

Review of the safety and efficacy of polar body transfer to avoid mitochondrial disease

Addendum to 'Third scientific review of the safety and efficacy of methods to avoid mitochondrial disease through assisted conception: 2014 update'

Report provided to the Human Fertilisation and Embryology Authority (HFEA), October 2014

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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 (mtDNA) can cause a range of rare but serious diseases that can be fatal. However, there are several novel treatment methods with the potential to reduce the transmission of abnormal mtDNA from a mother to her child, and thus avoid mitochondrial disease in the child and subsequent generations.

Such treatments have not been carried out in humans anywhere in the world and to do so is currently illegal in the UK. This is because the primary legislation that governs assisted reproduction, the Human Fertilisation and Embryology Act 1990 (as amended), only permits eggs and embryos that have not had their nuclear or mtDNA altered to be used for treatment. However, the Act allows for regulations to be passed by Parliament, which would allow techniques that alter the DNA of an egg or embryo to be used in assisted conception, to specifically prevent the transmission of serious mitochondrial disease due to mutations in mtDNA.

In June 2014 the Human Fertilisation and Embryology Authority (HFEA) conducted its third review for Government on the safety and efficacy of mitochondrial replacement (also referred to as mitochondrial donation) techniques. This provided a comprehensive overview of the scientific issues raised by mitochondrial replacement techniques and an assessment of the current state of the research. The panel's recommendations relating to the use of preimplantation genetic diagnosis (PGD), pronuclear transfer (PNT) and maternal spindle transfer (MST) to avoid the inheritance of mitochondrial disease are outlined in its June 2014 report¹ (and in previous reports of 2011 and 2013). The main conclusions were that MST and PNT are likely to be effective in avoiding mitochondrial disease caused by mutations in mtDNA; no evidence was found to suggest that the techniques would be unsafe in humans; and that the direction of travel of current research is consistent with both these findings.

In July 2014 the Government announced that it intends to proceed with putting mitochondrial donation regulations to Parliament, subject to giving further consideration to the panel's recommendations, refining the draft regulations to take account of changes identified during the recent consultation, and discussing an appropriate approval process with the HFEA².

¹ Human Fertilisation and Embryology Authority. Review of scientific methods to avoid mitochondrial disease: 2014 update. June 2014. Available at: www.hfea.gov.uk/8807.html

² Department of Health. Mitochondrial donation plans progress following consultation. July 2014. Available at: www.gov.uk/government/news/mitochondrial-donation-plans-progress-following-consultation

Subsequent to the third review, data describing a new technique for mitochondria replacement – polar body transfer (PBT) – were published (Wang et al 2014) and the HFEA was asked by the Department of Health to seek the panel's views on the safety and efficacy of this new technique and to provide a report. The panel note that PBT is at an early stage of development, with little or no human data publicly available for the methods, so consideration of the evidence is similarly at an early stage.

This review is written as an addendum to the panel's aforementioned third review. It focuses solely on the safety and efficacy of PBT to avoid the inheritance of mitochondrial disease, although it makes comparisons with MST and PNT where appropriate.

What are polar bodies?

Polar bodies are formed during the process of egg maturation and fertilisation; they contain mostly DNA (genomic) in the form of chromosomes, with very little surrounding material (cytoplasm). Unlike most cells in the body, which contain two sets of 23 chromosomes (one set inherited from the mother and one from the father), the DNA in the immature egg has already duplicated, so that it contains four sets, and has entered a process called meiosis. This process includes shuffling (recombination) of the maternally and paternally inherited DNA, which gives each individual a unique genetic inheritance. As the egg matures the four sets of chromosomes separate, half going into one relatively small daughter cell, which becomes the first polar body (PB1). This remains, within the transparent outer shell of the egg (the zona pellucida). The other two sets of chromosomes remain within the egg, whilst the first polar body does not form part of the resulting embryo.

The second polar body is produced during fertilisation, when the two sets of chromosomes remaining within the egg (after the first polar body was extruded) split again into two sets of 23 chromosomes. One set is packaged within another relatively small daughter cell, the second polar body (PB2), whilst the other set of chromosomes remains within the newly fertilised egg (known as a zygote), to become the maternal nuclear DNA of the resulting embryo. Sperm DNA, also containing a set of 23 chromosomes, becomes the paternal nuclear DNA. The first and the second polar bodies therefore contain genetic information that comes from the nuclear DNA of the egg.

What is polar body transfer?

There are two polar body transfer techniques (see Figures 2 and 3 for diagrammatic representations of the techniques). The first involves removing the first polar body from the unfertilised egg and transferring it to an unfertilised donor egg which has had its nuclear DNA removed. The second technique involves removing the second polar body after fertilisation and transferring it to a newly fertilised egg (a zygote), which has had its maternal nuclear DNA removed.

Recommendations

In writing this addendum, the panel assessed new evidence provided in relation to polar body transfer techniques and came to the view that developments in the coming years are likely to be rapid. The experiments recommended by the panel are broadly the same as those it previously recommended for MST and PNT.

Therefore, the panel agreed that the following experiments are also essential³ for assessing the safety and efficacy of PBT:

- Polar body 1 transfer (PB1T) using human oocytes that are then fertilised (not activated), and comparative follow up of development in vitro. *This could include a molecular karyotype analysis of PB2T.*
- Polar body 2 transfer (PB2T) using normally fertilised human oocytes, from which the maternal pronucleus has been removed, and development compared to normal ICSI-fertilised human oocytes. *The panel highlighted the importance of demonstrating a robust method for distinguishing the maternal and paternal pronuclei, such that the maternal pronucleus can be reliably selected for removal.*

In addition, the panel considers that:

- PBT in a non-human primate model, with the demonstration that the offspring derived are normal, is neither critical nor mandatory.

The panel considers the following to be desirable:

- 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 the use of any mitochondrial replacement technique using oocytes or zygotes with two different variants of mtDNA.

The panel noted that for some of the outlined experiments (see Annex C) additional data for any of the mitochondria replacement techniques would provide suitable evidence of safety and efficacy. It is not necessary for all of these to be carried out for each method because the panel are concerned with the properties of mtDNA in heteroplasmic situations, which should not be affected by the methods used.

³ In this, and in previous reports, we have described certain experiments as being “critical” or “essential”; the terms are used interchangeably and are not intended to convey any technical or medical meaning. Instead they simply indicate which experiments are, in the panel’s view, necessary to the consideration of the safety and efficacy of the technique.

Further to this, the panel considers that, even though PBT techniques are still at an early stage of development, these methods may offer possible advantages over MST and PNT techniques because they:

- (i) may reduce mtDNA carryover.
- (ii) reduce the risk, when compared to MST, of leaving chromosomes behind (as these are all packaged within the polar body).
- (iii) avoid the need to use cytoskeletal inhibitors to allow removal of the spindle or pronuclei from the patient's oocyte or zygote.
- (iv) involve the use of more conventional micromanipulation procedures, which (in the case of PB1T) can also be combined with ICSI. This will reduce the chance of damaging the patient's karyoplast or the donor's oocyte/embryo and therefore lead to greater efficiency.
- (v) raise the possibility of carrying out both PB1T and MST, or PB2T and PNT, to double the chances of success for each patient cycle.

However, additional studies on human material would need to be conducted to assess the extent to which these potential advantages are real.

1. Introduction, scope and objectives

1.1 Introduction

1.1.1 Mitochondrial malfunction has been recognised as the cause of a number of serious multi-organ diseases. The underlying defect can be due to mutations in nuclear DNA affecting gene products required within mitochondria, or to mutations in DNA carried within the mitochondria themselves (mitochondrial DNA, mtDNA). The latter encodes products required exclusively for the oxidative phosphorylation (OXPHOS) process of the electron transfer chain, which generates energy for cells in the form of ATP (an “energy molecule”)⁴. Although relatively rare, the seriousness of these diseases and particularly the inheritance pattern of mtDNA mutations have made them a focus for research into preimplantation methods to reduce or avoid such diseases in offspring.

1.1.2 The biology of mitochondria is complex and the attendant language is therefore technical in parts. This report tries to explain the issues, and Annex D provides a glossary including a definition of relevant terms used in this report.

1.2 Scope and objectives of this review

1.2.1 The terms of reference for the panel are to:

“produce a report outlining the following relating to the potential mitochondrial donation technique – polar body transfer (PBT):

- biology of polar bodies (e.g. explanation of what polar bodies are and the process of meiosis)
- whether PBT has the potential to avoid mitochondrial disease (e.g. relevant biological processes and background research)
- safety and efficacy of PBT (e.g. evidence from studies on human oocytes and animal models, and similarities/differences to MST and PNT).”

Accordingly, this report focuses exclusively on the science, and the safety and efficacy of PBT; it does not consider any ethical or legal issues that may be raised by this technique except when these are directly relevant to proposed research.

⁴ Although mitochondria have other functions within cells, such as in lipid metabolism and programmed cell death, these are encoded by nuclear genes.

- 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 considers the safety and efficacy of PBT to avoid mitochondrial disease and Section 3 sets out recommendations.

2. Review of polar body transfer

2.1 Polar body transfer (PBT) as an alternative or complementary method to MST and PNT as a way to avoid disease due to abnormal mtDNA

2.1.1 Mitosis and meiosis and DNA content in human cells

Mitosis and meiosis are processes that control the DNA content of cells through replication and segregation for cellular growth and sexual reproduction, respectively. In human cells, DNA is contained in 46 chromosomes comprising two sets of 23 homologous (similar) chromosomes of maternal and paternal origin; these sets come together during fusion of an oocyte with a sperm (N.B. for these purposes, the paternally-derived Y chromosome in males can be treated as a homologue of the X chromosome, albeit one that has lost most of the homology).

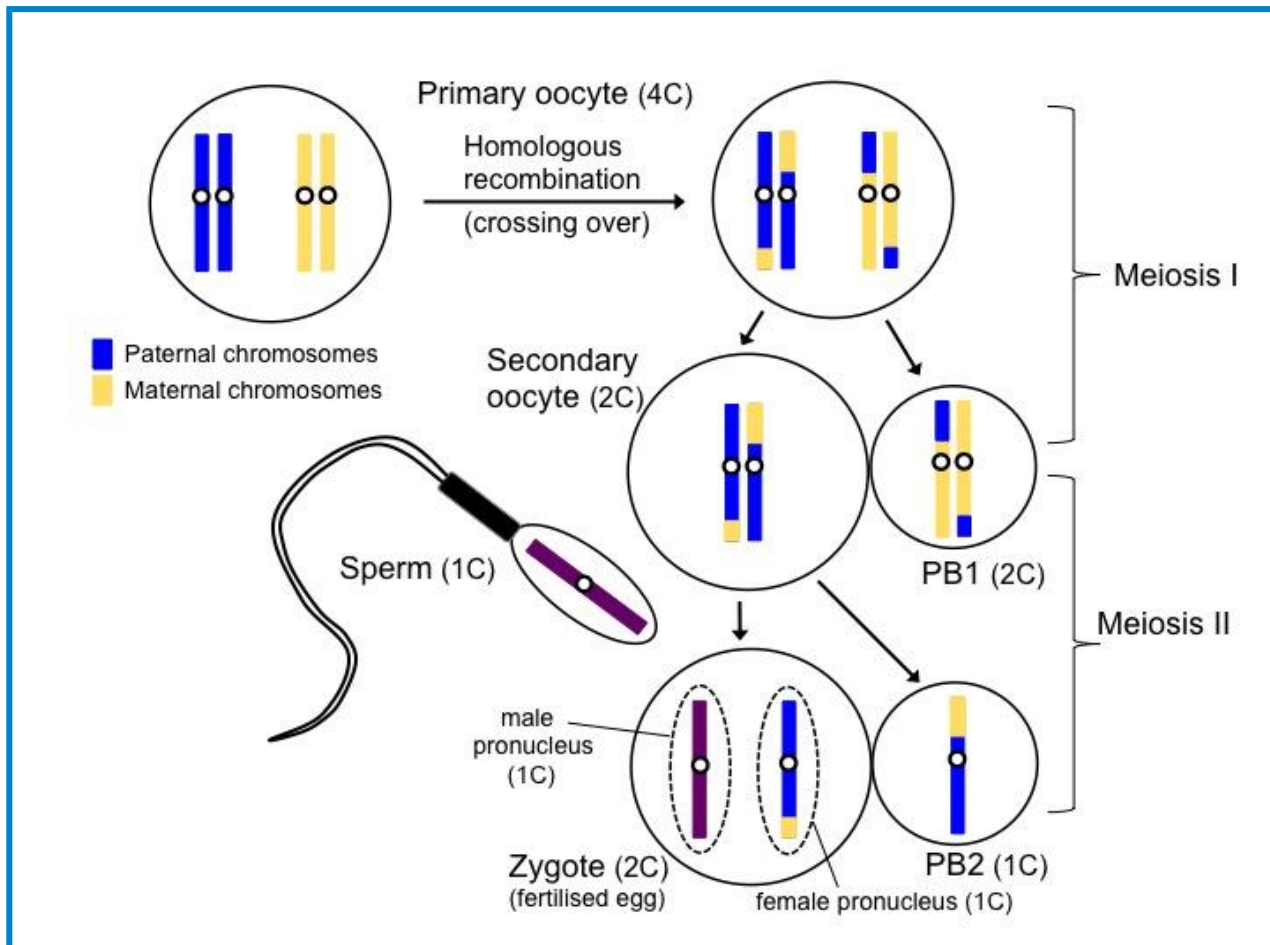
During **mitosis** these chromosome sets undergo cycles of DNA synthesis (replication) to form “bivalent” chromosomes, comprising two joined chromatids. At cell division, these chromatids separate and segregate into the new daughter cells. The DNA content of a mitotically dividing cell is said to be diploid (2N), because, after replication and division, it consists of two sets of chromosomes and a genomic copy number of 2.

The process of **meiosis** (see Figure 1) allows for recombination (shuffling of genes) and reduces the number of sets of chromosomes from diploid (2N) to haploid (1N), i.e. to a single set of chromosomes (1C), so that a normal diploid DNA content is restored at fertilisation. Following replication, (when the genomic copy number is x4 (4C)), meiosis begins with pairing of the replicated homologous chromosomes, which then exchange genetic information via recombination; this is an important mechanism for achieving random inheritance of parental traits. DNA content is then reduced by segregation of replicated homologous chromosomes (4C to 2C), and then segregation of replicated chromatids (2C to 1C).

2.1.2 Meiosis in the female germ line

The cellular precursors of human oocytes (known as oogonia) divide mitotically within the fetal ovary. Once they enter meiosis, which is generally accepted to occur within all the oogonia prior to or around birth, they are called primary oocytes and arrest in prophase of the first meiotic (MI) cell cycle, with DNA organised as paired replicated homologous chromosomes (4C). Primary oocytes persist in this state until after puberty, when some are “selected” in each menstrual cycle for growth and maturation.

Figure 1. Meiosis in the female



For simplicity only one maternal and paternal chromosome pair is indicated. DNA synthesis has already occurred in oocytes in the fetal ovary, therefore the chromosomes are already present as pairs of "chromatids" and DNA copy number is four (4C). The various components are not drawn to scale; notably the polar bodies and sperm are much smaller than the fully-grown oocyte and zygote.

During this process MI is completed with a very asymmetric cell division leading to formation of the secondary oocyte, which contain the majority of the cytoplasm and the first polar body (PB1), which has very little cytoplasm. The nuclear DNA content is now 2C in both the secondary oocyte and in PB1; due to crossing over during MI, the oocyte and PB1 each has a distinct (and unique) genetic make-up. No other differences with respect to the properties of the nuclear DNA have been noted (Hou et al, 2013 and discussion below).

The secondary oocyte arrests in meiosis II (MII) with replicated chromosomes aligned on the maternal spindle (Howe and FitzHarris, 2013). This is the stage at which the oocyte is ovulated as a mature oocyte, ready to be fertilised by a sperm.

Upon fertilisation with a sperm, MII is completed with a second asymmetric cell division such that the secondary oocyte extrudes a second polar body (PB2), which is haploid and contains a single copy of each DNA sequence (1C). This occurs about two hours after sperm-oocyte fusion.

The fertilised oocyte or zygote initially contains unreplicated DNA within each of the haploid maternal and paternal pronuclei (each 1C). The two pronuclei approach each other and undergo DNA synthesis such that the number of DNA copies in each doubles. This is followed by pronuclear membrane breakdown and the merger of the genomes before entry into mitosis during which the replicated chromosomes are segregated and cell division results in a two-cell embryo, each cell of which has the same diploid (2C) content, with a set of maternally and paternally-derived chromosomes. Subsequent mitotic divisions replicate the DNA and segregate these sets of chromosomes independently of each other (i.e. without crossing over) such that all cells will have both maternally and paternally-derived chromosomes. The majority of cells in the embryo and adult are diploid and 2C, although a few special cell types can have multiple copies (i.e. they become polyploid).

If the secondary oocyte is chemically or electrically activated, rather than fertilised, then the same events occur; however, in the absence of a sperm-derived paternal pronucleus the activated oocyte and resulting embryo will be haploid (1C). Occasionally PB2 may fail to be extruded upon such activation, which will lead to a wholly maternally derived diploid (2C) embryo. This can also be achieved experimentally by preventing PB2 formation, by fusing the PB2 back into the activated oocyte or by fusing together the blastomeres of a 2-cell haploid embryo.

2.1.3 Polar bodies and asymmetry

The mechanisms involved in the two asymmetric cell divisions leading to the formation of PB1 and PB2 have been studied extensively (Brunet and Verlhac, 2011; Maddox et al, 2012; Howe and FitzHarris, 2013; Sun and Kim, 2013; Li and Albertini, 2013). The asymmetry is achieved by components of the cytoskeleton that interact with the spindle and the plasma membrane of the oocyte, and bring the spindle from the centre of the oocyte close to its edge (within the “cortex”). In other respects, the process of cytokinesis, which leads to the formation of two daughter cells, appears to largely follow that of most other cell types. However, unlike most other asymmetric cell divisions where both daughters play roles within the tissue – such as a stem cell giving rise to another stem cell and to a differentiated cell type – the polar bodies are entirely dispensable; they can be removed or destroyed (e.g. with a laser) with no detectable effect on fertilisation or embryo development. A number of studies have therefore explored both the properties of polar bodies and the segregation of molecules, such as RNAs, proteins and organelles (including mitochondria) at both the completion of MI and MII.

The polar bodies – PB1 for a short period, but particularly PB2 – remain in the space between the embryo and the zona pellucida (the perivitelline space) during cleavage stages in several species, including human (Hertig et al, 1956; Van Blerkom and Davis, 1998; Bartholomeusz, 2003).

It has been reported (in the mouse) that the polar bodies show slow progression through the cell cycle, generally remaining in S-phase (DNA synthesis) without undergoing apoptosis (Hino et al, 2013). Indeed, PB2 can be viable for at least 72 hours after fertilisation in the mouse, and while there is some considerable variation reported for the persistence of human polar bodies, PB1 is generally thought to survive longer than it does in the mouse and PB2 can also survive in some cases to blastocyst stages (Hertig et al, 1956; Van Blerkom and Davis, 1998; Bartholomeusz, 2003). Zygotic genome activation occurs at the late 2-cell stage in the mouse and at around the 4- to 8-cell stage in humans; therefore, very early events in the embryo depend on maternal RNAs and proteins. These will be largely located in the cytoplasm, but as the polar bodies contain relatively little it is not surprising that they progress very slowly; indeed, because they also have reduced components required for cell death, such as caspases (nuclear gene encoded enzymes that are located within mitochondria), they are likely to remain largely in a state equivalent to that of the unfertilised oocyte (Telford, Watson and Schultz, 1990). VerMilyea et al (2011), also noted lower levels (per cytoplasmic volume) of mRNAs for genes involved in cell cycle progression in PB2 compared to the zygote, which is consistent with their remaining in S-phase for longer.

There have been several studies that have attempted to look at the mechanism of polar body degradation in mice and human preimplantation embryos. The DNA fragmentation detected by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) increases with time after ovulation or fertilisation, but this is not necessarily apoptosis or any other form of programmed cell death. DNA fragmentation also occurs in cells undergoing necrosis and other forms of cell death due to the lack of nutrients or metabolites. Attempts to use other specific markers of apoptosis, such as the presence of activated caspases, annexin V staining, and propidium iodide staining, have suggested that although apoptosis can occur it is not the main mechanism. For example, Fabian et al (2012) conclude that, in the mouse, the death of PB1 proceeds by a caspase-independent mechanism, indeed inhibitors of caspases fail to block PB1 degradation (Zakeri et al, 2005), while the death of PB2 can involve caspases, but these are not always evident even in late stage preimplantation embryos. Hino et al (2013), looked at PB2 in mice and found that only about 3% of PB2 showed caspase activity and none, even in blastocyst stages, showed TUNEL staining in normal culture conditions. Van Blerkom and Davis (1998) found TUNEL but not annexin V staining in PB1 of a relatively high proportion of mouse oocytes, whereas TUNEL was rarely detected in PB1 of human oocytes. However, they conclude that when DNA strand breaks occur, this may involve apoptosis-associated endonuclease digestion.

In summary (from the cited references above and others), there appear to be some species differences. Notably, human PB1s would appear to survive longer than mouse PB1s. PB2 survival seems variable in both humans and mice. In the mouse, genetic background effects are known to be relevant as in some strains PB2 always survive until blastocyst stages, whereas in others they persist only for a few cleavage divisions (Gardner, 2007). Perhaps there are also genetic effects on human PB2 survival, although variations in culture methods and media may also play a part. These intrinsic and extrinsic effects may also influence whether the death of any particular polar body is due to apoptosis or not. However, the evidence to date suggests that apoptosis is not the major mechanism employed; moreover, there does not seem to be any mechanism that specifically limits the survival of polar bodies in comparison to the oocyte or zygote, apart from their lack of cytoplasm.

There is nothing intrinsic in the process of meiosis itself that confers asymmetry. For example, meiosis in the male gives four functional haploid sperm from each diploid spermatocyte and the products of meiosis in single cell eukaryotes such as yeast have the same form, irrespective of the mating type (functionally equivalent to sex) from which they originate, and they are all equally able to participate in (the equivalent of) fertilisation

and subsequent development. However, the asymmetry in the female germ line of many animals serves several roles; two in particular:

- (i) The asymmetry confines almost all of the resources of the oocyte, in terms of energy supplies, RNA, proteins, and organelles, into one of the four possible daughter cells, conferring an advantage on this particular cell for early development. This would appear to be almost exclusively a quantitative mechanism, where it is simply a question of relative volumes of oocyte and polar bodies that determines the amounts of specific products. For example, the relative levels of most specific mRNAs present in single human polar bodies reflect those in the oocyte (Klatsky et al, 2010; Reich et al, 2011). The only exceptions are components associated with, or excluded from, the particular part of the oocyte where the spindle is located. To achieve segregation of chromosomes into both products of the asymmetric cell division, and the asymmetric division itself (to produce the large oocyte and small polar body), the spindle must be located close to the cell membrane of the oocyte at the position where cytokinesis will occur. This is achieved by components of the cytoskeleton that interact with the spindle and the membrane (Howe and FitzHarris, 2013; Li and Albertini, 2013). The region of the oocyte membrane overlying the spindle therefore has properties that distinguish it from the rest, and this persists after cytokinesis. One consequence of this is that polar bodies cannot normally be bound or fertilised by sperm (Fisk et al, 1996; Motosugi et al, 2006). Another consequence is that relatively few mitochondria are found in polar bodies (VerMilyea et al, 2011; Dalton and Carroll, 2013; Wang et al, 2014), and certainly in the case of PB1, fewer even than would be predicted by their volume of cytoplasm (see below).
- (ii) The asymmetry allows for the distribution of developmental cues within the oocyte, notably specific RNAs and proteins, including those that can be partitioned during early cell divisions and subsequently define embryonic patterning. This occurs in, for example, many invertebrates, such as the roundworm *C. elegans* and the fruitfly *Drosophila*, and in lower vertebrates, such as frogs (Riechmann and Ephrussi, 2001). However, there is little if any evidence for the presence of essential asymmetrically located determinants required for embryo patterning in mammalian oocytes (Brunet and Verlhac, 2011; VerMilyea et al, 2011). Instead, the early development of mammals is characteristically very regulative, such that cells can be removed, embryos can be split in two (as occurs in twinning), or two embryos can be combined together (as occurs in chimeras), and all result in normal development. Consistent with the lack of pre-pattern, experiments by VerMilyea et al (2011), failed to find any significant differences in transcripts

between early blastomeres. Some specific gene transcripts were found to have a higher or lower abundance in the region of the spindle compared to the rest of the oocyte and the same “asymmetry” was preserved into PB2 and the zygote. However, both the mechanism by which this occurs and its significance are unknown; the PBT experiments discussed below show that it is of little or no importance, at least in the species studied.

2.1.4 Polar bodies, genomic integrity, and PBT experiments

There is robust evidence in the mouse, and some evidence in humans, that the genome of polar bodies is intact and not distinguished in any way from that of the oocyte apart from its specific DNA sequence (differences in DNA sequence are due to recombination occurring in MI).

Genotyping of polar bodies is used as a diagnostic tool in PGD and PGS to infer the genetic status of the maternal DNA contribution to an embryo (e.g. Harton et al, 2011; Kuliev et al, 2011)⁵. This appears to be a rigorous method, which it would not be if the DNA sequence of the polar bodies were somehow degraded. Recently, whole genome analysis has been carried out on “triads” comprising of PB1, PB2 and the maternal pronucleus of single human oocytes (Hou et al, 2013). This revealed the genome integrity of all three products of meiosis, as well as the location of crossovers (due to recombination).

Array comparative genomic hybridization (aCGH) has also been performed to compare the genomes of individual PB1s and their counterpart spindle-chromosome complexes in human MII oocytes, and with individual PB2s with maternal pronuclei in human zygotes (Wang et al, 2014). No differences were found in chromosome copy number and there were no detectable alterations in the genome, with PB1 having a normal diploid and PB2 a normal haploid genome. DNA damage markers also showed no signal in either PB1s or PB2s.

The most conclusive evidence of the integrity of polar body genomes has come from mouse experiments showing that they are able to support normal development. Thus, when the maternal pronucleus of a fertilised oocyte (zygote) was removed and replaced by fusing in its own PB2 or a PB2 from another zygote, the reconstructed zygote (see Figure 3) could develop to the blastocyst stage and give rise to a normal live born animal (Evsikov and Evsikov, 1994; Wakayama et al, 1997).

⁵ This is a procedure regulated by the HFEA and methods are available for the isolation of polar bodies.

Wakayama et al (1997) showed that timing was important, with efficiencies of up to 70% when the recipient zygote was recently fertilised (shortly after pronucleus formation); a delay of four hours decreased this to 20%. The age of the PB2 was less relevant, suggesting that it progresses very slowly through the cell cycle (as discussed above). The poor developmental outcomes when older recipients were used probably reflect entry of the zygote into cell cycle before DNA synthesis has completed in the PB2 genome.

The integrity of the PB1 genome was also demonstrated in mice by Wakayama and Yanigamachi (1998). Although PB1 usually degenerates soon after ovulation in the mouse, some appear viable for more than 10 hours. If the PB1 nucleus was injected into a mature (secondary) oocyte after the maternal spindle was removed (with its associated nuclear DNA), then the PB1 chromosomes formed a new metaphase plate (spindle) (see Figure 2). After fertilisation and transfer to recipient females, 30-57% of such oocytes gave rise to live born mice.

Both PB1 and PB2 transfer have also been used to generate parthenogenetic embryos from which ES cell lines were derived (Wakayama et al, 2007), again demonstrating that the PBs must have an intact genome, without any need for rescue by a paternal genome.

Similar experiments have also been reported with PB1 transfer into enucleated oocytes from pigs (Wang et al, 2011). In these experiments some of the PBs had been stored frozen (vitrified) for two months before thawing and introduction into enucleated oocytes, which were then fertilised by ICSI. The resulting embryos were only followed in vitro to the 8- or 16-cell stage and they were not transferred to recipient females, but these experiments suggest that it is also possible to use frozen PBs.

That both PB1 and PB2 have an intact genome in the mouse was also effectively demonstrated very recently by Wang et al (2014); either PB1 could be shown to replace the maternal spindle in MII oocytes, or PB2 could replace the maternal pronucleus of zygotes. The group obtained birth rates of about 40% after embryo transfer in both the PB1 and PB2 transfer experiments, similar to various manipulated and unmanipulated controls, and all the pups were overtly healthy.

In addition, these authors found that the DNA damage markers phospho-p53 and phospho-H2AX were absent from PB1. For PB2, the authors also found that the nuclear membrane of the PB and that of the maternal PB were similarly intact, and that there was also no detectable DNA damage.

Figure 2. Polar body 1 transfer (PB1T) and maternal spindle transfer (MST)

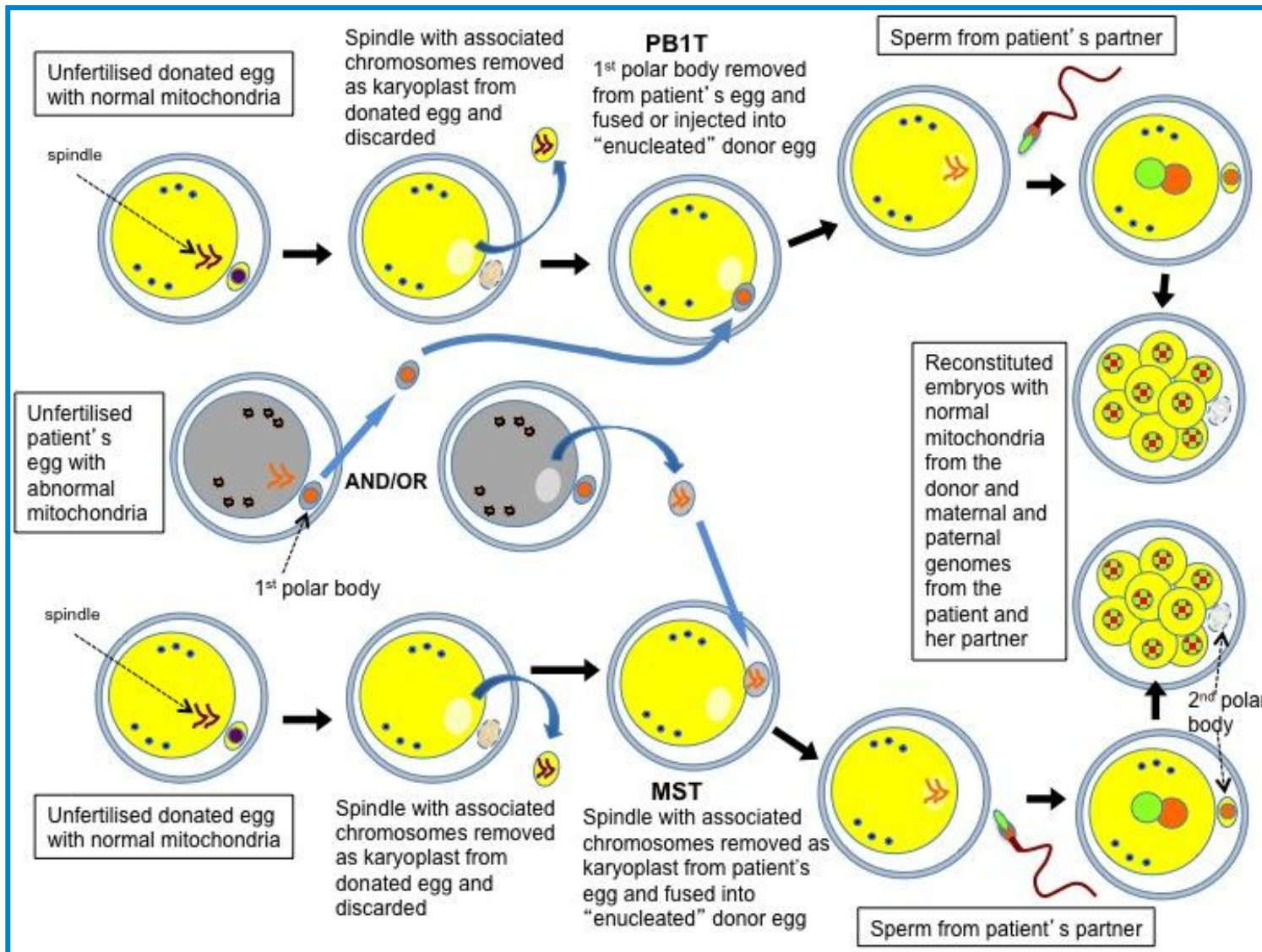
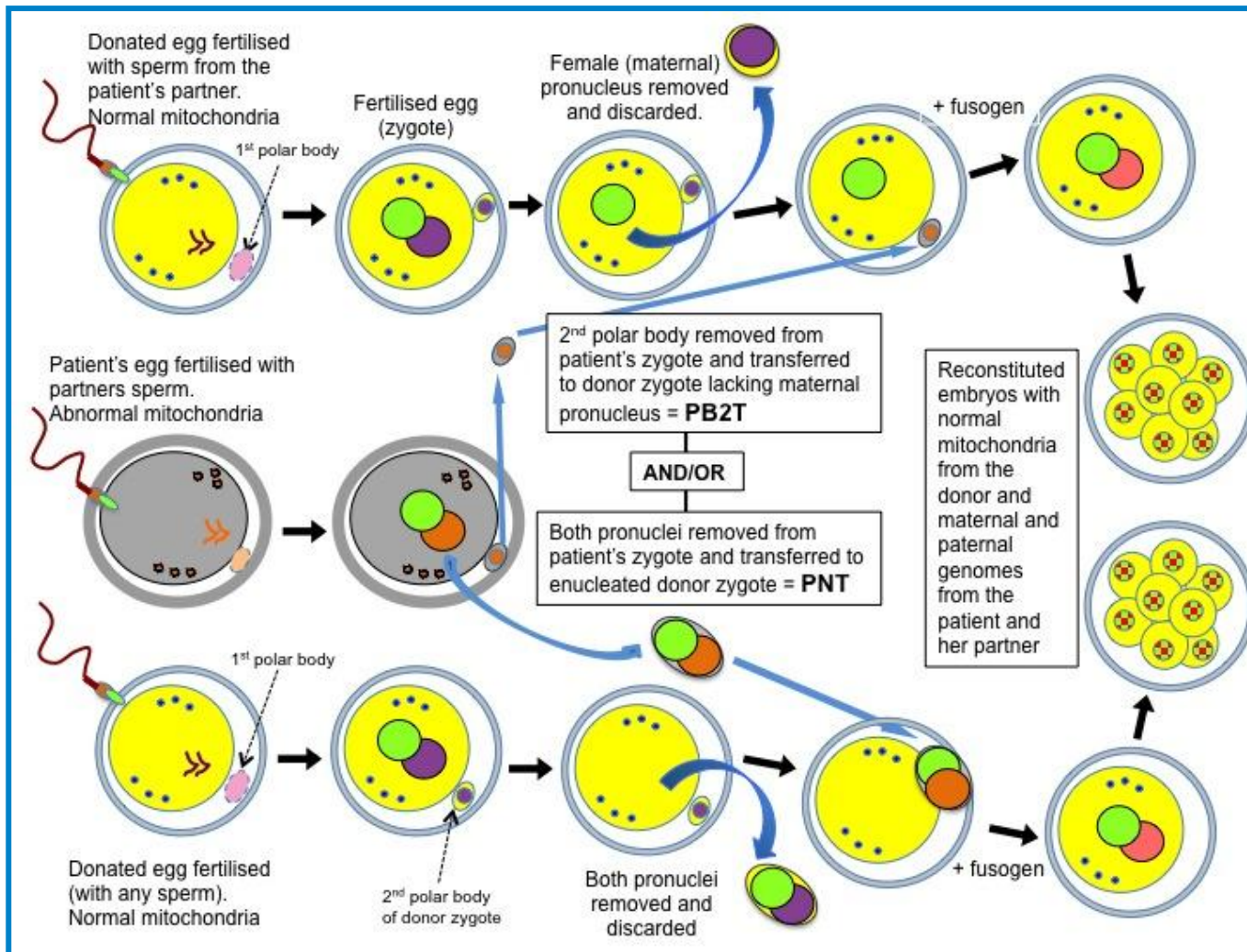


Figure 3. Polar body 2 transfer (PB2T) and pronuclear transfer (PNT)



For simplicity, but also because they may be removed to ensure they cannot fuse back into the zygote, PB2 from the donor zygote is not shown in the later stages.

To date, similar experiments have not been reported with human PBs, although in Tachibana et al (2013) a triploid ES cell line, HESO-ST6, was derived from an embryo arising from MST, which had either not extruded PB2 or this had fused back into the oocyte. There have also been rare cases in humans of mixed diploid/triploid children being born that are likely to be due to PB2 fusing back into one blastomere at the 2-cell stage (Hino et al, 2013).

If this is the mechanism, it would indicate that the genome of PB2 remains viable for at least 24 hours and that once incorporated into a blastomere, the slow cell cycle of PB2 can become synchronised with that of the host genome (Hino et al, 2013), as suggested in the mouse experiments of Wakayama et al (1997) and Hino et al (2013). The development of triploids is also noted to occur if PB2 fails to separate after fertilisation during IVF. Such “digynic” triploid embryos may also occur during natural mating (as will “diandric” triploids resulting from fertilisation of an oocyte with two sperm); however, they do not survive long as a result of being triploid.

In addition, it should be noted that cases of human XX/XY chimeras (giving rise to “true hermaphrodites”) have been shown to derive from the fertilisation of an oocyte and (probably) a larger than normal PB (a result of incorrect cytokinesis) by two different spermatozoa (Maddox et al, 2012).

Although no data have been reported on PBT with human oocytes or zygotes, the panel has been provided with some unpublished (and therefore confidential) data from attempts to perform both PB1T and PB2T. Although preliminary, the data suggest that these are both technically feasible, result in low carryover of mtDNA along with the PB nuclear genome, and allow early preimplantation development to proceed.

2.1.5 Errors at meiosis leading to abnormal oocytes and PBs

At MI (i.e. formation and extrusion of PB1), chromosomes should separate as “bivalents”, resulting in both the oocyte and PB1 having a complete set of chromosomes present as linked chromatids. Mis-segregation of chromosomes at meiosis, such that either the oocyte or a PB has an extra or missing chromosome, occurs both in natural reproduction and in IVF, and increases in frequency with maternal age. An additional mechanism leading to error is premature sister chromatid separation, leading to a chromatid (i.e. half of a bivalent) travelling to the PB1 at MI. This leads to an oocyte with only one copy of the chromatid in question, with three copies in PB1 (or vice versa). At MII, this error may be corrected, resulting in two abnormal PBs, and a normal oocyte, or, if uncorrected, to an abnormal zygote.

The panel is aware of the extensive literature on the use of PB1 and PB2 in PGD and preimplantation genetic screening (PGS), and the chromosome abnormalities found. Much of this information is based on data from older women with fertility problems who naturally have a higher rate of abnormalities in their oocytes, fuelling debate about the reliability of chromosome/chromatid segregation into the PBs (Capalbo et al, 2013; Fragouli et al, 2013).

Precise timing of the isolation of the PB for transfer, may be important because it can take one or two hours for PB1 or PB2 to completely separate from the oocyte or zygote, respectively (Montag et al, 2013). During this period there is a cytoplasmic bridge, which may also contain some spindle material. Premature PB biopsy may lead to some maternal chromosomes being pulled out of the oocyte or zygote.

2.1.6 Centrioles, centrosomes and microtubule organising centres (MTOCs) biology

An associated pair of centrioles, surrounded by an amorphous mass of dense pericentriolar material, makes up a compound structure called a centrosome. These are involved in the organisation of the spindle during mitosis, in correct chromosome segregation and in the completion of cytokinesis (cell division). The biology of spindle formation and activity is complex; however, it is clear from the recent experiments of Wang et al (2014), and the earlier experiments of Wakayama et al (1997), that formation of a new spindle apparatus and its functioning is not compromised after either PB1T or PB2T in mice. This is not unexpected given there is no requirement for centrioles during meiosis, because the spindle can form from MTOCs, of which there are many within the oocyte. In the mouse the same is true for the first mitotic division in the zygote. In the human, the first mitotic spindle has two centrioles, which usually originate from the sperm, although there are also multiple MTOCs that are thought likely to be able to compensate in their absence (Sathanathan 1996). All this suggests that, especially because both PB1T and PB2T involve fertilisation, spindle function should not be compromised after PBT in humans. If it were compromised, the resulting embryo would fail during cleavage divisions and it would be unable to make a normal blastocyst. Expert advice confirms that spindle formation and function following PBT are unlikely to be problematic. However, it would seem prudent to verify that early embryos, and/or ES cells derived from them, are euploid (i.e. have a normal diploid number of chromosomes).

2.2 The prospects for PBT as a method for mitochondrial replacement to avoid mitochondrial disease

2.2.1 Mitochondria and PBs

If PBT is to be explored as a method of mitochondria replacement to avoid mitochondrial disease, it is important to consider mtDNA copy number within PBs, and whether there are any mechanisms promoting the segregation of either normal or abnormal mitochondria amongst the products of the two meiotic cell divisions.

In comparison with the oocytes, which in the mouse contain at least 100,000 mtDNA molecules and in humans more than 300,000, PBs contain rather few. This is particularly so for PB1 where one study suggested an mtDNA copy number of about 1,000, as measured using 10 PB1s from healthy women (Steuerwald et al, 2000). Other unpublished data suggests that the numbers may be even lower; however, this may be influenced by the methodology used to determine mtDNA copy number (see section 2.2.3 below).

Wang et al (2014) examined mice for numbers of mitochondria and mtDNA copy number in PB1 and PB2. PB1 was found to contain very few mitochondria and in some cases none was visible (after staining with MitoTracker), while more were found in PB2 although they appear less dense than in the zygote. Making use of an accurate method to determine DNA copy number (chip-based digital PCR), PB1 was found to contain an average of 359 copies of mtDNA, whereas PB2 contained an average of 1,092 copies.

The low numbers of mitochondria might be consistent with the relatively short lives of PBs, especially of PB1, although some somatic cells can survive with fewer. Given that the PBs are mostly chromosomes (containing nuclear DNA), it is not surprising that they contain low mtDNA copy numbers and it is possible that this relates simply to the volume of “cytoplasm” they inherit. However, the data measuring mtDNA copy number from both human and mouse PBs in comparison to their volume suggest that mitochondria tend to be excluded from PBs, particularly from PB1, and preferentially end up in the oocyte and zygote.

Dalton and Carroll (2013) explored in detail the distribution and inheritance of mitochondria during the asymmetric cell divisions leading to the formation of both PB1 and PB2 in the mouse. The mitochondria were found to be enriched around the spindle during MI (by a dynein/cytoskeletal-mediated mechanism) and this persisted as the spindle migrated towards the edge of the oocyte (its cortex). The aggregation of the mitochondria around the spindle was found to be largely dependent on microtubules (which can be disrupted with nocodazole), rather than microfilaments (which can be disrupted with cytochalasin D or latrunculin A).

However, as MI continues (and this is observed already at anaphase) the mitochondria segregate towards the oocyte-directed spindle pole and are largely excluded from PB1. This is not a property of the spindle itself, but of the oocyte cytoskeleton within the cortex and how this interacts with the spindle. If the movement of the spindle to the cortex is prevented by disrupting microfilaments, then the mitochondria remain around the spindle with no segregation to one pole.

There is also an aggregation of mitochondria around the spindle as it forms during MII within the oocyte cortex, but this is no longer seen by the time of MII arrest (prior to fertilisation), although there are mitochondria in the vicinity of the spindle at this stage (N.B. similar observations were made by Wang et al, 2014).

There appears to be no specific mechanism to exclude mitochondria from PB2 during completion of MII and cytokinesis; however, the small volume of PB2, which is about 4.5% of the zygote volume (VerMilyea et al, 2011), generally ensures that there will be relatively few.

These mechanisms, notably those occurring during PB1 formation, seem to ensure that most mitochondria are inherited by the oocyte as they will be needed for subsequent embryo development, rather than by the PBs which normally have no role. Although individual mitochondria do not generate much energy in the form of ATP in either the oocyte or the early embryo, given that there are more than 100,000, they collectively generate sufficient to overcome the block in glycolytic activity⁶ that lasts until blastocyst stages (references within Dalton and Carroll, 2013).

2.2.2 Behavior of mutant and normal mtDNA during meiosis

To investigate the segregation of mutant and wild type (normal) mtDNA during human meiosis, Gigarel et al (2011) compared a total of 51 PB1s with their counterparts, namely oocytes, or, after fertilisation, isolated blastomeres or whole embryos. The oocytes were obtained from mothers carrying three different mtDNA mutations m.3243A>G (MELAS), m.8344A>G (MERRF) and m.9185T>G. Seven PB1s were found to be mutation-free, as were their counterparts, suggesting homoplasmy for wild-type mtDNA. For the remainder that were clearly heteroplasmic (with a mean mutant load of 37%), in about half the cases the proportion of mutant and wild type mtDNA tended to be similar between the PB1s and

⁶ This is an important mechanism for generating energy within cells. It is one of the reasons why, in patients carrying mutant mtDNA, cells that do not have high demands for energy can survive reasonably well (these are cell types with relatively few mitochondria).

their counterparts ($\pm 10\%$); however in the rest (most of which had higher mutation loads), the correlation broke down and the difference ranged from -34% to $+34\%$. This variability – which suggests that PB biopsy is not a reliable indicator of heteroplasmy and therefore is not a suitable method to avoid having a child with mitochondrial disease – contrasts with studies using mouse models (Dean et al, 2003; Sato et al, 2005) and with an independent study by Vandewoestyne et al (2011). The latter looked at PB1s and their counterparts from patients carrying one of the mutations (m.3243A>G) examined by Gigarel et al (2011), but they found a good correspondence between PB1 and the oocyte for mutation load.

This might suggest that there are some differences either in mtDNA copy number in PB1 between humans and mice or in the way that abnormal mtDNAs segregate, as the mouse mutation is different from the three studied by Gigarel et al (2011). Subsequently, Vandewoestyne et al (2012) repeated aspects of their original study, although this time also looked at PB2. The group again found a good correlation between PB1 and the oocyte; however, there was often a significant discrepancy between PB1 and PB2. They also found that there were discrepancies between the PBs and blastomeres in the corresponding cleavage stage embryos. They do not provide a mechanism, but changed their previous conclusion to agree with Gigarel et al (2011), namely, that PB biopsy is not a reliable indicator of the level of heteroplasmy in any resulting embryo.

Gigarel et al (2011) attributed the variability they found between PB1 and their counterpart oocytes and embryos to there being very low numbers of mtDNA molecules in PB1. They calculated that this could be as low as 10 copies per PB1. This number disagrees with the estimates derived from direct measurements in PB1s from normal women (Steuerwald et al, 2000); however, both approaches suffer from small sample sizes and technical difficulties. An additional complication, not raised by any of the authors, is that each mitochondrion may contain more than one copy of mtDNA. Consequently, in cases of heteroplasmy, some mitochondria may have mostly wild type, and some mostly mutant mtDNA, while others may have a mixture. If the mechanisms involved in selective partitioning of mitochondria to the oocyte depend in some way on the functional status of individual mitochondria, this might lead to higher than expected proportions of mutant mtDNA being associated with PB1 than if it was just due to chance. However, this does not explain the bias seen in the other direction in about 25% of the cases analysed by Gigarel et al (2011).

The most likely explanation for skewing is, therefore, one that is purely stochastic, i.e. mtDNA copy number is so low (as supported by direct measurements), particularly in PB1, that chance and random drift determine the outcome.

2.2.3 PBT with respect to reducing the burden of abnormal mtDNA

The experiments, in particular those of Wakayama and colleagues discussed previously, showing that live born mice could be obtained by transfer of PB1 to an enucleated oocyte, followed by fertilisation (PB1T), or by transfer of PB2 to replace the maternal pronucleus after fertilisation (PB2T), suggested to Wang et al (2014), that these methods might provide suitable alternative methods to MST and PNT as a way of effectively replacing abnormal with normal mtDNA. The group directly compared PB1T with MST and PB2T with PNT. They found that 87.5% of PB1T-derived embryos developed to blastocyst stages, the same rate as they obtained for MST-derived embryos (85.7%) and indeed similar to their figures for the development in vitro of intact fertilised oocytes. On the other hand, PB2T was less efficient than PNT at giving blastocysts (55.5% and 81.3%, respectively). After transfer to recipient females just over 40% of PB1T or MST embryos gave live-born mice, while for PB2T and PNT the rates were 40% and 53.8%, respectively. All pups were healthy and birth rates were similar to those they obtained for control, unmanipulated embryos.

These figures suggest that all of the methods are very efficient. Rates of development after PB2T appeared to be lower than the other method; however, this may be a question of timing and the precise stage of the recipient zygotes used by Wang et al (2014), as Wakayama et al (1997) reported up to 70% of PB2T embryos developing to blastocysts if the recipient zygote was manipulated shortly after formation of the pronuclei.

Wang et al (2014) also explored the potential for carryover of mtDNA with the various methods. They found that karyoplasts containing a spindle complex contained on average 2,318 copies of mtDNA, while karyoplasts containing both maternal and paternal pronuclei had on average 34,392 copies. This compares with averages of 359 and 1,902 for PB1 and PB2, respectively. The mtDNA carryover figures Wang et al (2014) obtained for MST, and especially for PNT, are considerably higher than those reported by Mitalipov et al, Egli et al and the Newcastle group for mtDNA carryover for MST and PNT with human oocytes and zygotes, where the estimates are <1% for MST and <2% for PNT⁷. The reason for this discrepancy is unclear, however it might reflect either differences between mouse and human, and how the methodology has to be adapted for the two species, or to comparative inexperience with MST and PNT by Wang et al (2014), compared to the other groups. Operationally, it is likely to be easier to

⁷ Human Fertilisation and Embryology Authority. Review of scientific methods to avoid mitochondrial disease: 2014 update. June 2014. Available at: www.hfea.gov.uk/8807.html

obtain and transfer PBs, as these are already distinct entities contained within their own cell membranes. In addition, the data show that levels of mtDNA carryover expected with PB1 and PB2 are likely to be consistently very low.

2.2.4 Similarities and differences between PB1T and MST and between PB2T and PNT

Conceptually, PBT1 is very similar to MST in that it is the nuclear genome product of MI (a diploid complement of chromosomes) that is transferred to a recipient (donor's) oocyte that has had its own removed, except that the nuclear DNA is derived from PB1 rather than from the oocyte itself. All other (downstream) aspects are identical. If used in treatment, the oocyte would be fertilised by sperm from the patient's partner, the second meiotic division would be completed (extruding PB2), and the resulting embryo would have a unique nuclear genetic contribution from both the patient and her partner as in normal reproduction. The mtDNA would be derived mostly from the donor oocyte, as with MST, except the proportion of carryover of mutant mtDNA with PB1 may be much lower than with the karyoplast containing the maternal spindle from the patient's oocyte.

Conceptually, PBT2 is similar to PNT, in that PB2 is a product of the second meiotic division as is the maternal pronucleus. The only difference is that, as described, PNT involves replacing both the maternal and paternal pronuclei, whereas with PB2T, only the maternal pronucleus of the donor zygote is replaced by PB2. In principle, the same could be done for PNT. However, this does mean that the donor oocyte must be fertilised by sperm from the patient's partner. All other (downstream) aspects are identical. Again, there would be a unique nuclear genetic contribution from both the patient and her partner as in normal reproduction, and the mtDNA would be derived mostly from the donor oocyte, as with PNT, except the proportion of carryover of mutant mtDNA along with PB2 may be lower than with the pronuclei from the patient's fertilised oocyte.

2.2.5 Safety and efficacy: possible issues novel to PBT

Most of the concerns about the potential use of either PBT1 or PBT2 will be common to those for MST and PNT; however, a few specific questions could be asked:

- (i) Is the genomic DNA in either PB1 or PB2 marked or imprinted in some way that differs from that in the oocyte? After all, the PBs are fated to die within a few days after they are generated during meiosis. As discussed, there is no evidence for such genetic or epigenetic abnormalities. This includes data from Hou et al (2013) and Wang et al (2014) who found no evidence for DNA damage in PB1 or PB2. Moreover, Wang et al (2014) specifically examined

various chromatin marks and found no difference between PB1 and the oocyte chromosomes or between PB2 and maternal pronuclear chromosomes. This was performed by immunostaining, which does not reveal the distribution of such marks along the chromosomes, information that would be very difficult to obtain. However, the results were consistent with equal treatment of the chromatids/chromosomes, irrespective of their PB-versus-oocyte fate during meiosis, by the chromatin modifiers present in the oocyte. Indeed, if the genomic DNA in either PB1 or PB2 was marked or imprinted in some way that differs from that in the oocyte it is highly unlikely that the PBT experiments in mice would have given normal live-born animals.

- (ii) Are there critical issues of timing, either because the PBs do not survive for long, or because they have to be synchronised with the recipient oocyte or zygote, notably with respect to spindle formation, DNA synthesis, and DNA or chromatin modification? It would clearly make sense to use the PBs for transfer soon after their formation (once chromosome segregation is complete) to minimise any chance of degradation, although the evidence for PB2 is that these can survive for several days. With respect to synchronisation, the evidence from Wakayama et al (1997) for PB2T suggested that success rates were higher if the recipient zygote was “younger”. Ideally the removal of the maternal pronucleus and its replacement by PB2 should be carried out soon after PB formation to allow DNA synthesis to be completed in time for the first cleavage division, however, not so soon that there is any risk of incomplete chromosome segregation. It should be noted that timing is also an issue for PNT, as mentioned in our third review of scientific methods to avoid mitochondrial disease. Timing is less of an issue for either PB1T or MST because the oocytes are blocked in MII waiting for fertilisation (or activation). As shown by Wang et al (2011) with PB1T in pig oocytes, freezing the PBs from the patient’s oocyte or zygote is also an option, and might make synchronisation easier⁸.
- (iii) With PB1T, the spindle is removed and has to reform with the chromatids from the introduced PB and function correctly to allow completion of MII and proper segregation of haploid sets of maternal chromosomes to PB2 and the zygote after fertilisation.

⁸ It is noted in earlier scientific reviews that it is possible to cryopreserve spindle and pronuclear karyoplasts and it was recommended that this be explored further for reasons of synchronisation between patient and donor, and to separate oocyte collection and reimplantation for the former.

This is clearly not a problem in mice, but it would need to be tested in human oocytes.

- (iv) For PBT2, can the maternal and paternal pronuclei be distinguished reliably within the donor (recipient) zygote such that it is the former that is removed and replaced by PB2 from the patient's zygote? If a mistake were made, then this would result in a parthenogenetic embryo (strictly speaking this would be defined as a "gynogenetic" embryo as it would have maternal genome contributions from two different women). Such embryos do not survive much beyond implantation. However, although it is relatively easy to distinguish the maternal and paternal pronuclei in mice, robust evidence for reliable distinction would have to be demonstrated in human zygotes.

2.2.6 Safety and efficacy: possible advantages of PBT

- (i) Technically, it may be simpler and safer to carry out PB1T or PB2T than MST and PNT, as the latter require treating the patient's oocyte or zygote with cytoskeletal inhibitors and extracting karyoplasts with a micropipette, where the nuclear DNA is contained within a portion of the cell membrane together with a small amount of cytoplasm. PBs are naturally formed karyoplasts, which can be removed simply with a micropipette and fused or injected into the recipient oocyte or zygote. PB biopsy is a technical procedure performed routinely by several centres offering PGD and PGS (Harton et al, 2011).
- (ii) Carryover of mtDNA from the patient's oocyte or zygote may be lower with PBT1 and PBT2 (especially the former) compared to MST and PNT. In addition to determining mtDNA numbers in isolated human PBs, this would need to be verified in human oocytes and zygotes generated after PBT, by determining, using sensitive methods, mtDNA copy numbers in embryos produced as a result of mitochondria replacement techniques.

For both (i) and (ii) the procedures should be compared carefully to explore whether these possible advantages are genuine or not, as the degree of damage to oocytes and carryover of mtDNA may be operator-dependent.

- (iii) As discussed and shown by Wang et al (2014), it should be possible to carry out both PB1T and MST with each oocyte from a patient into two donor (recipient) oocytes, or both PBT2 and PNT with each zygote from a patient into two donor (recipient) zygotes. This would effectively double the chance of success for each cycle of treatment undergone by the patient, since unlike reproductive

cloning, the nuclear genomes of the PBs are just as much products of meiosis as that of the oocyte and maternal pronucleus. Thus, should two children be born from the same oocyte or zygote they would have quite distinct genetic contributions from the patient, in addition to the distinct genetic contributions from the father that are provided by different sperm (as in non-identical twins).

- (iv) To avoid having to synchronise the patient and oocyte donor, cryopreservation of PBs may be easier than freezing oocytes, fertilised oocytes or karyoplasts. There may be some benefit for the patient in keeping her ovarian stimulation for oocyte collection distinct from “priming” her uterus for implantation and pregnancy.
- (v) The panel is aware of the extensive literature on the purported abnormalities in PB1 and PB2 found during aneuploidy testing, and that these abnormalities may be reflected in the oocyte, but it should be noted that this literature predominantly refers to older women and/or those with reproductive problems. There is no inherent reason to expect the risk of aneuploidy in PBs to be any different from that in oocytes (spindles or pronuclei).

2.3 Possible additional methods of avoiding mitochondrial disease

In addition to PBT the panel considered a number of other methods to avoid mitochondrial disease. Maternal pronuclear transfer is another form of PNT that would involve replacing only the maternal pronucleus of a zygote, not with PB2 but with a different (the patient’s) maternal pronucleus. This could be an option if the donor oocyte is fertilised by sperm from the patient’s partner. The advantage of this technique over transfer of both the maternal and paternal pronuclei is that the volume of cytoplasm, and therefore copies of mutant mtDNA accompanying one pronucleus, would be less than when transferring two pronuclei.

However, as is the case for PB2T, there would need to be a robust mechanism for distinguishing the maternal from the paternal pronucleus (e.g. time-lapse imaging). If the maternal and paternal pronuclei were misidentified, either in the patient or donor zygote, this could result in a gynogenetic embryo, which would not develop much beyond implantation; however, in the case of two paternal pronuclei (androgenetic) there is a risk of development into a hydatidiform mole (Jacobs et al, 1980)⁹.

⁹ In rare cases the panel noted that in maternal pronuclear transfer, the male pronuclei could, through human error, be swapped by accident resulting in a zygote generated from the nuclear genomes of the male partner and the oocyte donor. This event would require misidentification of the pronuclei twice in a row and would be mitigated by ensuring a robust process for identifying

The panel is also aware of additional methodologies to avoid mitochondrial disease that are based on targeted destruction of mutant mtDNA. These would involve using mitochondrially-targeted restriction enzymes or other DNA-editing/cutting methods (based on Zinc fingers, Talens or CrispR/Cas9) to specifically degrade mutant mtDNA, leaving normal mtDNA intact, with the aim of reducing levels of heteroplasmy (relevant studies are referenced at Annex B). The panel is aware that these methods have been conducted with some success in cell lines in vitro and that they are being attempted in mice¹⁰; however, the panel has not explored the safety and efficacy of these techniques in any detail because they involve alteration of mitochondrial DNA sequences and there are no proposals to permit their clinical use. Such approaches are very different to the techniques we discuss here and in previous reports, which involve transfer of entire, intact genomes that are the natural product of meiosis.

The panel also noted that, unrelated to mitochondrial disease, cryopreservation of PBs followed by transfer into an enucleated oocyte could be used for female fertility preservation (as suggested by Wakayama et al, 1997), although at present this would not be permitted under UK law.

the correct pronuclei. More generally, human error can occur whilst performing other in vitro techniques and hence this particular methodology is not unique in this respect.

¹⁰These methods are unlikely to be of use in cases of homoplasmy or where there is a high level of heteroplasmy for the mutant mtDNA, because there would either be no, or too few copies of, normal mtDNA for the resulting embryos to be viable. On the other hand, they could in theory be used in conjunction with MST, PNT or PBT with the aim of eliminating any carryover of mutant mtDNA.

3. Recommendations and further research

At each previous review, the panel has reached a view that the evidence it has seen does not suggest that maternal spindle transfer (MST) and pronuclear transfer (PNT) techniques are unsafe. On review of the evidence to date the panel extends this view to polar body transfer (PBT), while noting that PBT is novel and research in this area is in the early stages and should be monitored.

The panel recommends that additional studies be undertaken both in the basic research field to improve understanding of the biology of human mitochondria especially during development, and on translational research aimed specifically at providing further safety and efficacy information on PBT.

The panel suggest that, in order to show that PBT is safe and efficacious, further work should be carried out using normal human oocytes subjected to PBT and the embryos and embryonic stem (ES) cell lines derived from them, to explore whether they develop normally and have minimal carryover of mtDNA from the polar bodies. Further to this, exploration of nuclear-mitochondrial interactions and long lasting epigenetic modifications should be conducted and an examination of methods to prevent premature activation of oocytes or detect abnormally fertilised oocytes.

However, as stated in previous reports, complete reassurance will never come solely from experiments conducted in animal models or even with human material in vitro. Therefore, it should be accepted that there will always be some risk and unknowns associated with the use of any of the methods of mitochondrial replacement, including PBT, in humans until they are tried in practice, and that this should only be as part of regulated clinical use.

The panel therefore recommends the following (minimum) set of experiments to be undertaken and the results taken into account before PBT techniques can be assessed to be safe to use clinically:

- Polar body 1 transfer (PB1T) using human oocytes that are then fertilised (not activated), and comparative follow up of development in vitro. *This could include a molecular karyotype analysis of PB2T.*
- Polar body 2 transfer (PB2T) using normally fertilised human oocytes, from which the maternal pronucleus has been removed, and development compared to normal ICSI-fertilised human oocytes. *The panel highlighted the importance of demonstrating a robust method for distinguishing the maternal and paternal pronuclei such that the maternal pronucleus can be reliably selected for removal.*

In addition, the panel considers that:

- PBT in a non-human primate model, with the demonstration that the offspring derived are normal, is neither critical nor mandatory.

Studies should be carried out on:

- Mosaicism in human morulae (comparing individual blastomeres) and on human 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 the use of any mitochondrial replacement technique using oocytes or zygotes with two different variants of mtDNA.

The panel also recommends the following additional research to provide useful information on mitochondrial disease and PBT techniques:

- Karyotype analysis and comparative genomic hybridisation/copy number variation arrays of embryos derived from PBT (taking into consideration variation of karyotypes in polar bodies). In addition it would be useful to conduct analysis on PB2 following PB1T.
- Detailed analysis of epigenetic modifications and gene expression, with a range of markers for blastocyst cell types in embryos derived from PBT. Comparative examination for epigenetic variation between PB1/ PB2 and oocyte and any embryos created through PBT.
- PBT on unfertilised human oocytes that have abnormal mtDNA. *However, the panel recognises that these studies may be difficult (practically) to conduct, and as stated previously considers that the scientific justification for this does not outweigh the ethical concerns about performing such experiments. Comparative studies exploring carryover between the various techniques (MST, PNT, PB1T and PB2T) would be practically impossible and even more ethically contentious. This would also be unnecessary. Only one method should be sufficient to explore whether abnormal mtDNA has any (replicative) advantage after mitochondrial replacement.*
- As an alternative method for analysing the behaviour of mutant mtDNA, the use of induced pluripotent stem (iPS) cells derived from patients carrying different mtDNA mutations. *Any information gained would apply to all methods of mitochondrial replacement and is not specifically relevant to PBT.*
- As with other forms of mitochondria replacement techniques, studies on the mtDNA carryover in a non-human primate model into the possible heteroplasmy of tissues in the fetus would be advantageous. The possibility of carryover of even a small percentage of abnormal mtDNA

means that any females born from PBT should be considered at risk of transmitting the disease to their offspring. *This recommendation applies to all methods of mitochondrial replacement and is not specifically relevant to PBT.*

- Further studies on vitrifying oocytes, polar bodies and zygotes in order to allow synchronisation when carrying out PBT, as well as clinical management of patients.
- Tests for heteroplasmy should be carried out on primordial germ cells obtained from human ES cells derived from blastocysts created through PBT 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. *This applies to all methods of mitochondrial replacement and is not specifically relevant to PBT.*

Compared with both PNT and MST, it is clear that PBT is at an earlier stage of development, with little or no human data publicly available for the methods; however, the panel noted that progress in this area is rapid, and conclude that PBT does not introduce different principles to those of MST or PNT and the resulting embryos will be equivalent to those derived by MST and PNT.

Annex A: Methodology of the review

1. The HFEA was asked by the Government, in July 2014 to seek views of members of the panel on the safety and efficacy of the new PBT technique and provide a report by October.
2. In order to compile this report, the HFEA sought views of members of the panel¹¹ that provided the June 2014 review 'Third scientific review of the safety and efficacy of methods to avoid mitochondrial disease through assisted conception: 2014 update', which identified, collated and summarised relevant research and suggested additional experts to consult.
3. Panel members for this addendum are as follows:
 - Dr Andy Greenfield, Medical Research Council (MRC) Harwell and HFEA member
 - Professor Peter Braude, King's College London
 - Professor Robin Lovell Badge, MRC National Institute for Medical Research

A new panel member was appointed to advise on the addendum to ensure that specific cytogenetic and clinical molecular genetic expertise was provided:

- Professor Caroline Ogilvie, King's College London and Guy's & St Thomas' NHS Foundation Trust

Further to this, previous panel members were contacted and wider expert advice was sought to gather further evidence and to clarify issues.

4. Members met twice: on 26 August and 16 September to discuss drafting of the report, and discuss evidence in more detail with identified experts and researchers.

¹¹Panel members were selected for their broad-ranging scientific and clinical expertise, and for having no direct interests in the outcome of the review.

Annex B: Evidence reviewed

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Annex C: Summary of recommendations for further research

Essential research: PNT and MST (June 2014)	Essential research: PBT (September 2014)
<p>MST using human oocytes that are then fertilised (not activated).</p> <p><i>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.</i></p>	<p>(PB1T) using human oocytes that are then fertilised (not activated), and comparative follow up of development in vitro.</p> <p><i>This could include a molecular karyotype analysis of PB2T.</i></p>
<p>PNT using normally-fertilised human oocytes and development compared to normal ICSI-fertilised human oocytes.</p> <p><i>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.</i></p>	<p>PB2T using normally fertilised human oocytes, from which the maternal pronucleus has been removed, and development compared to normal ICSI-fertilised human oocytes.</p> <p><i>The panel highlighted the importance of demonstrating a robust method for distinguishing the maternal and paternal pronuclei, such that the maternal pronucleus can be reliably selected for removal.</i></p>
Desirable research: PNT and MST (June 2014)	Desirable research: PBT (September 2014)
<p>Studies on mosaicism in human morulae (comparing individual blastomeres) and on human 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 to demonstrate the degree of heteroplasmic mosaicism in morulae, and to provide data to address whether there was</p>	<p>Studies should be carried out on: mosaicism in human morulae (comparing individual blastomeres) and on human 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 the use of any mitochondrial replacement technique using oocytes or zygotes with two different variants of mtDNA.</p>

<p>any amplification of mtDNA carried over.</p> <p><i>The panel continues to recommend this is carried out.</i></p>	
<p>Removing the spindle or pronuclei and replacing them back into the same oocyte/zygote to better identify the impact of the manipulation technique.</p> <p><i>As in 2013, given the successful development to blastocyst stages after both MST and PNT with human oocytes and zygotes, the panel no longer considers this to be necessary.</i></p>	<p>Given the successful development to blastocyst stages after both MST and PNT with human oocytes and zygotes, the panel does not consider this to be necessary for PBT.</p>
<p>Karyotype analysis and comparative genomic hybridisation/copy number variation arrays of embryos derived from MST or PNT.</p> <p><i>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.</i></p>	<p>Karyotype analysis and comparative genomic hybridisation/copy number variation arrays of embryos derived from PBT (taking into consideration variation of karyotypes in polar bodies). In addition it would be useful to conduct analysis on PB2 following PB1T.</p>
<p>Detailed analysis of epigenetic modifications and gene expression, with a range of markers for blastocyst cell types in embryos derived from MST or PNT.</p> <p><i>This has been carried out for MST (further studies on mtDNA carryover have now been conducted in the Macaque model, as outlined above), and similar experiments on PNT-derived embryos are ongoing, but the panel continues to recommend these are completed.</i></p>	<p>Detailed analysis of epigenetic modifications and gene expression, with a range of markers for blastocyst cell types in embryos derived from PBT. Comparative examination for epigenetic variation between PB1/ PB2 and oocyte and any embryos created through PBT.</p>

MST on unfertilised human oocytes that have abnormal mtDNA and PNT on fertilised oocytes that have abnormal mtDNA.

The panel considers that the scientific justification for this does not outweigh the ethical concerns about performing such experiments. Whilst it might be argued that it is useful to perform such a study, especially if any evidence arises to suggest a specific mtDNA mutation may have a replicative advantage, the panel recognises that it may be impractical to obtain sufficient numbers of oocytes or zygotes with mutant mtDNA for research.

PBT on unfertilised human oocytes that have abnormal mtDNA.

However, the panel recognises that these studies may be difficult (practically) to conduct, and as stated previously considers that the scientific justification for this does not outweigh the ethical concerns about performing such experiments. Comparative studies exploring carryover between the various techniques (MST, PNT, PB1T and PB2T) would be practically impossible and even more ethically contentious. This would also be unnecessary. Only one method should be sufficient to explore whether abnormal mtDNA has any (replicative) advantage after mitochondrial replacement.

As an alternative method for analysing the behaviour of mutant mtDNA, the use of induced pluripotent stem (iPS) cells derived from patients carrying different mtDNA mutations.

The panel continues to recommend this is carried out.

As an alternative method for analysing the behaviour of mutant mtDNA, the use of induced pluripotent stem (iPS) cells derived from patients carrying different mtDNA mutations.

Any information gained would apply to all methods of mitochondrial replacement and is not specifically relevant to PBT.

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 or 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 were considered in the 2013 review notably by Lee et al (2012,) in the Macaque. On the basis of these however the panel recommends that further

As with other forms of MT, studies on the mtDNA carryover in a non-human primate model into the possible heteroplasmy of tissues in the fetus would be advantageous. The possibility of carryover of even a small percentage of abnormal mtDNA means that any females born from PBT should be considered at risk of transmitting the disease to their offspring.

This recommendation applies to all methods of mitochondrial replacement and is not specifically relevant to PBT.

<p><i>experiments are carried out to address this issue with human material. This recommendation still stands.</i></p>	
<p>Further studies on vitrifying oocytes, karyoplasts, and zygotes in order to allow synchronisation when carrying out MST and PNT, as well as clinical management of patients.</p> <p><i>The panel continues to recommend this, recognising that advances in cryopreservation are being made generally within the context of ART.</i></p>	<p>Further studies on vitrifying oocytes, polar bodies and zygotes in order to allow synchronisation when carrying out PBT, as well as clinical management of patients.</p>
<p>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.</p> <p><i>This recommendation still stands.</i></p>	<p>Tests for heteroplasmy should be carried out on primordial germ cells obtained from human ES cells derived from blastocysts created through PBT 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.</p> <p><i>The recommendation still stands – it applies to all methods of mitochondrial replacement and is not specifically relevant to PBT.</i></p>

Annex D: Glossary of terms

Term	Definition
Activate	To artificially trigger the processes that occur when an oocyte is fertilised
Adenosine triphosphate (ATP)	Molecule that transports energy in cells. It is involved in reactions of the electron transfer chain (ETC)
Anaphase	The stage of mitosis or meiosis when the sister chromatids move to opposite poles of the cell.
Apoptosis	Programmed cell death
Biopsy	Procedure of obtaining cells (e.g. from an embryo), in order to test them
Bivalent chromosome	A pair of homologous chromosomes held together by a process known as synapsis, following chromosome replication
Blastocyst	A 5-6 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
Carryover	The proportion of mitochondria that is carried over from the original oocyte or zygote during MST, PNT or PBT
Centriole	Cylindrical cell structure composed mainly of a protein called tubulin. An associated pair of these centrioles, surrounded by an amorphous mass of material, contributes to a structure called a centrosome
Centrosome	An organelle in the cytoplasm that organises microtubules (that maintain the structure of the cell and are involved in the formation of mitotic spindles – which pull apart chromosomes in cell division) and serves as a regulator of cell-cycle progression

Chromatid	A chromatid is one copy of a duplicated chromosome, which is generally joined to the other copy by a single centromere
Chromatin	The combination of DNA and other proteins in the nucleus, which packages the chromosomes
Chromosome	Structure in a nucleus that carries DNA
Comparative genomic hybridisation array (aCGH)	Screening method to detect abnormal chromosomes
Cytokinesis	The process in which the cytoplasm of a single cell is divided to form two daughter cells, following nuclear division
Cytoplasm	The jelly-like substance that fills a cell and contains the structures within it, including mitochondria
Cytoskeleton	A network of protein fibres contained within a cell's cytoplasm. Parts of the cytoskeleton are constantly destroyed, renewed or newly constructed
Diandric	Where triploidy occurs and the extra haploid set is from the father
Digynic	Where triploidy occurs and the extra haploid set is from the mother
Diploid	A cell or nucleus containing two complete sets of chromosomes (one paternal; one maternal)
Electron transfer chain (ETC)	The chain of chemical reactions that produce energy in a mitochondrion
Embryo transfer	Transferring embryo(s) into the uterus of a woman during a cycle of treatment
Embryonic stem (ES) cells	Cells, derived from an embryo and cultured in a laboratory, that have the potential to form all the different cell/tissue types in an animal or human and can replicate themselves indefinitely

Enucleated oocyte	An oocyte where the nucleus has been removed
Epigenetic	Relating to or arising from non-genetic influences on gene expression. Can result in inherited phenotypes through alteration of gene expression.
Eukaryotes	An organism consisting of a cell(s) in which the genetic material is DNA in the form of chromosomes contained within a distinct nucleus
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 result in a particular phenotype)
Germ cell	Any cell that will give rise to sperm or oocyte cells
Germ line	The sequence of cells that give rise to sperm or oocyte cells that will pass genetic information on to a child
Glycolysis	One of the main mechanisms by which cells use to generate energy in the form of ATP. This can occur in anaerobic conditions, however, it is less efficient than OXPHOS.
Haploid	A cell or nucleus containing a single set of unpaired chromosomes
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
Hydatidiform mole	A tumour of placental tissue that develops within the uterus following implantation of a zygote that has an excess of paternally-derived genetic material.

Induced pluripotent stem (iPS) cells	Adult cells that have been reprogrammed to act like embryonic stem cells
Karyoplast	A structure made up of the nuclear genetic material, surrounded by a small amount of cytoplasm, enclosed in a cell membrane, used to transfer a spindle or pronuclei during MST or PNT experiments
Karyotype	The number and appearance of chromosomes in a cell
Maternal spindle transfer (MST)	Technique to transfer the nuclear genetic material from a woman's oocyte into a donated oocyte with its nuclear genetic material removed
Meiosis	Type of cell division necessary for sexual reproduction
Microfilaments	Thin protein structures that contribute to the 'skeleton' and to the dynamics (movement) of the cytoplasm
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
Mitochondrial DNA (mtDNA)	The genetic material contained within the mitochondria
Mosaicism	When cells within the same individual have a different genetic makeup
Mutation	A permanent, heritable change in the DNA sequence
Nuclear DNA	The majority of the cell's genetic information, which is contained in the nucleus of the cell
Oocyte	An egg
Ooplasm	Cytoplasm contained in an oocyte

Organelle	Small structure within a cell
Oxidative phosphorylation	The reaction that produces ATP to generate energy in the cell
Parthenogenetic embryo	The fertilisation of an oocyte in the absence of a sperm-derived paternal pronucleus
Polar body	One of the small cells produced during the two meiotic divisions in the maturation of an oocyte. The first usually released just before ovulation and the second during the process of fertilisation
Polar body transfer	Techniques involving transfer of a mother's nuclear genetic material (taken from the polar body) from an oocyte into a donor oocyte with its nuclear genetic material removed (PB1T) or from a fertilised oocyte (zygote) to a donor zygote with the female pronucleus removed (PB2T).
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 genetic disease
Primordial germ cell	A specialised cell, formed at early stages of development, that is destined to give rise to oocyte or sperm cells
Pronuclei	The independent nuclei derived from the sperm and oocyte 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 a couple's nuclear genetic material from a fertilised oocyte (zygote) into an donor zygote with its nuclear genetic material removed
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
Sequence	Method for determining the order (sequence) of chemicals that make up DNA in an individual
Somatic cell	Any cell of an embryo or adult that is not a germ line cell
Spindle	A structure in a cell, made up of microtubules, that move the chromosomes when the cell divides
Triploid	A term used to describe a nucleus, cell or organism that carries three homologous sets of chromosomes
Vitrify	To freeze (an oocyte or embryo) by plunging into liquid nitrogen
Zygote	A fertilised oocyte
