Mitochondrial DNA content is associated with ploidy status, maternal age, and oocyte maturation methods in mouse blastocysts

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Received: 3 August 2017 / Accepted: 13 October 2017 / Published online: 24 October 2017
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Abstract

Purpose It was reported that mitochondrial DNA (mtDNA) was significantly increased in aneuploid human embryos compared to euploid embryos and was also associated with maternal age. In this study, we further established the mouse model of mtDNA quantitation in reproductive samples based on whole-genome amplification (WGA) and next-generation sequencing (NGS).

Methods WGA followed by NGS-based mtDNA quantitation was first performed on 6 single- and 100-cell samples from a tumor-derived mouse cell line, which was exposed to ethidium bromide to reduce mtDNA content. The relative mtDNA content was normalized to nuclear DNA. This method was then applied to mouse reproductive samples, including 40 pairs of oocytes and polar bodies from 8 CD-1 female mice of advanced reproductive age and 171 blastocysts derived via in vitro maturation (IVM) or in vivo maturation (IVO) from

young (6–9 weeks) and reproductively aged (13.5 months) female CF-1 mice.

Results Exposure to ethidium bromide for 3 and 6 days decreased mtDNA levels in both the single- and 100-cell samples as expected. Results demonstrated that the first polar body contained an average of 0.9% of mtDNA relative to oocytes. Compared to the cells in blastocysts, oocytes contained about 180 times as much mtDNA per cell. mtDNA levels were compared among blastocysts from reproductively young and old female mice that had either been produced by IVM or IVO. Cells in blastocysts from younger mice contained significantly lower amounts of mtDNA compared to aged mice ($P < 0.0001$). Cells in blastocysts produced via IVO had higher mtDNA content than IVM-derived blastocysts ($P = 0.0001$). Cells in aneuploid blastocysts were found to have significantly higher (1.74-fold) levels of mtDNA compared to euploid blastocysts ($P = 0.0006$).

Conclusion A reliable method for assessing mtDNA content in mouse gametes and embryos was established. Relative mtDNA levels were elevated in aneuploid embryos relative to euploid embryos, were higher in blastocysts from reproductively old mice relative to young mice, and were lower in embryos derived from IVM compared to IVO.

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Keywords Aging · Aneuploidy · Mouse blastocyst · Mitochondrial DNA · NGS

Introduction

The success of in vitro fertilization (IVF) has increased greatly with the application of comprehensive chromosome screening (CCS) in assisted reproductive technology (ART). The transfer of euploid embryos selected by CCS improved the

implantation rate from 47.9 to 66.4% and delivery rate from 67.5 to 84.7% in a well-designed randomized clinical trial [1]. However, since not every transferred euploid embryo results in implantation and delivery, simultaneous assessment of additional embryonic parameters could be pursued to help increase implantation rates.

Cytoplasmic content is important for embryo competence [2]. Mitochondria are well-defined cytoplasmic organelles that produce the energy currency (adenosine triphosphate; ATP) of the cell through respiration and regulate cellular metabolism [3]. Mitochondria are also an important source of reactive oxygen species (ROS) from complexes I and III of the electron transport chain [4], which cause oxidative stress in the mitochondria and may contribute to the decline in mitochondrial function associated with the aging process [5]. In addition to supplying cellular energy, mitochondria are involved in other tasks, such as signaling, cellular differentiation, and cell death, as well as maintaining control of the cell cycle and cell growth. Unlike other cell organelles, mitochondria contain their own genome. Each mitochondrion is estimated to contain 2–10 mitochondrial DNA (mtDNA) copies [6]. Eighty percent of mtDNA codes for mitochondrial RNA, and therefore, most mtDNA disorders lead to functional problems. The mtDNA copy number in early embryonic development has become the subject of a number of clinical and basic studies. It was reported that mtDNA was significantly increased in aneuploid human embryos compared to euploid embryos [7] and was associated with maternal age [8]. However, it has been controversial whether the quantification of mtDNA in trophoctoderm (TE) biopsy samples provides information predicting the implantation potential of a euploid blastocyst. Fragouli et al. and Diez-Juan et al. demonstrated that the less mtDNA content in embryos was an indicator for better embryonic implantation potential [8, 9]. However, Treff et al. found that relative mtDNA levels did not distinguish reproductive potential between the implanted and unimplanted embryos in a paired double-embryo transfer (DET) study [10]. Given the controversy over the use of mtDNA measurement to assess embryo viability, further research is needed to provide clarity for the clinical application of mtDNA quantification.

Research on early embryo development using model organisms obviates the need for using human eggs and preimplantation embryos, which would destroy their potential use for IVF and may not be feasible due to ethical and legal restrictions. Animal models also have the benefit of reduced genetic background variability. Mouse early embryo developmental stages are similar to those of the human embryo, and it is feasible to manipulate mouse embryos. Therefore, mice have the potential to be a useful model system to study the mtDNA levels as a biomarker in pre-implantation embryo development.

Previously, a method using whole-genome amplification (WGA) and next-generation sequencing (NGS) was developed for CCS of mouse reproductive samples [11]. We

observed that increased aneuploidy was associated with advanced reproductive age, which was consistent with other studies [12–14]. The origin of aneuploidy is primarily attributed to errors during maternal meiosis in the oocyte [15]. The number of mitochondria increases sharply during oogenesis and may play an important roles in the mechanisms underlying aneuploidy.

In the previous study, we also observed that blastocysts derived via in vitro maturation (IVM) had a higher aneuploidy rate than the blastocysts produced via in vivo maturation (IVO) [11]. Oocyte maturation is the final phase of oogenesis which involves both nuclear and cytoplasmic maturations [16]. In IVO, this process involves a number of signaling pathways and depends on the close interaction between the cumulus cells and the oocyte [17]. For IVM, while nuclear maturation is supported, cytoplasmic maturation is not assured [12]. The gradual accumulation of mitochondrial copy number and activity as oocyte development progresses is important for cytoplasmic maturation [18]. Understanding the association between blastocyst mtDNA level and the oocyte maturation methods will provide insight into cytoplasmic maturation under different maturation conditions.

In this study, we validated the WGA- and NGS-based mtDNA assessments on a tumor-derived mouse cell line with ethidium bromide (EtBr) treatment. EtBr impairs mtDNA replication, and mtDNA levels decrease upon exposure to EtBr [19]. After validating our method, relative mtDNA copy numbers in individual mouse reproductive samples including polar bodies, oocytes, and embryos were assessed, providing insight into mtDNA levels in the preimplantation embryo and their relationship with chromosomal ploidy status, maternal age, and oocyte maturation methods. A recent study demonstrated that relative mtDNA content was affected by embryo gender and ploidy [20]; therefore, we implemented the correction based on gender and ploidy when the relative mtDNA content was evaluated.

Material and methods

Experimental design

A two-phase design was implemented in this study. First, to validate mouse embryonic mtDNA assessment by WGA and NGS, single-cell and 100-cell samples from a tumor-derived mouse cell line were tested to validate the methodology for quantifying relative mtDNA content from limited and abundant cell numbers. Cells were exposed to ethidium bromide to reduce mtDNA content. In the second phase, mtDNA levels were assessed in oocytes, polar bodies, and blastocysts. Mitochondrial DNA content of blastocysts was compared between two age groups (young versus aged), oocyte maturation

methods (IVM versus IVO), and embryo ploidy status (euploid versus aneuploid).

Cell line

A tumor-derived mouse cell line (Coriell Cell Repository ID GM05384) was cultured in minimum essential media with 2X nonessential amino acids, 15% fetal bovine serum, and 1% penicillin–streptomycin–glutamine (Invitrogen Corp., Carlsbad, CA, USA) at 37 °C and 5% CO₂. Cultures were exposed to 25 ng/ml EtBr in full growth medium for 6 days to deplete mtDNA. Six single-cell and six 100-cell samples were picked up in 1 µl of media using a 100-µm stripper tip (Midatlantic Diagnostics, NJ, USA) under a dissecting microscope and placed into PCR tubes on day 3 and day 6. Six single cells and six 100 cells on day 0 without EtBr exposure were picked as baseline controls.

Cell lysis and WGA

Single-cell and 100-cell samples were lysed in alkaline lysis buffer prepared by adding 7 µl molecular biology grade water (Lonza, ME, USA) into PCR tubes, followed by 1 µl alkaline lysis buffer [200 mM KOH and 50 mM DTT]. Samples were incubated in lysis buffer at 65 °C for 10 min before 1 µl of neutralization buffer [0.9 M Tris–HCl, pH 8.3, 0.3 M KCl, and 0.2 M HCl] was added [21]. The lysates were stored at –20 °C until the DNA was amplified by WGA using the GenomePlex WGA4 kit as recommended by the supplier (Sigma Aldrich Inc. MO, USA). WGA DNA was purified using GenElute PCR cleanup columns (Sigma Aldrich Inc. MO, USA) and quantified using a Nanodrop 8000 spectrophotometer (Fisher Scientific Inc., MA, USA).

NGS

WGA DNA was normalized to 200 ng in a total volume of 35 µl of molecular biology-grade water. Ion Plus fragment library kit, Ion Xpress Plus fragment library kit, and Ion Xpress barcode adapters 1–96 kit were used to construct the WGA library as recommended by the supplier (Thermo Fisher Scientific, CA, USA). WGA DNA was fragmented with Ion Shear Plus reagent for 20 min to generate 150 to 250 base pair fragments. Fragmented DNA was then purified with Agencourt AMPure XP reagent beads as recommended (Beckman Coulter Inc., Brea, CA). Barcoded adapter ligation and nick repair were performed, followed by another Agencourt AMPure XP reagent bead purification. A peak size of 270 base pairs was selected with an E-Gel® SizeSelect™ agarose gel (Thermo Fisher Scientific, CA, USA). Size-selected DNA was amplified with 8 cycles using Platinum PCR SuperMix High Fidelity (Life Technologies, CA, USA). After Agencourt AMPure XP reagent bead

purification, 1 µl of amplified library was assessed with D1k ScreenTape (Agilent Technologies Inc., DE, USA). Individual libraries were diluted to 100 pM with low TE buffer (Thermo Fisher Scientific, CA, USA). An equal amount of each of the 24 samples was pooled together for one Ion PI Chip V2 (Thermo Fisher Scientific, CA, USA). Ion Sphere particles containing clonally amplified DNA were prepared with Ion PI Template OT2 200 Kit v3, and the template-positive Ion Sphere particles were then enriched with the Ion OneTouch ES (Thermo Fisher Scientific, CA, USA). The enriched template-positive Ion Sphere particles were sequenced with Ion PI Chip V2 and the Ion PI Sequencing 200 Kit v3 on the Ion Proton instrument (Thermo Fisher Scientific, CA, USA). Around 2 million reads were obtained for each barcoded sample.

Relative mtDNA assessment

Ten bases were trimmed from both ends of each read before the reads were aligned to the *Mus musculus* nuclear and mitochondrial reference genomes (mm10) using Bowtie2 version 2.1.0 with the “sensitive-local” preset mode. The number of reads mapped to each chromosome was counted using SAMtools version 0.1.19 while ignoring alignments with MAPQ < 20. Relative mtDNA content was assessed by dividing the number of reads aligned to the mitochondrial genome (R_m) by the count of reads aligned to nuclear genome (R_n) from the same sample: $Relative\ mtDNA = \frac{R_m}{R_n}$. This normalization strategy removes the impact of different cell numbers from the quantitative process and is therefore a relative measure of mtDNA per cell. Then, the resulting value from the blastocysts was multiplied by the correction factor *F* to account for genomic variation due to embryo gender and ploidy.

$$F = \frac{\sum_{i=1}^n (CL_i \times CN_i)}{Euploid\ female\ total\ chromosomal\ length},$$

where *i* is the chromosome number, CL is the chromosome length (Table 1), and CN is the chromosome copy number.

Mouse reproductive samples

Previous studies showed that CD-1 and CF-1 mouse strains had a reproductive age-associated deterioration in oocyte quality, including an increase in egg aneuploidy [13, 22–24]. Because of the limited availability of aged mice, we analyzed polar bodies and oocytes using CD-1 for methodology validation and CF-1 embryos to explore the association of mtDNA levels with ploidy, age, and oocyte maturation methods.

Oocyte and polar body samples were collected from a total of eight CD-1 female mice (Harlan Laboratories, IN, USA) of advanced reproductive age (16–19 months old) at Northwestern University. Ovaries were harvested from each animal, and cumulus-oocyte-complexes (COC) were isolated

Table 1 Mouse chromosome length

Chromosome	Length (bp)
1	195,471,971
2	182,113,224
3	160,039,680
4	156,508,116
5	151,834,684
6	149,736,546
7	145,441,459
8	129,401,213
9	124,595,110
10	130,694,993
11	122,082,543
12	120,129,022
13	120,421,639
14	124,902,244
15	104,043,685
16	98,207,768
17	94,987,271
18	90,702,639
19	61,431,566
X	171,031,299
Y	91,744,698

from antral follicles. Cumulus cells were mechanically removed, and oocytes were cultured in a-MEM-Glutamax (Life Technologies) medium containing 3 mg/ml bovine serum albumin (MP Biomedicals, CA, USA) at 37 °C in a humidified atmosphere of 5% CO₂ in air to induce spontaneous meiotic maturation. Only mature MII oocytes were used for subsequent processing. Polar bodies and mature oocytes were collected into PCR tubes for further analysis.

Blastocysts derived from IVM or IVO were collected from CF-1 mice (Harlan Laboratories, IN, USA) at Colorado Center for Reproductive Medicine. A total of 14 young females (6–9 weeks old) and 65 aged females (13.5 months old) were used for this application. To obtain COC for IVM, mice were stimulated with 5 IU of pregnant mare's serum gonadotropin (PMSG; Calbiochem, MA, USA). Ovaries were harvested 46 to 48 h after PMSG injection, and COCs were recovered and subsequently matured in vitro for 18 h and then used for IVF. To obtain in vivo-matured oocytes, 46 to 48 h after PMSG ovulation, mice were stimulated with 5 IU of human chorionic gonadotrophin (Calbiochem, CA, USA), and fully expanded COCs were collected from the oviduct 16 h later. Mature oocytes were fertilized using spermatozoa from B6D2F1 males (8 weeks old). After IVF and blastulation, the zona pellucida was removed from the embryos, and single whole blastocysts were collected into PCR tubes, followed by lysis, WGA, and NGS.

Sequencing data from polar bodies, oocytes and blastocysts were analyzed for relative mtDNA content with or without normalization by the correction factor *F*. To investigate the possible association of relative mtDNA level in blastocysts with ploidy status, maternal age, and oocyte maturation methods, statistical analysis was carried out with ANOVA using a linear model with the natural logarithm of the mitochondrial level as the dependent variable. Independent variables include the embryo ploidy status (aneuploid versus euploid), maternal age (young versus old), and the oocyte maturation method (IVM versus IVO).

All animal experiments were approved by the Institutional Animal Care and Use Committee (Northwestern University) or Colorado Center for Reproductive Medicine Ethics in Research Committee and were carried out in accordance with National Institutes of Health Guidelines and the Society for the Study of Reproduction's specific guidelines and standards.

Results

Reliability analysis of mtDNA content on cultured cells

Relative mtDNA levels were assessed in six single- and 100-cell samples following day 0, day 3, and day 6 treatments with EtBr. Exposure to EtBr for 3 and 6 days resulted in a decrease in relative mtDNA levels in both the single- and 100-cell samples as expected (Fig. 1). The standard deviation of six single-cell samples was higher than that of samples containing 100 cells, as would be expected based on the variation between different single cells.

Assessment of mtDNA content in mouse reproductive samples

The mtDNA level was determined and compared for 40 oocytes and their corresponding polar bodies as well as 171 blastocysts (Fig. 2). Polar bodies contained an average of 0.9% of mtDNA relative to oocytes due to asymmetric segregation of cytoplasmic content during meiosis. Oocytes contained 180-fold higher mtDNA content compared to the cells in blastocysts. This is consistent with current understanding of the timing of mitochondrial biogenesis during early development: mitochondria accumulate in the oocyte during oogenesis and reaches a peak in the fully grown oocyte. After fertilization, there is little mitochondrial biogenesis during preimplantation development, and mtDNA and mitochondria are split into daughter cells during post-fertilization cell division [25–27], so there is much less mtDNA in the blastocyst cells compared to oocytes.

Fig. 1 Relative mtDNA content in single- and 100-cell samples with EtBr treatment. Six single- and 100-cell samples were collected with EtBr exposure for 0, 3, and 6 days. EtBr treatment impairs the mtDNA replication; therefore, mtDNA content decreased in single- and 100-cell samples with longer exposure to EtBr as expected

Analysis of mtDNA content in mouse blastocysts

A total of 171 blastocysts in which CCS results had been obtained were analyzed for the relative mtDNA content with normalization by a correction factor which adjusts for differences in the nuclear DNA content due to gender and ploidy. In the young age group, 27 IVO blastocyst were analyzed, and 1 was aneuploid; 32 IVM blastocysts were analyzed, and 3 of them were aneuploid. In the old age group, 47 IVO blastocysts were assessed, and 6 of them were aneuploid; 65 IVM blastocysts were assessed, and 20 of them were aneuploid (Table 2). In both one-way ANOVA and multiple-factor ANOVA with interaction terms (model $\log(\text{mtDNA.level}) = \text{ploidy.status} + \text{age.group} + \text{oocyte.maturation.method} + \text{ploidy.status:age.group} + \text{age.group:oocyte.maturation.method} + \text{oocyte.maturation.method:ploidy.status}$), embryo ploidy status, maternal age group, and oocyte maturation method were all found to be

associated with embryo mtDNA level (p values were 0.0006, 2.0×10^{-6} , and 0.0001, respectively). Cells in aneuploid blastocysts contained higher quantities of mtDNA compared to euploid blastocysts (Fig. 3a). Younger age was associated with lower relative mtDNA levels (Fig. 3b), and IVO treatment for oocyte maturation was associated with higher relative mitochondrial levels (Fig. 3c).

Discussion

The mouse model has been widely used as a model system to study the human reproductive biology. Recently, we were the first to establish WGA- and NGS-based CCS methods to further inform our fundamental understanding of aneuploidy in mouse oocytes and preimplantation embryos. Here, we extended these studies by validating a method for simultaneous quantification of mtDNA content from limited amounts of starting material. Cultured mouse cells were treated with EtBr to generate cells with reduced levels of mtDNA. Single-cell and 100-cell samples were analyzed using the mtDNA quantification method, and the expected decrease in mtDNA content with longer exposure to EtBr was detected in both samples. Single cells showed higher variation than 100-cell samples, which may be due to cell cycle asynchrony or differences between individual cells in how they were affected by EtBr treatment.

Fig. 2 Box plots for relative mtDNA content level in mouse reproductive samples. The y-axis represented the natural logarithmic scale of relative mtDNA level. Relative mtDNA level from 40 oocytes had a median of 0.18, 40 polar bodies had a median of 0.0017, and 171 blastocysts had a median of 0.001

Table 2 Total of 171 blastocysts with CCS results

Age group	Oocyte maturation	Aneuploid	Euploid	Total
Young	IVO	1	26	27
	IVM	3	29	32
Old	IVO	6	41	47
	IVM	20	45	65

Fig. 3 Box plots for relative mtDNA content in mouse blastocysts. The y-axis represented the natural logarithmic scale of relative mtDNA level. **a** NGS analysis of 171 blastocysts showed a statistically significant increase in aneuploidy blastocytes ($P = 0.0006$). **b** Cells in blastocysts

from old mice contained a significantly higher level of mtDNA ($P = 2.0e - 6$). **c** NGS analysis also showed cells in blastocysts derived from IVO contained a significantly higher amount of mtDNA compared to IVM ($P = 0.0001$)

In addition to in vitro validation, our detection of a much higher level of mtDNA in oocytes as compared to polar bodies is consistent with the expectation that highly asymmetric division of the cytoplasm in meiosis results in accumulation of mtDNA in the oocyte for later embryo development [28]. The assay was further validated by the comparison of mtDNA content in oocytes and blastocysts. Most publications suggested that there is no or limited mtDNA replication until implantation, so each newly divided blastomere within the preimplantation embryo will possess fewer copies of the mtDNA at each stage of post-fertilization cell division [25–27]. In our study, the average mtDNA content in oocytes was 180-fold higher than the cells in blastocysts, indicating that there are about 7 to 8 rounds of cell division from fertilized oocytes to blastocysts. This finding is consistent with mouse embryonic development, in which a zygote undergoes 8 to 10 cell divisions to form a morula and then further develops into a blastocyst.

In this study, we found that relative mtDNA content was associated with ploidy status, maternal age, and oocyte maturation methods in mouse blastocysts. The difference of relative mtDNA level between the groups was significant with the implementation of the correction factor based on gender and ploidy.

Relative mtDNA content in aneuploid mouse embryos was found to be significantly higher than euploid embryos, which is consistent with observations in humans [7, 8]. Increased mtDNA copy number was observed in aneuploid oocytes from diabetic mice [29]. Mitochondrial biogenesis and adequate energy production are important for cytoplasmic maturation, embryogenesis, and placentation [30, 31]. There is a hypothesis that the mtDNA copy number in the embryo is not a direct indicator of energetic capability but rather is an indicator of energetic stress [8]. The observation of increased mtDNA content in aneuploid embryos in our study suggests

that aneuploid embryos may need more energy for chromosome segregation and activate mitochondrial biogenesis at an earlier stage of embryo development.

Mouse maternal age was associated with the relative mtDNA content of embryos; relative mtDNA content in mouse embryos increased with maternal age. Previous studies showed that reproductively old humans and animals tend to have lower mtDNA copy numbers or more dysfunctional mitochondria in their oocytes [32–34]. One explanation for the age-related mtDNA increase in the embryos is that a reduced number of mitochondria may produce an inadequate energy supply for embryonic development and therefore trigger early mitochondrial biogenesis and increased mitochondrial number in preimplantation embryos. Similar results were observed in human embryos [7, 8]. Another explanation is related to mitophagy, which is the selective degradation of mitochondria by autophagy and occurs to defective mitochondria following damage or stress. It promotes turnover of mitochondria and prevents accumulation of dysfunctional mitochondria [35]. It was found that mitophagy may play a key role in retarding accumulation of somatic mutations of mtDNA with aging [36]. In the process of embryogenesis, mitophagy might be impaired in the embryos from old mice, which causes the accumulation of dysfunctional mitochondria, and therefore, higher level of mtDNA is observed.

It has been reported that mouse oocytes produced via IVO had higher fertilization and blastocyst formation rates than IVM [37, 38]. Embryos derived via IVO also had a lower aneuploidy rate [11]. Those findings imply that the oocyte maturation method plays an important role in oogenesis and embryogenesis. In our study, we quantified the relative mtDNA level related to cytoplasmic maturation, and we found that relative mtDNA content was higher in embryos derived from IVO compared to IVM. Oocytes derived from IVO have higher quality compared to IVM, so they may accumulate

higher quantity of mtDNA during oogenesis, which could facilitate fertilization and embryo development [39]. Lower level of mtDNA in embryos derived from IVM, due to inadequate mitochondrial biogenesis or cytoplasmic maturation, may adversely affect oocyte quality, so age and IVM may have different mechanisms that underlie oocyte quality.

Quantification of mtDNA as a tool for embryo viability assessment in ART is an area of active research and debate. In a recent published blinded prospective nonselection study, Fragouli et al. concluded that embryos with elevated levels of mitochondrial DNA rarely implant [40]. Ravichandran et al. demonstrated that higher levels of mtDNA are associated with reduced implantation in a large blinded, retrospective study [41]. Treff et al. found that mtDNA quantification had no prediction value for implantation from embryos within a single cohort. Victor et al. also reported the lack of association of mtDNA with reproductive potential using a modified methodology that improved normalization strategies. Due to these conflicting results, further studies are needed before clinical use of mtDNA as a reproductive marker. Studies of mtDNA copy number in mouse oocytes and embryos may help to elucidate basic mechanisms of mitochondrial biogenesis and function related to reproductive competence. In our mouse model, we found that relative mtDNA levels were elevated in aneuploid embryos relative to euploid embryos, higher in blastocysts from reproductively old mice relative to young mice, and lower in embryos derived from IVM compared to IVO. The associations of mtDNA level with aneuploidy and age in mice were consistent with findings in human studies [8]. However, future work is needed to understand the relationship between mtDNA quantity and implantation success after embryo transfer in mice.

In conclusion, this study provides a validated method to assess the relative mtDNA content in mouse oocytes, polar bodies, and blastocysts which can be performed simultaneously and in conjunction with CCS. The method could be applied in future research to assess the effectiveness of therapeutic interventions involving mitochondrial copy number and distribution.

Compliance with ethical standards All animal experiments were approved by the Institutional Animal Care and Use Committee (Northwestern University) or Colorado Center for Reproductive Medicine Ethics in Research Committee and were carried out in accordance with National Institutes of Health Guidelines and the Society for the Study of Reproduction's specific guidelines and standards.

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