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Current strategies towards therapeutic manipulation of mtDNA heteroplasmy

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1. ABSTRACT

Mitochondrial disease is a multifactorial disorder involving both nuclear and mitochondrial genomes. Over the past 20 years, great progress was achieved in the field of gene editing which raised the possibility of partial or complete elimination of mutant mtDNA that causes disease phenotypes. Each cell contains thousands of copies of mtDNA which can be either wild-type (WT) or mutant, a condition called heteroplasmy. As there are multiple copies of mtDNA inside a cell, the percentage of mutant mtDNA can vary and a directional shift in the heteroplasmy ratio towards an increase of WT mtDNA copies would have therapeutic value. Gene editing tools have been adapted to translocate to mitochondria and were able to change heteroplasmy in a predictable manner. These include mitochondrial targeted restriction endonucleases, Zinc-finger nucleases, and TAL-effector nucleases. These procedures could also be adapted to reduce the levels of mutant mtDNA in embryos, offering an option to the controversial mitochondrial replacement techniques during *in vitro* fertilization. The current strategies to induce heteroplasmy shift of mtDNA and its implications will be comprehensively discussed.

2. INTRODUCTION

Mitochondria are unique organelles that actively participate in cell metabolism and homeostasis regulation. Most of the energy production is achieved by electron transfer in the respiratory chain through oxidative phosphorylation (OXPHOS) (1, 2). Aerobic tissues rely on OXPHOS for ATP production and the overall process is highly controlled involving the integration and balance of the oxidation of fatty acids, pyruvate, ketoacids and a range of other intermediary metabolites and regulatory ions such as calcium.

The human mitochondrial DNA (mtDNA) is a small double stranded circular genome which is maternally inherited (3). Each mammalian cell contains in average one thousand copies of mtDNA and each molecule contains 37 genes, encoding ribosomal RNAs (12S and 16S), 22 transfer rRNAs (tRNAs) and 13 OXPHOS complexes subunits (4). Defects in the mtDNA, both point mutations and large scale rearrangements, have been associated with severe mitochondrial syndromes, which cause diverse phenotypes including: stroke-like episodes, cardiomyopathies, ragged red fibers, optic neuropathies, neurodegenerative disorders, among others. When pathogenic mutations occur in the mtDNA most often both mutant and wild-type copies co-exist within the same cell, a phenomenon known as heteroplasmy (5), and, in general, only when the mutation load is higher than approximately 80% symptoms manifest (6). Mitochondrial diseases have a

prevalence of roughly one in 5000 live births, with only 8% of children with mitochondrial disease diagnosed before one year of age, while 28% are diagnosed by age 18 (7). Currently, there are no effective strategies to cure mitochondrial disease and, in spite of the advances in genetics and biotechnology, there are still some gaps in the understanding of mitochondrial genetics. For instance, we do not know what controls mtDNA copy number (8), and mechanisms of mtDNA replication are still controversial (9, 10). Moreover, there is an emerging need for *in vivo* models and studies that could be used to explore these crucial questions of mitochondrial biology.

During the past 16 years our lab and others have been focusing in the use of endonucleases to target mitochondria and induce double strand breaks (DSB) in the mtDNA. Taking advantage of the fact that mitochondria lack an established DSB repair mechanism, it has been shown that mtDNA is quickly degraded after a DSB (11). Therefore, heteroplasmy can be manipulated and the mutant genomes can be efficiently eliminated through cleavage of mutant mtDNA and repopulation of the cells with WT mtDNA. Recently, a new door has been open regarding translation of these techniques into the clinics by the development of precise DNA editing tools, which can be targeted to mitochondria to promote DSB in the mtDNA. The current gene editing tools to shift mDNA heteroplasmy, their molecular and cellular implications and possible applications will be comprehensively discussed in this review.

2.1. Mitochondrial energy production and genetics

Mitochondria are under a dual genetic control of both mitochondrial and nuclear genomes. Human mtDNA is a 16,569-bp double stranded circular genome which is located within the matrix and is packaged in protein-DNA complexes known as nucleoids. Each nucleoid contains one or two mtDNA molecules (12). Because of its proximity to the electron transport chain, mtDNA is highly exposed to oxidative damage and therefore prone to mutations (13, 14). In fact, many studies suggested that mtDNA has a higher mutation rate when compared to the nuclear genome (13) and repair is less efficient than in the nucleus (15, 16). Nonetheless, other studies contradict this concept. For instance, the biomarker 7,8-dihydro-8-oxoguanine (8-oxoG), the oxidized form of guanine, has been a pivotal marker for measuring the effect of endogenous oxidative damage to DNA and it is often associated with carcinogenesis and neurodegenerative diseases. Its highly mutagenic potential is believed to be caused by misincorporation of an adenine instead of cytosine causing G: C→T: A transversion mutations. It has been reported that repair of the damage caused by 8-oxoG in Chinese hamster ovary fibroblasts, was faster in the mitochondrial DNA than in the nuclear DNA, in a non-strand specific manner (17). Another study showed that the levels of oxidatively induced base lesions in mitochondrial DNA were not raised in livers of old wistar rats (18). The authors not only concluded that age is not playing a role in increasing the oxidative damage but also the mtDNA damage was not increased when compared to the nuclear DNA (18). The comparison of mtDNA damage with nuclear DNA, as well as the role of mitochondrial-induced oxidative damage in aging, are still topics of controversy, which will depend on the development of more accurate techniques and studies (19, 20). However, it is curious that by simply analyzing the whole tissue instead of isolated rat liver mitochondria, the levels of 8-oxoG were surprisingly lower than in isolated mitochondria and were also not higher than in the nuclear DNA (21). The authors of this study study and others concluded that it is then likely that the mitochondrial DNA oxidative damage levels have been overestimated due to technical artifacts, over the past years (19, 21).

Mitochondria are considered to be the powerhouse of the cells as they are the main energy producers. Shortly, pyruvate is channeled to enter mitochondria via pyruvate dehydrogenase (PDH) yielding acetyl CoA, NADH + H^+, and CO_2. Acetyl CoA enters the Krebs cycle or Tricarboxylic Acid (TCA) cycle generating NADH + H^+ (22). Fatty acids are oxidized in mitochondria via β-oxidation to generate Acetyl-CoA, NADH + H^+ and reduced FAD co-factor. Two electrons are then transferred from NADH to the OXPHOS complex NADH dehydrogenase (Complex I) or from $FADH_2$-containing enzymes as the electron transfer factor dehydrogenase or succinate dehydrogenase (SDH, complex II) to reduce ubiquinone (coenzyme Q10, CoQ) to ubiquinol ($CoQH_2$). The electrons are then transferred to complex III (*bc1* complex), cytochrome c, complex IV (cytochrome c oxidase, COX), and finally to oxygen, generating H_2O (23). Coupled to the electron flow down the chain is the pumping of protons across the mitochondrial inner membrane space by complexes I, III and IV, which creates a proton electrochemical gradient ($\Delta p=\Delta\Psi+\Delta\mu H^+$) which is acidic and positive in the intermembrane space and negative and alkaline in the matrix side. The potential energy (also called driving force) stored in the proton motive gradient (Δp) is used to aid in the import of proteins and calcium to the mitochondrial matrix, to generate heat and to synthesize ATP (2). The maximal proton motive force across the inner membrane is around 180-220 mV (24). The energy to convert ADP+Pi to ATP comes from the flow of protons through the ATP synthase (complex V) back into the matrix. The matrix ATP is also exchanged by cytosolic ADP through the ANT (adenine nucleotide translocator) located in the inner mitochondrial membrane (25). The production of ATP is dependent on the efficiency by which the protons are pumped out of the matrix by the electron transfer chain (ETC) complexes and by the efficiency of proton flux through complex V. However, this machinery is actually prone to proton leakage, leading to variations in the coupling efficiency

of the respiratory chain (26). Furthermore, alterations in the coupling efficiency can influence the generation of reactive oxygen species (ROS), ultimately leading to apoptosis (27). Finally, each of the ETC complexes incorporates multiple electron carriers. Complex I and II utilize flavins and iron-sulfur (Fe-S) centers (25). The Krebs cycle enzyme aconitase also utilizes a Fe-S group, susceptible to oxidative stress. Complex III encompasses a Fe-S center plus cytochromes b and c1-Complex IV encompasses two Cu centers plus cytochromes a and a3 (25). The importance of mitochondria in the cell cannot be overemphasized thus pathogenic mutations that affect any of the mitochondrial components will disturb cellular function.

As mentioned above, the 37 genes are encoded by the mtDNA include: the large and small RNA (12S rRNA and 16S rRNA), 22 transfer rRNAs (tRNA) and 13 key respiratory chain (RC) subunits. The structural proteins encoded by mtDNA include seven subunits from Complex I (ND1-ND6 and ND4L), one subunit from Complex III (cytochrome b), three subunits from Complex IV (COXI- III), and two subunits from Complex V (ATPase 6 and 8) (28). The remaining of the mitochondrial proteins, including the ones involved in maintenance, expression, transcription and translation, dynamics, are actually nuclear encoded and synthetized in the cytosol prior to mitochondrial import. Therefore, the optimal functioning of the mitochondria is crucially dependent of these two distinct genomes and their dynamic cross-talk. The mitochondrial genetic code also differs from the nuclear. In vertebrates, codons AUA (which require 5-formylcitidine (F^5C) present at the wobble position, (29)) and AUG code for the first methionine, UGA codes for tryptophan (in contrast to the nuclear genome whereas is a stop codon), and AGA and AGG were thought to read as stop codons (unlike arginine, as in the nuclear genome) (30), until recently. It has now been shown that at least in humans a -1 mitoribosome frameshift occurs at the AGA and AGG codons predicted to terminate the CO1 and ND6 ORFs, and consequently both ORFs terminate in the standard UAG codon (31). There are several other unique features of the mtDNA genome that are crucial to understand the mechanisms of mitochondrial disease, but the most important are: 1) mitochondrial DNA is maternally inherited, 2) there are multiple copies of mtDNA within each cell, and mutations can occur in all (homoplasmy) or in a proportion of the molecules (heteroplasmy); 3) in the presence of heteroplasmy, there is a threshold effect and mitotic segregation of the mtDNA, leading to different penetrance of bioenergetic defects; 4) the transmission of the heteroplasmy levels from the mother to the offspring is often random and unpredictable, determined by a "bottleneck" germline transmission. These concepts will be discussed and clarified in the next section.

2.2. Maternal inheritance of the mtDNA

There are multiple copies of mtDNA in both somatic and germ cells. As it was previously mentioned, the inheritance of mtDNA is exclusively maternal, although a rare exception has been reported (32). The clinical importance of this phenomenon is that pathogenic mutations in an affected mother are transmitted to all her offspring, but only females will transmit the mutation to their children. A disease that affects both genders but that is not transmitted by the paternal line is highly suggestive of a mtDNA deficiency (33, 34). There are two main processes that may explain why the mtDNA present in sperm is not transmitted to the next generation; first, there is a significant downregulation of the mtDNA copy number during spermatogenesis, the so-called "dilution effect". Second, sperm mitochondria undergoes ubiquitination, after fertilization. Consequently, the mtDNA content of the zygote was thought to be determined exclusively by the previously unfertilized egg (35, 36). Recently, a mathematical model suggested that the selection against heteroplasmy may explain the uniparental inheritance of mitochondria (37).

2.3. Heteroplasmy of mtDNA and mitotic segregation

Pathogenic mutant mtDNA often co-exists with wild-type mtDNA within the same cell (mtDNA heteroplasmy, (33)). The percentage of mutant mtDNA can vary between 0 and 100%. A minimum number of mutant mitochondrial genomes is required for the expression of a mitochondrial dysfunction or disease, and this is called the "threshold effect" (38). This effect is relative, as the percentage of mutated genomes necessary to impair mitochondrial function varies according to the metabolic requirements of the cell or tissue at any given time, the particular base pair/gene involved and, the nuclear genetic background (38-40). In general, the higher the levels of mutant mtDNA the more severe the disease state is, although occasional exceptions exist (41). Furthermore, mutations in the mtDNA may contribute to common age-related disease phenotypes including blindness, deafness, cardiovascular and neurological disease, renal and endocrine dysfunction (33). It is generally assumed that age-associated mtDNA mutations are caused by the accumulation of damage during aging (25); but, recent studies support the hypothesis that somatic mtDNA mutations created during embryogenesis may contribute to aging phenotypes in adult life (42, 43).

Mitochondria replicate and divide at any time during the cell cycle and the proportion of mutant mtDNA passed onto the daughter cell at cell division may not be identical to that of the parental cell (44). Besides, in response to metabolic demands of the whole cell, WT and mutant mtDNA may replicate at varying degrees during cell cycle. Therefore, the percentage of mutant mtDNA may change quickly between the parent and the daughter cells due to a random drift (a phenomenon known as mitotic segregation) (40). This phenomenon of mitotic segregation of heteroplasmic mtDNA mutations in somatic tissues, and, the principles for maternal transmission of mtDNA are cornerstones of mammalian

mitochondrial genetics (33). Unfortunately, the mechanisms of these events are not clearly understood at the cell or molecular levels despite their fundamental importance.

2.4. The mtDNA genetic bottleneck hypothesis and germline transmission

It has been reported that a phenotypically healthy mother harboring 50% of mutant mtDNA can produce both healthy and severely affected offspring (45). The reason behind this generational shift is designated as the "bottleneck effect" (46). A restriction in the number of mtDNA molecules or groups of molecules arranged in segregating units allows oocytes to inherit a sub-set of the mother's mtDNA pool. As a consequence, the heteroplasmy levels in children of an affected mother can be remarkably variable with respect to the maternal heteroplasmy, while the average heteroplasmy across the offspring is often comparable to that of the mother (46). Rapid intergenerational shifts in the level of mtDNA heteroplasmy levels were first observed in Holstein cows (47), and subsequently documented in human pedigrees transmitting pathogenic mtDNA mutations (35, 48). Observations in mice also supported this phenomenon of selective transmission (bottleneck effect), since it was observed that female mtDNA genomes repopulating the germline was restricted to homogeneous haplotypes (49, 50).

Random genetic drift mechanisms acting in all heteroplasmic mtDNA mutations were revealed by limited data analysis (35). Two laboratories have measured a dramatic reduction in the amount of mtDNA present in the first discernible germ cells (the primordial germ cells) that appear shortly after implantation in the female embryo (49, 50). By crossing heteroplasmic mice with others expressing fluorescent germ-cell markers, it was shown that the majority of heteroplasmy variation and migration, and the levels within individual oocytes, are actually determined before birth (51). These data are compatible with a preferential replication of a subpopulation of molecules either before or after birth, or some form of compartmentalization of the mtDNA, speeding up the genetic drift.

More recently, it has been hypothesized that there is a purifying selection mechanism in the maternal germ line that decreases the inheritance of mutant mtDNA (52, 53). In humans, females with high levels of mtDNA mutations in the germline have been associated with decreased fertility. Recently, the largest *in silico* analysis to date was performed in 577 mother-child pairs transmitting different mtDNA mutations. The study revealed that different mtDNA mutations segregate at different rates in human pedigrees (54). However, they found no evidence of selection during transmission. Certain pedigrees such as m.8993T>G/C segregated faster than the others analyzed in this study. These findings can help us understand the inheritance risks of heteroplasmic mtDNA diseases, and their prevention using conventional techniques such as prenatal and pre-implantation diagnosis. Furthermore, strategies that aim to reduce the mtDNA content could also lead to a faster segregation of heteroplasmic alleles increasing the probability of generating germ cells with very low mutant mtDNA levels or very high as well, which may not be viable at an early stage pregnancy (54). Interestingly, a very recent study corroborates this theory showing that gene editing strategies such us mitochondrial targeted-restriction endonucleases and mitoTALENs were able to prevent germline transmission of specific mtDNA haplotypes, by shifting the mtDNA ratio towards the wild-type mtDNA molecules, by the elimination of mutated mtDNA in oocytes and embryos (55). Moreover, this study provided a new road in the search for an effective treatment for individual mitochondrial diseases, and it will be discussed below in section 3.

3. MITOCHONDRIAL DISEASE AND HETEROPLASMY

Currently, more than 200 point mutations and large scale rearrangements in the human mtDNA are known to be associated with a large diversity of clinical symptoms, ranging from progressive muscle weakness to fatal infantile disorders (56). The first molecular evidence that heteroplasmic mtDNA mutations (deletions) could cause disease was demonstrated in 1988 by Holt and others (57). In addition, Wallace and others (58) also reported that a homoplasmic missense mutation in the ND4 gene (m.11778G>A) was maternally inherited and responsible for the Leber's hereditary optic neuropathy (LHON). It was also discovered that myoclonic epilepsy and ragged red fiber (MERRF) disease was caused by an heteroplasmic A>G transition mutation in position m.8344 in the $tRNA^{Lys}$ gene (59, 60). These interesting findings set the stage for the research of mitochondrial familial and age-related diseases. With the advance of molecular techniques, the following decade saw the description of several hundred different point mutations and deletions in patients with a wide range of clinical phenotypes (12). By contrast to what was thoughtmutations in the mtDNA are relatively common. It was reported that the incidence of clinical mitochondrial diseases is about one in 5,000, including the most common pathogenic mutations (60, 61). Furthermore, it was also revealed that one in 200 newborns harbored some level of one of 10 common pathogenic mutations (62, 63). Because of the high rate of mutations in the mtDNA, new pathogenic mutations are recurrently introduced into the human population. When a mutation arises in the mtDNA, there is an intracellular mixture of mutant and wild-type mtDNA molecules (as it was discussed previously), in some manner the initial mutant mtDNA becomes enriched within certain cells, and predominates influencing the cellular patient phenotype. In general, organs with

very high energy demand require a very efficient ATP production, consequently are the first ones to reveal the consequences of partial mitochondrial bioenergetic deficits due to pathogenic mutations. The most affected organs by these mutations are the brain followed by the heart, muscle, kidney, and endocrine systems (64). Therefore, a mitochondrial disease or syndrome can be defined as a chronic loss of cellular energy that results in a clinical phenotype. MtDNA point mutations are usually maternally inherited, with multiple individuals of the same family being affected, while mtDNA deletions are rarely inherited and are never homoplasmic. Actually, homoplasmic mtDNA point mutations cause in general a mild biochemical defect that typically affects only one organ or tissue (with some exceptions, (65, 66)). In contrast, heteroplasmic mutations affect multiple organs and the levels of heteroplasmy usually correlate with degree of the clinical phenotype, which is severe in the affected tissues. Recently, next-generation sequencing technology has been used to identify and quantify mtDNA mutations (67). However, these techniques have a high intrinsic error rate when applied to detection of low-level heteroplasmy. The data can be contaminated with pseudogene sequences present in the nuclear DNA which are derived from mitochondrial DNA, designated as nuclear mitochondrial DNA sequences (NUMTs), and because of this, it can be particularly challenging to accurately filter the data (68).

Despite all the technological difficulties, it is believed that mtDNA heteroplasmy exists in almost every healthy individual studied, even though at very low levels (69). These heteroplasmic variants can also be passed down the maternal lineage, raising the possibility that some presumably somatic mutations measured late in life are actually low-level heteroplasmies that have been inherited and somehow clonally expanded (e.g. in tumors).

Mitochondrial DNA polymerase gamma (POLG) is a heterotrimeric enzyme containing a Pol I-like catalytic core (PolgA) and an accessory subunit (70). A provocative report in 2013 showed that heterozygous mice for the mitochondrial DNA mutator allele (PolgA) have reduced fertility due to maternally transmitted mtDNA mutations (43). The phenotype is aggravated in PolgA (mut/mut) showing signs of premature ageing. The authors concluded that a pre-existing mutation load increases clonal expansion of mtDNA mutations, in a similar manner maternally transmitted mutations exacerbate the aspects of normal human aging (43).

3.1. Point mutations

Inherited point mutations primarily occur in protein coding tRNA genes and result in a reduction of cellular energy, via reduction in the activity of the electron transport chain respiratory complexes or due to an impairment of mitochondrial protein synthesis (71). Point mutations are usually rare and are often heteroplasmic. The threshold levels for the disease onset are usually between 80-90%. Many classical syndromes have been comprehensively described over the last few decades. A few examples of different syndromes and their associated-point mutations will be shortly described.

3.1.1. Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS)

Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) is typically manifested in childhood and the symptoms range from tonic-clonic seizures, recurrent headaches, and anorexia with vomiting and postlingual hearing loss but can also be associated with motor, vision and mental impairment due to the accumulation of stroke-like episodes (72, 73). The mitochondrial 3243 A>G in MT-TL1 accounts for 80% of the cases (74), but is also associated with variants in ND5 (75), ND1 complex I subunits and MT-CYB. Therefore, defects in complex I and IV activities have been described (76, 77).

3.1.2. Myoclonic epilepsy with ragged red fibers (MERRF)

Myoclonic epilepsy with ragged red fibers (MERRF) is a neuromuscular disorder associated with m.8344 A>G mt-tRNA^{Lys} (MTTK) gene mutation (60, 78). The clinical manifestations are myoclonus, epilepsy, muscle weakness, cerebellar ataxia and dementia, and neurological symptoms can evolve with age (59, 60). High heteroplasmic levels of this mutation have been associated with complex I and IV deficiencies (45, 59, 78). This point mutation has also been associated with Leigh's syndrome (79, 80).

3.1.3. Leber's hereditary optic neuropathy (LHON)

Leber's hereditary optic neuropathy (LHON) is a common cause of inherited blindness presenting bilateral, painless, sub-acute visual failure in young adult males (81). This was the first maternally inherited disease to be associated with mtDNA point mutations (58, 82, 83). There are three common point mutations associated with this syndrome that can be diagnosed by molecular genetic analysis, such as m.3460 G>A, m. 1178 G>A and m. 14484 T>C (84). These mutations are associated with complex I defects and LHON mutations are typically homoplasmic (85).

3.2. Large scale rearrangements and deletions

In contrast to point mutations, primary mitochondrial rearrangements of mtDNA are not inheritable, they are sporadic. Large-scale deletions are typically heteroplasmic and result in disease. To date, roughly 120 different mtDNA deletions have been found in patients with mitochondrial disease. In this case, the heteroplasmic threshold is reported to be lower than the one for point mutations, the patients manifest the

disease symptoms with as low as 50-60% heteroplasmic mtDNA levels (86). Two different models arise to explain deletions in the mtDNA, while one points to replication errors, the other one points to poor and inefficient mtDNA repair mechanisms. MtDNA deletions are associated with three main clinical phenotypes: Kearns-Sayre syndrome (KSS) (87), sporadic progressive external ophthalmoplegia (PEO) (88) and Pearson's syndrome (89). Mitochondrial dysfunction manifests usually with ragged red fibers (RRFs), which is the accumulation of dysfunctional mitochondrial in the sub-sarcolemmal region detected by Gomori trichrome staining (88). Person's bone marrow pancreas syndrome is also a rare infantile disorder characterized by sideroblastic anemia including severe exocrine pancreatic insufficiency (89). Pearson's patients have high levels of mtDNA deletions in affected organs, and might develop KSS later in life (90)

Finally, many mitochondrial disorders have been associated with reduction of the mtDNA copy number (91). Alpers syndrome, has been associated with mutations in POLG, which can impair mtDNA replication (92, 93). In addition, there is a plethora of mutations in nuclear genes coding for proteins associated with nucleotide metabolism and availability which affect mtDNA integrity. Recessive mutations in thymidine phosphorylase (TP) cause neurogastrointesinal encephalopathy with mtDNA depletion, multiple deletions and point mutations (94, 95). Mitochondrial DNA depletion has also been found in early onset of hypotonia with myopathy and hepatic involvement, caused by mutations in thymidine kinase 2 (TK2) or deoxyguanosine kinase (DGUOK), which reduced replication efficiency (96-99).

4. CURRENT STRATEGIES TO SHIFT MTDNA HETEROPLASMY: THE BRIDGE TOWARDS CLINICS

The concept of shifting the balance between healthy and mutated mtDNA as a treatment for heteroplasmic mtDNA disease has been under investigation over the past 20 years. Many publications demonstrated that it is possible to manipulate the mtDNA and shift heteroplasmy, either *in vitro* or *in vivo*. By simply reducing the levels of the mutant allele below a certain threshold, an improvement in pathology is achieved (11, 100-102).

Metabolic strategies to shift mtDNA heteroplasmy have been tested and evaluated throughout the last decades. It was shown that treating cybrids harboring a large-scale partial deletion with ketogenic supplements (deprived of glucose) was enough to shift mtDNA heteroplasmy towards wild-type mtDNA, rescuing metabolic function. This improvement in function though was not associated with any obvious decrease in the mutation levels (103). A possible explanation lies in the fact that the ketogenic stress diluted the deleterious effect of the mutation, because cells contained more mitochondria. Another example of heteroplasmy changes through metabolic intervention was trough exercise. Activation of satellite cells proliferation is known to occur in response to resistance exercise and muscle fiber injury (104, 105). In 1999, a report from a patient that started a program of resistance exercise showed an increase in the proportion of wild-type mtDNA molecules after the training (106). A larger study was performed years later by Murphy *et al*., showing that after 12 weeks of resistance training, many positive physiological changes occurred in the patients, including improved strength and oxidative capacity in the trained muscles; however no decrease in the proportion of mutated mtDNA was detected (107). Other studies followed these and the same conclusion was reached, no change in the heteroplasmy and if so, there was an increase in the mutant mtDNA instead of wild-type. In a similar manner, promoting mitochondrial biogenesis through overexpression of PGC-1α/β improved partially the OXPHOS in cells with a pathogenic mtDNA mutation (108). Again, this improvement was not associated with a heteroplasmic shift. Despite these concerns, a clinical trial was advanced investigating exercise versus inactivity in a cohort of patients with mitochondrial myopathy (109).

The idea of shifting the mtDNA heteroplasmy was finally efficiently demonstrated by the use of mitochondrial-targeted restriction endonucleases both in mouse and human-derived cell lines. This strategy has heralded a new era for mtDNA gene editing and heteroplasmy shift.

4.1. Mitochondrial-targeted restriction endonucleases (REs)

As it was previously discussed, mitochondria do not have an established/accepted double strand break repair mechanism and the little evidence that it may occur comes mainly from work from our lab and others using mitochondrial targeted restriction endonucleases (REs). These are recombinant REs with mitochondrial localization signals (MLSs) that are imported to the mitochondrial matrix, accessing mtDNA and creating double strand-breaks. A hybrid cell line containing both rat and mouse mtDNA was used to test if mitochondrial *Pst*I could alter mtDNA heteroplasmy. Because the mouse mtDNA has two *Pst*I restriction sites, and the rat none, upon expression of the endonuclease a significant shift in heteroplasmy was observed (110). These results suggested that cleavage of mtDNA leads to the degradation of the targeted mtDNA molecules and expansion of the residual mtDNA species (Figure 1). This line of research was applied to the pathogenic m.8993 T>G mutation in the mitochondrial ATP6 gene, which is known to cause Leigh's syndrome or neurogenic muscle weakness, ataxia and NARP (neuropathy, ataxia, and retinitis pigmentosa). This particular mutation creates a unique *Sma*I restriction site that could be

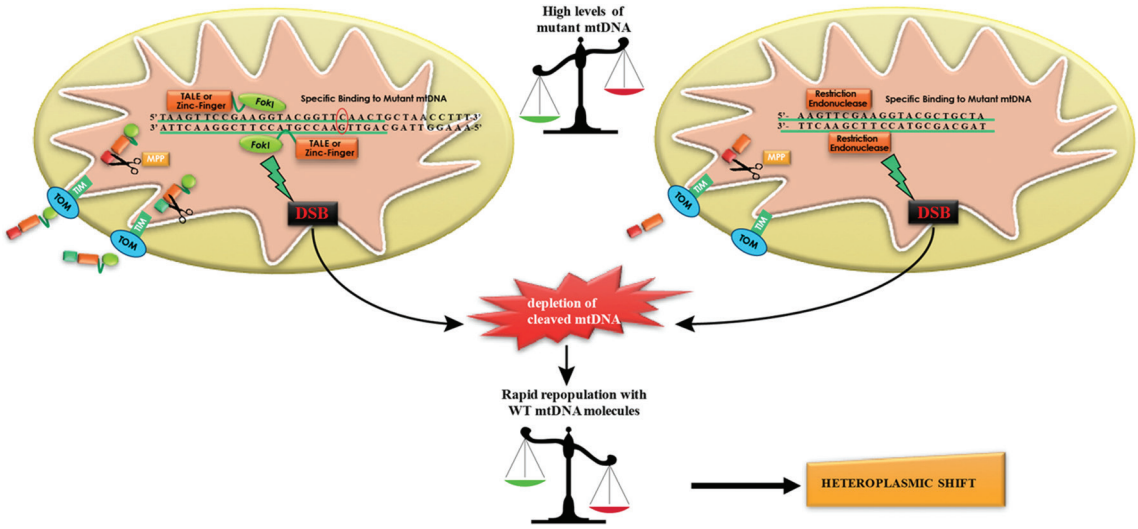

Figure 1. Schematic representation of the action mode of mitochondrial-targeted restriction endonucleases and designer endonucleases such as mitoZinc-fingers and mitoTALENs. First, the cytosolic synthesized unfolded proteins are imported into the mitochondrial matrix through the outer and inner mitochondrial import machinery (TOM/TIM complexes). When inside the matrix they are folded by molecular chaperones. The mitochondrial localization signal/sequence (MLS) is cleaved by a mitochondrial peptidase (MPP) and the mature protein is then ready to bind a specific mtDNA haplotype. When the two monomers are close enough, *Fok*I dimerizes and either the mitochondrial targeted-Zinc finger or TALEN cleave the specific mtDNA by inducing double-strand breaks. A similar pathway occurs with a mitochondrial targeted restriction endonuclease which will bind to the respective restriction site in the mtDNA. Since mitochondria do not have an effective double-strand break repair mechanism, most of the targeted mtDNA is degraded leading to a repopulation of the cell with the residual mtDNA (mostly WT), inducing a shift in heteroplasmy towards the WT.

targeted by a mitochondrially-targeted *Sma*I enzyme in heteroplasmic cells harboring m.8993 T>G mutation. This strategy resulted in the depletion of the mutated mtDNA, accompanied by an increase of WT mtDNA molecules (111). Later, *Xma*I (*Sma*I isoschizomer) was used to target the same mutation in human cybrids and in this study an adenovirus was used. At a viral multiplicity of infection of 50, the point mutation was effectively eliminated from cells with high mutation load, leading to the restoration of OXPHOS. However, it took five cycles of transfection to completely eliminate the mutated mtDNA (112).

The use of mitochondrial-targeted REs was also shown to be effective *in vivo*. Our group further advanced the potential of this concept and proceeded with *in vivo* studies utilizing the NZB/BALB heteroplasmic mouse model (11). This mouse model was created by cytoplasmic fusion in single cell embryos (113). The BALB mtDNA variant harbors a unique *Apa*LI site that is absent in the NZB mtDNA mouse variant. Inducible expression of mito-*Apa*LI in cells derived from these mice showed a rapid, directional and complete shift in mtDNA heteroplasmy, resulting in transient depletion. The initial depletion correlated with the initial levels of the target BALB mtDNA. By using recombinant viral vectors expressing the mito-*Apa*LI it was observed a significant shift in the mtDNA heteroplasmy, reflected by a decrease of BALB mtDNA levels in the muscle and brain transduced with recombinant viruses. It is also important to notice that in this study a shift in heteroplasmy seen in the brain was achieved with the use of AAV1,2-based viral vector (11). In addition, recombinant adenovirus (rAd5-Mito-*Apa*LI) transduction was very effective in the muscle fibers of 5-day-old heteroplasmic mice. This study showed for the first time that this strategy could be used *in vivo*. Nevertheless, this concept was limited to mutations that create unique restriction sites, such as NARP, which are unfortunately rare. Our group attempted to extend the approach to a multiple “cleavage site model”, by the use of a different restriction endonuclease- *Sca*I, which recognizes three sites in the BALB mtDNA and five in the NZB mtDNA (114). In a similar manner to the previous study, a “mammalianized” mito-*Sca*I enzyme construct was designed and Ad5 was injected in the jugular vein, to target the liver and local injection was used to target the muscle. A shift towards the BALB mtDNA that has fewer ScaI restriction sites was observed in both tissues. However, this approach resulted in a lower magnitude of the shift when compared with a single differential cleavage of mito-*Apa*LI. Moreover, it was more prone to trigger severe mtDNA depletion in treated cells. NZB/BALB mtDNA heteroplasmy could also be manipulated in heart, using a cardiotropic adeno-associated virus type-6 (AAV6) and in liver, using the hepatotropic adenovirus type-5 (Ad5) (115). Another study showed that mito-*Apa*LI expressed from AAV9 particles when delivered by intraperitoneal or intravenous injections in neonates, promoted dramatically shifts in heteroplasmy in all striated muscles, including heart (116).

4.2. Mitochondrial-targeted Zinc-fingers

One way to solve the limitation of REs is to develop sequence-specific nucleases that could be

designed to target any sequence of the mtDNA. Zinc-fingers technology allows the engineering of zinc-finger proteins that can bind any predetermined sequence. Dr Minczuk's group was a pioneer in this field and in 2006 the first paper was published showing that it was possible to deliver zinc-finger peptides (ZFPs) to mitochondria (117). They showed that when targeting ZFPs fused to the catalytic domain of a human methyltransferase it was possible to induce a site-specific alteration in the mtDNA, in cells harboring the m.8993 T>G mutation, responsible for NARP and maternally inherited Leigh's syndrome (MILS) (117). However, this approach revealed some limitations in terms of toxicity.

By fusing a particular zinc-finger to a nuclease domain, a zinc-finger nuclease can be created. The nuclease domain is *Fok*I, a type II restriction enzyme, which requires dimerization to induce a double strand break in the DNA (fig.1). In order to achieve dimerization, two zinc-finger endonuclease (*Fok*I) monomers need to be used, binding to adjacent sites on complementary DNA strands containing the target sequence. This technology has been successfully applied to nuclear DNA and site-specific gene correction and addition via homologous recombination was achieved by the induction of double-strand breaks (118, 119). In a similar manner, ZFNs could be targeted to mitochondria and selectively induce the depletion of mutated mtDNA molecules, shifting the heteroplasmy towards the intended direction. Engineered zinc finger nucleases (ZFNs) are chimeric enzymes utilizing the modular Cys2His2 zinc finger DNA-binding moiety conjugated to the C-terminal catalytic subunit of *Fok*I (120, 121). In 2008, it was described for the first time the application of ZFNs to mitochondria, targeting the same point mutation, m.8993 T>G. Initially, the authors used conventional pairs of heterodimeric ZFNs, however they were not able to find suitable pairs for that particular mtDNA sequence. Therefore, they have created a novel approach by designing a single-chain ZFN that recognizes 12 bp of the mtDNA target sequence, by conjugating two *Fok*I nuclease domains, connected to a flexible linker (of 35 aminoacids), to a ZFP with an N-term mitochondrial targeting sequence. This strategy proved to be effective in heteroplasmic cybrids through selectively cleaving the mutated mtDNA molecules (122) and increasing the ratio towards the wild-type mtDNA. However, this approach raised some cytotoxic concerns. To minimize nuclear-related cytotoxicity the authors used a nuclear export signal (NES) linked to the mito-ZFNs but this still resulted in cytotoxicity. In 2014, advances in the architecture of the dimeric mito-ZFNs were made by placing MTS-epitope tag-NES at the N-terminus. In contrast to the previous designs, the Myc tag of one of the monomers was replaced with a FLAG tag. Modified *Fok*I domains, which are paired as a heterodimer (ELD/KKR), were also incorporated in this new study (123). These *Fok*I variants exhibit efficiency comparable to the wild-type architecture, but with a greater than 40-fold reduction in homodimer activity (124-126). In addition, this approach not only showed to be effective to target the m.8993 T>G but also the common deletion (m.8483_13459del4977). With several combinations of mtZFNs capable of specifically degrading targeted mtDNA variants, these data anticipates its clinical application to patients with primary mitochondrial diseases.

4.3. Mitochondrial-targeted TALENs

Since the discovery of a naturally occurring family of proteins secreted by plant pathogens from the *Xanthomonas* species, another breakthrough was achieved for engineering programmable DNA-binding proteins. Transcription activator-like effectors or TALEs contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains, each of which recognizes a single base-pair in a DNA strand (127). This nucleotide specificity of each repeat domain is determined by the two amino acids at positions 12 and 13 designated as the repeat variable di-residues (RVDs). Four different RVD modules - namely Asn-Asn (NN), Asn-Ile (NI), His-Asp (HD) and Asn-Gly (NG) are most widely used to recognize guanine or adenine, adenine, cytosine and thymine, respectively. Using this code, targets of new TALEs have been correctly predicted, and functional targets of TALEs composed of randomly assembled repeats have been generated (128-130). Encouraged by the success of ZFNs architecture, a combination of TALEs fused to a non-specific nuclease (*Fok*I) lead to the creation of TAL effector nucleases (TALENs). TALENs can be designed to targ*et al*most any given DNA sequences posing only one constrain, the requirement for a thymine at the 5' end of the target sequence, which is recognized by two amino-terminal cryptic repeat folds. Recently, new TALE variants that can recognize other bases at the 5' end have broaden the range of targetable sites by TALENs (131). TALE-like proteins from *Ralstonia* spp. (which is another phytopathogenic bacteria) can also be engineered to bind to predetermined DNA sequences (132). Furthermore, and as it was previously mentioned, *Fok*I works as a dimer, so TALENs require two monomers to bind DNA and produce double strand breaks (fig.1). Even though they are potentially more powerful than ZFNs, their bulky size may limit their use with adeno-associated viral vectors (AAVs) *in vivo*. In 2013, our laboratory reported for the first time the use of mitochondrial-targeted TALENs- MitoTALENs (100). The constructs were designed to target the mtDNA "common deletion" (m.8483_13459del4977) and a point mutation m. 14459G>A in the MT-ND6 known to cause Leber's hereditary optic neuropathy plus dystonia (100, 133). The mitoTALENs were coded by the basic principles shown by Cermak and others; the DNA binding domain was kept in between 10-16 repeats and whenever possible, the critical discriminating base pairs were at position 0 or 1 (134). A typical TALEN monomer contains nuclear localization signals and in order to build mitoTALENs, these signals were replaced by

mitochondrial localization sequences, that differ between the two monomers used. It was found that some TALE domains lead to poor mitochondrial import for reasons that are still unknown. Thus, in this study ATP5B, COX8A and SOD2 were selected as they could efficiently direct the TALEN monomers to mitochondria. Immuno-tags were also included in this design in the N-terminus of each TAL domain, either –HA or FLAG. Fluorescent markers to select for expression, co-localization and sorting were also placed in the constructs, either eGFP in one monomer and mCherry in the other monomer. A T2A' (picornaviral 2A-like sequence) was also added between the mitoTALENs and the fluorescent markers, to allow their simultaneous expression under the same promoter (135). At last, *Fok*I heterodimeric domains were placed in all constructs (124, 126). Next, the constructs were first tested in either HeLa cells or COS-7 for expression and mitochondrial localization. To test if mitoTALENs were capable of shifting mtDNA heteroplasmy, trans-mitochondrial cybrids were produced by fusing human osteosarcoma 143B (TK^-) cells with the patient fibroblasts, generating cells with different levels of heteroplasmy. These heteroplasmic cells were transfected with both monomers and cell sorting was performed in order to separate the untransfected from the double-transfected cells. This was possible because of presence of eGFP or m-cherry tags in the two plasmids. The DNA from the cells was extracted, a PCR using mtDNA primers followed by a "last-cycle hot PCR" were performed, to avoid interference from heteroduplexes formed during the final PCR cycles. Restriction-digestion fragment length polymorphism (RFLP) was performed by using an appropriate restriction enzyme which enabled to distinguish between the wild-type and mutant mtDNA. It is also important to mention that when the dimers of *Fok*I are formed, the mitoTALENs cleaves the mtDNA haplotype which is subsequently degraded. To target the mutant mtDNA which differs by a single-base pair in the case of the point mutation, one of the TALEN monomers must be able to bind only to the mutated sequence and not to the wild-type. This goal was achieved efficiently in this study, demonstrating as well that the shift in heteroplasmy persisted 2 weeks after growing the sorted cells, and when the constructs were no longer detected by western-blot (100).

Another study from our group showed that it was possible to efficiently target two relatively common pathogenic mtDNA point mutations, the m.8344A>G $tRNA^{Lys}$ gene mutation associated with myoclonic epilepsy with ragged red fibers (MERRF) and the m.13513G>A *ND5* mutation associated with MELAS/ Leigh syndrome (136). MitoTALENs efficiently reduced the levels of the targeted pathogenic mtDNAs in the respective cells and improved their OXPHOS activity. To ameliorate the design in the context of the low complexity of mtDNA, the authors designed shorter versions of the mitoTALEN specific for the MERRF m.8344A>G mutation. These shorter mitoTALENs also eliminated the mutant mtDNA. This anticipates that reductions in size will improve the ability to package these large sequences into viral vectors, bringing the use of these genetic tools closer to the clinics (136). Recently, a breakthrough strategy to prevent germline transmission of mitochondrial diseases by inducing mtDNA heteroplasmy shift through the selective elimination of mutated mtDNA with mito nucleases has been demonstrated (55). The authors of this study took advantage of the NZB/BALB heteroplasmic mouse model and selectively prevented the germline transmission of one of the haplotypes, either by using endonucleases (mito-*Apa*LI) or TALENs, preventing their transmission to the next generation. Furthermore, they were also able to successfully reduce the mutation levels of human LHON and NARP- associated point mutations, in mammalian oocytes by the use of mitoTALENs (55). These results demonstrate the potential of germline heteroplasmy shift as a clinical strategy to prevent the transgenerational transmission of mitochondrial genomes.

The most recent addition to this group of gene editing endonucleases is the Cas9 nuclease. Cas9 is targeted using a very small short RNA sequence- clustered regularly interspaced short palindromic repeats (CRISPR) (137). CRISPRs are much easier to design in comparison to ZFNs or TALENs because the process requires only making a short RNA sequence. As an example of its potential role for nuclear mutations, Cas9/CRISPR has been shown to restore the weight loss phenotype of a mouse model of a *Fah* mutation in hepatocytes in a mouse model of the human disease hereditary tyrosinemia (138). However, its high off-target effects were a concern. There has been a report that FLAG-Cas9 can localize to mitochondria to edit mitochondrial DNA with sgRNAs targeting specific loci of the mitochondrial genome. Expression of FLAG-Cas9 together with gRNA targeting Cox1 and Cox3 led to the cleavage of the specific mtDNA loci. In addition, the authors observed disruption of mitochondrial protein homeostasis following mtDNA truncation or cleavage by CRISPR/Cas9. To overcome FLAG-Cas9 unspecificity they have created a mitochondrial-targeted Cas9 (mitoCas9) that showed specific cleavage of mtDNA together with expression of gRNA. MitoCas9-induced reduction of mtDNA and mitochondrial membrane potential disruption, as well as cell growth inhibition (139). However, this study did not show direct evidence that the Cas9 system was cleaving mtDNA, but rather the copy number of regions targeted to be cleaved (Cox1 and Cox3). Because mtDNA behaves as a genetic unit, it is difficult to envision how the levels of a specific mtDNA region would be different from other regions, unless there is a deletion, which would be observable by Southern blot. Such experiment was not presented. In our lab, we have tried to use Cas9/CRISPR for mtDNA heteroplasmy changes but failed. Although we were able to deliver Cas9

Table 1. Currently described mitochondrial-targeted gene editing tools

Gene editing tool	Nuclease	Target	Limitations	Advantages	References
Mitochondrial-targeted restriction endonucleases	*Pst*I, *Sma*I, *Apa*LI, *Xma*I, *Sca*I, among others	DNA	Limited to mutations that create unique restriction sites	Compact size Effective shift in heteroplasmy *in vivo*	11, 110-112, 114-116
Mitochondrial-targeted Zinc Fingers	Heterodimeric *Fok*I	DNA	Toxicity Large size (requires two monomers or quasi-dimeric architecture)	Virtually targets any mitochondrial DNA sequence	122, 123, 125
Mitochondrial-targeted TALENs	Heterodimeric *Fok*I	DNA	Large size (requires two monomers) T0 binding requirement (in general)	Virtually targets any mitochondrial DNA sequence	100, 152
Mitochondrial CAS9[1]/CRISPR[2]	Cas9	RNA	Problems with gRNA import to mitochondria. Potential off target effects	Compact and easy to design, it only requires the design of the short RNA	139, 152

[1]: CRISPR associated protein 9, [2]: Clustered regularly interspaced short palindromic repeats

to mitochondria with a targeting sequence, we suspect that the guide RNA was not efficiently imported, even though we used different RNA extensions to promote RNA import (140). The advantages and limitations of the described techniques are summarized in Table 1.

5. CLINICAL AVENUES AND PERSPECTIVES

There have been great advances for avoiding the transmission of mtDNA mutations. Pre-implantation genetic diagnosis (PGD) can assist females which carry mtDNA mutations, by preventing the transmission of deleterious mtDNA. After *in vitro* fertilization embryos are analyzed and only those with very low mutant levels are transferred to the uterus, but for woman that carry intermediate-levels of heteroplasmic mtDNA mutations, this technique may not be effective since there is uncertainty regarding the clinical mutation threshold remains (141, 142). Techniques such as "pro-nuclear transfer" involve the transfer of nuclear DNA from a donor zygote to an enucleated recipient zygote via fusion (143). The new "reconstructed zygote" retains the nuclear DNA from the mother, but the mtDNA from a donor. This approach has made significant steps towards treating mitochondrial disease at mtDNA level. Similarly, "spindle transfer" of nDNA to an enucleated donor was also successful in animal models and human embryos (144). Unlike pro-nuclear transfer, the nDNA isolation occurs pre-fertilization, meaning that it can be integrated into established *in vitro* fertilization techniques. Mitochondrial replacement techniques involve a series of complex technical manipulations of nuclear genome between patient and donor oocytes that result in the generation of embryos carrying genetic material from three different origins and for these reasons, these techniques raised different ethical and medical concerns. In spite of all the polemics, mitochondrial replacement was approved in the UK, early 2015 (145). Mitochondrial nucleases can be a powerful alternative for these techniques, as it does not require any mtDNA donor. Using a single mRNA or adenovirus injection into patient oocytes or embryos (respectively) expressing a mitochondrial nuclease is technically simpler and less traumatic compared to mitochondrial replacement techniques and it also does not require a third donor, circumventing some of the ethical issues associated with this matter.

There are currently at least two strategies for applying gene therapy to patients with mtDNA diseases: 1) Allotopic expression of mitochondrial genes; 2) Manipulation of mtDNA heteroplasmy. Allotopic expression of mitochondrial genes which consists in the synthesis of a wild-type version of the mutated protein in the nuclear-cytosolic compartment followed by its import into mitochondria has been a controversial approach because of the high hydrophobicity of mtDNA-encoded proteins and the competition with endogenous counterparts (146-149). Nonetheless, clinical trials for Leber's optic neuropathy are currently ongoing (150, 151). The use of mitochondrial endonucleases is still in its infancy, but hopefully will move into the clinics in the next few years.

To conclude, the manipulation of mtDNA heteroplasmy either by using mito restriction endonucleases, mito zinc-fingers or mitoTALENs could facilitate delivery and increase specificity of mtDNA editing, having the potential to eliminate mutant mitochondrial genomes from germline treated affected patients.

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