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REVIEW ARTICLE

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Mitochondrial replacement therapy and assisted reproductive technology: A paradigm shift toward treatment of genetic diseases in gametes or in early embryos

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Abstract

Background: Recent technological development allows nearly complete replacement of the cytoplasm of egg/embryo, eliminating the transmission of undesired defective mitochondria (mutated mitochondrial DNA: mtDNA) for patients with inherited mitochondrial diseases, which is called mitochondrial replacement therapy (MRT).

Methods: We review and summarize the mitochondrial biogenesis and mitochondrial diseases, the research milestones and future research agenda of MRT and also discuss MRT-derived potential application in common assisted reproductive technology (ART) treatment for subfertile patients.

Main findings: Emerging techniques, involving maternal spindle transfer (MST) and pronuclear transfer (PNT), have demonstrated in preventing carryover of the unbidden (mutated) mtDNA in egg or in early embryos. The House of Parliament in the United Kingdom passed regulations permitting the use of MST and PNT in 2015. Furthermore, the Human Fertilization and Embryology Authority (HFEA) to granted licenses world first use of those techniques in March 2017. However, recent evidence demonstrated gradual loss of donor mtDNA and reversal to the nuclear DNA-matched haplotype in MRT derivatives.

Conclusion: While further studies are needed to clarify mitochondrial biogenesis responsible for reversion, ruling in United Kingdom may shift the current worldwide consensus that prohibits gene modification in human gametes or embryos, toward allowing the correction of altered genes in germline.

KEYWORDS

germ line gene therapy, maternal spindle transfer (MST), mitochondrial bottleneck effect, mitochondrial diseases, mitochondrial replacement therapy (MRT)

1 | INTRODUCTION

In the recent era, many of pathogenic (causative) mutations associated with disease or disorders have been found due to the progress of the Human Genome Project (HGP). Mutation can be found both in the nuclear DNA (nDNA) and in the mitochondria DNA (mtDNA), and if pathogenic (causative) mutations exist in germ line, that is, present in the sperm or oocyte, they would be inherited by the offspring, and sometimes, even passed on to future generations. The patient who harbors pathogenic mutations that can cause progressive and lethal diseases with no available cure is often required to make difficult reproductive choices. In order to have a healthy baby,

they and their families are left to decide between options such as gamete/embryo donation, selecting normal embryos or conceptus by genetic testing, or adoption. In case of patients or carriers desire to have their own genetically related baby by eliminating the risk of disease development, reproductive choices become narrower and restricted to genetic testing or a correction of the causative mutation in gametes or in early embryos, called germ line gene therapy. Unlike somatic gene therapy, where genetic changes/fixes/replacements are performed in just one person, outcome of a germ line gene therapy is reflected in the entire body as well as in their descendants through their germ line. Thus, this type of modification would be passed on to their offspring, regardless of its effect being good or bad, similar to that of inherited diseases caused by germ line mutations.[1,2] Therefore, inappropriate germ line modification would inevitably also affect to future generations. However, if the techniques employed are safe and effective, and an appropriate level of information and support is offered, it might be considered a beneficial treatment for affected families. In recent times, some nuclear transfer techniques, also referred to as mitochondrial replacement therapy (MRT), are considered to be a potential germ line gene therapy for inherited mitochondrial diseases. Performing MRT requires changing law in the United Kingdom, and hence both the House of Parliament passed regulations permitting the use of MST and PNT in 2015. The Human Fertilization and Embryology Authority (HFEA), an independent statutory regulatory body for IVF clinics and projects involving research on human embryos in the United Kingdom, was the first in the world to grant licenses for the use of these techniques in March 2017. Thus, human germ line gene therapy is now underway. Furthermore, some clinicians and researchers are considering MRT as ART technique to solve cytoplasmic defects due to aging. In this review, mitochondrial biogenesis responsible for inheritance of genetic diseases, the research milestones of MRT, future research agenda prior to its application for clinical use and possible application of MRT in common ART for subfertile couples, are described.

2 | MITOCHONDRIAL DISEASES, THEIR INHERITANCE AND HETEROPLASMY

The primary role of mitochondria is aerobic respiration, that is, oxidative phosphorylation (OXPHOS) and the production of cellular energy in the form of adenosine triphosphate (ATP). MtDNA encodes 37 genes (13 genes for polypeptides, 22 genes for transfer RNAs, and two genes for ribosomal RNAs) that are crucial for production of cellular energy and programmed cell death (apoptosis).[3] Mitochondrial disorders or diseases attributable to defects in oxidative phosphorylation are mostly severe disorders and affect at least one in 8000 individuals.[4] Such conditions can be fatal or cause chronic morbidity and usually affect the most energy-demanding tissues such as the central nervous system, heart and skeletal muscles, liver, and kidney.[5] Mitochondrial diseases can be caused by genetic alterations of nuclear- or mitochondrial-encoded genes involved in the synthesis of ATP. While disorders resulting from nuclear DNA mutations follow a Mendelian pattern of autosomal recessive, autosomal dominant or X-linked inheritance, the inheritance patterns of conditions arising from mtDNA defects are quite different. First, affected individuals are usually heteroplasmic, that is, there is a mixture of normal and mutant mtDNA, the levels of which can differ among tissues. If the mutant load, that is, the ratio of mutant to normal mtDNA, exceeds a tissue- and individual-specific threshold, clinical features become evident, although exact genotype-phenotype correlations usually vary even within families.[5] Second, unlike the nuclear genome, mtDNA is transmitted maternally.[6] This is due to the significantly higher number of mtDNA molecules in a mature oocyte (200 000-300 000 copies) compared to the sperm (approximately 100 mtDNA copies).[7,8] In addition, sperm mitochondria that enter via fertilization are eliminated specifically during early embryo development.[9]

Diseases caused by mtDNA mutations were first described in 1988.[10-12] Since then, over 150 mutations (including 100 deletions and approximately 50 point mutations) associated with human diseases have been identified (see for review [13]). In addition, mtDNA mutations are also increasingly implicated in a range of socially recognizable conditions, including Alzheimer's, Parkinson's, and Huntington's diseases, obesity, diabetes, and cancer.[14-16] Interest in the study of these conditions has grown enormously due to the large number of patients diagnosed with these disorders and to the fact that they appear throughout life, from newborns to adults of all ages. These include Leber's hereditary optic neuropathy (LHON), which can result from mutations in the gene for the NADH-Q oxidoreductase, component of complexes I and III, myoclonic epilepsy with ragged-red fibers (MERRF) results in myoclonus, epilepsy, and ataxia and is caused by mutations in tRNA genes. Some mitochondrial diseases are caused by large-scale deletions in mtDNA. The best known in this group is Kearns-Sayre syndrome,[17] which includes symptoms of pigment retinopathy and cardiac disorders. As indicated above, the clinical phenotypes resulting from mtDNA mutations are dependent on the proportion of mutated mtDNAs. In the case of LHON, >60% mutant mtDNA load is required for the disease phenotype to manifest. In other cases, such as MERRF, over 85% mutant mtDNAs has to be present for symptoms to become apparent. At present, there are no widely approved fundamental cures for mitochondrial diseases and treatments currently available only alleviate symptoms and slow disease progression.

The percentage of mtDNA heteroplasmy in the offspring can reflect heteroplasmy in the mother, while extreme shifts in heteroplasmy are observed among children due to a phenomenon known as "genetic bottleneck." The bottleneck leads to a rapid segregation of mutant genotypes, which either are lost during transmission or reach very high levels and cause disease. Mitochondria are abundant in the female germ line (oocytes) and play an important role in oocyte maturation and subsequent early embryonic development via fertilization, where mitochondrial biogenesis is arrested. Rapid genetic drift in the germ line can be explained by the

TABLE 1 Features of MRT procedures

Classification	Cytoplasmic transfer	Nuclear Transfer				
Abbreviation	**CT**	**GVT**	**PNT**	**PB1T[a]**	**PB2T[b]**	**MST[c]**
Procedure name	Cytoplasmic Transfer	Germinal Vesicle Transfer	Pronuclear Transfer	Polar Body1 Transfer[a]	Polar Body2 Transfer[b]	Maternal Spindle Transfer[c]
Genetic material/nuclear ploidy	NA	GV/tetraploid: 4n	PN/diploid: nx2	PB1/diploid: 2n	PB2/haploid: n	MII spindle-chromosomal complex/diploid: 2n
Dilution/replacement	Dilution	Replacement	Replacement	Replacement	Replacement	Replacement
Type (stage) of recipient; patient or maternal	MII oocytes	GV intact	2PN	PB1	PB2	MII spindle
Type (stage) of donor; healthy donor	Minimum portion of MII cytoplasm (<5%)	GV stage cytoplasm	PN stage cytoplasm	MII cytoplasm	PN stage cytoplasm w/o female PN	MII cytoplasm
Applied to domestic animals?/Live birth?	Yes/yes	Yes/yes	Yes/yes	Yes/yes	Yes/yes	Yes/yes
mDNA Heteroplasmy in offspring (mouse/primate)	Evaluated for mtDNA segregation up on cleavage (primate)	NA	Heteroplasmic (up to 69%; mouse)	Homoplasmy to donor mtDNA (undetectable; mouse)	Nearly homoplamic to donor mtDNA (1.7%; mouse)	Heteroplasmic~Nearly homoplamic (5.5%/<1%)
Applied to human?/Live birth?	Yes/yes	Yes/no	Yes/no	Yes/no	No/–	Yes/yes
mDNA Heteroplasmy in embryos (human)	Heteroplasmic (nearly homoplamic to maternal mtDNA)	NA	Heteroplasmic (vary <2%~>5%)	NA	–	Nearly homoplamic to donor mtDNA (vary <1%~5%)

[a]Also referred to as Polar Body Nuclear Transfer 1 (PBNT1).
[b]Also referred to as Polar Body Nuclear Transfer 2 (PBNT2).
[c]Also referred to as Spindle-Chromosomal complex Transfer (ST).

progressive decline in mtDNA copies during preimplantation and immediate postimplantation development, resulting in a minimum number in the early primordial germ cells (PGCs) (~200 mtDNAs per cell) just before mtDNA replication in the embryo is initiated in mouse.[18] Resumption of mitochondrial biogenesis by E6.5 in the mouse results in a 10- to 20-fold increase in mtDNA copy number from the emergence of primordial germ cells until colonization of the gonad at E13.5.[19] Recent evidence shows that isolated human PGCs have profound reduction in mtDNA content and subsequent rapid replication in late PGCs, consistent with the observed bottleneck, and is narrower than that of mouse.[20] A study revealed that mtDNA mutations are present in PGCs within healthy female germ line in humans. The mtDNA genetic bottleneck has probably evolved to expose potentially deleterious mutations to selective forces. However, if a mutation is sufficiently mild enough to escape this selection, healthy mothers harboring a low-level heteroplasmic mtDNA mutation can have children with high levels of heteroplasmy and a severe disease.[20] Furthermore, although the consequences of heteroplasmy have not been thoroughly clarified,[21] a recent study in a primate model has shown that rapid mtDNA segregation during the early embryo development can result in nearly homoplasmic offspring and embryonic stem cells (ESCs) within a single generation, without going through germ line development.[22] Segregation of mtDNA is initiated as early as 4-cell stage embryos, implying that heteroplasmy in biopsied blastomeres from cleaving embryos may not be predictive of the total mutation load in the remaining blastomeres and thus in the embryo. However, notably, a "benign heteroplasmy" resulting from the mixing of two wild-type mtDNA populations may have different consequences from a "pathological heteroplasmy" that occurs during the aging process or in mitochondrial disease.[23] Indeed, it is known that the biogenetic feature of mtDNA differs in types of pathogenic mutations. For instance, the mutations *m*.8993 T>G and *m*.8993 T>C, responsible for NARP (Neurogenic muscle weakness, Ataxia, Retinis Pigmentosa) and Leigh syndrome, have strong genotype-phenotype correlation and show very little blastomeres, tissue-dependent, or age-dependent variations in mutant load.[24,25] In contrast, it has been reported that the *m*.3243A>G mutation leading to MELAS is unstable, with a non-uniform distribution in blastomeres or tissues, with no reliable genotype-phenotype prediction on the basis of mutant load.[26] Thus, both genetic testing and genetic counseling in most patients at risk of maternally inherited mtDNA mutations are challenging due to limitations in assessing the extent of mtDNA heteroplasmy and accurately predicting risks.

In summary, defects in mtDNA are clearly associated with a wide range of human diseases for which, at present, there is little or no available treatment. Given the fact that many of these disorders are dependent on the heteroplasmic state of the mtDNA and associated threshold effects, it is difficult to provide accurate genetic counseling based on preimplantation or prenatal genetic diagnoses. Affected families have been seeking and awaiting promising-assisted reproductive options, which could prevent transmission of mtDNA mutation to their children. Therefore, there is an urgent need to develop new therapeutic approaches that could prevent the transmission of mtDNA mutations from the mother to the child.

3 | REPRODUCTIVE CONCEPTS FOR MTDNA REPLACEMENT THERAPY

It is the unique feature of mitochondrial inheritance, (which is exclusively maternal through oocytes), which has been motivating researchers to develop new assisted reproductive techniques. The ultimate goal was to eliminate the transmission of faulty mtDNA by diluting or replacing defective cytoplasm with healthy cytoplasm containing healthy mtDNA. Several ART techniques have been proposed as a mean of eliminating the transmission of mitochondrial diseases in affected families by replacement of the cytoplasm, either of the egg, or at embryos of different stages. These technologies include cytoplasmic transfer (CT), germinal vesicle transfer (GVT), pronuclear transfer (PNT), polar body nuclear transfer (PBT), and maternal spindle transfer (MST). Features of each procedure is summarize in Table 1. In this section, we summarize the technical concepts, accumulated knowledge of CT, GVT, PNT, and PBT.

Cytoplasmic transfer was initially introduced as a procedure to supplement patient eggs with the donor cytoplasm. The rationale was to improve viability and developmental competence of compromised oocytes, where the underlying cause was determined as "ooplasmic deficiency." This procedure was offered to patients who had undergone repeated in vitro fertilization (IVF) cycles with implantation failure due to poor embryo development. Attempts to introduce sufficient amounts of donor cytoplasm by electrofusion were not successful due to low fertilization rates (23%), pronuclear anomalies, and poor embryo development.[27] However, CT as an extension of intracytoplasmic sperm injection (ICSI) involving coinjection of a small amount of donor cytoplasm with sperm has been more successful.[27-29] This approach was used to treat patients in several fertility clinics, and purportedly improved IVF outcomes and resulted in live birth.[28,30] Patients involved in these procedures were not suspected of having any mtDNA mutations, and only small volumes (1%-5%) of donor cytoplasm were injected. However, while donor cytoplasmic mtDNA was detected in small amounts in 6 of 13 embryos studied and in 2 of 4 fetal cord blood samples.[31] Thus, CT procedure is not suitable for MRT in patient with mitochondrial diseases because it would require transferring significantly larger cytoplasmic volumes (30%–50% of the final volume) to ensure adequate dilution of mutant mtDNA. It would be nearly impossible to inject such amounts without oocyte lysis. Furthermore, a relatively high number of chromosomal abnormalities and birth defects have been reported in infants resulting from the initial application of CT.[32] CT is now banned in the United States by the Food and Drug Administration (FDA) because of safety concerns. The FDA has advised practitioners that it has jurisdiction over the use of this procedure and has indicated that any further CT protocols must be done under Investigational New Drug (IND) exemptions and that an IND submission to the agency would be required to treat additional patients.

An alternative strategy to CT is to transfer the nuclear DNA from a mother with mtDNA disease to an enucleated oocyte containing normal mtDNA from a healthy female. Results of experiments conducted with mice suggest that it is technically feasible to transfer DNA between immature oocytes, in which nuclear DNA is enclosed in a clearly visible germinal vesicle.[33] The GV surrounded by a small amount of cytoplasm and a membrane (karyoplast) can be removed using a micropipette and placed into the perivitelline space of an enucleated GV oocyte (cytoplast). Fusion of the karyoplast and cytoplast is generally induced by electroporation. If this approach was applied to human oocytes, efficacy would be limited by the poor developmental competence of oocytes produced after in vitro maturation (IVM) of GV-intact oocytes. Moreover, GV oocytes have polarized cyto-architecture, with the mitochondria concentrated in the perinuclear space, possibly due to increased energy requirements of the nucleus.[34] Transplant of GVs would inevitably result in significant amounts of patient mtDNA in the donor cytoplasm. It is further possible that, due to its initial proximity to the nucleus, mutant mtDNA would be preferentially replicated in the reconstructed embryo.[35]

Pronuclear transfer is essentially the same procedure, except for the nuclear material being removed after fertilization. The 2PNs of a patient's zygote enclosed in the karyoplast is removed and transplanted to an enucleated comparable stage of zygote cytoplast, derived from donors containing healthy mitochondria. This technique has also been used in mice with considerable success, as determined by the birth of live offspring.[36-38] Similar to GVT, reconstructed embryos contained a significant portion of karyoplast mtDNA where the carryover mtDNA was detected in the pups at a range of 19%–35%. Derived fetuses were all heteroplasmic and heteroplasmy levels varied with tissue type, from 6% in the lung to 69% in the heart.[36,38] Recent PNT study with mouse also demonstrated significantly high level of 23.7% ± 11.1% on average heteroplasmy in F1 pups.[39] At the zygote-stage, mitochondria are accumulated as a conglomeration around the pronuclei.[40,41] Thus, the PNT procedure may result in cotransfer of mitochondria with abnormal mtDNA, even if isolated with only 2PN, encapsulated in small karyoplasts. However, PNT in humans has shown different outcomes than that in mice. Craven et al[42] reported a study using PNT with abnormally fertilized (unipronuclear or tripronuclear) human zygotes in 2010. Of the 36 reconstructed zygotes, three embryos (8.3%) developed to the blastocyst stage, and the average mtDNA carryover was 2% with the proportion of mtDNA genotype variation among blastomeres. Thus, the blastocyst development of human PNT with abnormally fertilized embryos was extremely inefficient. Further study utilizing normally fertilized human embryos along with the adjustment for timing to manipulation, that is, ePNT, exhibited improved blastocyst development.[43] Furthermore, heteroplasmy can be lowered by the omission of sucrose from the manipulation media and by utilizing the vitrifying donor cytoplast.[43] However, blastocyst formation of the ePNT embryos with vitrifying cytoplast was inferior to control autologous PNT embryos. Furthermore, there was inconsistency in significant portion of embryos, even with optimized ePNT embryos (21%) still exhibiting >5% heteroplasmy, whereas the initial attempts of PNT with abnormally fertilized embryos showed an average of 2% heteroplasmy. The author discussed that this could be attributed to technical problems, such as leakage from the cytoplast or inadequate shearing of cytoplasm form kyaroplast and such factors could be taken into account when selecting embryos for use in clinical treatment.[43] However, these inconsistencies have led to concerns that human PNT may also result in significant heteroplasmy in their offspring, sufficient for the disease to manifest, similar to that seen in mice PNT.[36] Although there are some concerns, PNT has been considered to be a potential MRT technique in the United Kingdom and clinical trial is now being conducted.

Polar body nuclear transfer has also been proposed as a potential MRT in mice.[39] Mammalian oocytes undergoing meiotic division sequentially extrude two sets of nuclear genomes as small polar bodies (PBs), that is, first and second polar body (PB1 and PB2). In the late 90 s, the developmental potential of transplanted PB1 and PB2, into an appropriate oocyte or zygote cytoplasm, which resulted in live birth, was investigated in mice.[44,45] In 2014, Wang et al expanded PBT as a potential MRT and compared both PB1 transfer (PB1T) and PB2 transfer (PB2T) with other MRT techniques, such as MST and PNT.[39] PBT, especially PB1T, demonstrated more favorable outcomes, where carryover of mtDNA from nuclear material was less compared to the other alternative strategies, not only in the F1 but also in the F2 pups. A recent study has also demonstrated that PB1T in humans and resulted in chromosomally normal ESC lines, however, the developmental potential of the reconstructed oocytes was lower than controls.[46] The results suggested that metaphase II-arrested human oocytes may have limited potential to properly assemble a normal bipolar spindle, following PB1T, compared to mice. The above results supported the notion that rescue of PB1, via introduction into the donor cytoplasm, may offer an MRT for mtDNA diseases. However, unsolved questions remain in PBT, such as the errors in segregation of chromosomes at meiosis, the best timing for transfer (since mammalian PBs show short lifetime due to apoptotic pressures that lead to DNA fragmentation or degradation) and a precise distinction from maternal pronucleus, in the case of PB2T. Importantly, the genetic composition of PB1 and PB2 is not identical to MII chromosomes and the female pronucleus because of meiotic recombination.[47]

4 | RESEARCH MILESTONE OF MATERNAL SPINDLE TRANSFER (MST)

Maternal spindle transfer (MST), also referred to as spindle-chromosomal complex transfer (ST), was first reported as a novel revolutionary approach to avoid transmission of inherited mitochondrial disease in 2009.[48] MST takes place in mature metaphase II (MII) oocytes where MII spindle-chromosomal complex is isolated as membrane-enclosed karyoplast and is transferred into an enucleated (nuclear material free) cytoplast from healthy donor MII oocyte (Figure 1).[48] Since 2009, MST has been thoroughly examined not only in primates, but also in humans carrying pathogenic mutations.

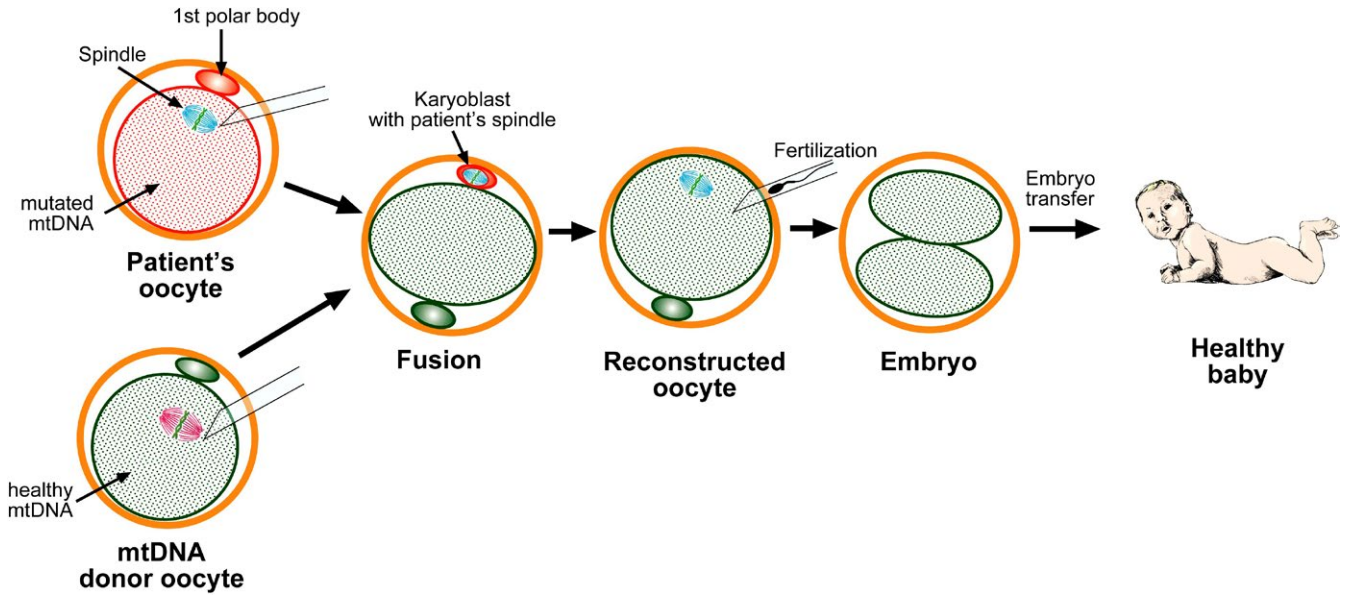

FIGURE 1 Schematic diagram representing mtDNA replacement by MST procedure. The spindle-chromosome complex (nuclear DNA) within membrane enclosed karyoplast from a patient's egg carrying mtDNA mutations is removed and transplanted into an enucleated oocyte donated by a healthy donor. The reconstructed oocyte is then fertilized with the husband's sperm and an embryo is transferred to a patient. The infant will be free of risk from maternal mtDNA mutations. Figure is adapted from Supplementary Figure 1 in Tachibana et al, Nature 2009[48]

Thus, MST can be considered as a leading technique for MRT and germ line gene therapy. In this section, research milestones of MRT are summarized.

Transferring of the nuclear material in MII oocytes offers many advantages for MRT over other approaches. First, primate and human MII spindle-chromosomal complex is relatively smaller than that of GV and PN stage interphase nuclei. Furthermore, MII spindle is devoid of mitochondria.[48] Therefore, transmission of unbidden mutated mtDNA can be minimized using MST. Second, compared to GVT, where IVM is required prior to fertilization, reconstructed oocytes via MST, are ready for fertilization. Third, MST may be ethically more acceptable than PNT, where cytoplasts are supplied from fertilized zygotes, and thus, requires destruction of normally fertilized zygotes, not gametes. Despite above known theoretical advantages, some technical obstacles have hampered the success of MST. However, researchers have successfully addressed some of the issues surrounding transferring MII spindles transfer, such as (a) visualization of MII spindle, utilizing the polarized microscope, (b) the development of a reliable technique for isolation and transfer of MII spindles by minimizing the cytoplasm surrounding the spindles, as kyaryoplast, via opening of the zona pellucida, by use of a laser objective, and (c) premature activation due to commonly used membrane fusion techniques attributed by susceptibility of the MII spindles, was solved by use of inactivated Sendai virus envelope (hemagglutinating virus of Japan-envelope; HVJ-E) for karyoplast and cytoplast fusion.[48,49]

Maternal spindle transfer was initially developed using rhesus macaque oocytes where two distinct genetically distant wild-type mtDNA haplotypes, of Chinese and Indian origin, were exchanged reciprocally. Reconstructed oocytes by MST showed normal fertilization via ICSI and yielded comparable blastocyst development, compared to control ICSI embryos. Developed MST blastocysts resulted in two novel ESC lines via derivation, and four healthy infants from three pregnancies, via embryo transfer into recipient females. Genetic analyses of both nuclear DNA and mtDNA revealed the proof of concept that there was successful replacement of cytoplasmic material from the cytoplast donor, whereas the maintenance of nuclear genetic inheritance of the original female and male was retained. The carryover of mtDNA from spindle donor (ie, degree of heteroplasmy), both in MST infants and in derived ESC lines, exhibited undetectable or below 3% heteroplasmy.[48] In contrast, MST in mice exhibited different outcomes that on average 5.5% ± 1.4% heteroplasmy were observed in F1 pups. This was probably attributed to relatively large MII spindle than that of humans. Furthermore, mouse oocyte has polarized cytoskeletal architecture that barrel-shaped large MII spindle is anchored under beneath of actin filament rich cortex.[50] Thus, isolation of an only MII spindle may be difficult, and thus, mice karyoplast contains excessive amount of cytoplasm to be transferred. In most mitochondrial diseases, a threshold of 60% or higher of mutated mtDNA is required for clinical features to manifest. In this regard, MST would be successful to prevent diseases, as well as sufficient in preventing the transmission of mitochondrial diseases to their offspring.

The MST procedure requires the cytoskeletal inhibitors, such as cytochalasin B, in order to render the cytoplasm and cell membrane less rigid and less prone to lysis, during both isolation and transfer of karyoplast. Furthermore, HVJ-E is also indispensable for inducing efficient membrane fusion by preventing premature activation.

Based on the results discussed in this review, brief exposure of oocytes to these reagents appears to be nondetrimental.[22,39,48,51] In addition, the cytoplasmic gene expression via HVJ-E is mediated without chromosomal integration of exogenous genes, as it is a negative sense, single-stranded RNA virus. Thus, viral infection or proliferation and integration of exogenous genes are less probable with this application. Indeed, RT-PCR for F-protein-coding region revealed the presence of no detectable viral genome in MST infants and ESC lines.[48]

It is important to validate the feasibility, safety, and efficacy in humans prior to application of a new technique for clinical use. In this regard, MST has been validated in humans both with oocytes carrying wild-type mtDNA and pathogenic mutations.[51,52] Initial attempts of MST with human oocytes from healthy donors demonstrated successful, nearly complete exchange of the cytoplasm containing mtDNA that resulted in karyotypically normal ESC lines, when embryos were obtained from normally fertilized 2PN zygotes. However, significant portion of zygotes (approximately half of MST zygotes) exhibited abnormal fertilization.[51] This was attributed by susceptibility of human MII oocytes to high concentrations of HVJ-E. However, an additional study has demonstrated that improved fertilization (similar to controls) was observed by lowering the HVJ-E concentration.[52] Stable results were observed, not only in wild-type mtDNA exchange but also in MST with oocytes carrying pathogenic mtDNA mutations.[52] Furthermore, both feasibility and efficacy of human MST have also been proven by another independent laboratory,[53] hence providing the ultimate proof, that the developed technique is not restricted only to an expert laboratory, but can be successfully replicated in many standard IVF laboratories, due to detailed protocol disclosure.[49]

Original MST protocols, using freshly acquired oocytes, would require both, patient and healthy mtDNA egg donors, to undergo synchronous ovarian stimulation regimens and oocyte retrievals to harvest, preferably, the same numbers of oocytes on the same day. However, it is difficult to manage the same-day oocyte retrieval from two women due to differences in their stages in the menstrual cycle and responses to gonadotropins. Thus, utilization of freeze-thaw oocytes is beneficial to clinical applications of the MST technique. Recent advances in oocyte vitrification procedures suggest that cryopreserved human MII oocytes can be used in clinical IVF practice with the same efficiency as fresh eggs.[54,55] In contrast, vitrification of monkey oocytes was detrimental, and reciprocal MST between freeze-thaw oocytes, and freshly obtained oocytes revealed that vitrification causes damage, primarily within the cytoplasm rather than the spindle apparatus.[51] On the basis of this data, transplant of vitrified spindles into the fresh cytoplasm yields the best results, comparable to controls. Developed blastocysts by MST, with vitrified spindles, resulted in healthy offspring and karyotypically normal ESC lines. Thus, patient oocytes can be vitrified prior to commencing MST and healthy donor cytoplasm should be harvested freshly on the day of MST procedure. However, human oocytes may behave differently from monkey oocytes; no differences in fertilization were noted between combinations involving vitrified karyoplast versus vitrified cytoplasts, in humans.[52] Thus, vitrified healthy donor oocytes may also be considered to be the cytoplast donor for human MST, but not on a priority basis.

There is a reciprocal interaction between the nuclear and mitochondrial genomes,[56] and it influences the stability of the nuclear genome and nuclear gene expression.[57] Mitochondrial replication is controlled by several nuclear-encoded genes, as well as mtDNA polymerase.[58] It has been shown that mitochondrial heteroplasmy, even without pathogenic mutations, may have a negative impact on cognitive and metabolic functions.[59,60] Furthermore, a recent study has demonstrated that reciprocal exchange of mtDNA between two different mouse strains, harboring distinct mtDNA haplotypes (B6 and PWD), exhibited different; PWD nuclear DNA with B6 mtDNA was embryonic lethal, whereas a stable transmission of the introduced PWD mtDNA to their descendants was observed in their counter parts.[61] On the other hand, mitochondria may regulate epigenetic modification of the nuclear DNA and affect the growth of offspring in PNT mice.[62,63] Thus, nuclear-mitochondrial incompatibility could be of concern, and inappropriate interactions between nDNA and mtDNA can negatively impact the process, causing impaired ATP production and/or a selective replication bias toward the undesired mtDNA haplotype. Longitudinal studies performed to assess the overall health of MST infants, generated by two genetically distant subpopulations of rhesus monkeys (Indian and Chinese macaque) showed normal growth comparable to age-matched juvenile rhesus macaque in the colony, at Oregon National Primate Research Center (ONPRC). The ATP production levels and mitochondrial membrane potential in skin fibroblasts from MST monkeys were similar to those of controls, indicating that adverse effects by nuclear-mitochondrial incompatibility are unlikely to occur in non-human primates.[51] Furthermore, the analysis of mtDNA carryover in monkey MST offspring showed no detectable mtDNA segregation into different tissues,[48] and longitudinal study in juvenile MST monkeys revealed no increased heteroplasmy.[51] In contrast, where majority of human MST resulted in ESC lines containing >99% mtDNA haplotype from donor, some ESC lines among these demonstrated gradual loss of donor mtDNA and reversal to the nDNA-matched haplotype (maternal mtDNA).[52] However, this finding could not be attributed to inappropriate methodology employed for MST, such that a significant amount of maternal mtDNA within the karyoplast might have been originally cointroduced with nDNA, because all embryo biopsies showed below 1% of maternal mtDNA heteroplasmy Kang et al,[52] explored a polymorphic D-loop region, called conserved sequence box II (CSBII), in which a polymorphism in CSBII (G5AG7) affects the efficiency of mitochondrial transcription termination and replication primer production. They observed that this polymorphism of CSBII in two reversed sibling ESC lines was G5AG8 in the donor, while the maternal haplotype was G6AG8. This single guanosine residue in donor mtDNA resulted in a fourfold reduction in replication primer synthesis. However, CSBII polymorphism was not observed in other reversed ESC lines, albeit other D-loop polymorphisms were observed in the mtDNA replication regulatory candidate of the core TAS region. A recent study also demonstrated that

mtDNA mutations are present in primordial germ cells (PGCs) within healthy females. Sequence variants in conserved regions within the D-loop were found to be accumulated in early PGCs (CS12), but not in late PGCs (CS20/21), suggesting the introduction of a transcriptional and replication bias in mtDNA, which thus eliminates low variants during early female germ cell development.[64] Although the exact mechanism by which polymorphisms affect replication of specific haplotypes in mtDNA remains unclear, it is speculated that these polymorphisms may contribute to a replication bias toward particular mtDNA haplotype.[52] 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 be considered while performing MRT in humans.

On the other hand, random genetic drift or segregation can also be causal for unexpected heteroplasmy change during early embryogenesis, as described above.[22] Rapid segregation of mtDNA variants between daughter blastomeres in preimplantation embryos may subsequently be transmitted to specific lineages of their progeny and result in heteroplasmy differences in each individual tissue and organ. However, fetuses and ESC lines derived from 50% mixture of two wild-type mtDNA haplotypes dramatically shifted toward homoplasmic condition within one generation, suggesting a new bottleneck in peri-implantation monkey embryos.[22] This bottleneck effect might reflect to a reversal phenomenon observed in human MST. Kang et al dissociated and subcloned reversed ESC lines into single clones and analyzed mtDNA heteroplasmy. The starting mtDNA levels varied in each isolated single-cell clone, but levels of heteroplasmy remained unchanged up on passaging and propagation. Subcloned ESC lines with higher maternal mtDNA levels exhibited faster growth, suggesting that matching nDNA and mtDNA may confer growth and proliferative advantages.[52] Furthermore, all reversed ESC lines exhibited substantial degree of heteroplasmy within early passage (2-3), whereas all MST preimplantation embryos originally harbored <1% of maternal mtDNA. Considering such a drastic shift within a very short peri-implantation period, it would be nearly impossible to propagate maternal mtDNA by only random segregation. This evoked a hypothesis that a very rapid genetic drift occurs in a very short peri-implantation period, leading to ESC derivation (where inner cell mass: ICM is plated on the fetal fibroblast) due to a new genetic bottleneck. Given that a very rapid stochastic or bottleneck mtDNA amplification in peri-implantation period is the main mechanism responsible for reversal, it would be difficult to prevent or overcome such event. If indeed, this truly occurs in peri-implantation human embryos, it absolutely raises an alert for PGD, where the selection of embryos carrying 30% or less mutation load, will most likely be unable to eliminate the possibility of disease transmission. In this regard, even though the prevalence of reversal is low, reliable evaluation for postimplantation MRT embryos would be needed until there is a clear understanding of the mechanism responsible for reversion and maternal mtDNA predominancy, from current clinical trials. However, the nature of reversion in in vivo human embryos is currently unknown; in vivo peri- and postimplantation embryos may behave differently from in vitro ESC derivation, because mtDNA reversion to the maternal haplotype has never been observed in any of the recovered MST monkey fetuses and offspring in the past. Concersely, mtDNA reversion might occur exclusively in humans as a species-specific attribute. To date, none of the above explanations for mtDNA reversion appear to be unlikely, including the MST procedure itself, the presence of pathogenic mutations and the genetic distance between donor and maternal mtDNA, measured in numbers of SNPs.[43,52] Additional studies evaluating suitable combination of donor and maternal mtDNA haplotype to avoid peri-implantation genetic drift, and new mitochondrial bottlenecks, would be required for future application of MRT.

Germ line modifications would inevitably affect the entire body, including transmission of genetic traits to the next generation, inducing heritable changes. Furthermore, drastic mitochondrial genetic drifts occur when the mtDNA goes through the female germ line, causing a "mitochondrial bottleneck effect." Therefore, exploration of the bottleneck effect in the female germ line would be a key safety measure for MRT in future generations, unless MRT is restricted only to male embryo transfer. Female germ line bottleneck obviously exists in nonhuman primates, where a wide range of heteroplasmy (3.7%-99.2%) has been observed in primordial oocytes recovered from a heteroplasmic female monkey fetus carrying 93.8% heteroplasmy.[22] A recent study has also demonstrated that isolated human PGCs in vivo exhibit a profound reduction in mtDNA content, and rare variants subsequently reach higher heteroplasmy levels in late PGCs, consistent with the observed genetic bottleneck.[64] Even though mtDNA carryover in MST oocytes is as low as 1%, heteroplasmy may be altered when mtDNA goes through the female germ line. Lee et al,[22] explored heteroplasmy in oocytes, where they found that female MST monkeys, having two oocytes (a twelfth from each fetus), exhibited a substantial degree of mtDNA carryover (16.2% and 14.1%, respectively), albeit both the somatic lineages and majority of the eggs displayed low or undetectable mtDNA heteroplasmy. Although it was not drastic shift, slightly increased heteroplasmy (on average 7.1% ± 6.8%) has been observed in F2 pups while the original heteroplasmy in F1 pups was 5.5% ± 1.4% in mice.[50] Thus, there is a possibility of disease manifestation in future generations of MRT, which will probably become evident after two generations. As noted above, limiting MRT to male embryos takes away the fear related to the transmission of mtDNA diseases as well as unexpected side effects of MRT to future generations, since mtDNA inheritance is exclusively maternal. In this regard, the institute of Medicine of the National Academies of Science, Engineering and Medicine published a report (referred to as "NAS report") addressing the border concerns of MRT, and the report recommended to move cautiously, limiting only to the transfer of male embryos, in future clinical trials.[65] However, this solution may raise some ethical questions regarding the fate of female embryos and the consequences of;"require parents to engage in sex selection".[66] In contrast to United States, the limitation to male embryos was rejected in the United Kingdom.

Despite some unsolved issues surrounding MRT, a group led by Dr John Zhang, New Hope Fertility Center New York city, briefly reported translational research on the use of MST in women carrying mtDNA mutation of Leigh syndrome (8993 T>G), resulting in childbirth, at the American Society for Reproductive Medicine (ASRM) annual meeting 2016.[67] However, as seen in a bioethical statement for MRT in United Kingdom (https://www.nuffieldbioethics.org/mitochondrial-dna-disorders), families using such techniques should commit to allowing very long-term follow-up of their children and families, in order to gather further knowledge about the outcomes of these techniques. To support this aim, the creation of a centrally funded register of any such procedures performed would be required, accessible to researchers over several decades. Unfortunately, it is unlikely that the first case of MRT will be offered such a benefit. The current legislation and regulation for human embryo research vary considerably in each country; however, it is essential to have an appropriate foresight to establish safety and efficacy requirements and guide government-funded clinical trials for future application of all MRT procedures.

5 | WIDER IMPLICATION OF MRT TECHNIQUE

In modern ART, maternal aging is the main cause of impaired outcomes. It has been reported that the 4977-bp deletion in mtDNA is commonly observed in unfertilized oocytes[68] or in oocytes from advanced maternal age.[69] Some point mutations and deletions in the mtDNA of oocytes increase with aging.[70] Either a significant loss of mtDNA or accumulation of defective mtDNA will affect oxidative phosphorylation and will negatively impact proper completion of meiosis, fertilization, and early preimplantation embryo development.[71] Embryo aneuploidy increases with maternal age as a result of errors in meiotic division I or II.[72,73] In addition, mitotic error during early embryonic cleavage division may occur and result in mosaic blastocyst formation.[74] Significant portion of chromosomally normal diploid embryos still fail to implant in preimplantation genetic testing for aneuploidy (PGT-A) cycles and implantation rate decreases with maternal age.[75] Furthermore, almost 10% of implanted chromosomally normal embryos still result in miscarriage. This might also be attributed to cytoplasmic deficiency, and thus the cytoplasmic factor may have an impact on the initiation of pregnancy, as well as on postimplantation development.

Mitochondrial replacement therapy could be considered ART technique to solve cytoplasmic defects due to aging. As described above, one of the MRT techniques, CT, was initially developed to improve viability and developmental competence of compromised oocytes, resulting from aging.[28] Hence, CT has already been applied to humans and has demonstrated improved ART outcomes.[27,30] However, this procedure has been banned by the FDA since 2001, due to safety concerns. We recently extended CT in PN stage zygotes named pronuclear stage cytoplasmic transfer (PNCT) (Figure 2). During conventional IVF, a number of abnormally fertilized oocytes are routinely discarded. We exploited such discarded zygotes as a potential source of cytoplasm for autologous CT. We focused on rescuing and enhancing subsequent development by the quantity of cytoplasmic volume exploiting from abnormally fertilized autologous zygotes, rather than the quality of heterologous mtDNA from young donors. PNCT allows filling the perivitelline space with a large amount of cytoplast (approximately one-third volume of whole cytoplasm), and preliminary results have shown acceptable in vitro development and also resulted in live birth (A. Fujimine, M.

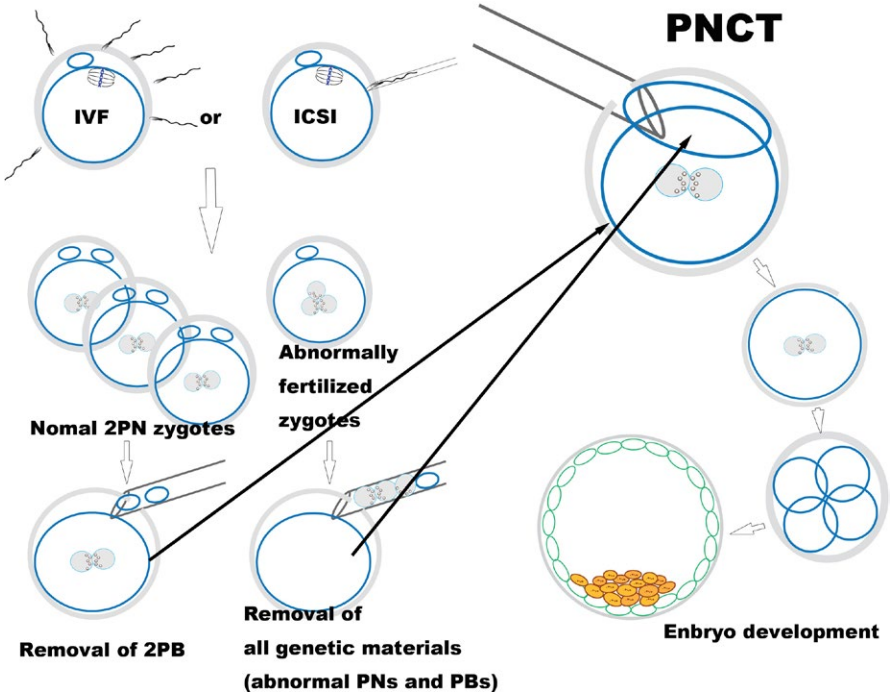

FIGURE 2 Schematic diagram of PNCT. Figure depicts PNCT procedure. Fertilized zygotes via IVF or ICSI, are classified to either normal 2PN zygotes or abnormally fertilized zygotes. While 2 PBs are removed from normally fertilized zygotes, all genetic materials, including abnormal numbers of PNs and PBs, are removed from abnormally fertilized zygotes to create nuclear free cytoplast. Large amount of cytoplast (approximately one third volume of whole cytoplasm) are isolated from nuclear free cytoplasm followed by brief exposure to HVJ-E. Cytoplast is then transferred into the perivitelline space of host 2PN zygotes. Followed by the fusion between 2PN cytoplasm and transferred cytoplast, embryos develop to the blastocyst stage

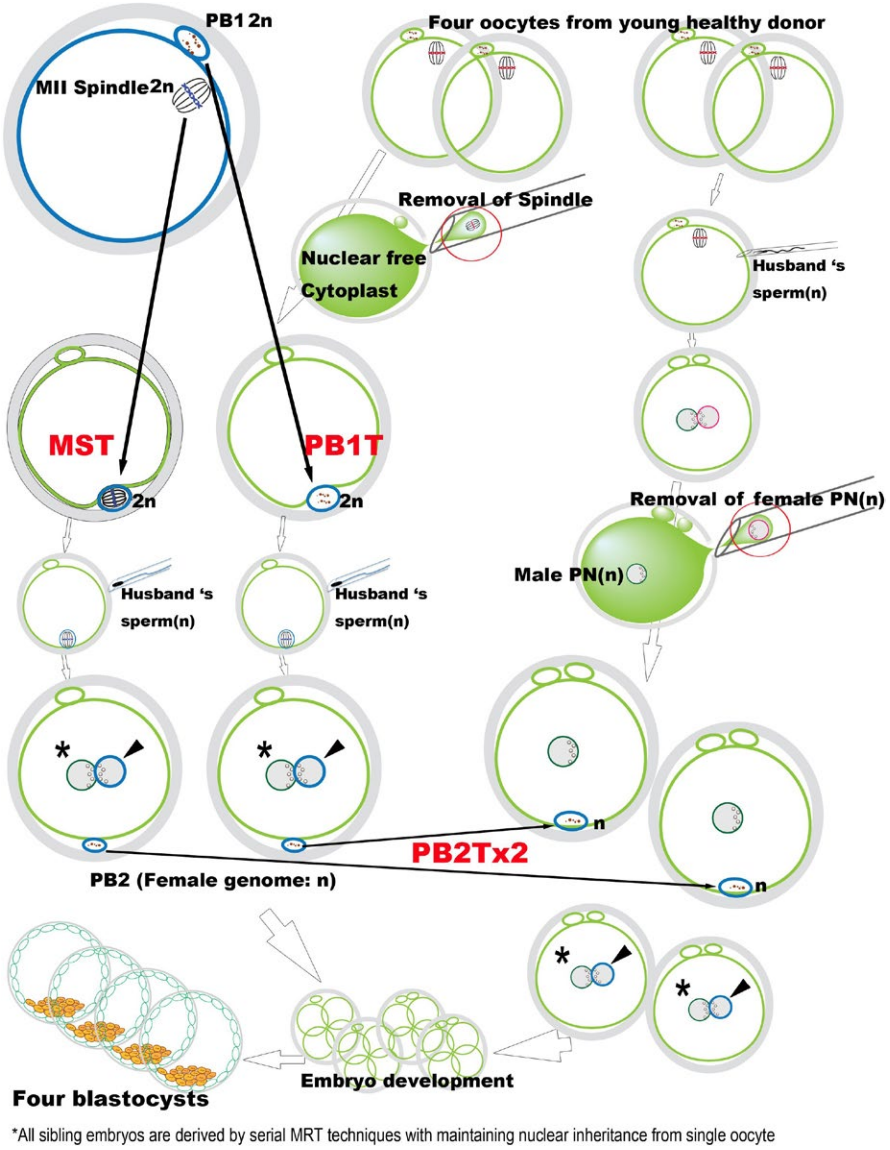

FIGURE 3 Schematic diagram representing creation of four sibling embryos from single oocyte. Theoretical possibility to exploit all genomic material from single oocyte, to creating four sibling embryos by MRT technique, is depicted. MII oocyte originally consists of two sets of diploid female genomes within MII spindle and PB1 (Blue single oocyte in upper left). Followed by enucleation of MII spindle from two donor MII oocytes (Green two oocytes in upper middle), MII Spindle and PB1 are transferred into donor cytoplast by MST and PB1T, respectively. MST can be omitted if cytoplasmic deficiency of original oocyte is not suspected. Newly reconstructed oocytes, by MST (or original oocyte w/o MST) and PB1T, are then fertilized by ICSI with husband's sperm. Meanwhile, two other donor oocytes are fertilized with husband's sperm to create 2PN zygotes (Green two oocytes in upper right). Followed by enucleation of female PN, PB2 x2 from PN stage zygotes created by MST (or original oocyte w/o MST) and PB1T, are then transferred into cytoplast with male PN left behind. While all four embryos harbor female haploid genome (arrowhead) from single oocyte and husband's genome (asterisk), these sibling embryos are not clone due to meiotic recombination

Tachibana, T. Kuno, K. Higashi, N. Shiga, Z. Watanabe, & N. Yaegashi, unpublished data). Such a strategy may be more acceptable owing its autologous cytoplasmic transplant, rather than germ line modification. Other than CT, PNT has already been applied to patients who have previously undergone repeated IVF cycle failures, but did not result in live birth due to premature delivery of twin pregnancy, albeit both babies were karyotypically normal.[76] On the other hand, impact on meiotic or mitotic chromosome segregation differs between the stage of zygotes or embryos, in which MRT is performed. Only GVT may have a chance to correct meiosis I error, while functional cytoplasm from young donors may reduce the incidence of aneuploidy resulting from meiosis II or mitotic errors in MST and PB1T. As PNT, PB2T and PNCT do not contribute to meiosis I and II, the expected effect is restricted to mitotic division during postzygotic cleavage. In this regard, GVT had been tested with human GV oocytes to evaluate its impact on meiotic maturation to the mature metaphase II oocytes. This purportedly showed feasibility of human GVT, where reconstructed GV oocytes utilizing GV intact from old oocytes (from women aged >38) with cytoplasm of young oocytes (from women aged <31) demonstrated normal polar body extrusion.[77] Liu et al[78] briefly reported potential of GVT oocyte to the blastocyst stage via fertilization, albeit limited data with small sample size. While none of GVT oocytes developed to the blastocyst stage, GVT followed by MST resulted in karyotypically normal blastocyst. Nevertheless, all other MRT techniques, including GVT, PBT and MST, potentially provide improved ART outcomes, with options of maintaining the nuclear DNA hereditary, as against the limitations of existing current ART applications, which are confined to donated embryos and oocytes. However, none of these have yet been evaluated with recurrent IVF failure and thus the concept has not been proven so far.

On the other hand, MRT may provide new options in ART for young cancer patients. Recent advances in the field of cryobiology have been contributed to the emerging fertility sparing treatment, called "Oncofertility treatment".[79] It has been demonstrated that spindles in suboptimally cryopreserved oocytes can be rescued by transplanting into fresh cytoplasts using MST in primates.[51] It is known that

cryopreserved unfertilized oocytes are more prone to poor ART outcomes compared to cryopreserved embryos. However, oocyte cryopreservation for young cancer patients is standard practice.[80] MST may confer better reproductive outcomes by replacing cryopreserved cytoplasm with fresh cytoplasm from young donors. Furthermore, MRT may rescue and solve fundamental thorny issues regarding the availability of oocytes for future ART, which is totally dependent on the number of originally cryopreserved oocytes. Unlike spermatogenesis where single spermatocyte produces four sperms, through consecutive meiosis, single oocyte produces only one mature oocyte and three-fourth of the genetic materials remains unutilized. However, BPT may enable the exploitation of the remaining three-fourth portion of the genetic material provided their integrity is maintained. In brief, PB1T provides an additional oocyte to be fertilized with the husband's sperm, and two zygotes can be theoretically created using two PB2T (one PB2 from original MII spindle and the other from PB1T spindle), with PN stage zygotes, created with husband's sperm and donor oocytes (Figure 3). This may be beneficial to patients of advanced maternal age, who have a low oocyte yield or are poor responders, commonly observed in ART cycles.[46]

While the theoretical advantages of MRT could be exploited in common ART, it is too premature to apply MRT for common infertility treatment at this point. Although, the use of MRT might be considered ethical for affected families, and in such cases, families are offered sufficient information, support, and comprehensive follow-up, the use of MRT in infertile patients does not guarantee those benefits. Furthermore, MRT may evoke another side of the ethical argument, with the fear of a slippery slope eventually creating the "designer baby," accompanied by the impending risk of violating the rights of the subsequent generation. However, if the technique is thoroughly validated in terms of safety and efficacy, and the rights for patient, baby and donors are legally secured, such as ICSI, MRT may also be accepted as standard practice in future. Obviously, further studies are needed to clarify some of the critical questions described above, especially mitochondrial biogenesis responsible for reversion.

6 | CONCLUSIVE REMARKS

In summary, a new assisted reproductive technique involving MRT, described here, could potentially prevent the transmission of mtDNA diseases and could be a new improvisation for modern ART. Since there are no fundamental cures for mitochondrial diseases so far, MRT could potentially provide significant health and social benefits to those affected families with the elimination of the risk of disease transmission, and thus, enable them to live a healthy life, free from progressive and lethal disorders. Meanwhile, a powerful genome editing tool called CRISPR has been applied to edit defective genes in human gametes/embryos or to investigate and understand human embryology.[81] Although mitochondrial diseases, caused by mutation in nuclear encoded genes, cannot be offered MRT, genome editing may offer curative treatment options for such patients in future.

With the advent of newer and safer technologies, a shift in the worldwide consensus on prohibition of gene modification in human gametes or embryos, toward allowing the correction of altered genes in gametes or in early embryos, is a possibility in the near future. Thus, the new era of ART, where cures can be provided in gametes or early zygotes, is underway. Authors would like to emphasize that recent fundamental discoveries were successfully made from indispensable and valuable quality human oocytes, donated for research. According to Jose Cibelli, "The need for human eggs for research is back. It seems like it never left the stage after all.".[82] We should realize that monkey is not a big mouse, as well as humans are not the exact counter parts of non-human primates. Therefore, further research using human gametes/zygotes, could be warranted to ensure the safety and efficacy of MRT.

7 | DECLARATION

For Mice PNCT, animal procedures were approved by the Institutional Review Board (IRB) approval at Tohoku university hospital (2016MdA-302). This article does not contain any studies with human subjects performed by the any of the authors.

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CONFLICT OF INTEREST

Authors have no conflict of interest to be declared.

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