Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease

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Mitochondrial DNA (mtDNA) mutations are maternally inherited and are associated with a broad range of debilitating and fatal diseases1. Reproductive technologies designed to decouple the inheritance of mtDNA from nuclear DNA may enable affected women to have a genetically related child with a greatly reduced risk of mtDNA disease. Here we report the first preclinical studies on pronuclear transplantation (PNT). Surprisingly, techniques used in proof-of-concept studies involving abnormally fertilized human zygotes2 were not well tolerated by normally fertilized zygotes. We have therefore developed an alternative approach based on transplanting pronuclei shortly after completion of meiosis rather than shortly before the first mitotic division. This promotes efficient development to the blastocyst stage with no detectable effect on aneuploidy or gene expression. After optimization, mtDNA carryover was reduced to <2% in the majority (79%) of PNT blastocysts. The importance of reducing carryover to the lowest possible levels is highlighted by a progressive increase in heteroplasmy in a stem cell line derived from a PNT blastocyst with 4% mtDNA carryover. We conclude that PNT has the potential to reduce the risk of mtDNA disease, but it may not guarantee prevention.

Predicting the risk of serious disease in children of women who carry mtDNA mutations is complicated by a number of factors. Mutations in mtDNA can be either homoplasmic (all copies of mtDNA are mutated) or heteroplasmic (mixture of mutated and wild-type mtDNA). In the case of heteroplasm, women produce oocytes with widely varying mutation loads3. While pathogenicity is generally proportional to the ratio of mutated to wild-type mtDNA, the severity of disease for a given mutation load can vary, even among homoplasmic individuals4. The resulting unpredictability in the risk of transmitting disease raises profoundly difficult reproductive decisions for women from affected families. While preimplantation genetic diagnosis (PGD) can be used to reduce the risk of mtDNA disease by identifying embryos with low mutation loads5, it is not useful for women who are homoplasmic for pathogenic mtDNA mutations6. In such cases, it may be possible to reduce the risk of transmission by transplanting the oocyte nuclear DNA to an enucleated donor oocyte free of pathogenic mtDNA mutations.

Progression through female meiosis offers a number of opportunities for transplanting nuclear DNA. Proof-of-concept studies7,8 indicate that transplantation of the nuclear genome between human oocytes arrested at metaphase of meiosis II (MII) is associated with a high incidence of abnormal fertilization7. An alternative approach is to transplant the nuclear genome after fertilization, when the haploid maternal and paternal genomes are separately packaged in large, clearly visible pronuclei. First performed in mouse zygotes more than three decades ago9, PNT is typically performed during the G2 phase of the 1st mitotic cell cycle. Using this approach, we have previously demonstrated that PNT between abnormally fertilized human zygotes is technically feasible2. However, their limited capacity for onward development has been a major barrier to further investigation of the therapeutic potential of PNT.

Here we investigate the effect of PNT on normally fertilized human zygotes. We found that the procedures (Extended Data Fig. 1a, b) previously used for abnormally fertilized zygotes2 resulted in reduced survival. Because developmental competence is correlated with accelerated division to the two-cell stage10, we asked whether the timing of PNT might be too close to the onset of 1st mitosis in normally fertilized zygotes (Fig. 1a). To address this, we undertook a series of experiments in which the pronuclei were transplanted shortly after they first appear (~8 h after insemination; Fig. 1b and Supplementary Videos 1, 2). Initially, we added sucrose to the enucleation medium to facilitate enucleation and fusion by inducing shrinkage of the cytoplasm (Fig. 1b). However, this was later abandoned to reduce the karyoplast mtDNA content and had minimal effect on survival (see later). Our data indicate that early PNT (ePNT) promotes survival (92% versus 59% for late PNT (ltPNT); P < 0.01; Fig. 1c). Moreover, ePNT zygotes showed normal pronuclei abutment and division to the two-cell stage (Extended Data Fig. 1c, d), indicating that sperm centriole function was not disrupted11.

Blastocyst formation, which is essential for implantation, occurs at 5–6 days after fertilization in vitro, and is marked by allocation of cells to the inner cell mass (ICM), or to an outer layer of trophectoderm cells12. The morphology of the ICM and trophectoderm correlates well with implantation and is used to assess blastocyst quality in clinical in vitro fertilization (IVF) programmes (Extended Data Fig. 2a–d). While the increased survival of ePNT zygotes (series I) resulted in improved blastocyst formation compared with ltPNT, both approaches produced few good quality blastocysts (Extended Data Fig. 2e, f). Control experiments in which pronuclei were replaced in the same zygote (autologous ePNT) indicated that blastocyst quality was compromised by the manipulations (Fig. 2a and Extended Data Fig. 2f). To address this, we modified the manipulation medium, removing Ca2+ and Mg2+ and reducing by tenfold the concentration of the fusogen, haemagglutinating virus of Japan envelope (HVJ)-E). In addition, we switched from a two-step to a single-step culture medium,
Early PNT promotes survival of normally fertilized zygotes after PNT. a, Progression from MII arrest to completion of the 1st mitosis showing timings of ePNT and ltPNT. ICSI, intracytoplasmic sperm injection. b, Images show the steps involved in ePNT. Left, arrowheads indicate the pronuclei (PN). Middle, enucleation pipette inserted through a laser-induced opening in the zona pellucida (arrow, bottom). Bottom, enucleated zygote (cytoplast). Inset shows two karyoplasts, each consisting of a single pronucleus surrounded by a small amount of cytoplasm. Right, karyoplasts treated with HVJ-E and inserted under the zona pellucida. Bottom, arrow indicates removal of excess cytoplasm (see Supplementary Video 2). c, Survival of reconstituted ePNT and ltPNT zygotes (P < 0.01). Comparisons by χ² test.

in which embryos remained for the duration of culture. Under these conditions (ePNT series II), blastocyst formation and quality did not differ between unmanipulated controls and technical controls (Fig. 2a, b). Similarly, heterologous ePNT, which involved reciprocal transfers between zygotes from fresh and vitrified oocytes, had no detectable effect on blastocyst quality (Fig. 2b, c). Consistent with the improved quality, nuclear counts indicated that ePNT blastocysts cell numbers were equivalent to controls (Extended Data Fig. 2g, h). However, heterologous ePNT resulted in reduced blastocyst formation (Fig. 2b), possibly due to an effect of vitrification at the MII stage, which was not ameliorated by delaying vitrification until after exit from MII (Extended Data Fig. 3a–f).

Analysis of aneuploidy by array-based comparative genomic hybridization (array-CGH) indicated that while the majority of poor quality ePNT blastocysts were aneuploid for multiple chromosomes (Fig. 2d and Extended Data Fig. 4), the overall incidence of aneuploidy was comparable between ePNT and control blastocysts, and was similar to a reference data set of IVF blastocysts, in which female age was matched to the karyoplast donors (Fig. 2e). These data indicate that the ePNT procedure does not result in an increased incidence of aneuploid blastocysts.

We next determined whether ePNT alters the pattern of gene expression in human blastocysts by performing RNA sequencing (RNA-seq) on single cells microdissected from ePNT and control blastocysts (Extended Data Fig. 5a, b). For reference, we also included a previously published series of unmanipulated blastocysts. The ePNT blastocysts included in these experiments were generated by fusion of cytoplasts and karyoplasts with the same (autologous and homologous ePNT), or different (heterologous ePNT), mitochondrial genotypes (Extended Data Fig. 5b).

To test for differences in global gene expression, we performed principal component analysis (PCA) on normalized RNA-seq data (Extended Data Fig. 5c). We first determined whether PCA is sufficiently sensitive to detect differences in global gene expression between good and poor quality blastocysts. Plotting PCI against PC2, which together account for the largest contributions to variation in global gene expression, revealed a high proportion of outliers among samples from poor quality blastocysts (Fig. 3a, b). By contrast, samples from good quality ePNT blastocysts clustered closely with controls (Fig. 3c). To determine whether additional principal components, however minor, might distinguish differences between good quality ePNT and control blastocysts, we plotted all combinations of the first ten principal components. In each combination we found that ePNT samples cluster with controls (Extended Data Fig. 6a).

Figure 2 | Blastocyst development after ePNT. a, Autologous ePNT (left) and heterologous ePNT (right). b, Blastocyst formation and quality (series II). Zygotes (n = 131 from 30 donors) were either unmanipulated (control (Ctr.), n = 30), or used for autologous (Altg.; n = 21) or heterologous (Het.; n = 80) ePNT. Heterologous ePNT blastocyst formation was reduced compared with control (P < 0.05; χ² test). Blastocyst quality is similar between the three groups (not significant; Fisher’s exact test). Lat A, latrunculin A; Noc, nocodazole. c, Images showing good quality control and ePNT blastocysts. Arrowheads, ePNT blastocyst hatching through laser-induced opening in the zona pellucida. Scale bars, 20 μm.

d, Aneuploidy in cells sampled from blastocysts (grades A–F) detected by array-CGH blind to sample origin (n = blastocysts; not significant, Fisher’s exact test). e, Aneuploidy in controls and ePNT blastocysts not significantly different from a reference population (Ref.) of IVF blastocysts (χ² test). Source data are available online for b.
we were able to distinguish distinct populations of cells corresponding to the three cell lineages of the mammalian karyoplast14 (Extended Data Fig. 6b). This was confirmed by t-distributed stochastic neighbour embedding (t-SNE), a nonlinear method for dimensionality reduction15 (Extended Data Fig. 6c). Consistent with this, unsupervised hierarchical clustering revealed that ePNT and control samples cluster together on the basis of lineage (Extended Data Fig. 7a). Together, these findings indicate that single-cell RNA-seq reliably detects differences in gene expression, and that global and lineage-associated gene expression is indistinguishable between control and ePNT blastocysts.

To address the question of whether ePNT specifically affects expression of mtDNA-encoded oxidative phosphorylation (OXPHOS) genes, we generated a heatmap after unsupervised hierarchical clustering. This revealed wide variation in the level of mtDNA OXPHOS gene expression within and between ePNT and control blastocysts. However, samples from both groups clustered together, irrespective of whether the karyoplast and cytoplast contained the same, or different, mitochondrial genomes (Extended Data Fig. 7b). This suggests that switching nuclear genomes does not alter mitochondrial gene expression.

On the basis of evidence from a range of pathogenic mutations, the probability of developing or transmitting disease is low when mutation loads are <18% (ref. 16) and <5% (ref. 17), respectively. Thus, reducing the contribution of karyoplast mtDNA to <5% has the potential to prevent transmission to subsequent generations. The level of mtDNA carryover during transplantation of pronuclei was measured by pyrosequencing (Extended Data Fig. 8a–c) using clumps of cells from day 6 ePNT blastocysts (n = 40) generated by reciprocal transfer between zygotes from fresh and vitrified oocytes (Extended Data Fig. 5b). The heterologous group is subdivided according to blastocyst grade. b. Graph shows an increased percentage of outliers in poor quality blastocysts (P values shown; Fisher’s exact test). c. PCA of single-cell RNA-seq data comparing control samples with good quality ePNT blastocysts. PCA was performed blind to sample origin. The numbers of samples and blastocysts are shown.

disease is likely to be increased by vitrifying patient rather than donor oocytes. On the basis of our findings, this approach results in <2% heteroplasmy in the majority (79%) of blastocysts and none with >5% heteroplasmy (Extended Data Fig. 8e, f). Notably, those with >2% heteroplasmy were predicted by technical problems such as leakage from the cytoplast or inadequate shearing of cytoplasm from the karyoplast. Such factors could be taken into account when selecting embryos for use in clinical treatment.

To assess the potential fate of karyoplast mtDNA under conditions in which it can replicate18, we derived human embryonic stem (hES) cell lines (n = 5) from ePNT blastocysts (Extended Data Fig. 9a–f). While all PNT-hES cell lines showed low levels of heteroplasmy at passage 1 (P1), one line (36PNT-hES), derived from a blastocyst with 4% mtDNA carryover, showed an upward drift with wide variation in heteroplasmy between colonies by P12 (Fig. 4d). This was confirmed by experiments in which individual colonies were subcloned and cultured for multiple passages (Extended Data Fig. 10). Interestingly,
the karyoplast and cytoplast donors for 36PNT belonged to the same mtDNA haplogroup (Extended Data Fig. 9f), however, we cannot exclude the possibility that sequence variants in the karyoplast donor mtDNA might have conferred a replicative advantage19. While the biological basis remains to be established, the relevance of the increased heteroplasmacy to development in vivo is unclear. For example, recent reports indicate that pluripotent cells derived from heteroplasmic fibroblasts exhibit a bimodal drift towards homoplasmacy, which is not observed in the parental line20,21. Moreover, with the exception of one controversial case22,23, a number of reports24,25, together with our own unpublished data, indicate that the level of heteroplasmacy in preimplantation embryos mirrors that in babies born after PGD. Nonetheless, the finding underscores the importance of reducing mtDNA carryover to the lowest possible levels and suggests that guaranteed prevention of disease will depend on complete elimination of karyoplast mtDNA.

The work reported here represents a considerable advance towards understanding the therapeutic potential of PNT in preventing transmission of mtDNA disease. Transplanting the pronuclei shortly after completion of meiosis resulted in improved survival. Further optimization of enucleation and embryo culture procedures promoted development of good quality blastocysts whose gene expression and incidence of aneuploidy did not differ from controls. Our findings also indicate that vitrification of patient rather than donor oocytes will probably minimize mtDNA carryover. This offers the added advantage of stockpiling patient oocytes before they become susceptible to age-related meiotic aneuploidy26. Given the low levels of mtDNA carryover using optimized procedures, we believe that ePNT has the potential to reduce risk of mtDNA disease. However, until more is known about the postimplantation fate of karyoplast mtDNA, it should be considered in combination with prenatal screening.

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

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Author Contributions M.H. and L.A.H. conceived and designed the PNT experiments. L.A.H., L.L., L.C. and L.A. performed PNT experiments and embryo manipulations. J.R., D.K. and Q.Z. performed cell counts. D.W., E.F. and S.A. performed whole-genome amplification and array-CGH. K.K.N., P.B., N.M.E.F. performed RNA-seq experiments. L.C., H.A.T. and D.M.T. measured mtDNA carryover and performed mtDNA haplogroup analysis. N.P., K.K.N., N.M.E.F., S.E.W., Y.T. and H.O.K. derived, cultured and characterized ES cell lines. K.K.N., P.B., M.L., J.R., L.A.H., L.C., Y.T., P.B. and M.H. analysed data. A.P.M. and M.C. coordinated the oocyte donation program. M.H. wrote the manuscript with input from D.M.T., D.W., K.K.N., J.R. and M.L.

Author Information Raw RNA-seq data and reads per kilobase per million mapped reads (RPKM) table have been deposited in the Gene Expression Omnibus under accession number GSE76284. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.H. (mary.herbert@ncl.ac.uk).

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METHODS

Human oocytes and manipulations. The study was approved by the Newcastle and North Tyneside Research Ethics Committee and was licensed by the UK Human Fertilisation and Embryology Authority (HFEA). Informed consent was obtained from all donors by research nurses who were not directly involved in the research, or in the clinical treatments of women participating in the study. Human oocytes (n = 523) included in this study were donated either by women undergoing infertility treatment (n = 44 oocytes from 6 donors, age range 25–36 years) as part of the ‘egg share’ programme or by non-patient donors (n = 479 oocytes from 57 donors, age range 21–36 years). Donors were compensated in accordance with current HFEA guidance on payments for donors. Non-patient donors received financial compensation of £500 per donation cycle. Compensation under the ‘egg share’ programme consisted of a subsidy (£1,500) from research funds towards the cost of treatment for self-funded patients, or an additional fully funded treatment cycle for those who did not become pregnant after NHS-funded treatment.

Oocytes were collected by ultrasound-guided follicle aspiration and the surrounding cumulus cells were removed using hyaluronidase (HYASE; Vitrolife). MII oocytes were identified by the presence of the 1st polar body and were fertilized by intracytoplasmic sperm injection (ICSI) using sperm donated specifically for this purpose. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment, except for aneuploidy and gene expression analysis.

Oocyte vitrification. MII oocytes were either vitrified or used immediately for PNT experiments. The majority (n = 107) of vitrified oocytes were vitrified at the MII stage. We also conducted a series of experiments in which vitrification was performed after completion of MII (at the 2PB stage; ~5.5 h post-ICSI; n = 34), to determine whether blastocyst development might be improved. Vitrification and warming were performed using the RapidVit and RapidWarm oocyte kits (Vitrolife, Sweden). Oocytes were stored in liquid nitrogen until required.

PNT. PNT was performed either at 16–20 h after ICSI (ltPNT), or at ~8–10 h after ICSI (ePNT). In the case of ePNT, two main series of experiments (series I and II) were performed. A total of 51 zygotes from 10 donors were used for ICSI (n = 12 controls; n = 39 ICSI) for ICSI experiments. The 58 zygotes from 13 donors in series I (n = 19 controls; n = 39 ICSI) and 131 zygotes from 30 donors in series II (n = 50 controls; n = 101 ePNT). Thirty-four zygotes from 13 donors were used for ePNT experiments involving oocytes vitrified at the 2PB stage. Two types of PNT experiments were conducted: (1) autologous PNT, which involved removal and replacement of pronuclei in the same zygote, was performed to distinguish between technical and biological effects; (2) heterologous PNT involved reciprocal transfer between pairs of zygotes, either from the same or different donors. Heterologous PNT between zygotes from different donors involved reciprocal transfer between zygotes originating from fresh and vitrified oocytes. This gave rise to reconstituted zygotes consisting of cytoplasm from fresh oocytes and karyoplasts from vitrified oocytes, or vice versa. These combinations are termed FreshCy and VirCy, respectively (see Extended Data Fig. 4a). In one set of ePNT experiments, which gave rise to a single ePNT blastocyst, the two donors were sisters and therefore have the same mitochondrial genotype. For the purposes of the gene expression experiments, these are referred to as homologous transfers.

The PNT procedure was performed in an isolator-based workstation (Vitrosafe) with temperature, CO2, and O2 control containing an inverted microscope (TE2000-U, Nikon) fitted with micromanipulators (Integra Ti, Research Instruments) and a laser objective (Saturn Active, Research Instruments). PNT procedures took ~15 min to complete and involved the following steps. First, zygotes in which 2PN were visible were placed in enucleation medium with cytoskeletal inhibitors. In all cases, nocodazole (10 μg ml−1) was used to depolymerize microtubules. In ICSI experiments we used either cytochalasin B (5 μg ml−1) or latrunculin A (2.5 μM or 5 μM) to destabilize the actin cytoskeleton. We subsequently used latrunculin A (2.5 μM) for all ePNT experiments. For ICSI and ePNT (series I) experiments, enucleation was performed in G-1 Plus medium (Vitrolife). We used Sydney IVF Embryo Biopsy Medium (Cook Medical), which does not contain Ca2+ and Mg2+ for ePNT (series II) experiments. Enucleation was performed in the presence or absence of sucrose (0.125 μM). Addition of sucrose increased the osmolarity of the enucleation medium from 280 mosm 1−1 to 449 mosm 1−1, which induced shrinkage of the cytoplasm, thereby facilitating enucleation. Second, a laser objective (Saturn Active, Research Instruments) was used to create an opening in the zona pellucida for insertion of the enucleation/passage pipette. The inner diameter pipette measurements and the outer diameter pipette measurements of 5 μm and 17 μm for ICSI, and 15 μm for ePNT. Third, the pronuclei, surrounded by a small amount of cytoplasm, were aspirated into the enucleation pipette, either as a single karyoplast, or as two separate karyoplasts (see Supplementary Videos 1 and 2). Fourth, karyoplasts were briefly exposed to a suspension of HVJ-E: GenOMONE-CEF Ex (Cosmo Bio). Undiluted suspension was used for ICSI and ePNT (series I) and a 1:10 dilution was used for ePNT (series II). Fifth, the pipette containing the karyoplasts was inserted through the laser-drilled opening in the zona pellucida and karyoplasts were gently expelled into the perivitelline space and allowed to fuse with the cytoplasm. Sixth, reconstituted and control zygotes were cultured either in a sequential medium, G-1 Plus (day 1–3)/G-2 Plus (day 3–6) (Vitrolife); ICSI and ePNT (series II), or in the single-step G-TL medium (Vitrolife; ePNT (series II)) from day 1 to 6. Time-lapse embryo imaging was performed for three sets of experiments using the Primo Vision Time-lapse monitoring system (Vitrolife).

Overview of experiments on PNT zygotes. Survival of reconstituted zygotes was initially compared between ICSI and ePNT (series I) and was subsequently recorded for all ePNT zygotes. In ePNT (series II), the first mitotic division was monitored by time-lapse imaging in three sets of experiments. All zygotes submitted to PNT were included in the analysis of development to the blastocyst stage. Zygotes (controls and PNT) that developed to the blastocyst stage were graded and included in the analysis of blastocyst quality. Blastocyst formation and grade were assessed on day 6 for ICSI, and on days 5 and 6 for ePNT, except in the case of two ePNT (series II) experiments, which were assessed only on day 6. These experiments are not included in the day 5 analysis shown in Fig. 2b and in Extended Data Fig. 3d–f.

Blastocyst cell counts were performed primarily to gain insight into the causes of poor blastocyst quality in the ICSI and ePNT (series I). Data on blastocyst cell counts were obtained from ICSI (n = 6) and ePNT: series I (n = 8) and series II (n = 5). Further analyses, including aneuploidy, gene expression, mtDNA copy number, and hES cell derivation were conducted on series II blastocysts only. Where possible, we performed multiple investigations on individual blastocysts. In accordance with our Local Research Ethics Committee approval and HFEA licence, these were performed on day 6. Unmanipulated control blastocysts and ePNT blastocysts were used for aneuploidy and gene expression, or aneuploidy and hES cell derivation. The blastocyst grades shown for each of these analyses refer to the grades on day 6. The numbers of blastocysts used for each set of experiment were: aneuploidy screening (ePNT: n = 30 from 20 donors; control: n = 11 from 10 donors), gene expression analysis (ePNT: n = 11 from 10 donors; control: n = 3 from 3 donors), mtDNA copy number (ePNT: n = 40 from 28 donors), hES cell derivation (ePNT: n = 15 from 13 donors; control: n = 6 from 4 donors).

Embryo grading. Embryos were graded using the UK National External Quality Assessment Scheme (NEQAS) grading schemes for embryos and blastocysts29. Blastocysts were assigned a three-digit grade representing a score of 1–6 for the extent of blastocoel expansion, 1–5 for the inner cell mass appearance and 1–3 for the trophectoderm appearance31. The grade was converted to a quality category using the table in Extended Data Fig. 2c.

Blastocyst nuclear counts. Day 6 blastocysts were fixed using 4% PFA at pH 7.4. Nuclear staining was carried out using DAPI (Vectashield). Blastocysts were imaged using an inverted confocal microscope (Nikon A1R) with a ×20 objective (Plan Apo, Nikon) and NIS-elements image software. Z-steps were taken at ~1 μm intervals and nuclear counts performed using ImageJ software. Aneuploidy screening. Clumps of cells were harvested from ePNT blastocysts for whole-genome amplification followed by microarray-CGH analysis according to a previously validated protocol using 24Sure Cytochip (Illumina). Cells were obtained from the trophoderm, ICM or both. Lysis and whole-genome amplification was performed using the SurePlex kit (Illumina) according to the manufacturer’s instructions and blind to sample origin. Samples from ePNT blastocysts were labelled with Cy3 while a commercially available reference 46,XY DNA was labelled with Cy5 (Illumina)32. A laser scanner (InnoScan 710, Innopsys) was used to analyse the microarrays after washing and drying. The resulting images were analysed using BlueFuse Multi analysis software (Illumina).

Gene expression analysis. The RNA was hybridized to a custom Oligoarray (STDM: 230K) and analysed on a GeneChip-25K scanner (Affymetrix) to determine the expression of 25,000 genes in all blastocysts followed by hierarchical clustering.

Blastocyst disaggregation. Blastocyst disaggregation was performed using an Olympus IX73 microscope and a Saturn 5 laser (Research Instruments) as described previously24. Embryos were placed in drops of G-MOPS solution (Vitrolife) on a Petri dish overlaid with mineral oil for micromanipulation. The separated ICM and polar trophectoderm were washed in Ca2+− and Mg2+-free PBS (Invitrogen) and incubated in 0.05% trypsin/EDTA (Invitrogen) for 5–10 min. Trypsin was quenched using Global Media supplemented with 5 mg ml−1 LifeGlobal Protein Supplement. Single cells were isolated using a 30-μm inner diameter blastomere biopsy pipette (Research Instruments).

DNA synthesis and amplification. cDNA was synthesized using SMARTer Ultra Low Input RNA for Illumina Sequencing-HV kit (Clontech Laboratories) according to the manufacturer’s guidelines and as previously published14. cDNA was sheared using Covaris S2 with the modified settings 10% duty, intensity 5, burst cycle 200 for 2 min. Libraries were prepared using Low Input Library Prep Kit.
VST-normalized count data were used as input for the R implementation of the t-SNE. DESeq2 was applied to the read counts for the ePNT and control data to identify differential expressed genes in the primitive endoderm, trophectoderm and epiblast samples.

**mtDNA carryover analysis.** 
mtDNA extraction and mtDNA sequencing. The control region of the mitochondrial genome from oocyte donors was sequenced using ovarian follicular cells harvested at the time of oocyte retrieval, or cumulus cells removed from the oocyte in preparation for ICSI. DNA extraction from follicular cells was performed using the QiAamp DNA Mini kit according to the manufacturer's instructions (Qiagen). Cumulus cells 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 proteinase K) at 55 °C. The enzyme was then inactivated by incubation at 95 °C for 10 min. The control region of the mitochondrial genome was amplified as described previously with the following modification: secondary PCR reactions were performed with four sets of overlapping M13-tailed primers (primer nucleotide positions, D1F: 15758–15777 and D1R: 019–001; D2F: 16223–16244 and D2R: 129–110; D3F: 16548–16569 and D3R: 389–370; D4F: 323–343 and D4R: 771–752) with an annealing temperature of 58 °C. PCR products were purified using TSAP (Promega) then sequenced on an ABI3130 Genetic Analyser (Applied Biosystems) with BigDye Terminator cycle sequencing chemistries (v3.1. Applied Biosystems). Sequences were directly compared to the revised Cambridge Reference Sequence for human mtDNA (GenBank accession number AC_000021.2) using Seqscape software (v2.1.1, Applied Biosystems).

Generation of heteroplasmic control DNA. The mtDNA control region containing either the wild-type or polymorphic nucleotide of interest was amplified using PCR primers (primer nucleotide positions: forward primer, 16016–1602; reverse primer, 571–552) with an annealing temperature of 58 °C. PCR products amplified from ovarian follicular cells were purified using the Agencourt Ampure XP purification system (Beckman Coulter) according to the manufacturer's instructions. PCR products amplified from cumulus cells were gel purified (QiAquick Gel Extraction kit, Qiagen) and cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions. Plasmid DNA was isolated using the QiAprep Spin Miniprep kit (Qiagen). Quantitative real-time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and PCR primers (forward primer, L16016–16016; reverse primer, H16168–16167) to accurately determine the DNA concentration. Equimolar concentrations of DNA containing the wild-type or polymorphic nucleotide of interest were then combined in varying ratios to generate a range of heteroplasmic controls.

Pyrosequencing. Quantitative pyrosequencing was used to measure mtDNA carryover in samples from ePNT blastocysts and PNT-hES cell lines. Locus-specific PCR and a pyrosequencing primer were designed for each polymorphic nucleotide of interest the mtDNA using PyroMark Assay Design Software v2.0 (Qiagen). Clumps of cells from ePNT blastocysts and from PNT-hES cell lines 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 proteinase K) at 55 °C. The enzyme was then inactivated by incubation at 95 °C for 10 min. mtDNA amplification was performed before pyrosequencing analysis and pyrosequencing performed on the PyroMark Q24 and PyroMark Q69 instruments according to the manufacturer's instructions. Quantification of the heteroplasmy level was achieved using the instrument software to directly compare the relevant peak heights of both the wild-type and polymorphic nucleotides at the relevant position. A standard curve was generated by plotting expected heteroplasmy level against actual heteroplasmy level for the control samples. The standard curve was used to determine the level of heteroplasmy in the blastocyst and PNT-hES cell samples.

**Mitochondrial haplogroups.** Haplogroups were determined by next-generation sequencing analysis of whole mtDNA, amplified in two overlapping 9-kb fragments (389–370 and 771–752). The forward and reverse pyrosequencing primer were designed for each polymorphic nucleotide in samples from ePNT blastocysts and PNT-hES cell lines. Locus-specific primers containing the wild-type or polymorphic nucleotide of interest were then compared to the revised Cambridge Reference Sequence for human mtDNA (389–370 and 771–752) with an annealing temperature of 58 °C. PCR products were purified using TSAP (Promega) then sequenced using BigDye Terminator cycle sequencing chemistries (v3.1, Applied Biosystems). Sequences were directly compared to the revised Cambridge Reference Sequence for human mtDNA (GenBank accession number AC_000021.2) using Seqscape software (v2.1.1, Applied Biosystems).

Statistical analysis. Data were analysed using one-way ANOVA with Tukey's HSD test, Mann–Whitney U-test, χ² test and Fisher's exact test, as indicated in the figure legends. RNA-seq data were analysed by PCA using either RPKM- or TPM-normalized counts.
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Extended Data Figure 1 | lpPNT and pronuclei centralization after ePNT. a, Schematic showing timing of lpPNT and ePNT. b, Images showing the stages of the lpPNT process: left, late pronuclear zygote; middle, enucleation; right, fusion. Scale bar, 20 μm. Note large pronuclei and pipette size compared with Fig. 1b and Supplementary Videos 1 and 2. c, Images show unmanipulated and ePNT zygotes at 16–18 h after fertilization. Scale bars, 20 μm. d, Pronuclei centralization and division to the two-cell stage assessed by live cell imaging in control and ePNT zygotes (not significant). Comparisons by $\chi^2$ test.
Extended Data Figure 2 | Blastocyst morphology and effect of PNT on blastocyst development and quality. a, Schematic showing cell lineages in a mammalian blastocyst: trophectoderm; primitive endoderm and epiblast. b, Morphological criteria and scoring system used for grading human blastocysts. Top, degree of expansion ranging from an early, unexpanded blastocyst (score 1) to fully expanded (score 6). Middle, range of ICM morphologies from absent (score 1) to large but tightly packed (score 5). Bottom, range of trophectoderm morphologies from scant and discontinuous (score 1) to a fully formed layer of tightly packed cells (score 3). Box colours correspond to the grades shown in c. c, Table used to assign blastocyst grades, according to levels of expansion, and morphology of the ICM and trophectoderm. Grade F (not shown) was assigned to embryos that developed to the blastocyst stage but subsequently showed signs of degeneration. d, Graph showing the relationship between blastocyst grade and implantation. Data obtained from clinical IVF cycles (n = 531) in which unmanipulated single blastocysts were replaced. There was no case in which a grade D or F blastocyst was replaced. P values are shown (χ² test). e, LtPNT experimental conditions, blastocyst formation (P < 0.01; χ² test) and quality. A total of 51 zygotes from 10 donors were allocated either to an unmanipulated control group (Ctr.; n = 12) or to LtPNT involving transfer between pairs of zygotes from the same donor (n = 29) or replacement back into the same zygote (autologous PNT (Atlg.) n = 10). f, ePNT (series I) experimental conditions, blastocyst formation and quality. This series of experiments involved a total of 58 zygotes from 13 donors. Zygotes were allocated to a control group (n = 19), or to ePNT involving either autologous (n = 18) or heterologous (Het.; n = 21) transfers. Differences are not significant (χ² test and Fisher’s exact test). g, Image of an ePNT blastocyst fixed on day 6 and stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 50 μm. h, Cell number assessed by nuclear counts showing comparable numbers in control and ePNT (series II) blastocysts (P = 0.001; one-way analysis of variance (ANOVA) with Tukey’s HSD test). Mean ± standard deviation (s.d.) calculated from individual blastocysts, numbers indicated on the x-axis.
Extended Data Figure 3 | Survival and blastocyst development after ePNT between zygotes obtained from freshly harvested and vitrified oocytes. a, Experimental scheme for heterologous ePNT in series II. Because of unpredictability in the response to ovarian stimulation, heterologous transfers involved reciprocal ePNT between zygotes generated from freshly harvested and vitrified oocytes. This resulted in reconstituted zygotes whose cytoplasts originated from a fresh oocyte (FreshCy), or from a vitrified oocyte (VitCy). Oocytes for these experiments were vitrified predominantly at the MII stage (blue box; \( n = 80 \) zygotes; 25 donors). We also conducted a series of experiments to determine whether vitrification at the 2PB stage (green box; \( n = 34 \) zygotes; 13 donors) would promote improved blastocyst formation.

b, Survival of reconstituted zygotes as a proportion of those submitted to autologous (Atlg.) and heterologous (Het.) ePNT according to the stage of vitrification (MII or 2PB) and according to whether the cytoplast was derived from a fresh (FreshCy) or a vitrified (VitCy) oocyte. Loss was generally due to karyoplast lysis, excessive leakage of cytoplasm, or degeneration of reconstituted zygotes during subsequent incubation. Differences are not significant (\( \chi^2 \) test).

c, Sucrose was initially included in the manipulation medium to facilitate enucleation and fusion, however, it was later removed because the data indicated that the osmotic effect resulted in increased mtDNA carryover (see Fig. 4b). Omission of sucrose from the enucleation medium had a small, but not significant, effect on survival of zygotes whose cytoplasts originated from vitrified MII oocytes (\( \chi^2 \) test).

d, Blastocyst formation as a percentage of zygotes submitted to the ePNT procedure recorded on days 5 and 6 after fertilization.

e, Blastocyst formation recorded on days 5 and 6 as a percentage of zygotes that survived the ePNT procedure. The numbers in each group and \( P \) values are shown, \( \chi^2 \) test.

f, Blastocyst quality grades (see Extended Data Fig. 2c, d) on days 5 and 6 (not significant; Fisher’s exact test). Source data files are available online.
Extended Data Figure 4 | Array-CGH results for PNT blastocysts. Summary of array-CGH results obtained from ICM and trophectoderm samples from control ($n=11$) and ePNT ($n=30$) blastocysts. Blastocysts are ordered by grade within experimental groups. The karyoplast donor age is also shown.
Extended Data Figure 5 | Experimental approach and bioinformatics analysis of single-cell RNA-seq data from ePNT and control blastocysts. a, Flow diagram showing the steps involved in RNA-seq of single cells microdissected from human blastocysts. b, Summary table of control and ePNT blastocysts submitted for RNA-seq analysis. For the purpose of gene expression analysis, we distinguish between ePNT blastocysts derived from fusion of cytoplasts and karyoplasts with the same, and different, mitochondrial genomes. Those with the same mitochondrial genomes included blastocysts from autologous ePNT and from a zygote pair donated by two sisters, which we refer to as homologous ePNT. Blastocysts arising from heterologous ePNT represent new combinations of nuclear and mitochondrial genome and are subgrouped according to the cytoplast origin (see Extended Data Fig. 4). c, Flow diagram outlining the bioinformatics analysis of RNA-seq data. Data were normalized either as reads per kilobase per million mapped reads (RPKM) or using DESeq2 (ref. 36). Normalized data were used to generate PCA plots, t-SNE plots and heatmaps.
Extended Data Figure 6 | Analysis of differential gene expression in good quality ePNT and control blastocysts. 

(a) PCA matrix using the first ten principal components of DESeq2 VST normalized data for the top 12,000 most variable genes. Global gene expression is indistinguishable between unmanipulated control and ePNT samples, PC1 versus PC2 highlighted in blue box.

(b) PCA matrix as shown in (a), distinguished by lineage, clearly seen in PC2 versus PC3 (pink box).

(c) t-SNE analysis after DESeq2 VST normalization of 6,000 of the most variably expressed genes, where samples were distinguished by lineage. Sample numbers and blastocyst grades are shown. Autologous and homologous ePNT samples are derived from blastocysts in which the karyoplast and cytoplast had the same mitochondrial genome. Heterologous ePNT samples were derived from pairs of zygotes with different mitochondrial genomes (Extended Data Fig. 5). Samples from experimental controls and reference population were combined for the purpose of the analyses shown in (a) and (b).
Extended Data Figure 7 | Expression of lineage-specific genes and mitochondrial OXPHOS genes in control and ePNT embryos.

a, Heatmap showing log2-transformed RPKM values of selected differentially expressed genes in trophectoderm (n = 10), epiblast (n = 10) and primitive endoderm (n = 10) lineages. 
b, Heatmap showing expression of mitochondrial OXPHOS genes after unsupervised hierarchical clustering. Expression of OXPHOS genes encoded by mtDNA is variable both within and between blastocysts. Control and ePNT samples cluster together, irrespective of whether the cytoplast and karyoplast had the same (blue font) or different (purple font) mtDNA. Sample numbers and blastocyst grades are shown. The reference population includes a previously published series14. Autologous and homologous ePNT samples are derived from blastocysts in which the karyoplast and cytoplast had the same mitochondrial genome. Heterologous ePNT samples were derived from pairs of zygotes with different mitochondrial genomes (Extended Data Fig. 5b). Expression levels are indicated on a high-to-low scale (purple–white–green). Source data files are available online for a and b.
Extended Data Figure 8 | Measurement of heteroplasmy due to mtDNA carryover during ePNT. a, Mitochondrial genotypes were determined by identifying polymorphic variants in the hypervariable mtDNA control regions of each donor. Sequence electropherograms of mtDNA non-coding control region with a sequence variant used for pyrosequencing (highlighted) (m.73A>G). b, Sequence pyrograms for the mtDNA variant (m.73A>G) in control samples. The expected level of variant is given along with the level determined by pyrosequencing (in brackets). c, Examples of the standard curve generated to increase accuracy in detecting low levels (0–25%) of heteroplasmy by pyrosequencing, which has previously been reported to accurately detect heteroplasmy at a level of 1% (ref. 42). Each data point represents the mean of 3–4 technical replicates. d, mtDNA carryover was measured by pyrosequencing using clumps of cells (n = 92) from day 6 blastocysts (n = 40; names shown on y axis). The cells were predominantly obtained from the trophectoderm (TE) layer (purple, n = 67). ICM cells (red, n = 5) and cells of mixed trophoderm/ICM origin (green, n = 20) were also analysed. Each data point represents the mean of 2–3 technical replicates. e, mtDNA carryover from individual blastocysts calculated from data shown in d. Each data point represents either the mean value where more than one sample was tested (n = 28 ePNT blastocysts), or a single value where only one sample was tested (n = 12 ePNT blastocysts). Horizontal lines show median values for each experimental group. Blastocysts arising from ePNT performed in the absence of sucrose and fused with a fresh cytoplast (FreshCy) had significantly reduced mtDNA carryover compared with blastocysts where ePNT was performed in the presence of sucrose (P values and blastocyst numbers are shown; two-sided Mann–Whitney U-test). f, Graph showing the proportions of blastocysts (n = 40) with mtDNA carryover measurements falling within the specified levels (not significant; χ² test). Source data files are provided for c–f.
Extended Data Figure 9 | Derivation and characterization of human ES cells from control and ePNT blastocysts. a, Examples of outgrowths formed following explantation of the ICM from ePNT (n = 15) and control (n = 6) blastocysts used for hES cell derivations. The dashed white circle indicates the region picked for initial passage of the ICM outgrowth. Bottom, examples of hES cell colonies. b, Example of a normal karyogram from a PNT-hES cell line (45PNT); 4/4 lines tested showed a normal karyotype. The remaining hES cell line did not grow beyond passage 2 and was derived from a uniformly aneuploid blastocyst (55PNT; Extended Data Fig. 4). c, Immunostaining of representative PNT-hES cells (grown in mTeSR1) for NANOG, SSEA4 (green), SOX2 and OCT4 (red) with DAPI (blue) merge. Graph shows quantitative polymerase chain reaction with reverse transcription (qRT–PCR) analysis of control and PNT-hES cell lines for pluripotency transcripts NANOG, POU5F1 and SOX2. Horizontal line shows the median value, which is similar between hES cells from unmanipulated control blastocysts (Ctr.; n = 2 hES cell lines) and ePNT-hES cells (n = 4 ePNT-hES cell lines). d, Immunostaining of representative PNT-hES cells after 20 days in basal MEF media, confirming differentiation into all three germ layers: endoderm (α-fetoprotein (AFP); SOX17), mesoderm (α-smooth muscle actin (SMA); desmin (DES)) and ectoderm (β-III tubulin (TUJ1); SOX1) in green or red, with DAPI (blue) merge. Scale bars, 50 μm. e, Table showing the mtDNA variants and primers used to measure mtDNA carryover in PNT-hES cell lines. f, Summary table showing details of blastocysts and the corresponding hES cells. Aneuploidy in PNT-hES cell lines was analysed by metaphase spreads, except for 31PNT-hES, which was determined by array-CGH.
Extended Data Figure 10 | Heteroplasmy in subclones of the hES cell line derived from 36PNT. a, The 36PNT hES cell line was frozen at passage 3 (after derivation), thawed and subcloned to monitor heteroplasmy arising from the karyoplast donor mtDNA. Six colonies (15–20) were randomly selected at the first post-thaw passage (P3) and clumps of cells were plated on 3 tissue culture wells; 5/6 colonies gave rise to 3 subclones, which were grown to P11. Subclones are distinguished by colour in the graphs. Each data point represents the mean of two technical replicates for a single cell sample. Source data file is available online.
Corrigendum: Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease

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We wish to clarify the statistical methods used in this Letter. Owing to the limited number of observations, blastocyst quality in Fig. 2d was analysed by pooling grades A and B to compare them with all the other grades combined. This provides a clinically relevant measure and increases the power of the test. In addition, owing to the exploratory nature of the experiments, the interdependence between tests and the scarcity of embryos, we did not apply a correction for multiple comparisons. Tests were limited to those of clinical significance only. Statistical analysis was performed in R, GraphPad Prism and IBM SPSS. In addition, the legend to Extended Data Fig. 3f should say that numbers were deemed too small for statistical analysis and the legend of Fig. 4c should say that groups with different letters have significantly different proportions of samples with undetectable and <2% carryover combined. (The online versions of this Letter have not been corrected.)