—Review—

Challenges towards establishing germline gene therapy for inherited mitochondrial diseases

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Abstract: Mitochondrial DNA (mtDNA) mutation is associated with serious human disorders and affects multiple organs and tissues with high-energy requirements. Since the transmission of mtDNA is complex and is not fully understood, an accurate estimation of mtDNA disease transmission by preimplantation genetic diagnosis (PGD) or by prenatal diagnosis (PND) remains challenging. Recently, nuclear transfer techniques, including maternal spindle transfer (MST), pronuclear transfer (PNT) and polar body transfer (PBT), have shown the promising results. These methods avoid the transmission of mutated mtDNA from mother to offspring, and are collectively known as the mitochondrial replacement therapy (MRT). Further, the United Kingdom Parliament approved the Human Fertilisation and Embryology Authority (HFEA) to grant licenses for experimental use of MST and PNT in humans in 2015. Thus, a new era of assisted reproductive technology (ART), in which cures can be provided at the gamete or early zygote stages, is realistically approaching. In this review, we summarize the methods and the challenges confronting the clinical application of MRT.

Key words: Mitochondrial diseases, Mitochondrial replacement therapy (MRT), Maternal spindle transfer (MST), Pronuclear transfer (PNT)

Introduction

Mitochondria are the cytoplasmic organelles responsible for the synthesis of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS) which provides an energy supply for the cell [1]. Mitochondrial functions and replication are controlled by both their own genome (mitochondrial DNA; mtDNA) and the nuclear genome. MtDNA encodes 37 genes (13 genes for polypeptides, 22 genes for transfer RNAs, and 2 genes for ribosomal RNAs) that are crucial for the production of cellular energy and programmed cell death (apoptosis) [2].

Mutations in mtDNA and nuclear DNA related to mitochondrial functions lead to mitochondrial diseases and disorders, including myopathies, neurodegenerative diseases, diabetes, cancer and infertility [2]. MtDNA mutations have two conditions: heteroplasmy, which is the co-existence of two or more types of mtDNA haplotype within the cell (i.e. as a mixture of wild-type and mutated mtDNA); and homoplasmy, which is a single type of mtDNA (i.e. wild-type or mutated) within the cell. Most inherited mitochondrial diseases correspond to the heteroplasmic condition, and the clinical manifestation and severity of mitochondrial diseases vary depending on the types of pathogenic mutation and the proportion of mutated mtDNA (i.e. degree of heteroplasmy). In the homoplasmic mtDNA mutation, diseases and disorders are absolutely passed from mother to child, whereas heteroplasmy also allows the lethal mutation to persist and be passed on to the next generation. The estimated minimum prevalence of mitochondrial diseases due to well-defined pathogenic mutations of mtDNA or nuclear genome is at least 1 in 5,000 live births, and could be much higher [3, 4]. Unfortunately, in Japan, there are no precise prevalence data derived from epidemiological study. Unlike the nuclear genome that is Mendelian inheritance, mtDNA inheritance is exclusively maternal and is only passed down from mother to child through the egg with no recombination [5, 6]. This unique feature of mitochondrial inheritance has been motivating researchers to develop new assisted reproductive techniques. The ultimate goal is to eliminate the transmission of faulty mtDNA by diluting or replacing defective cytoplasm with healthy cytoplasm containing healthy mtDNA.

The supplementation of donor cytoplasm to patient's

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eggs was introduced as cytoplasmic transfer (CT) in the late 1990's [7]. This approach was designed to improve the viability and developmental ability of potentially compromised patients' oocytes by adding a small amount (5–15%) of donor cytoplasm containing healthy organelles including mitochondria. Initial attempts of CT by electrofusion were not successful due to abnormal fertilization and compromised embryo development [8]. However, CT as an extension of intracytoplasmic sperm injection (ICSI) involving co-injection of a small amount of donor cytoplasm with sperm has been more successful [7, 9, 10]. This approach was extended to treat patients in several fertility clinics and purportedly improved IVF outcomes [7, 9]. However, it is not clear what defect is corrected by this technique, and moreover the active components of the transferred cytoplasm have not been identified. Thus, CT is not suitable for women carrying mutations since it would require transferring significantly larger amounts of cytoplasm to ensure adequate dilution of the mutated mtDNA. Further, a relatively high number of chromosomal abnormalities and birth defects have been reported in infants resulting from the initial application of CT [11]. Hence, this procedure was banned in the United States by the Food and Drug Administration (FDA) in 2001.

While the reproductive options mentioned above are currently unavailable, preimplantation genetic diagnosis (PGD) or prenatal diagnosis (PND) has been applied to identify embryos or foetuses which are devoid of risks of mitochondrial disease. However, some concerns remain over whether the diagnosis made with biopsied samples reflects the whole load of abnormal mtDNA in the embryo or foetus due to unknown mechanisms, such as the "mitochondrial bottleneck effect" and/or "selective replication" [12–14]. Therefore, PGD is not universally accepted to all types of mtDNA mutation and is only applicable to patients with heteroplasmic mutations that fulfills, (i) a close correlation between the mutation load and disease severity, (ii) a uniform distribution of mutant mtDNA in all blastomeres, and (iii) no change in mutant load over time (both prenatally and postnatally) [15]. Further, the risks to the next generation cannot be eliminated by PGD, unless the resulting baby is male. Unfortunately, PND including chorionic villus sampling and amniocentesis may be less effective at predicting mtDNA diseases than PGD. PND samples are taken from only a part of the external embryonic tissue which is a lower proportion of whole embryonic tissue than in PGD. On the other hand, oocyte donation or adoption is entirely different and is the only way to avoid the transmission of mitochondrial diseases to offspring from affected mothers. However, this method has the limitation of parental heredity.

At present, there are no widely approved fundamental cures for mitochondrial diseases, and the treatments currently available only alleviate symptoms and slow disease progression. Therefore, affected families have been seeking, and awaiting, promising assisted reproductive options, which could prevent transmission of mtDNA mutation to their children. Recently, a novel method, mitochondrial replacement treatment (MRT) has been proposed and has demonstrated promising results [16, 17]. MRT enables the maintenance of patient parental nuclear DNA heredity while eliminating the transmission of unbidden mutated mtDNA to offspring. Therefore, MRT presents a favorable balance of risks and benefits compared to the alternative methods mentioned above, especially in cases of homoplasmy or high levels of heteroplasmy. In this review, we summarize the methods of MRT, including maternal spindle transfer (MST), pronuclear transfer (PNT), germinal vesicle transfer (GVT), and polar body transfer (PBT). Furthermore, we also describe the ethical and technical problems, the global situation regarding MRT, and future research agendas for upcoming clinical application of MRT.

Maternal Spindle Transfer (MST)

In 2009, Tachibana *et al.* reported maternal spindle transfer (MST), also referred to as spindle-chromosomal complex transfer (ST), as a novel and revolutionary approach to avoid the transmission of mitochondrial diseases [18]. The MST procedure takes place in mature metaphase II (MII) oocytes in which MII spindle-chromosomal complex enclosed in karyoplast is isolated and transferred to an enucleated (spindle free) MII oocyte originated from a different female (Fig. 1A).

Transfer of the nuclear material in MII oocytes offers many advantages over other approaches. While GVT requires *in vitro* maturation (IVM) prior to fertilization, oocytes reconstructed by MST are ready for fertilization. MST may be ethically more acceptable than PNT in which cytoplasts are supplied from fertilized zygotes. Thus, PNT requires the destruction of normally fertilized zygotes, not gametes. Regarding technical issues, primate and human MII spindle-chromosomal complex is relatively smaller than that of GV and PN stage interphase nuclei. Further, MII spindle is devoid of mitochondria [18]. Thus, the transplantation of MII spindle-chromosomal complex would be suitable to reduce the carryover of unbidden mtDNA contained within the karyoplast that is transferred to the cytoplast of donor egg. Despite known theoretical advantages, technical obstacles have ham-

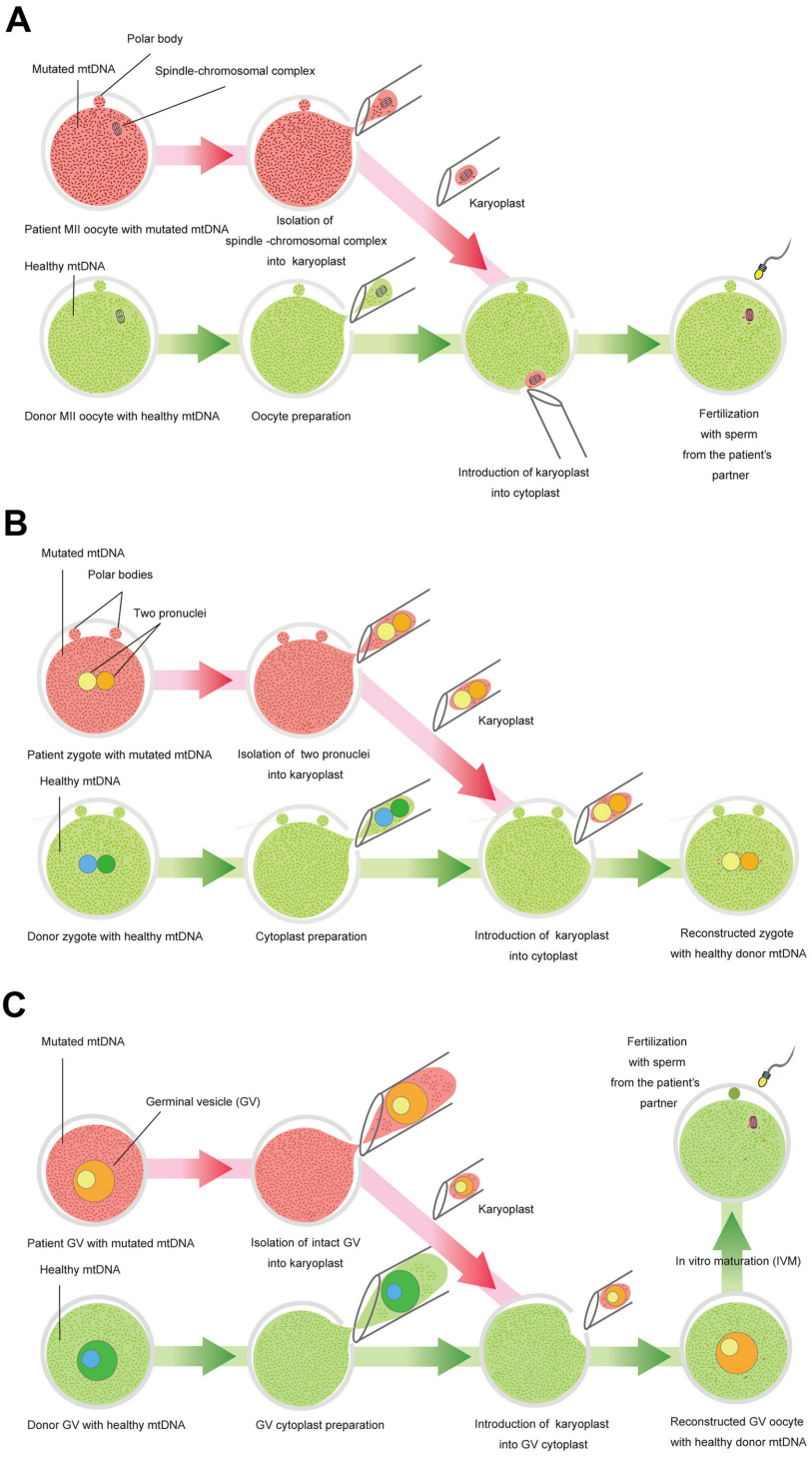

Fig. 1. Schematic representation of the MST, PNT and GVT procedures. Fig. 1A depicts the MST procedure. An isolated MII spindle enclosed in karyoplast from a patient egg is transferred into an enucleated (spindle-free) MII cytoplast from a healthy donor. The reconstructed oocyte is then fertilized with partner's sperm by intracytoplasmic sperm injection (ICSI). Fig. 1B depicts the PNT procedure. An isolated 2PN from a patient zygote is transferred into a comparable stage of pronuclei (PN)-free zygote stage cytoplast from healthy donors. Fig. 1C depicts the GVT procedure. An isolated intact GV from a GV stage oocyte from a patient is transferred into a GV-free cytoplast from a healthy donor. The reconstructed GV oocyte is subjected to *in vitro* maturation (IVM) and the matured MII oocyte is subsequently fertilized by ICSI. MST: maternal spindle transfer; PNT: pronuclear transfer; GVT: germinal vesicle transfer. *The figures in *Trends Mol Med; 21:68, 2015* was used as a reference.

pered the feasibility and success of the MST for many years. These include i) difficulties with visualization, ii) lack of an efficient technique for the isolation of intact MII spindle-chromosomal complex, and iii) the susceptibility of meiotic spindles and chromosomes to damage secondary to premature oocyte activation and resumption of meiosis during the manipulation. Indeed, initial attempts in which fusion of the karyoplast and cytoplast was induced by methods, such as electrofusion or chemicals (e.g. polyethylene glycol: PEG), were unsuccessful due to excessively low efficiency and abnormal fertilization. However, researchers have solved the issues concerning MII oocyte manipulation, and the key to this success was the implementation of several technical modifications addressing the above-mentioned obstacles, such as i) a non-invasive spindle visualization system, ii) laser objective, and especially, iii) the use of inactivated Sendai virus envelope (hemagglutinating virus of Japan-envelope;HVJ-E) for karyoplast and cytoplast fusion [18, 19].

A schematic description of the actual MST procedure is shown in Fig. 1A. A reconstructed oocyte is fertilized with partner's sperm by intracytoplasmic sperm injection (ICSI). In the initial attempts with rhesus macaque oocytes, MST was performed using mature MII oocytes of Chinese and Indian origin carrying different wild-type of mtDNA haplotypes, and the reconstructed oocytes developed to the blastocyst stage. The rate of blastocyst formation, and the number of total cells and the inner cell mass cells (ICMs) were comparable to controls, and 2 novel embryonic stem cell (ESC) lines were established from 8 MST blastocysts. The derivation efficacy (25%) was similar to controls with no detectable chromosomal anomalies. Fifteen MST blastocysts were transferred into 9 recipient females, resulting in 3 pregnancies and 4 live births [18]. Longitudinal studies of the MST infants have found that they have body weights, blood chemistries, mtDNA carryover heteroplasmy analysis results, ATP levels and membrane potentials in skin fibroblasts which are within normal ranges [18, 20]. The carryover of mtDNA from spindle donor (i.e. degree of heteroplasmy) both in MST infants and in derived ESC lines have been investigated using various methods, such as PCR-RFLP, subcloning technique and quantitative PCR (qPCR), with results of undetectable or below 3% heteroplasmy [18]. Karyoplast fusion was induced by HVJ-E in that study but the clinical application of a viral extract could be of concern. However, reverse transcription (RT)-PCR for the common F protein-coding sequence revealed HVJ-E viral genomic contamination in neither MST infants nor ES cell lines [18]. The detailed technical protocols were also reported [19].

The MST procedure requires that both patient and donor undergo synchronous egg retrievals. This may be a problem in clinical settings. Thus, the feasibility of using cryopreserved oocytes for MST was demonstrated using rhesus monkeys [20]. In that study, reciprocal MST in fresh and frozen-thawed oocytes was examined. Fertilization (88%) and blastocyst formation (68%) rates were similar to those of fresh controls when vitrified spindles were transferred into fresh cytoplasm. In contrast, the developmental competence of reconstructed oocytes with fresh spindle transferred into vitrified cytoplasm was compromised. Two ESC lines were established from 6 blastocysts, and 4 MST blastocysts were transferred into recipient females resulting in a healthy female infant [21]. Thus, patient eggs can be vitrified prior to commencing MST procedure, and the cytoplast of donor eggs need to be freshly available on the day of a MST procedure in a clinical setting. MST also offers the possibility of rescue of fertility in recurrent IVF failure due to oocyte deterioration caused by cytoplasmic dysfunction.

The results cited above were worthy of further investigation, and the feasibility, efficacy and safety of MST in humans were subsequently studied with oocytes from healthy volunteers harboring discernible SNP in mtDNA [20]. Among 106 human oocytes donated for research, 65 oocytes were subjected to reciprocal MST and 33 oocytes served as a control. The fertilization rate of MST oocytes (73%) was similar to controls (75%). However, approximately half of MST zygotes (52%) showed abnormal fertilization, primarily because of excessive pronuclear numbers. This was considered to have been the result of premature activation during manipulation leading to incomplete resumption of meiosis after fertilization. Among the normally fertilized MST zygotes, blastocyst formation (62%) and ESC line derivation rates (38%) were comparable to those of controls. Despite the risk of abnormal pronuclear formation in a portion of the MST zygotes, all ESC lines derived from 2PN/2PB MST zygotes had a normal euploid karyotype and inherited their mtDNA exclusively from the cytoplast of the donor eggs. Based on this result, it is estimated that retrieving on average 12 MII oocytes would be desirable to have at least 2 MST blastocysts suitable for transfer in a single cycle for each patient [20].

MST has been successfully replicated and independently verified using human oocytes by Paull *et al.* [21]. Although their oocytes were parthenogenically activated, reconstructed MST oocytes resulted in blastocysts which subsequently yielded stable ESC lines. The carryover mtDNA level of some parthenogenetic embryos was be-

low 1% in the early stage embryos, and subsequently decreased in blastocysts, eventually dropping to nearly undetectable levels in ESC lines [21]. The results are consistent with the conclusion that MST can be accomplished in humans with minimal risk of unbidden mtDNA carryover.

Pronuclear Transfer (PNT)

The PNT procedure takes place in zygote-stage embryos from a patient and donors, sperm donor and oocyte donor, where embryo possesses two distinctive visible pronuclei (PN) that contain haploid DNA from either sperm or oocyte. A schematic diagram of the PNT procedure is shown in Fig. 1B. The 2PN of the patient zygote enclosed in the karyoplast is removed and transplanted to an enucleated comparable stage zygote cytoplast derived from donors containing healthy mitochondria. The membrane fusion between 2PN and cytoplast is induced by either electric pulses or by HVJ-E.

The success of PNT was first reported in manipulated mouse zygotes that developed into live offspring in 1983 [22]. However, PNT has shown low efficacy of mitochondrial replacement in mice, and the carryover rate of mtDNA detected in pups is in the range of 19–35% [14, 23, 24]. At the zygote stage, mitochondria are accumulated as a conglomeration around the pronuclei [25, 26]. Thus, the PNT procedure may result in co-transfer of mitochondria with abnormal mtDNA even if only the 2PN encapsulated in a small karyoplast is isolated. Despite these concerns, Craven *et al.* reported the success of PNT using abnormally fertilized (unipronuclear or tripronuclear) human zygotes in 2010 [27]. Of 36 reconstructed zygotes, 3 embryos (8.3%) developed to the blastocyst stage, and the average mtDNA carryover was 2% for the proportion of mtDNA genotype variation among blastomeres. It is difficult to assess the efficacy and safety of PNT in normal human zygotes referring only to this report, because the reconstructed embryos were obtained from abnormally fertilized zygotes, and blastocyst development was extremely inefficient. Further investigations using normally fertilized embryos in non-human primates and/or humans are needed to evaluate the authentic potential of PNT in clinical use.

Germinal Vesicle Transfer (GVT)

GVT is essentially the same procedure as PNT, except that the nuclear material is removed and transferred in GV stage oocytes (Fig. 1C). The initial rationale of GVT is to rescue age-related aneuploidy caused by compromised cytoplasm. The GVT technique has been demonstrated in mice and humans indicating that it is technically feasible to transfer DNA between immature oocytes in which nuclear DNA is enclosed in a clearly visible germinal vesicle (GV) [28, 29]. This particular stage of oocyte requires the *in vitro* maturation (IVM) to the MII oocytes, and this procedure requires the removal of cumulus cells, which is thought to be critical for the success of IVM [30]. If this approach were applied to human oocytes, its efficacy would be limited by the poor developmental competence of the oocytes produced after IVM of GV-intact oocytes. Moreover, GV oocytes have a polarized cytoarchitecture with mitochondria concentrated in the perinuclear space surrounding GV [31]. Thus, transplantation of GVs would inevitably result in significant amounts of patient mtDNA in the reconstructed cytoplasm. In addition, there is the possibility that, due to its initial proximity to the nucleus, mutant mtDNA would be preferentially replicated in the reconstructed embryo [32]. Therefore, the GVT procedure carries the risk of introducing significant amounts of mtDNA carryover with karyoplast, and thus, GVT may not be a suitable for eliminating the transmission of mutated mtDNA.

Polar Body Transfer (PBT)

The mammalian oocytes undergoing meiotic division sequentially extrude two small polar bodies (PBs): the first polar body (PB1) which has a diploid set of chromosomes extruded after ovulation; and the second polar body (PB2) which has a haploid set of chromosomes extruded from a MII oocyte after fertilization or oocyte activation. Transplantation of PB1 and PB2 into an appropriate oocyte or zygote cytoplasm has resulted in live mice (Fig. 2A and B) [33, 34]. Moreover, the genome analyses of PB1 and PB2, using multiple annealing and a looping-based application cycle (MALBAC) based on sequencing technology, have revealed that PB1 and PB2 accurately possess the same settings of the genome in the oocyte pronuclei [35].

In 2014, Wang *et al.* reported PBT as a potential MRT in mice [36]. PBT contains PB1 transfer (PB1T) and PB2 transfer (PB2T) (Fig. 2 A and B). Their study demonstrated that the replacement of mtDNA is more effective in PBT than that of other MRT techniques, such as MST and PNT, and the mtDNA genotype remains stable in the F2 generation. Wang *et al.* stated the advantage of PBT is that PBs contain very few cellular organelles, including mitochondria with easily visualized cellular membrane, which facilitates micromanipulation. In addition, the efficient utilization of both PB1 and spindle-chromosomal

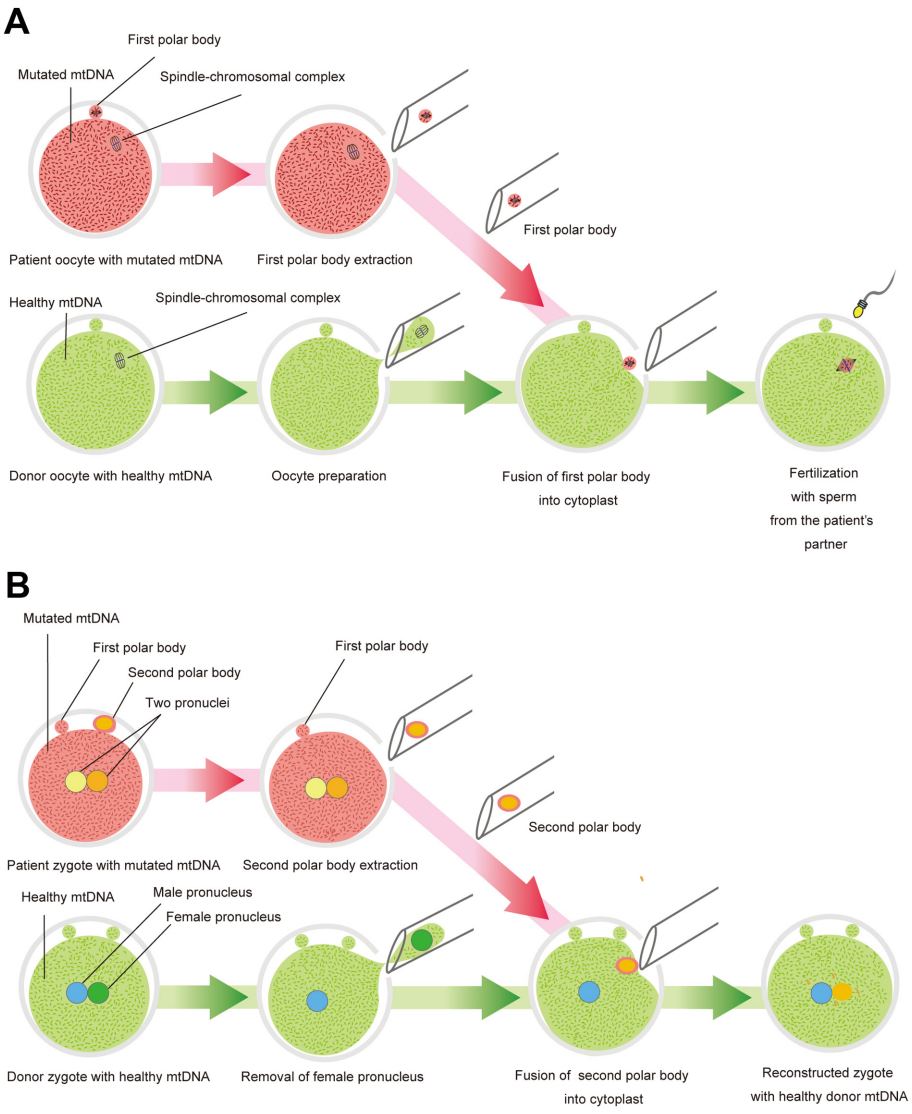

Fig. 2. Schematic representation of the PBT procedures. Fig. 2A depicts polar body 1 transfer (PB1T). An isolated PB1 containing dyad (2n) chromosomes from a patient oocyte is transferred into an enucleated MII cytoplast from a healthy donor. The reconstructed oocyte is then fertilized with partner's sperm by ICSI. Fig. 2B depicts polar body 2 transfer (PB2T). An isolated PB2 containing chromatids (n) from a patient zygote is transferred into a zygote stage cytoplast from healthy donors from which the female pronucleus has been removed. *The figures in *Trends Mol Med; 21:68, 2015* was used as a reference.

complex from a single patient oocyte, or PB2 and 2PN from a single patient zygote, can be utilized for the combination of "MST and PB1T" or "PNT and PB2T", respectively, and this was also considered as an advantage. *In vitro* development of PB1T, MST, PB2T and PNT embryos to the blastocyst stage were 87.5%, 85.7%, 55.5% and 81.3%, respectively. MtDNA heteroplasmy analysis of brain, heart, lungs, liver and kidneys from F1 pups revealed 0% for PB1T, 0–6.88% for MST, 3.08–3.62% for PB2T, 5.55–39.8% for PNT. In addition, mtDNA heteroplasmy analyses of F2 pups gave the following results: 0% for PB1T, 7.1 ± 6.8% for MST, 2.9 ± 4.3% for PB2T, and 22.1 ± 18.7% for PNT. Wang *et al.* concluded that PBT, especially PB1T, is appropriate for MRT.

Nevertheless, there are unresolved issues in PBT, such as errors in the segregation of chromosomes at meiosis, the best timing for transfer, since mammalian PBs have a short lifetime due to apoptotic pressures that

lead to DNA fragmentation or degradation, and the precise distinction from the maternal pronucleus in PB2T (http://www.hfea.gov.uk/9357.html). Importantly, the genetic composition of PB1 and PB2 is not identical to the MII chromosome and female pronucleus because of the meiotic recombination [37]. Furthermore, PBT has not been successfully replicated in other mammalian species including primates and humans. Future studies with non-human primates and humans are warranted to confirm the feasibility, efficacy and safety of PBT before the commencement of clinical trials.

The Future Research Agenda for Clinical Applications of MRT

It is important to validate each approach regarding the feasibility, safety and efficacy using non-human animal models, in order to determine the best approach for the clinical application of MRT. Thus, future research directions and agendas are described below.

The degree of mtDNA carryover by MRT is the most important concern for safety. In most mitochondrial diseases, a threshold of 60% or higher mutated mtDNA is required for clinical features to appear. In this regard, both MST and PNT would be sufficient for preventing diseases in offspring, because the mtDNA heteroplasmy level was undetectable or below 3% in monkeys and human MST embryos [18, 20, 21], and on average 2% for the proportion of mtDNA genotype variation in blastomeres in human PNT embryos. Further, the analysis of mtDNA carryover in monkey MST offspring discovered no detectable mtDNA segregation into different tissues [18], and a longitudinal study of juvenile MST monkeys revealed no increased heteroplasmy [20]. These results indicate that MST could resolve the concern of heteroplasmy in different tissues and organs by random genetic drift or segregation. However, there is a concern that mtDNA could be passed through the female germline due to an unknown genetic mechanism that is generally attributed to the "mitochondrial bottleneck effect". Heteroplasmy analysis of oocytes collected from female MST monkeys demonstrated that two oocytes (one from each foetus) contained substantial degrees of mtDNA carryover, 16.2% and 14.1%, even though the majority of eggs displayed low or undetectable mtDNA heteroplasmy [38]. This was unexpected since the mtDNA carryover in MST oocytes was estimated as low as 1%. However, the possibility exists that mtDNA heteroplasmy may change in subsequent generations through the female germline, probably due to the bottleneck effect. This raises another concern that in PGD, the selection of embryos carrying 30% or less mutation loads most likely would not eliminate the possibility of the future recurrence of mitochondrial diseases.

While much of the basic research concerning MST has been performed using non-human primates, the evaluation of mtDNA carryover levels in organs and tissues of offspring, and in oocytes of females generated by PNT and PBT is insufficient. Some inconsistencies in carryover mtDNA have been observed between different species, especially rodents and humans in PNT [14, 23, 24, 27]. Further, our present understanding relies on conclusions drawn a study with an unacceptably low yield of blastocysts that were produced using a limited number of abnormally fertilized zygotes in human PNT [27]. As described above, the technical feasibility of PBT has not yet been evaluated with primates or humans, despite a favorable result having been obtained with mice [36]. Also, we should note that oocyte freezing is critical for the success of MRT in clinical practice to avoid problems with synchronous ovarian stimulation. In this regard, the feasibility of PNT or PBT using cryopreserved oocytes or zygotes has not yet been evaluated [14, 20, 27, 36]. Thus, further extensive studies using non-human primates and/or humans are desirable prior to applying these techniques in clinical trials.

It is known that the biogenetic features of mtDNA differ in different types of pathogenic mutation. For instance, the mutations *m*.8993T>G and *m*.8993T>C, responsible for NARP (Neurogenic muscle weakness, Ataxia, Retinis Pigmentosa) and Leigh syndrome, have strong genotype-phenotype correlation and show very little blastomere, tissue-dependent or age-dependent variations in mutant load [39, 40]. In contrast, it has been reported that the m.3243A>G mutation leading to MELAS (Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, Stroke-like episodes), is unstable in time with a non-uniform distribution in blastomeres or tissues with no reliable genotype-phenotype prediction on the basis of mutant load [41]. Nevertheless the knowledge of mtDNA biogenesis in MRT obtained so far is based on oocytes from females harboring different wild-type mtDNA haplotypes, the details of mitochondrial biogenesis in early embryo development and/or embryonic development with mutated mtDNA in MRT remains largely unknown. Therefore, preclinical trials with patient oocytes harboring various mutation types will be necessary to establish the safety of MRT.

MRT techniques require the use of a number of indispensable reagents including cytochalasin B or nocadazole, which make the cytoplasm and cell membranes less rigid and less prone to lysis during the nuclear transfer procedure. Based on the results cited in

this review, brief exposure of oocytes to those reagents appears not to be detrimental [18, 20, 36]. In addition, MRT requires the extract from inactivated Sendai virus (HVJ-E). However, HVJ-E is completely inactivated and no detectable viral genome has been found in MST infants or ESC lines that were tested by RT-PCR [18]. Sendai virus has been widely used as a research tool in cell biology and recently it has been considered for use as a vector for gene therapy [42]. In addition, due to negative sense, single-strand RNA virus, cytoplasmic gene expression is mediated without chromosomal integration of exogenous genes. Thus, viral infection, proliferation, or integration of exogenous genes is less probable with this application.

Since mtDNA from donor oocytes may be required to interact with nuclei from different haplotypes in oocytes reconstructed by MRT, nuclear-mitochondrial incompatibility could be of concern. However, longitudinal studies of the overall health of MST infants generated by two genetically distant subpopulations of rhesus monkeys, Indian and Chinese macaques, reported normal growth comparable to that of age-matched juvenile rhesus macaques in the colony at Oregon National Primate Research Center (ONPRC), leading to speculation that adverse effects due to nuclear-mitochondrial incompatibility are unlikely to occur in humans [20]. Mitochondria may however regulate the epigenetic modification of nuclear DNA [43, 44]. The effects of mitochondria on epigenetic abnormalities and the growth of offspring in PNT mice have been studied, but the results are conflicting [45, 46]. Although further research is needed to establish genetic matching criteria for different mitochondrial haplotypes and epigenetics, matching patient and cytoplast donors for mitochondrial haplotypes might have to be considered when performing MRT for humans.

Ethical Issues and the Global Situation Concerning MRT

Clinical applications of MRT can be considered to germline gene therapy, in which genetic modifications to gametes or early embryos would be inherited by subsequent generations [20, 47]. Therefore, clinicians must give full consideration to ethical issues, and the development of the legal system and regulation of MRT is essential [48]. However, the legislation and the regulation of assisted reproductive treatments varies considerably across countries.

In the United Kingdom (UK), the Secretary of State for Health commissioned the Human Fertilization and Embryology Authority (HFEA), an independent regulatory agency established by the UK Parliament through the Human Fertilization and Embryology Act of 1990 (HFE Act), to convene an expert scientific panel to review methods to prevent mitochondrial diseases. From 2011 to 2014, the HFEA convened an expert panel to review the “safety and efficacy of methods to avoid mitochondrial diseases through assisted conception”, and concluded there was “no evidence which suggested that mitochondrial replacement is unsafe” (http://www.hfea.gov.uk/8807.html). The Nuffield Council on Bioethics, an independent body, published an ethical review of MRT which reached similar conclusions (http://nuffieldbioethics.org/project/mitochondrial-dna-disorders/). The House of Commons approved the conduct of MRT and the House of Lords followed suit in February 2015. HFEA has established a licensing system for applications from clinics wishing to carry out MRT which commenced on 29 October 2015 (http://www.hfea.gov.uk/9946.html). Thus, the UK will likely to be the first country to perform clinical trials of MRT as a germline gene therapy. Despite ethical issues surrounding the safety of MRT, the matter of germline modification and parental authority has been discussed and remains a matter of debate. Each application will be assessed on a case-by-case basis to take into account the ethical and technical complexities of MRT only for that particular patient.

In the United States, the FDA Office of Cellular, Tissue, and Gene Therapies of the Center for Biologics Evaluation and Research has the authority to regulate reproductive technologies. The FDA convened a meeting of the Cellular, Tissue, and Gene Therapy Advisory Committee to discuss the preclinical and clinical science regarding assisted reproductive methods, including MRT, for the prevention of mitochondrial diseases in February 2014 (http://www.fda.gov/AdvisoryCommittees/Calendar/ucm380042.htm). Subsequently, the FDA requested the National Academy of Science (NAS)/ the Institute of Medicine (IOM) to produce a consensus report regarding the ethical and social policy issues of genetic modifications of eggs and zygotes to prevent the transmission of mitochondrial diseases. Recently, the NAS made an announcement stating that it is ethically permissible to conduct clinical investigations of MRT (http://iom.nationalacademies.org/hmd/reports/2016/Mitochondrial-Replacement-Techniques#sthash.K2SGy0fX.dpuf).

Japan has not developed sufficient legislation regarding reproductive technologies. The Ministry of Health, Labour and Welfare, Japan Society of Obstetrics and Gynecology, and all related institutes have not officially considered or discussed the ethical and social policy issues of the genetic modification of eggs and zygotes to

prevent the transmission of mitochondrial diseases. In future, the ethical and social policy issues of ART including genome-editing technologies, will become increasingly important, therefore, new laws and legislation on this matter are urgently needed in Japan.

On the other hand, MRT could be considered an ART technique for overcoming cytoplasmic defects due to aging in regular fertility treatments. While the use of MRT would be considered ethical for affected families, and in such cases families are offered sufficient information, support, and comprehensive follow up, the use of MRT for infertile patients woluld not guarantee to provide those benefits. This may provoke another ethical argument about science descending the slippery slope leading to the creation of "designer babies". This course would carry a high risk of violating the rights of the subsequent generation. While MRT may provide options for maintaining nuclear DNA hereditary against the limitations of the present status of ART applications which are confined to donated embryos and eggs, it is premature to apply MRT to infertility treatments.

Conclusion

In this paper, we have summarized the various MRT techniques. Since there are currently no cures for mitochondrial diseases, the elimination of the risks of disease transmission could provide significant health and social benefits to affected families, enabling them to live free from what can be very severe and lethal disorders. As an alternative to MRT, a recent study demonstrated that a genome editing technique using TALEN (Transcription activator-like effector nuclease) could selectively reduce the desired haplotype of mtDNA including human mutated mtDNA [49]. This demonstrates that new approaches are likely to emerge in accordance with technological advances. A new era of ART where cures can be provided in gametes or early zygotes has begun. Therefore, we have to be careful to choose suitable treatments that can be trusted and relied upon by families affected by mitochondrial diseases and their descendants.

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