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

Report provided to the Human Fertilisation and Embryology Authority, March 2013

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Contents

Executive summary

Mitochondria are small structures present in cells that produce much of the energy required by the cell. They contain a small amount of DNA that is inherited exclusively from the mother through the mitochondria present in her eggs. Mutations in this mitochondrial DNA can cause a range of rare but serious diseases, which can be fatal. However, there are several novel methods with the potential to reduce the transmission of abnormal mitochondrial DNA from a mother to her child, and thus avoid mitochondrial disease in the child and subsequent generations.

The Human Fertilisation and Embryology (HFE) Act 1990 (as amended) only permits eggs and embryos that have not had their nuclear or mitochondrial DNA altered to be used for treatment. However, the Act allows for regulations to be passed by Parliament that will allow techniques that alter the DNA of an egg or embryo to be used in assisted conception, to prevent the transmission of serious mitochondrial disease.

The Secretary of State for Health asked the Human Fertilisation and Embryology Authority (HFEA), in February 2011, to carry out a scientific review to scope "expert views on the effectiveness and safety of mitochondrial transfer". In order to carry out this task, the HFEA established a small panel, with broad-ranging scientific and clinical expertise, to collate and summarise the current state of expert understanding on the safety and efficacy of methods to avoid mitochondrial disease through assisted conception. The panel reported its findings in April 2011.[1]

The panel noted that Preimplantation Genetic Diagnosis (PGD)[2] can only reduce, not eliminate, the risk of transmitting abnormal mitochondrial DNA that may lead to a mitochondrial disease. PGD is suitable for some, but not all, patients who suffer from mutations in their mitochondrial DNA. The panel made recommendations for centres carrying out PGD for mitochondrial disease to reduce the level of uncertainty around the diagnosis.

The panel concluded that the techniques of Maternal Spindle Transfer (MST) and Pronuclear Transfer (PNT)[3] are potentially useful for a specific and defined group of patients whose offspring may have severe or lethal genetic disease, due to

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[1] http://www.hfea.gov.uk/6372.html

[2] PGD involves removing and examining one or more cells from an early embryo, in the current context to identify those embryos that are unlikely to develop a mitochondrial disorder in the resulting child. PGD for mitochondrial diseases is licensed in the UK.

[3] Maternal spindle transfer and pronuclear transfer are two techniques, currently at the research stage that would involve transferring the nuclear genetic material from an unfertilised or fertilised egg that contains mitochondria with mutant mtDNA into an unfertilised or fertilised donor egg with normal mitochondria from which its nuclear genetic material has been removed. Neither technique is permitted for treatment under the HFE Act 1990 (as amended) because each replaces (and thereby alters) the mitochondrial DNA of the egg or embryo with that from the donor.

mutations in mitochondrial DNA[4], and who have no other option of having their own genetic child. As in every area of medicine, moving from research into clinical practice always involves a degree of uncertainty. The panel concluded that evidence available at that time (March 2011) did not suggest that the techniques are unsafe. Nevertheless, these techniques, especially applied to human embryos are novel, and have relatively few data to provide robust evidence on safety. The panel therefore urged that additional research be undertaken to provide further safety information and knowledge about the biology of human mitochondria and the panel proposed a set of experiments that it felt to be critical. Although optimistic about the potential of these techniques, the panel recommended a cautious approach and advised that this research be carried out, and the results taken into account, before the techniques can be considered safe and effective for clinical use.

Following receipt of this report the Secretary of State for Health and the Secretary of State for Business, Innovation and Skills asked the HFEA (together with Sciencewise[5]) to conduct a programme of public dialogue on the social and ethical impact of making these techniques available to patients. The findings of this public dialogue work, together with considerations of the practical implications of allowing these techniques to take place within regulations, will be reported back to the Government in spring 2013.

In anticipation of the outcomes of this public dialogue work the Secretary of State for Health asked the HFEA, in December 2012, to provide an updated view on the science to support the assessment of the efficacy and safety of MST and PNT techniques, including any recently published findings and the extent to which the panel's recommendations have been addressed. This report outlines the panel's updated view. It should be read alongside the panel's 2011 review. The remainder of this executive summary sets out the panel's conclusions regarding the safety and efficacy of MST and PNT (as of March 2013).

The panel's view still stands 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 or high levels of heteroplasmy, these are the only techniques that would make it possible for them to have a genetically related unaffected child.

There is currently more published work available to support MST than PNT, but there is still insufficient evidence to recommend one transfer technique over the other. Indeed, once an embryo begins to develop normally, the data accumulating from the two methods would appear to be very complementary.

Although the results with the two techniques are promising, further experiments need to be done before introducing either into clinical practice to provide further

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[4] Mitochondrial disease can also be due to mutations in nuclear genes that encode products required within mitochondria, for which these methods are not relevant, although PGD can ne used in these cases.

[5] The Sciencewise Expert Resource Centre (Sciencewise-ERC) is the UK's national centre for public dialogue in policy making involving science and technology issues.

reassurance with respect to efficiency and safety.

Once assessed as safe to use in clinical practice, the panel strongly recommends that permission is sought from the parents of the children born from MST or PNT to be followed up for an extensive period (then seek permission from the children themselves, when old enough). In the case of females, this ideally should be extended to the next generation. These recommendations should also apply to PGD for mtDNA genetic diseases.

Until knowledge has built up that says otherwise, the panel recommends that any female born following MST or PNT should be advised, when old enough, that she may herself be at risk of having a child with a significant level of mutant mtDNA, putting this child or (if a female) subsequent generations at risk of mitochondrial disease. Thus, we recommend that any female born following MST or PST is advised that, should she wish to have children of her own, that her oocytes 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 panel recommends the following regarding the minimum set of critical experiments set out in the 2011 report:

- MST using human oocytes that are then fertilised (not activated). This has now been carried out and published, but 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 appear to be well underway, but their results will need assessing before they can be incorporated into future recommendations.
- The panel no longer feels that PNT in a non-human primate model, with the demonstration that the offspring derived are normal, is critical or mandatory.

The panel now considers the following related set of experiments to also be critical:

- 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 ES cells and their derivatives with MST, further corroborative experiments would be valuable.

The panel makes a number of updated recommendations regarding additional recommended research, which are outlined at 3.9 and 3.10.

1. Introduction, scope and objectives

1.1 Introduction

1.1.1 Mitochondrial malfunction has been recognised as the significant cause of a number of serious multi-organ diseases. The underlying defects can be due to mutations in nuclear DNA affecting gene products required within mitochondria, or to mutations in DNA carried within the mitochondria themselves. The latter encode products required exclusively for the oxidative phosphorylation (OxPhos) process of the electron transfer chain, which generates energy for cells in the form of ATP. Although relatively rare, the seriousness of these diseases and particularly the unusual inheritance pattern of mitochondrial DNA (mtDNA) mutations have made them a focus for research into preimplantation methods to reduce or avoid a disease in offspring.

1.1.2 Section 2 of the 2011 report provides an overview of mitochondrial biology and disease including definitions of terms and a list of clinical disorders that are associated with mutations in mitochondrial DNA (mtDNA). Section 3 of the 2011 Report considered and made recommendations on the use of PGD to avoid mitochondrial disease and annex D outlined a glossary. This information has not been repeated within this update.

1.2 Scope and objectives of this review

1.2.1 The terms of reference for the panel are to "collate and summarise the current state of expert understanding on the safety and efficacy of maternal spindle transfer and pro-nuclear transfer in order to update their report of April 2011." Accordingly, this review focuses exclusively on the science and the safety and effectiveness of these techniques, and does not consider the ethical and legal issues that are raised by such techniques.

1.2.2 The methodology of this review is set out at annex A and the evidence reviewed is listed at annex B.

1.2.3 This report is structured as follows: section 2 and 3 consider the effectiveness and safety of MST and PNT, suggests further research and makes recommendations. In addressing its terms of reference, the panel has tried to set out the issues in as clear a manner as possible. However, as the biology of mitochondria is complex, the language used is necessarily technical in parts.

2. Review of maternal spindle transfer and pronuclear transfer to avoid mitochondrial disease

2.1 Recap summary of the methods

2.1.1 In cases where PGD is not appropriate, such as cases where the woman has high levels of mitochondrial heteroplasmy[6] or is homoplasmic[7] for mutant mtDNA, transmission of mtDNA disease can be avoided by using healthy donated oocytes. This method is safe, and has strong supporters[8]. However, whilst this guarantees the disease is not transmitted, it also means that any resultant child will not be genetically related to the mother. The novel methods that the panel reviewed allow the transmission of both parent's nuclear DNA but involve replacing abnormal mitochondria with normal mitochondria: maternal spindle transfer (MST) and pronuclear transfer (PNT).

2.1.2 MST uses micromanipulation techniques to transfer the nuclear genetic material (the spindle with maternally-derived chromosomes attached) from one oocyte into another from which its nuclear genetic material has been removed[9](Figure 1). The reconstituted oocyte is then fertilised to allow embryo development. PNT uses similar micromanipulation techniques to transfer the nuclear genetic material, in this case both the maternal- and paternal-derived pronuclei, from a fertilised oocyte (zygote) into an enucleated donor zygote (Figure 2). MST takes place between mature metaphase II oocytes. PNT takes place between fertilised oocytes, after the stage where the egg has been penetrated by sperm but prior to the first embryonic cell division. Both techniques are therefore carried out prior to the formation of an embryo when the maternal and paternal chromosomes come together within the same nucleus[10]. With either method, any resulting child would inherit nuclear genetic material from both parents, while the mitochondria would be derived largely or perhaps exclusively from the oocyte provided by the donor. These methods could therefore effectively substitute the mitochondria in the oocytes of a woman known to carry mutant mtDNA with mitochondria carrying normal mtDNA from the oocyte donor. If efficient, so that there is little or no transfer of abnormal mtDNA, this method could avoid mitochondrial disease not just in the resulting child, but also in subsequent generations (but see further detail on this below).

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[6] 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

[7] Where all the mitochondria in a cell contain the same mtDNA, which can either be all abnormal or all normal

[8] A statement from Joanna Poulton (Professor and Hon Consultant in Mitochondrial Genetics, University of Oxford), Joerg P Burgstaller (IFA Tulln and University of Veterinary Medicine Vienna) and Iain G. Johnston (Imperial College London)
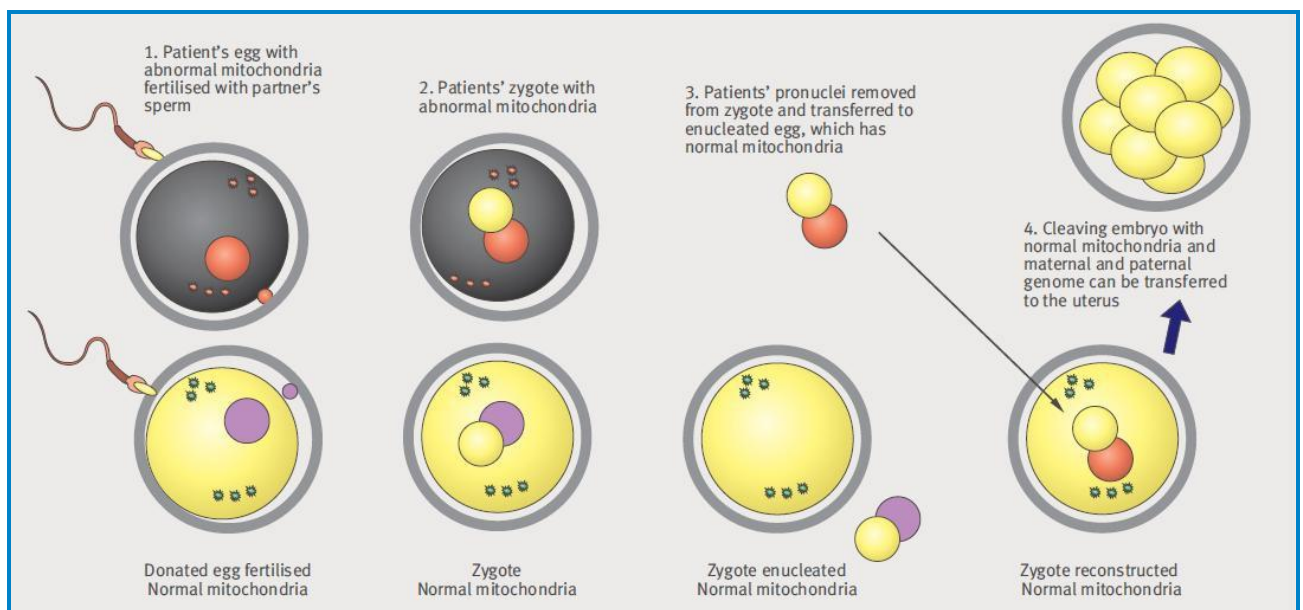
[9] This is equivalent to the oocyte being enucleated, and this term is used by some, although the chromosomes are not contained within a nuclear membrane at this stage.

[10] MST occurs pre-fertilisation and PNT occurs post-fertilisation but prior to the breakdown of the pronuclear membranes (syngamy)
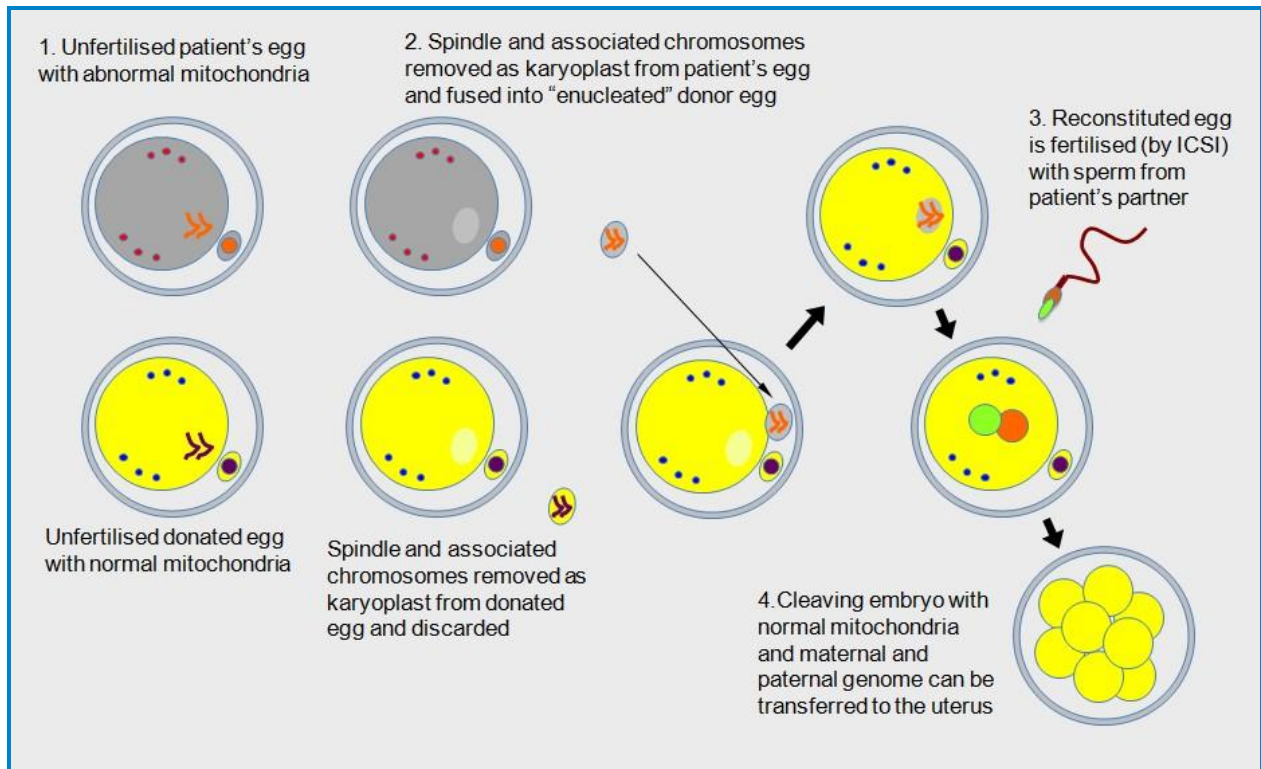
Figure 1. Maternal spindle transfer technique

Figure 2. Pronuclear transfer technique[11]

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[11] Bredenoord, A and P. Braude (2010) "Ethics of mitochondrial gene replacement: from bench to bedside" BMJ 341. Image reproduced with permission of Author

2.1.3 Although similar methodology is employed, it is important to stress that neither MST nor PNT is equivalent to reproductive cloning (somatic cell nuclear transfer, or SCNT). Any children resulting from MST or PNT would have arisen from fertilisation and be genetically unique. They would be the genetic child of the woman receiving treatment and her partner. MST and PNT do not involve reprogramming cells or nuclei as SCNT does, which is a relatively inefficient process and associated with significant risks of abnormal development[11].

2.2   Effectiveness of MST and PNT

2.2.1 A review of the effectiveness of MST and PNT, based on studies published up to March 2011, is outlined in section 4.2 of the original report.

2.2.2 Since 2011, several significant proof-of-principle studies with respect to the possible use of MST and PNT methods for treating mitochondrial disease have been carried out using human oocytes and zygotes and also with Macaque oocytes:

2.2.3 MST has been carried out on 65 human oocytes donated for research (a further 33 served as controls). Although some oocytes displayed clear evidence of abnormal fertilisation (53% - determined by an irregular number of pronuclei), remaining embryos were capable of developing to blastocysts and producing embryonic stem cell lines at rates similar to controls. All five of the embryonic stem cell lines derived from zygotes predicted to have undergone normal fertilisation after MST had normal euploid karyotypes and contained exclusively donor mtDNA[12] (Tachibana et al, 2013).

2.2.4 A second study has also demonstrated the use of MST with human oocytes, although these were parthenogenetically activated rather than fertilised. The primary purpose of the study was to assess the degree of mitochondrial DNA carryover rather than establishing a technique for creating embryos for clinical use. MST was shown not to reduce developmental efficiency to the blastocyst stage, and genome integrity was maintained, provided that spontaneous oocyte activation was avoided through the transfer of spindle–chromosome

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[11] The panel examined substantial evidence about SCNT as part of the 2011 review, including studies on heteroplasmy where mitochondria in the somatic cell persisted, sometimes at high levels, in the cloned embryo and offspring. This was usually associated with fusion of the somatic cell with an enucleated oocyte. This can introduce significant numbers of mitochondria that are in an active and replicating state, together with associated mitochondrial replication factors made by the somatic cell nucleus. In contrast, these factors are probably absent in mitochondria in mature oocytes or zygotes, as these mitochondria do not replicate until later. MST and PNT do not involve somatic cells.

[12] An ES cell line was also established from a zygote that had 3 pronuclei and one polar body (3PN/1PB) instead of the normal 2PN/2PB. This had a triploid karyotype consistent with a failure to extrude the second polar body and retention of its genetic material.

complexes that were incompletely assembled or partially disassembled (depolymerised). The authors claim to be able to achieve the latter by cooling the oocyte. Mitochondrial DNA transferred with the nuclear genome was initially detected at levels below 1%, decreasing in blastocysts and embryonic stem-cell lines to undetectable levels, and remained undetectable after passaging for more than one year, clonal expansion, differentiation into neurons, cardiomyocytes or pancreatic beta-cells, and after cellular reprogramming to derive iPS cells. Stem cells and differentiated cells had mitochondrial respiratory chain enzyme activities and oxygen consumption rates indistinguishable from controls. These cells were homozygous for all alleles (as they have become diploidised from an originally haploid state) and so would only give information about maternal imprinting. (Paull et al, 2013)

2.2.5   The panel was informed of unpublished findings regarding PNT and MST from the "Newcastle Group". Initial experiments using normally fertilised human zygotes for PNT have revealed the importance of timing of the various procedures and of matching developmental stage of the two zygotes. With optimisation, they have begun to obtain a significant proportion of manipulated embryos developing to blastocyst stages. Some zygotes resulting from PNT have been successfully vitrified and further work is being carried out to improve the quality and rate of development to blastocysts and to minimise mtDNA carryover at the blastocyst stage.

2.2.6   The panel noted that this information, together with comments from both the other groups interviewed, suggests that issues of timing may be relevant to any intended use of MST or PNT clinically since the cycles of the two egg donors will need to be synchronised. Egg retrievals will need to be carefully timed in order to be near coincident, the eggs need to be fertilised as soon as possible after collection, and for PNT the procedure needs to be carried out as soon as possible after normal fertilisation is confirmed. If there is a prolonged period of time between the two egg collections then one set of eggs may be over-mature, potentially leading to reduced development and an increase in abnormality rates. As this synchrony may not always be possible in a clinical setting, vitrification of eggs has been suggested as a solution. This is probably not an issue of safety, but one of efficiency, because the abnormalities are likely to be obvious and/or lead to early embryo lethality.

2.2.7   Data obtained from Macaques in the Tachibana et al (2013) study showed that cytoplasm is more sensitive to vitrification-induced damage than the spindle, which might suggest that the affected mother's oocytes should be frozen and thawed when a fresh donor oocyte become available. However, Macaque oocytes seem to be more sensitive than human oocytes to this freezing method. Paull et al (2013) also examined this, and found that they could freeze isolated "karyoplasts" from human oocytes (the spindle plus chromosomes, surrounded by oocyte membrane, but with little cytoplasm), and use these in MST after thawing.

2.2.8 Current research using PNT in Macaques has yet to be shown to be successful. From unpublished data[13] it appears that Macaque zygotes do not survive the PNT process well and published evidence suggests that there may be important differences between human and Macaque oocytes and early embryos; for example, different sensitivities to cryopreservation, and Macaque oocytes being less prone to abnormal activation/fertilisation following MST than human oocytes (as seen in Tachibana et al (2009) versus Tachibana et al (2013)). The panel now believes that the Macaque may not be a sufficiently good model for the human. Although this review is focused on the science, it is an ethical concern to carry out experiments on animals, especially non-human primates, if these are likely to not be informative. Therefore, 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 PNT and MST in other animal models reveals differences with the human, it would be not just reassuring, but important if such experiments revealed the underlying reasons, and did not merely state the problem.

2.3   Safety of MST and PNT

2.3.1 A review of the safety of MST and PNT, based on studies published up to March 2011 is outlined in section 4.3 of the original report. Based on the new evidence submitted the panel re-examined and commented on the following safety issues of the MST and PNT techniques: the carryover of mtDNA from the affected oocyte or zygote; the methods to prevent premature activation of oocytes or detect abnormally fertilised oocytes, the nuclear-mitochondrial interactions involved and the potential for long-lasting nuclear epigenetic modifications resulting from manipulation or altered mitochondrial states associated with mitochondrial disease. The panel did not specifically revisit previous discussions regarding the safety of reagents used to carry out the micromanipulation techniques. However, it was noted that the study by Paull et al (2013) relied on the use of several such reagents, the combination of which might have been expected to be deleterious, yet development of the (parthenogenetically activated) embryos, and ES cell lines derived from them, was apparently normal. It was felt by the panel, and by those it interviewed, that the number of reagents and their concentration should be kept to a minimum.

2.3.2 **mtDNA carryover:** Carryover of mtDNA from the affected oocyte or zygote might be expected with both techniques because the spindle or the pronuclei are enclosed in a karyoplast during the manipulation technique, which contains a small amount of surrounding cytoplasm enclosed in cell membrane in addition to the nuclear DNA. In theory, carryover of abnormal

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[13]Reported at a media briefing in October 2012 and reflected in a number of articles e.g.
http://www.sciencenews.org/view/generic/id/346024/description/Cloning-like_method_targets_mitochondrial_diseases

mtDNA may be an issue if abnormal mtDNA is preferentially replicated and if there is a marked difference in segregation across tissues. However evidence presented to the panel continues to be reassuring that neither occur, at least in somatic cell lineages (see 2.3.5 and 2.3.10 below for germ cells, where the situation is more complex).

2.3.3 It is relevant to note that a threshold of mitochondrial function is required for normal development, and despite developmental plasticity of the embryo, impaired mitochondrial function in the embryo affects subsequent fetal and placental growth (Wakefield et al, 2011).

2.3.4 One study suggested that (experimental) admixture of two normal but different mouse mtDNAs can be genetically unstable and can produce adverse physiological effects (Sharpley et al, 2012). These results could indicate that the differences between mtDNAs within a mammalian species may not be neutral and are suggested to explain the advantage of uniparental inheritance of mtDNA. This could be a concern for MST and PNT. However, the study used approximately equal amounts of mtDNA from two very different mouse strains, which could be considered distant subspecies. Also, another study, exploring mtDNA segregation during early embryogenesis in Macaques, produced distinctly different results - no such problems were observed with mixtures of mtDNA from two Macaque subspecies. However, the oocytes created to be heteroplasmic (50/50) for these two types of Macaque mtDNA variants resulted in embryos exhibiting significant partitioning of the mtDNA between different blastomeres and to some extent between trophectoderm and ICM. This partitioning seems to have resulted in some of the fetuses, or ES cell lines derived from such embryos, also showing a skewed ratio (in one case about 94% of one of the mtDNA variants was present). There was no evidence of preferential selection for 'resident' versus 'alien' mtDNA, suggesting that both variants work equally well with the resident nuclear DNA, even though the mtDNA sequence of the two sub-species of Macaque are as different from each other as they are from other primate species (Lee et al, 2012). The degree of heteroplasmy was so substantial that it could lead to homoplasmy. This could be an issue if one of the mtDNA variants is defective, with, by chance, either a beneficial or poor outcome for the individual born. However, the starting point in these experiments was about 50-50, whereas MST (or PNT) should give very low levels of carryover of mutant mtDNA, making homoplasmy for the normal mtDNA even more likely.

2.3.5 The authors also carried out MST between oocytes of the two Macaque subspecies to explore whether this preimplantation segregation of mtDNA variants could be a problem. They first determined that isolated karyoplasts carry "bound" mtDNA (there is no evidence that it is physically bound, just closely associated) at an average level of about 0.6% of the numbers within the cytoplasm. After MST, about 68% of fertilised oocytes developed to blastocysts, confirming earlier published data from the same group

(Tachibana et al, 2009)[14]. They then selected female blastocysts for embryo transfer, recovering two fetuses in which to survey levels of heteroplasmy. The mtDNA variant from the spindle donor oocytes was very low or undetectable in somatic tissues, suggesting a tendency towards homoplasmy. However, two out of 24 oocytes isolated from the fetal ovaries showed around 15% heteroplasmy. This difference between somatic and germ line was also evident in their earlier experiments, with segregation in oocytes appearing to be largely independent of that occurring in other tissues.

2.3.6　These findings largely support what is known about mtDNA levels and founding cell numbers of somatic tissues and the germ line during early postimplantation development and bottleneck theories as outlined in the panel's 2011 report. However, the observation of much earlier segregation of mtDNA, in cleavage stage embryos, is novel and contradicts evidence obtained from human embryos where blastomeres within an embryo tend to have very similar levels of heteroplasmy (as also demonstrated by Sallevelt et al, 2013 and Treff et al, 2012 - although with a few outliers). This could suggest that there is relatively little mixing of cytoplasm after spindle (or cytoplast) transfer, such that cleavage divisions are responsible for the segregation, whereas with heteroplasmy already existing in a growing oocyte, the mtDNA variants are likely to be distributed at random.

2.3.7　Modelling the inheritance of mtDNA has led to the conclusion that for a disease with a clinical threshold of say 60% mutant mtDNA (which is fairly typical) reducing the mutant mtDNA load to <5% with MST or PNT should dramatically reduce the chance of disease recurrence not just in the child, but in subsequent generations (Samuels et al, 2013). However, >5% carryover was associated with a significant chance of recurrence. Mutations with a lower clinical threshold were also likely to have a higher risk of recurrence, but reducing the amount of carryover would counteract this. If the threshold is very low, and the panel noted that there has been one report of heteroplasmy levels of less than 10% causing disease for a dominant mitochondria mutation initially detected in muscle[15], then the modeling may not be adequate. Moreover, it does not take account of the possibility of preferential replication or selection of mtDNA carrying specific mutations, however, there is little evidence of this occurring.

2.3.8　Publications and discussions with researchers indicate that PNT currently shows higher level of carryover than MST (up to 2% versus 0.3%) (Tachibana et al, 2013; Paull et al, 2013; Craven et al; 2010[16]). This may be due to

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[14] Tachibana M et al. (2009) Mitochondrial gene replacement in primate offspring and embryonic stem cells. Nature. 17;461(7262):367-72.

[15] Alston CL et al. (2010). "A novel mitochondrial tRNAGlu (MTTE) gene mutation causing chronic progressive external ophthalmoplegia at low levels of heteroplasmy in muscle."J Neurol Sci. 15;298(1-2):140-4.

[16] Craven L., H. A. Tuppen, et al. (2010). "Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease." Nature 465(7294):82-5

differences in the geometry and volume of the transferred structures, since two pronuclei are transferred in PNT rather than one spindle associated chromosome set in MST.

2.3.9 During the discussions, the panel was also minded to draw attention to parallels with PGD for mtDNA mutations in terms of acceptable levels of heteroplasmy in offspring. 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, issues to do with variable segregation of mutant mitochondria in their tissues and especially their gametes also apply here. Hence clear rules for acceptable levels of mtDNA heteroplasmy allowing transfer or not of an embryo should be developed for each disease by the specialist clinical team in conjunction with their patients, and follow up of such children and their offspring is strongly recommended, as the panel have recommended for offspring arising for MST and PNT – see 2.3.21.

2.3.10 In conclusion, any early segregation of a very low level of mutant mtDNA is unlikely to be a problem for children born as a result of MST (or PNT). Nevertheless, it would be reassuring to verify this with human preimplantation embryos generated as a result of MST and PNT for research purposes, and in ES cells and their differentiated derivative cell types obtained from such embryos. There is a potential concern, however, for subsequent generations if a female child born after the use of these techniques has a proportion of oocytes with a significant level of heteroplasmy. This could be researched by, for example, following differentiation protocols reported to generate primordial germ cells from human ES cell in vitro. Alternatively, it may be may be sufficient to explore these 'bottleneck' issues by looking at ES cell sub-lines derived from single cells ('clonal analysis'). If it turns out that there is 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 for her to make use of PGD to select for embryos homoplasmic for the normal mtDNA variant.[17]. From the data on macaques derived by MST, if the child is female, then it is possible that some of her oocytes may have a significant proportion of mutant mtDNA, considerably higher than her somatic tissues. The levels may still not be sufficient to cause her children to have a problem, but subsequent generations could be affected. Although diagnostic technology may well have advanced by then, by carrying out PGD (on embryos created from oocytes of female offspring resulting from MST or PNT, who might be carriers of the mutation in some of their oocytes) it ought to be possible to select embryos for implantation that have no abnormal mitochondria. This would guarantee that subsequent generations would be free from disease.

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[17] There is an accepted precedent for a method of ART having a known consequence for reproduction in the next generation. This is when ICSI is used for male infertility when the cause is known to be due to a Y chromosomal defect. Any son born as a result will carry the same defect and ICSI will be required for him to have a child – and so on.

2.3.11 **Methods to prevent premature activation of oocytes or detect abnormally fertilised oocytes:** The proof of principle studies, outlined in section 2.2, have demonstrated that nuclear genome transfer carried out in the process of MST can lead to premature oocyte activation and abnormal fertilisation. The panel explored the measures that could be put in place to address these risks.

2.3.12 The panel was reassured to hear that the abnormally fertilised eggs created followed MST can easily be identified using a standard stereo-microscope by looking for normal number of pronuclei and polar bodies which have failed to extrude at the PNT stage. As part of the tests to look for normality of development, array comparative genome hybridization (CGH) was used on trophectoderm biopsies from MST derived human blastocysts. Analysis did not detect abnormalities in uniformly triploid embryos suggesting some shortcomings of CGH approaches[18]. Some of the abnormalities associated with MST can be detected only after sperm fertilisation and some are likely to have been due to problems with oocyte ageing (Tachibana et al, 2013).

2.3.13 Paull et al (2013) reported that premature oocyte activation could be prevented by partial depolymerization of the spindle–chromosome complex through cryopreservation or cooling to room temperature, allowing normal polar body extrusion (Paull et al, 2013). The authors confirmed that they were satisfied that implementing a spindle chilling stage did not damage the spindle since they had not seen dispersion of the spindle, as had been suggested in a previous research paper (Pickering et al, 1990)[19]. The spindle came back to normal size on warming and the oocyte extruded a polar body.

2.3.14 **Nuclear-mitochondrial interactions:** A concern has been raised that there might be a failure of correct nuclear-mitochondrial interaction following MST or PNT because the donor mtDNA may be of a haplogroup different from that with which the maternal nuclear genome had been functioning. 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. This is typified by comparisons between European and African mtDNA. However, the panel maintains the view that there is no evidence for any mismatch between the nucleus and any mtDNA haplogroup, at least within a species (with the possible exception of the study by Sharpley et al (2012) mentioned in 2.3.4). Fifty per cent of nuclear genes are paternally inherited

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[18] Details relating to the shortcomings of CGH testing were provided by Mitalipov et al in supplementary information, to the core panel, and are not included in the Tachibana et al 2013 published article. The Panel was informed that CGH analysis of biopsied trophectoderm in blastocysts did not detect uniform triploidy. Therefore uniform triploidy was confirmed by deriving ESCs from the same blastocyst using conventional G-banding.

[19] Pickering SJ, et al (1990) "Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte." Fertility and Sterility 54(1):102-108

and are consequently 'alien' to the mtDNA; backcrossing can replace the nuclear DNA entirely in a few generations. Furthermore, mitochondrial disease has not been noted to be more frequent amongst mixed-race children. Tachibana et al (2013) also conducted a 3-year follow-up study on MST-derived macaque offspring born in 2009. The two species of Macaques used in these MST experiments have distinct mitochondrial haplotypes, yet neither the mixing of mitochondria nor swopping the haplotype with respect to their nuclear genome with which it normally resides, appears to result in any adverse effects in offspring. All four (males) were healthy and had normal mitochondrial function. Moreover, there were no significant changes with age in the degree of heteroplasmy in blood and skin cell samples, which remained less than 1% from the spindle donor. However, if further concerns were raised, it would be possible to match mtDNA haplogroups from the egg donor and the mother.

2.3.15 **Long lasting nuclear epigenetic modifications:** Concerns have been expressed relating to the potential for long lasting damaging effects on development or the health of offspring resulting from nuclear epigenetic perturbations resulting either from MST or PNT manipulations or associated with mitochondrial disease and manifest prior to manipulations. While the panel cannot rule out the possibility of epigenetic alterations in either instance, there is no evidence at present that such alterations have a significant or far reaching effect on development or health. One of the more recent studies reporting on MST in humans includes 3 year follow up health data on non-human primates created by this procedure which failed to reveal any adverse effects (Tachibana et al, 2013). It remains unknown whether there are aberrations in maternally epigenetically imprinted genes in oocytes linked to mitochondrial disease. If so, one would anticipate that this would perturb development of normally fertilised embryos and to the panel's knowledge there is no evidence that this is the case. For example, pathologies associated with typical imprinting defects, such as Angelman or Beckwith Wiedermann syndromes, have not been noted to occur in children with mitochondrial disease. Moreover, the mitochondria in growing oocytes are in a form that suggests that they are mostly inactive; therefore, on theoretical grounds the presence of mutant mtDNA in a growing and maturing oocyte is likely to be of little or even no consequence to the nuclear DNA.

2.3.16 **The panel's view still stands 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 or high levels of heteroplasmy, these are the only techniques that would make it possible for them to have a genetically related unaffected child.** Even where a proportion of embryos have levels of mutant mtDNA below the threshold known to lead to clinical disease, the evidence the panel has reviewed here, and in the original report, suggests that this does not always reflect the levels seen in offspring (due to bottleneck effects). Moreover, subsequent generations (if the embryos implanted after PGD are female), will continue to be at risk, even if the levels of heteroplasmy for the mutant

mtDNA are low. It might be hoped that improvement to MST or PNT might eventually lead to no or such minimal levels of carryover that the mtDNA disease has effectively been eliminated from the germline.

2.3.17 There is currently more published work available to support MST than PNT, but there is still insufficient evidence to recommend one transfer technique over the other. Indeed, once an embryo begins to develop normally, the data accumulating from the two methods would appear to be very complementary.

2.3.18 Although the results with the two techniques are promising, further experiments need to be done before introducing either into clinical practice to provide further reassurance with respect to efficiency and safety.

2.3.19 The frequency of premature activation/abnormal fertilisation noted by Tachibana et al (2013) is of concern, because when combined with what are probably methodological failures and the normal attrition of early human embryos, the number of normal blastocysts obtained is rather low and it might require more than one cycle to obtain a suitable embryo for transfer let alone to become a successful implantation. The cooling method used by Paull et al (2013) may assist, but this has not been tested with fertilisation. The data on PNT with normal fertilised zygotes are yet to be published, and the panel would be reassured if this included ES cell data of a comparable type to that in Tachibana et al (2013) and Paull et al (2013). More work needs to be done to ask whether mtDNA carryover associated with the spindle in MST or with the pronuclei in PNT becomes segregated in preimplantation development in a manner that is different with naturally occurring heteroplasmy. The panel is reassured both by the actual data on carryover of variant mtDNA, and by the modeling data showing that if carryover of mutant mtDNA is <2% then it is unlikely that any resulting child will show signs of mitochondrial disease. Nevertheless, there is still a concern about segregation and bottleneck issues leading to an unacceptably high level of abnormal mitochondria in the germ line of any female offspring, putting her children at risk. This can be explored with ES cell lines produced from MST and PNT embryos, preferably by deriving germ cells from them or by clonal analysis, as discussed above (2.3.2).

2.3.20 Once assessed as safe to use in clinical practice, the panel strongly recommends that permission is sought from the parents of the children born from MST or PNT to be followed up for an extensive period (then seek permission from the children themselves, when old enough). In the case of females, this ideally should be extended to the next generation. These recommendations should also apply to PGD for mtDNA genetic diseases.

2.3.21 Until knowledge has built up that says otherwise, the panel recommends that any female born following MST or PNT should be advised, when old enough, that she may herself be at risk of having a child with a

significant level of mutant mtDNA, putting this child or (if a female) subsequent generations at risk of mitochondrial disease. Thus, we recommend that any female born following MST or PST is advised that, should she wish to have children of her own, that her oocytes 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.

3. Further research

3.1   From the evidence received, the panel stands by the conclusions reached in 2011 and has not identified any new evidence that indicates that the MST and PNT methods are fundamentally unsafe. Nevertheless, these techniques are novel, especially as applied to human embryos, and with relatively few data. The panel therefore continues to recommend that additional studies be undertaken both on basic research to improve the knowledge about the biology of human mitochondria especially in development, and on research aimed specifically at providing further safety information on MST and PNT. However, 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 a risk associated with the use of MST or PNT in humans until it is tried in practice.

3.2   Basic research is needed into how the mitochondrial bottleneck functions and 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 eventually be possible to influence or control replication of abnormal mtDNA in the early embryo to affect its segregation or inheritance in subsequent development. This research may aid decisions about threshold levels when carrying out PGD, although it may be less relevant when considering the use of MST and PNT.

3.3   The panel discussed the usefulness of the development of embryonic stem cell lines to help understand the distribution of mitochondrial heteroplasmy after PNT, since it would be critical to know whether the anticipated low level of mutant mitochondria carryover following PNT (or MST) did not change adversely during development, nor that there was preferential amplification in different tissues. This could be established by examining individual blastomeres at the morula stage and potentially by examining various tissues (such as striated muscle, myocardium, neural tissue, etc; i.e. those tissues especially sensitive to mitochondrial defects), which are easily generated from embryonic stem cells cultured from blastocysts. These experiments are required to ask if heteroplasmy that occurred as a result of MST or PNT techniques (even if it is <2%) leads to more segregation than naturally occurring heteroplasmy, as discussed above in section 2.3.2 – 2.3.10, Although more difficult practically, analysis of single cell (clonally)-derived embryonic stem cell sublines or, preferably, of primordial germ cells derived from such embryonic stem cells in order to examine levels of heteroplasmy in these cells might give an indication of next generation heteroplasmy.

3.4   The panel noted an interesting development in disease modeling. Three strains of "mito-mice", carrying mitochondria with mutations in mtDNA known to be pathogenic in humans appear to be good models for use in a range of studies relevant to mitochondrial biology and disease. The authors focus the discussion on their use in trialing drugs for potential treatment rather than MST or PNT. The work is at a very early stage, but they propose some

interesting strategies that require generation of additional animal models and further trials (Nayada and Hayashi, 2011).

3.5 The panel reviewed its 2011 recommendations and the experiments that it considers are critical to assessing the effectiveness and safety of MST and PNT techniques as well as and experiments that will provide useful information on MST and PNT or mitochondria and disease. This research may also inform which of the two techniques is likely to be the most appropriate for clinical use. Many of the latter experiments, whilst of potential importance for basic research and for exploring alternative methods whereby abnormal mtDNA can be selected against, will not necessarily inform the decision as to whether it is safe to proceed to clinical application of MST and PNT methods.

3.6 **The 2011 report recommended the following (minimum) set of experiments to be undertaken and the results taken into account before MST and PNT techniques can be assessed as safe to use clinically:**

- **MST using human oocytes that are then fertilised (not activated)**
- **PNT using normally-fertilised human oocytes and development compared to normal ICSI-fertilised human oocytes**
- **PNT in a non-human primate model, with the demonstration that the offspring derived are normal.**

3.7 Experiments on the first of these have now been carried out and published. It is still important for some follow-up experiments to be carried out, notably to improve efficiency if possible, and confirmatory experiments would be valuable. Experiments on the second appear to be well underway, but it will be necessary to see full details (preferably published) before any assessment is possible. Due to the various issues outlined above, the panel no longer feels that their third recommendation is critical or mandatory. While it is of course possible that further experiments using non-human primates could provide some additional useful biological information, many of the important issues around heteroplasmy with variant mtDNAs have already been addressed or at least highlighted in Macaques, rodents and human studies. Others relating to the behaviour of mutant mtDNA may be better carried out in emerging mouse models or directly using human oocytes and zygotes and assays in preimplantation embryos and ES cells derived from them. But in terms of assessing both safety and efficacy of MST and PNT the panel is concerned that the differences between Macaque and human oocytes/early zygotes will be unhelpful. Indeed, if there are critical periods of development where the human is unique, such experiments may even be misleading if carried out in animals[20].

3.8 **In light of evidence and concerns about carryover of mutant**

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[20] Conducting experiments on non-human primates where they are not fully justified raises ethical issues.

mitochondria the panel considers it important to demonstrate the degree of heteroplasmic mosaicism in morulae[21], and to provide data to address whether there was any amplification of mtDNA carried over. Therefore the following is now considered to also be a critical experiment:

- **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[22]. Although experiments are already reported on ES cells and their derivatives with MST, further corroborative experiments would be valuable.**

3.9 **Given new published data and the panel's recent discussions with researchers, the following recommendation is no longer considered a critical experiment:**

- **PNT in a non-human primate model, with the demonstration that the offspring derived are normal.**

3.10 In the initial Report, the panel had also recommended the following additional research to provide useful information on mitochondrial disease and the MST and PNT techniques. The italicised text after each point outlines the panel's revised position:

- Removing the spindle or pronuclei and replacing them back into the same oocyte/zygote to better identify the impact of the manipulation technique: *Given the successful development to blastocyst stages after both MST and PNT with human oocytes and zygotes, the panel now considers this to be unnecessary.*

- Karyotype analysis and comparative genomic hybridisation/copy number variation arrays of embryos derived from MST or PNT: *this has been carried out for MST (further studies on mtDNA carryover have now been conducted in the Macaque model, as outlined above), but remain to be done after PNT, which the panel continues to recommend.*

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[21] The stage of an embryo just prior to blastocyst formation, where it is a mass of blastomeres

[22] ES cells have a low number of mitochondria that do not need to function. Differentiated cells derived from the ES cells, such as muscle, can have high numbers of mitochondria. These can be put in conditions requiring oxidative phosphorylation. It may also be possible to derive primordial germ cells in vitro to explore aspects of the mitochondrial bottleneck and whether certain abnormal mtDNA have a replication advantage.

- Detailed analysis of epigenetic modifications and gene expression, with a range of markers for blastocyst cell types or embryos derived from MST or PNT: *this has been carried out for MST (further studies on mtDNA carryover have now been conducted in the Macaque model, as outlined above), but remain to be done after PNT, which the panel continues to recommend.*

- MST on unfertilised human oocytes that have abnormal mtDNA and PNT on fertilised oocytes that have abnormal mtDNA : *the panel considers that this might be useful to perform, especially if any evidence arises to suggest a specific mtDNA mutation may have a replicative advantage, but the panel now recognises that it may be very impractical to obtain sufficient numbers of oocytes or zygotes with mutant mtDNA for research.*

- Similar experiments using induced pluripotent stem (iPS) cells derived from patients carrying different mtDNA mutations[23]: *the panel continues to recommend this is carried out.*

- Further studies on the mtDNA carryover in a non-human primate model into the possible heteroplasmy of tissues in the fetus. The possibility of carryover of even a small percentage of abnormal mtDNA, means that any females born from MST or PNT should be considered at risk of transmitting the disease to their offspring: *Some relevant experiments have now been published on this, notably by Lee et al, (2012) in the Macaque. On the basis of these, the panel recommends that further experiments are carried out to address this issue with human material, along the lines suggested below in 3.10.*

- Further studies on vitrifying zygotes created through PNT: *the panel continues to recommend this is carried out.*

3.11 The following additional research is also now recommended to provide useful information on mitochondrial disease and the MST and PNT techniques:

- Tests for heteroplasmy should be carried out on primordial germ cells obtained from human ES cells derived from blastocysts created through MST and PNT where the oocytes had variant or abnormal mtDNA. If primordial germ cell derivation is not possible or limitations in the model undermine its utility, clonal analysis of single cell-derived human ES cells could be used. Comparisons beginning with blastocysts known to be heteroplasmic for variant or abnormal mtDNA would be informative.

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[23] It is not possible for iPS cells to provide information on mitochondrial behaviour in the early embryo.

Annex A: Methodology of review

1. The Human Fertilisation and Embryology Authority (HFEA) agreed to a request from the Secretary of State for Health, in December 2012, to provide an updated view on the science to support the assessment of the efficacy and safety of pro-nuclear transfer and maternal spindle transfer techniques.

2. 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 pro-nuclear transfer and maternal spindle transfer techniques. Panel members, the majority of whom sat on the panel which produced the 2011 review, were selected for their broad-ranging scientific and clinical expertise, and for having no direct interests in the outcome of the review.

3. Membership of the panel:

    -   Professor Neva Haites (chair), University of Aberdeen

    -   Professor Peter Braude, King's College London

    -   Dr Paul De Sousa, University of Edinburgh

    -   Professor Robin Lovell-Badge, Medical Research Council National Institute for Medical Research

    -   Professor Anneke Lucassen, University of Southampton and formerly Human Genetics Commission

4. The panel put out a call for evidence on 4 January 2013. It asked for scientific evidence from experts in any relevant field on the safety or efficacy of pro-nuclear transfer and maternal spindle transfer techniques to avoid the transmission of mitochondrial disease, including published studies (published since March 2011), unpublished research or statements from individuals or organisations, to be submitted by 18 January 2013.

5. The call for evidence was sent directly to more than 30 experts in the field and to 25 professional bodies; the majority of whom had been sent the call for evidence for the original review. Recipients were invited to circulate the call to colleagues.

6. The Core Panel then reviewed the submitted evidence and spoke to the following researchers for additional information and clarification, via teleconferences on 30 January and 12 February:

    -   Dr Mary Herbert, Professor Alison Murdoch and Professor Douglas Turnbull, Newcastle University

    -   Dr Shoukhrat Mitalipov, Oregon Health and Science University

- Dr Dieter Egli and Dr Daniel Paull, The New York Stem Cell Foundation Laboratory

- Professor Michio Hirano, Columbia University

7. Annex B lists the written evidence reviewed by the panel.

Annex B: Evidence reviewed

1. Statements

- A statement from Dr David King, Director of Human Genetics Alert

- A statement from The Wellcome Trust

- A statement from Joanna Poulton (Professor and Hon Consultant in Mitochondrial Genetics, University of Oxford), Joerg P Burgstaller (IFA Tulln and University of Veterinary Medicine Vienna) and Iain G. Johnston (Imperial College London)

- A confidential statement regarding 'Progress towards experiments requested in the HFEA Core Panel Scientific Review' 'from Alison Murdoch, Mary Herbert and Doug Turnbull, Newcastle University

2. Published articles and reports (submitted)

- Paull, D, et al (2013) "Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants." Nature 31;493(7434):632-7.

- Treff, N. R. et al (2012) "Blastocyst preimplantation genetic diagnosis (PGD) of a mitochondrial DNA disorder." Fertil Steril. 98(5):1236-40.

3. Published articles and reports (identified by panel members)

- Amarnath, D. et al. (2011) "Nuclear–cytoplasmic incompatibility and inefficient development of pig–mouse cytoplasmic hybrid embryos." Reproduction 142 295–307.

- Jiang, Y. et al. (2011) "Interspecies Somatic Cell Nuclear Transfer Is Dependent on Compatible Mitochondrial DNA and Reprogramming Factors." PLoS ONE 6(4): e14805.

- Kemp, J.P. et al. (2011) "Nuclear factors involved in mitochondrial translation cause a subgroup of combined respiratory chain deficiency." Brain 134; 183 195.

- Lee, H-S. et al. (2012) "Rapid mitochondrial DNA segregation in primate preimplantation embryos precedes somatic and germline bottleneck." Cell Rep. 1(5): 506–515.

- McCormick, E. et al. (2012) "Molecular Genetic Testing for Mitochondrial Disease: From One Generation to the Next." Neurotherapeutics. DOI 10.1007/s13311-012-0174-1.

- Nakada, K. and Hayashi, J-I. (2011) "Transmitochondrial Mice as Models for Mitochondrial DNA-Based Diseases." Exp. Anim. 60(5):421-431.

- Payne, B.A.I. et al. (2013) "Universal heteroplasmy of human mitochondrial DNA." Human Molecular Genetics 22, 2: 384–390.

- Samuels, D.C. et al. (2013) "Preventing the transmission of pathogenic mitochondrial DNA mutations: can we achieve long-term benefits from germ line gene transfer?" Human reproduction 28(3):554-9.

- Saneto, R.P. and Sedensky, M.M.(2012) "Mitochondrial Disease in Childhood: mtDNA Encoded." Neurotherapeutics DOI 10.1007/s13311-012 0167-0.

- Sharpley, M.S. et al. (2012) "Heteroplasmy of Mouse mtDNA is Genetically Unstable and Results in Altered Behavior and Cognition." Cell 151: 333–343.

- Tachibana, M. et al. (2013) "Towards germline gene therapy of inherited mitochondrial diseases." Nature 493(7434):627-31.

- Wakefield, SL. et al. (2011) "Impaired Mitochondrial Function in the Preimplantation Embryo Perturbs Fetal and Placental Development in the Mouse." Biology of Reproduction 84, 572-580.