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LETTERS

Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease

Lyndsey Craven[1], Helen A. Tuppen[1], Gareth D. Greggains[3,4], Stephen J. Harbottle[3], Julie L. Murphy[1], Lynsey M. Cree[1], Alison P. Murdoch[3,5], Patrick F. Chinnery[1], Robert W. Taylor[1], Robert N. Lightowlers[1], Mary Herbert[3,4,5] & Douglass M. Turnbull[1,2,5]

Mutations in mitochondrial DNA (mtDNA) are a common cause of genetic disease. Pathogenic mutations in mtDNA are detected in approximately 1 in 250 live births[1–3] and at least 1 in 10,000 adults in the UK are affected by mtDNA disease[4]. Treatment options for patients with mtDNA disease are extremely limited and are predominantly supportive in nature. Mitochondrial DNA is transmitted maternally and it has been proposed that nuclear transfer techniques may be an approach for the prevention of transmission of human mtDNA disease[5,6]. Here we show that transfer of pronuclei between abnormally fertilized human zygotes results in minimal carry-over of donor zygote mtDNA and is compatible with onward development to the blastocyst stage *in vitro*. By optimizing the procedure we found the average level of carry-over after transfer of two pronuclei is less than 2.0%, with many of the embryos containing no detectable donor mtDNA. We believe that pronuclear transfer between zygotes, as well as the recently described metaphase II spindle transfer, has the potential to prevent the transmission of mtDNA disease in humans.

Mitochondrial DNA is present in all cells in multiple copies. Mutations in it are maternally transmitted[7]. In patients with mtDNA disease, either all mtDNA copies are mutated (homoplasmy) or there is a mixture of wild-type and mutated mtDNA (heteroplasmy)[8]. Studies of human pedigrees with transmitted mtDNA mutations have shown that clinical disease is only seen in those patients with high loads of mutated mtDNA in affected tissues (usually greater than 60% mutated mtDNA)[9,10]. There has been very limited success in developing effective treatments for mtDNA disease. Genetic counselling combined with prenatal or pre-implantation genetic diagnosis is increasingly being offered to women who carry pathogenic mtDNA mutations[11]. However, these techniques will only be of value to women who have low levels of mtDNA mutations in oocytes.

We used abnormally fertilized (unipronuclear or tripronuclear) human zygotes (one-cell embryos) generated from a human *in vitro* fertilization programme to study the feasibility of pronuclear transfer to prevent mtDNA disease transmission from mother to child. Unipronuclear and tripronuclear zygotes are not normally used in fertility treatment. Our studies involved the transfer of one or two pronuclei between abnormally fertilized zygotes (Fig. 1 and Supplementary Fig. 1). After treatment with cytoskeletal inhibitors (nocodazole and cytochalasin B), pronuclei were removed from a donor zygote within a karyoplast containing a small volume of cytoplasm. Karyoplasts were placed under the zona pellucida of a recipient zygote and were fused using inactivated viral envelope proteins of the haemagglutinating virus of Japan (HVJ-E). Reconstituted zygotes were cultured for 6–8 days to monitor development *in vitro*.

We first confirmed that pronuclear transfer between human zygotes was associated with a change in the nuclear genotype of the embryo by analysing microsatellite markers. In all embryos studied, informative markers confirmed that the reconstituted pronuclear-transfer embryo contained nuclear genotype of the donor embryo (Supplementary Table 1). We then determined if pronuclear transfer

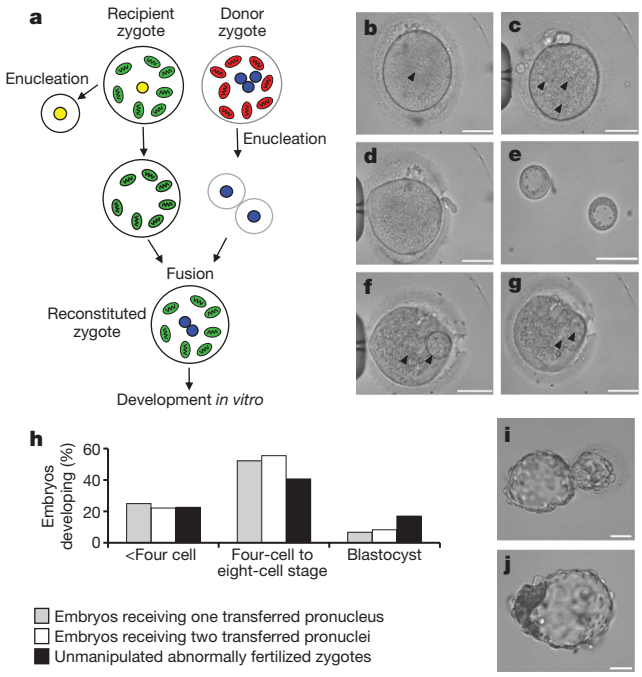


Figure 1 | Pronuclear transfer using abnormally fertilized human zygotes. **a–g**, Transfer of two pronuclei between human zygotes. **a**, Recipient zygote (one pronucleus removed) and donor zygote (three pronuclei, two of which are removed and fused with the recipient zygote). **b**, Recipient zygote containing a single pronucleus (marked with arrow), which is removed by a biopsy pipette to leave an enucleated zygote (**d**). **c**, Donor zygote with three pronuclei (marked with arrows), two of which are removed as karyoplasts (**e**). **f**, Enucleated recipient zygote with two pronuclear karyoplasts from the donor zygote (arrows) before fusion. **g**, Recipient zygote 20 min after transfer, already showing fusion of the karyoplast membranes (arrow). **h**, Development of unmanipulated abnormally fertilized zygotes ($n = 76$; black bars), embryos receiving one transferred pronucleus ($n = 44$; grey bars) and embryos receiving two transferred pronuclei ($n = 36$; white bars). **i, j**, Hatching blastocyst on day 6 (**i**) and hatched blastocyst on day 7 (**j**) containing two donor pronuclei. Scale bars, 50 μm.

[1]Mitochondrial Research Group, Institute for Ageing and Health, [2]Newcastle University Centre for Brain Ageing and Vitality, Institute for Ageing and Health, Newcastle University, Newcastle upon Tyne NE2 4HH, UK. [3]Newcastle Fertility Centre, International Centre for Life, [4]Institute for Ageing and Health, International Centre for Life, [5]North East England Stem Cell Institute (NESCI), Bioscience Centre, International Centre for Life, Newcastle University, Newcastle upon Tyne NE1 4EP, UK.

was compatible with onward development *in vitro*. This was complicated by the fact that abnormally fertilized zygotes have limited potential for development to the blastocyst stage *in vitro* (17%) compared with normally fertilized embryos (32%). Nonetheless, after pronuclear transfer, zygotes showed onward development with 10 out of 44 (22.7%) zygotes receiving one transferred pronucleus and 8 out of 36 (22.2%) zygotes receiving two transferred pronuclei developing to more than the eight-cell stage. We found no difference in embryo development at any stage, whether we transferred one or two pronuclei. After transfer of two pronuclei, 8.3% of abnormally fertilized embryos developed to the blastocyst stage (Fig. 1h–j). This is approximately 50% of the blastocyst rate for unmanipulated abnormally fertilized embryos; as there is no reliable morphological indicator to distinguish between the male and female pronucleus in the human zygote, it is likely that the decline in blastocyst formation is partly due to absence of either a maternal or paternal genome.

Having established that pronuclear transfer is compatible with onward development of human embryos, we next determined the carry-over of donor mtDNA genotype in the reconstituted pronuclear-transfer embryos (Fig. 2). We sequenced the non-coding mtDNA control region from both the pronuclear donor and pronuclear recipient embryos (Fig. 2b) and identified polymorphic mtDNA variants that were unique to donor or recipient embryo, thereby allowing the determination of mtDNA carry-over in the pronuclear-transfer embryo. Last hot cycle PCR restriction-fragment length polymorphism (RFLP) assays were developed specifically for these mtDNA variants (Fig. 2c) and used to analyse mtDNA extracted from whole embryos. We found that there was variation in the amount of mtDNA genotype from the donor zygote transferred to the embryos receiving two transferred pronuclei (8.1% ± 7.6 (mean ± s.d.), $n = 8$) (Fig. 2d).

There are many factors that could affect the carry-over of mtDNA after pronuclear transfer. We therefore studied the mtDNA copy number present in human oocytes. Similar to the results in mice and previous studies of human oocytes at various stages of development[12–14], we found marked variation in the mtDNA copy number (Fig. 2e), which may contribute to variation in the level of mtDNA carry-over.

Previous studies have investigated levels of heteroplasmy in blastomeres obtained from donated heteroplasmic embryos and have reported variations of 0–19% between individual blastomeres from the same embryo[11,15]. We therefore determined whether the proportion of donor mtDNA genotype also varied between blastomeres in the reconstituted embryos after transfer of two pronuclei (Fig. 3a, b). In one out of eight embryos there was no detectable donor mtDNA in any blastomere. In the other seven embryos that contained donor zygote mtDNA, there was variation in the level of donor mtDNA genotype between blastomeres (Fig. 3b). Although this variation is similar to previous reports on heteroplasmic human embryos[11,15], we wished to minimize the carry-over of donor zygote mtDNA and therefore explored techniques to reduce the amount of cytoplasm contained within the pronuclear karyoplast. We focused on careful manipulation

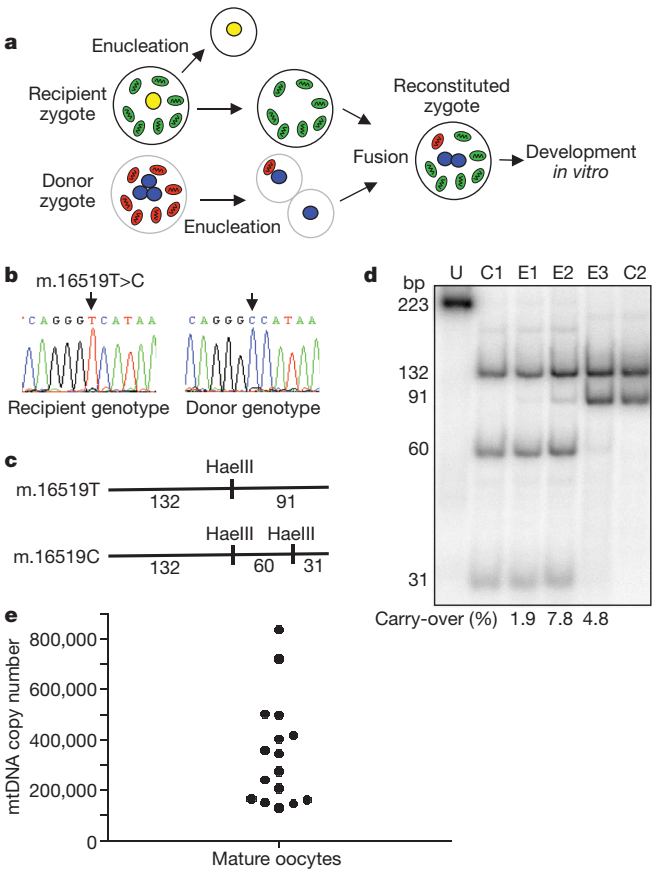
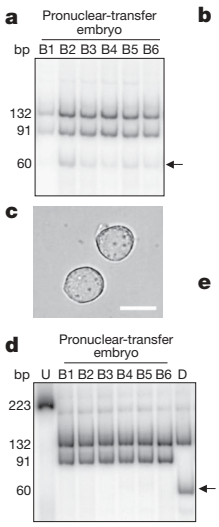


Figure 2 | Mitochondrial DNA analysis of pronuclear-transfer embryos. **a**, The potential transfer of donor zygote mtDNA to the recipient zygote. **b**, Sequence electropherograms of mtDNA non-coding control region in donor and recipient zygotes with the sequence variant used for the last hot cycle PCR RFLP assay highlighted. **c**, Scheme of RFLP designed using the sequence variant. **d**, Last hot cycle PCR RFLP analysis of donor mtDNA carry-over detected in embryos receiving two transferred pronuclei with products separated by 12% non-denaturing polyacrylamide gel electrophoresis. U, undigested; C1 and C2, controls (C1, donor embryo for E3, recipient embryo for E1 and E2; C2, donor embryo for E1 and E2, recipient embryo for E3). bp, base pairs. **e**, Mitochondrial DNA copy number in human mature oocytes.



b

Development stage	Blastomere number							
	1	2	3	4	5	6	7	8
Eight-cell	5.7	2.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eight-cell	30.2	11.1	10.8	10.7	10.1	5.1		
Eight-cell	15.6	5.8	n.d.	n.d.				
Six-cell	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
Six-cell	1.1	0.3						
Five-cell	10.5	n.d.	n.d.	n.d.				
Four-cell	39	18.9	14	8				
Four-cell	20	15.7	2.3					

e

Development stage	Blastomere number								
	1	2	3	4	5	6	7	8	9
Nine-cell	11.4	6.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eight-cell	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Seven-cell	8	2.5	1.9	n.d.	n.d.	n.d.	n.d.		
Six-cell	4.4	4.5	3.9						
Five-cell	11	3.3	3.3	2.4	1.4				
Five-cell	n.d.	n.d.	n.d.	n.d.	n.d.				
Three-cell	n.d.	n.d.	n.d.						
Three-cell	n.d.								
Two-cell	5.8								

Figure 3 | Mitochondrial DNA analysis of individual blastomeres disaggregated from pronuclear-transfer embryos. **a**, Last hot cycle PCR RFLP of individual blastomeres from a pronuclear-transfer embryo showing variable levels of mtDNA donor genotype in individual blastomeres. The arrow indicates the band representing carry-over mtDNA. **b**, Levels of donor mtDNA carry-over in individual blastomeres from eight embryos before modifications to minimize levels of donor mtDNA in pronuclear karyoplasts. In some embryos not all blastomeres could be collected. Figures represent the percentage mtDNA carry-over in individual blastomeres after pronuclear transfer. n.d., Non-detectable. **c**, Pronuclear karyoplasts after additional manipulation showing minimal amount of donor cytoplasm compared with Fig. 1e. Scale bar, 25 μm. **d**, Last hot cycle PCR RFLP of individual blastomeres from a pronuclear-transfer embryo showing no detectable levels of mtDNA donor genotype in individual blastomeres. The arrow indicates the band representing carry-over mtDNA. **e**, Levels of donor mtDNA carry-over in individual blastomeres from nine embryos after improvements to pronuclear karyoplast removal. In some embryos, not all blastomeres could be collected. Figures represent the percentage of mtDNA carry-over in individual blastomeres after pronuclear transfer. n.d., Non-detectable.

of the pronuclear karyoplast and were able to remove the pronuclei with a minimal amount of cytoplasm (Fig. 3c). Using last hot cycle PCR RFLP assays, we demonstrated that the mtDNA carry-over was significantly lower ($P < 0.005$), with four out of nine embryos containing undetectable levels of mtDNA carry-over (Fig. 3d, e). The average mtDNA carry-over in all remaining embryos was less than 2% ($1.68 \pm 1.81\%$ (mean $\pm$ s.d.), $n = 9$). These embryos also revealed much less variation in mtDNA carry-over between individual blastomeres (Fig. 3e). These levels of mtDNA are equivalent to those seen in unaffected individuals in epidemiological studies[1].

Very recently a related technique, metaphase II spindle transfer between unfertilized metaphase II oocytes, has been reported using oocytes from non-human primates[16]. This resulted in the birth of live offspring in which the authors were unable to detect donor mtDNA, albeit using a less sensitive assay than ours (the lower limit of detection was 3% compared with less than 0.5%). Although our optimized techniques of pronuclear extraction resulted in less than 3% carry-over, we nonetheless wanted to determine whether the technique of metaphase II spindle transfer might offer a further reduction in the level of mtDNA carry-over. We therefore measured the mtDNA copy number in karyoplasts containing the metaphase II spindle from freshly harvested human oocytes donated to research. We found no significant difference in the mtDNA copy number between metaphase II spindle karyoplasts ($13{,}222 \pm 5{,}733$ (mean $\pm$ s.e.m.), $n = 21$) and double pronuclear karyoplasts ($18{,}316 \pm 4{,}336$ (mean $\pm$ s.e.m.), $n = 12$). The wide variation within both groups of karyoplasts is probably due to the vastly different copy numbers observed in human oocytes (Fig. 2e). We conclude from this that both approaches would be effective in greatly reducing the risk of mtDNA disease.

Our studies show that in human zygotes, pronuclear transfer has the potential to 'treat' human mtDNA disease at a genetic level. The recent development of metaphase II spindle transfer has confirmed in non-human primates that this closely related method also holds great promise. The comparative value of both techniques has not been established in the same animal model or human oocytes, but both have potential advantages. The metaphase II spindle is smaller and technically easier to remove. However, it is not surrounded by a membrane, and without the use of a DNA stain it would be difficult to eliminate the possibility that some chromosomes may not be aligned on the metaphase plate or associated with the spindle as has been previously reported in human oocytes from older women[17] and in response to exposure to ambient conditions[18]. Studies in mice have shown that pronuclear transfer limits mtDNA transfer to subsequent generations[19]. In addition, the pronuclei are easier to visualize than the metaphase II spindle but they are also larger and their manipulation may induce more cellular trauma. Our studies have been particularly challenging because working with abnormally fertilized zygotes is technically more difficult than using those that are normally fertilized (two pronuclei) and is less likely to yield normal embryos owing to abnormal chromosomal constitution[20]. Despite these problems, we observed the rate of blastocyst development of the manipulated embryos to be approximately 50% that of the abnormal embryos that were not manipulated; the manipulated embryos also showed either no detectable or very low levels of mtDNA carry-over.

Because of the lack of available treatment for these patients and their families[21], preventing the transmission of mtDNA disease is a priority. Although mtDNA mutations are common, pronuclear or metaphase II spindle transfer is unlikely to be of value for asymptomatic individuals or those with mild mtDNA disease in the family. However, in some families, mtDNA disease can affect multiple family members with catastrophic consequences[22]. For these families, pronuclear transfer may be an option that mothers who carry mtDNA mutations would consider. Mitochondrial DNA mutations that are maternally inherited are either homoplasmic or heteroplasmic, and high loads of mutated mtDNA are necessary before there is clinical disease (usually more than 60% of total mtDNA)[8]. We have shown that we can generate human embryos with donor mtDNA carry-over at levels that are well below the disease threshold and unlikely to be detected except with very sensitive genetic techniques. With inherited mtDNA mutations there is little evidence of increasing levels of mutated mtDNA in tissues with time. In fact the opposite occurs, with loss of mutation in some tissues[23]. Thus the very low levels of mtDNA carry-over detected in some embryos will not cause mtDNA disease.

We believe the data presented in this paper on human zygotes and their development show that pronuclear transfer has the potential to prevent the transmission of mtDNA disease in humans. Manipulation of human oocytes and zygotes may cause chromosomal or epigenetic abnormalities[24] in the developing embryo. This requires further study to ensure the safety of different techniques. We believe our study in human zygotes and embryos represents a major advance towards preventing transmission of disease in patients with mtDNA mutations.

METHODS SUMMARY

Human embryos and manipulations. After receiving informed consent by the donors, we obtained abnormally fertilized human zygotes and metaphase II oocytes from patients undergoing fertility treatment at the Newcastle Fertility Centre at Life. The projects were licensed by the Human Fertilisation and Embryology Authority and approved by the Newcastle and North Tyneside Local Ethics Committee. Pronuclear transfer was performed using abnormally fertilized human zygotes generated after *in vitro* fertilization or intracytoplasmic sperm injection. Abnormal zygotes were identified on day 1 of development by the presence of one pronucleus (unipronucleate) or three pronuclei (tripronucleate) 18–19 h after insemination. Karyoplasts containing pronuclei and surrounding cytoplasm were removed from the donor zygote by using a biopsy pipette and transferred to a recipient zygote. After fusion, the reconstituted zygotes were either cultured for 6–8 days to monitor development to the blastocyst stage or were cultured before being disaggregated for analysis of mtDNA in individual blastomeres.

Analysis of mtDNA. To determine the carry-over of donor zygote mtDNA, we sequenced the non-coding control region and determined differences between donor and recipient mtDNA sequences. We devised last hot cycle PCR RFLP assays to differentiate between donor and recipient mtDNA. After cell lysis, extracted DNA was amplified, digested and separated on polyacrylamide gels. The relative amount of each genotype was determined by quantification of individual bands. Mitochondrial DNA copy number was determined in oocytes, early embryos and karyoplasts by real-time PCR using a probe to the *MT-ND1* gene of the mitochondrial genome.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.H., A.P.M., R.N.L. and D.M.T. conceived the project and designed the experiments. L.C., H.A.T., S.J.H., G.D.G., J.L.M., L.M.C., P.F.C. and R.W.T. performed experiments and analysed data. L.C., M.H., H.A.T. and D.M.T. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.H. (mary.herbert@ncl.ac.uk) or D.M.T. (d.m.turnbull@ncl.ac.uk).

METHODS

Human oocytes and embryos. Abnormally fertilized human zygotes and metaphase II oocytes were obtained from patients undergoing fertility treatment at the Newcastle Fertility Centre at Life after we received informed consent. The projects were licensed by the Human Fertilisation and Embryology Authority and approved by the Newcastle and North Tyneside Local Ethics Committee. Pronuclear transfer was performed using abnormally fertilized human zygotes generated after *in vitro* fertilization or intracytoplasmic sperm injection. Abnormal zygotes were identified on day 1 of development by the presence of one pronucleus (unipronucleate) or three pronuclei (tripronucleate) 18–19 h after insemination. For metaphase II spindle removal, freshly harvested metaphase II oocytes from consenting women were denuded of cumulus cells by using hyaluronidase (1× HYASE, Vitrolife). Mature (metaphase II) oocytes used for the study of mtDNA copy number included *in vitro* matured oocytes and oocytes that failed to undergo fertilization after intracytoplasmic sperm injection.

Pronuclear and metaphase II spindle karyoplast removal. Zygotes were transferred to G1v5 Plus medium (Vitrolife) containing cytochalasin B (5 μg ml^{-1}) and nocodazole (10 μg ml^{-1}) at 37 °C with 7% CO_2 immediately before manipulation or for 30 min before manipulation for improved karyoplast removal.

Zygotes were incubated in G1v5 Plus medium (Vitrolife) containing cytochalasin B (5 μg ml^{-1}) and nocodazole (10 μg ml^{-1}) at 37 °C with 7% CO_2 during the procedure. Manipulations were performed using an inverted microscope (Nikon Eclipse TE2000-U) equipped with a micromanipulation system (Integra Ti, Research Instruments). Zygotes were immobilized with a holding pipette, and a small ablation was made in the zona pellucida using a microsurgical laser (Saturn Active, Research Instruments). A customized biopsy pipette with an inner diameter of 25 μm (Rochford Medical) was inserted under the zona pellucida. The pronucleus and surrounding cytoplasm were then aspirated into the biopsy pipette as a membrane-bound karyoplast. For transfer of a single pronucleus, we removed a pronuclear karyoplast from either a unipronucleate or tripronucleate donor zygote and transferred this to a recipient zygote containing only one pronucleus. The recipient zygote was either a unipronucleate zygote, which required no manipulation before transfer, or a tripronucleate zygote from which two pronuclei had been removed. Thus, the reconstituted zygotes contained two pronuclei. In experiments involving transfer of two pronuclei, we removed pronuclei either as two individual pronuclear karyoplasts or a single karyoplast containing both pronuclei. These karyoplasts were then transferred to an enucleated recipient zygote such that the reconstructed zygote contained two pronuclei. For metaphase II spindle removal, oocytes were incubated in G1 medium (Vitrolife) containing 2.5 μg ml^{-1} cytochalasin B for 10 min before manipulation and throughout the procedure as above. The spindle was visualized using polarized light birefringence (Oosight Meta Imaging System, Cambridge Research and Instrumentation). Oocytes were immobilized with a holding pipette and the zona pellucida thinned using a microsurgical laser (Saturn Active, Research Instruments). A biopsy pipette with an inner diameter of 18–20 μm was inserted through the zona pellucida and the spindle and surrounding cytoplasm removed from the oocyte as a membrane-bound karyoplast.

Pronuclear karyoplast fusion. Pronuclear karyoplasts were transferred within a biopsy pipette to a 1-μl drop of HVJ-E (GenomONETM-CFEX HVJ Envelope Cell Fusion Kit, Cosmo Bio Co) and a small volume of the suspension approximately equal to the volume of the karyoplast aspirated into the pipette. The pipette was then moved to a drop containing a recipient zygote. The pipette was inserted into the zygote through a small ablation in the zona pellucida and the HVJ-E and pronuclear karyoplast aspirated into the perivitelline space, ensuring good contact between the karyoplast and plasma membrane. Fusion of the pronuclear karyoplast with the recipient zygote was confirmed visually and usually occurred within 10 min to 1 h after transfer. Manipulated zygotes were transferred to G1v5 Plus medium (Vitrolife) and cultured at 37 °C with 7% CO_2. Embryos were transferred to G2v5 Plus medium (Vitrolife) on day 3 of development and cultured at 37 °C with 7% CO_2 up to day 7. Embryos for mitochondrial DNA analysis were then transferred to sterile 0.5-ml microfuge tubes and stored at −80 °C until DNA extraction.

Manipulations to obtain individual blastomeres. Pronuclear-transfer embryos were disaggregated into individual blastomeres by micromanipulation or removal of the zona pellucida using acid Tyrode's solution.

For micromanipulation, the embryo was placed in G-PGD medium (Vitrolife) and immobilized with a holding pipette. A hole was made in the zona pellucida by using the microsurgical laser and individual blastomeres removed with a biopsy pipette. For removal of the zona pellucida, the embryo was placed briefly in acid Tyrode's solution until the zona pellucida had dissolved. The embryo was then transferred to G-PGD medium. Individual blastomeres were disaggregated by continual pipetting and transferred to sterile 0.5-ml microfuge tubes for analysis.

Embryo and blastomere lysis. Individual embryos or blastomeres were lysed for 2 h in a lysis buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% Tween-20 and 200 μg ml^{-1} proteinase K) at 55 °C. The enzyme was then inactivated by incubation at 95 °C for 10 min.

Mitochondrial DNA sequencing. The non-coding control region of the mitochondrial genome was amplified using two rounds of PCR amplification as described previously[25] with the following modification: secondary PCR reactions were performed with four sets of overlapping M13-tailed primers (primer nucleotide positions: D1F, 15758–15777; D1R, 019–001; D2F, 16223–16244; D2R, 129–110; D3F, 16548–16569; D3R, 389–370; D4F, 323–343; D4R, 771–752) with an annealing temperature of 58 °C. PCR products were purified using ExoSapIT (Amersham Biosciences) then sequenced on an ABI3130*xl* Genetic Analyzer (Applied Biosystems) with BigDye Terminator cycle sequencing chemistries (version 3.1, Applied Biosystems). Sequences were directly compared with the revised Cambridge Reference Sequence for human mtDNA[26] (GenBank accession number NC_012920) using SeqScape software (version 2.1.1, Applied Biosystems).

Amount of donor zygote mtDNA carry-over in pronuclear-transfer embryos. Level of donor zygote mtDNA carry-over was determined by last hot cycle PCR RFLP analysis. Separate assays were developed for each discriminatory mtDNA sequence variant identified and were performed as described previously[27,28], with modifications as listed in Supplementary Table 2. Fragments containing the sequence variants of interest were amplified by PCR using primers listed in the table and a last hot cycle performed with 5 μCi deoxycytidine triphosphate (3,000 Ci mmol^{-1}). Equal amounts (1,000 counts) of precipitated labelled products were digested overnight with 10 U of assay-specific restriction enzyme (New England Biolabs). Restriction fragments were separated by 12% non-denaturing polyacrylamide gel electrophoresis, dried onto a support, exposed to a Phosphorimager screen (Molecular Dynamics) and analysed with ImageQuant software (Molecular Dynamics). Carry-over donor zygote mtDNA was calculated as the percentage of total mtDNA in the recipient embryo.

Quantitative real-time PCR. Quantitative real-time PCR was performed using a previously designed TaqMan probe for the *MT-ND1* gene (*MT-ND1*L3506–3529) and PCR primers (forward primer, L3485–3504, reverse primer, H3532–3553)[29,30]. The reaction mixture consisted of 1 μl single cell lysate, 9.5 μl nanopure water, 12.5 μl TaqMan Universal MasterMix (2.5 μl 10× buffer A, 5 μl 10 mM $MgCl_2$, 0.5 μl each deoxyribonucleotide triphosphate (10 mM), 0.25 μl 1 U μl^{-1} AmpErase uracil-*N*-glycosylase, 0.13 μl 5 U μl^{-1} AmpliTaq Gold DNA polymerase, 2.62 μl nanopure water; Applied Biosystems), 300 nM forward and reverse primer and 100 nM fluorgenic probe. Each reaction was completed in triplicate and performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A template encompassing the *MT-ND1* region was amplified by PCR (forward primer, L3017–3036; reverse primer, H4057–4037) and the gel purified PCR product (QIAEX II Gel Extraction kit, Qiagen) used as a standard control. The concentration of the control template was determined using a spectrophotometer and this value used to calculate the copy number. Serial dilutions of the *MT-ND1* template were amplified in triplicate in the same experiment as the samples and a standard curve generated by plotting the logarithm of the copy number against the mean threshold cycle. The standard curve was then used to calculate the mtDNA copy number for each sample.

Genotyping nuclear DNA. Ovarian follicular cells and sperm were used for donor and recipient nuclear genotype analysis. DNA extraction from follicular cells was performed using the QIAamp DNA Mini kit according to the manufacturer's instructions (Qiagen). Sperm DNA was extracted in 200 μl 5% washed Chelex beads (Sigma), 2 μl proteinase K and 7 μl 10 mM dithiothreitol. After incubation for 4 h at 56 °C, the enzyme was inactivated by incubation at 95 °C for 10 min. The Chelex beads were removed from the DNA samples by centrifugation at 12,000*g* for 3 min. Whole-genome amplification from four individual embryos cultured to the two- to eight-cell stage was performed using the REPLI-g Mini kit (Qiagen). Briefly, embryos lacking the zona pellucida were lysed in 2.5 μl lysis buffer (200 mM NaOH, 50 mM dithiothreitol) for 10 min at 65 °C. Lysis was terminated with 2.5 μl 200 mM tricine. Genome amplification was achieved in a 50-μl reaction for 16 h at 30 °C according to the manufacturer's instructions. The reaction was terminated by incubation at 65 °C for 3 min. Follicular cell and sperm genomic DNA, and whole genome amplified embryo DNA, were analysed for 16 polymorphic microsatellite markers using the PowerPlex 16 System (Promega). PCR reactions were performed in a volume of 12.5 μl containing 1 ng of DNA, 1× Gold Star buffer, 1× PowerPlex 16 primer pair mix and two units of AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplification was done in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) as per manufacturer's instructions. One microlitre of PCR product was diluted in 9 μl of Hi-Di Formamide (Applied Biosystems) and 1 μl of ILS600 size standard (Promega), denatured at 95 °C for 3 min and immediately cooled on ice for 3 min. The same treatment was done with 1 μl of PowerPlex 16 System Allelic Ladder (Promega).

Amplified fragments were detected using a 3130*xl* Genetic Analyzer (Applied Biosystems) and data analysed using GeneMapper software (version 4, Applied Biosystems).

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