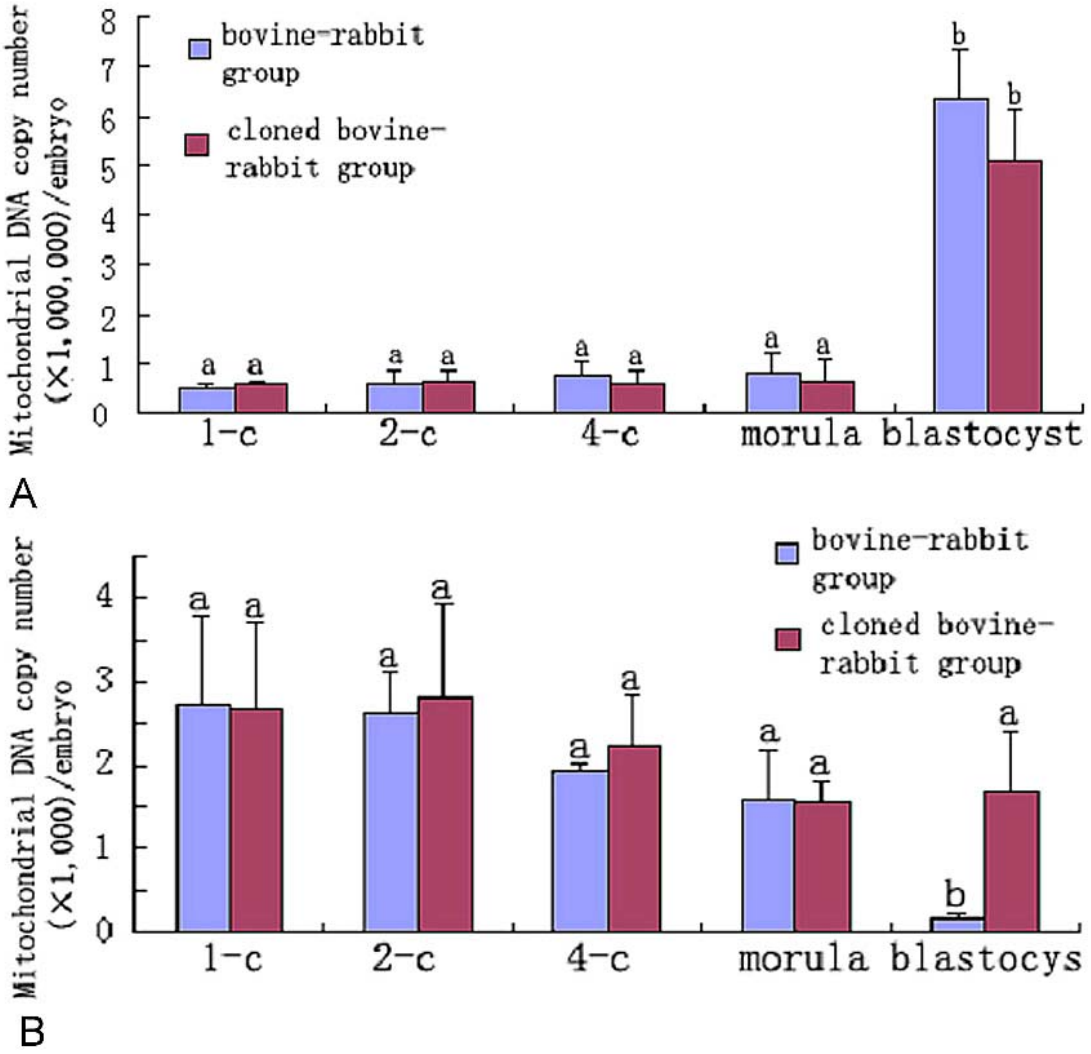

Figure 4. The dynamic changes in mtDNA composition during early development of bovine-rabbit group and cloned bovine-rabbit group. (A) Rabbit specific mtDNA and (B) bovine specific mtDNA.

ooplasm. The cloned and non-cloned fibroblasts exhibit similar developmental potentials reprogrammed by rabbit ooplasm. Our initial expectation was that the regenerated fibroblast cells would exhibit higher developmental capacity since it experienced reprogramming during the first NT. This scenario turned out not to be true, and critical nuclear-cytoplasmic interaction in interspecies NT is not known.

In the process of NT, mitochondria of donor cells together with the nucleus are transferred into the recipient oocyte. Disharmony between nuclear and mitochondrial genes is thus likely to complicate cloning. Consequently, NT offspring will exhibit one of the three patterns of mtDNAs transmission, namely homoplasmy derived solely from the recipient oocytes, homoplasmy derived exclusively from the donor somatic cell, or heteroplasmy resulting from the fusion partners (23). In our present study both bovine mtDNAs and rabbit mtDNAs could be detected throughout various development stages before implantation. This is contrast to the research of human-bovine reconstructed embryos, which reported that only the bovine (recipient) mtDNA was found beyond the 16-cell stage (24). It appears that the rabbit ooplasm has greater tolerance to foreign mitochondria. It mimics the mtDNA co-existing patterns in our previous research on panda-rabbit and chicken-rabbit reconstructed embryos (20, 25).

The number of mitochondria and the mtDNAs copy number are essential to oxidative phosphorylation capacity and the functional status of the mitochondria plays an important role in embryo development (26, 27). Because of the sensitivity and the high correlation between the quantity of mtDNA and the mitochondrial mass (28), we applied real-time PCR to examine the fate of mitochondria in single iSCNT embryo. A significant increase of rabbit mtDNA was detected in clone and non-clone groups, which is consistent with our previous analysis of monkey-rabbit

embryos (17). But our results are different from other reports, which demonstrated that oocyte mitochondria provided the energy needed by the embryo for the entire preimplantation phase of development, and there was no replenishment of mitochondria until the post-blastocyst stage (12, 29, 30). Many unknown factors are involved in the iSCNT embryos, and the only clear picture emerges is the species difference and the characteristics of rabbit oocyte.

As for the fate of the somatic cell derived mtDNA, in non-clone group, the bovine mtDNAs were probably degraded by an elimination system similar to that in natural fertilization (31). While in the clone group, the bovine mtDNA escaped the elimination check and maintained the quantity almost as much as initial level (at 1-cell stage). It is reasonable to assume that the escaping from extensive destruction is due to the mtDNA heteroplasmy in the donor cells which were regenerated from cloned bovine and showed mtDNA heteroplasmy as reported in our previous report (9). We speculate that in oocyte, the elimination systems recognize proteins in mitochondria encoded by nucleus but not by mitochondria. So the mitochondria from the cloned bovine fibroblast, which contained bovine oocyte-derived mitochondria different from that of somatic cells, were not recognized and not eliminated. Cross-talk between the nuclear genome and mitochondria genome are complicated, and further experiments and more data are needed to verify our hypothesis.

6. ACKNOWLEDGMENT

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Abbreviations: mtDNA: mitochondrial DNA; nDNA: nuclear DNA; iSCNT: interspecies somatic nuclear transfer

Key Words: Nuclear Transfer, mtDNA, Embryo Development, Real Time PCR

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