ARTICLES

Mitochondrial gene replacement in primate offspring and embryonic stem cells

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Mitochondria are found in all eukaryotic cells and contain their own genome (mitochondrial DNA or mtDNA). Unlike the nuclear genome, which is derived from both the egg and sperm at fertilization, the mtDNA in the embryo is derived almost exclusively from the egg; that is, it is of maternal origin. Mutations in mtDNA contribute to a diverse range of currently incurable human diseases and disorders. To establish preclinical models for new therapeutic approaches, we demonstrate here that the mitochondrial genome can be efficiently replaced in mature non-human primate oocytes (*Macaca mulatta*) by spindle–chromosomal complex transfer from one egg to an enucleated, mitochondrial-replete egg. The reconstructed oocytes with the mitochondrial replacement were capable of supporting normal fertilization, embryo development and produced healthy offspring. Genetic analysis confirmed that nuclear DNA in the three infants born so far originated from the spindle donors whereas mtDNA came from the cytoplast donors. No contribution of spindle donor mtDNA was detected in offspring. Spindle replacement is shown here as an efficient protocol replacing the full complement of mitochondria in newly generated embryonic stem cell lines. This approach may offer a reproductive option to prevent mtDNA disease transmission in affected families.

Mitochondria have important roles in cellular processes, for example, production of cellular energy in the form of ATP and programmed cell death (apoptosis). Each mitochondrion contains between two and ten copies of mtDNA, and because cells have numerous mitochondria, a cell may harbour several thousand mtDNA copies. Mutations in mtDNA occur at a tenfold or higher rate than in nuclear DNA, possibly due to a high concentration of free oxygen radicals, lack of histones and limited mtDNA repair mechanisms. Diseases caused by mtDNA mutations were first described in 1988[1–3]. Since then, over 150 mutations (including 100 deletions and approximately 50 point mutations) have been identified that are associated with serious human disorders, including myopathies, neurodegenerative diseases, diabetes, cancer and infertility[4]. Interest in mtDNA mutations has grown owing to the increasing number of associated diseases and because they can affect patients throughout life. In addition, mtDNA mutations are increasingly implicated in a range of prevalent public health conditions, including Alzheimer's, Parkinson's and Huntington's diseases[5–9].

Typically, a cell contains only one type of mtDNA (homoplasmy). If an individual cell contains two or more types of mtDNA—that is, as a mixture of normal and mutant mtDNA—the phenomenon is known as heteroplasmy. Heteroplasmy allows lethal mutations to persist and most importantly to pass to the next generation. MtDNA is maternally inherited through the egg's cytoplasm, whereas sperm mitochondria constitute a minor fraction of the zygote's cohort and are rapidly eliminated after fertilization[10]. It is estimated that 1 in 3,500–6,000 people has either mtDNA disease or is at risk for development of mtDNA-based disorders[11–13]. At present, there are no cures for mitochondrial disorders and available treatments only alleviate symptoms and slow disease progression. Pre-implantation genetic diagnosis has previously been applied, in a few cases, to identify and transfer embryos devoid of pathogenic mtDNA mutations and resulted in the birth of a healthy baby[14]. However, genetic counselling in most patients at risk of maternally inherited mtDNA mutations is challenging due to limitations in assessing the extent of mtDNA heteroplasmy and accurately predicting risks[15]. Therefore, there is a significant need to consider new therapeutic approaches that could prevent transmission of mtDNA mutations from mother to child.

Spindle–chromosomal complex transfer

The complete replacement of mutant mtDNA in patients' eggs with healthy mtDNA would be the most reliable method to avoid recurrence of mtDNA diseases, but neither the feasibility nor safety of such a substitution has been evaluated. We hypothesized that mtDNA can be efficiently replaced by a novel approach, that is, spindle–chromosomal complex transfer (ST) in mature eggs (metaphase II, or MII, oocytes) without perturbing subsequent fertilization and developmental competence. To this end, the nuclear genetic material from a patient's egg containing mtDNA mutations could be removed, and transplanted into an enucleated egg containing normal mtDNA donated by a healthy female. A child born after fertilization with the partner's sperm would be free of risk from maternal mtDNA mutations as well as being the biological child of the patients (Supplementary Fig. 1).

Initially, we investigated the distribution of active mitochondria in rhesus macaque oocytes by labelling with MitoTracker red and monitoring with confocal laser scanning microscopy. In mature MII-stage oocytes, mitochondria were distributed relatively uniformly throughout the cytoplasm but spindles and metaphase chromosomes were

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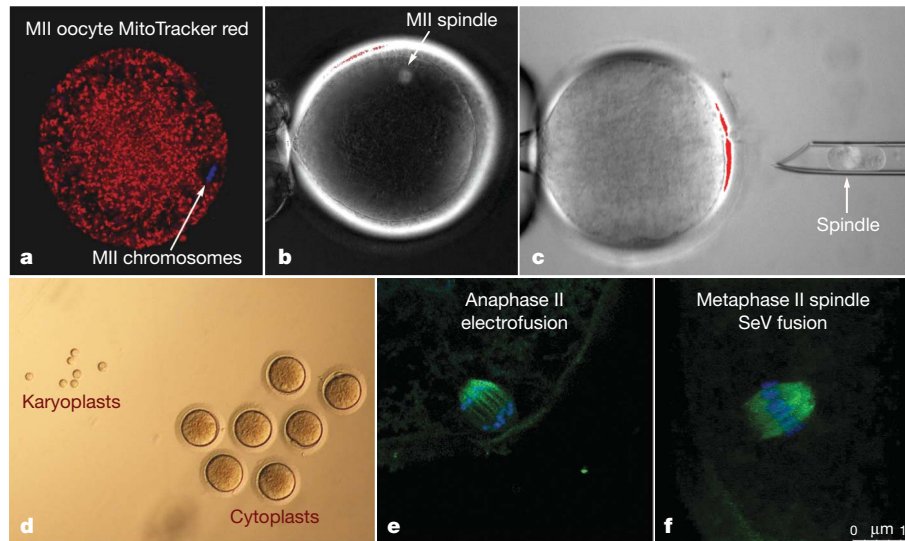
Figure 1 | Spindle–chromosomal complex transfer and meiotic analysis of reconstructed monkey oocytes. a, Confocal microscopy of a rhesus macaque MII oocyte labelled with DAPI (blue) to depict chromosomes and MitoTracker red to label active mitochondria. **b**, MII spindle visualization with Oosight Imaging System. **c**, Karyoplast isolation. **d**, Isolated karyoplasts and cytoplasts. Original magnifications: **a–c**, ×300; **d**, ×40. **e**, Confocal microscopy of progression to the anaphase II stage induced by electrofusion. **f**, Intact metaphase II spindle after SeV fusion. Spindles are labelled with DAPI (blue) to depict chromosomes and with α/β-tubulin (green) to show microtubules.

devoid of mitochondria (Fig. 1a). These results suggested that the isolation and transfer of MII spindle–chromosomal complexes can be accomplished without significant mtDNA carryover from the nuclear donor oocyte. Until now, two major technical obstacles have hampered the feasibility and success of this approach: (1) difficulties in visualization and isolation of intact MII chromosomes; and (2) the susceptibility of meiotic spindles and chromosomes to damage secondary to premature oocyte activation during manipulations. The visualization of DNA in mature oocytes is difficult because the nucleus is no longer evident after re-initiation of meiosis and breakdown of nuclear membrane. However, enucleating MII oocytes became routine after development of techniques for DNA staining with fluorophores (such as Hoechst 33342) and subsequent visualization under ultraviolet light, notably during cloning of embryos by somatic cell nuclear transfer (SCNT). However, unlike in cloning where the extracted chromosomes are discarded, the viability and integrity of the oocyte spindle-associated DNA during ST must be maintained.

We recently accomplished successful SCNT in primates with adult skin cells as nuclear donors and went on to isolate functional embryonic stem (ES) cells[16,17]. The key to this success was implementation of several technical modifications to avoid damage to the cytoplast during spindle removal. This involved non-invasive visualization of the metaphase spindle during enucleation with polarized microscopy to avoid deleterious effects of Hoechst staining and ultraviolet exposure (Fig. 1b). We reasoned that the modified enucleation technique should be applicable to the isolation of intact MII spindle–chromosomal complexes and their subsequent transfer to spindle-free, donor cytoplasts. Indeed, we were able to isolate intact spindle–chromosomal complexes surrounded by a small amount of cytoplasm and cell membrane (karyoplast) with 100% efficiency (Fig. 1c and Supplementary Movie). We measured diameters of both karyoplasts and enucleated oocytes (cytoplasts) and calculated that the average volume of a karyoplast was 11.3 ± 1.2 pl (mean $\pm$ s.e.m.; $n = 7$) whereas the average volume of a cytoplast was 752.1 ± 18.3 pl (Fig. 1d). Thus, a karyoplast contained approximately 1.5% of the volume of a cytoplast, suggesting that a negligible amount of mitochondria/mtDNA surrounding the spindle would be carried over during ST.

Next, we investigated the re-introduction of spindle–chromosomal complexes into enucleated oocytes derived from unrelated females. Karyoplasts were placed into the perivitelline space of cytoplasts, on the side opposite the first polar body, and fusion with the cytoplast was accomplished by electroporation. Approximately 1 h after fusion, reconstructed oocytes were fixed and analysed by immunocytochemistry for spindle–chromosomal complex integrity. A spindle was present in all analysed oocytes, but the majority of oocytes unexpectedly resumed the meiotic division and progressed to the anaphase of meiosis II or completed meiosis and formed a second polar body (Fig. 1e).

We hypothesized that fusion by electroporation may have triggered premature activation and subsequent resumption of meiosis. To test this assumption, we used an alternative karyoplast fusion technique using an extract from Sendai virus (SeV). Isolated karyoplasts were briefly exposed to SeV extract and then placed into the perivitelline space of cytoplasts (Supplementary Movie) where fusion was observed within 20–30 min. Analysis of reconstructed oocytes demonstrated that spindle–chromosomal complexes were maintained in the MII stage and had morphology similar to intact controls (Fig. 1f). Resumption of meiosis and separation of the second polar body was observed in the SeV group only after fertilization. Thus, these results indicate that electrofusion pulse induces premature activation and resumption of meiosis during spindle introduction. In contrast, this artefact was prevented by using SeV-assisted fusion.

Developmental potential of reconstructed oocytes

We next determined the developmental competence of ST-generated oocytes produced by electrofusion or SeV, following fertilization by intracytoplasmic sperm injection (ICSI) and *in vitro* embryo culture. Karyoplast–cytoplast fusion rates were comparable between the two ST approaches. However, pronuclear formation in the electrofusion group was not observed; instead, sperm-injected oocytes cleaved prematurely by the morning after ICSI and arrested between the 8-cell and morula stages. In contrast, fertilization, cleavage and blastocyst rates in the SeV group were similar to those of intact control oocytes (Table 1). These results are consistent with the conclusion that spindle transfer in MII oocytes using SeV does not compromise subsequent fertilization and *in vitro* embryonic developmental competence. In order to assess the quality of the blastocysts produced, we carried out cell counts by labelling with DAPI (all cells) and NANOG (the inner cells mass, ICM) (Supplementary Fig. 2). Five expanded or hatched blastocysts for each ST and corresponding control group were analysed. The mean number of total cells and ICM cells in ST

Table 1 | *In vitro* development of embryos after ST into enucleated oocytes and ICSI

Treatment	n	Fusion (%)	Fertilization (%)	8-cell (%)*	Morula (%)*	Blastocyst (%)*
ST (SeV)	87	78 (90)	74 (95)	69 (93)	58 (78)†	45 (61)†
ST (elect.)	15	11 (73)	11 (100)	8 (73)	2 (18)‡	1 (9)‡
Control	72	NA	68 (94)	57 (84)	51 (75)†	41 (60)†

SeV and elect. indicate fusion with Sendai virus extract or electrofusion, respectively. Different footnote symbols (dagger and double dagger) indicate significant difference ($P < 0.05$). Data were analysed using χ^2 test. ICSI, intracytoplasmic sperm injection; NA; not applicable (no fusion step).
* The percentage of 8-cell-, morula- and blastocyst-stage embryos was calculated based on the number of fertilized embryos.

blastocysts were 152 ± 34 and 21 ± 10, respectively, and similar to controls (127 ± 79 and 23 ± 10, respectively; $P > 0.05$).

To define further the developmental potential, we isolated ES cell lines from ST blastocysts. Two stable ES cell lines were established from eight ST embryos (designated as STES-1 and STES-2) with derivation efficiency (25%) similar to controls (Supplementary Table 1). Both cell lines exhibited typical primate pluripotency markers and were able to differentiate into neuronal cell types and spontaneously contracting cardiomyocytes (Supplementary Fig. 3). To determine if the ST procedure induced lasting chromosomal abnormalities, we conducted a cytogenetic analysis of STES cell lines by G-banding. The analysis revealed that these cell lines contained normal rhesus macaque karyotypes (one male 42 XY and one female 42 XX) with no detectable chromosomal anomalies (Supplementary Fig. 4). Finally, we tested the *in vivo* developmental potential of ST embryos by transfer into the reproductive tract of recipient females. We transferred 15 ST embryos into oviducts of 9 recipients: 6 females received either 1 or 2 blastocysts each whereas three recipients received 2 cleavage stage (4–8-cell) embryos each (Table 2). Three females became pregnant—one carrying twins and two with singletons—all derived from the transfer of ST embryos at the blastocyst stage (Table 2). Remarkably, the pregnancy (33%) and implantation rates (27%) with ST embryos were even higher than those previously reported for non-manipulated, ICSI-produced embryos (Supplementary Table 2)[18]. On 24 April 2009 the first pregnant monkey delivered a set of healthy twins by caesarean section (called Mito and Tracker; Fig. 2). To our knowledge, these infants represent the first animals to be born after MII spindle–chromosomal complex transfer. The second pregnant monkey gave birth to a healthy infant on 8 May 2009 whereas the third is ongoing at time of publication. All three ST infants are healthy and their birth weights and gestational lengths were within normal ranges for rhesus macaques (Supplementary Table 3)[18]. Overall, these results demonstrate that MII spindle–chromosomal complexes can be efficiently isolated and transplanted into enucleated oocytes. Reconstructed oocytes produced with this technology were suitable for fertilization and developed to blastocysts at rates similar to controls. Moreover, the isolation of normal ES cells and the birth of healthy offspring support further evaluation of this approach for mitigating the consequences of mtDNA defects.

Figure 2 | Mito and Tracker, the first primates to be produced by spindle–chromosomal complex transfer (ST) into enucleated oocytes followed by fertilization and embryo transfer. Twin pregnancy was established by transfer of two ST-derived blastocysts into a recipient. Both infants are healthy and their growth and development is within a normal range for rhesus macaques. The photo was taken at 6 days of age.

Analysis of nuclear and mtDNA in ST offspring

Finally, we investigated the degree of heteroplasmy extant in infants and cell lines as a result of mtDNA carryover during spindle transfer. We isolated DNA samples from ST infants and STES cell lines to analyse genomic DNA by microsatellite parentage analysis and mtDNA by direct sequencing of the mitochondrial D-loop hypervariable region 1 (rhDHV1) as previously described[16]. We also collected and analysed DNA from four differentiated cultures derived from the outgrowth of plated ST blastocysts (designated as ST1, ST2, ST3 and ST4). Detailed analysis of nuclear DNA using 41 microsatellite markers confirmed that all three infants and all cell lines inherited their nuclear genome from the spindle donor animals (Fig. 3a and Supplementary Table 4). Spindle and cytoplast donor animals contained multiple single nucleotide polymorphisms (SNPs) within the rhDHV1 region (nucleotides positions 15974–16516 of the *Macaca mulatta* NCBI reference sequence NC_005943) that were informative and permitted to distinguish the mtDNA origin of infants and cell lines (Fig. 3a). Initially, we performed direct sequencing of rhDHV1 and the results confirmed that in all samples the mtDNA originated from the cytoplast donors (Fig. 3b). No contribution of spindle donor mtDNA was detected in any ST offspring using this approach. As indicated above, a small amount of cytoplasm is usually carried over with the spindle during the ST procedure. Therefore, we conducted three independent quantitative mtDNA assays. The first involved cloning of mtDNA PCR products containing rhDHV1 region and the direct sequencing of multiple individual clones. We randomly selected and sequenced 20–25 clones for each sample and the results indicated that mtDNA in all analysed clones was from cytoplast donors (Supplementary Table 5). Thus, if
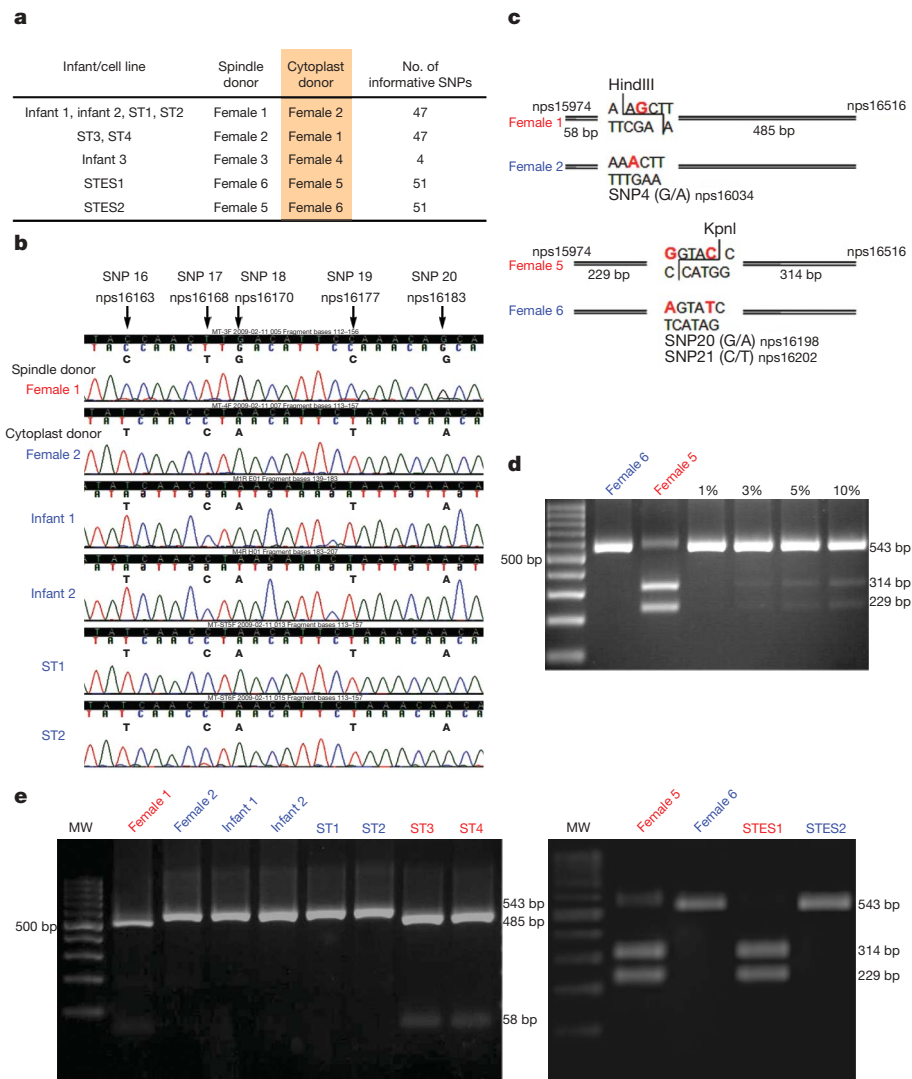
Table 2 | Embryo transfers and pregnancies with ST embryos

Spindle donor	Cytoplast donor	Embryo stage (age)*	Number of embryos transferred	Recipient stage†	Pregnancy
Female 1	Female 2	ExB (d7)	2	Day 4	Twin
Female 2	Female 1	ExB (d8)	2	Day 2	No
Female 4	Female 3	4-cell (d2)	2	Day 2	No
Female 3	Female 4	EB (d7)	1	Day 4	Single
Female 13	Female 14	8-cell (d3)	2	Day 2	No
Female 14	Female 13	8-cell (d3)	2	Day 2	No
Female 13	Female 14	ExB (d8)	2	Day 4	No
Female 15	Female 16	ExB (d8)	1	Day 4	No
Female 8	Female 7	ExB (d7)	1	Day 4	Single

EB, early blastocyst; ExB, expanded blastocyst.
* Day of ICSI is calculated as day 0 (d0).
† The next day after oestrogen surge is considered as ovulation day or day 0. Embryo transfers were scheduled to synchronize the stage of early embryo development with uterine development leading to the implantation interval.

Figure 3 | MtDNA analysis in ST offspring. a, Origin of ST offspring and informative mtDNA SNPs. **b,** MtDNA chromatogram demonstrating SNPs. Infants 1 and 2 and ST1 and ST2 cell lines were produced by transfer of spindles from female 1 to cytoplasts from female 2. **c,** Restriction enzyme recognition sites within SNP area. Female 1 mtDNA can be digested by HindIII whereas G to A nucleotide change precludes restriction of female 2 mtDNA. KpnI digests mtDNA from female 5 but not from female 6. **d,** MtDNA from females 5 and 6 were mixed at various proportions and were detectable at the level of 3%. **e,** RFLP analysis of ST offspring demonstrating undetectable contributions of spindle donor mtDNA. MW, molecular mass marker.

heteroplasmy existed, any contribution of spindle mtDNA in ST infants and cell lines is below detectable levels.

The second approach used mtDNA restriction fragment length polymorphism (RFLP)[19]. SNPs in the mitochondrial genome can result in the loss of a restriction site; thus digestion of PCR fragments with endonucleases can be used to reveal the presence of a population of mtDNA originating from the spindle donors. We identified unique restriction enzyme recognition sites within SNP areas for all ST samples except for the third infant. Specifically, mtDNA of female 1 (a spindle donor for infants 1 and 2 plus ST1 and ST2 cell lines) possessed a unique HindIII digestion site that includes the G/A SNP site (Fig. 3c). In contrast, female 2 (a cytoplast donor) carried mtDNA with an A allele at this SNP precluding recognition and digestion by HindIII (Fig. 3c). To confirm the sensitivity of this approach, we performed RFLP analysis of serial mixtures (1 to 100%) of mtDNA from females 5 and 6. The results indicate that RFLP can detect as little as 3% of heteroplasmy (Fig. 3d). MtDNA heteroplasmy was undetectable in any experimental sample by RFLP (Fig. 3e).

Finally, we used a quantitative real-time PCR analysis based on existing SNP differences between spindle and cytoplast donor animals.

Two different fluorescent probes were designed for identification of mtDNA from females 5 and 6 based on SNPs and specificity was validated (Supplementary Fig. 5a). To build a standard curve and establish sensitivity, we conducted real-time PCR analysis of mtDNA mixtures from the two females at various ratios. The technique detected heteroplasmy at the minimal level of 3% (Supplementary Fig. 5b). Next, we analysed STES1 and STES2 cell lines derived from these females and results confirmed that contribution of the spindle donor mtDNA is undetectable (Supplementary Fig. 5b). Similarly, we performed a real-time PCR analysis of mtDNA from infants 1 and 2 as well as ST1–ST4 cultures; the results indicated that all ST offspring are nearly homoplasmic.

These results provide strong support for the notion that mtDNA can be efficiently replaced in mature primate oocytes by ST, resulting in offspring with undetectable or negligible amounts of spindle-derived mtDNA.

Karyoplast fusion using SeV extract was instrumental in our study but clinical applications of the viral extract may be problematical. Therefore, we analysed SeV extract, ST infants and STES cells for the presence of the SeV genetic material. We used reverse transcription (RT)–PCR assay using commonly used primers for the F protein-coding sequence[20]. We

did not detect the presence of viral genome in analysed samples (Supplementary Fig. 6), indicating that the extract is inactivated and purified from the genomic RNA.

Discussion

Assisted reproductive technologies that could eliminate the transmission of mitochondrial diseases in affected families include cytoplasmic, germinal vesicle and pronuclear transfer[15,21,22]. Cytoplasmic transfer—the augmentation of patient eggs with a small amount of donor cytoplasm—was designed to improve viability and developmental competence of compromised, 'ooplasmic deficient' oocytes. The introduction of large amounts of donor cytoplasm by electrofusion was unsuccessful[23]; however, the co-injection of a small volume (1–5%) of donor cytoplasm with sperm as an extension of ICSI has been successful with acceptable IVF outcomes[23–26]. Although heteroplasmy was detected in small amounts in six of thirteen embryos studied and in two of four fetal cord blood samples[27], this procedure is not suitable for women carrying mtDNA mutations because it would require transferring significantly larger, practically impossible, cytoplasmic volumes (30–50% of the final volume) to ensure adequate dilution of mutant mtDNA. A relatively high number of chromosomal abnormalities and birth defects in infants resulting from the initial application of this technique[28] led to it being banned in the United States by the Food and Drug Administration because of safety concerns.

Another possibility is transferring nuclear DNA from a mother with mtDNA disease to a cytoplast (or spindle-free oocyte) containing normal mtDNA as obtained from a healthy egg donor. Experiments in mice suggested that it is technically feasible to transfer DNA between immature oocytes where nuclear DNA is enclosed in a clearly visible germinal vesicle[29]. However, if this approach were applied to human oocytes, efficacy would be limited by the poor developmental competence of oocytes produced after the *in vitro* maturation of germinal-vesicle-intact oocytes. Moreover, germinal vesicle oocytes have polarized cyto-architecture with mitochondria concentrated in the perinuclear space[30] and germinal vesicle transplantation would inevitably result in the introduction of significant amounts of patient mtDNA into the donor cytoplasm. Pronuclear transfer is essentially the same procedure, except that the nuclear material, both the male and female pronucleus, is removed after fertilization. This technique in the mouse has resulted in the birth of live offspring; however, reconstructed embryos contained karyoplast mtDNA (on average 19%) and progeny were all heteroplasmic[31–33].

Thus, these nuclear transfer approaches are associated with the transmission of a significant amount of mtDNA from the nuclear donor (patient), resulting in heteroplasmy and rendering them inappropriate for patients with mtDNA-associated disease.

Chromosome transfer in mature oocytes was thought to be unattainable because of the unique biological characteristics of MII-arrested oocytes until we introduced several procedural innovations that overcame these biological barriers[12]. Here we also provide evidence that the ST approach is efficient, safe and does not generate any detectable heteroplasmy. This could be due to the fact that spindle–chromosomal complexes were isolated into karyoplasts containing very small amounts of cytoplasm. Moreover, in primates, karyoplasts are mostly occupied by a spindle–chromosomal apparatus that is free of mitochondria. Another advantage is that ST does not require the *in vitro* oocyte maturation and can be performed with minimum procedural upgrades in a standard clinical IVF setting.

Assuming that our protocols are applicable to human oocytes, ST with cryopreserved oocytes would undoubtedly benefit future clinical applications. Current technology requires that the oocyte donor and recipient undergo synchronous ovarian stimulation protocols; however, the increasing availability of oocyte cryopreservation protocols will abrogate this requirement.

As we show here, the ST strategy will probably contribute the least amount of spindle-associated mtDNA compared to the other approaches discussed above. The sensitivity of our assays did not allow detection of mtDNA heteroplasmy levels below the 3% threshold. However, owing to random genetic drift and segregation of mtDNA, some tissues and organs of ST offspring may contain a higher degree of heteroplasmy. Another concern is the possibility of recurrence of mtDNA diseases in the next generation, in children of heteroplasmic females produced by ST, owing to the phenomenon known as 'genetic bottleneck'[34,35]. Future clinical applications of mtDNA replacement therapy will largely depend on the safety and efficiency of this technology. Thus, appropriate long-term preclinical studies addressing these important concerns in a clinically relevant non-human primate model are warranted before this technology can be applied to humans.

MtDNA mutations contribute to a diverse range of human diseases, encompassing both tissue-specific and multiple system disorders, with the onset of disease appearing throughout life. We demonstrate here that the transmission of mtDNA mutations from mother to child can be abrogated by the efficient transfer of mother's spindle-associated chromosomes into donor cytoplasts. In the rhesus macaque, spindle-associated chromosomal transfer did not show adverse effects on fertilization or on subsequent embryo/fetal development to term. Hence, we propose that this procedure may represent a new, reliable therapeutic approach to prevent the transmission of mtDNA mutations in affected families.

Note added in proof: The third pregnant monkey gave birth to a healthy infant on 6 July 2009 while this paper was under review.

METHODS SUMMARY

Mature MII oocytes were recovered from females undergoing controlled ovarian stimulations. Oocytes were transferred to a manipulation chamber containing medium with $5 \mu g \, ml^{-1}$ cytochalasin B and MII spindle–chromosomal complexes were aspirated and isolated using Oosight imaging system (http://www.cri-inc.com) as described before[16]. Isolated karyoplasts were briefly exposed to SeV extract (http://www.cosmobio.co.jp) and placed into perivitelline space on the side opposite to the 1st polar body (Supplementary Movie). During manipulations, the oocyte's zona pellucida was penetrated by laser-assisted zona drilling (http://www.hamiltonthorne.com). After fusion, reconstructed oocytes were fertilized by ICSI and cultured as described previously[16]. Detailed methods are described in Supplementary Information.

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Author Contributions S.M. and M.T. conceived the study, designed experiments and conducted ST micromanipulations. M.S. performed ICSI, mitochondrial staining and analysis in oocytes. H.S., J.W. and Y.L. conducted ES cell derivation, characterization and differentiation. M.T., H.M., L.C., H.S. and Y.L. performed DNA/RNA isolations and mtDNA analyses. C.R. and O.K. conducted ovarian stimulations, oocyte recovery, ICSI and embryo transfers. S.M. and M.T. analysed the data and wrote the paper.