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Recent Advances in Mitochondrial Disease

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Abstract

Mitochondrial disease is a challenging area of genetics because two distinct genomes can contribute to disease pathogenesis. It is also challenging clinically because of the myriad of different symptoms and, until recently, a lack of a genetic diagnosis in many patients. The last five years has brought remarkable progress in this area. We provide a brief overview of mitochondrial origin, function, and biology, which are key to understanding the genetic basis of mitochondrial disease. However, the primary purpose of this review is to describe the recent advances related to the diagnosis, genetic basis, and prevention of mitochondrial disease, highlighting the newly described disease genes and the evolving methodologies aimed at preventing mitochondrial DNA disease transmission.

INTRODUCTION

The Origin of Mitochondria

Mitochondria, essential organelles that are present in almost all eukaryotic cells (8, 49), are thought to have originated from free-living bacteria following a symbiotic event with a host cell approximately two billion years ago (4). In this hypothesis, known as the endosymbiotic theory, a proteobacterium was engulfed by endocytosis, providing the host with the ability to produce cellular energy in the form of ATP. The resulting double-membrane-bound organelle lacked a nucleus (a consequence of its prokaryotic origin) but contained its own genetic material. Over time, most of this genetic material was either transferred to the host's nuclear genome or lost as a consequence of functional redundancy. The resulting human mitochondrial genome is a double-stranded, circular molecule comprising 16,569 base pairs that was first sequenced more than 35 years ago (2, 3). This sequencing revealed that only 37 genes remain in the mitochondrial genome: 13 structural subunits required for oxidative phosphorylation, together with 2 mitochondrial rRNAs (12S and 16S) and 22 mitochondrial tRNAs necessary for the synthesis of these subunits.

The Dual Genetic Control of Mitochondrial Function

As described in MitoCarta2.0, the current estimate of the number of proteins required for mitochondrial function stands at 1,158 (9). The majority of these proteins are encoded by nuclear DNA (nDNA), but 13 are encoded by mitochondrial DNA (mtDNA); consequently, mitochondria are under the dual genetic control of both the mitochondrial and nuclear genomes. Reflecting their diverse functions, only ~150 mitochondrial proteins are directly involved in oxidative phosphorylation and ATP production (28). The remaining mitoproteome components include proteins involved in the assembly of the complexes, the maintenance and expression of mtDNA, intraorganellar protein synthesis, and mitochondrial dynamics (57). These nuclear-encoded mitochondrial proteins are translated on cytosolic ribosomes before import into the mitochondria through the TIM/TOM system (51).

Unlike the nuclear genes, which have coding exons interspersed with noncoding intronic sequence, mtDNA lacks intronic sequences, and the only major noncoding region is the 1.1-kb displacement loop, which contains two promoters for mtDNA transcription and an origin of replication (69). Another key difference between mtDNA and nDNA, and one that is critical for understanding the pathogenesis of mitochondrial disease, is the fact that mtDNA is a multicopy genome, ranging from just ~100 copies in sperm to >100,000 copies in mature oocytes. These mtDNA genomes can be either homoplasmic (where all genomes have an identical mtDNA genotype) or heteroplasmic (where there is a combination of genomes with different mtDNA genotypes) (**Figure 1**). Although nuclear genes are inherited in a Mendelian manner, the inheritance of mtDNA is strictly maternal, most likely because paternal mtDNA is actively eliminated from the oocyte after fertilization (72). Although the exact mechanism remains unknown, this elimination is thought to involve a mitochondrial endonuclease that degrades mtDNA within paternal mitochondria after fertilization (106).

Mitochondrial Function

Mitochondria are involved in several important cellular processes, including biosynthesis of iron-sulfur clusters (86), calcium homeostasis (42), and apoptosis (98), but the production of cellular ATP by oxidative phosphorylation is their most recognized role. This process requires five multisubunit protein complexes, four of which make up the mitochondrial respiratory chain (complex

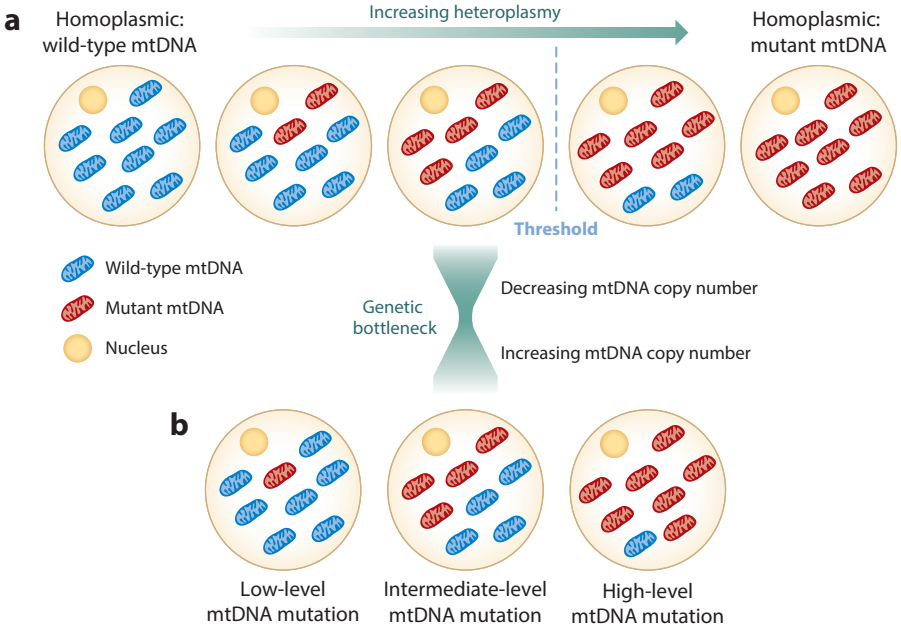

Figure 1

Mitochondrial DNA (mtDNA) heteroplasmy, the threshold effect, and the mitochondrial bottleneck. (*a*) Most pathogenic mtDNA mutations are heteroplasmic, with individual cells having different proportions of mutant and wild-type mtDNA. Increasing levels of heteroplasmy are often associated with increasing severity of mitochondrial disease. The cell can usually tolerate high levels of mutant mtDNA, and a critical threshold must be exceeded before a biochemical defect in the respiratory chain is detected. This threshold level is dependent on the mtDNA mutation but is generally thought to be between 60% and 80%. (*b*) The mitochondrial genetic bottleneck is thought to involve transmission of a restricted number of mtDNA molecules during development of the female germline, followed by mtDNA amplification that can result in human oocytes containing >100,000 copies of mtDNA. The presence of the bottleneck can give rise to oocytes that contain very different levels of heteroplasmy when compared with the levels of the mother, making genetic counseling very challenging.

I to complex IV) and are involved in transporting electrons through the complexes to the final electron acceptor—molecular oxygen. This transfer of electrons generates a proton gradient across the inner mitochondrial membrane that is harnessed by complex V (also known as ATP synthase) to synthesize ATP. These complexes have long been recognized as discrete entities embedded within the inner mitochondrial membrane, but they are also found as respiratory supercomplexes. The crystal structures of two functional supercomplexes (supercomplexes I and III) and the fully functional respirasome (consisting of complexes I, III, and IV) have recently been elucidated (12, 32, 55).

Mitochondrial Disease

Mitochondrial disease is the collective term for a heterogeneous group of genetic disorders characterized by defective oxidative phosphorylation. These disorders are clinically diverse and can manifest in the neonatal phase, childhood, or adulthood (28). Given that mitochondria are present in all cells of the body with the exception of red blood cells, the resulting clinical symptoms can present in isolated organs, but they often occur with multiple system involvement in organs with

high energy demands, such as the brain, skeletal muscles, and heart (28). Often there are limited genotype-phenotype correlations to direct molecular genetic diagnosis, and many phenotypes can be caused by defects involving numerous different genes. A good example of the latter is Leigh syndrome, a progressive neurodegenerative disorder of childhood and the most common clinical phenotype seen in pediatric mitochondrial disease, which can be caused by mutations in almost 80 different genes (53). The most detailed estimate of mitochondrial disease prevalence in adults is based on a cohort study in northeast England that revealed that the prevalence of mitochondrial disease is 2.9 cases per 100,000 individuals for nDNA mutations and 9.6 cases per 100,000 individuals for mtDNA mutations, with a further 10.8 per 100,000 individuals at risk of developing symptoms in later life or transmitting a pathogenic mutation (30). The frequency of mtDNA mutations in the population is much higher, with ~1 in 200 healthy individuals thought to carry a pathogenic mtDNA mutation at low levels of heteroplasmy (18). Although this low level of mtDNA mutation is below the critical threshold for disease expression, it does have potential implications for future generations. The prevalence of childhood-onset (<16 years) mitochondrial diseases has been estimated to range from 5 to 15 cases per 100,000 individuals (16, 81). Because childhood mitochondrial disease is predominantly due to nuclear gene defects, considerable variation in prevalence results from the presence of genetic founder mutations and high consanguinity, as in other nuclear gene defects.

There remain many challenges in our understanding of mitochondrial diseases, as highlighted in a recent review (28). Although our ability to obtain a genetic diagnosis has vastly improved (see below), there remains little in the way of effective treatment. Several treatment approaches have been successful in animal models (11, 23, 46, 47, 104), but there is little evidence that this has progressed to effective clinical trials in humans. The lack of effective treatment highlights the importance of developing methods to prevent transmission of mitochondrial disease.

THE GENETIC DIAGNOSIS OF MITOCHONDRIAL DISEASE

The dual involvement of the mitochondrial and nuclear genomes results in all possible inheritance patterns—maternal, X linked, autosomal recessive, autosomal dominant, and de novo occurrence. The application of next-generation sequencing (NGS) is particularly effective for heterogeneous conditions such as mitochondrial disease, in which a large number of genes can be interrogated simultaneously and in the diagnostic setting. NGS technologies are revolutionizing genetic testing, with prioritized Sanger sequencing of candidate nuclear genes being replaced with powerful, high-throughput analysis. A variety of options are currently being implemented, including targeted panels of candidate genes (1), unbiased whole-exome sequencing (33), and whole-genome sequencing (37).

Custom, panel-based NGS strategies can be very successful for providing a rapid genetic diagnosis in the clinical setting, but this success depends on the degree of characterization to ensure the appropriate candidate genes are targeted. Stratification by respiratory chain defect can be appropriate for many patients for whom muscle biopsy is available, but even then it may be misleading; several patients with an isolated complex I deficiency in fact have a defect of mitochondrial translation (34). This strategy can also be ineffective for genes that exhibit inconsistent biochemical profiles (91) or patients whose presumed mitochondrial presentation is subsequently established to have a (presumed) non-mitochondrial etiology [e.g., *MECP2* mutations that cause Rett syndrome (50)]. Stratification by clinical phenotype is similarly complicated by genetic heterogeneity (7).

One solution to the stratification dilemma—and one that has been successfully implemented for the analysis of other heterogeneous Mendelian disorders—is a combination of unbiased whole-exome sequencing with targeted analysis of virtual gene panels (56, 102), which allows informative

reporting of negative results and removes the possibility of incidental findings. Further analysis of whole-exome sequencing data for patients who lacked a diagnosis following virtual panel analysis could be subsequently undertaken in a research setting. Indeed, most of the candidate genes included on diagnostic virtual panels have their origins in research, which has been incredibly fruitful in elucidating genes involved in human pathology, including heterogeneous mitochondrial clinical phenotypes such as cardiomyopathy, with associated mutations identified in *AARS2* (31), *MRPL3* (22), *MTO1* (24), and *ACAD9* (33). The recent report of both biallelic and de novo dominant mutations affecting the novel mitochondrial disease gene *ATAD3A* (36) highlights the importance of thorough analysis of single heterozygous variants in presumed recessive etiologies and the concurrent analysis of parental samples; this is further highlighted by the recent expansion of the *SLC25A4* mutation spectrum, which now includes de novo dominant mutations (92) in addition to the dominant and recessive mutations previously reported.

The clinical and genetic diversity of mitochondrial disease results in a complicated diagnostic algorithm that is very much tailored to the specific patient using the referral information alongside all available test results. For example, an adult patient who presents with chronic progressive external ophthalmoplegia, and in whom numerous cytochrome *c* oxidase–deficient fibers (a pathological hallmark of mtDNA dysfunction) have been identified on muscle histochemistry, would undergo common mtDNA point mutation and rearrangement screening, whereas a pediatric patient who presented with Leigh syndrome and evidence of complex IV deficiency, following muscle histochemistry and biochemistry, would undergo mtDNA genome analysis and sequencing of the common nuclear Leigh syndrome gene *SURF1* (107) prior to whole-exome sequencing.

Given the small size of the mtDNA genome, it is often sequenced in patients with a clinical diagnosis of mitochondrial disease to exclude a primary mtDNA defect before the nuclear genes are scrutinized. Sanger sequencing was previously the preferred method for most diagnostic laboratories, but NGS-based testing is becoming more prevalent (90); this testing not only identifies a primary mtDNA defect, but also provides an accurate measure of heteroplasmy. Indeed, the routine diagnostic pipeline used within our National Health Service (NHS) Highly Specialised Mitochondrial Diagnostic Laboratory in Newcastle for mtDNA analysis has recently been revised, with long-range polymerase chain reaction and NGS replacing Sanger sequencing as the standard procedure for sequencing mtDNA genomes.

THE EVOLUTION OF MITOCHONDRIAL DISEASE GENE DISCOVERY

In addition to vastly improved diagnosis of mitochondrial disease caused by nuclear gene defects, there has been considerable progress in identifying the nuclear genes that contribute to mitochondrial function. This research has involved a variety of different approaches. The mitoproteome was originally thought to contain approximately 1,500 proteins; as mentioned above, however, the current inventory in MitoCarta2.0 details 1,158 proteins (9). These proteins were ascribed mitochondrial function using machine learning protocols that were trained to predict mitochondrial localization using a known mitochondrial protein data set. Additionally, functional evidence was provided by tandem mass spectroscopy and homology between predicted proteins and those of *Rickettsia prowazekii* and yeast (9). This number will probably be refined, because there is an estimated false positive rate of 5% and some genes are likely to be absent owing to the limitations of the system; a detection pipeline focused on mitochondrially localized proteins will likely miss proteins with extramitochondrial localization or those that are only transiently localized to the mitochondrion (9).

Of the proteins cataloged in MitoCarta2.0, the vast majority have not yet been linked to human disease. In fact, as of December 15, 2016, mutations in just 281 mitochondrial proteins

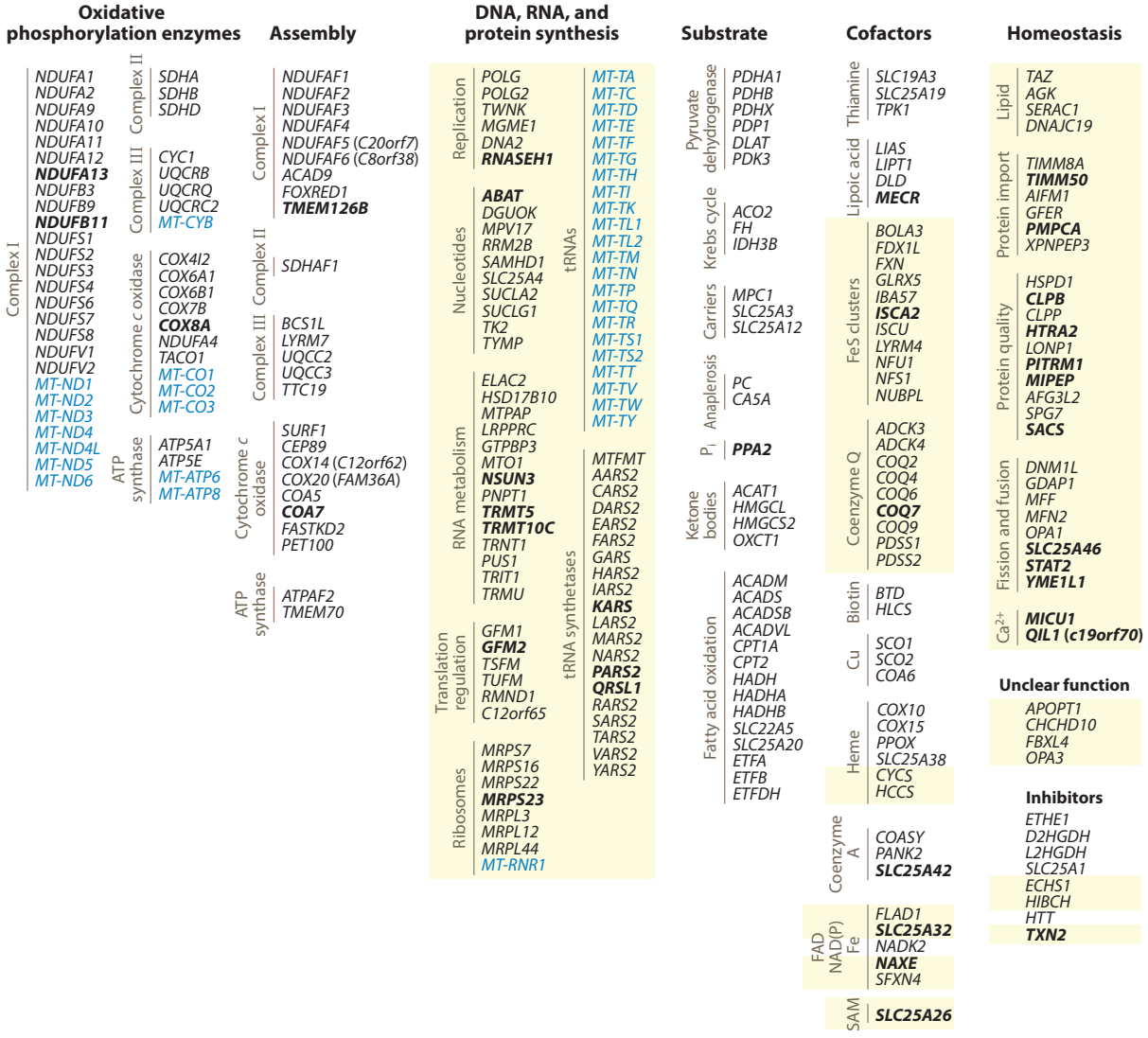

Figure 2

Monogenetic defects associated with human mitochondrial disorders. As of December 15, 2016, defects had been reported in 281 genes in patients with mitochondrial disease, including in 36 genes from the mitochondrial genome (shown in *blue*). The genes that are most commonly associated with multiple oxidative phosphorylation complex deficiencies are highlighted in yellow. Genes in boldface indicate recent additions to the original figure. Adapted from Reference 63 with the kind permission of Professor Johannes Mayr.

had been established to cause human disease (63) (**Figure 2**). Analysis of patient samples remains an important method for identifying and characterizing novel mitochondrial disease genes, but it represents just one method in an increasingly diverse range of techniques. The current "-omics" era continues to provide a wealth of novel candidate genes, several of which have been implicated in human mitochondrial disease pathology since their discovery.

Even within the current version of MitoCarta2.0, a significant proportion remain unclassified (83), and the characterization of these orphan proteins remains a key objective. Recent successes

in characterizing these proteins have used various methodologies. In the field of complex I assembly alone, novel disease genes have been identified by three different methods, including CRISPR/Cas9 knockout and profiling to identify a novel complex I assembly factor, DMAC1 (87); complexome profiling to identify *TMEM126B* (38); and proteomic profiling of protein-protein interactions to identify *C17orf89* (21). Another novel technique that has proved effective for novel mitochondrial disease gene discovery is the genome-wide CRISPR/Cas9 screening of cells that are viable on glucose but unable to grow on galactose, consistent with defective oxidative phosphorylation (5). Dying cells are selected on the basis of expressing a cell death marker, annexin V, and subjected to NGS to characterize the CRISPR/Cas9-induced genetic lesions. Although the sensitivity of the assay was 50%, it identified a further five genes encoding mitochondrial proteins, including *FASTKD2*, which had been previously identified through analysis of affected patients (25). Interestingly, this so-called death screen also identified *TMEM261*, encoding DMAC1, as a mitoproteome constituent despite its absence in MitoCarta2.0.

The interface between research and patient diagnostics remains crucial to linking novel disease candidates with human disease. Following identification of *TMEM126B* as a complex I assembly factor, two groups reported biallelic mutations in a cohort of undiagnosed complex I–deficient patients. Mutations were identified by either whole-exome sequencing or targeted NGS, and their pathogenicity was validated by blue native polyacrylamide gel electrophoresis and Western blotting of patient tissues (1, 77).

In addition to identifying novel disease genes, these high-throughput -omics strategies have recently identified a potential therapeutic agent for mitochondrial disease patients with complex I deficiency (6). CRISPR/Cas9 knockout and compound library screening using complex I–deficient cells highlighted the same potential target in the form of a bromodomain protein. CRISPR/Cas9 knockout of BDR9 cells showed increased gene expression, whereas complex I–deficient cells survived following treatment with the repurposed cancer compound I-BET 525762A, a bromodomain protein inhibitor. The mechanism is consistent with upregulation of mitochondrial gene expression, thereby remodeling oxidative phosphorylation to bypass the deficient complex I. Although this technique remains untested in the clinical setting, it represents a welcome therapeutic possibility for patients with mitochondrial disease.

MITOCHONDRIAL DNA DISEASE

The mitochondrial genome has a high mutation rate, 10–17 times that of the nuclear genome (94), which is assumed to be the result of increased oxidative damage caused by reactive oxygen species produced during oxidative phosphorylation. The first pathogenic mtDNA mutations were reported in 1988 (41, 97); since then, an increasing number of mtDNA mutations associated with a wide variety of clinical symptoms have been identified in patients with mitochondrial disease. To date, more than 260 different pathogenic mutations have been characterized [see MITOMAP (59)], and the number continues to rise (52, 64). These mutations can be classified into three types: point mutations in protein-coding genes, point mutations in genes involved in protein synthesis (tRNA or rRNA genes), and mtDNA rearrangements, including mtDNA deletions and insertions. Point mutations in mtDNA are typically maternally inherited, although de novo mutations can occur (75), whereas mtDNA rearrangements are not usually inherited and tend to be de novo large-scale mtDNA deletions.

Some patients with mitochondrial disease are homoplasmic for the mtDNA mutation, but it is more common for patients to be heteroplasmic and have a mixture of both mutant and wild-type mtDNA. In the presence of heteroplasmy, the proportion of mutant to wild-type mtDNA is important in disease expression, with higher levels of mutant mtDNA often associated with more

severe clinical symptoms. This is referred to as the threshold effect, whereby the level of mtDNA mutation must exceed a critical threshold before a biochemical defect in the respiratory chain is detected (**Figure 1**). The threshold level is generally thought to be 60–80% mutant mtDNA but may vary among different organs and individuals. The threshold level also depends on the particular mtDNA mutation, with a mutation load of 60–80% often required for the onset of clinical symptoms (17, 79, 100). In addition, heteroplasmy levels may change over time within an individual. In dividing cells, random segregation of mitochondria can result in daughter cells containing different levels of the mtDNA mutation. The mechanisms behind this segregation are not fully understood, but in vitro studies have demonstrated that the mutation level in single cells can fluctuate in either direction (up or down) between periods of stable heteroplasmy (73). The mutation level also decreases in fast-dividing tissues, such as blood (74). In nondividing (postmitotic) cells, mutant genomes can reach high levels within individual cells owing to clonal expansion. This expansion is thought to be dependent on relaxed replication of the mitochondrial genome caused by random genetic drift of mutant genomes (19).

Rapid shifts in heteroplasmy levels can occur both within and between generations. This phenomenon, first described in Holstein cows transmitting heteroplasmic mtDNA variants (70), can be explained by the transmission of a restricted number of mitochondrial genomes and is known as the mitochondrial genetic bottleneck (**Figure 1**). Evidence to support the existence of the bottleneck was provided for the first time by studies in mice, which revealed a dramatic reduction in the amount of mtDNA present within the germline (10, 15, 96). Although the precise mechanism remains to be defined, for women who carry pathogenic heteroplasmic mtDNA mutations, the bottleneck can result in mature oocytes containing vastly different levels of heteroplasmy. Studies of human pedigrees transmitting pathogenic mtDNA mutations confirm this variable inheritance but also reveal that different mtDNA mutations can segregate at different rates (101). This implies that the size of the bottleneck can vary depending on the particular mtDNA mutation, which has obvious implications when considering the most appropriate reproductive option to reduce the risk of mitochondrial disease in the offspring. For women who carry homoplasmic mutations, the mtDNA mutation will inevitably be transmitted to all offspring, but the risk of disease can still be difficult to predict because of the variable penetrance of the mutation. The risk is even more difficult to predict for heteroplasmic mutations, however, because the bottleneck can result in offspring with levels of heteroplasmy that are very different from those of the mother, making genetic counseling extremely complicated.

REPRODUCTIVE OPTIONS FOR FAMILIES WITH MITOCHONDRIAL DISEASE

In the absence of a cure for mitochondrial disease, reproductive options that aim to reduce the risk of severe disease in the offspring are a valuable resource for at-risk couples. Where a genetic diagnosis exists, genetic counseling enables calculation of the recurrence risk and stresses the importance of making a genetic diagnosis in patients with mitochondrial disease. For inherited nuclear mitochondrial disorders, the recurrence risk depends on the pattern of inheritance. Risk calculation for women who harbor mtDNA mutations is considerably more challenging, partly because of the unpredictable transmission of mtDNA mutations through the genetic bottleneck, but several reproductive options are available (**Figure 3**). One such option, which prevents inheritance of the mtDNA mutation and eliminates any risk of mitochondrial disease, is oocyte donation. For women who wish to have a genetically related child, however, other options are available, including prenatal diagnosis and preimplantation genetic diagnosis. The availability

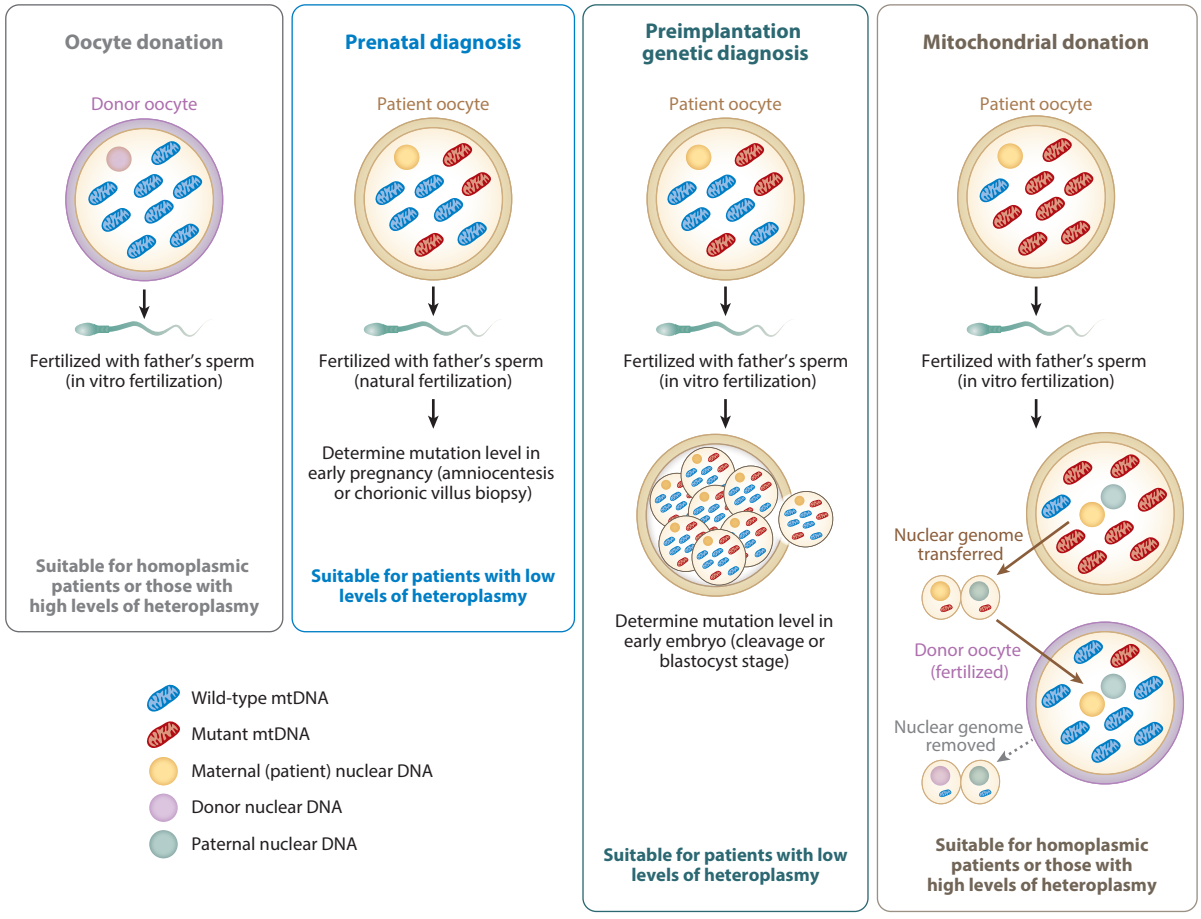

Figure 3

Reproductive options for women with pathogenic mitochondrial DNA mutations.

of another technique, known as mitochondrial donation, represents a significant advance in the prevention of mitochondrial disease.

Prenatal Diagnosis

Prenatal diagnosis for mitochondrial disease is a current reproductive option for couples at risk of transmitting nDNA or mtDNA mutations to their offspring. Prenatal diagnosis is typically performed by biopsy of fetal material from an ongoing pregnancy (either chorionic villus biopsy at 10–12 weeks' gestation or amniotic fluid sampling at 15–22 weeks' gestation) and allows the risk of mitochondrial disease in the developing fetus to be assessed. Risk calculation for ongoing pregnancies is relatively straightforward for nDNA-associated mitochondrial disease but can be more complex for mtDNA mutations. For this calculation to be accurate, it is important that the heteroplasmy level measured in the prenatal sample represents the heteroplasmy level within the fetus and that this level does not change over time. Although available data are limited, heteroplasmy levels determined in prenatal samples do appear to correlate with fetal tissues obtained

following termination of a pregnancy based on the prenatal result (20, 35, 85), although exceptions can occur (67). Variation in mutation levels within different regions of the placenta has also been observed, which has obvious implications for prenatal testing by chorionic villus biopsy (62), but does not appear to be a consistent finding (67). A small number of studies have reported postnatal follow-up data after prenatal diagnosis (45, 54, 67, 85, 100), indicating that the mutation level remains relatively constant during prenatal development. A recent review of 17 prenatal tests performed for several different mtDNA mutations concluded that prenatal diagnosis is a reliable option, and demand has been increasing among families affected by mitochondrial disease (68). In addition, prenatal diagnosis is useful for reassurance in subsequent pregnancies following the identification of a de novo mtDNA mutation in a previously affected child (75).

Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis for mitochondrial disease is another reproductive option for couples at risk of transmitting nDNA or mtDNA mutations to their offspring (26, 27, 80, 82). This technique involves genetic testing of cells removed from early embryos obtained during in vitro fertilization procedures and allows the selection of embryos with a reduced risk of mitochondrial disease for implantation. Although experience is still limited, preimplantation genetic diagnosis has been used for a small number of different mtDNA mutations and has successfully identified suitable embryos for transfer with undetectable or low levels of mtDNA mutation (39, 67, 76, 84, 93). For the majority of preimplantation genetic diagnosis cases, the biopsy has been performed on cleavage-stage embryos at the eight-cell stage of development, which involves removing one or two blastomeres for testing. For this technique to be reliable, the mtDNA mutation level in the biopsied blastomere must be representative of the mutation level in the remaining embryo, assuming that the mutant mtDNA segregates uniformly across all blastomeres during early embryo cleavage. Limited studies in human embryos have revealed that this is often the case, and although exceptions have been reported (76, 95), there are generally low levels of variation in heteroplasmy among blastomeres of cleavage-stage embryos (67, 76, 93).

Alternatively, the biopsy can be performed at the blastocyst stage of development, which involves removing a small number of trophectoderm cells. This technique requires that the mutation level is consistent throughout the trophectoderm and corresponds to the level in the inner cell mass, which limited data suggest is the case in human embryos (93). There are only two reported cases of blastocyst biopsy for mitochondrial disease caused by an mtDNA mutation (39, 93), and the outcome of one is subject to some disagreement (66). Even so, preimplantation genetic diagnosis for mitochondrial disease has resulted in the birth of healthy children with a lower risk of mitochondrial disease and so provides a promising option for some affected families. For this approach to be successful, however, the mtDNA mutation carrier must produce embryos with heteroplasmy levels below the critical threshold for disease expression; some guidelines have suggested a threshold of 18% (40), but the level will vary depending on the mtDNA mutation.

Mitochondrial Donation

Mitochondrial donation is the broad name for a novel in vitro fertilization–based technique that has the potential to prevent transmission of mitochondrial disease caused by an mtDNA mutation and could benefit ~150 women each year in the United Kingdom (29). The technique involves removing the nuclear genome from an oocyte or zygote taken from a woman with an mtDNA mutation and transferring it to an enucleated oocyte or zygote from a healthy donor that has had its own nuclear genome removed. This results in a reconstituted oocyte or zygote that contains

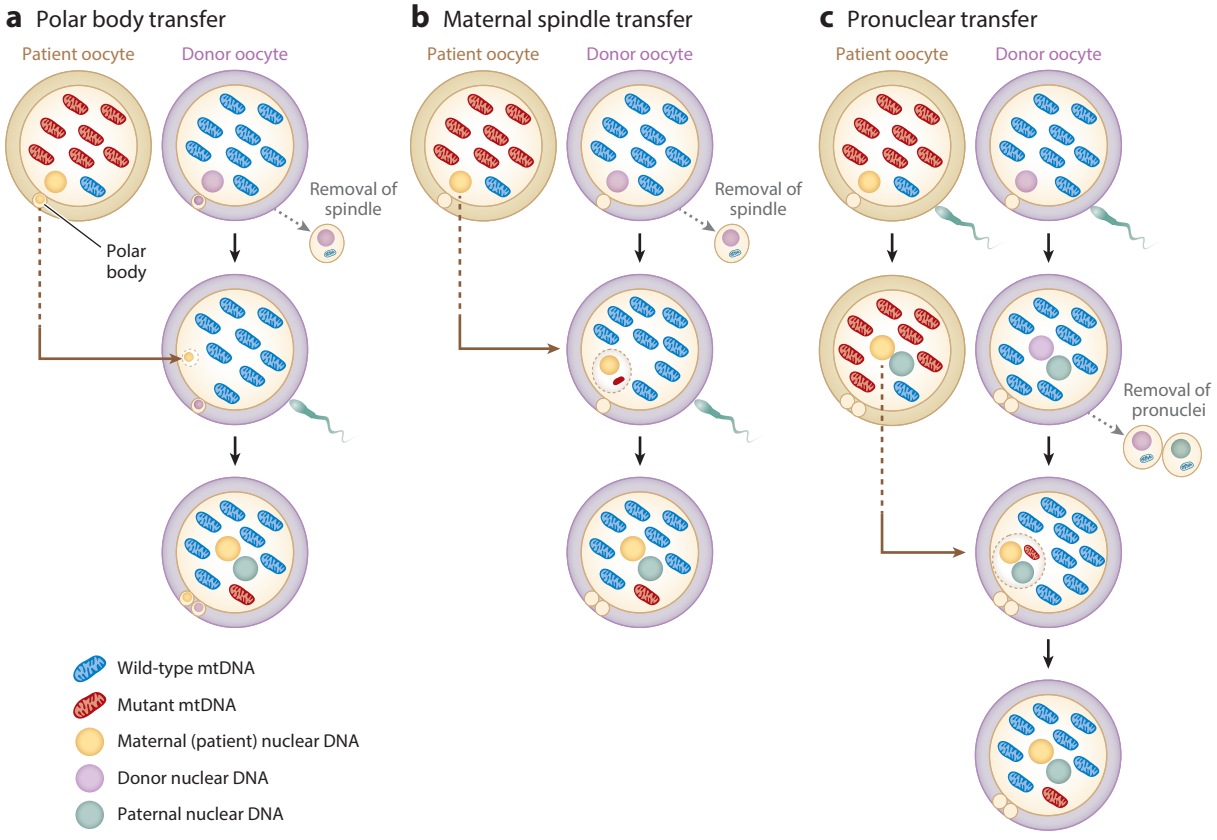

Figure 4

Mitochondrial donation techniques. Mitochondrial donation involves the transfer of nuclear DNA (nDNA) from an oocyte or zygote carrying a pathogenic mitochondrial DNA (mtDNA) mutation to an enucleated donor oocyte or zygote with wild-type mtDNA. The resulting embryo contains predominantly wild-type mtDNA associated with a much lower risk of mitochondrial disease. nDNA can be transferred between unfertilized oocytes using (*a*) polar body transfer or (*b*) maternal spindle transfer, or between fertilized zygotes using (*c*) pronuclear transfer.

nDNA from the prospective parents but predominantly wild-type mtDNA from the donor. These manipulations can be performed between oocytes or zygotes at the one-cell stage of development, either before fertilization using metaphase II oocytes [polar body transfer (PBT) or maternal spindle transfer (MST)] or immediately after fertilization using pronucleate stage zygotes [pronuclear transfer (PNT)] (**Figure 4**). The resulting embryo is then transferred for implantation, resulting in a child that is genetically related to both parents but has a reduced risk of mitochondrial disease.

The potential for mitochondrial donation to prevent transmission of mitochondrial disease has been demonstrated in both animal and human experiments. Early studies in mice developed an efficient method for transferring pronuclei between zygotes (65), which was subsequently used to prevent transmission of mitochondrial disease in mice harboring an mtDNA rearrangement (mito-mice) (78). Later, PNT was optimized for the first time using human zygotes (14). Although this study used abnormal zygotes with limited developmental potential, the proof-of-principle experiments revealed that manipulated zygotes were compatible with onward development and capable of reaching the blastocyst stage in culture. The authors concluded that, although some

mtDNA will inevitably be transferred with the nuclear genome during the procedure, PNT had the potential to prevent transmission of mtDNA disease in humans because the average level of mtDNA carryover was <2%, with many embryos having undetectable levels.

The first preclinical study to address the safety and efficacy of PNT was recently published and revealed that a refined technique, early PNT (ePNT), was required when using normally fertilized human embryos, resulting in efficient development to the blastocyst stage and <2% mtDNA carryover in the majority of ePNT blastocysts (44). In addition, there were no detectable effects on aneuploidy or gene expression, providing further information on the safety of the technique. However, an embryonic stem cell line derived from a PNT blastocyst exhibited a progressive increase in heteroplasmy, suggesting that PNT may reduce the risk of mitochondrial disease but may not guarantee prevention; as such, PNT should be considered in combination with prenatal testing.

The potential for MST to prevent transmission of mtDNA disease has been demonstrated in nonhuman primates following development of an efficient method to transfer the metaphase II spindle between rhesus macaque oocytes (89). The study revealed that reconstituted oocytes developed to the blastocyst stage at a rate similar to that of control oocytes and were capable of producing healthy offspring that contained undetectable levels of spindle donor mtDNA. A three-year follow-up study reported that the overall health of the MST offspring was comparable to that of age-matched controls and that the level of mtDNA carryover did not change significantly with age (88). The same technique was subsequently performed using human oocytes, and although the spindle transfer procedure was highly efficient, fewer than half of the oocytes fertilized normally and developed to the blastocyst stage at a rate similar to that of controls (88). However, the level of mtDNA carryover was always 1% or lower, consistent with the results of several similar studies (71, 103), indicating that MST has the potential to prevent transmission of mtDNA disease. Further confirmation of this finding was provided in a recent study that performed MST using human oocytes harboring pathogenic mtDNA mutations and found that the resulting embryos contained <1% mtDNA carryover (48). This level of mtDNA carryover remained stable in the majority of embryonic stem cell lines derived from MST blastocysts but appeared to gradually increase in some (48, 103). Several factors could contribute to this increase in heteroplasmy, including the possibility that specific mtDNA variants could lead to preferential amplification of one particular mtDNA over another, but the underlying mechanisms remain unknown and require further research. The clinical relevance of this observation in embryonic stem cell lines is also contentious, but selection of compatible mtDNA donors based on their mtDNA sequence could help minimize any potential risk.

The potential for PBT to prevent transmission of mtDNA disease has not been investigated as extensively as the other techniques. Studies in mice revealed that the first polar body can be used as a source of the nuclear genome for transfer, producing reconstituted oocytes that are capable of fertilization and efficient development to the blastocyst stage (99). Furthermore, live offspring were obtained with undetectable levels of mtDNA carryover in all tissues tested, suggesting that PBT has the potential to prevent mtDNA disease. Translation of this technique into human oocytes, however, has revealed some limitations that may preclude it from clinical application, including a low blastocyst rate and embryonic stem cell derivation rate, possibly because of poor blastocyst quality (60).

There have been only limited preclinical studies for MST, but in 2016, the first child was born in which this technique was used to prevent transmission of mitochondrial disease. This did cause controversy, however, because the United States–based team performed the technique in a different country owing to the current lack of approval for MST in the United States. Although little scientific detail is available about the procedure, a published abstract reported that an MST

blastocyst containing a 5.73% mutation load was transferred for implantation and that the child was apparently healthy at five months of age (105). In the United Kingdom, mitochondrial donation has undergone an extensive review over many years; although the technique was permitted for human use by the UK Parliament in 2015 (13), it has not yet been clinically applied.

The latest review on the safety of mitochondrial donation was recently published by the Human Fertilisation and Embryology Authority (HFEA) (43), which recommended that mitochondrial donation can now be offered as a "clinical risk reduction treatment for carefully selected patients" (p. 7). The review also highlighted some further recommendations, including that consideration be given to the mtDNA sequence of the mitochondrial donor as a precautionary step to limit any potential mismatch, and that prenatal testing be offered to anyone who becomes pregnant following mitochondrial donation. The review also advised that patients and their offspring should be encouraged to participate in long-term follow-up. Following the publication of this report, the HFEA approved the use of mitochondrial donation in the United Kingdom, allowing fertility clinics for the first time to apply for a license to perform the procedure. The HFEA must approve every clinic, and licenses are offered only on a patient-by-patient basis.

Potential Gene Therapy to Prevent Transmission of Mitochondrial Disease

The possible use of gene therapies to prevent transmission of mitochondrial disease is an area of research that has advanced over the last few years. The approach involves the elimination of mutant mtDNA from oocytes using nucleases engineered to bind and remove (through double-strand breaks) specific mutant mtDNA sequences. These nucleases include mitochondrially targeted zinc-finger nucleases (mtZFNs) and mitochondrially targeted transcription activator–like effector nucleases (mitoTALENs), both of which selectively remove mutant mtDNA and reduce heteroplasmy levels in patient-derived transmitochondrial cybrid models (5, 25, 26, 42, 68). The same strategy has been applied to heteroplasmic mouse oocytes containing two different mtDNA genomes, revealing that nucleases can be used to prevent germline transmission of specific mtDNA sequences and reduce the level of pathogenic mtDNA mutations, as performed in heteroplasmic mouse oocytes generated by fusion with patient cells (79). Although the potential of these techniques to reduce transmission of mtDNA mutations is clear, it is important that the selective removal of mutant mtDNA in oocytes be followed by subsequent repopulation with wild-type mtDNA, resulting in an overall decrease in heteroplasmy. Given the apparent absence of mtDNA replication in oocytes and preimplantation-stage embryos, the eliminated mutated mtDNA may not be effectively replaced with wild-type mtDNA, which could limit the use of these techniques. This would be especially true for oocytes with high heteroplasmy levels, because it could result in embryos that are no longer viable owing to a depletion in mtDNA copy number.

The CRISPR/Cas9 system is an alternative gene therapy approach that uses a specific single guide RNA (sgRNA) to direct a Cas9 nuclease to the target mutation, where it induces a double-stranded DNA break that is subsequently repaired (75). The widespread use of CRISPR/Cas9 for nuclear genome editing confirms the value of this technology (58, 61), but it has yet to be applied to mtDNA. The challenge of the CRISPR/Cas9 system to prevent transmission of mitochondrial disease is that there is no evidence that the sgRNA species can cross the mitochondrial membrane, and there have been no reports of a successful application of this technique to correct mtDNA mutations.

CONCLUSION

Research into mitochondrial diseases has seen remarkable advances over the last five years. The ability to identify and characterize novel mitochondrial genes and mutations within these genes

has improved dramatically and provided a better understanding of both mitochondrial function and dysfunction. The application of NGS technologies has vastly improved the genetic diagnosis of mitochondrial disease, which in turn has allowed patients to consider an expanding number of reproductive options to reduce the risk of having a severely affected child. Women who transmit high levels of an mtDNA mutation have had limited options, but recent advances have confirmed that mitochondrial donation can prevent disease transmission, providing an alternative reproductive choice.

Many challenges remain. Although we are increasingly able to establish a genetic diagnosis in patients with mitochondrial disease, there are still patients for whom diagnosis is challenging. Cases involving late-onset, autosomal dominant conditions for which familial samples are not available are perhaps the most difficult group. There is little doubt that further advances in NGS, in particular the bioinformatics support, will lead to changes in the diagnostic algorithm and an increasing reliance on genetics rather than more conventional studies based on, for example, muscle biopsy.

One of the other challenges for the field is understanding the clinical variation in mitochondrial disease. A good example is Leber hereditary optic neuropathy, a maternally inherited form of blindness that affects retinal ganglion cells. More than 90% of patients have one of three, often homoplasmic, pathogenic mtDNA mutations (m.3460G>A, m.11778G>A, and m.14484T>C). The causal mutation is present in all family members, but only 50% of males and 10% of females develop the subacute visual loss. The same remarkable clinical variability is seen in patients with the common m.3243A>G mtDNA mutation: Patients with similar levels of heteroplasmy have very different phenotypes. Identifying the cause of this variation is crucial in terms of providing advice to patients and offering potentially preventative treatment strategies.

The biggest challenge in mitochondrial disease, however, is the lack of effective treatments. Despite the major advances highlighted in this review, for the vast majority of patients, therapy is limited to management of the complications of mitochondrial disease. The development of treatments may be highly disease specific (for example, bone marrow transplantation in patients with thymidine phosphorylase deficiency resulting from *TYMP* mutations) or may involve more generic strategies aimed at improving oxidative phosphorylation in patients with many different genetic defects. The development of large patient cohorts and registries will facilitate clinical trials in the future, and we hope that the next five years will see major advances in this area.

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