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doi:10.1038/nature11647

Towards germline gene therapy of inherited mitochondrial diseases

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Mutations in mitochondrial DNA (mtDNA) are associated with severe human diseases and are maternally inherited through the egg's cytoplasm. Here we investigated the feasibility of mtDNA replacement in human oocytes by spindle transfer (ST; also called spindle–chromosomal complex transfer). Of 106 human oocytes donated for research, 65 were subjected to reciprocal ST and 33 served as controls. Fertilization rate in ST oocytes (73%) was similar to controls (75%); however, a significant portion of ST zygotes (52%) showed abnormal fertilization as determined by an irregular number of pronuclei. Among normally fertilized ST zygotes, blastocyst development (62%) and embryonic stem cell isolation (38%) rates were comparable to controls. All embryonic stem cell lines derived from ST zygotes had normal euploid karyotypes and contained exclusively donor mtDNA. The mtDNA can be efficiently replaced in human oocytes. Although some ST oocytes displayed abnormal fertilization, remaining embryos were capable of developing to blastocysts and producing embryonic stem cells similar to controls.

Mitochondrial DNA is localized in the cell's cytoplasm, whereas chromosomal genes are confined to the nucleus. Each cell may have thousands of mtDNA copies, which may all be mutated (homoplasmy) or exist as a mixture (heteroplasmy). The clinical manifestations of mtDNA diseases vary, but often affect organs and tissues with the highest energy requirements, including the brain, heart, muscle, pancreas and kidney[1]. The expression and severity of disease symptoms depends on the specific mutation and heteroplasmy levels[1].

An estimated prevalence of inherited mtDNA diseases is 1 in every 5,000–10,000 live births, suggesting that, in the United States alone, between 1,000 and 4,000 children are born every year with mtDNA diseases[2,3]. Based on other estimates, the frequency of pathogenic mtDNA mutations is even higher—1 in 200 children inherit mutations[4]. However, not all of these children develop the disease at birth, because mtDNA mutations are present at low heteroplasmy levels.

At present, there are no cures for mitochondrial disorders and available treatments only alleviate symptoms and delay disease progression. Therefore, several strategies for preventing transmission of mtDNA mutations from mothers to their children have been actively pursued.

One approach is to completely replace the mutated mtDNA of a patient's oocyte with the healthy mitochondrial genome from an oocyte donated by another woman using spindle transfer (ST)[5]. The technique isolates and transplants the chromosomes (nuclear genetic material) from a patient's unfertilized oocyte into the cytoplasm of another enucleated egg, containing healthy mtDNA as well as other organelles, RNA and proteins. A child born as a result of the ST procedure will be the genetic child of the patient but carry healthy mitochondrial genes from the donor. Our prior studies in a monkey model demonstrated not only the feasibility of the ST procedure but also that ST is highly effective and compatible with normal fertilization and birth of healthy offspring[6]. This strategy has been considered clinically to be a highly important future gene therapy to avoid transmission of serious mitochondrial diseases (http://www.hfea.gov.uk/6372.html).

Here we present a comprehensive study demonstrating the feasibility and outcomes of ST with human oocytes donated by healthy volunteers. To measure success, we fertilized reconstructed oocytes *in vitro* and assessed the normality of fertilization and embryo development to blastocysts. In addition, we derived embryonic stem cells (ESCs) and carried out detailed genetic analyses to assess efficacy of gene replacement and possible chromosomal abnormalities associated with ST. We also conducted additional studies in a rhesus macaque model to investigate the feasibility of using cryopreserved oocytes for ST and postnatal development of ST offspring.

Mitochondrial DNA replacement in human oocytes

Seven volunteers (aged 21–32 years) underwent ovarian stimulation and a total of 106 mature metaphase II (MII) oocytes were retrieved (range of 7–28, or a mean of 15 oocytes per donor cycle). Participants were synchronized in three separate experiments, so that at least two fresh oocyte cohorts were available on the same day for reciprocal ST. We selected a total of 65 MII oocytes for the ST procedure and 33 served as non-manipulated controls (Fig. 1a). We successfully transferred the spindle surrounded by a membrane and a small amount of cytoplasm (karyoplast) between 64 oocytes (98%; Fig. 1a, b). Sixty oocytes survived fertilization by intracytoplasmic sperm injection (ICSI; 94%) and 44 formed visible pronuclei (73%). These outcomes were similar to the results for controls; 32 oocytes survived ICSI (97%) and 24 (75%) formed pronuclei (Fig. 1b). Microscopic evaluations determined that almost half of ST zygotes (21/44, 48%) contained normal two pronuclei and two polar bodies (2PN/2PB) (Fig. 2a). However, the remaining ST zygotes had an irregular number of pronuclei and/or polar bodies (Fig. 2a). Abnormal fertilization was also observed in the intact control group, albeit at a lower incidence (3/24, 13%).

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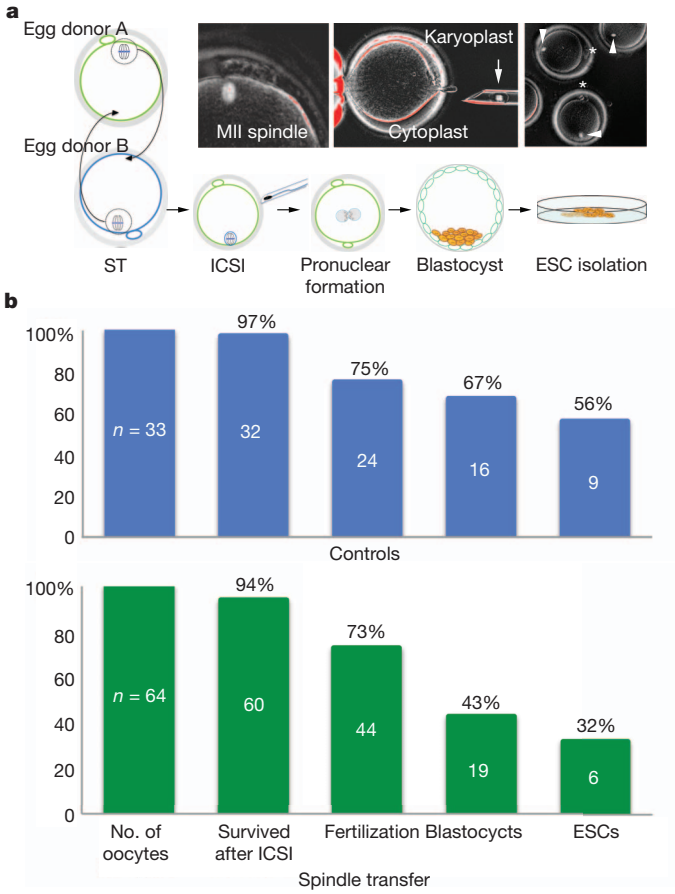

Figure 1 | Experimental design and main outcomes after ST with human oocytes. **a**, Oocytes were retrieved from two unrelated donors and spindle–chromosomal complexes were reciprocally exchanged. Reconstructed oocytes were fertilized by ICSI and monitored for *in vitro* development to blastocysts and ESCs. Images in boxes depict a human mature MII oocyte with the spindle visualized under polarized microscope (left), isolated cytoplast and karyoplast (middle) and intact spindles inside recipient cytoplasts after transfers (right, arrowheads). Original magnifications: left and middle, ×200; right, ×100. Asterisks indicate first polar bodies. **b**, Experimental outcomes after ST in human oocytes. The top and bottom graphs represent fertilization, blastocyst and ESC isolation rates for intact control and ST embryos, respectively. No statistical differences were found between ST and controls in survival after ICSI, fertilization, blastocyst development and ESC derivation rates ($P > 0.05$).

Blastocyst formation rate in the normally fertilized ST group (13/21, 62%) was statistically similar to controls (16/21, 76%). However, the majority of abnormal ST zygotes arrested, with only 26% (6/23) reaching blastocysts (Supplementary Table 1). Interestingly, blastocyst development of 3PN/1PB zygotes was noticeably higher (4/11, 36%) than that of other abnormally fertilized ST groups (Supplementary Table 1).

Derivation and genetic analysis of human ESCs

To provide additional insights into the developmental competence of ST-produced human embryos and to obtain sufficient material for molecular and cytogenetic analyses, we derived ESCs from blastocysts. Nine ESC lines (HESO lines) were established from 16 control blastocysts (56%; Fig. 1b). This ESC derivation rate is significantly higher than currently reported for embryos donated by IVF patients[7]. Similarly, 13 ST blastocysts developed from normally fertilized zygotes produced five ESC lines (38%; HESO-ST lines). We also plated four ST blastocysts that originated from 3PN/1PB zygotes and derived one ESC line (25%; Supplementary Table 1).

The ESCs derived from the ST embryos had normal morphology and were indistinguishable from controls (Supplementary Fig. 1a, b).

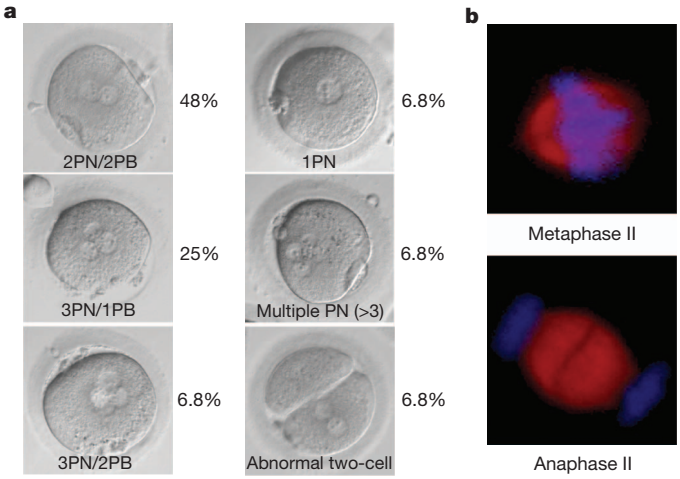

Figure 2 | Abnormal pronuclear formation and spindle morphology in human ST zygotes. **a**, Proportion of normally fertilized zygotes with two pronuclei and two polar bodies (2PN/2PB) compared with abnormal zygotes (3PN/1PB, 3PN/2PB, 1PN, multiple PN and two-cell) after ST. **b**, Integrity of meiotic spindles in human ST oocytes depicting normal metaphase II (top), and premature progression to the anaphase II (bottom).

All ESCs expressed standard pluripotency markers, including OCT-4, SOX2, SSEA-4, TRA-1-81 and TRA-1-60 (Supplementary Fig. 1a, b). Following injection into immunodeficient mice, experimental ESCs formed teratoma tumours consisting of cells and tissues representing all three germ layers (Supplementary Fig. 1c). Detailed analysis of nuclear DNA using microsatellite markers confirmed that all HESO-ST lines inherited their chromosomes from the spindle donor oocytes (Fig. 3a, Table 1 and Supplementary Fig. 2a, d). Analysis of mtDNA confirmed that all HESO-ST cell lines derived their mtDNA from the cytoplast donors (Fig. 3b, Table 1 and Supplementary Figs 2b, e and 3)[6,8].

As reported previously[6], small amounts of mtDNA are usually co-transferred with the karyoplast during the ST, causing a low carryover heteroplasmy. In clinical situations this may result in the transmission of mutant mtDNA to ST embryos and children. Therefore we conducted both qualitative and quantitative mtDNA assays to determine the degree of mtDNA carryover in ST embryos and ESC lines. We identified mtDNA sequence differences between oocyte donors and unique restriction enzyme recognition sites for restriction-fragment length polymorphism (RFLP) assay[6]. For example, mtDNA of the egg donor 1 (spindle contributor for HESO-ST2, -ST3 and -ST4) possessed a unique *EcoRV* digestion sequence GATATC (Supplementary Fig. 4a). In contrast, egg donor 2 (mtDNA contributor) carried a single nucleotide polymorphism GATACC precluding enzyme recognition. The results confirmed that mtDNA in ST cell lines was exclusively derived from the cytoplast donors with no detectable mtDNA carryover (Table 1 and Supplementary Figs 2c and 4a). We also used more sensitive ARMS–qPCR (amplification refractory mutation system–quantitative polymerase chain reaction) that enables the measurement of heteroplasmy below 1%[9,10]. The mean mtDNA carryover in ST oocytes and embryos was 0.5% (s.d. ± 0.4; range 0–0.9%) and in HESO-ST cell lines was 0.6% (s.d. ± 0.9; range 0–1.7%) (Supplementary Fig. 4b and Supplementary Table 2). These results are consistent with our previous data from a nonhuman primate[6] and suggest negligible mtDNA carryover in ST offspring.

Cytogenetic analyses, using G-banding, indicated that all five HESO-ST lines derived from normal ST embryos contained diploid male or female karyotypes, with no evidence of detectable numerical or structural chromosomal abnormalities (Table 1 and Supplementary Fig. 5). However, two out of nine lines derived from control embryos had numerical aberrations. Notably, HESO-6 carried a 47 XYY karyotype, whereas HESO-9 was 45 XO (Supplementary Table 3 and

a Nuclear DNA genotyping

	AME	D7S513	D6S1691
Sperm donor	XY	191/193	227/229
Egg donor 1	XX	191/199	211/223
Egg donor 2	XX	187/201	225/225
HESO-ST2	XX	193/199	223/229
HESO-ST3	XX	191/193	211/227
HESO-ST4	XY	193/199	211/229

b MtDNA genotyping

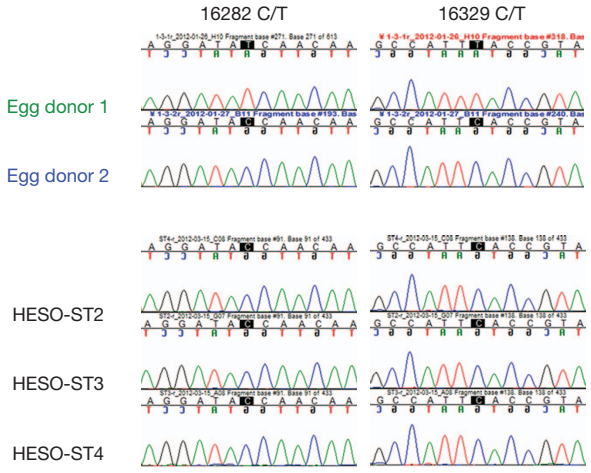

Figure 3 | Genetic analysis of ESCs derived from human ST embryos. **a**, Nuclear DNA origin of HESO-ST2, -ST3 and -ST4 determined by microsatellite parentage analysis. The microsatellite markers for D7S513 and D6S1691 loci demonstrate that the nuclear DNA in these ESC lines was from the egg donor 1 (the spindle donor). **b**, mtDNA genotyping by direct sequencing show that the mtDNA in HESO-ST2, -ST3 and -ST4 is originated from the egg donor 2.

Supplementary Fig. 6). G-banding revealed that HESO-ST6, the cell line derived from abnormally fertilized ST zygote, contained abnormal triploid female chromosome complement (Supplementary Fig. 5). In addition, detailed microsatellite analysis of nuclear DNA in this cell line confirmed the presence of three alleles for most short tandem repeat (STR) loci (Supplementary Table 4). On the basis of allele inheritance, we concluded that the triploid karyotype was caused by retention of the genetic material of the second polar body. This was consistent with observation of the extra pronucleus but lack of the second polar body in the zygote.

Abnormal fertilization in human zygotes produced by ST

Abnormal fertilization observed in some human ST oocytes was unexpected because this was not observed in monkey studies[6]. Therefore, additional experiments were conducted to investigate possible underlying mechanisms. Genetic analysis of the HESO-ST6 cell line hinted that some ST oocytes and embryos retain extra chromosomes that are normally extruded into the second polar body. This was likely to be caused by sub-optimal conditions during the ST procedure that disturbed spindle integrity. Initially, we focused on the effect of cytochalasin B (CB), a microfilament inhibitor known to inhibit cytokinesis and to block the second PB extrusion in oocytes[11,12]. CB is used acutely during the ST procedure and oocytes are thoroughly rinsed before fertilization, but residual CB could interrupt the second PB extrusion. Therefore, we extended incubation time between ST and ICSI, or decreased CB concentration. However, abnormal fertilization persisted even in the absence of CB (Supplementary Tables 5 and 6), indicating that abnormal meiotic segregation is not likely to be caused by CB exposure.

We next addressed whether oocyte polarity during displacement of spindles leads to abnormal meiosis. Typically, spindles in MII oocytes are adjacent to the first polar bodies. However, during the ST procedure, karyoplasts are reintroduced on the opposite side (referred to as 180 degree)[5,6]. We reintroduced spindles next to or at the 90 degree from the first PB (Supplementary Fig. 7). However, ST zygotes had similar pronuclear abnormalities (Supplementary Fig. 7).

Lastly, we reasoned that human meiotic spindles may undergo premature activation during the ST manipulations leading to incomplete resumption of meiosis after fertilization. Spindle morphology and meiotic stage was analysed in intact and ST human oocytes following immunolabelling with α- and β-tubulin. Analysis demonstrated that in some ST oocytes spindles already progressed to the late anaphase II, whereas all control oocytes maintained uniform metaphase II (Fig. 2b).

Oocyte cryopreservation before ST

Current ST protocols use fresh oocytes and require that both patient and healthy mtDNA egg donors undergo synchronous retrievals. However, it is difficult to manage the same-day egg retrievals owing to differences in the ovarian cycle and responses to gonadotropins. In addition, an equal number of patient and donor eggs retrieved would be ideal to avoid oocyte wastage. Therefore, oocyte freezing, storage and thawing will be critical for clinical applications of the ST. Recent advances in oocyte vitrification procedures suggest that cryopreserved human MII oocytes can be used in clinical IVF practice with the same efficiency as fresh eggs[13,14]. To evaluate the feasibility of using cryopreserved oocytes for ST, we turned to the nonhuman primate model. We tested a commercially available vitrification kit (CRYOTOP) and determined that survival and recovery of rhesus macaque MII oocytes post-thaw is high. After ISCI, 72% formed pronuclei, but only 6% developed to blastocysts ($P < 0.05$) (Table 2). Thus, this cryopreservation method compromises blastocyst development, because blastocyst formation of fresh oocytes from the same cohort was 52% (Table 2)[6,9].

We next conducted reciprocal ST between fresh and frozen-thawed monkey oocytes and examined fertilization and embryo development (Supplementary Fig. 8). When fresh spindles were transplanted into vitrified cytoplasts, fertilization after ICSI was impaired (50%)

Table 1 | Genetic analysis of human ESCs derived from ST blastocysts

Cell line	HESO-ST-2	HESO-ST-3	HESO-ST-4	HESO-ST-5	HESO-ST-6	HESO-ST-7
Nuclear donor	Donor 1	Donor 1	Donor 1	Donor 4	Donor 3	Donor 6
Cytoplast donor	Donor 2	Donor 2	Donor 2	Donor 3	Donor 4	Donor 7
Fertilization	2PN/2PB	2PN/2PB	2PN/2PB	2PN/2PB	3PN/1PB	2PN/2PB
Karyotype (passage no.)	46 XX, P4	46 XX, P7	46 XY, P7	46 XX, P4	69 XXX, P4	46 XY, P3
Nuclear DNA origin (by STR)	Donor 1	Donor 1	Donor 1	Donor 4	Donor 3	Donor 6
mtDNA origin	Donor 2	Donor 2	Donor 2	Donor 3	Donor 4	Donor 7
mtDNA carryover (RFLP)	Undetectable	Undetectable	Undetectable	Undetectable	Undetectable	NT
mtDNA carryover (ARMS–qPCR)	0.20%	0.01%	1.70%	NT	NT	NT

NT, not tested.

Table 2 | Fertilization and embryo development of frozen rhesus oocytes

Experiment	Group	*n*	Survived after ST (%)	Survived after ICSI (%)	Fertilized (%)	Blastocysts (%)
1	Fresh oocytes	32	NA	30 (94)	29 (97)	15 (52)*
	Vitrified oocytes	26	NA	25 (96)	18 (72)	1 (6)
2	Control fresh oocytes	34	NA	33 (97)	30 (91)†	17 (57)‡
	Fresh cytoplasts Vitrified spindles	36	34 (94)	32 (94)	28 (88)†	19 (68)‡
	Vitrified cytoplasts Fresh spindles	35	35 (100)	34 (97)	17 (50)	0

* Blastocyst rate statistically different from that for vitrified oocytes ($P < 0.05$).
† Fertilized rate statistically different from that for vitrified cytoplasts with fresh spindles ($P < 0.05$).
‡ Blastocyst rate statistically different from that for vitrified cytoplasts with fresh spindles ($P < 0.05$).
Data were analysed using analysis of variance (ANOVA).
NA, not applicable.

compared to controls (91%) (Table 2). Moreover, all embryos in this ST group arrested before reaching blastocysts, whereas 57% controls progressed to blastocysts. These ST results were similar to those seen with frozen-thawed intact controls (Table 2). However, when spindles from vitrified oocytes were transferred into fresh cytoplasts, fertilization (88%) and blastocyst formation (68%) rates were similar to fresh controls (Table 2). These results indicate that vitrification causes damage primarily within the cytoplasm rather than to the spindle apparatus.

To further evaluate developmental potential, we plated six ST blastocysts derived from vitrified spindles onto feeder cells and established two ESC lines (33%). We transplanted four ST blastocysts from vitrified spindles into a recipient that resulted in the timely birth of a healthy infant (Supplementary Fig. 8).

Postnatal development of monkey ST offspring

Although the technical feasibility of mtDNA replacement is documented for human embryos and ESCs, questions remain regarding whether a 'mismatch' between mtDNA and nuclear DNA haplotypes may cause mitochondrial dysfunctions in ST children[15]. To address these concerns, we conducted a 3-year follow-up study on monkey ST offspring born in 2009 (ref. 6).

The growth and development of four healthy infants following ST procedure was evaluated during the postnatal period (Supplementary Fig. 9a). Their overall health, including routine blood and body-weight measurements monitored from birth to 3 years were comparable to age-matched controls. The values for haemoglobin, red blood cell and white blood cell counts, mean corpuscular volume and haemoglobin concentrations were all within normal ranges (Supplementary Table 7). We also measured blood chemistry and arterial blood gas parameters and demonstrated that metabolic status of ST offspring is comparable to controls (Supplementary Table 7). In addition, the body-weight gain for the ST juvenile monkeys was similar to that of age-matched controls (Supplementary Fig. 9b). We also confirmed that ATP levels and mitochondrial membrane potential ($\Delta\Psi$m) in skin fibroblasts were similar to those of controls (Supplementary Fig. 10). Finally, there were no significant changes in mtDNA carryover and heteroplasmy in blood and skin samples with age (Supplementary Fig. 9c).

Discussion

This report summarizes our effort to test an mtDNA replacement in unfertilized human oocytes, initially developed and optimized in a monkey model. The results demonstrate that the ST procedure can be performed with high efficiency in human oocytes. Manipulated oocytes also supported high fertilization rates similar to those of controls. However, approximately half of the human ST zygotes had abnormal fertilization, primarily as a result of excessive pronuclear numbers. This was an unexpected outcome that was not observed with monkey oocytes. Our follow-up studies indicated that this is caused by the failure to complete meiosis and segregate chromosomes into the second PB, probably owing to premature activation. A set of haploid genetic material is normally discarded during asymmetrical cell division into the second PB, while the other half forms the female pronucleus. By genetic analysis of ESCs derived from abnormally fertilized zygote, we confirmed the triploid nature and presence of two sets of female chromosomes.

The spindle-chromosomal apparatus in MII oocytes is an extremely sensitive structure that can easily be perturbed by physical or chemical manipulations. Our initial attempts to isolate and transplant monkey MII spindles were unsuccessful owing to similar problems with spontaneous resumption of meiosis[6]. Procedures were optimized to avoid this negative outcome and current ST protocols allow maintenance of an intact MII spindle and normal fertilization. It seems that human MII oocytes are more sensitive to spindle manipulations and further improvements and optimizations will be required for future clinical applications. Maintenance of meiotic spindles in MII oocytes is dependent on the activity of M-phase-specific kinases including maturation-promoting factor (MPF) and mitogen-activated protein kinase[16]. Under normal conditions, sperm entry triggers degradation of kinase activities and chromosome segregation mediated by oscillations of intracellular Ca^{2+} concentrations[17]. However, an influx of calcium induced by mechanical or chemical manipulations can induce parthenogenetic activation of oocytes and resumption of meiosis[18]. Thus, ST manipulations in a medium without Ca^{2+} or supplementations with MG132 could potentially avoid problems with spontaneous activation[19,20].

Morphological evaluation of fertilization and early detection of abnormal pronuclear and/or polar body formation seems to be critical to separate normal and abnormal ST embryos. Blastocyst development and ESC isolation in normally fertilized ST zygotes were similar to controls. We also confirmed that all ESC lines derived from these ST embryos are karyotypically normal.

Two of the nine ESC lines (22%) derived from non-manipulated oocytes also showed chromosomal abnormalities. Because aberrations were confined to the sex chromosomes (47 XYY and 45 XO), it is possible that this was induced by sperm carrying either two Y chromosomes or no Y chromosome.

Despite the risk of abnormal pronuclear formation and aneuploidy in a portion of ST zygotes, embryo development and ESC isolation rates in normal ST zygotes are comparable to intact controls. Based on our estimates of retrieving on average 12 MII oocytes, 35% normal (2PN/2PB) fertilization rates, and 60% blastocyst development, at least two ST blastocysts suitable for transfers can be generated during a single cycle for each patient.

The safety of the ST procedure is also dependent on the amount of mutated mtDNA co-transferred with spindles. Importantly, mtDNA carryover in ST embryos and ESC lines is technically undetectable or below 1%. In most patients with mtDNA diseases, a threshold of 60% or higher of mutated mtDNA must be reached for clinical features to appear. Thus, it is unlikely that low mtDNA carryover during ST would cause disease in children. Segregation of mutated mtDNA to specific tissues during development and ageing may hypothetically result in a significant accumulation of the mutant load. However, analysis of mtDNA carryover in monkey ST offspring discovered

no detectable mtDNA segregation into different tissues[9]. In addition, there were no changes in heteroplasmy levels during postnatal development of monkeys. Thus, carryover, segregation and tissue-specific accumulation of mutant mtDNA molecules in ST children seem unlikely to be major concerns.

Birth of a healthy monkey infant after oocyte freezing marks an important milestone in applying the ST technology to patients. Transplantation of vitrified spindles into fresh cytoplasms yields the best results, comparable to controls. However, fertilization of vitrified cytoplasts even with fresh spindles was compromised. These remarkable findings indicate that the damage after cryopreservation is confined mainly to the eggs' cytoplasm, not to the chromosomes and spindles as commonly believed[21]. Our observations also reveal another unexpected potential clinical application of the ST technique, suggesting that spindles in sub-optimally cryopreserved oocytes can be rescued by transplanting into fresh cytoplasts.

Follow-up postnatal studies in four monkeys produced by ST provide convincing evidence that oocyte manipulation and mtDNA replacement procedures are compatible with normal development. These monkeys were derived by combining nuclear and mtDNA from the two genetically distant subpopulations of rhesus macaques. Mitochondrial and nuclear genetic differences between these monkeys are considered to be as distant as those between some different primate species[22], thus imitating haplotype differences between humans. Concerns have been raised that nuclear and mtDNA incompatibilities between mtDNA patients and cytoplast donors may cause a 'mismatch' and mitochondrial dysfunctions in ST children even in the absence of mutations[15]. On the basis of our long-term observations, it is reasonable to speculate that nuclear–mtDNA interactions are conserved within species.

Pioneering work in nonhuman primates is critical for the development, and safety and efficacy evaluations, of new treatments[23,24]. It is important that scientists and clinicians further optimize ST protocols for human oocytes and ensure that these procedures are safe. It is also crucial that the US Food and Drug Administration initiates careful review of these new developments. Such oversight will be important to establish safety and efficacy requirements and guide clinical trials. Current US National Institutes of Health funding restrictions surrounding these innovative reproductive technologies will also require amendments to support federally funded clinical trials.

METHODS SUMMARY

The study protocols were approved by both the Oregon Health & Science University Embryonic Stem Cell Research Oversight Committee and the Institutional Review Board.

Mature oocytes were donated by volunteers and ST procedures were carried out as described[5,6]. Oocytes were fertilized, cultured to blastocysts and used for ESC isolation. Detailed methods are described in Supplementary Information at www.nature.com/nature.

Received 9 June; accepted 3 October 2012.
Published online 24 October 2012.

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Supplementary Information is available in the online version of the paper.

Acknowledgements The authors would like to acknowledge the Oregon Health & Science University (OHSU) Embryonic Stem Cell Research Oversight Committee and the Institutional Review Board for providing oversight and guidance. We thank oocyte and sperm donors and staff at the Women's Health Research Unit at the Center for Women's Health, University Fertility Consultants and Reproductive Endocrinology & Infertility Division at the Department of Obstetrics & Gynecology of Oregon Health & Science University for their support and procurement of human gametes. The Division of Animal Resources, Surgery Team, Assisted Reproductive Technology & Embryonic Stem Cell Core, Endocrine Technology Core, Imaging & Morphology Core, Flow Cytometry Core and Molecular & Cellular Biology Core at the Oregon National Primate Research Center provided expertise and services for the nonhuman primate research. Hamilton Thorne Inc., donated an XYClone laser system for this study. We are grateful to W. Sanger and D. Zaleski for karyotyping services, C. Penedo for microsatellite analysis and J. Hennebold for consulting on metabolic assays. We are also indebted to A. Steele, R. Cervera Juanes and E. Wolff for their technical support. The human oocyte/embryo research was supported by grants from the OHSU Center for Women's Health Circle of Giving and other OHSU institutional funds, as well as the Leducq Foundation. The nonhuman primate study was supported by grants from the National Institutes of Health HD063276, HD057121, HD059946, EY021214 and 8P51OD011092.

Author Contributions M.T., P.A., J.J. and S.M. conceived the study, designed experiments and wrote institutional review board protocols. P.A., M.S. and N.M.G. coordinated recruitment of participants. P.A., K.M., D.B., D.L., D.W. and P.P. performed ovarian stimulation and oocyte recovery. M.T. conducted ST micromanipulations. M.S., K.M. and S.M. performed ICSI. M.T., M.S., J.W., D.M.S., N.M.G., R.T.-H. and E.K. conducted ESC derivation and characterization. S.G. analysed teratoma tumours. M.T., H.M. and D.M.S. performed DNA/RNA isolations, metabolic and mtDNA analyses. C.R., M.T., M.S.,H.-S.L., R.S. and S.M. conducted monkey studies. M.T., R.S., J.J., P.P. and S.M. analysed data and wrote the paper.

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