



Human
Fertilisation &
Embryology
Authority

Scientific review of the safety and efficacy of methods to avoid mitochondrial disease through assisted conception: 2016 update

**Report to the Human Fertilisation and Embryology Authority (HFEA)
November 2016**

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Executive summary

The panel continues to see clinical value in maternal spindle transfer (MST) and pronuclear transfer (PNT) to mitigate or prevent the inheritance of mitochondrial disease. It recommends that, in specific circumstances, MST and PNT are cautiously adopted in clinical practice where inheritance of the disease is likely to cause death or serious disease and where there are no acceptable alternatives. The report describes the reasoning behind this decision and provides guidance regarding circumstances in which MST and PNT could be considered.

In February 2015 the UK Parliament approved regulations to permit the use of maternal spindle transfer (MST) and pronuclear transfer (PNT), collectively 'mitochondrial donation', to avoid serious mitochondrial disease. The regulations, which came into force on 29 October 2015, enable licensed fertility clinics in the UK to apply to the Human Fertilisation and Embryology Authority (HFEA) for a licence to perform mitochondrial donation treatments.

Although the regulations make it lawful to use mitochondrial donation in the clinic, the HFEA must be satisfied that the techniques involved are sufficiently safe and efficacious before any clinic can apply for a licence to offer mitochondrial donation. This report to the HFEA considers the scientific data relevant to an assessment of the safety and efficacy of MST and PNT and makes recommendations on whether either technique should be introduced into clinical practice. The final decision will rest with the HFEA board.

The process leading to a change in the law in the UK has a long history. The Human Fertilisation and Embryology (HFE) Act 1990 was amended in 2008 to allow for regulations to be passed to permit techniques that prevent the transmission of serious mitochondrial disease due to deleterious mutations in mitochondrial DNA (mtDNA), in recognition of research that had taken place over several years. In 2011, the Government asked the HFEA to examine the safety and efficacy of these techniques and in response the HFEA established a scientific panel, with broad-ranging scientific and clinical expertise, to examine the evidence¹. Two further reviews were carried out in 2013² and 2014^{3,4}. In addition, the HFEA was asked in January 2012 to carry out a [public dialogue](#)⁵ on the social and ethical impact of making these techniques available to patients.

MST and PNT have the potential to avoid transmitting serious mitochondrial disease from mother to child. In its clinical application, MST involves transferring the nuclear DNA from an oocyte with abnormal mitochondria and placing it into an oocyte with healthy mitochondria. PNT involves transferring the pronuclei from an embryo that has abnormal mitochondria and placing them into an embryo that has healthy mitochondria.

¹ HFEA 2011 Scientific review of the safety and efficacy of methods to avoid mitochondrial disease through assisted conception. Accessed at: http://www.hfea.gov.uk/docs/2011-04-18_Mitochondria_review_-_final_report.PDF.

² Annex VIII: Scientific review of the safety and efficacy of methods to avoid mitochondrial disease through assisted conception: update. Accessed at: www.hfea.gov.uk/docs/Mito-Annex_VIII-science_review_update.pdf.

³ Third scientific review of the safety and efficacy of methods to avoid mitochondrial disease through assisted conception: 2014 update. Accessed at: http://www.hfea.gov.uk/docs/Third_Mitochondrial_replacement_scientific_review.pdf.

⁴ Addendum to the 2014 update - Review of the safety and efficacy of polar body transfer to avoid mitochondrial disease. Accessed at: http://www.hfea.gov.uk/docs/2014-10-07_-_Polar_Body_Transfer_Review_-_Final.PDF.

⁵ HFEA Public Dialogue: Medical frontiers: debating mitochondria replacement. Accessed at: <http://www.hfea.gov.uk/9359.html>.

Reconvening the expert panel in 2016

This fourth scientific review of the safety and efficacy of mitochondrial donation follows a similar structure to the earlier reviews. As before, the aim is to provide a comprehensive overview of the scientific issues raised by mitochondrial donation and an assessment of the current state of the research. The panel was tasked with reviewing the latest evidence of safety and efficacy for the two mitochondrial donation techniques – MST and PNT, with particular reference as to whether the recommendations outlined in the 2014 scientific report have been met. However, whereas the previous reports were commissioned by the Government to inform a decision about whether to change the law, the context for this report is different: Parliament has changed the law and this report has been commissioned by the HFEA as part of an internal assessment of whether mitochondrial donation is ready for clinical practice.

In deciding to change the law Parliament has always been clear that neither MST nor PNT should be introduced into clinical practice until they were judged sufficiently safe. Before the first application for licensing can be received, the outstanding safety and efficacy experiments recommended in the 2014 scientific mitochondria review (see Box 1) need to be considered by the panel and then by the HFEA³.

Box 1: The conclusions and recommendations listed in the 2014 scientific review were as follows:

- MST using human oocytes that are then fertilised (not [artificially] activated): It is still important for some follow-up experiments to be carried out, notably to improve efficiency, if possible, and further corroborative experiments would be valuable.
- Experiments comparing PNT using normally-fertilised human oocytes with normal ICSI fertilised human oocytes: The method continues to be developed and appears promising. Further work will be published in the near future and those results will need assessing before they can be incorporated into recommendations.
- PNT in a non-human primate model, with the demonstration that the offspring derived are normal, is not critical or mandatory.
- MST and PNT should both be explored and that, as yet, it did not consider one technique to be preferable to the other.
- Consideration should be given to mtDNA haplogroup matching (see section 3.7.20 of the 2014 report) as a precautionary step in the process of selecting donors.

This fourth review was commissioned in response to recent publications reporting significant progress in addressing the recommendations above^{6,7,8}. Whilst these publications were the trigger, the review also

⁶ Hyslop LA, Blakeley P, Craven L, Richardson J, Fogarty NM, Fragouli E, Lamb M, Wamaitha SE, Prathalingam N, Zhang Q, O'Keefe H. Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease. *Nature*. 2016 Jun 8 534: 383-386.

Hyslop LA, Blakeley P, Craven L, Richardson J, Fogarty NM, Fragouli E, Lamb M, Wamaitha SE, Prathalingam N, Zhang Q, O'Keefe H. Corrigendum: Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease. *Nature*. 2016 Jul 27 538, 542.

⁷ Yamada M, Emmanuele V, Sanchez-Quintero MJ, Sun B, Lallo G, Paull D, Zimmer M, Pagett S, Prosser RW, Sauer MV, Hirano M. Genetic Drift Can Compromise Mitochondrial Replacement by Nuclear Transfer in Human Oocytes. *Cell stem cell*. 2016 Jun 2;18(6):749-54.

⁸ Kang E, Wu J, Gutierrez NM, Koski A, Tippner-Hedges R, Agaronyan K, Platero-Luengo A, Martinez-Redondo P, Ma H, Lee Y, Hayama T, Van Dyken C, Wang X, Luo S, Ahmed R, Li Y, Ji D, Kayali R, Cinnioglu C, Olson S, Jensen J, Battaglia D, Lee D, Wu D, Huang T, Wolf DP, Temiakov D, Izpisua Belmonte JC, Amato P, Mitalipov S. Mitochondrial replacement in human oocytes carrying pathogenic mitochondrial DNA mutations. *Nature* 2016 DOI: 10.1038/nature20592

took into account other relevant studies conducted since the previous review, in order to consider whether the techniques are now ready to be used in clinical practice and, if so, what clinical issues should be taken into account.

This review builds on the findings of the previous three reviews (including the addendum to the third review, exploring polar body transfer techniques), and is not written as a stand-alone summary of current scientific knowledge. For full details of areas reviewed, please refer to the previous reports. An updated timeline highlighting the key developments in the consideration of mitochondrial donation in the UK is at Annex B.

As noted above, the science relevant to the safety and efficacy of mitochondrial donation has been considered in detail by this panel a number of times since 2011. This has allowed the direction of travel to be assessed in addition to the current state of the methodology. On each occasion, the panel has reviewed evidence from experts directly in the field, both in the UK and abroad, and taken account of unpublished as well as published data and opinions. The panel's reports to Government in 2011, 2013, 2014, and now this one to the HFEA in 2016, reflect this composite evidence.

In 2016 the panel considered submissions received as a result of the call for evidence and reviewed new literature in this area, as set out in Annex C. The panel met on five occasions to allow some of those who had submitted evidence to present their work and take part in a roundtable discussion. These individuals or groups had been selected because of the direct relevance of their work to the methods being considered. The panel was therefore able to consult with a number of relevant research groups and additional experts in order to inform their conclusions on the progress of current research.

Progress on essential experiments and clinical data

The panel agreed that good progress had been made in experiments recommended in previous reviews and that additional clinical data should also be considered:

- MST using human oocytes that are then fertilised (not artificially activated): There has been significant progress in this area of research from the group of Professor Shoukrat Mitalipov and his collaborators⁸ demonstrating an increase in the efficiency of these methods using oocytes carrying pathogenic mtDNA mutations as well as from controls. Carryover of mtDNA was below 1% in MST blastocysts (Kang et al 2016). Whilst Yamada et al. (2016) used artificially activated rather than fertilised oocytes following MST, they report that the process of oxidative phosphorylation (OXPHOS) was normal in differentiated cell types from embryonic stem (ES) cells derived from MST embryos, despite using mtDNA combinations from distinct haplogroups. Similar results were also obtained by Kang et al (2016). Yamada et al. (2016) further corroborated these conclusions by studying ES cells derived from somatic cell nuclear transfer (SCNT) blastocysts.
- Experiments comparing PNT using normally-fertilised human oocytes with normal ICSI-fertilised human oocytes: Hyslop et al., (2016) demonstrated that PNT undertaken shortly after *in vitro* fertilisation resulted in the generation of embryos that were competent to develop to the blastocyst stage. Carryover of mtDNA was shown to be less than 2% and detailed characterisation of these blastocysts indicated no detectable increase in the incidence of aneuploidy or disruption to normal profiles of gene expression in isolated cells.
- The use of PNT to establish a pregnancy for a 30-year old nulligravid woman with unexplained infertility was published by Zhang et al. (2016a)⁹. After transfer of five embryos, a triplet pregnancy resulted that was surgically reduced to twins. The two remaining fetuses survived only to mid-

⁹ Zhang J, Zhuang G, Zeng Y, Grifo J, Acosta C, Shu Y, Liu H. Pregnancy derived from human zygote pronuclear transfer in a patient who had arrested embryos after IVF. *Reproductive BioMedicine Online*. 2016a Oct 31;33(4):529-33.

gestation, probably due to the obstetric complications of the multiple pregnancy, rather than the PNT itself. Neither fetus had detectable levels of the maternal (karyoplast-derived) mtDNA haplotype.

- A recent abstract, entitled “First live birth using human oocytes reconstituted by spindle nuclear transfer for mitochondrial DNA mutation causing Leigh syndrome” by Zhang et al. was published online in the programme for the October 2016 American Society for Reproductive Medicine (ASRM) meeting. The baby is reported to be doing well and was found to have low levels of mutated mtDNA in several tissues. Some details of the MST methods used are given by the authors in another abstract in the same programme¹⁰. However, the full details of the methods and treatment cycle have not yet been published.

The fate of mtDNA carried over after MST and PNT

In previous reviews the panel recommended experiments on mtDNA behaviour in human embryonic stem (ES) cells (and their differentiated derivatives) derived from blastocysts generated by MST and PNT. Although such experiments do not accurately model the developing fetus, they do permit an analysis of the mtDNA carried over in the karyoplast in conditions where mtDNA replication occurs. There have been three studies^{6,7,8}, all of which report that in the majority of ES cell lines the karyoplast-derived mtDNA haplotype remained at a similarly low level as in the blastocysts from which the cells were derived, even after many passages, or was lost altogether.

However, each study also reported exceptions to this, in which the karyoplast-derived mtDNA haplotype increased in proportion to that of the cytoplasm (one out of eight in Yamada et al., 2016; one out of five in Hyslop et al., 2016; and three out of 18 in Kang et al. (2016)). This “reversion”¹¹ was also seen in two out of 12 ES cell lines derived after SCNT by Yamada et al. (2016) and in one out of eight such lines described in Kang et al. (2016). Whilst the degree of reversion was variable and seemed stochastic when subclones of the ES cells were analysed, it could approach 100%. Data indicate that reversion may also occur in cells differentiating from ES cells^{7,8}, suggesting that it is not an issue specific to ES cells maintained in a pluripotent state.

Moreover, reversion can occur with normal or mutant mtDNA. Therefore, despite the findings of Zhang et al. (2016a and b), the panel could not rule out the possibility of reversion occurring in clinical application of these techniques. These experiments (Yamada et al., 2016; Hyslop et al., 2016; Kang et al., 2016), and their possible significance, are considered in detail in sections 3 and 4. Ideally, it would be possible to avoid any chance of reversion, an eventuality which may depend on understanding the underlying cause. Some possibilities are discussed in section seven.

MST or PNT: is there a preferred technique?

The panel continues to note that available data do not indicate whether one technique, MST or PNT, is preferable to the other at this stage and recommends that both should be considered. However, it notes that PNT is currently more refined within the UK. The panel also reiterates its opinion that polar body

¹⁰ Zhang J, Liu H, Luo S, Chavez-Badiola A, Liu Z, Munne S, Konstantinidis M, Wells D, Huang T. First live birth using human oocytes reconstituted by spindle nuclear transfer for mitochondrial DNA mutation causing Leigh syndrome. *Fertility and Sterility*. 2016b Sep 1;106(3):e375-6.

Liu H, Lu Z, Luo S, Chavez-Badiola A, Blazek J, Munne S, Huang T, Zhang J. In vitro fertilization and development of human oocytes reconstituted by spindle nuclear transfer to replace mutated mitochondrial DNA. *Fertility and Sterility*. 2016 Sep 1;106(3):e21.

¹¹ Different authors have used different terms to describe this phenomenon. In Kang et al. (2016) this is described as “reversion” because the predominant mitochondrial DNA haplotype found is that of the karyoplast. In Yamada et al. (2016) this phenomenon is termed “genetic instability” and in Hyslop et al. (2016) “genetic drift”.

transfer (PBT) techniques, discussed in the addendum to the 2014 report⁴, showed great promise as a means to minimise mtDNA carryover and avoid mitochondrial disease, but notes that current regulations and UK legislation do not permit this technique.

Recommendations

The key recommendations are conditional on a number of considerations, including a requirement for appropriate levels of skill being demonstrated by named practitioners within a named clinic, and relevant key performance indicators being met, parameters that will be assessed by the HFEA.

From a medical point of view, all novel treatments pose essentially the same question: when is a treatment sufficiently safe to offer to patients? Research cannot answer every question before a new treatment is offered, nor can it be expected to guarantee safety or efficacy when applied for the first time. It can only serve to reduce the risk; in this case of a child being born with symptomatic mitochondrial genetic disease, but with caveats concerning for whom this type of risk reduction strategy might be suitable and highlighting areas that need close attention. Patients must understand and accept the potential limitations of any proposed treatment, and possible risks, before proceeding. With this in mind, **the panel recommends that it is appropriate to offer mitochondrial donation techniques as clinical risk reduction treatment for carefully selected patients.**

In coming to this decision, the panel makes some key recommendations for using these techniques in clinical practice.

Patient selection

The panel suggests that MST and PNT should in the first instance be offered to selected patients for whom preimplantation genetic diagnosis (PGD) would be inappropriate, or unlikely to succeed. Like PGD undertaken for mtDNA mutations, MST or PNT can be used as a risk reduction strategy¹², but initially only in those patients in whose germ line there are likely to be high levels of heteroplasmy or homoplasmy for the abnormal (pathogenic) mtDNA, and who are thus unlikely to have any suitable embryos for transfer.

Pre-treatment assessment would need to take into account the particular mutation involved, the inheritance pattern in the family, the likely clinical manifestations of disease, the efficacy of any previous treatments such as PGD, and the patient's understanding of the risks and limitations of what is being offered. If the techniques prove to be safe when used in these patients, including the absence of any significant reversion to the carried-over mtDNA haplotype, their application could be extended to other patients.

Prenatal testing and follow-up

The panel advises that (i) all patients should be offered prenatal testing if they become pregnant following MST or PNT treatment, and (ii) centres offering MST or PNT should encourage patients and their offspring to take part in long-term follow-up.

Haplogroup matching

The panel continues to recommend that consideration is given to mtDNA haplogroup¹³ matching as a precautionary step in the process of selecting donors. As highlighted in the 2014 report, this is a complex

¹² Bredenoord AL, Dondorp W, Pennings G, De Die-Smulders CE, De Wert G. PGD to reduce reproductive risk: the case of mitochondrial DNA disorders. *Human reproduction*. 2008 Nov 1;23(11):2392-401.

¹³ A haplogroup is a term used to define a group of similar haplotypes. Mitochondria from separate human lineages can be classified according to similarities or differences in their DNA sequence into many different haplogroups. The more evolutionary distant the separation of two maternal lineages, the greater the differences between mitochondrial haplogroups.

topic, with some potential risks or benefits associated with choosing a specific donor mtDNA haplogroup/haplotype donor. At present, the panel believes any risks associated with a mtDNA-nuclear DNA mismatch remain theoretical; the recent studies examining embryonic cells and stem cells generated from MST- and PNT-derived human embryos reported no evidence of any complications or compromise of mitochondrial function arising from unmatched mtDNA haplogroups (see section 4.14 – 4.20). However, the panel recommends that when these techniques are used clinically, the latest evidence regarding how mtDNA haplotypes affect mitochondrial-nuclear (mito-nuclear) interactions, including replicative behaviour of mtDNA, should be considered in order to inform the donor selection process. Such evidence might even indicate the selection of a specific, unmatched donor in any given case.

Whatever decision is made, the panel recommends that haplotype information on the recipient and the donor is recorded. The panel also noted that in assessing this risk the treating clinician should be mindful of parallels with potential mito-nuclear mismatch in natural reproduction. Evidence for any effects associated with particular combinations of mtDNA and specific nuclear alleles in natural reproduction, perhaps together with any influence of environment, may come from large-scale genome studies linking DNA sequence with health outcomes^{14,15}.

Further research

The panel also highlights some promising areas for continuing research, particularly the exploration of methods to further reduce or eliminate mtDNA carryover, through refinements of the techniques, and possible development of new techniques, as discussed above. Furthermore, the panel concludes that it will be important to decide whether reversion towards karyoplast-derived mtDNA in some ES cell lines derived from embryos following MST or PNT is clinically relevant and if so, what underlying mechanisms are responsible. Further research possibilities are discussed in section 7.

¹⁴ Horikoshi M, Beaumont RN, Day FR, Warrington NM, Kooijman MN, Fernandez-Tajes J, Feenstra B, van Zuydam NR, Gaulton KJ, Grarup N, Bradfield JP. Genome-wide associations for birth weight and correlations with adult disease. *Nature*. 2016 Oct 13;538(7624):248-52.

¹⁵ Johnson SC, Gonzalez B, Zhang Q, Milholland B, Zhang Z, Suh Y. Network analysis of mitonuclear GWAS reveals functional networks and tissue expression profiles of disease-associated genes. *Human Genetics*. 2016 Oct 4:1-1.

1. Introduction

- 1.1.** Mitochondrial malfunction is a significant cause of several serious multi-organ diseases. These disorders can be due to mutations in nuclear DNA affecting gene products required within mitochondria, or mutations in DNA carried within the mitochondria themselves. Mitochondrial DNA (mtDNA) encodes 13 of the 90 or so products required exclusively for oxidative phosphorylation (OXPHOS), which generates energy for cells in the form of the energy-storing molecule, adenosine triphosphate (ATP)¹⁶. Although relatively rare, the seriousness of these diseases and, in particular, the unusual inheritance pattern of mtDNA mutations has made them a focus for research into preimplantation methods to reduce or avoid such diseases in offspring.
- 1.2.** The biology of mitochondria is complex and the attendant language is technical in parts. This report tries to explain the issues in as straightforward a manner as possible and Annex D provides a glossary with a definition of relevant terms.
- 1.3.** The terms of reference for the panel are: ‘to review the latest evidence of safety and efficacy for the two mitochondrial donation techniques – maternal spindle transfer (MST) and pronuclear transfer (PNT), with particular reference to whether the recommendations outlined in the 2014 scientific report have been met.’ Accordingly, this review focuses exclusively on the science and the safety and effectiveness of these techniques; it does not consider the ethical and legal issues that are raised by such techniques, except when they are directly relevant to proposed research and/or potential clinical applications.
- 1.4.** This report does not contain an in-depth review of preimplantation genetic diagnosis (PGD) to avoid mitochondrial disease because this can be found in the 2014 report (section 2); however, much of the discussion around recommendations and further research draws on a direct comparison between MST/PNT and PGD and their suitability for particular patients.
- 1.5.** In February 2015, Parliament approved regulations to permit the use of MST and PNT to avoid serious mitochondrial disease. The regulations came into force on 29 October 2015. Since then the HFEA has designed a system to license and regulate mitochondrial donation. Before a clinic will be allowed to carry out either of these techniques it will need to follow a two-stage licensing process: firstly, to apply to the Authority for a general licence for MST and/or PNT, and then to seek authorisation to undertake the treatment in the case of a particular patient. In making such an application, clinics will be required to submit information to the Authority about the patient being treated, including details of the sperm provider and of the mitochondria donor. The clinic will also be required to have a follow-up scheme in place to monitor any children born as a result of MST or PNT.

¹⁶ Although mitochondria have other functions within cells, such as in lipid metabolism and programmed cell death, these are exclusively encoded by nuclear genes.

2. Mitochondrial donation techniques

- 2.1.** Although prenatal testing and the option of pregnancy termination can be used when the fetus is at risk of mitochondrial disorder due to mutations in mtDNA, this is not acceptable to some couples. In addition, because of the peculiarities of inheritance of mtDNA, it is difficult to determine a safe threshold below which disease is unlikely to manifest; this threshold will differ according to the specific mutation carried by the patient and, moreover, levels of mutant mtDNA present in one tissue at one stage may differ from the level elsewhere and at other stages.
- 2.2.** Currently, PGD is used as one of the primary methods to avoid the birth of a child who would be born with, or develop, mitochondrial disease. However, PGD is not suitable for women who carry a high level of abnormal mtDNA in their oocytes, especially when the oocytes are homoplasmic¹⁷ for a given mutation¹⁸ (estimated to be the case in about 20% of patients at risk), since it is unlikely or impossible that there will be any embryos with a level of abnormal mtDNA low enough to avoid disease in any resulting children. Therefore, the reliability of the embryo biopsy result, and the level of abnormal mtDNA per embryo, are important factors when assessing the likely effectiveness and safety of PGD for mtDNA disorders.
- 2.3.** In cases where PGD is not appropriate, the transmission of mtDNA disease can be avoided by using healthy donated oocytes. This method is safe and has strong supporters; however, whilst this guarantees the disease is not transmitted, it also means that any resultant child will not be genetically related to the mother.
- 2.4.** The novel methods that are the focus of this review, MST and PNT, allow the transmission of both parents' nuclear DNA but in effect involve replacing abnormal mitochondria with normal mitochondria from a healthy donor oocyte. Since the donor oocyte is the intended source of all (or at least a large majority of) mitochondria in the prospective offspring, these techniques have come to be known as mitochondrial donation techniques, although many still use the term 'mitochondrial replacement techniques'¹⁹.
- 2.5.** MST uses micromanipulation techniques to transfer the woman's nuclear genetic material (the spindle with maternally-derived chromosomes attached) from one oocyte into another from which the nuclear genetic material has been removed²⁰ (Figure 1). The reconstituted oocyte is then fertilised with her partner's sperm (or donor sperm) to trigger embryo development.
- 2.6.** PNT uses similar micromanipulation techniques to transfer the nuclear genetic material, but in this case both the maternal- and paternal-derived pronuclei from a fertilised oocyte (zygote) are transferred into an enucleated one-cell embryo (zygote) derived from a fertilised donated egg (Figure 2). MST takes place between metaphase II oocytes (mature eggs) whilst PNT takes place

¹⁷ Where all the mitochondria in a cell contain the same mtDNA, which can be either all abnormal or all normal.

¹⁸ A woman may have very low levels in her somatic tissues, but homoplasmic oocytes. If the woman herself was homoplasmic, it is very likely that she would be suffering from mitochondrial disease. The only exception is where this is conditional on other factors, such as gene variants in nuclear DNA or environmental effects (e.g. sensitivity to gentamycin-induced deafness).

¹⁹ Mitochondrial Replacement Techniques: Ethical, Social, and Policy Considerations, The National Academies Press 2016.

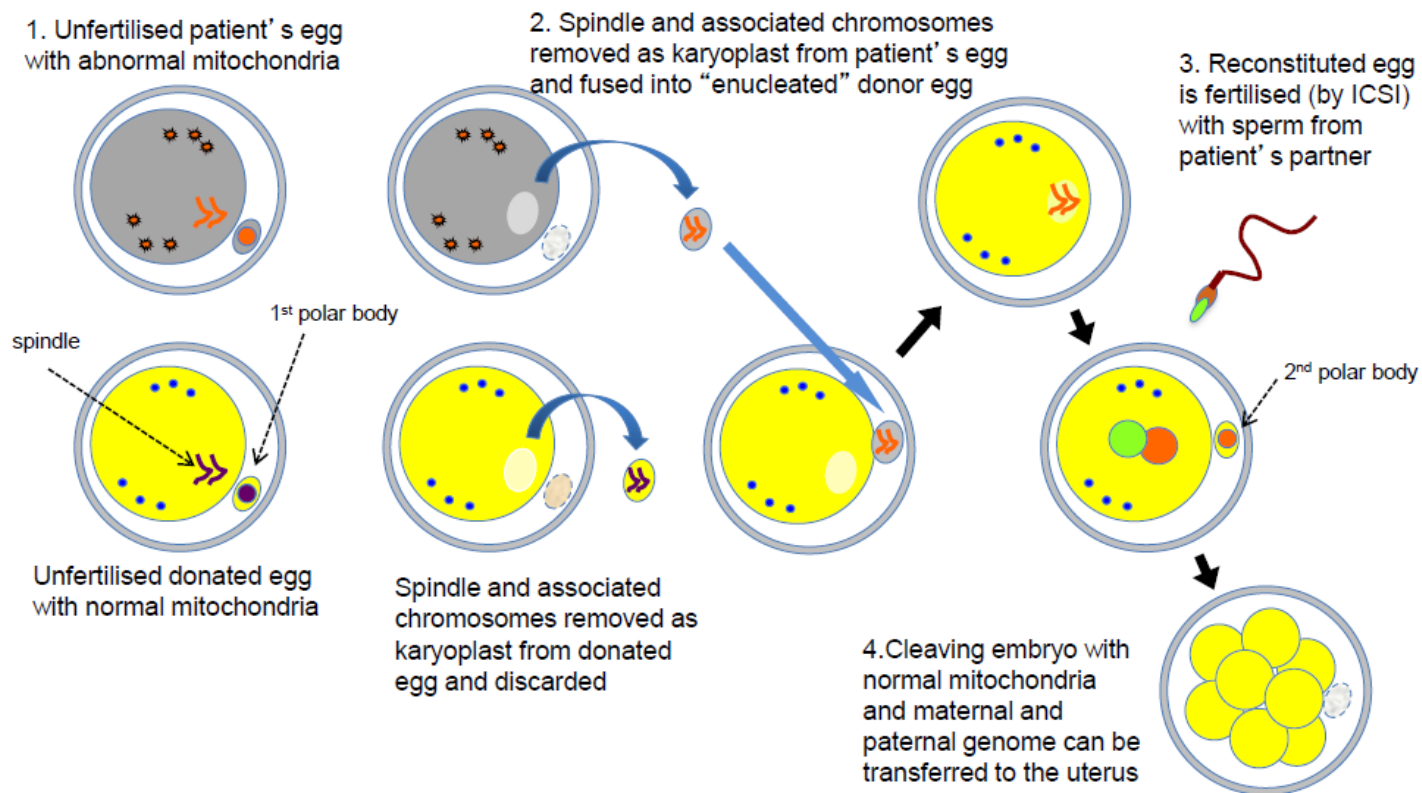
²⁰ This is equivalent to the oocyte being enucleated, and this term is used by some, although the chromosomes are not contained within a nuclear membrane at this stage.

between zygotes, after the stage at which the egg has been penetrated by sperm and the maternal and paternal pronuclei have formed, but prior to the breakdown of the pronuclear membranes, and the first embryonic cell division.

- 2.7.** Both techniques are therefore carried out prior to the mingling of maternal and paternal chromosomes within a one-cell embryo²¹. With either method, any resulting child would be genetically unique, inheriting one nuclear genome from each parent, whilst the mitochondria would largely be derived from the oocyte provided by the donor. These methods could therefore effectively substitute the mitochondria from the affected woman with mitochondria carrying normal mtDNA from the oocyte donor. If there were little or no transmission of abnormal mtDNA, this method could avoid mitochondrial disease not just in the resulting child, but also in subsequent generations. Detailed diagrams can be found in Figure 1 and Figure 2.

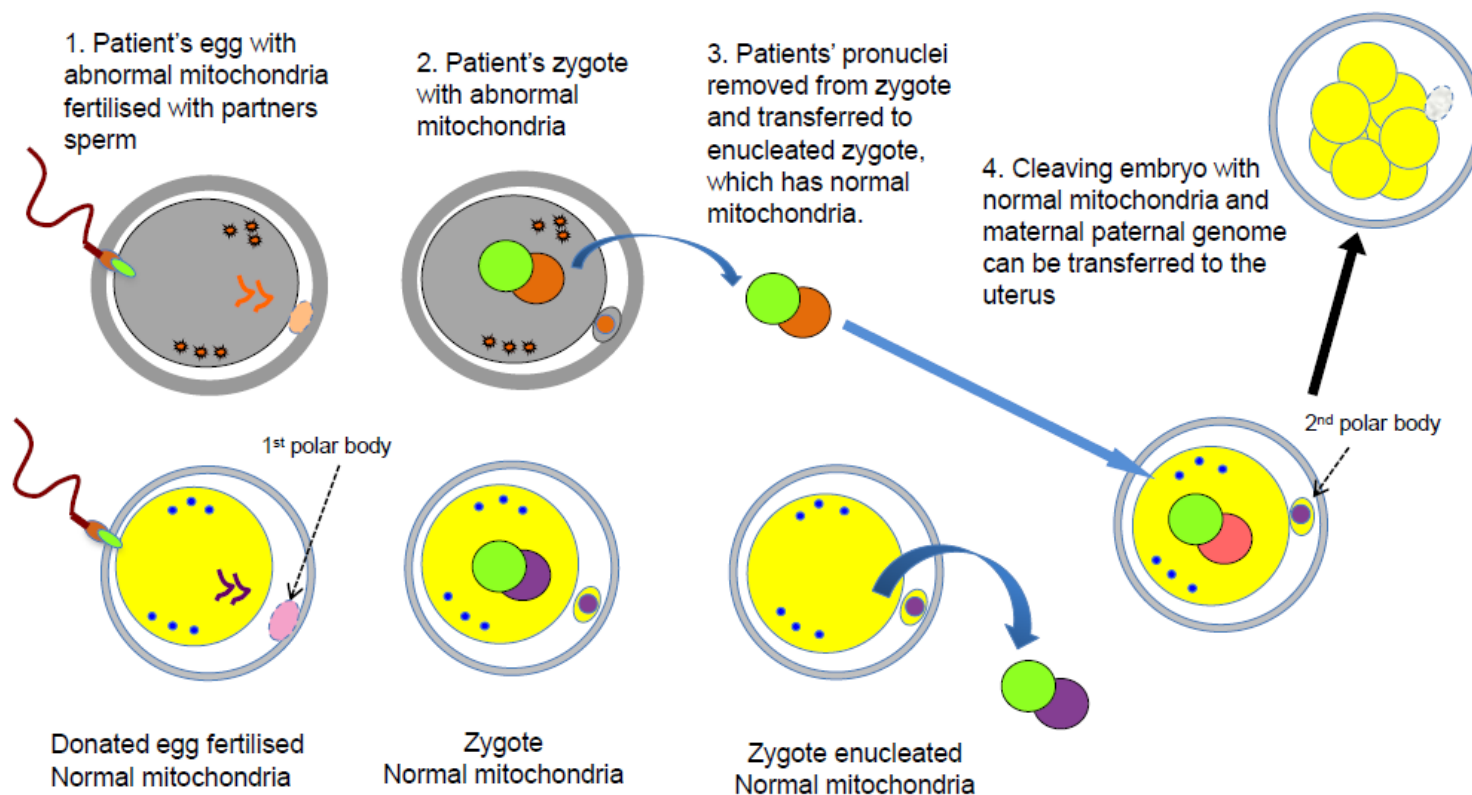
²¹ MST occurs pre-fertilisation and PNT occurs post-fertilisation but prior to the breakdown of the pronuclear membranes and syngamy.

Figure 1. Maternal spindle transfer (MST)



The 1st polar body is a diploid product of the first meiotic division, which occurs at ovulation. The 2nd polar body is a haploid product of the second meiotic division, which occurs on fertilisation (or after activation). The polar bodies fail to persist and do not contribute to the resulting embryo.

Figure 2. Pronuclear transfer (PNT)



The 1st polar body is a diploid product of the first meiotic division, which occurs at ovulation. The 2nd polar body is a haploid product of the second meiotic division, which occurs on fertilisation (or after activation). The polar bodies fail to persist and do not contribute to the resulting embryo.

3. Effectiveness of MST and PNT

Developments in maternal spindle transfer (MST)

Summary of previous considerations

In 2014 the panel considered evidence from the research group led by Professor Shoukrat Mitalipov at the Oregon Health and Science University. The panel was updated on progress with the macaques produced previously as a result of MST techniques and was informed that the four male macaques had then reached, or were approaching, adulthood (five years of age) and that they had shown no signs of abnormalities, but no further follow-up of health outcomes had been conducted.

The group was seeking to establish the fertility status of the macaques via a breeding programme and undertaking more focussed studies looking at physiological impact. At that time, there remained one female macaque who was then 2-3 years old (from a second set of experiments), but had not yet reached sexual maturity. In unpublished data, the Oregon group had further explored details of the methods used for MST with human oocytes, notably to reduce the rates of abnormal fertilisation and to increase efficiency.

- 3.1.** During this 2016 review, the panel considered new evidence from the New York group of Professor Dieter Egli, including their recently published work⁷ and discussion via video conference; from the Oregon group, via written submissions⁸ and video conference with Professor Shoukhrat Mitalipov; and from the New York-based group of Dr John Zhang, via video conference.
- 3.2.** Yamada et al. (2016) used artificially activated rather than fertilised oocytes following MST. They made use of vitrification (a cryopreservation technique) to avoid having to collect oocytes from two donors at the same time and allow synchronisation of stages. Although either karyoplasts (the spindle plus chromosomes, but inevitably also some cytoplasm, enclosed in plasma membrane), or cytoplasts (the oocyte minus the spindle and chromosomes) could be cryopreserved and used to obtain embryos after MST, cryopreservation of the former was more efficient. High quality blastocysts were used to derive a total of eight ES cell lines. Although all steps were reasonably efficient, the use of artificial activation is not clinically relevant; therefore, these statistics are not discussed here. However, it was considered that other aspects of the data obtained were comparable to experiments where fertilisation has been used (see below).
- 3.3.** The percentage of mtDNA carried over with the karyoplast was found to be between 0 and 2.2%, with an average of 0.2%, remaining constant throughout development to the blastocyst stage. Of the eight ES cell lines at early passages, four had lost all trace of the karyoplast-associated mtDNA haplotype. The other four contained between 0.2 and 1.7 %. By passage six,

no karyoplast-derived mtDNA could be detected in any of the cell lines except one, and this did not change for at least 30 passages.

- 3.4.** However, in the one exceptional cell line in this study, heteroplasmy for the karyoplast-derived mtDNA increased from 1.3% at derivation to 53.2% at passage 36. Subsequently, bulk cultures from this line or subclones derived from it showed seemingly random drift of heteroplasmy by around passage 40, with some subclones even becoming homoplasmic for either the karyoplast-derived or the cytoplasm-derived mtDNA haplotype (H1 or L3, respectively). However, the range of heteroplasmy reduced in the clonal lines around passage 50, and by passage 59, the karyoplast haplotype returned in bulk culture to the low level (1%) detected in early passages.
- 3.5.** Yamada et al. (2016) also made use of ES cell lines derived from blastocysts made by somatic cell nuclear transfer cloning (SCNT)^{22,23,24}. The mtDNA present within the somatic cell is introduced along with its nucleus into the enucleated donor oocyte. These SCNT-ES cell lines can therefore also be used to explore heteroplasmy and its consequences. While ten of the lines appeared homoplasmic for the donor oocyte mtDNA haplotype, two others showed similar unstable heteroplasmy as in the one MST-derived ES cell line, with a significant increase in the somatic cell-derived mtDNA haplotype.
- 3.6.** Yamada et al. (2016) obtained differentiated cell types *in vitro* from both MST-derived and SCNT-derived ES cell lines, both to monitor levels of heteroplasmy and to test mitochondrial function. They found that mtDNA instability could also occur following differentiation, implying that this phenomenon is not unique to ES cells. See section 4 for a more detailed discussion of the topic of reversion of mtDNA derived from the karyoplast.
- 3.7.** With respect to mitochondrial function, Yamada et al. (2016) analysed respiratory chain enzyme (RCE) activities, oxygen consumption, including coupling efficiency, cell respiratory control ratio, and spare capacity. The results suggest that the process of OXPHOS was normal in differentiated cell types from ES cells derived from both MST-derived and SCNT-derived embryos. This was the case even when using mtDNA combinations from distinct haplogroups.
- 3.8.** Yamada et al. (2016) conclude: “the maternal genome is fundamentally compatible with different mitochondrial genotypes and vertical inheritance is not required for normal mitochondrial function. Like the paternal genome at fertilisation, the maternal genome can form a novel combination of nuclear and mitochondrial alleles that is fully functional. This is not unexpected because maternal nuclear alleles may re-segregate with a new mtDNA genotype after inheritance through a son. The continuous re-combination in human reproduction therefore requires fundamental compatibility of nuclear and mitochondrial genomes.”
- 3.9.** In his published article⁷ and during the interview, Prof. Egli discussed potential issues of heteroplasmy. He pointed out that if MST or PNT were used clinically in cases where the patient's oocytes were heteroplasmic for mutant and normal mtDNA, and if one or both persisted and amplified, any resulting child could contain the donor (cytoplasm)-derived mtDNA haplotype, patient-derived mutant mtDNA and/or patient-derived normal mtDNA, which will be the same haplotype but differ in the specific mutation. Prof. Egli was concerned not only for the possibility of reversion to mutant mtDNA, which could lead to mitochondrial disease, but also because of

²² Chung YG, Eum JH, Lee JE, Shim SH, Sepilian V, Hong SW, Lee Y, Treff NR, Choi YH, Kimbrel EA, Dittman RE. Human somatic cell nuclear transfer using adult cells. *Cell Stem Cell*. 2014 Jun 5;14(6):777-80.

²³ Ma H, Folmes CD, Wu J, Morey R, Mora-Castilla S, Ocampo A, Ma L, Poulton J, Wang X, Ahmed R, Kang E. Metabolic rescue in pluripotent cells from patients with mtDNA disease. *Nature*. 2015 Jul 15

²⁴ Noggle S, Fung HL, Gore A, Martinez H, Satriani KC, Prosser R, Oum K, Paull D, Druckenmiller S, Freeby M, Greenberg E. Human oocytes reprogram somatic cells to a pluripotent state. *Nature*. 2011 Oct 6;478(7367):70-5.

concerns that two normal mtDNA genotypes at approximately equal levels could result in altered mitochondrial function. Such findings have been reported in mice²⁵, although, as was argued in the panel's previous reports, their relevance to humans is unclear given that the two mtDNA haplotypes used were very different and the mice were highly inbred.

Whilst in a distinct genetic context, it is worth noting that a recent study of 12 tissues obtained at autopsy from each of 152 individuals, ranging in age at death (due to causes other than mitochondrial disease) from three days to 96 years suggests that tissue- and allele-related mtDNA heteroplasmy is widespread in human tissue. That this heteroplasmy can be so extensive is consistent with positive selection for the specific somatic mtDNA mutations, and stochastic or ratchet effects^{26,27}.

- 3.10.** Professor Mitalipov briefed the panel, via video conference, on the status of the macaques created previously by MST. The five offspring showed no signs of abnormalities and were developing comparably with controls. Reversion to the mtDNA haplotype associated with the transferred spindle had not been observed in peripheral tissues (skin, blood and cells from urine). There appeared to be no effect on fertility of the males: several pregnancies are ongoing. The single female MST macaque is due to reach sexual maturity in the coming year and will also be used as part of a breeding program to examine fertility and the transmission of mitochondria to offspring.
- 3.11.** The group of Prof. Mitalipov has further explored details of the methods used for MST with human oocytes that were then fertilised. Some of this work was provided in the form of a draft manuscript (now published)⁸ and this, together with additional unpublished work, was discussed with Prof. Mitalipov in the video conference. These experiments sought to increase the efficiency of the method, to further test the use of cryopreservation, which could be used to manage the treatment of the patient independently of the donor (and separate induced ovulation from implantation), and to investigate *in vitro* reversion to spindle-associated mtDNA. They were able to include a small number of patients carrying pathogenic mtDNA mutations (causing Leigh Syndrome or MELAS) and at risk of having affected children to explore how MST might work clinically. Karyoplasts (the spindle plus chromosomes and cytoplasm enclosed in plasma membrane) were introduced by fusion into 'enucleated' oocytes (cytoplasts) donated by women with normal mtDNA. The reconstructed oocytes were then fertilised by ICSI and the resulting embryos allowed to develop to the blastocyst stage. Some of the latter were used to derive ES cells for further analysis, notably of karyotypes and to explore the behaviour of mtDNA from both the patient (carried over with the spindle), and from the donor. Two sets of controls were used for comparisons: embryos produced after MST between normal donated oocytes, and unmanipulated oocytes (all fertilised by ICSI).
- 3.12.** The cytoplasts that were left over after removing the karyoplasts from the mutant mtDNA carrier oocytes were analysed to provide a measure of the level of heteroplasmy in each. This was compared with that seen in biopsies from the patients, in cells biopsied from the blastocysts after MST, and in ES cell lines were derived from the latter.

²⁵ Sharpley MS, Marciniak C, Eckel-Mahan K, McManus M, Crimi M, Waymire K, Lin CS, Masubuchi S, Friend N, Koike M, Chalkia D. Heteroplasmy of mouse mtDNA is genetically unstable and results in altered behavior and cognition. *Cell*. 2012 Oct 12;151(2):333-43.

²⁶ Li M, Schröder R, Ni S, Madea B, Stoneking M. Extensive tissue-related and allele-related mtDNA heteroplasmy suggests positive selection for somatic mutations. *Proceedings of the National Academy of Sciences*. 2015 Feb 24;112(8):2491-6.

²⁷ Wallace DC, Chalkia D. Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. *Cold Spring Harbor perspectives in biology*. 2013 Nov 1;5(11):a021220.

3.13. The observations made can be divided into: (1) those that concern the patients and their management compared with controls, and (2) the outcome of using MST:

1.(a) Estimates of fertility potential (ovarian reserve measured by antral follicle count, Anti Mullerian Hormone (AMH) and yield of oocytes) in patients suggested they were compromised in comparison with controls; however, as the authors note, the numbers were very low: just four patients were involved. This contrasts with reports of PGD for mtDNA disorders that show reasonable numbers of embryos suitable for biopsy can be obtained^{28,29,30,31}.

(b) High fertilisation rates, comparable to controls, were observed using MST oocytes. Abnormal fertilisation or activation events (e.g. those giving rise to unipronucleate or tripronucleate embryos) appeared more frequent when using oocytes obtained from the patients compared with either MST or unmanipulated controls, perhaps related to the lower oocyte yield, and quality; but again the numbers were low and differences not statistically significant.

(c) Vitrification was used in some cases to allow oocytes from donors and carriers to be obtained at different times, but this seems not to have been as useful as in the recent studies from the Newcastle and New York groups^{6,7}.

(d) As anticipated from previous studies³¹ the levels of heteroplasmy within oocytes from a given patient correlated better with the clinical picture seen in her previous children than it did with the levels found in her somatic tissues.

2. (a) All karyoplasts, whether from patients or controls, and cytoplasts, survived the MST procedures, except for one in the control group. Whilst several embryos produced by MST showed karyotype abnormalities, the frequency was similar to that seen in unmanipulated controls. The proportion developing to blastocysts, ~75%, and their quality was also similar between MST-derived embryos and controls; moreover, this was irrespective of the number of differences (SNPs) distinguishing the mtDNA haplotypes of the two original oocytes used for MST. The latter could suggest that the potential for such differences to disrupt mito-nuclear interactions, as proposed to occur in a number of experimental situations involving exchange of mtDNA haplotypes in inbred animals³², is not realised during human embryo development, at least until the blastocyst stage.

(b) Out of a total of six blastocysts obtained after MST, using oocytes from the four patients, four were judged to be of sufficient quality for transfer, one was of low quality and one was aneuploid.

(c) Measurement of levels of karyoplast and cytoplast-derived mtDNA haplotypes in biopsies from embryos up to blastocyst stages showed that the degree of carryover of the former was consistently less than 1%, and this was irrespective of whether the karyoplast came from patients or controls.

²⁸ Thorburn DR, Wilton L, Stock-Myer S. Healthy baby girl born following pre-implantation Genetic diagnosis for mitochondrial DNA m. 8993t> g Mutation. In MOLECULAR GENETICS AND METABOLISM 2009 Sep 1 (Vol. 98, No. 1-2, pp. 5-6). ACADEMIC PRESS INC ELSEVIER SCIENCE.

²⁹ Monnot S, Gigarel N, Samuels DC, Burlet P, Hesters L, Frydman N, Frydman R, Kerbrat V, Funalot B, Martinovic J, Benachi A. Segregation of mtDNA throughout human embryofetal development: m. 3243A> G as a model system. Human mutation. 2011 Jan 1;32(1):116-25.

³⁰ Steffann J, Gigarel N, Samuels DC, Monnot S, Borghese R, Hesters L, Frydman N, Burlet P, Frydman R, Benachi A, Rotig A. Data from artificial models of mitochondrial DNA disorders are not always applicable to humans. Cell Rep. 2014 May 22;7(4):933-4.

³¹ Sallevelt SC, de Die-Smulders CE, Hendrickx AT, Hellebrekers DM, de Coo IF, Alston CL, Knowles C, Taylor RW, McFarland R, Smeets HJ. De novo mtDNA point mutations are common and have a low recurrence risk. Journal of Medical Genetics. 2016 Jul 22:jmedgenet-2016.

³² Morrow EH, Reinhardt K, Wolff JN, Dowling DK. Risks inherent to mitochondrial replacement. EMBO reports. 2015 May 1;16(5):541-4.

(d) The level of the karyoplast-derived mtDNA haplotype remained very low or was absent in the majority of the ES cell lines, but after prolonged culture, three out of 18, including one from a patient-derived blastocyst, showed a loss of mtDNA of the donor oocyte (cytoplasm) haplotype and “reversion” to the mtDNA haplotype associated with the karyoplast. See below for a more detailed discussion of the topic of reversion of mtDNA derived from the karyoplast.

- 3.14.** The group of J. Zhang used MST with human oocytes¹⁰. Reconstituted oocytes were fertilised by ICSI and cultured until blastocyst stages after which a trophectoderm biopsy was performed and tested for mtDNA heteroplasmy carryover and for aneuploidy. Frozen donor oocytes not employed for MST were used as controls. In contrast to the groups of Mitalipov, Egli and Herbert, they used an electric pulse to trigger membrane fusion between the karyoplast and cytoplasm rather than a viral fusogen³³. Only minimal details of the adapted electrofusion method used were given in the Zhang et al ASRM abstracts, which report normal rates of development and that 56% of MST-derived blastocysts were euploid (which would not be expected if there had been premature activation), and this was comparable with control blastocysts (53%). These techniques were applied to oocytes from a patient who carried 24.5% mutated mtDNA at position of 8993 T>G (Leigh syndrome), but whose oocytes probably had a much higher level. (It was reported that: “She had 4 pregnancy losses and 2 deceased children at age 8 months and 6 years from Leigh syndrome as confirmed by >95% mutation load.”) Despite a large number of poor quality oocytes, five MST oocytes were fertilised and four developed to blastocysts, although in this case only one was euploid (46 XY) with 5.7% of mutated mtDNA. It should be noted that this appears to represent a degree of carryover somewhat higher than others have obtained with MST. This embryo was transferred to the patient who, it is reported, gave birth to an apparently healthy boy at 37 weeks gestation. The mutation load in different tissues ranged from undetectable to 9.23%.
- 3.15.** Overall these studies indicate that the MST methods worked well, with reasonable success rates, even with oocytes from women carrying pathogenic mtDNA. Several steps during oocyte retrieval may have been more inefficient for carriers compared with controls, but this may also reflect their age distribution and perhaps BMI, and the numbers were too low to reach firm conclusions. Although there was little evidence of mito-nuclear mismatch in their experiments, Prof. Mitalipov also suggested that matching donor and recipient mtDNA haplotypes may be desirable.

³³ Electrofusion has been used successfully in experiments with mice and other mammals (Tsunoda, Kato and Shioda, 1987; Kona and Tsunoda, 1988). It had also been tried by other groups working on MST with human oocytes. However, it was abandoned because it led to poor viability of the resulting embryos. Notably, in experiments by Tachibana et al (2009) on MST with macaques, it was found to promote activation (and premature completion of meiosis) without fertilisation at much higher frequencies than Sendai viral fusogen; and similar results were reported by Paull et al (2013) using MST with human oocytes.

Developments in pronuclear transfer (PNT)

Summary of previous considerations

In 2014 the panel was updated on the progress of the Newcastle group's work, in both written submissions and presentations at the panel workshop. The panel was informed that the group had made considerable progress modifying their experiments from those using zygotes that were the product of abnormal fertilisation, which they were originally constrained to use, to those involving normal fertilisation.

Although the products of abnormal fertilisation tend to arrest early in development, irrespective of the time of fertilisation, this problem does not typically occur after normal fertilisation, which allowed the group to establish new procedures and working protocols to improve PNT efficiency. These and other modifications to the PNT procedures gave reproducibly high rates of development to the blastocyst stage. A manuscript describing these data was being prepared by the group for publication.

The group had also proceeded with experiments required to demonstrate the safety and efficacy of these techniques, by determining chromosome make-up, cell numbers, markers of cell type, and mtDNA carryover. This work had identified some subtle differences in embryo development that were being investigated, but nothing had been found to raise safety concerns. Pyrosequencing analysis had allowed the group to demonstrate that PNT using normally fertilised human eggs showed very low or absent levels of carryover of mtDNA – less than 2%. The group was in the process of deriving ES cells from human embryos created by PNT.

The Newcastle group was also in the first phase of using MST on human eggs in order to compare MST and PNT; however, they explained that due to the limited number of human eggs available for research, it was difficult to carry out comparative MST and PNT experiments in parallel. The group was open-minded as to which technique should be used clinically.

In 2014 the panel concluded that good progress had been made in developing PNT and that researchers looked likely to make further progress in refining the technique over the short term to ensure efficacy and in establishing safety (within the constraints of *in vitro* conditions). As stated in the 2013 report, the panel still believed that at that time there was insufficient evidence to choose between MST and PNT as a preferred technique.

- 3.16.** During this 2016 review, the panel received further information from the Newcastle group, both in written submission and in a presentation in person. The group has improved their PNT protocol, by altering the timing of pronuclear transplantation (with respect to the cell cycle stage of both the pronucleus donor and recipient oocytes) and changing the media used in the process. This work, in progress during 2014, has now been published⁶. By transplanting pronuclei soon after completion of meiosis (approximately eight hours after insemination by ICSI - early PNT, ePNT), rather than shortly before the first mitotic division (late PNT, ltPNT), they improved survival of reconstituted zygotes to 92% (vs 59% for ltPNT). Omission of sucrose used to induce shrinkage of the cytoplasm, and changes to the composition of the medium, also improved blastocyst survival, but not quite to the levels of controls or autologous PNT. Carryover of mtDNA was reduced to <2% in 79% of PNT blastocysts; none had carryover >5%. No significant differences in either aneuploidy rates or profiles of gene expression were found in comparisons between ePNT-

derived and control blastocysts, both parameters being examined extensively⁶. This analysis included single cell RNAseq data, which was clearly able to distinguish cells from the different lineages present within blastocysts, and to distinguish cells obtained from normal versus aneuploid embryos. Following modification of the manipulation and embryo culture medium, the group found that the PNT manipulations did not have a detrimental effect on blastocyst formation or quality. Although a manuscript posted on the PeerJ preprint server³⁴ has challenged aspects of the statistical analysis presented in Hyslop et al (2016), further details provided by the authors of the latter paper argue that their original analysis and conclusions hold.

- 3.17.** The panel concluded that there has been good progress in PNT techniques, which have been refined towards optimisation.

Experiments using abnormal mitochondria

Summary of previous considerations

In 2014 the panel and consulted experts reflected on whether it was necessary to conduct studies using oocytes from women affected by mitochondrial disease. Some experts recommended that as a “gold standard” they would like to see experiments conducted using oocytes from women affected by mitochondrial disease to see if pathogenic mutations behave differently from polymorphisms.

The panel discussed the rationale and the extent of this testing, i.e. would it be required for every type of mutation given that about 300 have been reported to date, which would be impractical, or for only a few? Moreover, the choice of which to test might depend on the specific issues to be examined, such as segregation and bottleneck effects, or consequences on mtDNA-nuclear gene interactions, and whether it is reasonable to extrapolate from one type of mutation to another.

In 2014 the panel considered evidence from an animal model demonstrating that certain mtDNA haplotypes may have an advantage on specific nuclear DNA backgrounds. Any study on pathogenic mutations in the context of MST or PNT would have to control for this, which may be impossible. Proof-of-principle experiments have been carried out using PNT with mice carrying mutant mtDNA and these rescued respiratory diseases in the derived progeny. There are now additional mouse models with other types of mtDNA mutation, but while similar PNT experiments could be carried out with these, they would still be open to the criticism that they might not reflect the human situation.

The panel raised the considerable ethical and practical issues around such research; whether it would be reasonable to request women who are keen to achieve an unaffected pregnancy using MST or PNT to donate eggs to this type of experiment that would not necessarily benefit their aim, and would require additional exposure to ovarian stimulation. The scientific rationale would have to be sufficiently strong to ask for this step, especially as some of the women may

³⁴ The panel received an open access, non-peer-reviewed article [submission](#) from EH Morrow and FC Ingleby, which claims that the Hyslop conclusions are premature until sufficient data are available to carry out statistical modelling. The panel notes that this degree of discriminatory power would likely require large numbers of human embryos, which would be neither feasible nor ethically unproblematic. The panel also received a submission from some authors of Hyslop et al. 2016 rebutting the arguments of Morrow and Ingleby.

already have had one or more affected children. Furthermore, as they get older the fertility of the women is likely to decline and treatment is less likely to succeed.

The panel concluded, in 2014, that this is an area where a recommendation could be revisited in the transition between the current research on the safety and efficacy of the techniques and any future consideration of how early clinical use might be conducted. It does not consider this an obligatory step at present. Moreover, and bearing in mind the ethical issues outlined above, if incorporated prior to early clinical use it would be reasonable to choose only one or a few of the most common types of mutation. In addition, clear experimental objectives would need to be defined, which would depend on the nature of any prevailing questions and data concerning any potential replicative advantage attributed to the specific mutation in question.

- 3.18.** In 2016 the panel considered the evidence from Prof. Mitalipov and his team (as described in Kang et al. 2016), reporting experiments using MST with oocytes obtained from four patients who carried abnormal mtDNA and were at risk of having affected children. This was clearly a useful exercise for his team, and it revealed some of the complications of conducting IVF procedures with the patients. However, apart from the need to coordinate oocyte collection from donors as well as the patients, which can be managed through cryopreservation, these issues would be similar to those encountered when using PGD to avoid mitochondrial disease. In terms of the use of the MST techniques and results obtained with them, these were no different from the experiments conducted in parallel with oocytes from normal donors. There is also a report of the recent birth of a child after MST for a patient carrying a mtDNA mutation (8993 T>G) (see 3.15 above); however, insufficient details are currently available, and the numbers involved are too low to draw any general conclusions.
- 3.19.** **The panel continues to believe that conducting studies using oocytes from women affected by mitochondrial disease is not an essential area of research, in advance of clinical use.**

Experiments using non-human primates

Summary of previous considerations

In 2014, following further discussion with researchers, the panel upheld the view that the use of non-human primate experiments was no longer critical and raised this for discussion at the scientific workshop in 2014. One expert thought that currently successful macaque trials exploring MST techniques do not invalidate the possibility of mito-nuclear incompatibilities, despite the use of two genetically distant subpopulations, and that the current studies have insufficient statistical power to detect mito-nuclear incompatibilities.

Demonstrating that mito-nuclear interactions are not of practical concern would require a much larger sample of macaques. An experimental design that would be statistically significant would involve >5 genotypes, with replicate observations, resulting in approximately 40 offspring in total. Trans-generational effects would require breeding and monitoring of subsequent generations.

Although these reviews are focused on the science, there are ethical concerns about carrying out experiments on animals, especially non-human primates, if these are likely to not be informative. Therefore, as stated in 2013, given that the most critical species in which to obtain

results is the human, and because there are differences in the very early embryology between mammalian species, the panel also concludes that if any additional experiments on MST and PNT in other animal models reveals differences from humans, it would be not just reassuring, but important if such experiments revealed the underlying reasons, and did not merely state the problem.

It is therefore the panel's view that such experiments would be difficult to justify with respect to cost, ethical considerations and the length of time required, especially as any risks were considered to be small. The panel suggested that a more informative experimental approach would be based on the derivation of embryonic stem cell lines and subsequent differentiation of these to examine oxidative phosphorylation, gene expression levels, and other physiological parameters in distinct human lineages derived from MST/PNT embryos.

- 3.20.** During this 2016 review, the panel did not receive any evidence to indicate that other groups have had success with PNT in macaques, nor that anyone is attempting such experiments. The ethical arguments above still stand: it would be a major undertaking to embark on a study of PNT in macaques, or other non-human primates, when the methods need to be established, when there are clear differences in the biology of early macaque and human embryos that may prevent this, and when PNT does work with human zygotes, which is of direct relevance. Furthermore, many of the questions that these experiments might address, namely issues of mtDNA carryover and its subsequent behaviour in offspring, can be addressed by studying the macaques derived by MST. Other work, for example, studying the behaviour of mutant mtDNA after mitochondrial replacement, is best performed in a non-primate animal model where this has already been derived, namely the mouse, or in human embryos and cell lines *in vitro*.
- 3.21.** As in previous years, the panel believes that PNT in a non-human primate model, with the demonstration that the offspring derived are normal, is not required.

4. Safety of MST and PNT

- 4.1.** The safety of MST and PNT has been a central concern of each review. The first review undertaken in 2011 was based on studies published up to March 2011 (outlined in section 4.3 of the original report). In 2013, the panel re-examined and commented on the following safety issues: the carryover of mtDNA from the affected oocyte or zygote; the methods to prevent premature activation of oocytes or detect abnormally fertilised oocytes; the mito-nuclear interactions involved and the potential for long-lasting nuclear epigenetic modifications resulting from manipulation or altered mitochondrial states associated with mitochondrial disease. The panel revisited these topics in 2014, updating their views on each strand, where applicable³⁵. In 2016 the panel also considered these areas, with an additional focus on considering work using embryonic stem (ES) cell lines to understand the fate of mtDNA that had been carried over with the karyoplast.

mtDNA carryover

Summary of previous considerations

In previous reports the panel highlighted the potential concern for subsequent generations if a female child born after the use of these techniques had a proportion of oocytes with a significant level of heteroplasmy. It suggested that this could be investigated by, for example, following differentiation protocols reported to generate primordial germ cells from human ES cells *in vitro*. Alternatively, it may be sufficient to explore these 'bottleneck' issues by looking at clonal ES cell lines. If it turned out that there was a significant risk that a proportion of oocytes, and therefore any resulting embryos from a women born after MST or PNT, could be heteroplasmic, then a recommendation might be made for her to make use of PGD to select for embryos homoplasmic for the normal mtDNA variant, or with very low heteroplasmy.

In 2013, the panel concluded that any early segregation of a very low level of mutant mtDNA was unlikely to be a problem for children born as a result of MST (or PNT). In 2014, the panel established that no new evidence had been presented in relation to this area. The panel continued to recommend that, if any uncertainty about the degree of heteroplasmy in oocytes remains when women born as a result of MST or PNT wish to have children, then either this should be examined directly in their unfertilised oocytes collected after ovarian stimulation, and/or PGD should be carried out on fertilised embryos prior to selecting those with no, or very low levels of, abnormal mtDNA for transfer. This should minimise the chance of transmission of abnormal mtDNA to subsequent generations.

- 4.2.** Carryover of mtDNA from the affected oocyte or zygote is expected with both MST and PNT because, during micromanipulation, the spindle or the pronuclei are surrounded by a small amount of cytoplasm that contains mitochondria. Evidence presented to the panel in 2013, and since, confirms that carryover after mitochondrial replacement is very low, and refinements in

³⁵ In 2014, the panel discussed the role of reagents used in both techniques and concluded that the MHRA would need to be satisfied about the provenance and clinical safety of all reagents. The panel were satisfied, in 2016, that the clinical safety of all reagents was being considered, and validated.

techniques of karyoplast extraction have continued to reduce it for PNT (including ePNT), and MST, such that the levels are <2% in the vast majority of embryos tested^{6,7,8}. Levels of abnormal mtDNA of 2% would not be expected to cause pathologies; indeed, many individuals probably exhibit similar levels in somatic tissues without any obvious consequences^{36,37}. Moreover, much higher levels of heteroplasmy than 2% are deemed acceptable in PGD for mtDNA mutations. However, carryover of abnormal mtDNA following MST or PNT may cause problems subsequently if it is preferentially amplified (perhaps by effects on mtDNA replication) and/or if there is a marked difference in segregation across tissues, either because of factors specific to these methods or because of the presence of two different mtDNA haplogroups/haplotypes.

- 4.3.** The panel also considered that by extrapolating data on macaques derived by MST, if the child were female, then while some of her oocytes may have very low or undetectable levels of mutant mtDNA, it is possible that others have a significantly higher proportion, considerably higher than her somatic tissues. These levels may still not be sufficient to predispose her children to manifest disease, but subsequent generations could be affected. Although diagnostic technology may well have advanced by then, the use of PGD (on embryos³⁸ from oocytes of female offspring resulting from MST or PNT, who might be carriers of the mutation in some of their oocytes) would enable the preferential selection for implantation of embryos whose tested blastomeres or trophoblast cells had low levels of abnormal mitochondria³⁰. This could reduce the risk of mitochondrial disease in subsequent generations.

Derivation of embryonic stem cell lines to explore the fate of carried-over mtDNA

- 4.4.** In this 2016 review, the panel considered recent data by Yamada et al. (2016) and the Newcastle group⁶ on ES cells obtained from parthenogenetic MST embryos and normally-fertilised PNT embryos, respectively. It also considered the data from the laboratory of Prof. Mitalipov⁸ that described MST in human oocytes that were normally fertilised (now published⁸). All three groups sought to assess the potential fate of mtDNA that had been carried over with the karyoplast by deriving human embryonic stem (ES) cell lines. Hyslop et al. (2016) derived five ES cell lines from ePNT blastocysts, having also assessed the levels in the blastocysts from trophectoderm biopsies.

While all PNT-ES cell lines showed low levels of heteroplasmy at passage 1 (P1), and three of the lines retained this low level after extensive culture, one line (36PNT), derived from a blastocyst with 4% mtDNA carryover, was unique in showing an upward drift with wide variation in levels of heteroplasmy between colonies by P12. The group confirmed this by experiments in which individual colonies from the original line were subcloned and cultured for multiple passages (the fifth hES cell line was not studied beyond passage 2). Hyslop et al. highlighted that the karyoplast and cytoplasm donors for the cell line showing this upward drift in karyoplast-derived mtDNA belonged to the same mtDNA haplogroup, whereas the other three lines studied all involved

³⁶ Greaves LC, Nooteboom M, Elson JL, Tuppen HA, Taylor GA, Commane DM, Arasaradnam RP, Khrapko K, Taylor RW, Kirkwood TB, Mathers JC. Clonal expansion of early to mid-life mitochondrial DNA point mutations drives mitochondrial dysfunction during human ageing. *PLoS Genet.* 2014 Sep 18;10(9):e1004620.

³⁷ Ye K, Lu J, Ma F, Keinan A, Gu Z. Extensive pathogenicity of mitochondrial heteroplasmy in healthy human individuals. *Proceedings of the National Academy of Sciences.* 2014 Jul 22;111(29):10654-9.

³⁸ Another possibility would be to test polar bodies for levels of heteroplasmy, however, this is known to be very unreliable – see Gigarel N, Hesters L, Samuels DC, Monnot S, Bulet P, Kerbrat V, Lamazou F, Benachi A, Frydman R, Feingold J, Rotig A. Poor correlations in the levels of pathogenic mitochondrial DNA mutations in polar bodies versus oocytes and blastomeres in humans. *The American Journal of Human Genetics.* 2011 Apr 8;88(4):494-8.

combinations of distinct haplogroups. This suggests that there is no simple relationship between degree of match of mtDNA haplogroups and risk of amplification of carried-over mtDNA.

- 4.5.** Yamada et al. (2016) independently assessed the potential for changes in the degree of heteroplasmy following human mitochondrial donation, using proliferating stem cell lines and *in vitro* differentiation models, in which mtDNA replication is common. They utilised human oocytes reconstructed by MST that were subsequently activated without fertilisation to yield parthenogenetic embryos. These embryos, which were capable of forming high quality blastocysts, had consistently low levels of karyoplast-derived mtDNA carryover (average of 0.2%).

To assess whether this low level heteroplasmy was stable following cell division, stem cell lines were derived from blastocysts. For all but one of the eight stem cell lines derived, heteroplasmy levels decreased below the limit of detection from passage 6 and all subsequent passages (more than 6 months of culture)³⁹. However, in one cell line, levels of karyoplast-derived mtDNA increased during culture to 53.2% at passage 36. At passage 59, however, levels had reduced to 1.0%. Fluctuation was also observed when affected ES cell lines were differentiated.

Additional experiments in the same study used SCNT to introduce diploid nuclei from human somatic cells into human oocytes. SCNT also introduces approximately one thousand somatic cell mitochondria into the oocytes, therefore ES cell lines derived from SCNT-derived blastocysts were also examined for heteroplasmy. They examined four diploid SCNT ES cell lines, in which the oocyte had been enucleated prior to fusion with the somatic cell, and eight lines that had retained the oocyte genomic DNA. Nine of the lines showed very low heteroplasmy, with the somatic cell mtDNA haplotype ranging from 0-0.6 %.

However, two of the diploid SCNT ES lines that had arisen from the same somatic cells and oocyte donor showed a rapid upward drift of the somatic mtDNA haplotype within 10 passages and had reached homoplasmy between passage 15 and 25. Clonal analysis confirmed that the drift was from haplotypes L0 to K1 in both cases. However, a third SCNT ES cell line from the same original haplotype combination contained no K1 mtDNA and remained homoplasmic for the L0 haplotype from passage 1 to 40. An additional SCNT ES cell line (H1 with L3) also showed mtDNA instability. When the L0/K1 heteroplasmic ES cells prone to drift were differentiated *in vitro* or in teratomas, there was a similar directional shift to the K1 haplotype, whereas the line containing H1 and L3 showed drift in either direction. Homoplasmic ES cell lines remained so on differentiation, as expected.

Further experiments showed no survival or proliferative advantage in culture of homoplasmic K1 ES cells over homoplasmic L0 ES cells, nor were there any significant differences in mitochondrial respiratory chain enzyme (RCE) activity.

- 4.6.** Kang et al. (2016) also investigated the stability of low-level heteroplasmy in human embryos generated by MST followed by normal fertilisation. All MST zygotes and cleaving embryos (n=22) contained <1% karyoplast-derived mtDNA and similar outcomes were observed in 15 out of 18 ES cell lines derived from MST blastocysts. However, in three of the cell lines (two controls and one from a patient), karyoplast-derived mtDNA predominated, and after extended passaging was the only detectable mtDNA. They also found that one out of 8 SCNT-derived ES cell lines showed a gradual increase from 19% at passage two to 100% at passage 10.

- 4.7.** Thus, three reports of human mitochondrial donation in distinct contexts – including MST followed by normal fertilisation (Kang et al., 2016), parthenogenetic activation (Yamada et al., 2016), and PNT of normally-fertilised oocytes (Hyslop et al., 2016) - report the successful generation of

³⁹ This result is similar to their earlier finding for all of four pluripotent cell lines derived from human spindle transfer blastocysts (Paull et al., 2013).

embryos exhibiting very low levels of heteroplasmy (carryover typically <1-2%). In the context of embryonic stem cell culture, in most cases the level of heteroplasmy remained stable or the karyoplast-derived mtDNA haplotype became undetectable throughout repeated passaging.

However, in a minority of cell lines analysed (in the order of 15-25%) instability of heteroplasmy was demonstrated, resulting in either a significant increase of, or even complete reversion to, the mtDNA haplotype found in the karyoplast. This has a clear implication: even though the methods achieve the desired aims in the preimplantation embryo, were such drift or reversion to occur in the context of embryonic/fetal development following clinical application of MST or PNT, such that the levels of pathogenic mtDNA increased throughout the fetus or in particular tissues, this could lead to disease in the child born⁴⁰.

4.8. The panel considered several possible explanations for changing levels of heteroplasmy in stem cells, some of which were suggested in the relevant publications and in discussions with those interviewed by the panel. These relate either to some aspect of the methods used or to properties of mtDNA, and they are not mutually exclusive.

(i) Level of carryover: In the report by Hyslop et al. (2016), the one ES cell line derived after PNT that showed reversion on passaging was that with highest level of carryover (4%), in comparison with <2% for those that did not. However, for the MST-derived ES cell lines (Yamada et al., 2016; Kang et al. 2016) there was no obvious correlation between reversion and levels of carryover; moreover, these tended to be lower, mostly ranging from 0.1-1.0%.

It is also worth noting that none of the embryos and early passage ES cell lines from any of the studies had carryover levels above 5%, the proposed level above which embryos are theoretically at risk of expansion through stochastic/bottleneck effects^{27,41,42,43}. While initial levels of carryover may not have been a major factor in determining the risk of subsequent reversion in stem cells, the panel recognises the importance of keeping carryover levels in embryos to a minimum. If carryover could be eliminated altogether, any risk of reversion would be removed, whether *in vitro* or *in vivo* (see section 7 for possible methods to eliminate or reduce mtDNA carryover).

(ii) Methodology: It is possible that aspects of the methods used in the papers discussed above could give the karyoplast mtDNA an advantage in some circumstances. For example, the process of SCNT is known on occasion to induce a round of mtDNA replication in the early embryo at a time when this does not normally occur^{44,45}. This may be due to the presence of nuclear-encoded mtDNA replication factor DNA polymerase γ (Pol γ) within the somatic cell, which will be introduced into the oocyte on fusion. Also, while most proteins present in the oocyte are destroyed during cleavage stages, some persist, and it is possible that any pol γ may remain associated with the mtDNA that originated in the somatic cell, giving this an early advantage when replication begins in earnest, after establishment of ES cell lines *in vitro*. However, this mechanism is unlikely to be

⁴⁰ In cases where the patient's oocytes are heteroplasmic, there is a chance that reversion could involve the normal mtDNA, in which case the child would be healthy. Indeed, this was the case in the one patient-derived ES cell line that showed reversion, as reported in Kang et al., 2016.

⁴¹ Johnston IG, Burgstaller JP, Havlicek V, Kolbe T, Rülcke T, Brem G, Poulton J, Jones NS. Stochastic modelling, Bayesian inference, and new *in vivo* measurements elucidate the debated mtDNA bottleneck mechanism. *Elife*. 2015 Jun 2;4:e07464.

⁴² Samuels DC, Wonnapijit P, Chinnery PF. Preventing the transmission of pathogenic mitochondrial DNA mutations: can we achieve long-term benefits from germ-line gene transfer? *Human Reproduction*. 2013 Mar 1;28(3):554-9.

⁴³ Wilson IJ, Carling PJ, Alston CL, Floros VI, Pyle A, Hudson G, Sallevelt SC, Lamperti C, Carelli V, Bindoff LA, Samuels DC. Mitochondrial DNA sequence characteristics modulate the size of the genetic bottleneck. *Human molecular genetics*. 2016 Mar 1;25(5):1031-41.

⁴⁴ Bowles EJ, Campbell KH, John JC. Nuclear transfer: preservation of a nuclear genome at the expense of its associated mtDNA genome (s). *Current topics in developmental biology*. 2007 Dec 31;77:251-90.

⁴⁵ Lloyd RE, Lee JH, Alberio R, Bowles EJ, Ramalho-Santos J, Campbell KH, John JC. Aberrant nucleo-cytoplasmic cross-talk results in donor cell mtDNA persistence in cloned embryos. *Genetics*. 2006 Apr 1;172(4):2515-27.

responsible for reversion after MST or PNT, because neither of these involve mtDNA in a replicative state. Yet, the reversion phenomenon occurred in ES cell lines derived after MST, PNT and SCNT. Could there be something else in common?

One possibility might be segregated or partitioned distribution of karyoplast-derived mtDNA after MST or PNT or SCNT, which could lead to some blastomeres having a level of carryover higher than others, and perhaps sufficiently high that stochastic/bottleneck effects could operate to skew the proportion even further during ES cell culture. Relatively few cells of the inner cell mass (ICM) give rise to ES cells, which could explain why reversion is seen only in a proportion of ES cell lines. Single cell analysis of blastocysts should reveal if this is the case. This was carried out by Hyslop et al. (2016), and it is apparent that a few cells are outliers with up to 12% carryover (see their Fig. 3). This is in contrast to evidence reported from PGD of human embryos where blastomeres within an embryo tend to have very similar levels of heteroplasmy^{46,47}. One obvious implication of this is that tissues deriving from such blastomeres may have sufficiently high levels of karyoplast-derived mtDNA that they could be prone to increase to even higher levels, as seen with one case of PGD^{47,48} and as predicted by theory⁴⁹.

The panel discussed the possibility of partitioning followed by segregation after MST and PNT in its 2013 report, following the studies of Lee et al. (2012)⁵⁰ who had deliberately created macaque oocytes that were heteroplasmic (50/50) for two mtDNA variants. These resulted in embryos exhibiting significant partitioning of the mtDNA between different blastomeres and to some extent between trophectoderm and ICM. Some of the fetuses, or ES cell lines derived from embryos, also showed a skewed ratio (in one case about 94% of one of the mtDNA variants was present). There was no evidence of preferential selection for either mtDNA, suggesting that both variants function equally well with the resident nuclear DNA, even though the mtDNA sequences of the two sub-species of macaque were as different from each other as they are from other primate species.

Lee et al (2012) also claimed that isolated karyoplasts from macaque oocytes carry “bound” mitochondria (there is no evidence that they are physically bound, just closely associated) at an average level of about 0.6% of the numbers within the cytoplasm. Mitochondria interact with the cytoskeleton and this could be involved in subsequent partitioning of the karyoplast-associated mitochondria. While cytoskeletal inhibitors are used during MST, PNT and SCNT to allow extraction of the spindle or pronuclei from oocytes or zygotes, which may allow mitochondria to mix freely, the inhibitors are no longer present when the karyoplast containing the spindle or pronuclei (or an intact somatic cell in the case of SCNT), is first washed though a drop of viral fusogen and then fused to the enucleated oocyte or zygote. Perhaps this could allow the karyoplast cytoplasm and associated mitochondria to remain in a localised region of the zygote, such that they could fail to be partitioned equally amongst blastomeres during cleavage divisions. It should be possible to follow this using MitoTracker labelling of mitochondria in the karyoplasts,

⁴⁶ Sallevelt SC, Dreesen JC, Drüsedau M, Spierts S, Coonen E, van Tienen FH, van Golde RJ, de Coo IF, Geraedts JP, de Die-Smulders CE, Smeets HJ. Preimplantation genetic diagnosis in mitochondrial DNA disorders: challenge and success. *Journal of medical genetics*. 2013 Feb 1;50(2):125-32.

⁴⁷ Treff NR, Campos J, Tao X, Levy B, Ferry KM, Scott RT. Blastocyst preimplantation genetic diagnosis (PGD) of a mitochondrial DNA disorder. *Fertility and sterility*. 2012 Nov 30;98(5):1236-40.

⁴⁸ Mitalipov S, Amato P, Parry S, Falk MJ. Limitations of preimplantation genetic diagnosis for mitochondrial DNA diseases. *Cell reports*. 2014 May 22;7(4):935.

⁴⁹ Chinnery PF, Craven L, Mitalipov S, Stewart JB, Herbert M, Turnbull DM. The challenges of mitochondrial replacement. *PLoS Genet*. 2014 Apr 24;10(4):e1004315.

⁵⁰ Lee HS, Ma H, Juanes RC, Tachibana M, Sparman M, Woodward J, Ramsey C, Xu J, Kang EJ, Amato P, Mair G. Rapid mitochondrial DNA segregation in primate preimplantation embryos precedes somatic and germline bottleneck. *Cell reports*. 2012 May 31;1(5):506-15.

and perhaps to prevent it by incubating the reconstituted oocyte or zygote after fusion in cytoskeletal inhibitors for a brief period.

(iii) Peculiarities of embryonic stem cells: Among other differences (see box 2 below), ES cells have a relatively small amount of cytoplasm compared with most somatic cell types and typically have few copies of mtDNA⁵¹. They may, therefore, be particularly prone to “bottleneck” effects where just by chance there is drift in the level of heteroplasmy. This would not, however, be expected by itself to give a consistent directional drift, as seen in the cases of reversion found by Yamada et al. (2016), Hyslop et al. (2016), and Kang et al. (2016). But it is noteworthy that the karyoplast-derived mtDNA haplotype disappeared altogether in some ES cell lines, and in one line studied by Yamada et al. (2016), which underwent a significant reversion to the karyoplast-derived mtDNA haplotype, the effect was completely reversed in bulk culture and subclones also showed an over-all drift back towards the cytoplasm-derived haplotype.

However, even a very small advantage of one mtDNA haplotype over the other could lead to an additional “ratchet” effect. This could operate within cells, if, for example, one mtDNA haplotype replicated faster than the other, and/or between cells, if, for example, the advantage involved energy production, such that cells with slightly higher copy number of the advantageous mtDNA type within its mitochondria could contribute slightly more of the cells in the next passage, and so on.

Yamada et al. (2016) tested this latter possibility by mixing cells homoplasmic for haplotype L0 or haplotype K1 at different ratios (in heteroplasmic ES cell lines, drift was consistently in favour of the K1 haplotype). They found that the proportion of each mtDNA genotype remained stable over 8 passages (6 weeks). This suggests that reversion to the K1 haplotype in cultures of heteroplasmic ES cells does not involve cell competition⁵² and that it is more likely to be due to a replicative advantage of K1 over L0 within cells.

Prof. Mitalipov expressed the view that human embryonic stem cells may not act in the same way as embryos. Moreover, he informed the panel that he has not observed the same reversion phenomenon in mouse embryonic stem cells or in those from macaques, suggesting that human ES cells may be unusual in this regard. Kang et al (2016) report that derivation of ES cell clones from isolated individual cells from two cell lines exhibiting reversion in whole culture (3243ST-ES1 and NT-ES8) revealed faster growth rates in clones with higher levels of maternal (karyoplast-derived) mtDNA. The authors conclude that certain mtDNA haplotypes confer a proliferative advantage on ES cells and that this is independent of detectable mitochondrial activity. It remains unclear how such an effect might occur, given that mitochondria are not very active within ES cells; moreover, this observation is not consistent with the results of the cell mixing experiments of Yamada et al. (2016), as described above.

In addition to following mtDNA heteroplasmy in ES cells over many passages, Yamada et al. (2016) looked at differentiated cell types derived from the same ES cell lines that had shown dramatic shifts. Differentiation of the two SCNT-derived lines to fibroblasts or cardiomyocytes resulted in a directional shift to the K1 haplotype, while the MST-derived line showed both decrease or increase of the H1 haplotype during differentiation, mirroring what had happened with further passaging of the undifferentiated ES cells. Cells with homoplasmy remained stable as stem cells and during differentiation *in vitro* or *in vivo* as teratomas. The authors conclude: “mtDNA genotype instability was not specific to pluripotent stem cells”.

However, rather than beginning with early passages before any shift was obvious, they used

⁵¹ Folmes CD, Ma H, Mitalipov S, Terzic A. Mitochondria in pluripotent stem cells: stemness regulators and disease targets. *Current opinion in genetics & development*. 2016 Jun 30;38:1-7.

⁵² Sancho M, Rodríguez TA. Selecting for fitness in mammalian development. *Cell Cycle*. 2014 Jan 1;13(1):9-10.

those that had already shown significant reversion. This is, therefore not an ideal test of what would happen in somatic cells if embryos derived by MST had been implanted. Nevertheless, Yamada et al. (2016) also measured the function of mitochondria in the differentiated cells, using a range of parameters, finding no significant differences between any mtDNA haplogroup/haplotype. This again suggests that preferential amplification of one haplotype over another is more likely due to differences in replication and not selection for improved function.

Box 2: Embryonic stem (ES) cells as a model of *in vivo* development: caution required

The panel recommends caution when attempting to interpret data from ES and other cells cultured *in vitro* in order to make inferences about the risks of mtDNA reversion during embryonic development *in vivo*. ES cells are epigenetically anomalous compared with most somatic cells, with pan-genomic loci co-occupied by active (H3K4me3) and silent (H3K27me3) bivalent marks. Unlike differentiating and differentiated cells, ES cells do not require DNA methylation⁵³ and ES and somatic cells produce (in the mouse, at least) markedly different developmental readouts in nuclear transfer, for unknown reasons⁵⁴. These differences could, of course, influence (or be influenced by) mitochondrial behaviour.

As noted above, ES cells are morphologically atypical, with large nucleus-cytoplasm ratios and they have an anomalous cell cycle, with a truncated G1 in which Geminin escapes degradation due to atypical suppression of the E3 ubiquitin ligase, APC^{55,56,57}. The cell cycle is linked to mitochondrial segregation⁵⁸ and coupled with the unusual morphology of ES cells and the unusual regulation of proteasomal degradation (which is relevant for mitophagy), obviously has the potential to influence sorting of cytoplasmic factors, including mitochondria, in an unusual manner.

However, it is not known if these fundamental biological differences between ES and somatic cells are relevant to mitochondrial behaviour. If ES cells are to be used as a model for what might happen *in vivo* it is necessary to have a closer consideration of the way mitochondria behave in ES cells compared to somatic cells. The mitochondria of human and mouse ES cells, unlike their differentiated somatic cell counterparts (e.g. fibroblasts) are immature, with a perinuclear localisation entirely concordant with different mechanisms of mitochondrial homeostasis.

Moreover, ES cells are derived from a transient compartment in preimplantation embryos that lasts only a few hours. In addition, no *in vitro* culture system available today can fully account for exogenous (e.g., circulating) factors (cytokines, growth factors, steroid and peptide hormones, neurotransmitters, etc.), programmed lineage differentiation, including apoptosis, immune surveillance, tissue-level reactive oxygen species (ROS) homeostasis, the role of niches, cross-

⁵³ Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*. 1992 Jun 12;69(6):915-26.

⁵⁴ Jackson-Grusby L, Klemm M, Rideout WM 3rd, Yanagimachi R, Jaenisch R. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci U S A*. 2001 May 22;98(11):6209-14. Epub 2001 May 1.

⁵⁵ Yang VS, Carter SA, Hyland SJ, Tachibana-Konwalski K, Laskey RA, Gonzalez MA. Geminin escapes degradation in G1 of mouse pluripotent cells and mediates the expression of Oct4, Sox2, and Nanog. *Current Biology*. 2011 Apr 26;21(8):692-9.

⁵⁶ Gonzalez MA. *Curr Biol*. 2011 Apr 26;21(8):692-9.; Distinct activities of the anaphase-promoting complex/cyclosome (APC/C) in mouse embryonic cells.

⁵⁷ Yang VS, Carter SA, Ng Y, Hyland SJ, Tachibana-Konwalski K, Fisher RA, Sebire NJ, Seckl MJ, Pedersen RA, Laskey RA, Gonzalez MA. *Cell Cycle*. 2012 Mar 1;11(5):846-55.

⁵⁸ Mishra P, Chan DC. Mitochondrial dynamics and inheritance during cell division, development and disease. *Nature reviews Molecular cell biology*. 2014 Oct 1;15(10):634-46.

talk between juxtaposed heterologous cells and tissues, morphogenesis and numerous other systems-level complexities.

Another complicating factor is that the conditions currently used for establishing and culturing human ES cells are unlikely to be optimal, because they do not mimic any *in vivo* environment. This may mean that ES cells in culture are prone to more stresses than are epiblast cells *in vivo*. Whilst in general, *in vitro* culture of stem cell lines, or their differentiated derivatives, may provide a reasonable model for studying bottleneck effects, such as those occurring around gastrulation when mtDNA copy number per cell declines to a minimum and replication is initiated (one of the original reasons for the panel suggesting these experiments), they may not offer a fully suitable model of the mtDNA replicative dynamics that obtain during embryonic/fetal development *in utero*. In summary, although experiments with ES and other cells cultured *in vitro* may be a tractable way of modelling the behaviour of human mtDNA in post-implantation embryos, this does not mean the experiments are reliable (see section 7 for suggested further research).

(iv) Differences between haplotypes in mtDNA replication: The panel reviewed data concerning specific combinations of mtDNA haplotypes for evidence that one is preferentially amplified over the other. Kang et al. (2016), reported that 3 of 18 (17%) ES cell lines derived from blastocysts produced by MST displayed reversion to the carryover mtDNA haplotype. It seems unlikely that this is related to the presence of defective mitochondria because it occurred with ES cell lines derived from two control MST blastocysts as well as for one mutation carrier MST blastocyst. From the data in their manuscript, it is possible that U5a mtDNA tended to predominate over H1b and perhaps other haplotypes, but this appears to be independent of the number of SNP differences between the two haplotypes.

Yamada et al. (2016) also found that some haplotype combinations were associated with reversal or instability, although the drift was consistent with some combinations and random with others (see above); however, they were unable to offer a mechanistic explanation for this. The differences in mtDNA sequence between the various haplotypes were noted, but again, no obvious explanation was identified.

Similarly, in the one case of reversion seen in an ES cell line by Hyslop et al. (2016) following PNT, the karyoplast and cytoplast mtDNAs were within the same haplogroup, whereas combinations of different haplogroups did not show this effect. An additional complication, which could affect whether genetic drift is seen or not, might involve interactions between specific mtDNA haplotypes and the maternal and/or paternal nuclear genome. This has been proposed as the reason underlying the drift seen in experiments where heteroplasmy has been introduced into mouse and macaque embryos (Johnston et al., 2014; Lee et al., 2012). But the mouse data suggest that drift is positively correlated with genetic distance between mtDNA haplotypes, so this is unlikely to explain reversion observed specifically in this human ES cell line. In addition, in the SCNT experiments of Yamada et al. (2016), where the same nuclear DNA and mtDNA haplotype was used, the outcome with respect to drift was inconsistent. Moreover, they could detect no effects on mitochondrial function that correlated with mtDNA haplotype and the nuclear genome.

If it is not the overall differences between mtDNA sequences that are responsible for reversion, another possible cause is variation within the displacement-loop (D-loop) region, in sequences that interact with the mtDNA replication machinery. Human mtDNA replication is not fully understood^{59,60}, but it is known to involve the nuclear-encoded mtDNA polymerase γ (Poly, the

⁵⁹ Young MJ, Copeland WC. Human mitochondrial DNA replication machinery and disease. *Current opinion in genetics & development*. 2016 Jun 30;38:52-62.

POLG product) and several other associated proteins, which interact with specific sequences within the D-loop together with an RNA primer. In human mitochondria these RNA primers occur at very low frequency, but evidence suggests that they are transcribed by the nuclear-encoded mitochondrial RNA polymerase (mtRNAP or POLRMT). This normally directs polycistronic transcription from H-strand and L-strand promoters located in the mtDNA control region, whereas the primers arise from a switch in its activity that causes RNA transcripts to remain bound to the template as discontinuous but persistent RNA:DNA hybrids, termed 'R-loops'.

Variation in the sequences to which the replication machinery binds, in the RNA primer sequences or the transcription initiation sequences to which POLRMT interacts, are all likely to affect the rate of mtDNA replication (e.g. see: Tan et al., 2016)⁶¹. Indeed, mutations within these sequences are often associated with certain cancers or other pathologies in which mtDNA copy number is dysregulated⁶². Epigenetic modifications can also have an effect: demethylation of D-loop sequences has been associated with increased mtDNA copy number in colorectal cancer⁶³, whereas methylation was linked with reduced copy number in obesity⁶⁴. However, it is not clear how these differences in methylation occur, or whether they are a cause or consequence with respect to altered mtDNA replication.

Whether or not reversion occurs could in theory be associated with allelic variants in the nuclear genes encoding the factors involved in mtDNA replication, which may affect how their protein products interact with haplotype-specific D-loop sequences, allowing one to replicate preferentially over the other. However, Yamada et al. (2016) found no evidence for such mito-nuclear interactions.

In order to account for reversion in ES cells in their study, Kang et al (2016) focussed on the highly polymorphic D-loop region called conserved sequence box II (CSBII). They examined a CSBII sequence polymorphism (G5AG7) known to be associated with altered efficiency of mitochondrial transcription termination and replication primer generation. When they analysed maternal and donor mtDNA in a total of 26 ES cell lines, comprising 18 different haplotype combinations, they noted that two lines exhibiting reversion (ST-ES7 and ST-ES8) carried donor mtDNA with a G5AG8 polymorphism and maternal mtDNA of G6AG8. Using *in vitro* assays they showed that the G5AG8 polymorphism of donor mtDNA resulted in a 4-fold reduction in replication primer synthesis. They conclude that the effect of such a D-loop polymorphism (G6AG8), arising from the addition of just a single guanosine residue, may be to confer a replicative advantage to mtDNA harbouring it. Two other ES cell lines showing reversion did not reveal CSBII polymorphisms in donor/maternal mtDNA, but a number of other D-loop polymorphisms in the core TAS region were detected, and the authors speculate that these may also contribute to replication bias of a particular mtDNA haplotype over another.

It is worth noting that in cases of heteroplasmy for mutant and normal mtDNA not generated by MST or PNT, i.e. where there is no heterologous mtDNA nor haplotype mismatch, drift towards

⁶⁰ Ciesielski GL, Oliveira MT, Kaguni LS. Chapter Eight-Animal Mitochondrial DNA Replication. The Enzymes. 2016 Dec 31;39:255-92.

⁶¹ Tan BG, Wellesley FC, Savery NJ, Szczelkun MD. Length heterogeneity at conserved sequence block 2 in human mitochondrial DNA acts as a rheostat for RNA polymerase POLRMT activity. Nucleic Acids Research. 2016 Sep 19;44(16):7817-29.

⁶² Damas J, Samuels DC, Carneiro J, Amorim A, Pereira F. Mitochondrial DNA rearrangements in health and disease—a comprehensive study. Human mutation. 2014 Jan 1;35(1):1-4.

⁶³ Gao J, Wen S, Zhou H, Feng S. De-methylation of displacement loop of mitochondrial DNA is associated with increased mitochondrial copy number and nicotinamide adenine dinucleotide subunit 2 expression in colorectal cancer. Molecular medicine reports. 2015 Nov 1;12(5):7033-8.

⁶⁴ Zheng LD, Linarelli LE, Liu L, Wall SS, Greenawald MH, Seidel RW, Estabrooks PA, Almeida FA, Cheng Z. Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. Clinical epigenetics. 2015 Jun 10;7(1):1.

higher levels of one has also been noted to occur during development after PGD. For example, in the pregnancy reported by Treff et al., (2012), the 12% heteroplasmy level for the m.3243A>G mutation detected by PGD rose to 52% at 1.5 months after birth⁴⁶. This suggests that chance effects resulting in genetic drift can occur. These effects could exacerbate an initial bias caused by preferential mtDNA replication.

- 4.9.** In conclusion, the panel views the stability of the mtDNA profiles found in most of the stem cell cultures generated from human MST/PNT embryos discussed above as reassuring, but the unusual reversion (upward drift) in the levels of carried-over mtDNA seen in a minority of ES cell lines after more or less prolonged culture, is of concern. However, its relevance to what might happen in human embryos and fetuses *in utero* following MST/PNT is still unclear. It is notable that Zhang et al. (2016a) found no evidence of reversion in the two fetuses studied after PNT (which was carried out in an attempt to treat apparent infertility, not to avoid transmission of mitochondrial disease), nor was there obvious reversion in multiple neonatal tissues following the first case of MST reported in recent abstracts¹⁰.
- 4.10.** **From a consideration of all the recent data made available to us concerning reversion of mtDNA in vitro, and in particular the study of Kang et al (2016), it seems theoretically possible that variations in the sequence of the D-loop may confer a replicative advantage to one haplotype over another in specific combinations. However, further research is required, particularly because the numbers of MST- and PNT-derived embryos studied so far remains rather too low to conclusively rule out the other potential mechanisms discussed above, and, of course because there could be others that have not been considered here. Finally, the panel emphasises the importance of systematically collecting data from the long-term follow-up of any pregnancies and subsequent births created by MST/PNT, in order to establish potential risks to any children born as the result of treatment.**

Transfer of XY (male) embryos only

Summary of previous considerations

In 2014, the panel discussed the strategy of transferring only XY (male) embryos following MST/PNT, to ensure no carryover of maternally inherited mutated DNA to subsequent generations. In 2014, it did not support such a proposal, even though it would avoid the possibility of trans-generational inheritance of pathogenic mtDNA, as well as circumvent objections made by some that the techniques are a form of germ line genetic alteration.

Selecting only XY embryos for transfer would require PGD, an additional step that is likely to compromise early development of already manipulated embryos; moreover, it would (on average) immediately reduce by half the number of embryos available for transfer. This would decrease the efficiency of the techniques and make it likely that patients would have to undergo repeated cycles of treatment, which may never be successful. Determining embryo sex after implantation would require termination of normal XX (female) embryos as discussed as part of the Nuffield Council on Bioethics ethical review of mitochondrial donation techniques in 2012⁶⁵.

⁶⁵ Nuffield Council on Bioethics. (2012). Novel techniques for the prevention of mitochondrial DNA disorders: an ethical review. Retrieved August 22, 2016, from <http://nuffieldbioethics.org/project/mitochondrial-dna-disorders>.

There already exists an accepted precedent for assisted reproduction with predictable, deleterious genetic consequences for the next generation: when ICSI is used to overcome male infertility in patients with a Y chromosome defect, any son born as a result will carry the same defect, and ICSI will be required for him to have a child. This will be true for all subsequent generations of males of this lineage. In contrast, for MST and PNT, the risks of mitochondrial disease in the next generation are likely to be low. The subsequent use of PGD in this next generation, to select embryos with very low or no levels of abnormal mtDNA, may rid all subsequent generations of the need to have interventions to avoid mitochondrial disease.

- 4.11.** During this current review in 2016, the panel noted the recent report on mitochondrial replacement techniques (MRT) from the National Academies of Sciences, Engineering and Medicine¹⁹, which had been commissioned by the US Food and Drug Administration (FDA). A committee was convened to examine and analyse issues relating to techniques for avoiding some types of inherited mitochondrial DNA (mtDNA) diseases and make recommendations for further clinical investigations.

This report recommended limiting clinical research to the transfer of MRT XY (male) embryos, so as to avoid heritable genetic modification, with no transfer of female embryos until there “is clear evidence of safety and efficacy from male cohorts, using identical MRT procedures, regardless of how long it took to collect this evidence”. The panel had difficulty in supporting this view due to the requirement for additional manipulation of the embryo, the reduction in embryos available for transfer, the deliberate exclusion of normal XX (female) embryos and the impracticalities of attempting to deliver absolute safety. Importantly, it should be noted that under the HFE Act, sex selection is only permitted for sex-linked diseases, which do not include diseases due to mutations in mtDNA.

- 4.12.** In 2016, the panel agreed that their view on this matter has not changed and therefore they do not support any proposal to select XY (male) embryos only.

Methods to prevent premature activation of oocytes or to detect abnormally fertilised oocytes

Summary of previous considerations

In 2014 the panel received little new evidence in relation to this area. However, alternative methods to prevent abnormal activation were described in a series of recent papers reporting SCNT with human oocytes. These made use of low levels of caffeine, putatively as a protein phosphatase inhibitor, in the medium during spindle removal and fusion of the somatic cell. In addition, it was also clear that care needs to be taken to prevent fusion of polar bodies back into the oocyte or zygote, as this could produce aneuploid embryos^{66,67,68}

⁶⁶ Yamada M, Johannesson B, Sagi I, Burnett LC, Kort DH, Prosser RW, Paull D, Nestor MW, Freeby M, Greenberg E, Goland RS. Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells. *Nature*. 2014 Jun 26;510(7506):533-6.

⁶⁷ Chung YG, Eum JH, Lee JE, Shim SH, Sepilian V, Hong SW, Lee Y, Treff NR, Choi YH, Kimbrel EA, Dittman RE. Human somatic cell nuclear transfer using adult cells. *Cell Stem Cell*. 2014 Jun 5;14(6):777-80.

⁶⁸ Tachibana M, Amato P, Sparman M, Woodward J, Sanchis DM, Ma H, Gutierrez NM, Tippner-Hedges R, Kang E, Lee HS, Ramsey C. Towards germline gene therapy of inherited mitochondrial diseases. *Nature*. 2013 Jan 31;493(7434):627-31.

- 4.13.** In 2016 the panel received no new evidence specifically related to this topic. However, the success rates described in Kang et al. (2016) for blastocyst generation and levels of aneuploidy suggest distinct improvements have been made in this area.

Mito-nuclear interactions

Summary of previous considerations

During the 2014 considerations an oral presentation was given to the panel by Dr Iain Johnston, of Imperial College London and subsequently a manuscript was provided (now published as Burgstaller et al., 2014). The group explored data that investigated mtDNA segregation and its possible dependence on genetic distance between distinct haplogroups, where mito-nuclear interactions may, theoretically, play an important role in the mechanism. These experiments were designed to explore, in an artificially induced heteroplasmic situation in mice, whether some mtDNA haplotypes experience a selective advantage over others in certain tissues and over time. The notion that this might happen was first highlighted by Jenuth et al., (1996) who investigated mice carrying mtDNA from both NZB and BALB/c strains that had been derived from founders made by ooplasm transfer. The panel also considered the separate issue of whether it is possible that an incompatibility could arise between nuclear and mitochondrial genomes in an embryo generated by MST or PNT.

In a submission and presentation from Dr Edward Morrow, University of Sussex, some of their group's concerns relating to the safety of mitochondrial replacement techniques, expressed in the recent article by Reinhardt et al., (2013) and in a follow-up manuscript submitted as evidence³², were revisited. The presentation highlighted their views on nuclear-mtDNA interactions and the anterograde and retrograde signalling between these DNA genomes, emphasising this signalling cross-talk and the possible effects on oxidative phosphorylation, metabolic pathways and other processes that they propose might result from disrupting these interactions. Discussions concerning these two separate issues relating to mito-nuclear interactions are detailed in the 2014 report (page 30-31).

In summary, the panel reviewed two separate arguments for considering haplotype/haplogroup matching. The concern of Morrow and co-workers is based on evolutionary arguments that some nuclear-haplotype combinations can lead to a failure of co-evolved mito-nuclear interactions with adverse consequences. They propose haplotype, or at least haplogroup, matching to reduce the risk of any such effect occurring. Their arguments did not address pathogenic (disease pre-disposing) mutant mtDNA *per se*. Moreover, Morrow and colleagues did not address the possibility that an exchange to a different haplotype/haplogroup might, in some cases, confer advantages⁶⁹. The panel considered that the likelihood of such hypothetical problems occurring would be low. Given the high degree of variation in the nuclear genome that can have an impact on embryo fitness, whether this is due to effects on mitochondrial function or not, the consequences of pursuing any strategy involving matching are likely to be minimal. The *Drosophila* research cited in the 2014 report⁷⁰ used inbred strains, which makes it more likely that any effects of a mismatch will be detected, whereas humans are much more outbred so that

⁶⁹ Latorre-Pellicer A, Moreno-Loshuertos R, Lechuga-Vieco AV, Sánchez-Cabo F, Torroja C, Acín-Pérez R, Calvo E, Aix E, González-Guerra A, Logan A, Bernad-Miana ML. Mitochondrial and nuclear DNA matching shapes metabolism and healthy ageing. *Nature*. 2016 Jul 28;535(7613):561-5.

⁷⁰ Innocenti P, Morrow EH, Dowling DK. Experimental evidence supports a sex-specific selective sieve in mitochondrial genome evolution. *Science*. 2011 May 13;332(6031):845-8.

such effects are unlikely to be detectable.

Data from Johnston et al.^{71,72} raised the concern that, after MST or PNT, the now rare mtDNA variant carrying the mutation would preferentially expand (due to some unknown mechanism) in some tissues. This could occur if that variant possesses some "selectable" advantage in those tissues in comparison to the mtDNA variant provided by the donor oocyte. If a selective mechanism were operating, it could do so within (mtDNA competition) or between cells (cell competition) in the developing embryo or newborn. Any post-natal manifestation following MST or PNT might be deleterious for the child and/or subsequent generations if the child were a girl and the selection process operated in the germ line.

This may be different from the considerations of Morrow et al., which concern failure of mito-nuclear interaction; but any selective advantage conferred by a mitochondrial variant could depend on mito-nuclear interactions. Haplotype/haplogroup matching was proposed to remove any putative selective advantage. In addition, it might one day be possible to choose a donor carrying a haplogroup that has a selectable advantage (considered in the context of the child's nuclear DNA), which would beneficially out-compete the variant carrying the mutation. However, as with Morrow et al., the arguments of Johnston et al. are based on an inbred animal model. The outbred nature of humans makes it much less likely that any effect on mtDNA segregation would occur⁷³ or, if it did, that any reliable correlation could be made.

In conclusion, the panel considered evidence presented concerning the two issues relating to mtDNA haplotypes: (i) that mito-nuclear mismatch as a result of MST or PNT might lead to unexpected adverse effects on the offspring - albeit that this does not seem to occur naturally, and (ii) possible segregation in favour of any carried-over mutant mtDNA, possibly exacerbated by unusual mito-nuclear interactions. Whilst the panel acknowledges that haplogroup matching of donors could be a possible precautionary step that might ameliorate such effects should they operate in humans, they appreciate that it may not always be possible, especially when close relatives might also be at risk of transmitting the mtDNA mutations, or when the patient has an unusual haplotype compared with the possible donor population. Further evidence would be needed to understand whether and how the proposed effects might be operative.

In 2014 the panel recommended that consideration should be given to mtDNA haplogroup matching when selecting donors, although the panel considered that the risks of not doing so would be very low, and acknowledged that there may be practical factors preventing it. In addition, the panel recommended that the follow-up of children born as a result of these new methods should include assessment of whether any preferential expansion of the mitochondria carrying the mutations occurs over time, and comparison between the general state of health of the child and the degree of haplotype mismatch. This follow-up could help to inform future decisions about matching.

4.14. During this 2016 review, the panel discussed issues arising from possible haplotype mismatching and noted several contributions to the topic of how conserved mito-nuclear compatibility may be in the human population:

⁷¹ Burgstaller JP, Johnston IG, Jones NS, Albrechtova J, Kolbe T, Vogl C, Futschik A, Mayrhofer C, Klein D, Sabitzer S, Blattner M. MtDNA segregation in heteroplasmic tissues is common in vivo and modulated by haplotype differences and developmental stage. *Cell reports*. 2014 Jun 26;7(6):2031-41.

⁷² Burgstaller JP, Johnston IG, Poulton J. Mitochondrial DNA disease and developmental implications for reproductive strategies. *Molecular human reproduction*. 2015 Jan 1;21(1):11-22.

⁷³ James JE, Piganeau G, Eyre-Walker A. The rate of adaptive evolution in animal mitochondria. *Molecular ecology*. 2016 Jan 1;25(1):67-78.

- i) Ma et al. (2015) used somatic cell nuclear transfer from patient fibroblasts, harbouring mitochondria associated with Leigh syndrome and MELAS, to donor oocytes (with normal mitochondria) and derived pluripotent stem cell lines (PSC) from these. Patient mtDNA haplogroups and donor haplogroups varied. They were able to derive PSCs containing no residual patient-derived mtDNA and showed that transcriptional and epigenetic profiles of these were similar to conventional ES cells. Despite haplogroup differences between patient and donor, at 47 nucleotide positions of the mtDNA, normal metabolic function in PSCs was observed. They conclude that normal nuclear-to-mitochondrial interactions are highly conserved within species.
- ii) In their study of MST followed by parthenogenetic activation of reconstructed oocytes, Yamada et al. (2016) examined stem cells that had identical nuclear genomes, but were homoplasmic for either the L0 or K1 haplotypes. No significant differences in mitochondrial function were observed. Yamada et al. (2016) conclude that the maternal nuclear genome is fundamentally compatible with different mtDNA haplotypes. They also note that this is not unexpected since maternal nuclear alleles may re-segregate with a new mtDNA haplotype after transmission through a son.
- iii) In their examination of the transcriptomes of cells isolated from ePNT and control blastocysts, Hyslop et al. (2016) examined expression of mtDNA-encoded oxidative phosphorylation (OXPHOS) genes. They note that, although there was wide variation in mtDNA OXPHOS gene expression within and between ePNT and control blastocysts, samples from both groups clustered together after unsupervised hierarchical clustering, irrespective of whether the karyoplast and cytoplasm contained the same or different mtDNA haplotypes. They conclude that switching nuclear genomes does not result in a detectable effect on mitochondrial gene expression.
- iv) In their study of MST followed by normal fertilisation of reconstructed human oocytes, Kang et al. (2016) analysed development of MST embryos with differing degrees of divergence between the karyoplast and cytoplasm mtDNA haplotypes, ranging from closely related (six single nucleotide polymorphisms (SNPs)), to medium (33 SNPs) to distant (57 SNPs). Fertilisation and blastulation rates were similar among all three groups. They conclude that MST does not adversely affect mtDNA-nuclear interactions during human embryonic development *in vitro*.

- 4.15.** The above reports, all investigating the possibility of mito-nuclear incompatibility in human embryo-derived cell lines, embryo-derived cells or whole embryos following mitochondrial donation techniques conclude that such a possibility remains theoretical. All focus on *in vitro* studies, and thus their relevance to the whole adult organism is unclear. But they are notable in studying this possibility in the context of human outbred genomes and conclude that such genomes can robustly interact with a variety of common, but genotypically distinct, mtDNA haplotypes that presumably evolved to be so compatible. The panel also continued to monitor the use of model organisms to address possible mito-nuclear incompatibility.
- 4.16.** Latorre-Pellicer et al. (2016) studied mice in which the C57BL/6 (B6) nuclear genome is combined with either the B6 mitochondrial genome as usual (B6-C57), or the mitochondrial genome from the NZB/OlaHsd strain of mouse, to yield the conplastic strain, B6-NZB. (N.B. this was carried out by repeated backcrosses, not by MST or PNT.) The level of divergence between the B6 and NZB mtDNA genotypes is reported to be comparable to that between human Eurasian and African mtDNAs. They characterised both strains throughout their lifespan using a range of phenotypic parameters. Unexpectedly, the conplastic B6-NZB strain exhibited an extended median lifespan of 16%. Two-year old B6-C57 mice manifested more signs of ageing than conplastic counterparts. Respiration in B6-C57 mice also declined steadily with age, whilst B6-NZB remained constant between days 20 and 300. The authors conclude that NZB mtDNA promotes healthier ageing in the B6 nuclear background. They also report that mtDNA haplotype influences mitochondrial proteostasis and reactive oxygen species generation, insulin signalling and obesity. On the basis

of these observations, the authors suggest that consideration should be given to matching the mtDNA of patient and mitochondria donor if performing mitochondrial donation.

- 4.17.** While these data are interesting, the relevance to humans, who are outbred and undergo regular exchange of mtDNA haplotype and nuclear genomes, is unclear; these points were made in the 2014 report. But in addition, the finding that there is a significant advantage to B6 mice in exchanging their mtDNA to that of NZB adds an additional complication. This could be seen not as an argument for matching haplotypes, but for deliberately searching for one that is advantageous. Moreover, because the reciprocal conplastic strain (NZB-C57) was not generated, it is not known if these would have fared less well or have had the same or other advantages.
- 4.18.** Ma et al. (2016) performed reciprocal mitochondrial donation using PNT between two subspecies of mouse that have diverged from a common ancestor for over 500,000 years, B6 and PWD. As expected, these strains are highly divergent in their mtDNA genotypes, differing by 391 SNPs (2.4% of the mtDNA genome). Remarkably, post-implantation development of embryos with a B6 nuclear genome and PWD mtDNA (B6-PWD) was not significantly different to controls. Male fertility in adults was reduced, but not in females. In contrast, embryos with PWD nuclear DNA and B6 mtDNA (PWD-B6) were much less viable: only one live pup out of 359 transferred blastocysts was obtained; the rest were stillborn. This lethality likely reflects incompatibility between PWD nuclear DNA and B6 mtDNA. The authors propose that these data constitute direct evidence that mtDNA genotype divergence plays a major role in reproductive isolation.
- 4.19.** The significance for humans of the mtDNA-nuclear DNA incompatibility revealed by this study is unclear. Average mtDNA haplotype divergence in humans is bimodal, with a main peak at 45 SNPs and a secondary peak at 100 SNPs⁷⁴. The PWD-B6 mtDNA divergence (391 SNPs) is far greater - and it is striking that B6-PWD conplastic mice are still viable despite this. Nevertheless, the authors suggest that matching haplotypes of the patient and mitochondrial donor in clinical application of mitochondrial donation would be judicious. It is also worth noting that inbred mouse strains are commonly used because they allow the consequences of particular mutations to be studied without additional confounding genetic variation, and because phenotypes in general are much more highly penetrant on such backgrounds. This is probably due to the fact that the almost all loci in such mice are homozygous at all nucleotide positions, apart from those affected by *de novo* mutation. Outbred genomes, such as that of humans, provide a greater degree of functional 'buffering' due to common heterozygosity.
- 4.20.** **The panel's view on mito-nuclear interactions has not been altered by recent data. An argument can be made for deliberately choosing a donor haplotype (or at least D-loop sequence) based on its ability to out-compete that of the patient to avoid any possibility of reversion. However, this would require further research and an understanding of the outcome of clinical applications of the methods. The panel maintains its earlier conclusion that consideration should be given to haplotype/haplogroup matching based on the outcome of future research and clinical experience.**

⁷⁴ Blanco R, Mayordomo E, Montoya J, Ruiz-Pesini E. Rebooting the human mitochondrial phylogeny: an automated and scalable methodology with expert knowledge. BMC bioinformatics. 2011 May 19;12(1):1.

Long-lasting nuclear epigenetic modifications

Summary of previous considerations

In 2014, the panel received no new evidence on epigenetic effects to alter their conclusions reached in 2013. One of the experts the panel consulted during the 2014 review highlighted that the nuclear DNA of mothers carrying a proportion of mutant mtDNA could undergo some remodelling of genes (e.g. epigenetic modification), notably those involved in mitochondrial function, with the result that these might then compensate for the deficiency in the mutated gene on the mtDNA and thereby reduce the phenotypic consequences of the mutant mtDNA. Such epigenetic effects might also affect subsequent mito-nuclear communications in a novel way; however, to be relevant, this would have to occur in the germline and particularly in oocytes, which is unlikely given the rather inactive state of mitochondria in these cells. The experts considered this to be an unlikely eventuality, but of low risk if it did occur.

The panel also considered the possibility of epigenetic or other deleterious effects on embryos as a result of oxidative stress during the mitochondria replacement procedures. They concluded that there is substantial evidence that mitochondria with different haplotypes vary in their tolerance to oxidative stress; however, this is likely to be more relevant in mature tissues, where mitochondrial function is critical, and less of a concern to early embryos in culture.

- 4.21.** The panel's view on potential epigenetic effects remained unchanged from those expressed in previous reports. Any risks with respect to MST and PNT would appear to be low. However, the panel recognised the need to monitor research in this area.

5. Clinical considerations

Implications of mtDNA reversion in human stem cells

- 5.1.** It seems clear from the experiments of Hyslop et al. (2016), Yamada et al. (2016) and Kang et al. (2016) that some clones of stem cells derived from human embryos constructed by MST or PNT can accumulate significantly elevated levels of karyoplast-associated mtDNA with increased passaging. Should this phenomenon be replicated *in utero* following clinical application of mitochondrial donation, it could lead to the birth of an affected child. Without a definitive mechanism to account for this reversion, the panel considered it difficult to determine the risk of occurrence *in vivo*. It may be that these effects were peculiar and specific to embryonic stem cell populations and their differentiated derivatives that were being used *in vitro* as a model of the developing embryo. Or, it may be that they were effects that might occur in embryos developing *in utero* in their usual three dimensional morphology and particular niche, as a result of carryover of karyoplast-associated mitochondria that preferentially replicate and cause unexpected symptomatic heteroplasmy. This theoretical possibility was raised in section 3 of the 2014 report.

Comparisons with PGD

- 5.2.** In considering the potential for adverse effects of pathogenic mtDNA carryover following MST or PNT, the panel made comparisons with mutation levels (heteroplasmy) in the embryo when PGD is undertaken for mitochondrial disease (see Table 1). It is apparent from the published literature discussed below, and from interviews conducted by the panel, that decisions about safe levels of heteroplasmy vary widely between clinics practicing PGD for mitochondrial disease. Depending on the specific mutation, it is not uncommon for there to be few, if any, tested embryos with undetectable levels of heteroplasmy; most embryos contain 'low' levels of pathogenic mtDNA within a range purported to have a low or reduced likelihood of manifesting symptoms in the future child⁷⁵, but these levels are usually higher than the levels of carryover achieved (or limits proposed) following MST or PNT. Moreover, there are cases with homoplasmy or very high levels of heteroplasmy, where PGD can never give an embryo with acceptable levels of pathogenic mtDNA.
- 5.3.** Whereas some clinics use a theoretical consideration of a general level of mutant heteroplasmy below which symptoms are unlikely in order to set a maximum for transfer, it is clear that the 'safe' level will vary with the nature of the mutation and the particular clinical features it gives, and the likelihood that the symptoms will be serious⁴⁶. Estimates of these 'safe levels' may well be improved as more information becomes available with use of PGD for mitochondrial disorders, although this will take time to accumulate. In addition, some centres prefer to take into account the patients' wishes, especially when there are no 'safe level' embryos to replace. One view is that it might even be acceptable to replace an embryo with a higher-than-threshold level of mutant heteroplasmy, recognising that, even at such levels, this would nevertheless represent a risk reduction from the high levels that would probably be present should the couple have decided to reproduce naturally¹².

⁷⁵ Hellebrekers DM, Wolfe R, Hendrickx AT, de Coo IF, de Die CE, Geraedts JP, Chinnery PF, Smeets HJ. PGD and heteroplasmic mitochondrial DNA point mutations: a systematic review estimating the chance of healthy offspring. Human reproduction update. 2012 Jul 1;18(4):341-9.

Table 1: Mutation levels in live births following PGD and MST for mitochondrial disease^{76, 77}

PGD data (2005-2016)					
	Mutation	At biopsy	At birth	Comments	Reference
1	m.8993T>G	0% & 0%	0%	First report. Two embryos transferred	Steffann et al., 2006
2	m.8993T>G	2.5%	4%	3-5% cord blood & placenta; buccals 5% at age 4½y	Thorburn et al 2010*
3	m.3243A>G	5% & 13%	5%	Two embryos transferred; 15±5% placenta, 5±1% cord blood	Monnot et al., 2011
4	m.3243A>G	12%	15%	47% blood, 52% urine @ 1½m; 46/42% @18m	Treff et al., 2012/ Mitalipov et al., 2014
5	m.8993T>G	0%	0%	'Healthy son', no further details	Sallevelt et al., 2013
6	m.8344A>G	53% & 59%	63%	Two embryos transferred; no further details	Steffann et al., 2014
7	m.3243A>G	0%	0%	Male; measured in cord blood, urine, saliva	Heindryckx et al., 2014
8	m.36**G>A	2%	7%	Female, measured buccal and urine cells	Newcastle group 2016*
9	m.83**A>G	48%	Not available	Male; <60% generally asymptomatic	Newcastle group 2016*
10	m.130**T>C	1%	0%	Male; undetectable in cord and peripheral blood	Newcastle group 2016*
11	m.101**T>C	1%	1-2%	Male; cord blood	Newcastle group 2016*
MST data (2005-2016)					
	Mutation	At biopsy	At birth	Comments	Reference
1	8993T>G	5.7%	1.6%	'Healthy' male; 1.60 ± 0.92% measured in buccal cells, hair follicles, foreskin, urine, placenta, amnion, cord blood, and cord tissue.	Zhang et al., 2016b, Liu et al., 2016***

⁷⁶ Each row corresponds to one live birth.

⁷⁷ * personal communication

** characters hidden to respect confidentiality

*** abstract.

- 5.4.** The particular mutation has a bearing on what is considered a 'safe' level. Whilst embryos from 3243A>G carriers manifest a full range of mutation loads and thus are associated with more in the intermediate, and potentially symptomatic range, and have fewer embryos with low or absent levels, other mutations (e.g. 8993T>G) show skewed distributions with embryos tending to fall in either the very high or the very low or absent range, allowing a greater margin of safety in the low range^{78,79}.
- 5.5.** However, there is still some uncertainty when deciding on 'safe' levels of mutant mtDNA for embryo transfer, since the actual proportion of pathogenic mtDNA present in the child once born could be somewhat higher or lower than that predicted from the embryo biopsy. This might be due to effects of the mitochondrial bottleneck in early development, and the possibility of non-random genetic drift or preferential amplification of abnormal mtDNA¹¹. The panel is aware of the PGD case report (Treff et al. and 15; see Table 1, case 4) where levels rose from 12% at blastocyst biopsy, 15% in buccal cells at birth, to 52% in urine cells at 1½ months of age⁴⁸. Therefore, in addition to reviewing published cases, the panel made contact with centres internationally that practise PGD for mitochondrial disease in order to ascertain how frequently this happens. The panel noted that although there seem to have been very few babies born as a result of PGD for mtDNA mutations (see table 1), in general, heteroplasmy at birth is not very different from that at embryo biopsy, even when levels of mutation load are much greater than the <5% recommended for PNT and MST. However, it clearly is possible that significant drift can occur after birth, even in situations where the mutation resides in an otherwise genetically homogeneous population of mtDNA, as illustrated by the case described above.
- 5.6.** The possibility that drift similar to that seen in PGD could arise after MST or PNT cannot be excluded. In addition, as discussed at length above (notably in section 4), there may be other effects specific to MST and PNT due to the transfer process itself, which may occasionally 'activate' the mtDNA that was associated with the karyoplast, or as a result of the highly asymmetric distribution of karyoplast-associated mtDNA that is generated in the reconstructed embryo, or because of the presence of two different haplogroups/haplotypes, where one has a replicative advantage over the other. In this regard, the panel was mindful of the concerns expressed in submissions, and implicit in the recent publications, about reversion towards or to the mtDNA haplotype associated with the karyoplast in a small percentage of stem cell lines. However, it is noteworthy that in the report of Zhang et al., (2016a), where PNT was undertaken for treatment of infertility rather than avoidance of genetic disease, examination of the two mid-trimester fetuses showed no presence of the maternal mtDNA mutation. Moreover, in the recent abstracts describing the use of MST for the avoidance of transmission of m.8992T>G¹⁰, the report of low heteroplasmy levels in the baby suggests that substantial reduction of mutant mtDNA by PNT and MST is possible.
- 5.7.** Our understanding of mitochondrial donation techniques will doubtless improve as more clinically relevant models are developed and further data are obtained from human embryos. It is to be hoped that this will lead to a mechanistic understanding of likely behaviours of mitochondria and mtDNA in vivo, and therefore strategies to avoid adverse effects, if necessary. For these reasons, the panel recommends that it would be useful to examine the behaviour of mtDNA in stem cells derived from PGD embryos (unsuitable for transfer) with low levels of heteroplasmy.

⁷⁸ Steffann J, Monnot S, Bonnefont JP. mtDNA mutations variously impact mtDNA maintenance throughout the human embryofetal development. *Clinical genetics*. 2015 Nov 1;88(5):416-24.

⁷⁹ Blok RB, Gook DA, Thorburn DR, Dahl HH. Skewed segregation of the mtDNA nt 8993 (TRG) mutation in human oocytes. *The American Journal of Human Genetics*. 1997 Jun 30;60(6):1495-501.

Clinical use of mitochondrial transfer techniques

- 5.8.** In making a decision about the efficacy and clinical safety of mitochondrial transfer techniques, the panel noted:
- (i) PNT methods can now efficiently produce good quality blastocysts based on a number of criteria
 - (ii) much higher levels of mutation heteroplasmy are accepted for PGD than are recommended as a target for PNT and MST (<5%)
 - (iii) reversion to the karyoplast-associated mtDNA haplogroup occurred in only a proportion (around 10-25%) of stem cell lines cultured from MST embryos in vitro
 - (iv) even use of PGD may not eliminate transmission of mtDNA mutations, but it is still useful for risk reduction in the offspring. In addition, the panel considered the recent report of a three-month old, apparently healthy baby born after MST, with low levels of pathogenic mtDNA in several tissues, to be reassuring. The panel thus felt that MST and PNT are now at an acceptable stage for cautious clinical use as a risk reduction strategy in carefully selected cases⁴⁹: those where PGD is unlikely to be successful or impossible (e.g. due to high levels of maternal pathogenic mtDNA), or where PGD has been unsuccessful. In these circumstances the patients would have to be counselled as to the risks, and offered prenatal testing should they become pregnant.
- 5.9.** The panel discussed ways of predicting the phenotype prenatally^{29,31}. Prenatal testing by CVS, amniocentesis or fetal blood sampling was considered. Amniocentesis may be scientifically preferable, as the cell types present in amniotic fluid come from all three embryonic lineages, whereas fetal blood cells are entirely mesodermal in origin, and the risk of miscarriage following amniocentesis is extremely low. However, the greater yield of cells from CVS compared with that from amniocentesis means that CVS is the preferred option in most diagnostic laboratories. None of these sample types for prenatal testing would necessarily be representative of outcome, so it would be important to counsel patients about the pros, cons and limitations of prenatal testing before any decisions are made.
- 5.10.** Due to the difficulties in predicting whether an embryo will undergo reversion to the mutant mtDNA haplotype carried over from eggs or zygotes from patients, and the possibility of other unpredictable outcomes of the treatment, the panel concludes that it would be prudent to restrict mitochondrial donation treatment to patients whose clinicians believe that PGD is not a feasible option because of predicted homoplasmy or high levels of heteroplasmy in their embryos. This restriction may be revised following analysis of data from treatment cycles. Indeed, as the panel has suggested previously, if MST and/or PNT prove safe and reliable, then they could be considered preferable to PGD as a risk reduction strategy for mitochondrial disease, not just in the children born, but for subsequent generations. The panel also advises that prenatal testing should be offered to all women undergoing treatment, but recognises that it is unlikely that all women will accept this offer. Long-term follow-up of the children is strongly recommended.

6. Recommendations

- 6.1.** The panel's view is that the methods of PNT and MST are sufficiently safe to proceed cautiously and in restricted circumstances. The science in this area has not stood still. Much progress has been made since the last review in 2014 when the panel offered the view that these methods were not unsafe but that further research was still needed. Since that time, significant work has been carried out, both in the basic research field to improve understanding of the biology of human mitochondria, especially in development, and on translational research aimed specifically at providing further safety information on MST and PNT. The panel's previous requests for additional experiments have largely been met and many of the concerns that had been raised are, in its view, even less likely to be a problem.
- 6.2.** One new issue that has arisen in the recent work from three groups is reversion towards the karyoplast-derived mtDNA haplotype after prolonged culture of ES cell lines derived from a minority of embryos made using MST or PNT. It remains unclear whether reversion will occur *in vivo* and be a problem if the techniques are applied clinically. However, this is a potential risk that the panel takes seriously and hence it recommends restricted application in the first instance. An alternative for the panel was to recommend that clinical application should not proceed until there was better understanding of the causes of the reversion and approaches designed to reduce or eliminate it. However, the panel felt that this approach was too cautious, especially when the need is real, and the risks may be small and manageable to some extent via prenatal testing, an option that may be acceptable to some patients.
- 6.3.** As stated in the previous reports and above, complete reassurance will never come from experiments conducted in animal models and with human material *in vitro*. Therefore, it should be accepted that there will always be some risk and uncertainties associated with the use of MST or PNT in humans until it is tried in practice⁸⁰. It is with due consideration of this point that **the panel recommends that techniques to avoid mitochondrial disease (MST and PNT) could be initially implemented as a risk reduction treatment for carefully selected patients.**

Patient selection

- 6.4.** For the time being, **the panel suggests limiting the scope of MST/PNT treatment to patients for whom PGD may be inappropriate and probably unsuccessful**, such as women whose germ line is likely to be homoplasmic or have high levels of heteroplasmy; such treatment will provide these patients with the choice of an alternative intervention for risk reduction. MST/PNT is currently the only option for producing a genetically related child for these patients. The panel does not recommend that the clinical use of MST or PNT should be extended to otherwise healthy individuals with fertility problems, because a strong causal link between infertility and impaired mitochondrial function has not been made; in addition, such clinical use is not legal in the UK.
- 6.5.** Pre-treatment assessment would need to take into account the particular mutation involved, the inheritance pattern in the family, the likely clinical manifestations of disease, patient age and the efficacy of any previous treatments such as PGD, and the patient's understanding of the risks and limitations of what is being offered. If the techniques prove to be safe when used in these

⁸⁰ Bredenoord AL, Braude P. Ethics of mitochondrial gene replacement: from bench to bedside. *Bmj*. 2010 Nov 8;341:c6021.

patients, including the absence of any significant reversion to the carried-over mtDNA haplotype, their application could be extended to other patients.

Prenatal testing and follow-up

- 6.6.** The panel recommends that if mitochondrial donation treatments are implemented, **patients should be offered prenatal testing following the transfer of any embryo that has been created as a result of these techniques**; however, it recognises the likelihood that not all women will accept this offer.
- 6.7.** The panel also strongly recommends rigorous long-term follow-up of children born as a result of these techniques in order to gather further information about safety and efficacy.

Parallels with PGD for mtDNA mutations

- 6.8.** As highlighted in the 2014 report, the panel drew attention to the parallels with PGD for mtDNA mutations in terms of acceptable levels of heteroplasmy. Although the intention of such therapy is to select embryos for transfer with as low a level of mutant mtDNA as possible to avoid the birth of a child who would manifest the disease in their lifetime, concerns related to variable segregation of mutant mitochondria in their tissues, and especially their gametes, also apply here.
- 6.9.** Hence, the panel recommends that for PGD of mtDNA mutations, clear rules are developed that establish acceptable levels of mtDNA heteroplasmy, indicating when transfer (or not) of an embryo is advised for each disease and each mutation, managed by the specialist clinical team in conjunction with their patients.

As with MST and PNT, follow-up of children and their offspring is strongly recommended for individuals arising from PGD for mtDNA mutations.

Haplogroup matching

- 6.10.** The panel continues to recommend that consideration is given to mtDNA haplogroup¹³ matching as a precautionary step in the process of selecting donors. Although the panel believes that any risks associated with a mtDNA-nuclear DNA mismatch remain theoretical, it recommends that when these techniques are used clinically, the latest evidence regarding how mtDNA haplotypes affect mitochondrial-nuclear (mito-nuclear) interactions, including replicative behaviour of mtDNA, should be considered in order to inform the donor selection process. Such evidence might even indicate the selection of a specific, unmatched donor in any given case. Whatever decision is made, **the panel recommends that haplotype information on the recipient and the donor is recorded.**

Further research

- 6.11.** Basic research is still needed to explore how the mitochondrial bottleneck functions, addressing the critical parameters involved in the segregation of normal and any specific abnormal mitochondria amongst cell types in humans, because this is generally not well understood. For example, in the long term it may be possible to influence or control replication of abnormal mtDNA

in the early embryo to affect its segregation or inheritance in subsequent development⁸¹. This is discussed in more detail in section 7.

- 6.12.** Our understanding of mitochondrial donation techniques will doubtless improve as more clinically relevant models are developed and further data are obtained from human embryos. It is to be hoped that this will lead to a mechanistic understanding of likely behaviours of mitochondria and mtDNA in vivo, and therefore strategies to avoid adverse effects, if necessary. For these reasons, the panel recommends that it would be useful to examine the behaviour of mtDNA in stem cells derived from PGD embryos (unsuitable for transfer) with low levels of heteroplasmy.

⁸¹ Gammage PA, Rorbach J, Vincent AI, Rebar EJ, Minczuk M. Mitochondrially targeted ZFNs for selective degradation of pathogenic mitochondrial genomes bearing large-scale deletions or point mutations. *EMBO molecular medicine*. 2014 Apr 1;6(4):458-66.

7. Further research

- 7.1.** In reaching a view that MST and PNT are sufficiently safe that they could now be offered to certain patients, the panel also recognises that further research is still desirable. Together with safety and efficacy data from first attempts, the data generated by such future research may result in more patients benefitting from these techniques. It is also important to obtain more basic knowledge about the biology of mitochondria in humans and model systems. This may shed light on the safety and efficacy of mitochondrial donation, lead to novel ways to prevent mitochondrial diseases in offspring, and/or enable treatment for individuals already affected by them. The suggestions below are not intended to be exhaustive.

Disruptions to mito-nuclear interactions

- 7.2.** Although the panel continues to consider these unlikely based on the evidence it has seen, any deleterious or beneficial effects of altering mito-nuclear interactions should be explored, where possible, in scenarios as relevant as possible to human physiology. The panel recommend that genome-wide association studies (GWAS) and other large-scale human genome sequencing projects that are linked to health, which may have been initiated for other reasons, should include an analysis of mtDNA sequences and how these are inherited. Detailed knowledge of the structure of mitochondrial complexes involved in OXPHOS could also be used to help understand and perhaps even predict how nuclear and mtDNA-encoded components interact and how variations in the gene sequences might have a bearing on their function⁸²

Whole genome sequencing approaches to investigating mitochondrial disease genetics

- 7.3.** Existing whole genome sequencing projects linked to health and lifestyle records for broad human populations, such as the 100,000 genomes project⁸³ provide useful information relevant to clinical manifestation of mitochondrial disease and options for treatment or its avoidance. In addition, focussed studies involving whole genome sequencing of patients with mitochondrial disease and unaffected family members (as well as comparisons with the general population) might reveal whether there are any predisposing nuclear genomic alleles or specific combinations of nuclear and mtDNA variants that are more likely to be associated with disease (with the caveat that numbers may be too low to give robust answers). A recent review³¹ by Sallevelt et al (2016) reported that in 24.6% of patients with (likely) pathogenic mtDNA point mutations, the mutation had arisen *de novo*. This group presents an opportunity to assess whether one or more nuclear genome sequences, perhaps in combination with specific mtDNA variants, predispose to mtDNA mutations. Genes involved in mtDNA replication or which lead to high levels of ROS are likely candidates – indeed they are already known to be involved: one of the six *de novo* cases discussed by Sallevelt et al. (2016) carried a *POLG* mutation.

⁸² Fiedorczuk K, Letts JA, Degliesposti G, Kaszuba K, Skehel M, Sazanov LA. Atomic structure of the entire mammalian mitochondrial complex I. *Nature*. 2016 Sep 5.

⁸³ The 100,000 Genomes Project. Accessed at: <https://www.genomicsengland.co.uk/the-100000-genomes-project>.

mtDNA behaviour in ES or iPS cells and their differentiated derivatives

- 7.4.** It is not yet clear whether the reversion phenomenon is specific to ES cells in culture and/or whether it would be seen when heteroplasmy involves the same haplotype (i.e. after mtDNA mutation) or if it only occurs when heteroplasmy involves a mix of two (or more) mtDNA haplotypes (e.g. after MST, PNT or SCNT). Even if it turns out to be mostly specific to ES cells, it is still worth investigating if it reveals information about mtDNA bottlenecks, upward drift in the proportion of mutant mtDNA often seen with ageing in specific cell types, and the effects of specific variants on mtDNA dynamics (such as replication) in combination with specific nuclear alleles. Some studies have been performed on iPS cell lines derived from patients with heteroplasmy²³, but it would also be worth studying ES cell lines derived from embryos known to carry different levels of heteroplasmy after PGD. It would also be possible to create large panels of ES or iPS cell lines with different combinations of mtDNA haplotype by making cybrids (fusing enucleated cells of one haplotype with ES/iPS cells of another).

The possibility of deriving oocytes from pluripotent stem cells *in vitro*

- 7.5.** The recent demonstration that it is possible to derive functional mouse oocytes entirely *in vitro* from ES cells and iPS cells suggests several future prospects that might help avoid mitochondrial disease⁸⁴. The panel acknowledges that the generation of human oocytes *in vitro* will pose many problems, not least how to deal with issues of timing, where development of the first oocytes to a fully grown stage in humans usually takes over a decade (in comparison with less than two months in mice). Moreover, given that both stochastic and quality control processes are thought to lead to the death (atresia) of the vast majority of oocytes *in vivo*, it will be a challenge to determine how to select those that are normal. However, the ability to derive oocytes *in vitro* would open up several possibilities for research and even treatment, including:

(i) By following the development of oocytes from iPS cells derived from patients heteroplasmic for mtDNA, more could be learned about the bottleneck and how this can lead to significant increases in the proportion of mutant mtDNA such that oocytes can even become homoplasmic, and how this contrasts with purifying selection that is also thought to occur in the germ line. There are also questions about mechanisms underlying such shifts, which could involve the loss or gain of cells with poorly performing mitochondria, mitophagy to remove abnormal mitochondria, and/or differences in rates of mtDNA proliferation and ratchet effects, that could favour mutant or normal mtDNA^{27,85}. All of these parameters may be influenced by the environment of the cells (availability of nutrients, oxygen, etc), which can be manipulated in culture in ways that are difficult *in vivo*.

(ii) By fusing cytoplasts from cells with one mtDNA haplotype with iPS cells carrying another, it would be possible to create heteroplasmy deliberately with a range of haplotype combinations to test when and how reversion occurs and whether oocytes are prone or resistant to this.

(iii) iPS cells from the patient could be used to derive oocytes, which could be used for MST with oocytes from normal donors providing the cytoplasts. In cases of heteroplasmy, because the iPS cells would be derived from somatic cells of the patient they are likely to have relatively low levels

⁸⁴ Hikabe O, Hamazaki N, Nagamatsu G, Obata Y, Hirao Y, Hamada N, Shimamoto S, Imamura T, Nakashima K, Saitou M, Hayashi K. Reconstitution *in vitro* of the entire cycle of the mouse female germ line. *Nature*. 2016 Oct 17.

⁸⁵ Malena A, Pantic B, Borgia D, Sgarbi G, Solaini G, Holt IJ, Spinazzola A, Perissinotto E, Sandri M, Baracca A, Vergani L. Mitochondrial quality control: Cell-type-dependent responses to pathological mutant mitochondrial DNA. *Autophagy*. 2016 Nov.

of the mutant mtDNA, even if her oocytes consistently have very high levels or are homoplasmic. It may even be possible to select iPS cell clones from the patient with very low levels of mutant mtDNA. This might even remove the need to carry out MST; although if the quality of the cytoplasm of in vitro-derived oocytes were low, providing new cytoplasm from a normal donor oocyte would be beneficial.

(iv) If in vitro-derived oocytes have high quality cytoplasm, but poor nuclear DNA, then it may be possible to derive oocytes from the patient via iPS cells with very low proportions of mutant mtDNA, and then use these to provide autologous cytoplasts for MST using karyoplasts from the patients naturally produced oocytes. (v) If patients have reduced fertility as suggested in some of the evidence we received (although this was at odds with the data from the use of PGD), then the ability to derive oocytes *in vitro* may overcome this problem.

Initiation of mtDNA replication and the reversion phenomenon

- 7.6.** There is no evidence in all the studies so far that reversion begins prior to late blastocyst stages, yet it can begin in relatively early passages of ES cells derived from such blastocysts. mtDNA replication must occur in ES cells (even though they have very low levels of OXPHOS), but there is currently little understanding of how and when mtDNA replication resumes in the early human embryo and whether it is similar in the various cell types (epiblast and extraembryonic). There is also evidence that replication of normal and mutant mtDNA differs in distinct cell types⁸⁴, but not when this arises during development. Very recently it has been demonstrated that it is possible to culture human embryos from late blastocysts, at about 7 days post fertilisation, in a system that mimics aspects of implantation and allows their development in three dimensions up to around 13 days^{86,87}. It should now be possible to use such cultures to explore mtDNA dynamics and mitochondrial function during this critical period when extra-embryonic tissues begin to elaborate and the epiblast forms the embryonic disc, just prior to gastrulation. In as much as these systems faithfully recapitulate this early developmental phase, they might also address whether mtDNA haplotype reversion occurs after MST or PNT. Although it may be feasible to modify the cultures to permit development through and beyond gastrulation, this would contravene the current legal limit of 14 days. However, isolated regions could, in principle, be maintained for longer, to explore what happens in cell populations that give rise to the germ layers and to primordial germ cells.

Research to eliminate risks of carryover and reversion

- 7.7.** The panel suggests that research in the areas below may eliminate the risks of carryover or reversion and may be useful for future refinements of the techniques.

There are essentially two categories of options that might be used to reduce or potentially eliminate reversion to karyoplast-derived mtDNA, if this turns out to be a concern following clinical use of MST and/or PNT. One does not require knowledge of the underlying mechanism, but would involve methods to reduce or eliminate carryover of karyoplast-associated mtDNA. The other is predicated on at least some knowledge of the mechanism and is based on subsequent management or manipulation of this to prevent reversion. It seems likely that the mechanism is due either to some feature of the methodology that is common to MST and PNT (and SCNT), or

⁸⁶ Deglincerti A, Croft GF, Pietila LN, Zernicka-Goetz M, Siggia ED, Brivanlou AH. Self-organization of the in vitro attached human embryo. *Nature*. 2016 May 4.

⁸⁷ Shahbazi MN, Jedrusik A, Vuoristo S, Recher G, Hupalowska A, Bolton V, Fogarty NM, Campbell A, Devito LG, Ilic D, Khalaf Y. Self-organization of the human embryo in the absence of maternal tissues. *Nature cell biology*. 2016 May 4.

to preferential replication of the karyoplast-derived mtDNA haplotype. The latter could be a consequence of specific differences in the sequence of its D-loop, compared to that in the cytoplasm (donor)-derived mtDNA haplotype. Notably, all these options should reduce or eliminate normal as well as abnormal mtDNA if both are present within the karyoplast and lead towards homoplasmy for the cytoplasm (donor)-derived mtDNA haplotype. However, the safety and efficacy of these proposed methods will need exploration.

Methods to reduce or eliminate carryover of karyoplast-associated mtDNA

Mechanical methods

- 7.8.** It may be possible to refine methods of micromanipulation when removing the karyoplast containing the spindle or pronuclei in order to reduce the volume of the cytoplasm containing mitochondria. This has already been done to some extent by the Newcastle Group using the pipette to pinch off excess cytoplasm as the karyoplast is being introduced into the recipient zygote (Hyslop et al., 2016 and Professor Mary Herbert, unpublished evidence presented to the panel). However, it would be challenging to avoid any transfer of mitochondria at all, and there is the concern that even very low levels of mtDNA with a replicative advantage could eventually predominate.

Alternative methods for performing mitochondrial donation

- 7.9.** Whilst acknowledging that they are currently unlawful in the UK, the panel continues to support the investigation of polar body transfer (PBT) methodologies⁴. The very low numbers of mitochondria found particularly in the 1st, but also the 2nd polar bodies make them ideal, ready-made karyoplasts for transfer to a donor egg or zygote. In conjunction with the carry-over elimination approaches discussed above and below, they offer great potential for near elimination of karyoplast-derived mtDNA. Following a detailed study in mice⁸⁸, a recent publication described the successful generation of human blastocysts following polar body transfer and showed that the levels of aneuploidy were similar between PBT and control blastocysts⁸⁹, underlining the promise of this approach for treatment of women at risk of passing on mitochondrial disorders.

Induction of mitophagy for selective elimination of karyoplast-derived mitochondria

- 7.10.** Mitophagy is the natural process by which cells eliminate damaged or unwanted mitochondria. It falls within the general term of autophagy, which is a regulated process by which cells eliminate part of their cytoplasm to cope with stress or damage, from which they can then recover. The mechanisms make use of pathways within cells to eliminate proteins, macromolecular structures, or organelles, where these are marked by ubiquitination and broken down by proteasomes or destroyed after fusion with lysosomes. There are at least two types of mechanism leading to mitophagy:
- (i) The mechanism that operates in most cells, and which is often triggered by misfolded proteins

⁸⁸ Wang T, Sha H, Ji D, Zhang HL, Chen D, Cao Y, Zhu J. Polar body genome transfer for preventing the transmission of inherited mitochondrial diseases. *Cell*. 2014 Jun 19;157(7):1591-604.

⁸⁹ Ma H, O'Neil RC, Gutierrez NM, Hariharan M, Zhang ZZ, He Y, Cinnioglu C, Kayali R, Kang E, Lee Y, Hayama T. Functional Human Oocytes Generated by Transfer of Polar Body Genomes. *Cell Stem Cell*. 2016 Nov 10. In Press.

inside the mitochondrial compartment, involves the proteins PINK1 and Parkin, which label dysfunctional mitochondria for degradation^{90,91,92,93}. Mitochondrial fission contributes to this process by separating damaged segments of mitochondria from healthy ones⁹⁴. The use of agents to disrupt protein folding within mitochondria, or target misfolded proteins, or PINK1 and Parkin, or other triggers of mitophagy of the mitochondria specifically in the karyoplast (or in the oocyte or zygote prior to its removal), might lead to the specific destruction of the karyoplast-derived mitochondria in the early embryo. Recent data suggest that pp242, an ATP-competitive mTORC1/mTORC2 kinase inhibitor, can trigger mitophagy, at least in tumor cells⁹⁵. This, or similar small molecules, could be used if shown to be safe and their action can be restricted to the elimination of karyoplast-derived mitochondria. Diot et al (2016) suggest that autophagy (including mitophagy) occurs in early cleavage stage mouse embryos and that mitochondria removed by this process may tend to be those with the highest levels of mutant mtDNA and the least capacity for oxidative phosphorylation. They also suggest that two known activators of mitophagy, rapamycin and phenanthroline, can function in two-cell stage mouse embryos. Perhaps they would also work in oocytes or zygotes.

(ii) The other mechanism of mitophagy is that used to eliminate the mitochondria that are present in the sperm after fertilisation. There are two reasons proposed for why this happens naturally: to prevent heteroplasmy in the resulting embryo, and/or to eliminate mtDNA that is likely to be damaged by high levels of ROS in the sperm^{96,97}. The mechanism relies on ubiquitination of mitochondrial membrane proteins within the sperm, although the details of how and exactly when this occurs is not well understood. After fertilisation, however, recent evidence suggests that mitophagy of sperm mitochondria in the zygotes of higher mammals relies on a combined action of canonical ubiquitin-like autophagy receptors, notably sequestosome 1 (SQSTM1), and valosin-containing protein (VCP)-mediated dislocation and presentation of ubiquitinated sperm mitochondrial proteins to the 26S proteasome. This may explain how all the sperm mitochondria are degraded inside fertilised oocytes by a protein recycling system usually involved in degradation of single protein molecules. If the mechanism for ubiquitinating the mitochondria in sperm could be hijacked and repurposed to specifically ubiquitinate those in the karyoplast (or in the oocyte or zygote prior to its removal), then this should automatically lead to their degradation in the early embryo in an analogous manner.

It may be possible to harness or modify these natural processes of mitophagy to eliminate karyoplast-derived mitochondria prior to MST or PNT. Following refinement in cell lines and animals, the safety and efficacy of these approaches could be assessed in human embryos.

⁹⁰ Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of cell biology*. 2008 Dec 1;183(5):795-803.

⁹¹ Pickrell AM, Youle RJ. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron*. 2015 Jan 21;85(2):257-73.

⁹² Pellegrino MW, Haynes CM. Mitophagy and the mitochondrial unfolded protein response in neurodegeneration and bacterial infection. *BMC biology*. 2015 Apr 3;13(1):1.

⁹³ Haroon S, Vermulst M. Linking mitochondrial dynamics to mitochondrial protein quality control. *Current opinion in genetics & development*. 2016 Jun 30;38:68-74.

⁹⁴ Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G, Alroy J. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal*. 2008 Jan 23;27(2):433-46.

⁹⁵ Gordeev SA, Bykova TV, Zubova SG, Bystrova OA, Martynova MG, Pospelov VA, Pospelova TV. mTOR kinase inhibitor pp242 causes mitophagy terminated by apoptotic cell death in E1A-Ras transformed cells. *Oncotarget*. 2015 Dec 29;6(42):44905.

⁹⁶ Song WH, Yi YJ, Sutovsky M, Meyers S, Sutovsky P. Autophagy and ubiquitin-proteasome system contribute to sperm mitophagy after mammalian fertilization. *Proceedings of the National Academy of Sciences*. 2016 Sep 6;113(36):E5261-70.

⁹⁷ Song WH, Ballard JW, Yi YJ, Sutovsky P. Regulation of mitochondrial genome inheritance by autophagy and ubiquitin-proteasome system: implications for health, fitness, and fertility. *BioMed research international*. 2014 Jun 17;2014.

Nuclease-mediated elimination of the karyoplast mtDNA haplotype

7.11. Experiments on cells in culture and in mice suggest that specific restriction enzymes, zinc finger nucleases (ZFN) and TALE nucleases (TALENs), each of which can be designed and constructed in the lab, can be targeted to mitochondria (using one of the peptide sequences involved in the normal transport of nuclear-encoded proteins to mitochondria) in order to make a double-stranded break in specific sequences within mtDNA leading to its degradation^{98,99,100,101,102}. Notably, TALENs have been used to eliminate a specified mtDNA haplotype in heteroplasmic 1-cell mouse embryos, which on transfer can give healthy mice that are homoplasmic for the mtDNA haplotype that was not targeted¹⁰⁰. Professor Ispizua-Belmonte submitted evidence to the panel, in which he discussed these methods and their possible uses. These approaches were originally developed with the idea of eliminating mutant mtDNA from early embryos or mature tissues in cases of heteroplasmy.

However, it was recognised they would be inappropriate for cases of homoplasmy, or where the level of heteroplasmy for the abnormal mtDNA was very high, because they would reduce mtDNA levels below those required for viability¹⁰⁰. One potential way to overcome this problem, assuming there was at least a proportion of normal mtDNA, would be to introduce mtDNA replication factors (that are normally absent in the early embryo) at the same time as the targeted nuclease. This should promote several rounds of replication of the normal mtDNA, while the mutant version is being destroyed; but this approach is untested and may be difficult to control.

One other complication is that the method originally proposed and tested in mice requires targeting the specific sequence that is mutated in the abnormal mtDNA while leaving the normal version untouched. This will depend on the specific mutation and may be technically challenging. Nevertheless, these nuclease-based methods might be an ideal way to eliminate carryover after MST or PNT. This will require detailed knowledge of the mtDNA sequences in both the patient and the oocyte donor in order to design, for example, a TALEN that will specifically cut the mtDNA haplotype from the former. However, it does not need to distinguish the specific mutation, but only one or more of the several variant sequences that will distinguish the two haplotypes, making it easier to design.

There will be a concern that the nucleases could also affect “off-target” sequences within the nuclear genome. If these were cut, then processes of DNA-repair, such as non-homology end joining (NHEJ) would operate, with the likelihood that they will lead to a small insertion or deletion (INDEL), which could have deleterious consequences if it affected a protein coding region or critical regulatory region of a gene. (N.B. the mechanisms of NHEJ and homology-directed repair (HDR), which both operate on double-stranded breaks in DNA within the nucleus, do not occur within mitochondria.) It is therefore critical to establish that the targeting of the nucleases to mitochondria is highly efficient, and/or that the target sequence within the mtDNA is absent from both the patient’s nuclear genome and that of her partner. Again, the safety and efficacy of these

⁹⁸ Because there is no obvious means by which guide RNAs could be targeted to mitochondria, the CRISPR/Cas9 system is currently unlikely to be useful in these approaches and mtDNA repair doesn’t operate efficiently.

⁹⁹ Gammage PA, Gaude E, Van Haute L, Rebelo-Guiomar P, Jackson CB, Rorbach J, Pekalski ML, Robinson AJ, Charpentier M, Concordet JP, Frezza C. Near-complete elimination of mutant mtDNA by iterative or dynamic dose-controlled treatment with mtZFNs. *Nucleic Acids Research*. 2016 Sep 19;44(16):7804-16.

¹⁰⁰ Diot A, Dombi E, Lodge T, Liao C, Morten K, Carver J, Wells D, Child T, Johnston IG, Williams S, Poulton J. Modulating mitochondrial quality in disease transmission: towards enabling mitochondrial DNA disease carriers to have healthy children.

¹⁰¹ Bacman SR, Williams SL, Pinto M, Peralta S, Moraes CT. Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. *Nature medicine*. 2013 Sep 1;19(9):1111-3.

¹⁰² Reddy P, Ocampo A, Suzuki K, Luo J, Bacman SR, Williams SL, Sugawara A, Okamura D, Tsunekawa Y, Wu J, Lam D. Selective elimination of mitochondrial mutations in the germline by genome editing. *Cell*. 2015 Apr 23;161(3):459-69.

approaches – including potential effects on the sequence of nuclear DNA - should be carefully assessed in human embryos generated by MST/PNT.

Although these methods might raise ethical and regulatory concerns about the use of genome editing techniques to make heritable changes, in the scenarios being discussed here, the goal is to eliminate the targeted mtDNA haplotype so that it cannot be inherited. Moreover, the methods involve the introduction of proteins (or perhaps mRNAs encoding them) in the oocyte or zygote, such that they will be present only transiently.

Mitochondria are often moved around within cells and form strong associations with organelles, including the nucleus. This is presumably to maintain a ready source of ATP for processes that require energy. These associations, which involve microtubules and/or microfilaments, are often dynamic^{103,104,105,106}. For example, mitochondria are largely excluded from the 1st polar body and the set of chromosomes it contains, while perhaps remaining more closely associated with the set that remains within the oocyte^{107,108}.

There is also evidence that they are closely linked with the pronuclei (perhaps to facilitate DNA replication)⁹³. Any physical link might be difficult to disrupt by simple mechanical means. While the removal of the karyoplast from either the oocyte or zygote makes use of cytoskeletal inhibitors, perhaps the amounts used are insufficient to break these links. The panel therefore sees value in research aimed at identifying the molecular nature of the putative links, as a means of shedding light on the biology of mitochondria in the oocyte and zygote, and to determine whether they can be disrupted using agents like nocodazole (which disrupts microtubules), cytochalasin B or D and Latrunculin A (that disrupt microfilaments) at levels that do not interfere with viability, including meiotic and mitotic cell divisions.

Methods relying on knowledge of the mechanism(s) that underlie reversion

Disruption of mitochondrial networks or clusters

7.12. Repeated fusion and fission events involving mitochondria promote the equal distribution of their contents, which would include mtDNA variants within a cell. In the fully-grown oocyte and early embryo, mitochondria tend to be small and round, suggesting that fusion is infrequent^{93,109}. If so, this might lead to retention of karyoplast-derived mtDNA in the subset of mitochondria carried over after MST or PNT, rather than to their re-distribution throughout all the mitochondria. If the

¹⁰³ Katayama M, Zhong Z, Lai L, Sutovsky P, Prather RS, Schatten H. Mitochondrial distribution and microtubule organization in fertilized and cloned porcine embryos: implications for developmental potential. *Developmental biology*. 2006 Nov 1;299(1):206-20.

¹⁰⁴ Shoji S, Yoshida N, Amanai M, Ohgishi M, Fukui T, Fujimoto S, Nakano Y, Kajikawa E, Perry AC. Mammalian Emi2 mediates cytotstatic arrest and transduces the signal for meiotic exit via Cdc20. *The EMBO journal*. 2006 Feb 22;25(4):834-45.

¹⁰⁵ Koutsopoulos OS, Laine D, Osellame L, Chudakov DM, Parton RG, Frazier AE, Ryan MT. Human Mitons associate with mitochondria and induce microtubule-dependent remodeling of mitochondrial networks. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 2010 May 31;1803(5):564-74.

¹⁰⁶ Chaigne A, Campillo C, Voituriez R, Gov NS, Sykes C, Verlhac MH, Terret ME. F-actin mechanics control spindle centring in the mouse zygote. *Nature communications*. 2016 Jan 4;7. Pages/volume?

¹⁰⁷ Dalton CM, Carroll J. Biased inheritance of mitochondria during asymmetric cell division in the mouse oocyte. *J Cell Sci*. 2013 Jul 1;126(13):2955-64.

¹⁰⁸ Wang T, Sha H, Ji D, Zhang HL, Chen D, Cao Y, Zhu J. Polar body genome transfer for preventing the transmission of inherited mitochondrial diseases. *Cell*. 2014 Jun 19;157(7):1591-604.

¹⁰⁹ Takahashi Y, Hashimoto S, Yamochi T, Goto H, Yamanaka M, Amo A, Matsumoto H, Inoue M, Ito K, Nakaoka Y, Suzuki N. Dynamic changes in mitochondrial distribution in human oocytes during meiotic maturation. *Journal of assisted reproduction and genetics*. 2016 Apr 27:1-0.

karyoplast-derived mitochondria were to become distributed equally throughout the cytoplasm of the zygote, then the two haplotypes would become mixed and remain at a low level once fusion/fission events begin in the late blastocyst or around gastrulation (assuming no difference in the onset of mtDNA replication or its rate). However, if the karyoplast-derived mitochondria tend to remain clustered, this mixing would be inefficient. This is in fact what tends to occur, at least in mouse zygotes and 2-cell embryos¹⁰⁴. There is then a possibility that the phenomenon of reversion is due to the karyoplast-derived mitochondria remaining associated in a cluster, such that during cleavage divisions they tend to end up in one or a few blastomeres, which by chance may be those that give rise to the epiblast. (The ICM is derived from a few blastomeres that first become inside cells in the morula; the epiblast is derived from a subset of the ICM cells, and the embryo proper (or ES cells) arise from only from a subset of the epiblast cells.)

Mitochondria are often associated with the cytoskeleton, which is used to move them around from one part of a cell to where they are needed most. The cytoskeleton is also likely to be involved in their fusion and fission¹⁰⁷. Although the oocytes and zygotes used in MST and PNT are treated with cytoskeletal inhibitors to allow removal of the spindle or pronuclei as karyoplasts, the inhibitors are not present in the medium when the karyoplasts are put in a drop of fusogen or when the cytoplasm plus karyoplasts are incubated to allow fusion and subsequent development. This means that the karyoplast-derived mitochondria may well begin to re-cluster before associating with those in the cytoplasm, and then the two populations may tend to remain separately clustered during subsequent cleavage divisions.

Using MitoTracker, or a fluorescent protein tagged so that it labels mitochondria, it should be possible to follow distribution and segregation of karyoplast-derived mitochondria during cleavage divisions¹¹⁰. If clustering and segregation into a subset of blastomeres is seen, then it might be possible to disrupt this in the zygote by brief incubation in cytoskeletal inhibitors, or to at least to include these along with the karyoplast with the fusogen, while being careful to avoid mis-segregation of chromosomes and aneuploidy.

Pronuclear stage zygotes seem fairly resistant to disruption of cytoplasmic components, given that rabbit, sheep, cow and pig zygotes can withstand centrifugation in order to visualise the pronuclei when making transgenic animals via microinjection^{110,111, 112}. Indeed, centrifugation would concentrate all mitochondria to one pole of the zygote and these would then become redistributed, presumably at random throughout the cytoplasm¹¹³.

¹¹⁰ Yamochi T, Hashimoto S, Amo A, Goto H, Yamanaka M, Inoue M, Nakaoka Y, Morimoto Y. Mitochondrial dynamics and their intracellular traffic in porcine oocytes. *Zygote*. 2016;1-2.

¹¹¹ Tatham BG, Sathananthan AH, Dharmawardena V, Munesinghe DY, Lewis I, Trounson AO. Fertilization and early embryology: Centrifugation of bovine oocytes for nuclear micromanipulation and sperm microinjection. *Human reproduction*. 1996 Jul 1;11(7):1499-503.

¹¹² Hirabayashi M, Hirao M, Takahashi R, Kimura K, Hirasawa K, Ueda M, Hochi S. Production of transgenic rabbits using centrifuged pronuclear zygotes. *J Vet Med Sci*. 2000 Oct;62(10):1047-52.

¹¹³ Ferreira CR1, Burgstaller JP, Perecin F, Garcia JM, Chiaratti MR, Méo SC, Müller M, Smith LC, Meirelles FV, Steinborn R. Pronounced segregation of donor mitochondria introduced by bovine ooplasmic transfer to the female germ-line. *Biol Reprod*. 2010 Mar;82(3):563-71.

Establishing a hierarchy of D-loop sequences

- 7.13.** It may be possible to choose a compatible donor according to likely dominance over the patient haplotype, thereby reducing the risk of reversion. Whilst Kang et al (2016) tested only a limited number of combinations, they propose that there could be a hierarchy of replication advantage amongst mtDNA haplotypes (H56>H1b, U5A>H1b>F1a, U5a>X2c>D4a and H49>B2k). Further research is required to substantiate and expand such a hierarchy in order to determine its applicability to donor selection. It would be necessary, first, to systematically explore which D-loop sequences confer a replicative advantage over others *in vitro* by making cybrid cell lines where cytoplasts carrying one mtDNA haplotype are fused with cells carrying another. This could even be done with iPS cells derived from patients and potential donors. Once sufficient knowledge is available, it might be possible to predict simply on the basis of the D-loop sequences which combinations would favour the donor haplotype. However, it may also be necessary to make allowance for variation in the nuclear-encoded mtDNA replication factors¹¹⁴, although this can also be tested. It is also worth noting that effects mediated by mtDNA sequence variants are likely to operate at the same time as stochastic or bottleneck effects.

TALEN-mediated methylation of relevant D-loop sequences in the patient's oocyte or karyoplast

- 7.14.** As noted in Section 4.8, methylation of specific D-loop sequences has been linked with reduced mtDNA copy number. Rather than targeting a nuclease to a specific DNA sequence, TALENs with an inactive or “dead” form of the Fok1 nuclease (dFok1) linked to a DNA methyltransferase protein should result in DNA methylation around their target sequence¹¹⁵. If this approach can be used to target the D-loop sequences involved in mtDNA replication specifically of the karyoplast-associated haplotype, this should make them less likely to replicate, allowing the cytoplasmic mtDNA haplotype to predominate. This could rely on differences in the D-loop sequences of the two haplotypes, or by introducing the TALENs into the patient's oocyte or zygote prior to MST or PNT. However, this assumes that the methylation will persist until mtDNA replication begins. Methylation-based disruption of mtDNA replication has the advantage of not introducing a DNA-cutting enzyme into the cell, with attendant risks of unwanted cutting. Again, however, whether such approaches would be efficacious, safe and lawful is unclear.

¹¹⁴ Akman G, Desai R, Bailey LJ, Yasukawa T, Dalla Rosa I, Durigon R, Holmes JB, Moss CF, Mennuni M, Houlden H, Crouch RJ. Pathological ribonuclease H1 causes R-loop depletion and aberrant DNA segregation in mitochondria. *Proceedings of the National Academy of Sciences*. 2016 Jul 8;201600537.

¹¹⁵ Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, Shu J, Dadon D, Young RA, Jaenisch R. Editing DNA Methylation in the Mammalian Genome. *Cell*. 2016 Sep 22;167(1):233-47.

Annex A: Methodology of the review

The purpose of this review is to evaluate, in light of new evidence, whether techniques to avoid mitochondrial disease are sufficiently safe to be offered to patients in a clinical setting.

In order to carry out this review, the HFEA convened a small panel to collate and summarise the current state of expert understanding on the efficacy and safety of pronuclear transfer and maternal spindle transfer techniques. Panel members, some of whom sat on the panel which produced the 2011 and 2013, and 2014 review, were selected for their broad-ranging scientific and clinical expertise, and for having no direct interests in the outcome of the review.

Panel members for this review are as follows:

- Dr Andy Greenfield (Chair), Medical Research Council (MRC) Harwell Institute and HFEA member
- Professor Peter Braude, King's College London, UK
- Professor Frances Flinter, Guy's & St Thomas' NHS Foundation Trust, UK
- Professor Robin Lovell-Badge, Francis Crick Institute, UK
- Professor Caroline Ogilvie, King's College London and Guy's & St Thomas' NHS Foundation Trust, UK
- Dr Tony Perry, University of Bath, UK.

The panel published a call for evidence on 13 July 2016. It asked for scientific evidence from experts in any relevant field on the safety or efficacy of pronuclear transfer and maternal spindle transfer techniques to avoid the transmission of mitochondrial disease, including published studies (which have not been submitted in previous calls for evidence or have been published since January 2013), unpublished research or statements from individuals or organisations.

The call for evidence was published on the HFEA website and sent directly to more than 60 professional bodies and experts in the field, the majority of whom had been sent the call for evidence for the original review. The deadline for submissions was 21 July 2016.

The panel then reviewed the submitted evidence. It also spoke to the following researchers and experts for additional information and clarification, at meetings held on 18 and 19 July and 14 October:

- Professor Dieter Egli, Columbia University, USA
- Professor Mary Herbert, Newcastle University, UK
- Professor Shoukhrat Mitalipov, Oregon Health and Science University, USA
- Dr Sherman Silber, St Luke's Hospital, St Louis, Missouri, USA
- Professor Hubert Smeets, University of Maastricht, The Netherlands
- Professor Douglas Turnbull, Newcastle University, UK

- Dr John Zhang, New Hope Fertility Centre, New York, USA.

The panel contacted a number of researchers after the call for evidence, to gather further expertise and clarify issues. Annex C lists the written evidence that the panel reviewed.

Annex B: Timeline of considerations

Date	Consideration
2005	Research licence for pronuclear transfer granted.
May 2010	The Authority's Scientific and Clinical Advances Advisory Committee considered research developments.
February 2011	The Secretary of State for Health asked the HFEA to carry out a scientific review to scope "expert views on the effectiveness and safety of mitochondrial transfer".
April 2011	The panel of experts, co-ordinated by the HFEA, reported to the Secretary of State for Health on the safety and efficacy of methods to avoid mitochondrial disease.
June 2011	The Authority's Ethics and Law Committee considered ethical issues.
January 2012	The Secretary of State for Health and the Secretary of State for Business, Innovation and Skills asked the HFEA to carry out public dialogue work on the ethics and public attitudes towards mitochondrial replacement.
January 2012 – August 2012	Public dialogue and consultation work planning and preparation. Public dialogue work took place (deliberative public workshops and public representative survey took place).
September 2012 – December 2012	Open consultation ran (open consultation questionnaire, open consultation meetings and patient focus group).
December 2012	The Secretary of State for Health asked the HFEA to provide an updated view of the science to support the assessment of the efficacy and safety of MST and PNT.

January 2013

The panel of experts was reconvened and call for evidence issued.

Key findings include:

- The panel's view remained as it was in 2011: that MST and PNT have the potential to be used for all patients with mtDNA disorders, which may make them preferential to PGD in the future. In patients with homoplasmy (all containing mutant mtDNA) or high levels of heteroplasmy (partially containing mutant mtDNA), these are the only techniques that would make it possible for them to have a genetically related unaffected child.
- The panel was of the view that there was more published work available to support MST than PNT, but there was still insufficient evidence to recommend one transfer technique over the other.
- Once assessed as safe to use in clinical practice, the panel strongly recommended that permission should be sought from the parents of the children born from MST or PNT to allow them to be followed up for an extensive period (and that permission should then be sought from the children themselves, when old enough). The panel recommended that any female born following MST or PST is advised, should she wish to have children of her own, that her oocytes (eggs) or early embryos are analysed by PGD in order to select for embryos free of abnormal mtDNA. This has the potential to eliminate risk in subsequent generations.
- The 2013 panel continued to recommend the set of minimum critical experiments first outlined in the 2011 report. However, they highlighted that the recommended work in understanding MST using fertilised oocytes and PNT using normally fertilised oocytes was underway and noted that progress was good. The panel's comments on the progress made on the recommended research are summarised in Annex C of the 2013 report.
- Further studies on mosaicism in human morulae (comparing individual blastomeres) and on human embryonic stem (ES) cells (and their differentiated derivatives) derived from blastocysts, where the embryos have (i) originated from oocytes heteroplasmic for mtDNA and (ii) been created through MST and PNT using oocytes or zygotes with two different variants of mtDNA. Although experiments are already reported on embryonic stem (ES) cells and their derivatives with MST, further corroborative experiments would be valuable.
- A recommendation made by the panel in 2011 to carry out PNT in non-human primate models was considered, in the light of new evidence, to be both difficult and unnecessary. Such experiments were therefore no longer mandatory.

March 2013

The findings of the public dialogue and the 2013 scientific review update were submitted to Government, together with considerations of how the techniques might be regulated. The public dialogue work concluded that the public were generally supportive of these techniques, although concerns around safety, the donor role and the regulation of the techniques were highlighted.

June 2013	The Government announced that, based on the findings of the HFEA’s public dialogue and consultation exercise and the views of the panel, it would move forward with draft regulations for public consultation.
February 2014	The Department of Health opened a consultation on draft regulations for the use of these techniques to prevent mothers passing on serious mitochondrial diseases to their children.
June 2014	Alongside the public consultation on draft regulations for the use of these techniques, the Secretary of State for Health asked the HFEA to carry out a further scientific review to evaluate the latest evidence on the effectiveness and safety of mitochondrial transfer.
February 2015	Parliament approved the regulations that permitted the use of mitochondrial donation, by PNT or MST.
October 2015	<p>The Regulations on mitochondrial donation to avoid serious mitochondrial disease officially came into force. The statutory provisions governing this new treatment are set out in the HFEA licensing and regulatory framework and prescribe the steps UK clinics must take before they can offer this new treatment.</p> <p>In order to trigger the licensing process and enable clinics to apply to be licensed to offer the treatment, a scientific panel would need to be reconvened to assess whether the outstanding three tests have been met and there is sufficient evidence to proceed.</p> <p>The HFEA agreed a draft licensing framework that would be put in place if the techniques were deemed sufficiently safe and efficacious.</p>

Annex C: Evidence reviewed

Statements

- A statement identifying studies by Yamada et al. (2016) and Cagone et al. (2016). Professor J Poulton (University of Oxford, UK) and Professor J St John (Centre for Genetic Diseases, Clayton, Australia).
- A statement expressing opinion of the findings of Yamada et al. (2016) and Hyslop et al. (2016). (anonymous, confidential).
- A statement responding to 2014 report focussing on comparator experiments for PNT and mtDNA haplotyping. Professor D Thorburn (University of Melbourne and Murdoch Childrens Research Institute, Australia).
- A statement, with references, expressing concerns about the use of MST/PNT in treatment. Dr E Allan, UK.
- A statement regarding alternative techniques to prevent germline transmission of mitochondrial disease. Professor JC Izpisua Belmonte (Salk Institute for Biological Sciences, USA).
- A preprint manuscript: Differential gene expression in blastocysts following pronuclear transfer, 2016. Dr E Morrow and Dr F Ingleby (University of Sussex, UK).
- A statement in response to PeerJ non-peer reviewed article from Morrow and Ingleby. Professor M Herbert (University of Newcastle, UK), Dr P Blakeley and Dr K Niakan (The Francis Crick Institute, UK), (confidential).
- A published manuscript: mtDNA diversity in human populations highlights the merit of haplotype matching in gene therapies. Dr I Johnston (University of Birmingham, UK).

Unpublished articles and data

- Unpublished manuscripts and data from Professor M Herbert and Professor Sir D Turnbull (University of Newcastle, UK), (confidential):
 - 1. mtDNA carryover in individual cells from cleavage stage embryos/ morulae.
 - 2. Heteroplasmy in embryonic stem cell lines from ePNT blastocysts.
 - 3. Analysis of variant mitochondrial genotypes in hESCs lines.
- Unpublished manuscripts from Professor S Mitalipov (Oregon Health and Science University, USA), (confidential):
 - 1. Mitochondrial replacement in human oocytes carrying pathogenic mtDNA mutations.
 - 2. Functional Haploid Human Oocytes Generated from Polar Body Genomes.
- Unpublished manuscript: Mitochondrial DNA inheritance is determined by genetic drift and negative and positive selection from Professor H Smeets (University of Maastricht, The Netherlands), (confidential).

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Annex D: Glossary

Activate	To artificially trigger the processes that occur when an egg is fertilised.
Adenosine triphosphate (ATP)	Molecule which transports energy in cells. It is involved in reactions of the electron transfer chain (ETC).
Apoptosis	Programmed cell death.
Autophagy	Where a cell begins to digest part of itself.
Backcross	Formally, breeding a hybrid animal with one of its parents. In this context, where a hybrid breeds with one of its parental types, such as a particular strain.
Biopsy	Procedure of obtaining cells, eg, from an embryo, in order to test them.
Blastocyst	A 5 to 6 day-old embryo, with both an outer trophectoderm cell layer and an inner cell mass.
Blastomere	A cell of an embryo prior to the blastocyst stage.
Chromosome	Structure in a nucleus that carries DNA.
Cytoplasm	An oocyte where the nuclear material has been removed.
Cytoplasm	The jelly-like substance that fills a cell and contains the structures (organelles) within it, including mitochondria.
De novo mutation	A spontaneous new mutation (as opposed to one that is inherited).
Displacement (D)-loop	A highly variable, non-coding region of mtDNA required for the initiation of replication and transcription.
Electron transfer chain (ETC)	The chain of chemical reactions that produces energy in a mitochondrion.

Embryonic stem (ES) cells	Cells, derived from an embryo and cultured in a laboratory, that have the potential to form all different cell/tissue types in an animal or human and can replicate themselves indefinitely.
Epigenetic	Relating to or arising from non-genetic influences on gene expression. Can result in inherited phenotypes through alteration of gene expression.
Gene expression	The process by which information from a gene is used in the synthesis of a functional gene product (which may or may not contribute to a particular phenotype).
Genome editing	A term that covers a collection of related techniques that aims to introduce specific alterations to DNA by the use of programmable enzymes. Pre-eminent amongst these at present is CRISPR-Cas9, though other techniques are available or in development. Edits may be subtle, affecting single nucleotides, or large, resulting in the complete removal of particular regions of DNA. These edits may be restricted to somatic tissues or, if affecting germ line cells, be heritable.
Germ cell	Any cell that will give rise to sperm or egg cells.
Germ line	The sequence of cells that give rise to sperm or egg cells that will pass genetic information on to a child.
Germ line mosaicism	Also known as gonadal mosaicism, a condition in which the precursor (germ line) cells to ova or spermatozoa are a mixture (mosaic) of two or more genetically different cell lines. If the mosaicism is present in only a small proportion of cells, then it may lead to the inheritance of a condition from a parent who is unaffected by it.
Heteroplasmy	Where two or more different mtDNA types coexist in a single cell, commonly used (as in this report) where one type is abnormal and the other normal.
Homoplasmy	Where all the mitochondria in a cell contain the same mtDNA, which can either be all abnormal or all normal.
Induced pluripotent stem (iPS) cells	Adult cells that have been reprogrammed to act like embryonic stem cells.
Inner cell mass	The mass of cells contained within a blastocyst stage mammalian embryo that will give rise to all cell and tissue types of the fetus.

Karyoplast	A structure made up of the nuclear genetic material, surrounded by a small amount of cytoplasm and enclosed in a portion of the cell membrane, used in the current context to transfer the spindle or pronuclei.
Karyotype	The number and appearance of chromosomes in a cell.
Lineage	Term used to describe cells with a common ancestor.
Maternal spindle transfer (MST)	Technique to transfer the nuclear genetic material from a woman's egg into a donated egg with its nuclear genetic material removed.
Meiotic division	Type of cell division necessary for sexual reproduction.
Metaphase II oocyte	Mature oocytes or unfertilised eggs (that have undergone the first meiotic cell division).
Micromanipulation	Use of physical techniques to add or remove cellular components.
Microtubules	Structural components of the cytoplasm, contributing to its 'skeleton' and involved in many cellular processes including cell division.
Mitochondria	Small structures present in cells that produce the cell's energy (see Introduction).
Mitochondrial bottleneck	The description of a mechanism to explain how during the development of the embryo the low number of mitochondria per cell, and the small number of cells that go on to form each tissue type, can by chance produce different proportions of heteroplasmy in tissues.
Mitochondria carryover	The proportion of mitochondria that is carried over from the original egg or zygote during MST or PNT.
Mitochondrial DNA (mtDNA)	The genetic material contained within the mitochondria.
Mitochondrial haplogroup	A group of similar mitochondrial DNA types as defined by DNA sequence.

Mitophagy	Cellular components are commonly broken down and recycled in a process known as autophagy. Mitophagy describes the controlled destruction of mitochondria. Turnover of mitochondria, especially if damaged, is important for maintaining cellular health.
MitoTracker	Fluorescent dye that specifically stains mitochondria and which can persist in living or fixed cells.
Mosaicism	When cells within the same individual have a different genetic makeup.
Mutation	A permanent, heritable change in the DNA sequence.
Oocyte	An egg.
Ooplasm	Cytoplasm contained in an egg.
Organelle	Small structure within a cell.
Oxidative phosphorylation	The reaction that produces ATP to generate energy in the cell.
Parthenogenesis	The generation of embryos without fertilisation of an egg. Because eggs (and sperm) contain only half of the usual complement of human chromosomes, parthenogenetic embryos will also be haploid. Haploid individuals are non-viable, but haploid cells may be cultured <i>in vitro</i> .
Phenotype	The set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.
Preimplantation genetic diagnosis (PGD)	A technique that removes and examines one or more cells from an early embryo to identify those embryos that are unlikely to develop a specific genetic disease.
Prenatal testing	Testing for diseases or conditions in a fetus before it is born.
Primordial germ cell	A specialised cell, formed at early stages of development, that can give rise to egg or sperm cells.
Pronuclei	The independent nuclei derived from the sperm and egg during the process of fertilisation, as distinct from the nucleus formed from the combination of the maternal and paternal genomes that is present in every cell type from the 2-cell stage onwards.

Pronuclear transfer (PNT)	Technique to transfer male and female parental nuclear genetic material from a fertilised egg (zygote) into a donor zygote with its nuclear genetic material removed.
Reagents	Chemicals used in experiments.
Segregation	The process that determines the genetic information present in different tissues or organisms.
Selection	The process by which particular entities, such as genes, or cells, are favoured.
Sequencing	Method for determining the order (sequence) of chemicals that make up DNA in an individual.
Sex selection	Selecting an embryo of a particular sex to avoid passing on a serious sex-linked condition.
Somatic cell	Any cell of an embryo or adult that is not a germ line cell.
Somatic cell nuclear transfer (SCNT)	Fusion of an adult (somatic) cell with an egg that has had its nucleus removed.
Spindle	A structure in a cell, made up of microtubules, that move the chromosomes when the cell divides.
Stochastic	Describes an event that is unpredictable due to one or more random variables.
Transcription	The first part of the process of gene expression, whereby a complementary RNA-copy of a sequence of DNA is made. This may then be translated to make the protein encoded by the DNA.
Vitrify	To freeze (an egg or embryo) by plunging into liquid nitrogen.
Zygote	A fertilised egg.

