Dominant distribution of mitochondrial DNA from recipient oocytes in bovine embryos and offspring after nuclear transfer

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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 embryo. The distribution of mitochondrial DNA heteroplasmy in nuclear transfer bovine embryos and resultant offspring was investigated by measuring polymorphism in the displacement loop region of mitochondrial DNA using PCR-mediated single-strand conformation polymorphism. Most offspring (20 of 21 calves) from recipient oocytes of undefined mitochondrial DNA genotypes showed different genotypes from the mitochondrial DNA of donor cells. The single calf that was an exception showed heteroplasmy, including the donor mitochondrial DNA genotype. Six cloned calves were produced from oocytes of a defined mitochondrial DNA genotype. All of these clonal members and various tissues showed only the mitochondrial DNA genotype derived from the oocyte. The mitochondrial DNA from donor cells appeared to be eliminated during early embryonic development; it gradually decreased at the early cleavage stages and was hardly detectable by the blastocyst stage. These results indicate that the genotype of mitochondrial DNA from recipient oocytes may become the dominant category of mitochondrial DNA in calves resulting from nuclear transfer.

Introduction

Mammalian mitochondrial DNA (mtDNA) is a double-stranded circular molecule of about 16.5 kb, the genetics of which differ from that of the nuclear genome. mtDNA molecules are present in mitochondria that occupy a substantial portion of the cell cytoplasm. The number of mtDNA molecules in most types of cell is about 2×10^3 copies (Michaels *et al.*, 1982). In contrast, the number of mtDNA molecules in a single oocyte, which has the largest amount of mitochondria in the cytoplasm, is about 2×10^5 (Michaels *et al.*, 1982; Hauswirth and Laipis, 1985). At fertilization, mtDNA heteroplasmy occurs as a consequence of the spermatozoon, which has a small number (approximately 50–75) of mtDNA molecules, entering the mature oocyte (Hecht *et al.*, 1984). However, after the egg has cleaved several times, mtDNA heteroplasmy is erased by the mechanism of exclusion of the sperm mtDNA from the egg cytoplasm (Kaneda *et al.*, 1995; Sutovsky *et al.*, 1996). The mtDNA shows inheritance by predominantly maternal cytoplasmic exchange (Gyllenstein *et al.*, 1985).

Spontaneous mutation of mtDNA has been observed and may cause mtDNA heteroplasmy. However, it appears that mtDNA heteroplasmy becomes homoplasmy by random distribution during oogenesis (bottleneck effect) (for review,

see Lightowers *et al.*, 1997). Pedigree analysis of a heteroplasmic displacement loop (D-loop) sequence variant in Holstein cows has demonstrated a return to homoplasmy within only one (Koehler *et al.*, 1991) or two generations (Ashley *et al.*, 1989). Jenuth *et al.* (1996) created lines of heteroplasmic mice carrying two mtDNA genotypes and showed that the bottleneck effect can be explained by random genetic drift in early oogenesis. Thus, it is likely that mtDNA heteroplasmy is unstable and shifts rapidly to a homoplasmic condition.

The procedure of nuclear transfer has been used to produce live offspring of several species, including mice, sheep, cattle, rabbits and pigs (for review, see Prather and First, 1990; Bondioli, 1993). Nuclear transfer embryos are produced by fusion of a nuclear donor cell with an enucleated recipient oocyte, giving rise to mitochondrial heteroplasmy in the reconstituted embryo. In these offspring, the nuclear genome is the same among clonal members, but presumably the mtDNA is different. Plante *et al.* (1992) reported that mtDNA polymorphisms were found among four cloned Holstein bulls and suggested that the mtDNAs originated from recipient oocytes that were collected from different ovaries with different mtDNA genotypes. Steinborn *et al.* (1998a) reported that mtDNAs originating from donor cells were detected in cloned cattle, although one clone did show a reduction in donor cell mtDNA. Optimally, the origin of mtDNA should be determined in both the donor cell and the recipient oocyte to investigate the mtDNA genotype in nuclear transfer animals.

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Received 6 July 1998.

The D-loop in mtDNA has a striking pattern of nucleotide substitution and is useful for evolutionary investigation of genetic variations (Greenberg *et al.*, 1983). The D-loop region contains the regulatory sequences responsible for replication and transcription of the mitochondrial genome, such as the origin of heavy-strand replication (Clayton 1984). The lower stream of this replication origin is the most variable region in the D-loop (Horai and Hayasaka, 1990). Both Holstein and Japanese Black cattle are breeds classified as *Bos taurus* by mtDNA analysis (Watanabe *et al.*, 1985; Kikkawa *et al.*, 1995; Takeda *et al.*, 1997). Although identification of the polymorphism on mtDNA has been conducted to distinguish between these breeds, no unique difference is observed, as is the case for other European cattle (Loftus *et al.*, 1994). However, polymorphic variation was detected in all the cattle by direct sequencing or single-strand conformation polymorphism (SSCP) analysis in these reports. SSCP analysis allows detection of an unknown substitution, even if only a single base substitution has occurred (Orita *et al.*, 1989; Suzuki *et al.*, 1991), and can also easily detect heteroplasmy in genomes. This method can be used to identify reliably the mtDNA genotype of nuclear transfer embryos and cloned calves reconstituted from different mtDNA sources.

In this study, mtDNA heteroplasmy was determined in bovine nuclear transfer embryos and their offspring by detecting polymorphism in the D-loop using the PCR-SSCP method (Harumi *et al.*, 1994; Takeda *et al.*, 1997). In the first experiment, SSCP patterns in recipient oocytes and their ovaries were compared to determine whether the mtDNA genotypes were the same or different. In the second experiment, nuclear transfer embryos were produced using a recipient oocyte and a blastomere of a previously defined mtDNA genotype, and mtDNA heteroplasmy and the origin of mtDNA in the nuclear transfer embryos and resultant offspring were analysed by PCR-SSCP.

Materials and Methods

Collection of embryos and oocytes

Donor embryos were recovered non-surgically on day 5 or day 6 after oestrus from Japanese Black cows after conventional superovulation treatment (Kojima *et al.*, 1989). Blastomeres from embryos at the morula stage were used as nuclear donors.

Recipient oocytes were collected from several ovaries (mostly from Holstein and a few from Japanese Black) obtained from an abattoir. In these experiments, nuclear transfer was performed using recipient oocytes of undefined origin (UD-NT).

In a second series of experiments, the mtDNA origin of recipient oocytes was determined. Three pairs of ovaries were obtained and recipient oocytes were collected individually from each pair of ovaries. Nuclear transfer was performed using recipient oocytes of defined origin (D-NT). The mtDNA genotype of these ovaries was also determined.

Four additional pairs of ovaries were obtained and their oocytes were collected to compare the mtDNA genotype of an individual ovary with that of its oocytes.

Nuclear transfer procedure

Follicular oocytes collected from ovaries were matured in TCM-199 (Gibco BRL, Rockville, MD) supplemented with 10% (v/v) heat-treated fetal calf serum (FCS). After 24 h maturation, the oocytes were enucleated and pretreated with a combination of 10 mmol calcium ionosphere A23187 l⁻¹ (Calbiochem, San Diego, CA) for 5 min and 10 µg cycloheximide ml⁻¹ (Sigma Chemical Co., St Louis, MO) for 6 h, before fusion with a donor cell. Electrofusion was performed at 75 V DC pulse mm⁻¹ for 2 × 50 µs in Zimmerman mammalian cell fusion medium. The reconstituted embryos were cultured in CR1aa medium (Rosenkrans and First, 1994) for 7 days, as reported by Takahashi *et al.* (1996).

Nuclear transfer embryos that developed to the blastocyst stage *in vitro* were transferred non-surgically into recipient cows. D-NT embryos were produced using 30-cell stage morulae as donor cells to determine mtDNA heteroplasmy during early embryonic development. Each of three D-NT embryos was removed from culture at a different stage of embryo development: immediately after fusion, 48 h after fusion (the four- to eight-cell stage), and 7 days after fusion (the blastocyst stage). The mtDNA genotype of these embryos was analysed by the method described below.

Nuclear transfer calves for analysis

Twenty-one nuclear transfer calves were produced from UD-NT embryos. Eight of these cloned calves were selected for determination of mtDNA genotypes in the leucocytes or tissues (skeletal muscle, heart, testis or ovary, uterus, brain, liver, kidney, lung and spleen).

In addition, six nuclear transfer calves, consisting of two sets of triplets, were produced from D-NT embryos. These calves resulted from transfer into four and three recipients of eight and six D-NT blastocysts, respectively. Blastomeres from two embryos at the morula stage, consisting of 18 and 21 cells, were used as nuclear donors. The mtDNA genotypes of four D-NT calves were determined in various tissues (skeletal muscle, heart, testis, brain, liver, kidney, lung and spleen), and in the leucocytes of the other two calves. Genetic identity of triplet cloned calves was also confirmed by blood-type analysis.

The mtDNA genotypes of donor cells were determined by analysis of the blood of Japanese Black cows from which the donor embryos had been collected. The mtDNA genotypes of recipient oocytes were determined by analysing the ovaries yielding the recipient oocytes. The mtDNA genotypes of nuclear transfer calves were compared with those of the donor cells and recipient oocytes.

DNA extraction for mtDNA analysis

Leucocytes were purified from fresh blood treated with EDTA, followed by centrifugation (2000 g for 30 min) and low hypotonic (37.5 mmol NaCl₂ l⁻¹) treatment. The leucocytes and tissue samples were disrupted with lysis

Fig. 1. mtDNA genotypes of bovine ovaries and their oocytes analysed by PCR–single-strand conformation polymorphism (SSCP) of SF and SR fragments. Lanes 01, 02 and 03: genotype of ovaries; lanes 1: oocytes collected from 01; lanes 2: oocytes collected from 02; and lanes 3: oocytes collected from 03.

buffer (1% (w/v) SDS, 10 mmol Tris-Cl l^{-1} , pH 8.0, 5 mmol EDTA l^{-1} , 100 μ g proteinase K ml^{-1} and 10 μ g RNase A ml^{-1}) at 37°C overnight. Total DNA was purified by phenol–chloroform extraction and ethanol precipitation, and 100 ng of each total DNA was used as templates for PCR–SSCP analysis.

Each sample from the reconstituted embryos, blastomeres and oocytes was washed nine times in Dulbecco's PBS without magnesium and calcium (Takara Shuzo Co., Tokyo) containing 1% (w/v) polyvinylpyrrolidone and placed into 10 μ l H₂O in 0.5 ml PCR tubes. The samples were frozen and thawed three times before PCR–SSCP analysis.

mtDNA genotype analysis using PCR–SSCP

mtDNA genotypes were determined by the PCR–SSCP method using two sets of primers, situated in the variable region and the whole region of the D-loop, respectively, to confirm the result from each PCR reaction. PCR–SSCP of the variable region allows detection of mutations and heteroplasmy on mtDNA, and PCR–SSCP of the whole D-loop allows detection of mutations that are not detected in the variable region.

The sequences of oligonucleotide primers used for PCR amplification of the variable region in the D-loop were as follows: SF (3'-CCATGCATATAAGCAAGTACATGAC-5') and SR (3'-CTGATTAGCCATTAGTCCATCGAG-5'). The PCR amplification under 'hot start' conditions was performed in a 25 μ l reaction volume containing 12.5 pmol of each primer, 1 $\times$ PCR buffer (Promega Co., Madison, WI), 0.9 U Taq DNA Polymerase (Promega) pre-reacted with 0.23 μ g TaqStart antibody (Clontech Laboratories Inc., Palo Alto, CA), 0.2 mmol dNTP l^{-1} and 1.5 mmol MgCl₂ l^{-1} . The reaction mixture was incubated in a thermal cycler (Program temp control system PC-700, Astec Inc., Fukuoka) under the following conditions: 96°C for 1 min for first denaturing, 30 cycles of denaturing, annealing and extending (95°C for 1 min for denaturing, 65°C for 1 min for annealing and 72°C for 1 min for extending) and 72°C for 4 min for final extension. Each PCR product (1 μ l; SF and SR fragment: 330 bp) was diluted one to three times with TE buffer (10 mmol Tris-Cl l^{-1} , pH 8.0, 1 mmol EDTA l^{-1}) for SSCP analysis.

Two primers (M. P. Sabour, personal communication) were used for PCR amplification of the whole D-loop: LF (3'-TAATATACTGGTCTTGTAAC-5') and LR (3'-AACA-

GTTAACAGGAAGGCTGGGACCAAACC-5'). The PCR procedure was as described by Harumi *et al.* (1994). The DNA fragments amplified by LF and LR primers were digested using the restriction enzyme *HpaII* (LF and LR fragments: 547 bp, 353 bp and 230 bp) and used for SSCP analysis.

For SSCP analysis, 1 μ l of each PCR product was mixed with 5 μ l 95% deionized formamide solution containing 20 mmol EDTA l^{-1} , incubated at 95°C for 5 min and chilled on ice for 10 min. Each sample (1 μ l) was analysed on 5.5% (w/v) polyacrylamide gel (8 cm $\times$ 8 cm $\times$ 0.1 cm) containing 5% (v/v) glycerol. The electrophoresis was performed in 0.5 $\times$ TBE buffer (45 mmol Tris-borate l^{-1} , 1 mmol EDTA l^{-1}) at 120 V for 5 h under controlled gel temperature at 4°C (Takeda *et al.*, 1997). The resulting band patterns were visualized by silver staining (Silver Stain Plus Kit, Bio Rad Laboratories, Hercules, CA).

Results

The mtDNA genotypes of eggs were compared with those of the ovaries from which they were obtained by the PCR–SSCP method. Among four ovaries analysed, three ovaries showed homogeneous mtDNA patterns (Fig. 1). All the eggs from these ovaries had the same mtDNA genotype as the relevant ovary. The fourth ovary showed a heteroplasmic pattern and two different mtDNA genotypes, but oocytes obtained from the ovary had only one of the two types of mtDNA (Fig. 1).

The mtDNA genotype of each of the 21 UD-NT offspring was compared with the mtDNA genotype of the donor cell. All the mtDNA genotypes of UD-NT offspring were different from those of donor cells (Fig. 2), with only one exception (Fig. 2c). Most of the UD-NT offspring (17 calves) showed homoplasmy in their mtDNA genotypes (Fig. 2a), and three of the other four UD-NT offspring (two sets of cloned calves) had two kinds of mtDNA pattern (heteroplasmic), which were also different from the mtDNA of donor cells (Fig. 2c). However, one of these calves showed heteroplasmy and contained the same mtDNA genotype as its donor cell (Fig. 2c). Eight UD-NT calves with homoplasmic mtDNA genotypes were analysed to determine the mtDNA genotypes in various tissues (skeletal muscle, heart, testis or ovary, uterus, brain, liver, kidney, lung and spleen). The mtDNA band patterns of all of the tissues were the same in each cloned calf (Fig. 2b).

The mtDNA genotypes of six D-NT offspring (two sets of triplets) produced from oocytes with pre-determined mtDNA genotypes were compared with those of the recipient oocytes and donor cells. In all of the D-NT calves, only mtDNA genotypes that were also found in their oocytes were observed (Fig. 3a). The same results were obtained with the mtDNA genotypes of various tissues from four of the D-NT calves. In these calves, the mtDNA genotypes were the same as the oocytes and not the donor cells (Fig. 3b).

Patterns of mtDNA in D-NT embryos during early embryonic development were examined. The mtDNA genotypes of the embryos recovered immediately after fusion were heteroplasmic, consistent with the view that mtDNAs from both donor cells and recipient oocytes were

Fig. 2. mtDNA genotypes of calves produced by nuclear transfer using recipient oocytes of undefined origin (UD-NT) analysed by PCR-single-strand conformation polymorphism (SSCP). (a) Unknown mtDNA genotype in UD-NT calves: (i) SF and SR fragments; (ii) LF and LR fragments. Lanes 1 and 2: UD-NT calves produced from the same embryo as nucleus donors; N1: cow (blood) offered nucleus donor cells of 1 and 2. UD-NT calf 2 and its nucleus donor cell N1 appeared to be the same genotype by analysis (i), but were shown to be of different genotypes by analysis (ii). (b) mtDNA genotypes of various tissues of UD-NT calf 2 (shown by an asterisk on (a)), analysed by PCR-SSCP using LF and LR fragments. Bl, blood; SM, skeletal muscle; H, heart; Ov, ovary; U, uterus; Br, brain; Lg, lung; Sp, spleen; K, kidney; lane N1: the nucleus donor cell of calf 2. (c) mtDNA heteroplasmy in UD-NT calves: (i) SF and SR fragments; (ii) LF and LR fragments. Lanes 3 and 4: UD-NT calves produced from the same embryo as nucleus donors, lane N2: cow (blood) offered the nucleus donor cells of 3 and 4. Arrows show the same band patterns as the donor cells.

present. The amount of donor cell mtDNA decreased at the four- to eight-cell stage and was almost absent at the blastocyst stage (Fig. 4).

Discussion

Transmission of mtDNA genotypes to offspring is thought to be controlled by strict maternal inheritance (Gyllenstein *et al.*, 1985; Birkey, 1995). Moreover, different types of mtDNA segregate evenly in the female germ line due to a genetic bottleneck in early oogenesis. As a result, most individuals are homoplasmic and have a single mtDNA genotype (Hauswirth and Laipis, 1985; Jenuth *et al.*, 1996). Hauswirth and Laipis (1982) reported two mitochondrial genotypes

within one maternal lineage in Holstein cows. Pedigree analysis of mtDNA variants indicates that a rapid change of mtDNA genotypes occurs within only one or a few generations (Ashley *et al.*, 1989; Koehler *et al.*, 1991). A similar phenomenon was also observed in the present study: one of the ovaries analysed showed mtDNA heteroplasmy. However, different mtDNAs became segregated in each oocyte as a single mtDNA genotype, indicating that a rapid bottleneck might occur in mtDNA sequences within one generation. Although, this may be a rare occurrence, this phenomenon should be considered when mtDNA maternal lineage is analysed.

Steinborn *et al.* (1998b) detected a wide range (0.0004–18%) of donor cell mtDNA in cloned calves using the allele-specific TaqMan PCR method. In the present study, only the

Fig. 3. mtDNA genotypes of calves produced by nuclear transfer using recipient oocytes of defined origin (D-NT) analysed by PCR-single-strand conformation polymorphism (SSCP). (a) Comparison of mtDNA genotypes with two sets (a and b) of D-NT triplets, the nucleus donor cells (N) and the recipient oocytes (Oa and Ob, respectively) analysed by PCR-SSCP of SF and SR fragments (i) and LF and LR fragments (ii). (b) mtDNA genotypes of various tissues of a D-NT calf (shown by an asterisk on (a)), analysed by PCR-SSCP of SF and SR fragments. SM, skeletal muscle; H, heart; Br, brain; T, testis; Lv, liver; K, kidney; Lg, lung; Sp, spleen; lane N, the nucleus donor cell; lane Oa, the recipient oocyte.

mtDNA genotype of recipient oocytes, not of donor cells, was detected in each of the D-NT calves, indicating that the mtDNA of D-NT calves originated from the recipient oocytes. Furthermore, the mtDNA genotype of donor cells was not detected in most of the UD-NT calves. Analysis of mixtures of DNA at several ratios revealed that the limit of detection of this method was 3–4%, which was considered to be adequate for detection of the mixture of mtDNA in the reconstituted embryos in this study.

Four of 21 UD-NT offspring (representing two sets of clones) showed mtDNA heteroplasmy in their tissues. One set of clonal members came from the same litter and showed exactly the same band patterns, which were different from the donor mtDNA genotype. It is possible that rapid genetic

Fig. 4. Heteroplasmy in nuclear transfer bovine embryos analysed by PCR-single-strand conformation polymorphism (SSCP) of SF and SR fragments. (a) mtDNA genotypes of nuclear transfer embryos immediately after fusion (lanes a), 48 h after fusion (the four- to eight-cell stage) (lanes b), and 7 days after fusion (the blastocyst stage) (lanes c). Lane O: an oocyte collected from the same ovary; lane N: a blastomere collected from the same embryo. Arrows show the same patterns as the donor cells. (b) Analysis of mixtures of oocyte (O) and blastomere (N) DNAs at several ratios. Each PCR product was mixed and used as template DNA for the PCR-SSCP.

mutations are responsible for the heteroplasmic mtDNA genotype of these UD-NT calves. Alternatively, the heteroplasmy may be transmitted from the recipient oocyte, the mtDNA genotypes of which were originally heteroplasmic. The other clonal members were not from the same litter and showed different band patterns. One of these was the same as the donor mtDNA genotype. It is possible that the recipient oocyte had mtDNA heteroplasmy, including the same genotype as that of the donor cell. However, a similar phenomenon has been observed for intraspecific nuclear transplantation in mice (Meirelles and Smith, 1997) and cattle (Steinborn *et al.*, 1998a). These investigations showed that the mtDNA from the karyoplast persists in the reconstituted embryos and in the resultant progeny.

mtDNAs from both the recipient oocyte and from the donor cell were detected in the D-NT embryos collected immediately after fusion of the karyoplast and the oocyte. However, the donor mtDNA decreased at the four- to eight-cell stage and was hardly detectable at the blastocyst stage. These results explain why donor cell mtDNAs were not detected in most of the nuclear transfer calves, and indicate that the mtDNA originating from the donor blastomere was nearly eliminated during early embryonic development. This finding does not support the observations of Steinborn *et al.* (1998a), who reported neutral segregation of parental mtDNA in cloned bovine embryos during embryogenesis using embryos from Simmental cattle as donor cells. However, Steinborn *et al.* (1998b) also reported a reduction in donor cell mtDNA in one of the clones using embryos from

the Brown Swiss breed as donor cells. In the present study, nuclear transfer embryos were produced using donor cells derived from Japanese Black cattle, the mtDNA sequence of which is also classified as *Bos taurus*; no unique differences are observed between Holstein and Japanese Black breeds (Watanabe *et al.*, 1985; Kikkawa *et al.*, 1995; Takeda *et al.*, 1997). In these cases, it is possible that some unknown factors cause a reduction in donor cell mtDNA. This observation is similar to the finding that in bovine embryos fertilized *in vitro* the sperm mitochondria become undetectable at the late four-cell stage (Sutovsky *et al.*, 1996). It has also been reported that specific elimination of sperm mtDNA from the fertilized egg occurs at the pronuclear stage in intraspecific mice hybrids (Kaneda *et al.*, 1995). During bovine embryonic development, mitochondria show extensive morphological transformation (Plante and King, 1994). In bovine embryos, mitochondrial maturation begins at the eight-cell stage and is complete by the blastocyst stage. King *et al.* (1996) reported that in bovine nuclear transfer embryos, mature mitochondria that were probably carried along with the donor cells were observed at the one- to four-cell stages. It is speculated that mature mitochondria transferred with donor cells or spermatozoa may be broken down during morphological transformation of mitochondria in early development. In most cases in the present study, the mtDNA in the nuclear transfer calves was not derived from donor cells, but rather the mtDNA from the recipient oocytes was dominant.

There are reports on the relationship between mtDNA mutations and phenotypic expressions, such as disease (Wallace, 1992) and ageing (Soong *et al.*, 1992) in humans, milk production in Holstein cows (Schutz *et al.*, 1994) and carcass traits in Japanese Black cattle (Mannen *et al.*, 1998). Nagao *et al.* (1998) reported that a mitochondrial congenic mouse carrying *Mus spretus* mtDNA on a background of *Mus musculus* (C57BL/6J) nuclear genome showed a phenotype of growth and physical performance, as measured by running on a treadmill until exhaustion, which was different from that of C57BL/6J mice. Possible interactions between mtDNA and the nuclear genome in intraspecific nuclear transfer are under investigation in nuclear transfer calves.

The authors wish to thank T. Kojima, H. Takahashi and H. Hanada for their support and suggestions, Y. Izaiki, T. Suzuki and the staffs of the cattle-breeding farm for their help, and S. P. Leibo, C. S. Langham and M. P. Sabour (University of Guelph, Canada) for their assistance in preparing the manuscript. S. Takahashi and H. Imai were supported by a grant from the Livestock Technology Association, Japan.

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