Review

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Mitochondrial replacement therapy in reproductive medicine

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Mitochondrial dysfunction is implicated in disease and age-related infertility. Mitochondrial replacement therapies (MRT) in oocytes or zygotes, such as pronuclear (PNT), spindle (ST), or polar body (PBT) transfer, could prevent second-generation transmission of mitochondrial DNA (mtDNA) defects. PNT, associated with high levels of mtDNA carryover in mice but low levels in human embryos, carries ethical issues secondary to donor embryo destruction. ST, developed in primates, supports normal development to adults and low mtDNA carryover. PBT in mice, coupled with PN or ST, may increase the yield of reconstructed embryos with low mtDNA carryover. MRT also offers replacement of the deficient cytoplasm in oocytes from older patients, with the expectation of high pregnancy rates following *in vitro* fertilization.

MtDNA and its role in pathologies

Mitochondria are cytoplasmic organelles with their own genome that have a major role in energy generation by oxidative phosphorylation (OXPHOS; see Glossary). The enzymatic machinery involved in OXPHOS requires both nuclear and mtDNA participation and, while the latter encodes only 37 genes, there are large numbers of mitochondria per cell, particularly in the oocyte. MtDNA in eukaryotes derives evolutionarily from bacteria and may have roles far beyond those described here [1]. Mutations in mtDNA, either alone or in conjunction with certain nuclear DNA mutations, can result in serious disorders that are often difficult to diagnose and for which there are currently no cures. The frequency of either inherited or acquired (somatic) mtDNA mutation is surprisingly high, reflecting vulnerability to replication errors and susceptibility to damaging reactive molecules confounded by limited DNA repair mechanisms. Diseases caused by mtDNA mutations were first described in 1988 [2–5]. Since then, over 700 mutations, both germline and somatic, have been identified, some of which have been associated with human disorders, including myopathies, neurodegenerative diseases, diabetes, cancer, and infertility (http://www.mitomap.org/bin/view.pl/MITOMAP/WebHome).

Glossary

ATP: an important biological molecule that contains high-energy chemical bonds. Energy, for metabolic purposes, is released when one or more of the bonds in ATP are broken, yielding AMP or ADP.
Blastocyst: a young, spherically shaped embryo typically formed 5–6 days after fertilization, and before implantation. The blastocyst contains an outer layer of cells called the trophectoderm and an inner cluster called the inner cell mass (ICM).
Cumulus cells: an extensive, adherent cluster of cells surrounding the ovulated oocyte. These cells have a nurturing role during the maturation process, but are shed during oocyte and/or embryo transport via the oviduct.
Cytoplast: cell membrane enclosed substance of the cell excluding the nucleus formed after the cell enucleation or spindle removal procedure.
Germline: the line of cells terminating in gametes (eggs and sperm) used by sexually reproducing organisms to transmit their genes to offspring. Mutations in germ cell DNA pass from generation to generation.
Heteroplasmy: a term used to describe the mixture of two or more different mitochondrial genomes within a cell. The ratio of mutated to healthy mtDNA determines the severity of mtDNA-based diseases.
***In vitro* fertilization (IVF):** a process in which the egg is fertilized by sperm outside the body. Embryos produced by this technique are used in the treatment of families with fertility issues.
Intracytoplasmic sperm injection (ICSI): a method of fertilizing an oocyte by direct injection, ensuring sperm incorporation and avoiding multiple sperm penetration.
Karyoplast: a cell spindle or nucleus, along with a small amount of cytoplasm, enclosed by cell membrane created during the nuclear transfer procedure.
Meiotic spindle: the spindle-shaped structure of the oocyte containing the nuclear genome and comprising microtubules attached to chromosomes. Spindles are important for chromosome alignment and segregation during reductive divisions.
Metaphase II oocyte: an egg arrested at the metaphase stage of meiosis, typically representative of a mature oocyte collected after ovarian stimulation during IVF.
Mitochondrial carryover: mutant or patient mitochondria (and, hence, mtDNA) that are transferred with a karyoplast during MRT. The number of mtDNA molecules carried over is a function of the amount of cytoplasm and the density of mitochondria in the spindle or pronuclear region.
Mitochondrial DNA (mtDNA) mismatch: detrimental interaction between the donor mitochondrial and patient nuclear genomes after MRT due to sequence differences in mtDNA.
Oxidative phosphorylation (OXPHOS): the transfer of electrons through several protein complexes that occurs inside mitochondria supporting the production, storage, and release of chemical energy in the form of ATP.
Parthenogenesis: the development of embryos from unfertilized eggs. In mammals, parthenogenesis can be induced artificially and, following diploidization, can result in development of preimplantation stage embryos.
Polar bodies: small cells containing DNA formed during meiotic, reductive divisions of the oocyte. PB1 is formed before fertilization and can be visualized readily in the ovulated oocyte, while PB2 is formed after fertilization and can be seen in the zygote. Both polar bodies eventually disintegrate.
Preimplantation genetic diagnosis (PGD): a reproductive technique used in the diagnosis of genetic abnormalities in early embryos. Typically, one or two cells (blastomeres) from an eight-cell embryo (3-days old) are removed for testing and only normal embryos at 5-days of age are transferred to the patient.
Pronuclei: membrane-enclosed entities in the zygote that house the male and female chromosomal contributions. Their fusion completes fertilization and immediately precedes the mitotic division that results in the two-cell embryo
Zygote: the one-cell embryo formed soon after fertilization containing two pronuclei, one from the egg and one from the sperm.

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Box 1. Mitochondrial DNA heteroplasmy threshold for disease

Mitochondrial production of ATP by OXPHOS occurs in almost every cell in the body and the number of mitochondria per cell varies between cell types, depending on their energy requirements. Therefore, deficits in mitochondrial function are likely to be experienced differently throughout the body, with the potential for multitissue and/or organ involvement. This leads to a complex situation when cause and effect relations are sought. In heteroplasmic mtDNA disease, there are differences in threshold levels not only between tissues in the same carrier, but also between siblings in an affected family or between families affected with the same mutation. Consequently, it is impossible to establish a single, uniform threshold for disease and its transmission. However, in the monkey and human studies summarized here, the mtDNA carryover levels following MRT were consistently at or below 2%, a value almost certainly below the threshold for disease. For an extensive review of these issues, see [2]. In the context of initial clinical trials for MRT, patient selection could be restricted to families carrying homoplasmic or high heteroplasmy mtDNA mutations, who have already given birth to an affected child with early disease onset.

Transmission of germline mtDNA mutations that potentially cause disease in the next generation has been reported at a frequency of 1 in 200 in newborns [6]. Symptoms can begin at any age and severity of disease relates to the specific mutation and its penetrance, the percentage of mtDNA with the mutation per cell (the heteroplasmy level), modulation by nuclear genome background, and other parameters (Box 1). Second-generation transmission of mtDNA-based disease can be circumvented, at least in preclinical animal and human *in vitro* studies, predicated on the knowledge that mtDNA is exclusively inherited from the egg (i.e., maternally), and that safe, efficient MRTs are in place. MRTs intervene at the oocyte or one-cell embryonic stage (zygote) and are accomplished by extracting the nuclear DNA from the patient's egg or embryo, leaving behind cytoplasm (cytoplast) with mutated mtDNA, followed by transplantation into a donor cytoplast containing wild type mtDNA and cytoplasm.

Techniques for MRT

Alternatives to germline gene therapy have been described for couples at risk of transmitting mtDNA-based disorders, including prenatal and preimplantation genetic diagnosis (PGD). Although potentially useful for low heteroplasmic conditions, these alternatives are inappropriate for homoplasmic conditions where the patient mutant load is 100% [7]. Novel approaches for circumventing mtDNA-based disease transmission that involve germline gene therapy are described here, including PNT, ST, and PBT.

PNT

The zygote stage in mammals is characterized by the presence of two pronuclei (PN), each clearly visible and containing a haploid chromosomal complement of nuclear DNA from either sperm or oocyte (Figure 1). Transfer of both PN from one zygote to another was first accomplished during the early 1980s, demonstrating that manipulated mouse zygotes could develop into live offspring [8]. More recently, PNT in the mouse has been used to model MRT [9]. However, the efficacy of PNT in mice has been adversely affected by high mtDNA carryover levels in the pups, at approximately 24% [10–12]. This is due to the inevitable cotransfer of a small amount of cytoplasm containing mitochondria and mtDNA (Figure 1). As an example, in the most recent report [12], an average heteroplasmy level of 24% was associated with biopsied tissues from seven pups and a similar level was sustained in the F2 generation. This could reflect the large size of PN and uneven mitochondrial distribution [13,14]. Thus, isolation of PN, even if encapsulated in small karyoplasts, may result in the cotransfer of unacceptable numbers of mitochondria**.** Although most common inherited human mtDNA diseases are typically associated with high mutated mtDNA thresholds (Box 1) [15], these results in mice do not bode well for MRT in humans. Nevertheless, the feasibility of PNT in humans was reported in 2010 using abnormal zygotes with either one or more than two PN, which are normally discarded during routine *in vitro* fertilization (IVF) [16]. Zygotes containing 2PN were created by transfer of one PN from a poly PN zygote into a 1PN zygote. Given that male and female PN in the human zygote cannot readily be differentiated by visual observation, only half of the reconstructed zygotes would contain both a male and female PN. Reconstructed zygotes (n = 36) were then placed in culture and eight developed beyond the eight-cell stage. Three, or 8%, of these embryos developed to the blastocyst stage with low mtDNA carryover (<2%). Despite the low yield of blastocysts, the authors concluded that this approach carries potential for MRT. However, even with this proof of principle, it is currently impossible to evaluate the safety and efficacy of PNT in normal human zygotes based on this preliminary report, and further investigation in nonhuman primates is warranted before reaching a final conclusion on PNT viability as a MRT technique in humans.

ST

In 2009, ST was pioneered in the rhesus monkey, demonstrating that MRT could be accomplished at the unfertilized oocyte stage (Figure 2) where efficacy and safety was demonstrated by live births, normal growth curves of offspring, and low levels of mtDNA carryover [17,18]. A step-by-step technical description of the ST protocol has been published [19,20], supporting independent replication and verification [12,21]. Briefly, Chinese and Indian-origin rhesus females carrying different wild type mtDNA haplotypes were identified based on sequence polymorphism. Unlike zygotes, the distribution of mitochondria in oocytes is uniform, allowing ST without significant mtDNA carryover from the nuclear donor oocyte. Meiotic spindles surrounded by a small volume of cytoplasm and membrane were extracted into karyoplasts containing only 1.5% the volume of cytoplasts. Karyoplasts from Chinese-origin rhesus macaques were then fused to donor cytoplasts from Indian-origin macaques and vice versa to produce reconstructed oocytes with donor mtDNA. Fertilization by intracytoplasmic sperm injection (ICSI) was followed by embryo culture to the blastocyst stage**.** Blastocyst development and quality was comparable to controls, and two ESC lines were established from eight ST embryos; a derivation efficiency similar to controls. Fifteen ST blastocysts were transferred into nine females, resulting in three pregnancies and four live births. These efficiencies