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Live birth derived from oocyte spindle transfer to prevent mitochondrial disease

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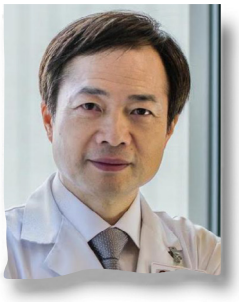

Dr John Zhang completed his medical degree at Zhejiang University School of Medicine in China, and subsequently received his Master's Degree at Birmingham University in the UK. In 1991, Dr Zhang earned his PhD in IVF, and, after researching the biology of mammalian reproduction and human embryology for nearly 10 years he completed his fellowship training in Reproductive Endocrinology and Infertility at New York University's School of Medicine in 2001. Dr. Zhang continues his clinical research in minimal stimulation IVF, non-embryonic stem cell, long-term oocyte cryopreservation, and oocyte reconstruction by nuclear transfer.

KEY MESSAGE

We report a live birth after oocyte spindle transfer to prevent transmission of the mitochondrial disease, Leigh syndrome.

A B S T R A C T

Mutations in mitochondrial DNA (mtDNA) are maternally inherited and can cause fatal or debilitating mitochondrial disorders. The severity of clinical symptoms is often associated with the level of mtDNA mutation load or degree of heteroplasmy. Current clinical options to prevent transmission of mtDNA mutations to offspring are limited. Experimental spindle transfer in metaphase II oocytes, also called mitochondrial replacement therapy, is a novel technology for preventing mtDNA transmission from oocytes to pre-implantation embryos. Here, we report a female carrier of Leigh syndrome (mtDNA mutation 8993T > G), with a long history of multiple undiagnosed pregnancy losses and deaths of offspring as a result of this disease, who underwent IVF after reconstitution of her oocytes by spindle transfer into the cytoplasm of enucleated donor oocytes. A male euploid blastocyst was

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obtained from the reconstituted oocytes, which had only a 5.7% mtDNA mutation load. Transfer of the embryo resulted in a pregnancy with delivery of a boy with neonatal mtDNA mutation load of 2.36–9.23% in his tested tissues. The boy is currently healthy at 7 months of age, although long-term follow-up of the child's longitudinal development remains crucial.

Introduction

Mitochondria provide energy for most eukaryotic cells and are assembled with proteins encoded by both nuclear and mitochondrial DNA (mtDNA). At least 1 in 5000 people in the general population has one mutation in mtDNA, which can cause mitochondrial dysfunction and maternally inherited diseases (Gorman et al., 2015). When both wild type (normal) and mutant mitochondrial genomes co-exist, a condition called heteroplasmy, the severity of the symptoms is associated with the level of mtDNA mutation load (Freyer et al., 2012). Leigh syndrome is a devastating childhood disease caused by mitochondrial deficiency. About 20–25% of Leigh syndrome cases are caused by mtDNA mutations (Swalwell et al., 2011). The mtDNA 8993T > G mutation, one of the most common of such mutations, impairs the function of the F0 portion of ATPase causing ATP-synthetic defects. In cells harbouring 8993T > G mutation, mitochondrial ATP synthesis is reduced by 50–70% (Nijtmans et al., 2001), thereby causing failure of the mitochondrial respiratory chain. Patients with Leigh syndrome often develop regression of both mental and motor skills leading to disability and rapid progression to death, often owing to seizures and respiratory failure (Nijtmans et al., 2001; Swalwell et al., 2011). When mtDNA 8993T > G mutation load is less than 30%, the carrier is expected to be asymptomatic. A large cohort study showed that the probability of having severe symptoms, i.e., pathological phenotype, is low until the mutant load (heteroplasmy level) reaches 60–70% for the 8993T > G mutation (White et al., 1999), indicating a high tolerance threshold for mutation load.

Current clinical options to prevent transmission of mtDNA mutations to offspring are limited. A couple could adopt a child, use donor oocytes or use prenatal diagnosis and abort an affected pregnancy. There is no reliable way of pre-selecting embryos with pre-implantation genetic diagnosis for most cases of mtDNA mutation (Mitalipov et al., 2014), particularly for a woman with a high level of heteroplasmy. Nuclear transfer has been proposed as a novel approach to minimize the transmission of mutant mtDNA from a carrier mother to her child at the gamete or zygote level (Craven et al., 2010). Experimental nuclear transfer in both animals and humans has been reported, and our group as well as others has worked on this for over 2 decades (Liu et al., 1999, 2003; Zhang et al., 1999; Tachibana et al., 2009; Tachibana et al., 2013; Zhang and Liu, 2015; Hyslop et al., 2016; Zhang et al., 2016). The tragic consequences of childhood mitochondrial disease, in particular those of Leigh syndrome, prompted the current experimental effort.

Recent studies show that two technicques can be used to carry out nuclear transfer for mitochondrial replacement therapy: metaphase II (MII) spindle transfer and pronuclear transfer (Craven et al., 2010; Tachibana et al., 2009). These techniques, however, have yet to be conducted clinically owing to regulatory constraints in countries in which reproductive techniques are subject to legal and regulatory oversight. Pronuclear transfer leads to discarding of zygotes and may raise religious and ethical concerns in certain populations. This makes the spindle transfer technique preferable to pronuclear transfer. The intent of the treatment described here was to allow a woman, who carries a mitochondrial DNA mutation (Leigh syndrome), and who has a demonstrated history of transmission of the disease to her offspring, to have a male child with minimal pathogenic mitochondria and with no risk of transferring the disease to his offspring.

Materials and methods

Case description

A 36-year-old asymptomatic woman with a history of four pregnancy losses (between 6–16 weeks of gestation, reasons unknown), and two deceased children (at ages 8 months and 6 years) (**Figure 1**) from Leigh syndrome as confirmed by over 95% mutation load, sought assistance to conceive a healthy baby. The patient was asymptomatic and carried the mitochondrial genome mutation 8993T > G in subunit six of the ATPase gene, which is known to cause Leigh syndrome (Holt et al., 1990). Whole mtDNA sequencing analysis using next-generation sequencing revealed 8993T > G heteroplasmy levels of 23.27%, 24.50% and 33.65% in her hair follicles, blood and urine precipitate, respectively (**Supplementary Table S1**) (**Figure 2**). The

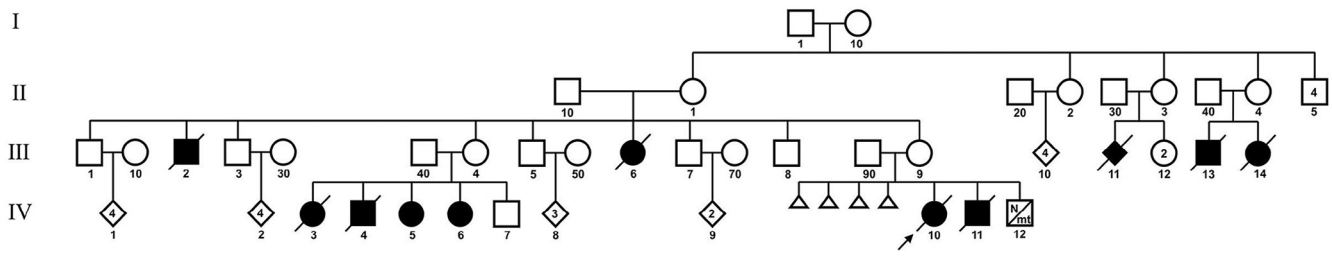

Figure 1 – Pedigree of the family with Leigh Syndrome (black fill indicates clinically affected individuals, blank triangle for miscarriage). The product of the nuclear transfer procedure was indicated by N/mt in order to indicate that the nuclear (N) genome and mitochondrial (mt) genome were from different individuals.

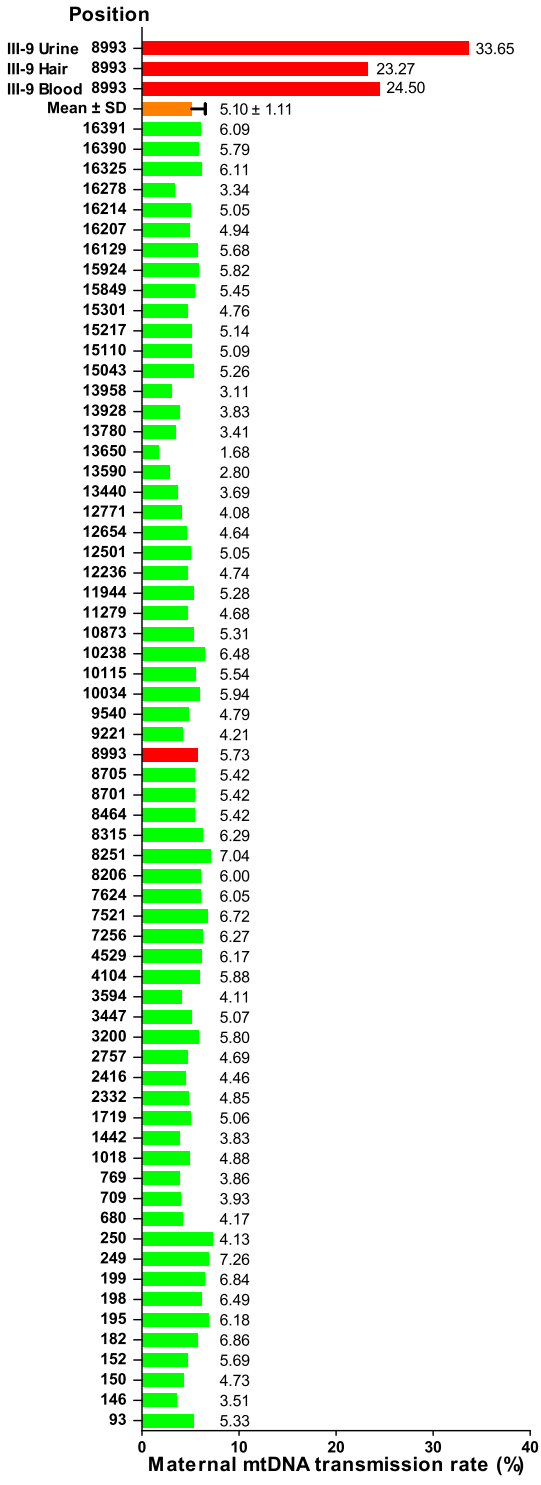

Figure 2 – Detection of maternal mitochondrial DNA transmission in the trophectoderm cells of IV-12 blastocyst. The maternal transmission rate was assessed at each of the 64 informative mitochondrial DNA single nucleotide polymorphisms (green bars). The average transmission rate was 5.10% ± 1.11% (Mean ± SD, orange bar). The heteroplasmy level at the mutation site (8993T > G) was 5.73% (red bar) in the blastocyst of IV-12. Mother's (III-9) levels of heteroplasmy (8993T > G) were 24.50%, 23.27% and 33.65% in the blood, hair follicles and urine precipitate (red bars), respectively. For the screen shot of the sequences alignment for m.8993T > G, see Supplementary Figure S3.

Table 1 – Oocyte retrieval results and fate of oocytes.

N	First IVF cycle	Second IVF cycle
Cumulus–oocyte–complexes	11	18
MII (spindle visualized)	4 (4)	5 (3)
MII without zona (spindle visualized)	1 (1)	1 (1)
Germinal vesicle	1	2
Degenerate	5	10
Vitrified	5	0
Survived after vitrification (spindle visualized)	2 (1)	NA
with spindle transfer	1	4
ICSI	1	4
2PN	0	4
Blastocyst	NA	4
Euploid	NA	1
Male euploid and transferred	NA	1

ICSI, intracytoplasmic sperm injection; MII, second metaphase; NA, not applicable; 2PN, two-pronuclear.

patient's mtDNA haplogroup was I (**Supplementary Figure S1**). The patient's parents were both healthy and asymptomatic. She had brothers and sisters; one brother and one sister had died of Leigh syndrome, whereas the other siblings were asymptomatic. Her surviving sister had one asymptomatic son and four children with Leigh syndrome (**Figure 1**). Two of the four affected children have died as a result of the disease at ages 9 months and 1 year.

The patient opted to proceed with spindle transfer over pronuclear transfer for religious reasons (to avoid disrupting a zygote). After cautious counselling for mitochondrial replacement therapy, the patient consented to a protocol approved by the New Hope Fertility Center, Mexico Institutional Review Board (January, 2015). Oocyte donor screening and consenting were conducted according to the New Hope Fertility Center protocols. Twenty-eight donor oocytes were vitrified using a kit from Kitazato BioPharma Co., Ltd., and a total of eight oocytes were warmed for spindle transfer. The donor's mtDNA, taken from other oocytes of the same donor, was sequenced, and no pathogenic mutation was found (**Supplementary Table S1**). The donor's mtDNA haplogroup was L2c (**Supplementary Figure S2**).

During two cycles of minimal ovarian stimulation of the patient, using previously established protocols for MII oocyte collection, a total of 29 cumulus–oocyte complexes was retrieved (11 from the first cycle and 18 from the second cycle): 15 oocytes were degenerate, three oocytes were at germinal vesicle stage and two oocytes were zona pellucida-free (**Table 1**). Because of the overall poor quality of the oocytes, only nine of the 29 harvested oocytes were found to have a polar body (MII oocytes). Five oocytes, including one zona-free oocyte from the first cycle, were vitrified after a birefringent spindle was visualized by polar microscopy. One vitrified warmed oocyte and four fresh oocytes underwent spindle transfer. Membrane fusion between removed karyoplast (spindle) and cytoplasm (enucleated donor oocyte) is usually achieved by either electrofusion or by use of an extract from Sendai virus. To avoid introducing foreign proteins, such as that from Sendai virus, a modified electrofusion procedure was used, as described below.

Spindle transfer

Spindle transfer was commenced for the four fresh oocytes 3 h after oocyte retrieval. For the one surviving vitrified-warmed oocyte, spindle

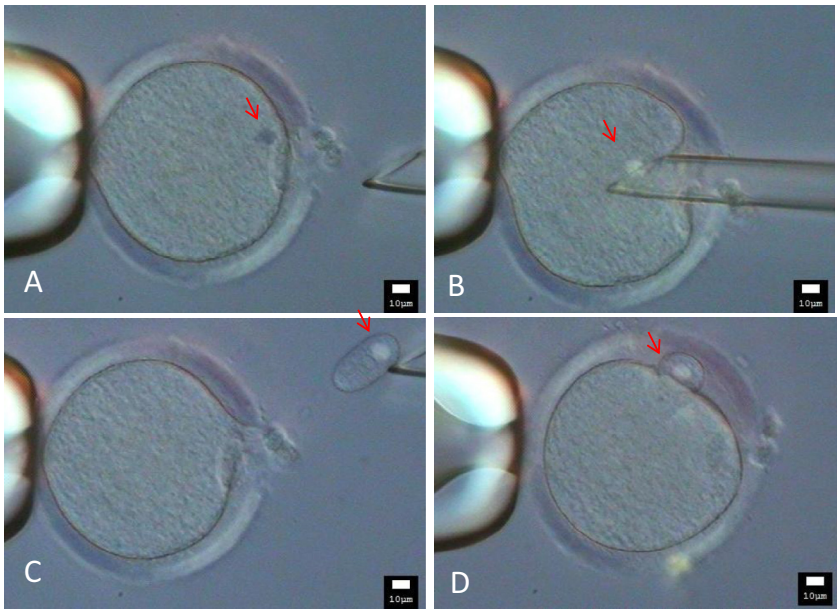

Figure 3 – Spindle transfer from the patient's metaphase II oocyte into the cytoplasm of a donor oocyte. (A) The patient's oocyte immobilized by a holding pipette and approached by a transfer pipette; (B) suction of spindle into biopsy pipette; (C) spindle removed from the patient's oocyte; (D) transfer of the removed spindle into the perivitelline space of the enucleated donor oocyte. Arrows indicate the spindle. Scale bar, 10 µm.

transfer was carried out 3 h after oocyte warming using electrofusion (**Figure 3**) (Zhang, 2014; Zhang et al., 2016). Briefly, the oocytes were exposed to HEPES buffered human tubal fluid medium (HTF w/Hepes, Life Global, reference LGGH-500) with 10% protein supplement (Life Global, reference LGPS-50) with 7.5 µg/ml cytochalasin B (Sigma, St Louis, MO, USA) for 8 min at 37°C to disrupt the microfilaments and increase plasma membrane flexibility before manipulation. The dish was then placed onto the warm stage of an Olympus IX71 inverted microscope equipped with micromanipulators. A slot was made in the zona, using the LYKOS Laser System (Hamilton Thorne, Beverly, MA, USA) with several pulses of 100–200 µs at 100% power. This allowed the pipette to pass through the zona opening and approach the spindle before gently applying negative pressure to aspirate the spindle with minimal ooplasmic content. Once the spindle was separated from the cytoplasm, it was transferred into the perivitelline space of the vitrified and warmed enucleated donor oocyte cytoplast, from which only the large size first polar body had been removed (but not recorded for specific oocytes). Membrane fusion between the patient spindle and the donor cytoplast was initiated by placing it into fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, and 0.05 mM $MgSO_4$) between platinum electrodes. A single electrical pulse (1.4 kV/cm DC for 50 µs) was delivered by an Electro Cell Manipulator (BTX Model 2001, Holliston, MA) at room temperature as modified from a previous report (Zhang and Liu, 2015). The reconstituted oocytes were rinsed three times and then incubated in Global Total for Fertilization medium (Life Global Ref# LGTF-100) at 5% CO_2, 5% O_2 and 37°C (Astec, Japan). Membrane fusion usually occurred within 30 min after the application of the electric pulse. Each reconstituted oocyte was injected with a single sperm from the patient's partner, which had been frozen and thawed; only motile sperm with normal morphology were immobilized and selected for injection within 1 h of fusion. After intracytoplasmic sperm injection, reconstituted oocytes were cultured in Global Total medium in an EmbryoScope Time-Lapse System incubator (Vitrolife, Sweden) in 5% CO_2 and 5% O_2 at 37°C. The medium was refreshed every 48 h until blastocyst formation.

Blastocyst biopsy

All blastocysts underwent biopsy for aneuploidy testing by array comparative genomic hybridization (aCGH). The biopsy was carried out on day 5 for two embryos and on day 6 for the other two embryos. Blastocysts were placed in HEPES medium supplemented with 10% protein and held by a holding pipette. Using gentle suction through a biopsy pipette with 30 µm inner diameter, three to five trophectoderm cells were dissected and removed using repeated 400 µs laser pulses at 100% power. The trophectoderm cells were then loaded into 2 µl non-stick wash buffer in a 0.5 ml sterile polymerase chain reation (PCR) tube and stored in a freezer at –80°C until aCGH test and whole mtDNA sequencing analysis. All these procedures were conducted in the New York City clinic, and the embryos were then vitrified to await the outcome of tests before transfer. The euploid embryo was shipped to our clinic in Mexico for transfer to the patient.

aCGH test

DNA samples, from blood and saliva of the mother and different tissues of the boy (as described below) were processed according to manufacturer's instructions and hybridized to a single nucleotide polymorphism (SNP) array (HumanKaryomap-12 BeadChip; Illumina, USA), targeting around 300,000 SNPs spread across the entire human genome. The BeadChip data were evaluated and genotyping raw data were obtained and further analysed with software (BlueFuse Multi V4.2, Illumina).

Whole mtDNA sequencing analysis

Genomic DNA was extracted from whole blood and tissue samples, including buccal swabs, skin tissues from circumcision, hair follicles, placenta, umbilical stalk and urine precipitate using Gentra DNA extraction kit (Qiagen, Hilden, Germany). Entire mtDNA was amplified by single long-range PCR reaction as previously described (Ma et al., 2015). Briefly, the primers specifically recognize genuine mtDNA F-2120 GGACACTAGGAAAAAACCTTGTAGAGAGAG and R-2119 AAAGAGCTGTTCCTCTTTGGACTAACA. TAKARA LA Taq polymerase (Takara Biotechnology) was used for PCR amplifications. The conditions for PCR were 94°C for 1 min; 98°C for 10 s and 68°C for 16 min, 30 cycles; 72°C for 10 min. DNA samples from biopsied trophectoderm cells used for preimplantation genetic screening were re-amplified through Rolling Circle Amplification using a REPLI-g Mitochondrial DNA Kit (Qiagen, Hilden, Germany). The biopsied oocyte cytoplasm from the donor (obtained in exactly the same way as the spindle transfer procedure mentioned above except for the absence of a spindle in the biopsy) was lysed using the SurePlex whole genome amplification kit (BlueGnome, Cambridge, UK) and then amplified using a REPLI-g Mitochondrial DNA Kit (Qiagen, Hilden, Germany). All samples were amplified and sequenced in duplicate to rule out the possibility of artifactual mutations created by polymerase errors during the PCR or sequencing processes.

Whole mtDNA sequencing analysis by MiSeq

Whole mtDNA sequencing was carried out as detailed previously (Kang et al., 2016a; Tang and Huang, 2010) and modified (Ma et al., 2015). Briefly, the amplified mtDNAs were used for library preparation by Nextera XT DNA kit (Illumina). Sequencing was carried out on the Illumina MiSeq platform (DNA Core Facility, Cincinnati Children's Hospital Medical Center) and the data were analysed using NextGENe software (Softgenetics, USA). Briefly, sequence reads ranging from 100–200 bps were quality filtered and processed using NextGENe software and an algorithm similar to BLAT (Kent, 2002). Sequence error correction feature (condensation) was carried out to reduce false-positive variants and produce sample consensus sequence and variant calls. Alignment without sequence condensation was used to calculate the percentage of the mitochondrial genome with depth of coverage of 1000. Starting from quality FASTQ reads, the reads were quality filtered and converted to FASTA format. Filtered reads were then aligned to the human mitochondrial sequence reference NC_012920.1 followed by variant calling (Kang et al., 2016a). Variant heteroplasmy was calculated by NextGENe software as follows: base heteroplasmy (Mutant Allele Frequency %) = mutant allele (forward + reverse) / total coverage of all alleles C, G, T, A (Forward + Reverse) *100. The clinical significance of the variants was analysed as described before (Tang and Huang, 2010) with MitoMaster (http://www.mitomap.org/MITOMASTER/WebHome) (Lott et al., 2013).

Results

All five oocytes with birefringent spindles were successfully reconstituted and exposed to intracytoplasmic sperm injection, and four out of five oocytes were fertilized and developed into blastocysts (**Table 1**). After blastocyst trophectoderm biopsy, embryos were vitrified, awaiting both aneuploidy testing results as well as mutated mtDNA levels. aCGH results showed that one male blastocyst was euploid. The other three blastocysts were aneuploid, showing monosomy 22, monosomy 15, and partial monosomy 15q24.2-qter.

The mtDNA sequence of the mother, the oocyte donor and the embryo were compared. As shown in **Figure 2** (**Supplementary Table S1**), all 65 sites (including 8993T > G) in the mitochondrial genome that distinguished the mother from the oocyte ooplasm donor were assessed. The patient's oocytes had almost 100% of 8993T > G mutation load (based on the calculation of 64 SNP's), consistent with her history of previous affected pregnancies, and also indicating a mitochondrial bottleneck. In contrast, the average transmission rate of maternal mtDNA in the biopsied blastocyst was 5.10 ± 1.11% (mean ± SD) and the heteroplasmy level for 8993T > G was 5.73%. The vitrified and warmed euploid embryo derived from spindle transfer was transferred, and led to an uneventful pregnancy with vaginal delivery of a boy at 37 weeks of gestation. The neonate weighed 3180 g and measured 51.5 cm in length, and the Apgar score was 9 at both 1 and 5 min. Physical examination, including a thorough neurological investigation at birth, 2 weeks, 4 weeks, 2 months, 3 months, and 4 months has been normal. The boy is still under close monitoring with a long-term follow-up plan.

The mtDNA 8993T > G mutation load in the baby was assessed in multiple tissues collected within 2 days of birth, including buccal epithelium, hair follicles, circumcised foreskin, urine precipitate, placenta, amnion, umbilical blood and umbilical cord. In addition, single nucleotide polymorphism (SNP) array (HumanKaryomap-12 BeadChip) targeting around 300,000 SNPs spread across the entire human genome was used for genotyping the mother (III-9) and son (IV-12). Our results showed a normal karyotype of 46, XY. No deletion, duplication or uniparental was observed (**Supplementary Table S2**). The mtDNA samples underwent whole mtDNA sequencing analysis and all 15 variants from the mother were detected in the different tissues of the neonate that displayed a much lower heteroplasmy level than that observed in the mother (**Supplementary Table S3**). The mutation load for 8993T > G varied from undetectable in the placenta, umbilical blood and umbilical cord to 2.36% in the urine precipitate, 3.52% in the buccal epithelium, 5.59% in the hair follicles, 6.77% in the amnion, and 9.23% in the circumcised foreskin (**Figure 4**). Our results suggest that spindle transfer may minimize the transmission of mutant mtDNA from a carrier mother to her child.

Discussion

This study strongly suggests that spindle transfer can significantly reduce the load of mutated mtDNA. Furthermore, the male gender of the child eliminates the risk of transferring the disease to his offspring (Falk et al., 2016). It is inevitable that a certain amount of cytoplasm will be carried over into the enucleated donor oocyte in order to maintain nuclear integrity, thereby introducing mtDNA along with the nucleus, leading to heteroplasmy in the reconstituted oocyte (Yabuuchi et al., 2012). The mutated mtDNA load observed here, however, is low, and we expect the offspring to remain asymptomatic (Craven et al., 2010). Our spindle transfer technique accomplished a less than 6% carryover rate. Most studies, even our own preliminary in-vitro studies (unpublished data), reported a carryover rate of less than 3%. Although 6% carryover still significantly reduces the transmission of maternal mutated mtDNA to the offspring, further studies are needed to effect consistency in carryover rate during spindle transfer.

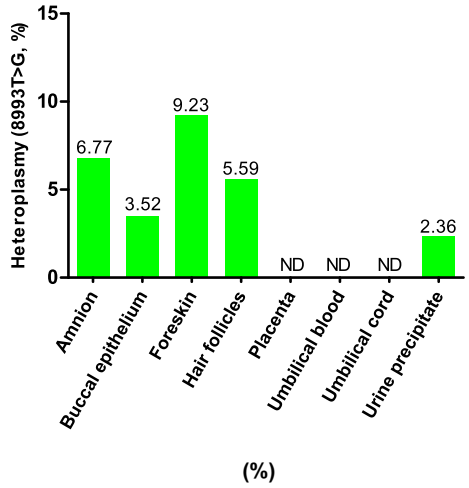

Figure 4 – : The heteroplasmy level of the maternal mitochondrial DNA 8993T > G mutation in the boy (IV-12). The amnion, hair follicles, neonatal buccal epithelium, circumcised foreskin, neonatal urine precipitate, placenta, umbilical blood and umbilical cord were collected within 2 days of birth. Total DNA was extracted, polymerase chain reaction amplified for the full-length mitochondrial genome and subjected to full maternal mitochondrial DNA sequencing and analysis with NextGen software. The percentages of 8993T > G were calculated in each sample. For the screen shot of the sequences alignment for 8993T > G, see Supplementary Figure S3. **ND, not detectable.**

A concern has been raised by Yamada et al. (2016) about 'drift' in mtDNA heteroplasmy level. In that study, however, pluripotent human embryonic stem cell lines derived from blastocysts were used to determine the replicative stability of the mtDNA genotype. They found, in all eight cell lines except for one (haplogroup karyoplast:cytoplast, H1:L3), that mitochondrial heteroplasmy decreased below the limit of detection by passage 6 and remained stable for more than 30 passages or more than 6 months of culture. For the one exceptional cell line, the carryover was 1.3% at derivation and remained low until more than 20 passages, then suddenly, expanded to 53.2% at passage 36. For no obvious reason, the level then decreased to 1%. The mechanism underlying this drift is unknown. As mitochondria may behave differently in embryonic stem cells than in normal human development (Callaway, 2016), these in-vitro results may not represent mtDNA behaviour *in vivo* where cell-cell interaction is important. Several groups tested the hypothesis that specific mitochondrial or nuclear genotype combinations might confer cellular survival, proliferative advantages, or both, because of differences in mitochondrial function. In the study by Ma et al. (2015), compatibility of the nucleus from D4a haplotype and mitochondria from F1a with 47 different SNPs in mtDNA between the two haplogroups was examined. Two such distant nuclear and mtDNAs had normal nuclear–mitochondrial interactions as demonstrated by lineage-specific differentiation and restoration of metabolic activity (Ma et al., 2015). It was reported that one out of five lines from a blastocyst with 4% mtDNA carryover showed an upward drift to about 20% by passage 12. The karyoplast and cytoplast donors for that particular cell line, however, were from the same mtDNA haplogroup. All these studies concluded that the haplotype did not confer mtDNA drift (Hyslop et al., 2016; Ma et al., 2015; Yamada et al., 2016). In a recent study, however, a conserved sequence block II (CSBII) sequence in the D-loop region of mtDNA was identified that may provide more efficient synthesis of the replication primer and so confer a replicative advantage on the mtDNA, faster growth and proliferative advantages to embryonic stem cells independent of mitochondrial activity, or both (Kang et al., 2016b).

In the present study, the mtDNA haplogroup of the patient and the donor oocyte were I and L2c, respectively. The CSBII SNP sequences were G6AG7 in both the patient and the donor. To exclude the possibility of the drift of mtDNA heteroplasmy during pregnancy, the mtDNA 8993T > G mutation load in the baby was assessed in multiple tissues collected within 2 days of birth. Although these results suggested that the maternal mtDNA levels varied among tissues, in general, they were minimal or at a very low level. Comparing the 5.73% mtDNA 8993T > G mutation load in the trophectoderm biopsy of the embryo with a mutation load in the neonate's tissues which fluctuated around this value, it is suggested that the mtDNA 8993T > G mutation has no significant selective advantage.

An adult human has a total number of 3.72×10^{13} cells with 1x 10^{16} cell divisions (Bianconi et al., 2013). Therefore, a slight advantage of cell survival or proliferation with mutant mtDNA can dramatically increase the final level of heteroplasmy. So, the trend of the shift in mtDNA heteroplasmy is much more important than the actual heteroplasmy level in the blastocyst in determining the final level of mtDNA mutation in the baby. To exclude the possibility of the drift of mtDNA heteroplasmy during pregnancy, the mtDNA 8993T > G mutation load in the baby was assessed in multiple tissues collected within 2 days of birth (**Figure 4**). Although these results suggest that the maternal mtDNA levels vary among tissues, in general, they are minimal or at a very low level. Comparing the 5.73% mtDNA 8993T > G mutation load in the trophectoderm biopsy of the embryo with a mutation load in the neonate's tissues which fluctuated around this value, it is suggested that the mtDNA 8993T > G mutation has no significant selective advantage.

The clinical significance of heteroplasmy level is often mutation-dependent (White et al., 1999). For this particular heteroplasmic 8993T > G mutation, the genotype-phenotype correlation has been well studied in a large cohort (White et al., 1999). The data from 48 8993T > G mutation pedigrees and 178 individuals showed that the probability of having severe symptoms is low until the mutant load reaches 60–70% for 8993T > G. It has also been reported for this particular mutation that no substantial tissue variation and no increase in heteroplasmy level occurs over time (Dahl et al., 2000). In our study, the mother's whole mtDNA sequencing analysis revealed 8993T > G heteroplasmy levels of 23.27%, 24.50%, and 33.65% in her hair follicles, blood, and urine precipitate, respectively and she is also asymptomatic. Hence, with a heteroplasmy level of less than 10%, there is hope that medical problems in the boy related to the mtDNA mutation will not appear. Most importantly, at the time of writing, the baby is healthy at 7 months of age.

The effect of mitochondrial heteroplasmy on meiosis completion in reconstituted human oocytes remains unclear, although a study in mice demonstrated that mitochondrial heteroplasmy of reconstituted oocytes did not influence their maturation *in vitro* or their preimplantation development (Kobayashi and Sato, 2008). In the present case, the normal karyotype in one embryo along with normal development *in vitro* and *in vivo* of one reconstituted oocyte indicates that normal meiosis completion could occur after spindle transfer in humans. Nuclear-mitochondrial compatibility is a concern in relation to mitochondrial function (Lee et al., 2008). A recent study, however, has shown that transcriptomic profiles were not affected

after somatic cell nuclear transfer between donor and recipient of different mtDNA haplogroups, indicating that normal nuclear-mitochondrial interaction could be preserved (Ma et al., 2015).

Therefore, we report the birth of a healthy boy derived from IVF using a successfully reconstituted human oocyte from a female carrier of mitochondrial disease with a history of two deceased children from a mutation in mtDNA 8993T > G. Long-term follow-up of the child's development remains crucial. Because of the novelty of the procedure and the concern of mtDNA heteroplasmy drift, we are still following the baby closely (every 3 months in the first year of age) even though the baby is asymptomatic at 7 months old now. In the second year, we plan to examine the child every 6 months and from the third year on, annually until 18 years old if the child is asymptomatic. After 18 years, we hope to assess his fertility function. At this point there is no evident abnormality, and his mutation load (tested at birth) seems to be well below the safe lower limit. The family does not wish the baby to undergo retesting for mutant mitochondrial DNA load after the initial test, unless there is a clinical benefit. Should any clinical manifestations be observed, we will follow the protocol and test the possibility of mtDNA heteroplasmy drift.

This first live birth in the current report represents our first attempt at clinical nuclear transfer to minimize the transmission of maternal mutated mtDNA to offspring. There is certain to be much controversy over this treatment, and further study is mandatory.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2017.01.013.

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