Mitochondrial DNA disease: new options for prevention

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Very recently, two papers have presented intriguing data suggesting that prevention of transmission of human mitochondrial DNA (mtDNA) disease is possible. [Craven, L., Tuppen, H.A., Greggains, G.D., Harbottle, S.J., Murphy, J.L., Cree, L.M., Murdoch, A.P., Chinnery, P.F., Taylor, R.W., Lightowlers, R.N. et al. (2010) Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. Nature, 465, 82–85. Tachibana, M., Sparman, M., Sritanaudomchai, H., Ma, H., Clepper, L., Woodward, J., Li, Y., Ramsey, C., Kolotushkina, O. and Mitalipov, S. (2009) Mitochondrial gene replacement in primate offspring and embryonic stem cells. Nature, 461, 367–372.] These recent advances raise hopes for families with mtDNA disease; however, the successful translational of these techniques to clinical practice will require further research to test for safety and to maximize efficacy. Furthermore, in the UK, amendment to the current legislation will be required. Here, we discuss the clinical and scientific background, studies we believe are important to establish safety and efficacy of the techniques and some of the potential concerns about the use of these approaches.

MITOCHONDRIAL GENETICS AND DISEASE

Mitochondrial DNA (mtDNA) is the only constitutive extra chromosomal DNA within human cells (1). It is a small genome (16.6 kb) with a very limited number of genes. All 37 genes (13 protein encoding and 24 RNA genes) are involved with the synthesis of proteins which form crucial subunits of the mitochondrial respiratory chain. The mitochondrial genome is also present in multiple copies in all cells. Defects of the mitochondrial genome take the form of point mutations and deletions, and many hundreds of different mutations associated with disease have been described. These mtDNA mutations may be either homoplasmic, in which all copies are mutated, or heteroplasmic, with a mixture of mutated and wild-type mtDNA present. The majority of mtDNA mutations described are heteroplasmic and in patients there is a clear link between the levels of mutated mtDNA versus wild-type mtDNA; patients with large amounts of mutated mtDNA (associated with lower amount of wild-type) have the most severe symptoms (2).

The clinical features of mtDNA disease are very variable and relate in part to the severity and amount of the mtDNA mutation (3). The clinical features usually affect tissues in which there is a high metabolic demand such as central nervous system, skeletal muscle or heart. However, other tissues are frequently involved such as the β-cells of the pancreas leading to diabetes, the inner hair cells of the cochlear causing deafness or the renal tubules leading to kidney dysfunction. There are a number of well-defined clinical syndromes but the majority of patients do not fall into easily defined clinical groups, and definition by a genotype is often helpful (4,5).

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The incidence of mtDNA disease is not known but there have been a number of epidemiological studies either looking for the presence of specific mutations or the incidence of mtDNA disease within a limited population. The frequency of pathogenic mtDNA mutations in the population is high (~1 in 200 people) (6–8). The majority of these individuals will not be affected by disease because the mtDNA mutation is present at low levels of heteroplasmia or mutation detected is only pathogenic in the presence of a specific insult. The other epidemiological approach which focuses solely on known cases is inevitably an underestimate but suggests that the incidence of mtDNA disease is at least 1 in 10,000 affected with many more at risk (9).

mtDNA in mammals is strictly maternally inherited with all homoplasmic mtDNA mutations transmitted to offspring (2). The transmission of heteroplasmic mtDNA mutations is complicated by both a selection process and a genetic bottleneck present during development of the female germline. Mice expressing an error-prone mtDNA polymerase provide an opportunity to study the changes able to pass though the bottleneck and reveal a very strong selection against mutations in the protein-encoding genes of mtDNA, which were eliminated rapidly (10). There is also marked variation between the offspring of heteroplasmic mothers due to an extreme reduction in mtDNA copy number during germ cell development and/or selective genome replication (11–13).

TREATMENT OF MTDNA DISEASE

Unfortunately, advances in treatment for patients with mtDNA disease have not advanced at the same pace as the ability to establish a diagnosis. For the vast majority of patients with mtDNA disease, therapy is limited to symptomatic relief and early detection of treatable symptoms such as epilepsy, cardiac disease and diabetes. There have been limited numbers of clinical trials despite numerous reports of studies in the literature (14). However, a recent randomized study has shown that idebenone, a ubiquinone analogue, is helpful in patients with Leber’s Hereditary Optic Neuropathy (Chinnery, P.F. et al., unpublished data). Evidence from animal models and from clinical studies indicates that increasing mitochondrial biogenesis by exercise and drugs also holds promise (15,16). Such treatments are most likely to be effective in those patients with milder mtDNA disease, where small increases in wild-type mtDNA will improve function. More fundamental treatments to either remove mutated mtDNA by specifically targeting restriction endonucleases (17) or inhibiting replication of mutated mtDNA (18) have either worked in vitro or in animal models, but are some way from use in patients. Other approaches of bypassing the biochemical defect using alternative oxidases, for example, also hold promise for the future (19).

CURRENT REPRODUCTIVE OPTIONS FOR WOMEN CARRYING MTDNA MUTATION

There are now a number of options available (20). Genetic counselling is important to explain risks involved, but must be given carefully, taking account of the specific mutation and the number of affected family members. More recently, pre-natal and pre-implantation genetic diagnoses have become an option particularly relevant to those mothers carrying mutations which segregate widely between offspring such as the m.8993T>C NARP mutation. With both techniques, there is a concern that cells taken for genetic analysis may not reflect the level of heteroplasmia in the embryo or fetus. However, recent studies suggest that, at least during early stages of development, there is relatively little variation in heteroplasmia between cells (21). Nonetheless, for women with homoplasmic or high levels of heteroplastic mtDNA mutations, the only option at present to ensure an unaffected child is oocyte donation. These oocytes tend to be in short supply and have the limitation of not being genetically related to the mother.

PREVENTING TRANSMISSION OF MTDNA DISEASE

In view of the lack of curative treatment and the extremely disabling phenotype in many patients, preventing transmission of mtDNA defects is an important goal for families. One approach to achieving this is to transplant the nuclear genome from the oocyte or embryo of an affected woman to those from a healthy donor (Fig. 1). This has the potential to enable women to have a genetically related child without transmitting mutated mtDNA. Early experiments in mouse, using fertilized eggs (1 cell zygote), indicated that transplantation of the male and female pronuclei between zygotes was compatible with onward development and birth of normal offspring (22,23). It was later demonstrated that pronuclear transfer is effective in preventing transmission of an mtDNA rearrangement in a mouse model (24). More recently, proof of principle experiments have been conducted using abnormally fertilized human zygotes (25) (Fig. 2). A related technique involving transplantation of the metaphase II spindle between unfertilized oocytes (Fig. 1) has resulted in live born rhesus monkeys (26).

The efficacy of these techniques in preventing transmission of mtDNA disease is highly dependent on minimizing the transfer of mitochondria during transplantation of the nuclear genome. Both metaphase II spindle transfer and pronuclear transfer involve fusion of a karyoplast containing the nuclear material surrounded by a small amount of cytoplasm, which may well contain mitochondria. Thus, there is the potential for transmission of the affected mitochondria to the offspring. Techniques employed to minimize the size of the karyoplast have been successful in limiting this transmission such that there is no detectable carryover of mtDNA or very low levels detected in the developing embryo (≤2%). In the presence of heteroplasmy, there is a threshold of mutated to wild-type mtDNA which must be reached before clinical features are observed. In the vast majority of transmitted mtDNA mutations, this threshold is high and thus low levels of mutated mtDNA transferred during nuclear transplantation are highly unlikely to generate a phenotype. There is a slight risk that the mutated genotype may segregate to specific tissues, but marked tissue-specific segregation of mtDNA mutations seems to be confined to mutations which are
rarely passed through the germline (such as large-scale mtDNA deletions) and thus unlikely to be a major concern.

It will also be important to determine whether the manipulations associated with nuclear genome transplantation are detrimental to embryo development. While the wealth of evidence from mouse studies indicates that pronuclear transfer is compatible with normal development, evidence from human studies indicates a 50% reduction in the proportion of embryos developing to the blastocyst stage (25). However, it should be noted that the human studies were conducted on abnormally fertilized zygotes containing either one or three pronuclei, which show considerable variation in chromosome constitution and have reduced developmental capacity. While the experimental design included restoration of diploidy, evidence from studies on human oocytes indicate that chromosome scattering can occur. This has also been reported in human oocytes from older women (28) and in response to exposure to ambient conditions (29). Therefore, it will be important to perform the manipulations in tightly controlled environmental conditions and to develop techniques to minimize the risk of inducing aneuploidy during transplantation of the metaphase II spindle.

Unlike pronuclear transfer, the chromosomes of the metaphase II oocyte are not enclosed within a nuclear membrane. Instead, they are attached to the spindle, which can be visualized by polarized light birefringence. However, it is not possible to see the chromosomes without the use of a fluorescent dye, which may affect onward development. While correctly aligned chromosomes are positioned on the spindle equator, evidence from studies on human oocytes indicate that chromosome scattering can occur. This has also been reported in human oocytes from older women (28) and in response to exposure to ambient conditions (29). Therefore, it will be important to perform the manipulations in tightly controlled environmental conditions and to develop techniques to minimize the risk of inducing aneuploidy during transplantation of the metaphase II spindle.

While pronuclear transfer offers the advantage of having the maternal and paternal genomes enclosed in clearly visible pronuclei, the ability to faithfully transmit chromosomes to daughter cells during division to the two-cell stage depends on the assembly of a normal bipolar spindle during the first mitosis. It will therefore be important to ensure that reconstituted zygotes contain only two centrioles, which in humans are derived from the sperm and are tightly apposed to the pronuclear membrane (30). Thus, in reducing the size of the karyoplast to minimize mtDNA carryover, it will be important to ensure that there is sufficient cytoplasm to include the centrioles and, possibly other spindle assembly components, in the karyoplast.

Comparison between metaphase II spindle transfer and pronuclear transfer

These two techniques have separately been shown to be technically feasible and lead to low levels of carryover mtDNA, but the question of which is the better technique for patients with mtDNA disease remains unresolved. Metaphase II spindle transfer is performed at an earlier stage and the karyoplast is smaller than with pronuclear transfer, with the potential for less carryover of mtDNA. However, recent studies in human embryos show equivalent levels of carryover (unpublished data), providing further evidence that the risks of offspring developing disease are likely to be minimal.

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Figure 2. Pronuclei removal from an abnormally fertilized human embryo and transfer to an enucleated abnormally fertilized human embryo. (A) The tripronucleate human embryo (two pronuclei can be seen, arrowheads). A hole has been made in the zona pellucida using a microsurgical laser (arrow). (B) The biopsy pipette is inserted into the embryo and a pronucleus aspirated within a karyoplast (arrowhead). (C) The pronuclear karyoplast can be seen within the biopsy pipette (arrowhead). (D) A single pronuclear karyoplast removed from the embryo. (E) The pronuclear karyoplasts can be seen within the biopsy pipette (arrowheads). (F) The biopsy pipette is inserted through the hole in the zona pellucida. (G) The pronuclear karyoplasts are transferred into the enucleated embryo along with a small amount of HVJ-E. (H) The pronuclear karyoplasts (arrowheads) are allowed to fuse with the enucleated embryo.
Nuclear transplantation also requires the use of reversible cytoskeletal inhibitors, the effect of which has not been rigorously tested in human oocytes or zygotes. Metaphase II spindle transfer requires inhibition of the actin cytoskeleton only, whereas pronuclear transfer requires inhibition of actin and tubulin polymerization. While both treatments are reversible, further studies are required to determine whether they have a detrimental effect on subsequent embryonic development.

In addition to the potential impact of the manipulations on embryo development, logistical considerations will also have an impact on the choice between metaphase II spindle transfer and pronuclear transfer. For example, clinical application will involve ovarian stimulation of the oocyte donor and the affected woman. However, due to biological variation in response to ovarian stimulation, it will not be possible to guarantee that both the donor and the recipient will be ready for oocyte collection on the same day. It may therefore be necessary to cryopreserve oocytes or zygotes until nuclear genome transplantation can be performed. Thus, the efficacy of cryopreservation and/or vitrification techniques at the different stages of development will also need to be considered. In this regard, cryopreservation of zygotes is a long-established procedure in assisted conception; however, vitrification techniques for the successful storage of metaphase II human oocytes are a relatively recent development in clinical assisted reproduction (31).

POTENTIAL DISADVANTAGES OF NUCLEAR GENOME TRANSPANTATION TECHNIQUES TO PREVENT TRANSMISSION OF MTDNA DISEASE

In addition to the risk of aneuploidy and other effects of the technical procedures, concern has been raised about the implications of possible incompatibilities between the nuclear genotype of the parents and the donor mitochondrial genomes. The potential biological significance of this stems from the fact that the majority of proteins involved in mitochondrial metabolism are encoded by the nuclear genome (32,33). However, in the vast majority of non-consanguineous births, the paternal nuclear genome comes from a different mitochondrial background. As there is no evidence of parental imprinting of nuclear-encoded mitochondrial proteins, it is assumed that paternally encoded genes contribute to mitochondrial function. Thus, in the process of fertilization, biology appears to make provision for ‘incompatibilities’ between the nuclear and mitochondrial genomes. This may be explained by the relative sequence similarity between different mitochondrial genotypes. For example, only a small number of amino acid substitutions (ranging from just a few to 20) are observed when two human mtDNAs are compared. It is also worth noting that the largest incompatibility between nuclear and mitochondrial backgrounds will be between people with heritages rooted in different continents and we are not aware of any evidence of increased mitochondrial disease in children with mixed heritage.

Assisted reproduction techniques, particularly invasive techniques such as intracytoplasmic sperm injection, have been reported to be associated with an increased incidence of epigenetic abnormalities such as Angelman syndrome and Beckwith–Wiedemann syndrome (34). The question of whether the manipulations associated with nuclear genome transplantation might induce epigenetic anomalies remains to be resolved. One study in mouse following transfer of the female pronucleus showed transcriptional repression and methylation of liver proteins associated with impaired growth (35). However, a recent report describing intra-strain maternal pronuclear transfers, metaphase-II spindle transfers and ooplasms transfers between C57BL/6 and DBA/2 mice found no major, long-term growth defects or epigenetic abnormalities, in either males or females, associated with intergenotype transfers (36). Furthermore, ongoing studies of the rhesus monkey offspring born following metaphase-II spindle transfer (26) indicate that they are growing normally (unpublished data).

FURTHER STUDIES REQUIRED TO EVALUATE SAFETY AND EFFICACY ON NUCLEAR TRANSFER TECHNIQUES

Legislation relating to embryo research varies considerably between different countries—from an outright ban on all research to no legislation at all. The UK has been at the vanguard of permissive legislation combined with strict regulation of research in this area; however, translation to clinical treatment to prevent the transmission of mtDNA disease will require that the UK Parliament introduces ‘regulations’ so that an embryo is considered a ‘permitted’ embryo if it has undergone manipulation using material provided by two women. A ‘permitted’ embryo could then be transferred to the uterus for fertility treatment. As a first step, the Secretary of State for Health recently commissioned the Human Fertilisation and Embryology Authority (HFEA) to convene an expert scientific panel to review methods to prevent mitochondrial disease. The expert panel has now completed their report (http://www.hfea.gov.uk/6372.html). This report states, ‘Although optimistic about the potential of these techniques, the panel recommends a cautious approach and advises that this research is carried out, and the results taken into account, before the techniques can be considered safe and effective for clinical use’. In other countries that regulate human embryo research, there is very little legislation that refers specifically to nuclear transfer techniques to prevent transmission of mtDNA disease. However, other countries are considering these issues and, for example, a legislative review of the law related to embryo research is being undertaken in Australia and recommendations have been made to allow nuclear genome transfer between embryos for mitochondrial studies (The Australian Academy of Science, 2011). Further detail of legislation in different countries is available in a recent background paper prepared for the Nuffield Council on Bioethics www.nuffieldbioethics.org/ Germline_therapies_background_paper.pdf.

All nuclear genome transplantation studies so far have either used animal (including non-human primate) oocytes or zygotes or abnormally fertilized human zygotes. To assess safety and efficacy, it will be important to perform a systematic analysis of metaphase-II spindle transfer and pronuclear transfer in normal human oocytes and zygotes. This should include
optimizing the manipulations to limit the size of the karyoplast and the fusion techniques between the karyoplast and the recipient oocyte/zygote. In addition, it will be important to optimize vitrification and cryopreservation procedures in order to minimize loss of oocyte/zygote viability during cryostorage and subsequent transplantation of the nuclear genome. The efficacy of the two techniques should also be evaluated by studying embryo development, and epigenetic and genome stability after karyoplast transfer. Finally, since the goal is to minimize mtDNA carryover during karyoplast transfer, this will have to be evaluated as well as establishing whether residual karyoplast-associated mtDNA partitions equally between cells during embryo development.

CONCLUSIONS

This is an exciting time in terms of research to provide treatments and prevent disease transmission for patients with mtDNA disease. It is important that the field moves from simply documenting new diseases and mutations to effectively helping patients with new therapeutic approaches. Most recent progress has been made in the area of disease prevention but further work in this area is essential before such interventions are made available to patients. However, whilst these studies are ongoing, it is crucial that there is continued debate about the ethical and regulatory issues surrounding these innovative IVF-based techniques so that any advances can rapidly be converted to improved patient care.

Conflict of Interest statement. None declared.

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