

Pronounced Segregation of Donor Mitochondria Introduced by Bovine Ooplasmic Transfer to the Female Germ-Line¹

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ABSTRACT

Ooplasmic transfer (OT) has been used in basic mouse research for studying the segregation of mtDNA, as well as in human assisted reproduction for improving embryo development in cases of persistent developmental failure. Using cattle as a large-animal model, we demonstrate that the moderate amount of mitochondria introduced by OT is transmitted to the offspring's oocytes; e.g., modifies the germ line. The donor mtDNA was detectable in 25% and 65% of oocytes collected from two females. Its high variation in heteroplasmic oocytes, ranging from 1.1% to 33.5% and from 0.4% to 15.5%, can be explained by random genetic drift in the female germ line. Centrifugation-mediated enrichment of mitochondria in the pole zone of the recipient zygote's ooplasm and its substitution by donor ooplasm led to elevated proportions of donor mtDNA in reconstructed zygotes compared with zygotes produced by standard OT (23.6% ± 9.6% versus 12.1% ± 4.5%; $P < 0.0001$). We also characterized the proliferation of mitochondria from the OT parents—the recipient zygote (*Bos primigenius taurus* type) and the donor ooplasm (*B. primigenius indicus* type). Regression analysis performed for 57 tissue samples collected from the seven OT fetuses at different points during

fetal development found a decreasing proportion of donor mtDNA ($r^2 = 0.78$). This indicates a preferred proliferation of recipient taurine mitochondria in the context of the nuclear genotype of the OT recipient expressing a *B. primigenius indicus* phenotype.

bovine, developmental biology, embryo, gamete biology, mitochondrial DNA, ooplasmic transfer

INTRODUCTION

Microinjection of ooplasm containing mitochondria into another oocyte is called ooplasmic transfer (OT) or, less precisely, cytoplasmic transfer. Ooplasmic transfer was initially developed to treat infertility in patients exhibiting persistent poor embryonic development and recurrent implantation failure after in vitro fertilization (IVF). The transfer of 5% to 15% of ooplasm from a presumptively young fertile donor into a putatively defective recipient oocyte resulted in heteroplasmic human offspring [1]. The inheritance of the donor- and recipient-derived mitochondrial genotypes (mitotypes) and possible physiological consequences of this deviation from the normal, uniparental maternal mtDNA inheritance have been addressed in animal studies [2].

In mouse, intraspecies OT [3] or karyoplast transplantation [4] performed to study the transmission and segregation of heteroplasmy produced offspring with moderate proportions of the donor-derived mitotype (7%–19%). Heteroplasmic mice have been used to study the persistence of heteroplasmy over generations [4], the mitochondrial bottleneck [5], and the identification of genes regulating mtDNA segregation [6].

Common *Mus musculus domesticus* mitotypes determine differences in respiration capacity per mtDNA molecule, mitochondrial oxidative phosphorylation performance, and the production of reactive oxygen species (ROS) [7]. Production of ROS was found to cause complex phenotypes associated with murine mitotypes [7]. The mitotypes NZB/BinJ and BALB/c, which belong to different phylogenetic subbranches [8] and differ in amino acid residues in the respiratory chain complexes I, III, and IV, are subject to different tissue-specific selection attributed to factor(s) involved with mtDNA maintenance [9]. Furthermore, they show significantly altered basic physiological functions when in a heteroplasmic state [10].

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Ooplasm donation between the subspecies *M. musculus musculus* and *Mus musculus domesticus* generated heteroplasmic offspring in which the former mitotype was selected regardless of nuclear background in most of the tissues [11].

Somatic cell nuclear transfer (SCNT) represents another way to produce mitochondrial heteroplasmy in mammals. The segregation of the mitotypes derived from the SCNT partners has been studied in cattle [12, 13], mouse [12], sheep [13], and pig clones [14]. The cytoplasm of a somatic cell contains only around 1% of the mtDNA amount of an oocyte. Thus, SCNT generates clones with no or low-level heteroplasmy if the parental mitotypes segregate neutrally [13, 15]. Rarely, cases exhibiting a marked elevation of the donor mtDNA proportion have also been observed in each of the four mammalian species studied in this respect [13, 15, 16]. The donor-derived mitotype was generally elevated in its proportion [13, 16, 17] or was preferentially selected only in a specific tissue [12, 14].

Here, we performed OT in cattle to provide a large-animal model with higher relevance to human physiology and longevity. Humans and cattle are much more similar to each other than humans and mice regarding mitochondrial ROS production and basal metabolic rate resulting from mitochondrial oxidative phosphorylation [18, 19]. Using recipient and donor mitotypes from two subspecific origins, *Bos primigenius taurus* and *Bos primigenius indicus*, we addressed the conceptual issue of a putative female germ-line transmission of the donor mtDNA. In addition, we tested two alternative OT strategies to further increase the donor mtDNA proportion transferred. One of the modifications of standard OT used centrifugation of the recipient zygote to generate a phase enriched in mitochondria that was subsequently substituted by a fraction of donor ooplasm. The other manipulation tested was an attempt to block the replication of recipient mtDNA with ethidium bromide (EB). The moderate levels of heteroplasmy produced by transferring 10% to 15% of ooplasm during OT allowed us to study the selection of parental mtDNAs during fetal development and in the offspring.

MATERIALS AND METHODS

Biological Material

Two nucleomitochondrial combinations of Nellore cattle (*B. primigenius indicus*) were used for OT. For the production of recipient zygotes, ovaries of Nellore cattle with a *B. primigenius taurus* mitotype generated by backcrossing *B. primigenius indicus* males to *B. primigenius taurus* females were obtained from a local slaughterhouse (Ferreira et al. [20] and the current study). Nellore cows possessing a *B. primigenius indicus* mitotype based on their Pure Imported Origin pedigrees [21] were used as ooplasm donors.

Cattle were maintained on pasture with free access to water and mineral supplements. The procedures for the use of animals for the investigations performed in this work were approved by The Institutional Animal Care and Use Committee at the Jaboticabal Campus of São Paulo State University (protocol no. 017256-06).

Recipient Zygote Production

Oocytes were obtained postmortem by follicular aspiration from the ovaries of unregistered Nellore cows. Follicles with diameters between 3 and 8 mm were aspirated using an 18-Gauge needle attached to a 20-ml syringe. In vitro maturation (IVM) of cumulus-oocyte complexes (COCs) was performed as described previously [20]. Oocytes and embryos (see below) were cultured in vitro at 38.5°C under a humidified atmosphere of 5% carbon dioxide and 95% air. Chemicals and culture media were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Oocytes were fertilized in Tyrode Albumin-Lactate-Pyruvate (TALP)-IVF medium [22] supplemented with 0.6% (w/v) bovine serum albumin (BSA), 30 µg/ml heparin, 18 µM penicillamine, 10 µM hypotaurine, and 1.8 µM epinephrine as described previously [23]. Oocytes and sperm were incubated

for 10–12 h for IVF. Fertilized oocytes were washed in synthetic oviduct fluid medium (SOF) [24] supplemented with 10% fetal calf serum (FCS) and maintained in the incubator in this medium until used as recipient zygotes in OT.

Production of Donor Ooplasm

In vivo-derived oocytes were obtained by ovum pickup (OPU) [25] from Nellore cows with Pure Imported Origin pedigrees. Ovarian follicular waves were synchronized with dominant follicle ablation 96 h prior to OPU. At 24 h prior to OPU, animals received 50 units of follicle-stimulating hormone and 50 units of luteinizing hormone (Pluset; Laboratorios Calier S.A., Barcelona, Spain). The COCs recovered by OPU were transported in PBS at 25°C to 30°C to the laboratory and submitted to IVM for 21 h. The OPU-collected COCs were denuded of cumulus cells by gentle pipetting in 0.5% hyaluronidase solution and were selected by the presence of the first polar body (PB). Denuded oocytes were maintained in the incubator in IVM medium until enucleation and aspiration of donor ooplasm.

Microsurgery, Parthenogenetic Activation, and Standard OT

Microsurgery was performed using an inverted microscope (Olympus IX-70; Tokyo, Japan) equipped with a stage maintaining a temperature of 38.5°C, micromanipulators, and microinjectors (Narishige, Tokyo, Japan). Denuded oocytes having a first PB were incubated in SOF medium [24] supplemented with 10% FCS, 10 µg/ml Hoechst 33342, and 7.5 µg/ml cytochalasin B for 15 min. Removal of metaphase II-arrested chromosomes was performed in a 300-µl drop of SOF buffered with 20 mM HEPES (HSOF). HSOF was then supplemented with 10% FCS and 7.5 µg/ml cytochalasin B under mineral oil in a plastic Petri dish. Using a 25-mm (external diameter) glass pipette, metaphase II chromosomes and the first PB were removed and exposed to ultraviolet light to confirm enucleation. At 24 h after IVM, enucleated oocytes were then chemically activated by incubation in HSOF supplemented with 5 µM ionomycin for 5 min, followed by incubation for 4 h in SOF supplemented with 2 mM 6-dimethylaminopurine, 2.5% FCS, and 0.5% BSA. After activation, ooplasts were transferred to 100-µl drops of SOF supplemented with 2.5% FCS and 0.5% BSA for 10–12 h before OT. Immediately before OT, cumulus cells were removed from presumptive zygotes by gentle pipetting in 0.5% (w/v) hyaluronidase solution and were selected for the presence of the second PB. For microsurgery, selected zygotes and activated ooplasts were preincubated in SOF with 10% FCS and 7.5 µg/ml cytochalasin B for 30 min. In order to provide space for donor ooplasm, the ooplasm surrounding the second PB was removed from recipient zygotes using the same micromanipulation system described above. Subsequently, approximately 10%–15% of donor ooplasm was introduced into the perivitelline space of the recipient zygote. Each single, enucleated donor oocyte was used to reconstruct up to seven zygotes. The resulting couplet was placed in electrofusion solution (0.28 M mannitol, 0.1 mM CaCl₂·2H₂O, 0.1 mM MgSO₄·7H₂O, and 0.3% BSA) and exposed to two electrical pulses of 1.5 kV/cm for 30 µs (ECM-2001; BTX, San Diego, CA). Fused zygotes were selected before in vitro culture (IVC).

OT Modifications

Two OT modifications involving centrifugation or chemical treatment (Supplemental Fig. S1, all Supplemental Data are available online at www.biolreprod.org) were tested in comparison with the standard (control [CO]) protocol to increase the proportion of donor mtDNA in reconstructed embryos.

The physical approach used centrifugal force to generate a mitochondria-enriched ooplasm fraction. In detail, recipient zygotes extruding the second PB were placed for 30 min in SOF medium containing 10% FCS and 7.5 µg/ml cytochalasin B. Centrifugation for 15 min at 10 000 × g [26] concentrated the mitochondria at one of the zygote's poles. This part of the recipient zygote's ooplasm was removed by micromanipulation and discarded. Therefore, the resulting recipient zygotes were regarded as partially depleted (PD) of their mtDNA because they contained less mitochondria than their CO group counterparts. The integrity of mitochondria in the mitochondria-enriched zone was demonstrated by staining with the mitochondrion-selective red-fluorescent dye chloromethyl-X-rosamine (MitoTracker Red CMXRos; Invitrogen), which accumulates dependent upon membrane potential in mitochondria of live cells. For staining, the zygote was incubated for 30 min in SOF medium supplemented with 500 nM MitoTracker Red CMXRos and 7.5 µg/ml cytochalasin B. After centrifugation at 10 000 × g for 15 min, mitochondria enrichment and cellular subfractionation were visualized by epifluorescence microscopy using excitation and emission at 579 nm and 599 nm, respectively

(Fig. 1; a video illustrating the key points of the standard OT and PD treatment is provided as Supplemental Movie S1).

The chemical approach (EB group) used exposition of recipient oocytes to 7 $\mu\text{g/ml}$ EB, a known inhibitor of mtDNA synthesis [27], during the last 4 h of IVM in the presence of 50 $\mu\text{g/ml}$ uridine and 100 $\mu\text{g/ml}$ pyruvate. The EB dose slightly exceeded the range of low concentrations (0.1–2 $\mu\text{g/ml}$ [27, 28]), resulting in either partial or complete inhibition of mtDNA replication but having no effect on the replication of nuclear DNA (King and Attardi [27] and Hashiguchi and Zhang-Akiyama [28], and references therein). The EB concentration and the duration of administration were chosen based on a pilot experiment in which different doses were tested during IVM and evaluated with respect to their effect on oocyte maturation and embryonic development (data not shown). After 18–22 h of IVM, oocytes usually reached the metaphase II stage (expanded cumulus and first PB). To avoid hampering meiotic resumption and the migration of cortical granules, exposure of oocytes to EB was restricted to the last 4 h of IVM (Hours 20–24). At this time, most of the oocytes had completed maturation, and thus had reached their final mtDNA content.

After EB treatment, oocytes were washed thoroughly with TALP-IVF medium and submitted to IVF to be used later as recipient zygotes for OT.

Embryo Culture and Sampling

After OT, reconstructed zygotes were washed in HSOE and cocultured in vitro with a monolayer of granulosa cells in 100- μl drops of SOF supplemented with 2.5% FCS and 0.5% BSA, under mineral oil. For determination of the content and proportion of mtDNA, zygotes and blastocysts of each treatment group were collected after fusion or at Day 7 of IVC, respectively.

Embryos were placed individually in 0.2-ml PCR microtubes containing 5 μl of ultrapure water and were stored at -80°C until DNA extraction.

Embryo Transfer and Tissue Collection

A total of 43 blastocyst-stage embryos from all groups (CO: $n = 12$; PD: $n = 12$; and EB: $n = 19$) were transferred nonsurgically to the uterus of 32 recipient cows (CO: $n = 9$; PD: $n = 9$; and EB: $n = 14$) synchronized as reported previously [29]. Animals were diagnosed by ultrasound examination at Day 30 of pregnancy. A total of 6 of the 10 pregnancies obtained were interrupted for fetal collection (CO: $n = 1$; PD: $n = 1$; and EB: $n = 4$). The other four pregnancies were allowed to complete fetal development (CO: $n = 1$; PD: $n = 1$; and EB: $n = 2$).

Fetuses at different stages of gestation were recovered from pregnant uteri within 1 h after slaughter. Tissue samples were isolated and stored at -80°C until DNA extraction. Placenta and somatic tissue samples taken by needle biopsy (skin, muscle, and blood) or laparoscopy (liver) were recovered at birth. Somatic tissue biopsies of OT calves were also sampled at the ages of 6, 10, and 16 mo. Tissue samples were placed in 1.8-ml cryotubes and stored at -80°C until DNA extraction. The COCs recovered from OT heifers by OPU without hormonal stimulation were submitted to IVM. After removal of cumulus cells, single oocytes were placed in 0.2-ml microtubes containing 5 μl of ultrapure water and were stored at -80°C until DNA extraction.

A tissue sample list is given as Supplemental Table S1.

Quantification of the mtDNA Content and the Heteroplasmy Level by Real-Time PCR

Total DNA was isolated from oocytes, zygotes, blastocysts, and tissues using the NucleoSpin Tissue Kit (Macherey & Nagel, Dueren, Germany) according to the protocol provided by the manufacturer.

The mtDNA content of zygotes and blastocysts was measured by a quantitative real-time PCR (qPCR) assay targeting an *MT-RNR2* consensus sequence present in the recipient and donor mtDNAs of *B. primigenius taurus* (GenBank no.: AY526085) and *B. primigenius indicus* (AY126697).

The proportion of donor-derived mtDNA was determined by *B. primigenius indicus*-specific qPCR and normalized to changes in the input mtDNA amount measured by the latter consensus assay. A second set of qPCR assays targeting the mitochondrial control region and consisting again of a *B. primigenius indicus*-specific assay and a subspecies-consensus assay for normalization were used for confirmation of heteroplasmies in the tissues. Application of this assay was more demanding because it required sequence analysis of all samples because of the higher genetic variation in the control region compared with the highly conserved *MT-RNR2* gene.

Primers and TaqMan probes (Table 1) were designed using Primer Express version 1.5 (Applied Biosystems) and were synthesized at Invitrogen (Lofer, Austria) and Metabion (Martinsried, Germany), respectively. Duplicate qPCR reactions of 25- μl volume contained 80 mM Tris-HCl (pH 9); 20 mM

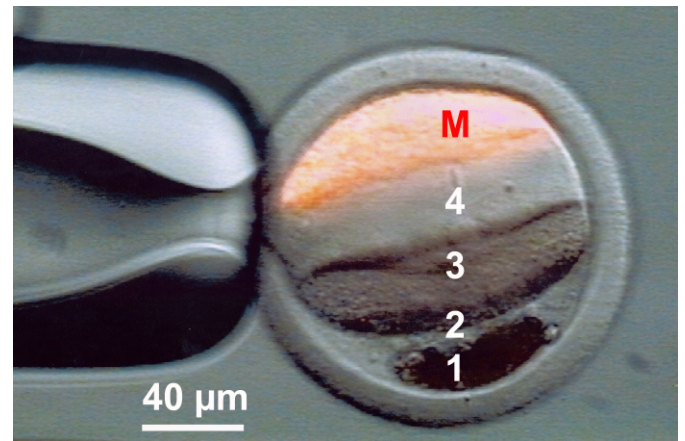


FIG. 1. Membrane potential-maintaining mitochondria were fractionated in a mature bovine zygote by centrifugation at $10\,000 \times g$. For the purpose of demonstration only, mitochondria were visualized by staining with chloromethyl-X-rosamine (MitoTracker Red CMXRos; Invitrogen). This mitochondrion-selective red fluorescent dye stains mitochondria in live cells and accumulates dependent on membrane potential. Zones formed are: 1, lipid; 2, membrane-bound vesicles; 3, smooth endoplasmic reticulum; 4, organelle-free ooplasm; and M, mitochondria [26]. Full OT procedure is shown in Supplemental Movie S1. Bar = 40 μm .

(NH_4)₂SO₄: 0.02% w/v Tween-20; 300 nM each primer; 100 nM probe; 0.2 mM each dinucleotide triphosphate (MBI Fermentas, St. Leon-Rot, Germany); 4.5 or 3.5 mM MgCl₂ for the consensus and the donor mtDNA-specific assays, respectively; 1 unit HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia); and 1.5 μl of target DNA or water in the case of the no-template control. DNA denaturation and enzyme activation were performed for 15 min at 95°C . DNA was amplified over 40 cycles consisting of 95°C for 20 sec and 63°C or 60°C for 1 min for the *MT-RNR2* assay and the control region assay, respectively. Amplification was performed on the ABI PRISM 7900HT Sequence Detection System (SDS) run under the SDS software version 2.3 (Applied Biosystems). The standard curve method [13], which yielded amplification efficiencies between 87% and 92% ($r^2 > 0.990$) for the four qPCR assays, was used for quantification.

The mtDNA content of zygotes and blastocysts was given as the mean calculated from three qPCR runs using the consensus *MT-RNR2* assay. Heteroplasmy levels of oocytes and tissue samples were determined by a single qPCR assay run targeting *MT-RNR2* and normalization by *MT-RNR2* consensus assay data.

In order to calculate relative mtDNA amounts, C_T values of the runs per sample were entered into the regression formula of the respective standard curve, giving rise to logarithmic DNA amounts in arbitrary units. Finally, the mtDNA content was expressed as mean \pm SEM in relation to the CO zygote mean.

Sequence Analysis

Partial sequences for the *MT-RNR2* gene and the D loop were determined for the five Nellore cows used as ooplasm donors to exclude interferences from mutations in the primer and probe-binding regions. The primer pairs bMT2773-f (5'-GGTTTACGACCTCGATGTT)/bMT3174-r (5'-CCACTAACGTAAG GAATGCT) and D-f (AGTCTCACCATCAACCCCA)/D-r (AGGATTTT CAGTGCCTTGC) were used to amplify PCR products for determination of partial *MT-RNR2* and D-loop sequences, respectively. BigDye terminators (Applied Biosystems, Foster City, CA) were used for cycle sequencing of PCR products. Sequences were submitted to the GenBank under the accession numbers GQ412276 to GQ412280 and GQ412281 to GQ412285 for *MT-RNR2* and D loop, respectively.

Statistical Evaluation and Data Presentation

Embryo data (percentage of donor mtDNA and total mtDNA amount) were analyzed as a factorial distribution considering OT group (CO, PD, and EB) and stage of development (zygote and blastocyst) as main factors. When necessary, means were compared by least square means. Heteroplasmy data determined for fetuses and calves were compared within the same group by ANOVA using the Statistical Analysis System version 8.3 (SAS Institute Inc.,

TABLE 1. Primers and TaqMan probes used for mtDNA quantification of *B.p. indicus* and *B.p. taurus* mitotypes.

Assay type ^a	Target site	Primer or probe	Sequence (5' to 3') ^b
ND	<i>MT-RNR2</i>	bMT3010-f bMT3096-r bMT3030-FAM	GCCCTAGAACAGGGCTTAGT GGAGAGGATTTGAATCTCTGG FAM-AAGGTGGCAGAGCCCGGTAATTGC-BHQ1
D	<i>MT-RNR2</i>	iMT2974-f iMT3095-r bMT3030-FAM	CCAATGACAGCATCTCAATCA GAGAGGATTTGAATCTCTGGG FAM-AAGGTGGCAGAGCCCGGTAATTGC-BHQ1
ND	Control region	bMT16162-f bMT16287-r bMT16204-FAM	TAATTACCATGCCGCGTGAAA GGCCCTGAAGAAAGAACCAGA FAM-TCCCTCTTCTCGCTCCGGGCC-BHQ1
D	Control region	iMT16185-f iMT16264-r bMT16204-FAM	CAACCCGCTAAGCAGAGG GCCTGGTAA <u>A</u> TTTCAT <u>TAA</u> FAM-TCCCTCTTCTCGCTCCGGGCC-BHQ1

^a ND, nondiscriminative (consensus) assay for *B.p. taurus* and *B.p. indicus*; D, discriminative (*B.p. indicus*-specific) assay.

^b Specific nucleotides are underlined.

Cary, NC). If necessary, the Tukey posthoc test was performed subsequently. Percentage data were transformed using arcsine. In all analyses, $P = 0.05$ was considered as the level for statistical significance. Exponential regression analysis of the donor mtDNA proportion throughout embryogenesis and generation of box whiskers plots were performed with the software package Microcal Origin version 6 (Microcal Software Inc., Northampton, MA).

RESULTS

Here, we used OT to generate heteroplasmic embryos. Two strategies based on physical or chemical treatment (Fig. 1 and Supplemental Fig. S1) were evaluated to increase the level of ooplasmic donor mitochondria. The physical treatment based on centrifugation-mediated enrichment of mitochondria in a fraction of the recipient ooplasm which was subsequently substituted with normal ooplasm was performed to generate recipient zygotes with partially depleted mitochondria (PD group). The chemical treatment was intended to impair the recipient mtDNA replication by exposition of recipient oocytes to EB (EB group). Standard OT (CO group) was performed for comparison.

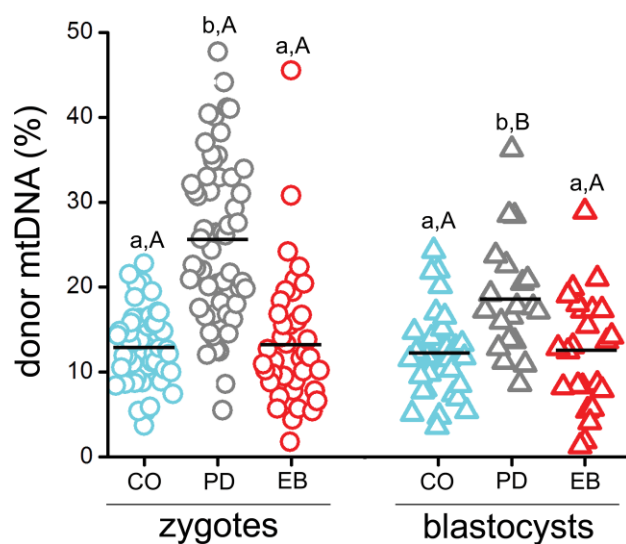


FIG. 2. Percentage of donor mtDNA in postfusion zygotes and blastocysts. Statistical significance ($P < 0.05$) is denoted by different superscript letters when difference was observed between groups of a development stage (a, b) or between developmental stages (A, B). The mean of individual proportions is depicted by a bar.

mtDNA Heteroplasmy in Early Embryos

A total of 225 embryos were collected for quantification of the donor:recipient mtDNA ratio (no. of zygotes/blastocysts in CO group: $n = 49/36$, PD group: $n = 55/20$, and EB group: $n = 41/24$, respectively). Overall, mean rates for embryo cleavage and blastocyst development of 69.7% and 31.7% were obtained, respectively. No difference in the two parameters was found among the groups ($P = 0.73$ and 0.42, respectively).

The percentage of donor mtDNA (e.g., the level of heteroplasmy) was analyzed at the zygote and the blastocyst stages (Fig. 2). The standard OT protocol, in which approximately 10% to 15% of the donor oocyte volume is transferred to a recipient zygote, yielded similar levels of heteroplasmy for zygotes and blastocysts, respectively ($12.1\% \pm 4.5\%$ and $11.2\% \pm 4.8\%$, respectively; $P = 0.52$). An increase in the level of donor mtDNA was achieved in the PD group. The removal of a mitochondria-enriched fraction of ooplasm from the recipient zygote before OT yielded zygotes

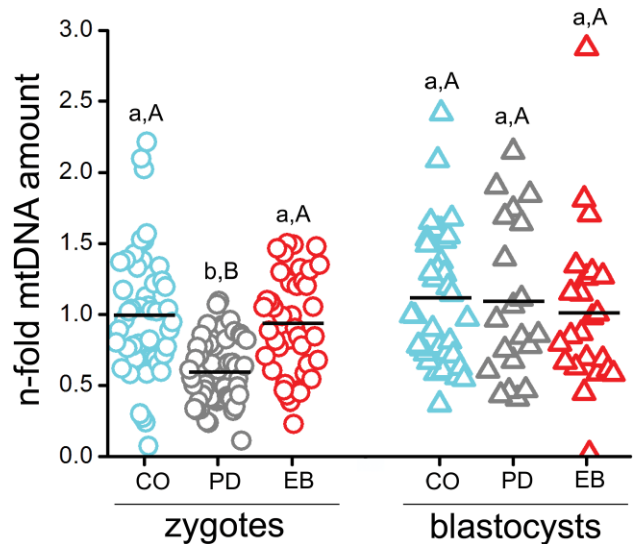


FIG. 3. Reconstructed zygotes with partially depleted mtDNA produced by OT into recipient zygotes being removed of a mitochondria-enriched fraction. The mtDNA content is expressed as mean \pm SEM in relation to the CO zygotes' mean. Statistical significance ($P < 0.05$) is denoted by different superscript letters when difference was observed between groups of a development stage (a, b) or between developmental stages (A, B). The mean of individual data is depicted by a bar.

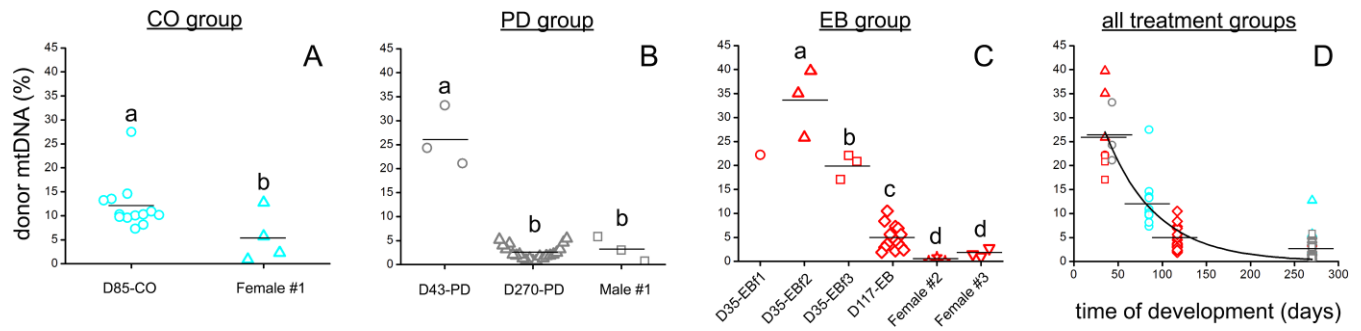


FIG. 4. The proportion of donor mtDNA decreased exponentially throughout fetal development. Samples from the three OT protocols (CO [A], PD [B], and EB [C] groups) are displayed individually or summarized (D). Different animals are indicated by different symbols. Bars represent the mean donor mtDNA proportion. Different letters (a, b, c, d) within the same graph denote statistical significance ($P < 0.01$). The coefficient of determination (r^2) of 0.78 obtained by regression analysis across all samples supports a negative exponential function.

and blastocysts with a highly significant elevation of the donor:recipient mtDNA ratio ($23.6\% \pm 9.6\%$, $P < 0.0001$; $17.6\% \pm 6.6\%$, $P < 0.001$, respectively). Surprisingly, the level of heteroplasmy decreased during early embryogenesis, as indicated by the comparison of the two embryonic stages analyzed for the PD group ($P < 0.001$). In contrast, treatment of recipient zygotes with EB did not affect the heteroplasmy level. The proportion of donor mtDNA found in EB zygotes and blastocysts ($P = 0.57$) was similar to that measured in their OT counterparts (means of $11.4\% \pm 7.9\%$ and $10.2\% \pm 6.6\%$; $P = 0.94$ and 0.93 , respectively).

mtDNA Quantity in Early OT Embryos

Before the blastocyst stage of early mammalian embryogenesis, a decrease in mtDNA content and neosynthesis of mitochondrial genomes is assumed [30–33]. This phenomenon would explain the decreased proportion of donor mtDNA in PD blastocysts compared with PD zygotes (see above). Therefore, we quantified the mtDNA content in zygotes and blastocyst-stage embryos.

The removal of the mitochondria-enriched fraction from the recipient ooplasm in the PD group decreased the mtDNA content of zygotes to an average of 60% ($P < 0.0001$; Fig. 3). The mtDNA amounts for EB and CO zygotes were not different ($P = 0.80$). The recovery of the amount of mtDNA in the PD group at the blastocyst stage to levels found in CO and EB blastocysts ($P = 0.23$) is in accordance with the mtDNA increase seen between four/eight-cell and blastocyst stages of

bovine embryogenesis [30], and the conserved ratio of mtDNA molecules per unit volume of cytoplasm [34]. Zygotes and blastocysts of the CO or EB groups did not differ in their mean amount of mtDNA ($P = 0.19$ and $P = 0.69$, respectively), but a 1.8-fold increase was found in the PD group between the two embryonic stages ($P < 0.001$).

Selection Against Donor Mitochondria During Fetal Development

Cell multiplication during embryogenesis can lead to stringent selection against donor- or recipient-derived mitotypes.

In the CO group, 12 blastocysts were transferred to nine recipient cows. At Day 85, one fetus was collected that exhibited heteroplasmy of $10.6\% \pm 2.2\%$ in all 12 tissues analyzed (Supplemental Table S2). This level is similar to the level found at the blastocyst stage (see above).

In the PD group, 12 embryos were transferred to nine recipient cows. Skin and liver of a Day 43 fetus showed heteroplasmy of 24% and 33%, respectively, comparable with those determined for PD blastocysts ($24\% \pm 10\%$; see above). In one case, the transfer of a single blastocyst resulted in a monozygotic twin pregnancy (fetus D270-PD and male 1, Supplemental Tables S2 and S4)—a phenomenon reported previously [35]. One of the male twins (D270-PD), which died intrapartum because of amniotic fluid aspiration, showed heteroplasmy ranging from 1% to 5% across 20 tissues

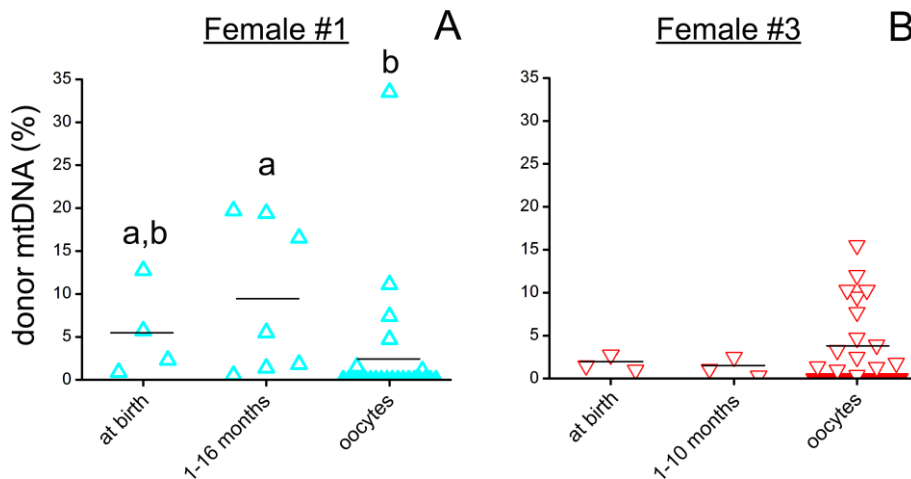


FIG. 5. Heteroplasmy produced by OT is transmitted to the female germ line. Donor mtDNA levels in tissues (see Supplemental Table S4 for types) during postterm development (first two data sets) and in oocytes (third set) of the animals Female 1 (A) and female 3 (B) are shown. Bars depict mean donor mtDNA for each time. Filled symbols indicate homoplasmic oocytes. Statistical significance ($P < 0.01$) is denoted by different superscript letters.

(mean: $2.5\% \pm 1.5\%$; Supplemental Table S2). See below for heteroplasmy levels found in the live twin (male 1).

In the EB group, 19 blastocysts were transferred to 14 recipient cows. Heteroplasmy levels quantified in fetal tissues of three Day 35 fetuses (22% , $36\% \pm 6.5\%$, and $19\% \pm 2.7\%$; Supplemental Table S2) included outliers with elevated heteroplasmy not covered by heteroplasmy levels found in the EB blastocysts analyzed ($n = 24$; mean: $13\% \pm 7\%$). Low heteroplasmy values were seen in a Day 117 fetus from this group (mean: $4.9\% \pm 2.6\%$ across 14 tissues).

Regression analysis performed throughout fetal development using all heteroplasmy data determined for fetuses of all three OT protocols demonstrated a negative exponential relationship between the time of gestation and the proportion of donor mtDNA ($r^2 = 0.78$; Fig. 4 and Supplemental Table S2).

Heteroplasmy During Postterm Development

Next, we quantified heteroplasmy after term in three of the four live OT animals showing a donor-derived mtDNA proportion of above 1% in blood and skin at birth (e.g., in male 1 and females 1 and 3, but not female 2). The three heteroplasmy live animals exhibited completely normal development and had no obvious health problems until manuscript submission (4 yr). An additional analysis of blood parameters performed only for the female animals yielded values within the reference range (Supplemental Table S3).

First, the issue of a tissue-specific segregation of donor mtDNA was addressed in female 1 (CO group). The blood of this animal exhibited higher heteroplasmy at birth and at 6 and 16 mo (13% , 20% , and 17% , respectively) compared with skin (6% and 4%) and muscle (2% and 1%) at birth and 6 mo after term, respectively (paired t -test: $P < 0.05$).

We also asked whether the level of heteroplasmy changed in a given tissue of an individual over time. Samples collected at birth and during the first months of postnatal development indicated a trend for a decreasing proportion of donor mtDNA in muscle and skin but not blood (Supplemental Table S4 and data not shown). The limited sample size ($n = 3$) precluded rigorous statistical analysis.

Transmission of Heteroplasmy to the Female Germ-Line

The heteroplasmy OT animals female 1 and female 3 belonging to the CO and EB groups, respectively, were analyzed for a putative transmission of the mixed mtDNAs from mother to offspring (e.g., through the mitochondrial genetic bottleneck). The donor mtDNA was detectable only in 25% and 65% of the oocytes collected ($n = 24$ or 23 , respectively; Supplemental Table S4). In cases of germ-line transmission, its proportion varied considerably (1.1% to 33.5% and 0.4% to 15.5% , respectively; Fig. 5).

DISCUSSION

Elevation of the Donor mtDNA Proportion Introduced by OT

Mitochondrial heteroplasmy levels of only a few percent are produced by SCNT if genotypes are transmitted neutrally [12, 13, 15, 36]. These low levels are sometimes lost before birth, preventing further segregation or (patho)physiological studies. The microinjection of 5%–15% ooplasm donates more mtDNA than a somatic cell, and thus generates animal models with higher levels of heteroplasmy (Jenuth et al. [3] and the current study). In humans, the transfer of small amounts of up

to 15% of donor ooplasm to the recipient's oocyte [37] resulted in the transmission of donor mtDNA into human offspring [1, 36]. Here, we provided the first quantitative data for the mtDNA proportion transferred in a standard OT protocol (Fig. 2). The 10% to 15% of donor ooplasm introduced into a recipient zygote by standard OT resulted in zygotes and blastocysts exhibiting heteroplasmy levels of $12.1\% \pm 4.5\%$ and $11.2\% \pm 4.8\%$, respectively. To further enhance the level of OT-generated heteroplasmy, we explored ways like EB treatment and partial mitochondria depletion of recipient zygotes.

Given that an mtDNA replication inhibitor (2',3'-dideoxycytidine) applied during IVM successfully decreased the mtDNA amount of porcine oocytes [32], we attempted to reduce the proportion of recipient zygote-derived mtDNA by treating recipient oocytes with the DNA-intercalating dye EB during the last hours of IVM. The lack of effect in the blastocysts of the EB group could be due to a rapid reversion of the drug effect after its removal from culture medium [38], or species-specific limitation in causing mtDNA depletion [28]. Alternatively, this could be due to a rapid mitochondrial fusion process creating a connected compartment that facilitates content exchange and access to mtDNA products [39], thus distributing the drug between donor and recipient mitochondria and resulting in equal access to their replication machinery between the morula and blastocyst stages of bovine embryogenesis [30].

Elevation of the donor mtDNA proportion, however, was achieved by PD treatment. Using centrifugation of recipient zygotes, a recipient ooplasm fraction enriched in mitochondria is produced at the pole (Fig. 1) and is subsequently substituted by donor ooplasm having a normal density of mitochondria. Consequently, the ratios of donor:recipient mitotypes at the zygote and the blastocyst stages increased significantly (Fig. 2). The reestablishment of normal mtDNA levels in PD blastocysts (Fig. 3) is consistent with the maintained ratio of mtDNA copies per unit ooplasm volume reported for early embryogenesis up to the blastocyst stage [34]. The effect of elevated heteroplasmy was less pronounced in the resulting blastocysts (Fig. 2). This decrease can be explained by the turnover of mtDNA (e.g., decrease and neosynthesis of mtDNA before the blastocyst stage [30–33]) and selection against donor mtDNA (Fig. 4, and see below). It was not due to a difference in the blastocyst development rate, thus excluding a developmental selection of embryos with different mtDNA content as a cause.

Selection Against Donor Mitochondria

Here, we used OT to mix donor and recipient mitochondria from a similar developmental/differentiation stage. We demonstrated that throughout fetal development, the donor-derived *B. primigenius taurus* mitotype is selected over the *B. primigenius indicus* recipient mtDNA in the context of a phenotypically *B. primigenius indicus* genetic background derived by repeated backcrossing of *B. primigenius indicus* males to "native" Brazilian *B. primigenius taurus* cows (Fig. 4).

First, mtDNA transmission and segregation experiments of this kind have been performed in mice. The introduction of a considerable amount of donor ooplasm during OT performed between *Mus musculus domesticus* and *M. musculus molossinus* mice led to low heteroplasmy levels in most tissues in the offspring (references in Smith and Alcivar [40]). Authors regarded the reduced proportion of transmitted donor mtDNA compared with its input as indication that the introduced

mtDNA was lost at some stage between fetal development and adulthood by random drift or by selective replicative disadvantage of the transplanted mtDNA. A deviation from the original parental mtDNA ratio in the offspring is not surprising, considering that 390 amino acid residues are substituted between these *Mus musculus* subspecies [8]. This number exceeds the number of residue changes determined for the *B. primigenius taurus* and *B. primigenius indicus* parental mtDNAs of this work by more than 20-fold [41], and therefore could explain the presumed nonneutral transmission of parental mitotypes.

In mice, the *M. musculus musculus* mtDNA of the RR strain was selected over the *M. musculus domesticus* mitotype of C57BL/6 in the two nuclear genetic constellations analyzed [8, 11, 42]. Generally, the proliferation of mitotypes should be considered an interplay between the mitochondrial and nuclear genomes. In fact, there is genetic evidence that the nucleus controls mammalian mtDNA segregation [6].

In cattle, SCNT between donor and recipient cells possessing *B. primigenius taurus* mitotypes [16, 43] generated three clones with elevated levels of the donor-derived mitotype. This finding was attributed to a replicative advantage of the donor mtDNA over recipient mtDNA during the course of embryogenesis [16].

In humans, the surprisingly high proportions of 36%, 43%, and 70% donor mtDNA found in the blood of three OT infants, respectively [44], could have been caused by random genetic drift, but could also be indicative of a difference in the proliferative potential of mitotypes under the given nucleomito-chondrial interactions.

In sheep, the high heteroplasmy of an SCNT clone indicated that the recipient oocyte-derived mitotype was negatively selected [13]. This putatively mildly deleterious mtDNA possessed three nonconservative amino acid substitutions, one of which was found at an evolutionary conserved site.

OT Donor Mitochondria Are Transferred to the Female Germ Line, Thereby Segregating Considerably

In mammals, mtDNA variants are observed to segregate rapidly between generations, despite the high mtDNA copy number in the oocyte (reviewed in Cree et al. [45]). Early in prenatal development, a restriction in the mtDNA content to about 200 copies per early primordial germ cell has been documented [5, 46]. At this time of development, in our bovine OT model, the levels of donor mtDNA were still moderate and began to diminish as a result of selection against it (see above). Here, we asked whether the OT donor mtDNA is transmitted to and segregated by the female germ line.

For a representative number of oocytes collected by OPU at the age of 14 or 20 mo from two females, we demonstrated donor mtDNA transmission in 25% and 65% of oocytes, and its pronounced segregation ranging from 0.3% to 33.6% in heteroplasmic oocytes. In higher mammals, the segregation of donor mtDNA through the germ-line bottleneck was assessed previously only indirectly, using tissues of a few SCNT offspring [47].

The finding of germ-line transmission of donor mtDNA and its pronounced segregation are consistent with published data reporting random genetic drift of heteroplasmy through the female germ line in mice generated by ooplasm donation [3]. Our data from a higher mammalian species formally answer the question of whether the moderate amount of mtDNA introduced in case of OT will be passed to the next generation, thereby solving the important conceptual issue of classification

of OT as an assisted reproductive technology modifying the germ line [48].

Further Perspectives

As outlined above, we proved the expectation that the moderate amount of 10% to 15% of donor ooplasm transferred to the recipient zygote by standard OT produces (only) moderate mtDNA heteroplasmy levels of about 11% to 12% (see blastocyst-stage data in Supplemental Table S1). Even the PD pretreatment before transferring the donor ooplasm yielded only slightly higher values of 18% to 24%. However, to prevent the transmission of mtDNA disease to offspring from women suffering from mtDNA disorders, a larger amount of up to 50% of donor ooplasm is needed. It is questionable whether it is possible to introduce such an amount of ooplasm into the oocyte. The consequence is that the relative proportion of mutant to wild-type mtDNA is unlikely to change enough to prevent clinical disease (reviewed in Bredenoord et al. [48]). It remains to be seen whether future research in the fields of mtDNA transmission and segregation and nucleomito-chondrial interaction will open a way to reach this threshold (e.g., by using a donor mtDNA of “lagging” proliferative potential). To fall short of this physiologically critical threshold of pathological mitotypes is currently only conceivable using spindle-chromosomal complex transfer developed in the nonhuman primate *Macaca mulatta*. This technique was reported to decrease the mtDNA proportion transplanted along with nuclear genetic material into an enucleated egg containing normal mtDNA below a threshold of 3% [49], thus reaching at least 97% of the normal mitotype in the reconstituted embryo.

In addition to the PD treatment studied in this work, we believe that centrifugation of the donor oocyte and transfer of aspirated donor ooplasm enriched in mitochondria to PD zygotes partially depleted in mitochondria could be a way to further elevate the proportion of donor mtDNA in OT embryos.

Future research in a large-animal system like cattle should also assess the potential of OT to affect paternal genome function and for more subtle phenotypic changes or transient effects [50–52].

Model Prospects

Because of the ethical issues involved with medical research involving human embryos, an appropriate animal model is needed for studying early embryogenesis, to demonstrate the safety of human infertility therapeutics related to OT [53], and to assess ways to prevent transmission of human mitochondrial disorders [48, 54]. The only large-animal models of relevance are the nonhuman primates *Callithrix jacchus* (common marmoset) and *Macaca mulatta* (rhesus monkey) [49, 55]. In the nonhuman primate, basic research is at the frontiers in the field of spindle replacement [49], but the success of other micromanipulation techniques is restricted to two offspring obtained by embryonic cell nuclear transfer [56]. All efforts to produce clone monkeys by SCNT for biomedical research have failed so far [53]. Because of this lack of SCNT success and numerous ethical and welfare issues regarding nonhuman primates compared with the economic and well-established breeding of cattle, basic research in the bovine model could assist and precede experiments in the nonhuman primate.

First, there is mounting evidence that the bovine model is better than the murine model for the study of human embryonic development with respect to the timing of genome activation, intermediate metabolism, and interaction with the culture medium [57]. Second, human and mouse differ in their

maximum lifespan potential. Evidence shows that long-lived vertebrates consistently have low mitochondrial free radical generation rates, a crucial factor determining their aging rate [58, 59]. Oxidative damage to mtDNA is also lower in long-lived vertebrates than in short-lived vertebrates [60]. Therefore, the longer-lived cow (30 yr) would be much more adequate concerning these issues than the mouse as a shorter-lived species (2–4 yr in mice, depending on the strain).

Third, for determining the efficacy of any therapeutic for human mtDNA disease, a higher mammalian model in addition to mouse models heteroplasmic for pathogenic mtDNA mutations would be beneficial. The bovine model represents an appropriate candidate in this regard. This large-animal model is an intensively studied model for mtDNA transmission and segregation [61], provides a large reservoir of different mitotypes [41, 62], would allow the use of a number of cost-efficient dwarf breeds [41, 63–66], and would respect ethical concerns regarding trapping and sampling of endangered species, especially apes.

In conclusion, here we documented germ-line transmission of the OT-generated heteroplasmy in a large-animal model for the first time. Our OT modification based on partial depletion of mitochondria in the recipient ooplasm to elevate levels of heteroplasmy extends the repertoire of strategies for introduction of mitotypes. It might be especially important in light of gene therapy of human mitochondrial disease requiring demonstration of efficacy in animal models before starting clinical trials.

Generally, OT can assist in broadening understanding of nucleomitochondrial interaction and identifying mitochondrial and nuclear genotypes which can influence proliferation of mitochondria and/or cells. In this regard, it can help to fine tune new therapeutic approaches that could prevent transmission of mtDNA mutations from mother to child, thus avoiding recurrence of mtDNA diseases.

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