Fixing the powerhouse: genetic engineering of mitochondrial DNA

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Mitochondria are complex factories that provide our cells with most of the energy we need to survive and perform daily tasks. They comprise their own small genome, mitochondrial DNA (mtDNA), which contains genes for parts of the energy-producing machinery. Mutations in mtDNA can lead to mitochondrial diseases, which are a devastating group of heterogenous inheritable diseases that can develop at any stage of life. Despite rapid developments in genome engineering for nuclear DNA, the incompatibility of certain techniques in mitochondria has meant that the field of mitochondrial genome modification has been impeded for many years. However, recent advances in mtDNA engineering techniques, such as programmable nucleases and base editors, will allow for a deeper understanding of the processes taking place in mitochondria and improve the prospects of developing treatments for mitochondrial diseases.

A tale of two genomes

Mitochondria are complex, multifaceted organelles involved in many processes within the cell – most notably, the production of the cell's energy currency (ATP) via the process of oxidation phosphorylation (OXPHOS). While mitochondria host multiple other crucial metabolic processes, their essential requirement in providing the cell with energy has popularly deemed them the 'powerhouse of the cell'. Mitochondria contain their own small genome (mtDNA) that is exclusively inherited through the maternal line. The mtDNA contains genes for only 13 proteins of the OXPHOS system and all the 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) required to translate their genes. The remainder of the proteins for OXPHOS and the expression of mtDNA (such as the ribosome and transcription factors), amongst over 1000 other proteins, are encoded by the nucleus and imported into mitochondria across their double membrane by a variety of dedicated transporter proteins. Thus, mitochondria are under the genetic control of two distinct genomes and as a consequence of this, mitochondrial diseases can arise from mutations in both the nuclear and mitochondrial genomes.

Mitochondrial diseases, traditionally categorized as a group of genetic disorders disrupting the production of ATP, are collectively one of the most prevalent inherited neurological disorders, affecting approximately 1 in 4300 adults in the UK. The diseases mostly affect organs with high energetic demand, such as the heart and the brain, but can affect many organs at once leading to severe symptoms, which can in the worst cases cause premature death. The symptoms are extremely wide ranging and can affect people of any age, ranging from childhood to older adults. This makes mitochondrial diseases notoriously difficult to diagnose and treat, and they are currently incurable. An example of a mitochondrial disease family is MELAS, which causes a wide range of symptoms including muscle weakness, organ failure and dementia. It normally develops in early childhood and patients have a life expectancy of 10–35 years. MELAS can be caused by mutations in either the nuclear or the mitochondrial genome, but in most cases is caused by a mutation in a tRNA encoded in mtDNA. Mutations in mtDNA are also increasingly associated with some age-related diseases, including neurodegenerative diseases and cancer. Therefore, there is an ever more urgent need to develop new approaches to investigate and hopefully treat the root causes of mitochondrial dysfunction.

The importance of proportions

In contrast with nuclear DNA, each cell contains multiple copies of circular mtDNA molecule. Depending on the cell type and its energy requirement, each human cell contains anywhere between hundreds to thousands of mtDNA molecules, of which each individual mitochondrion contains 1–15 copies. Unlike the nuclear genome which only replicates during cell division, mtDNA is replicated continuously and thus has an increased chance of generating errors. These errors can range from point mutations to large deletions of regions of mtDNA. However, due to the multi-copy nature of the mtDNA, the mutated mtDNA molecules frequently coexist with unaffected wild-type mtDNA molecules in a condition known as heteroplasmy (Figure 1). Generally, for disease symptoms to present in humans, the

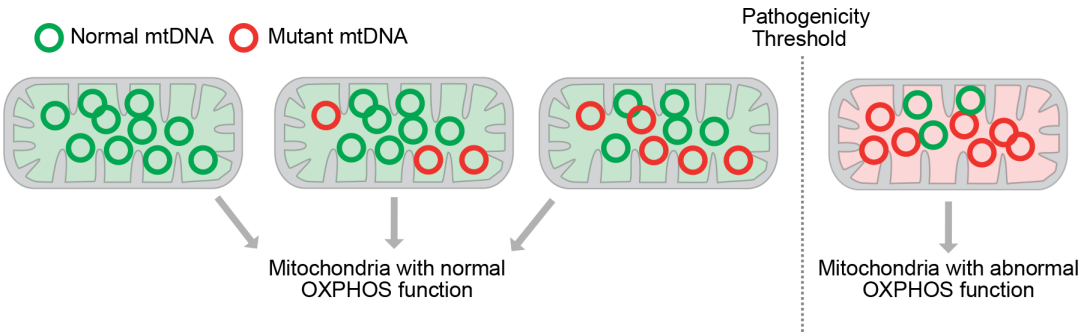

Figure 1. The heteroplasmy and the mitochondrial biochemical/pathogenicity threshold

percentage of mutant mtDNA molecules within a tissue needs to exceed between approximately 60% and 90%, known as the pathogenicity threshold. Interestingly, a high proportion of the healthy population has mutant mtDNA molecules, but below the threshold to show symptoms of disease, which explains why mitochondrial diseases can develop at any age; certain mutant mtDNA molecules can expand over time in specific tissues and reach the pathogenicity threshold. This unique complex nature of heteroplasmy makes therapeutic intervention of mitochondrial diseases challenging.

Shifting the balance: cut it, paste it, cure it

Given the advent of CRISPR/Cas gene editing technologies, it may seem that correcting mitochondrial mutations is simple. But, a prerequisite for CRISPR/Cas systems is delivery of guide RNA complementary to the region of interest to allow for site-specific modification or cleavage. Unfortunately, there is no known mechanism by which RNA (or DNA for that matter) can be imported efficiently across the mitochondrial membrane; therefore CRISPR/Cas-mediated DNA modification in mitochondria is not currently possible. However, proteins are able to enter mitochondria, and proteins perform a variety of functions including those used for genetic engineering. The mitochondrial genome engineering approaches using such proteins fall into two main categories: DNA cutting and base editing (Figure 2).

The unique multiple-copies-per-cell nature of the mitochondrial genome, and the requirement to keep the number of mtDNA copies stable, means that by removing enough of the mutant mtDNA, you can allow the remaining healthy wild-type mtDNA to replicate and replace it, and hence by reducing the heteroplasmy level below the pathogenic threshold. This process is known as 'heteroplasmy shifting'. The approach relies on the use of site-specific cutting of the mutant mtDNA, leading to its degradation. By attaching a nuclease (enzyme that cleaves DNA) to a DNA-binding protein that binds to specific DNA regions or by evolving naturally occurring nucleases, it is possible to selectively cut the mutant mtDNA molecule. There are a variety of proteins that are able to bind specific DNA sequences. The theory behind DNA-binding proteins is based upon a simple relationship between the side chains of amino acids being able to interact with specific DNA bases. It is therefore possible to alter the amino acid sequences of the protein

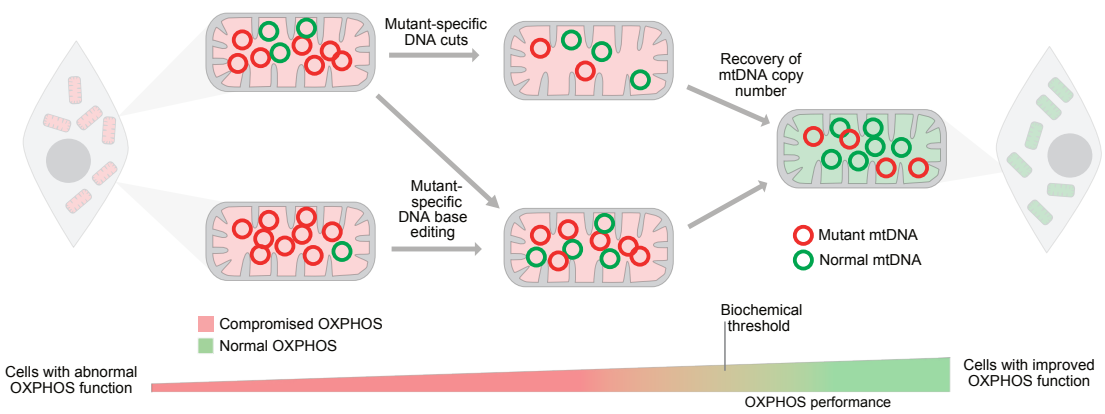

Figure 2. The therapeutic strategies to correcting mitochondrial diseases

Figure 3. The biochemical approaches to mitochondrial genome engineering

to bind a specific region of DNA. These proteins can then be given permission to enter mitochondria by the addition of a short amino acid sequence known as a mitochondrial targeting sequence (MTS) (Figure 3). Using these proteins, known as mitoTALENs, mtZFN or mitoARCUS, to reduce mutant mtDNA heteroplasmy has been proven to be successful in living mice, where nucleases were packaged into an adeno-associated virus (AAV), in order to penetrate cells, and injected into the animals. The mice showed a reduction in the mutant mtDNA heteroplasmy levels, which was accompanied by rescue of the cardiac disease phenotypes. This proof-of-concept in a living animal is an important step forward for the treatment of mitochondrial diseases.

More recently, important new tools have been developed using bacterial deaminases, enzymes that allow for specific C-to-T and A-to-G base conversions. The technology uses DNA-binding protein pairs binding to specific opposing DNA strands, attached to deaminase domains, and an MTS to target mitochondria (Figure 3). The technology has since been used to base edit mouse embryos, zebrafish embryos, rats and the hearts of neonatal and adult mice. The latter demonstrates editing can be achieved in developed tissues, allowing for the potential to treat human disease using specific base correction, which, given the fact mitochondrial diseases can develop at any age, is a very substantial breakthrough for prospective treatments. Also, the use of nucleases to degrade mutant mtDNA is not suitable in homoplasmic diseases (where the mutant mtDNA is 100%), as there is no remaining healthy WT mtDNA to repopulate after the mutant is degraded. Base editors are much more applicable in this instance.

Nobody said it was easy

While the pre-clinical data using designer nucleases and base editors are promising, with both showing positive *in vitro* and *in vivo* results, there are still substantial challenges before these technologies can

be applied to patients with mitochondrial diseases. Both techniques have limitations in their abilities. Nucleases can bind not only to non-specific DNA regions in the mitochondria, but also to the nuclear DNA. Whilst this has been shown to be at low level in cells, if not carefully monitored it has the potential to cleave or edit otherwise healthy DNA sites, which may expand in certain tissues and lead to unwanted health implications. Similarly, the current base editing technology using deaminases also has limitations in its ability to specifically target a single base and not adjacent sites on the mtDNA, which may lead to off-target editing.

In order for mutations in patients to be corrected, the DNA encoding the base editing or DNA cutting proteins has to be delivered to specific organs. Currently, only AAV delivery has been clinically approved. However, developing a safe and cost-effective AAV delivery approach for mitochondrial diseases is a significant challenge. The dosage currently used in base editing mice studies would equate to potentially unsafe levels in humans. Concerns around immune responses to AAV gene delivery should also not be underestimated, and there are alternatives that should be explored. There are, however, promising clinical trials underway that have reported successful transgene delivery to muscle and the central nervous system.

Recent developments in basic understanding of mitochondrial diseases have improved our knowledge and the potential of treating these diseases. There are many promising options being explored, including programmable nucleases and base editors covered here, amongst others. The advancement of these technologies through clinical trials and beyond provides hope for the treatment of human mitochondrial diseases. ■

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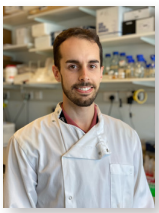

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