Review

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Modeling mitochondrial DNA diseases: from base editing to pluripotent stem-cell-derived organoids

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Abstract

Mitochondrial DNA (mtDNA) diseases are multi-systemic disorders caused by mutations affecting a fraction or the entirety of mtDNA copies. Currently, there are no approved therapies for the majority of mtDNA diseases. Challenges associated with engineering mtDNA have in fact hindered the study of mtDNA defects. Despite these difficulties, it has been possible to develop valuable cellular and animal models of mtDNA diseases. Here, we describe recent advances in base editing of mtDNA and the generation of three-dimensional organoids from patient-derived human-induced pluripotent stem cells (iPSCs). Together with already available modeling tools, the combination of these novel technologies could allow determining the impact of specific mtDNA mutations in distinct human cell types and might help uncover how mtDNA mutation load segregates during tissue organization. iPSC-derived organoids could also represent a platform for the identification of treatment strategies and for probing the *in vitro* effectiveness of mtDNA gene therapies. These studies have the potential to increase our mechanistic understanding of mtDNA diseases and may open the way to highly needed and personalized therapeutic interventions.

Keywords iPSCs; mitochondria; mitochondrial diseases; mitochondrial genome editing; organoids

Subject Categories Genetics, Gene Therapy & Genetic Disease; Molecular Biology of Disease; Stem Cells & Regenerative Medicine

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Introduction—mitochondria and mitochondrial DNA diseases

Mitochondria are double-membrane organelles essential for the execution of key cellular functions including bioenergetics, redox balance, calcium homeostasis, and overall intercellular signal transduction (Picard & Shirihai, 2022). The vast majority of genes needed to produce more than 1,000 proteins important for mitochondrial activities are encoded in the nucleus (Pagliarini *et al*, 2008; Rath *et al*, 2021). In addition, mitochondria harbor their own genome. Mitochondrial DNA (mtDNA) is a small, circular molecule approximately 16,500 base pairs (bp) in size, encoding 13 proteins, 22 tRNAs, and two rRNAs (Anderson *et al*, 1981). These 37 mtDNA genes are vital to ensure proper oxidative phosphorylation (OXPHOS) functionality, which thus depends on the concerted action of both nuclear and mitochondrial genomes. Unlike nuclear DNA, mtDNA is polyploid, which means that within each cell there are multiple copies of mtDNA (Magnusson *et al*, 2003; Gustafsson *et al*, 2016).

The number of mtDNA copies can vary greatly in different cell types and organs (Filograna *et al*, 2021). For example, in mammals, sperm may contain around 100 copies of mtDNA while oocytes contain 150,000 copies (Chen *et al*, 1995; Wai *et al*, 2010). In humans, blood leukocytes contain around 150–600 mtDNA copies (Picard, 2021; Rausser *et al*, 2021) and the heart around 4–6,000 (D'Erchia *et al*, 2015). mtDNA content may even vary within the same tissue, as in the case of mammalian brain, where region-specific variability can be observed (Brinckmann *et al*, 2010; Fuke *et al*, 2011). Although tissues with greater energy demands appear to contain more mtDNA copies per cell (Picard, 2021), the underlying mechanisms are not fully understood. It has been suggested that methylation of the mitochondrial DNA polymerase gamma (PolG) may contribute to the regulation of mtDNA copy number in tissues (Kelly *et al*, 2012). The relation with cell cycle progression remains not clear, as contrasting evidence exists with respect to the correlation between mtDNA initiation/replication and cell cycle phases (Pica-Mattoccia & Attardi, 1972; Antes *et al*, 2010; Chatre & Ricchetti, 2013).

Mitochondrial diseases are genetic conditions caused by mutations in either nuclear or mitochondrial genes, ultimately impairing OXPHOS and mitochondrial function (Vafai & Mootha, 2012). Mitochondrial diseases are multi-systemic, but often affect tissues with high energy demands, such as the nervous system, the heart, and skeletal muscles (Wallace, 1999; Russell *et al*, 2020). In this review, we focus on those mitochondrial diseases that are specifically caused by mtDNA alterations, including point mutations and large-scale deletions. Given the multi-copy nature of mtDNA, mtDNA point mutations or large-scale deletions can affect a portion of mtDNA copies (i.e. heteroplasmy) or virtually all mtDNA copies (i.e., homoplasmy). mtDNA diseases comprise different clinical syndromes and are estimated to occur in 1:5,000 individuals (Cree *et al*, 2009; Table 1).

Point mutations in mtDNA constitute a large part of mtDNA diseases and result in various clinical entities. Leigh syndrome (LS,

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Table 1. mtDNA diseases.

Clinical syndromes caused by mtDNA point mutations					
Disease	**Main clinical features**	**Frequent genes affected**	**Frequent mutations**	**Gene function**	**Mutation load**
Leigh Syndrome (LS)	Psychomotor regression, seizures, difficulty in breathing, hypotonia, ataxia, lactic acidosis, and fatigue	*MT-ND1* *MT-ND2* *MT-ND3* *MT-ND4* *MT-ND5* *MT-ND6* *MT-ATP6* *MT-ATP8*	m.3890G > A m.13513G > A m.8993T > C m.8933T > G m.9185T > C m.9176T > G	Protein subunits of Complex I or Complex V	Heteroplasmy >20% for Complex I mutations; Heteroplasmy >90% or homoplasmy for Complex V mutations
Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS)	Seizures, stroke-like episodes with encephalopathy, possible presence of myopathy, cardiomyopathy, and ataxia	*MT-TL1* *MT-TQ* *MT-TH* *MT-TK* *MT-TS1* *MT-TS2* *MT-ND1* *MT-ND5* *MT-ND6*	m.3243A > G m.5541C > T	tRNAs and protein subunits of Complex I	Heteroplasmy 50–90%
Myoclonic Epilepsy and Ragged-Red Fibers (MERRF)	Myoclonus, epilepsy, progressive ataxia, muscle weakness seizures, ataxia, and cardiomyopathy	*MT-TL1*, *MT-TH* *MT-TK* *MT-TS1* *MT-TF* *MT-TH* *MT-TI* *MT-TP* *MT-TS2*	m.8344A > G	tRNAs	Heteroplasmy 20–90%
Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP)	Pigmentary retinopathy, ataxia, and neuropathy	*MT-ATP6* *MT-ATP8*	m.8993T > C m.8933T > G	Protein subunits of Complex V	Heteroplasmy <90%
Leber's Hereditary Optic Neuropathy (LHON)	Subacute visual failure	*MT-ND1* *MT-ND4* *MT-ND5* *MT-ND6*	m.11778G > A m.14484T > C m.3460G > A m.13513G > A	Protein subunits of Complex I	Homoplasmy

Clinical syndromes caused by mtDNA large-scale deletions			
Disease	**Main clinical features**	**Type of mtDNA deletions**	**Mutation load**
Chronic Progressive External Ophthalmoplegia (CPEO)	Ptosis, ophthalmoparesis, and myopathy	Single large-scale mtDNA deletions (SLSMDs)	Variable heteroplasmy (also < 50%)
Kearns–Sayre Syndrome (KSS)	Progressive External Ophthalmoplegia, ptosis, pigmentary retinopathy, ataxia, sensorineural hearing loss, and myopathy	SLSMDs	Variable heteroplasmy (usually > 50%)
Pearson Marrow–Pancreas syndrome (PMPS)	Sideroblastic anemia, neutropenia and thrombocytopenia, diabetes, and failure to thrive	SLSMDs	Variable heteroplasmy (usually > 70%)

OMIM # 256000) is a pediatric progressive retardation of psychomotor function associated with incoordination of eye movements, recurrent vomiting, epilepsy, pyramidal and extrapyramidal signs, central abnormalities of pulmonary ventilation, and lactic acidosis (Baertling *et al*, 2014; Lim *et al*, 2022a). Magnetic resonance imaging showing bilateral calcification in the basal ganglia region is particularly important and is pathognomonic of the disease. The most common mtDNA mutations affect genes encoding for components of Complex I (e.g., mutation m.3890G > A in gene *MT-ND1* or mutation m.13513G > A in gene *MT-ND5*) or components of Complex V (e.g., mutation m.8993T > G or m.8993T > C in gene *MT-ATP6*). Forms due to Complex V defects are also referred to as maternally inherited Leigh syndrome (MILS; Ciafaloni *et al*, 1993). Although the heteroplasmy can be highly variable in LS patients, Complex I mutations have been reported to be causative of LS even at a level of 20%, while MILS develops for heteroplasmy higher than 90% (Thorburn *et al*, 1993; Stenton *et al*, 2022; Lim *et al*, 2022a). Neuropathy, ataxia, retinitis pigmentosa, and ptosis (NARP,

OMIM #551500) includes epilepsy and mental decline in addition to the clinical signs that make up the acronym, and usually occur in adulthood (Holt *et al*, 1990). NARP is caused by the same mutations that would cause MILS, but present at lower heteroplasmy (Thorburn *et al*, 1993). Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS, OMIM #540000) is defined by the presence of stroke-like episodes, lactic acidosis, and ragged-red fibers (Pavlakis *et al*, 1984). Other signs of central nervous system involvement include mental decline, recurrent headaches, focal epilepsy, and sensorineural deafness. The onset of the disease is variable, ranging from early childhood to young adulthood. MELAS is typically due to mtDNA mutations in tRNA genes; the most frequent one is m.3243A > G in gene *MT-TL1* (Goto *et al*, 1990; Nesbitt *et al*, 2013). The heteroplasmy in MELAS patients is variable but typically around 50–90%, with higher levels in the central nervous system compared to peripheral muscles (Scholle *et al*, 2020). Myoclonus, epilepsy, and ragged-red fibers (MERRF, OMIM #545000) is characterized by myoclonus, muscle hyposthenia, hypotrophy, cerebellar ataxia, hearing loss, and mental deterioration (Shoffner & Wallace, 1992). The extent of clinical manifestations can vary widely even within the same family. Like MELAS, MERRF is caused by mutations in tRNA genes (e.g., m.8344A > G in gene *MT-TK*) with variable heteroplasmy around 20–90% that is typically higher in the central nervous system (Lertrit *et al*, 1992; Hameed & Tadi, 2022). Leber's hereditary optic neuropathy (LHON, OMIM #535000) is hallmarked by acute or sub-acute loss of central vision with outcome in optic atrophy, with onset in youth and higher prevalence in males (Yu-Wai-Man *et al*, 2002). Visual impairment is usually the only clinical manifestation of the disease. The three most frequent mutations affect genes encoding for components of Complex I (i.e., m.11778G > A in gene *MT-ND4*; m.3460G > A in gene *MT-ND1*; and m.14484T > C in gene *MT-ND6*) and are mainly present in the patients at homoplasmic level (Wallace *et al*, 1988).

Single large-scale mtDNA deletions (SLSMDs) cause clinical syndromes comprising three overlapping phenotypes that are rarely observed in more than one member of the same family: chronic progressive external ophthalmoplegia (CPEO), Kearns–Sayre syndrome (KSS), and Pearson marrow–pancreas syndrome (PMPS; Harding & Hammans, 1992; Goldstein & Falk, 1993; Pitceathly *et al*, 2012; Broomfield *et al*, 2015). CPEO is characterized mainly by weakness or paralysis of the muscles that move the eye (ophthalmoplegia), and myopathy may also be present. Besides SLSMDs, CPEO can also be caused by single-nucleotide substitutions in specific transfer (t) RNA genes (Moraes *et al*, 1993; Taylor *et al*, 2002) and pathogenic mutations in nuclear genes leading to multiple mtDNA deletions (Heighton *et al*, 2019). KSS (OMIM #530000) is characterized by a combination of ophthalmoplegia, retinal pigmentary degeneration, and cardiomyopathy (Tsang *et al*, 2018). PMPS (OMIM #557000) presents as refractory sideroblastic anemia with vacuolization of bone marrow precursors and exocrine pancreatic dysfunction (Rotig *et al*, 1989). Affected patients, who recovered spontaneously from infantile sideroblastic anemia, may later develop features of KSS (Larsson *et al*, 1990). SLSMDs are usually around 5,000 bp long. The most frequent deletion is the so-called "common deletion" which is 4,977 bp long and starts from position 8,470 until position 13,446 (Cortopassi & Arnheim, 1990). The level of heteroplasmy for SLSMDs is variable and is typically higher in KSS and PMPS than in CPEO (López-Gallardo *et al*, 2009; Broomfield *et al*, 2015).

Here, we provide an overview of the challenges associated with studying mtDNA diseases. In addition to clinical complexity, mtDNA diseases have been hard to model due to specific difficulties in mtDNA engineering, which have hampered the generation of a vast repertoire of *in vitro* and *in vivo* models. We describe how recent breakthrough findings could offer unprecedented opportunities for tackling mtDNA diseases. The possibility to perform base editing of mtDNA and significant advances in induced pluripotent stem cells (iPSCs) modeling using three-dimensional (3D) organoids could lead to the development of innovative model platforms for assessing the impact of specific mtDNA mutations in different human tissues, and for evaluating the effectiveness of targeted treatments or mtDNA gene therapy *in vitro*. We anticipate that the combination of such technologies could pave the way to highly needed disease-modifying and personalized interventional strategies for families affected by mtDNA diseases.

Challenges in handling and treating mtDNA diseases

A major challenge in developing treatments for mtDNA diseases is the extreme variability in the genotype–phenotype correlation (Wallace, 1999; Gorman *et al*, 2016). Besides LHON, which only affects the optic nerve, mtDNA diseases are clinically heterogeneous and typically involve multiple organs. Each defined clinical syndrome can be caused by different mtDNA mutations (Table 1). At the same time, certain individual mtDNA mutations can be associated with various clinical presentations. For example, mutations m.8993T > G or m.8993T > C in the gene *MT-ATP6* can cause LS or NARP depending on the heteroplasmy level, and mutation m.13513G > A in the gene *MT-ND5* can be associated with MELAS, LS, or LHON (Bannwarth *et al*, 2013). Furthermore, individuals affected by the same clinical syndrome might show distinct symptomatology and severity, irrespective of the underlying mtDNA mutation (Gorman *et al*, 2016).

Given this complexity, it has been challenging to identify treatments that are effective for a defined clinical phenotype or a defined causative genotype. Patients affected by mtDNA diseases may be pharmacologically treated with a combination of vitamins and antioxidants known as "mitochondrial cocktail," although the efficacy of such intervention appears to be limited and unspecific (Bottani *et al*, 2020; Russell *et al*, 2020; Weissig, 2020). In addition, given the variable organ impairment, different patients may require organ-specific therapies.

One notable exception is LHON, which is a unique mtDNA disease with restricted symptomatology (i.e., degeneration of retinal ganglion cells in the optic nerve). The antioxidant drug Idebenone has been officially approved for the treatment of LHON patients by the European Medicines Agency (EMA; Klopstock *et al*, 2011). LHON is the first mtDNA disease treated with mtDNA-based gene therapy. The allotopic expression of wild-type *MT-ND4* through intra-ocular delivery using adeno-associated viruses (AAV) has been proposed as a means to ameliorate vision in LHON (Guy *et al*, 2017). Remarkably, a phase III trial based on this gene therapy approach indeed reported improved eye function in treated LHON patients (Yu-Wai-Man *et al*, 2020; Falabella *et al*, 2022). For other mtDNA diseases, however, there are currently no approved therapies. Nonetheless, several treatment strategies are being pursued and different clinical trials are ongoing. These interventions include

the modulation of mitochondrial biogenesis, NAD/NADH ratio, mitochondrial turnover, redox balance, or hypoxia (Bottani *et al*, 2020; Russell *et al*, 2020; Weissig, 2020).

One positive recent development stemming from advances in *in vitro* fertilization technologies is the possibility to prevent the transmission of pathogenic mtDNA to offspring. This procedure, known as mitochondrial replacement therapy (MRT), is based on transferring the nuclear DNA from an affected mother carrying mtDNA mutations into an enucleated oocyte or zygote of a donor woman with healthy mtDNA (Greenfield *et al*, 2017). In this way, the nuclear DNA of the offspring comes from the actual mother, while the mtDNA comes from the healthy female donor. There have been positive studies for MRT in rodents and primates (Tachibana *et al*, 2013; Kang *et al*, 2016b). MRT has apparently already been successful in a Mexican family (Zhang *et al*, 2017). However, this technology is currently only approved in a small number of countries, and some countries may never approve it because of ethical reasons. Moreover, it has been shown that a reversion to pathogenic mtDNA heteroplasmy can occur in some cases (Hudson *et al*, 2019). The reason for this reversion is not clear and may be potentially linked to unwanted carryover of the maternal mutated mtDNA taken up in the process of extracting the nucleus, or to possible selection of some mutations or mtDNA haplotypes (Chinnery *et al*, 2014). There are also concerns related to the long-term consequences of MRT. Studies in mice and flies implied that subtle nuclear–mitochondrial DNA mismatch could cause late-onset cardiac defects or infertility (Latorre-Pellicer *et al*, 2016). Lastly, MRT cannot be used as a means to treat patients who are already affected by mtDNA diseases.

Challenges in understanding mtDNA diseases

The diagnosis and clinical handling of mtDNA diseases are hindered by the complexity of mtDNA genetics. mtDNA mutations (point mutations and single large-scale deletions) are typically functionally recessive. This means that in order to cause a biochemical and functional impairment, they need to be present in a significant amount of mtDNA molecules (Gorman *et al*, 2016). Higher heteroplasmy is usually associated with more severe conditions, but the critical threshold for pathogenicity is different for distinct mtDNA mutations. For example, *MT-ATP6* mutations cause NARP at low level of heteroplasmy and MILS when the level is higher than 90% (Thorburn *et al*, 1993). The mutation load can also vary between individuals carrying the same mtDNA defect and even among different tissues in the same individual. For example, the mutation m.3243A > G causative of MELAS can be present at higher levels in the central nervous system than in the periphery, and with varying heteroplasmy in different brain regions of the same patients (Scholle *et al*, 2020). At the cellular level, increasing mutation load may lead to progressive changes in transcriptional response (Picard *et al*, 2014). At the same time, it has been proposed that mtDNA mutation load alone may not be sufficient to explain a defined clinical phenotype, as the individual nuclear background could modulate the presentation of mtDNA diseases (D'Aurelio *et al*, 2010; Pickett *et al*, 2018).

How mtDNA mutations are inherited is a matter adding further complexity to the study of mtDNA diseases. mtDNA mutations in humans are considered to be almost invariably only maternally inherited, as sperm mitochondria are degraded upon fertilization (Stewart & Chinnery, 2015). However, there have been reports suggesting bi-parental inheritance of mtDNA mutations (Luo *et al*, 2018). The occurrence of such paternal inheritance of mtDNA is highly debated. One alternative explanation involves the transmission of nuclear-encoded mitochondrial sequences (NUMTs) that may create the impression of mtDNA heteroplasmy (Wei & Chinnery, 2020). Indeed, recent findings showed that NUMTs are frequent in the general population (Wei *et al*, 2022a).

The difference in heteroplasmy observed among offspring of the same mother is usually explained by the presence of a genetic bottleneck taking place during oocyte development (Stewart & Chinnery, 2015). According to this hypothesis, there is a reduction in mtDNA copies in the early oocytes, which can inherit a different proportion of mutated mtDNA molecules from maternal mitochondria. During oocyte maturation and cell division, the mutation load can then increase, decrease, or remain the same, thereby leading to the development of offspring with variable heteroplasmy (Zaidi *et al*, 2019; van den Ameele *et al*, 2020). In addition, there is evidence suggesting the presence of selection for or against defined mtDNA variants (Wei & Chinnery, 2020). In particular, it has been reported that non-synonymous changes in mtDNA protein-coding genes are selected against in the germline, thereby preventing most mtDNA mutations from reaching homoplasmy (Stewart *et al*, 2008). A developmentally programmed mitochondrial degradation may be instrumental for such mtDNA selection (Palozzi *et al*, 2022). The mode of inheritance appears even more complex for large-scale mtDNA deletions, as they seem to be rarely transmitted to offspring in humans (Chinnery *et al*, 2004).

To further complicate the study of pathological mtDNA variants, it has been reported that mtDNA defects also occur somatically in different tissues and organs later in life (Payne *et al*, 2011). The accumulation of mtDNA mutations may not be linked to oxidative-mediated mtDNA damage (Kennedy *et al*, 2013). Conversely, as the mitochondrial genome undergoes continuous replication even in post-mitotic cells (Magnusson *et al*, 2003; Gustafsson *et al*, 2016), mtDNA might accumulate mutations over time at higher rates compared to nuclear DNA, despite the high fidelity of PolG (Nissanka *et al*, 2018; Stewart & Chinnery, 2021). Among acquired mtDNA defects, multiple large-scale mtDNA deletions have been found associated not only with genetic defects in nuclear genes encoding for proteins involved in mtDNA replication and mitochondrial dynamics but also with physiological aging (Payne *et al*, 2011; Vincent & Picard, 2018). Although the mechanisms underlying the tissue-specific and temporal distribution of such somatic mtDNA mutations remain unclear, it has been suggested that the diversity in mtDNA heteroplasmy might contribute to the development of late-onset disorders such as neurodegeneration, diabetes, and cancer (Hahn & Zuryn, 2019). Since mtDNA heteroplasmy can be a common phenomenon even in healthy conditions (Payne *et al*, 2013; Wei & Chinnery, 2020), it has been proposed that mtDNA mutations observed in older individuals might have been present since birth although at lower heteroplasmy and that they might have been clonally expanded throughout life (Keogh & Chinnery, 2013). Heteroplasmic mtDNA mutations seen in multiple tissues may thus represent a mixture of mutations that are germline inherited and mutations accumulated somatically upon aging due to continuous mtDNA replication (Stewart & Chinnery, 2021).

Challenges in engineering mtDNA

A major obstacle in the study of mtDNA mutations has been the difficulty of engineering mtDNA. While the quickly evolving clustered regularly interspaced short palindromic repeats, (CRISPR)/CRISPR-associated proteins (Cas) field produces novel applications with astonishing speed, enabling nowadays the introduction of virtually any type of mutations in the nuclear DNA (Anzalone *et al*, 2020), manipulation of mtDNA remained difficult to achieve until very recently (Silva-Pinheiro & Minczuk, 2022).

Several peculiar features of the mitochondrion and its genome complicate editing approaches. First, the organelle double membrane represents an important barrier that is not easily crossed by nucleic acids. This lack of efficient import into mitochondria represents a crucial challenge, as the delivery of a DNA template is essential for precise genome editing with CRISPR nucleases (Gammage *et al*, 2018a). Furthermore, all CRISPR/Cas systems require guide RNAs (gRNAs) in order to direct the genomic scissor to a specific target site. Thus, impaired nucleic acid import precludes the application to mtDNA editing even for CRISPR/Cas base editors that do not require template DNA (Jinek *et al*, 2012).

Even if DNA delivery might be achieved, it appears quite difficult for this DNA to integrate into the host mitochondrial genome. This is because mtDNA does not exhibit the same repairing mechanisms as nuclear DNA. Upon a DNA double-strand break, mtDNA does not attempt to initiate a repair process, but rather undergoes degradation (Nissanka *et al*, 2018; Peeva *et al*, 2018). Although there is evidence of base excision repair (BER) and single-strand break (SSB) repair in mtDNA, mitochondria do not seem to possess efficient double-strand break (DSB) repair mechanisms, such as homologous recombination (HR) or non-homologous end joining (NHEJ; Kazak *et al*, 2012). This lack of efficient recombination hampers the possibility for exogenously delivered DNA to be readily integrated into the host mtDNA (Hagström *et al*, 2014).

The multi-copy nature of mtDNA adds an additional layer of complexity. In order to achieve effective correction, it is necessary to find a way to select the desired mtDNA variant among a high number of mtDNA molecules (Silva-Pinheiro & Minczuk, 2022). The possibility of exogenously modifying the mutation load, known as "heteroplasmy shift," has been exploited by several groups using different models (Jackson *et al*, 2020). The heteroplasmy shift is achieved by selectively eliminating mutant mtDNA molecules and allowing the remaining wild-type mtDNA molecules to repopulate the mtDNA pool. Given the threshold effect of mtDNA diseases, shifting mtDNA heteroplasmy toward the wild-type sequence could allow restoration of proper mitochondrial functionality.

Heteroplasmy shift has been accomplished following different strategies. First, scientists used mitochondrially targeted restriction endonucleases (mitoREs) that specifically cut mutant mtDNA copies and thereby induce their selective degradation (Srivastava & Moraes, 2001; Tanaka *et al*, 2002; Bacman *et al*, 2012; Reddy *et al*, 2015). Although effective, the use of mitoREs is, however, limited by the scarcity of unique restriction sites specific for pathogenic mtDNA mutations. A more versatile heteroplasmy shift can be obtained by employing mitochondrially targeted transcription activator-like effector nucleases (mitoTALENs; Bacman *et al*, 2013; Hashimoto *et al*, 2015; Yang *et al*, 2018) and mitochondrially targeted zinc finger nucleases (mtZFNs; Minczuk *et al*, 2008; McCann *et al*, 2018). These approaches can target both point mutations and deletions by introducing a DSB. This targeted heteroplasmic shift was successfully demonstrated in various models (Bacman *et al*, 2013; Gammage *et al*, 2014, 2018b; Hashimoto *et al*, 2015; Reddy *et al*, 2015). Variations of these techniques include smaller-sized constructs to facilitate packaging into adeno-associated virus (AAV) vectors, mito I-CreI homing endonuclease-based designer meganuclease (mitoARCUS) that is smaller in size but with relatively long recognition site (Zekonyte *et al*, 2021), and mitoTALENickase that is capable of introducing single large-scale mitochondrial deletions (Phillips *et al*, 2017).

Despite these progresses, heteroplasmy shift approaches cannot be applied to cells carrying mtDNA mutations at homoplasmic level or at very high levels of heteroplasmy, as they require the presence of a sufficient number of wild-type mtDNA molecules to repopulate and restore function (Silva-Pinheiro & Minczuk, 2022). Furthermore, none of the techniques described above are capable of introducing precise mitochondrial genome modifications.

Models of mtDNA diseases

The difficulty of engineering the mitochondrial genome significantly hindered the development of animal and cellular models of mtDNA diseases. Despite these challenges, relevant cellular and animal models of mtDNA diseases have been generated (Fig 1).

Peripheral cells such as fibroblasts have been extensively used for investigating the pathogenesis of mtDNA diseases. Peripheral cells can be obtained with minimally invasive procedures, and can maintain patient-specific mtDNA mutations (Bourgeron *et al*, 1993). Patient-derived fibroblasts have been used to evaluate the functionality of the respiratory chain activity (Invernizzi *et al*, 2012; Saada, 2014), the integrity of the mitochondrial network (Caporali *et al*, 2020; Del Dotto *et al*, 2020), and to measure calcium metabolism or oxidative stress (Venco *et al*, 2015; Granatiero *et al*, 2016). Nonetheless, their restricted proliferative capacity and inter-individual variability hamper the application of peripheral cells in mechanistic disease-modeling studies.

A remarkable advance came with the development of transmitochondrial cytoplasmic hybrids (cybrids; King & Attardi, 1989). Cybrids are produced by fusing enucleated donor cells (cytoplasts) obtained from patients (e.g., fibroblasts or platelets) with recipient cells devoid of mtDNA (ρ^0). Cybrids have been instrumental in dissecting the pathogenicity of individual mtDNA defects *in vitro* (King & Attardi, 1989). Using cybrids, it is possible to verify the consequences of mtDNA point mutations and large-scale deletions and to correlate the heteroplasmy level of a specific mutation with the biochemical impairment (Picard *et al*, 2014; Cavaliere *et al*, 2022). Cybrids allow to determine the potential pathogenicity of any newly identified mtDNA mutations in patients. This information could play a role in genetic diagnosis: if the investigated mtDNA mutation does not show defects in cybrids, it is then possible that the patient phenotype may be caused by an unknown nuclear DNA mutation. Cybrids have been used in countless of *in vitro* works (Bugiardini *et al*, 2020; Hernández-Ainsa *et al*, 2022; Schaefer *et al*, 2022). In addition, they have also been crucial for the derivation of animal models (see below). Nonetheless, cybrids may hold some limitations as *in vitro* models of mtDNA diseases. Recipient cells are often

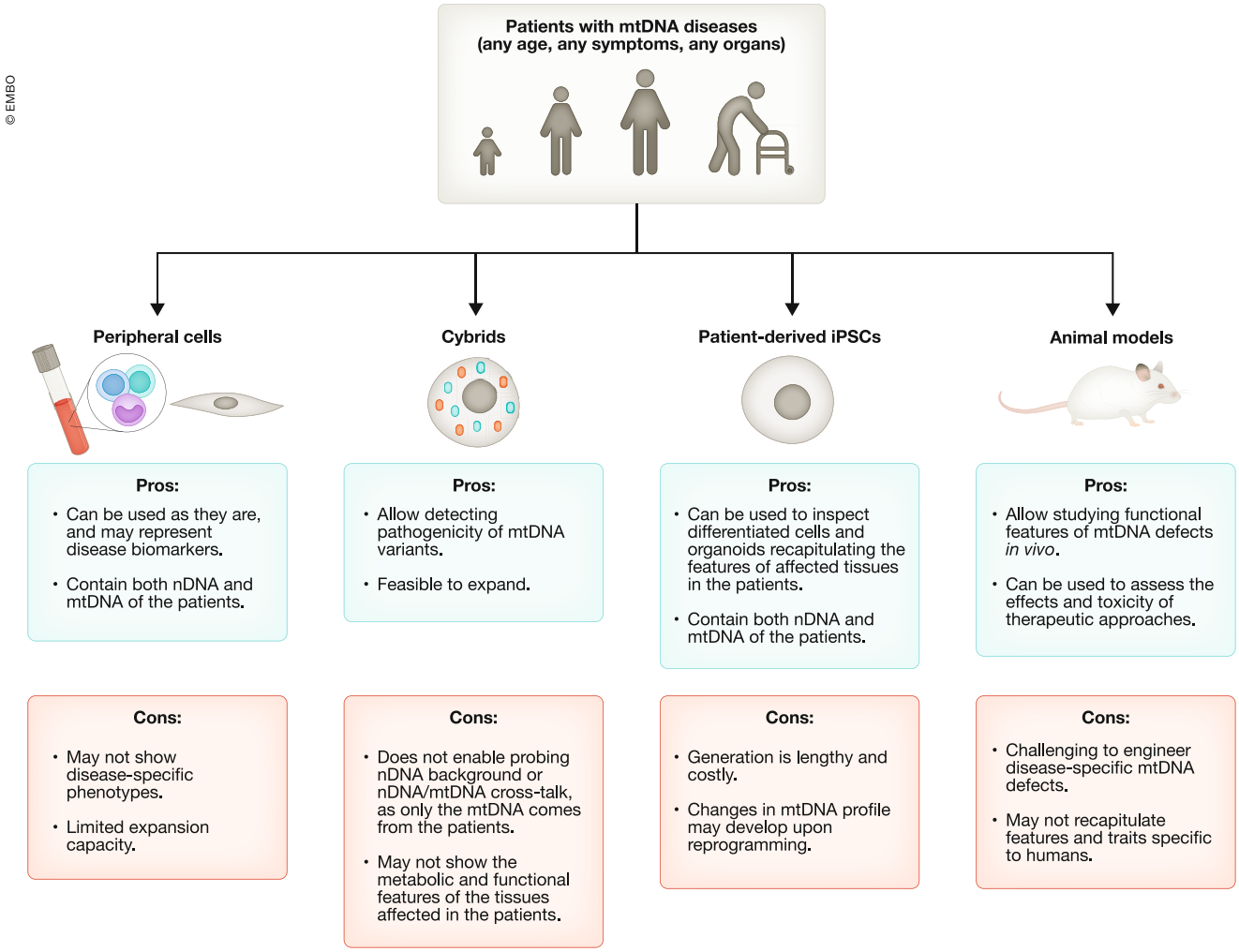

Figure 1. Modeling tools for mtDNA diseases.

Despite the challenges associated with mtDNA engineering, it has been possible to develop approaches for modeling mtDNA diseases. Comparison (pros and cons) of these modeling strategies are presented here as a schematic drawing. See text for details. nDNA, nuclear DNA; mtDNA, mitochondrial DNA; iPSCs, induced pluripotent stem cells.

represented by immortalized cancer-like cells to enable effective growth and expansion. Such cancer-like immortalized cells mainly rely on glycolysis and not OXPHOS for energy production (Kondoh *et al*, 2005; Giang *et al*, 2013; Wilkins *et al*, 2014). Hence, cybrids, as well as peripheral fibroblasts, cannot faithfully reproduce the functional features of OXPHOS-dependent neurons or cardiac cells, which are the cell types typically impaired in patients affected by mtDNA diseases (Pfeffer *et al*, 2013; Carelli & Chan, 2014).

Different mammalian animal models of mtDNA diseases have been successfully developed (Stewart, 2021). One strategy to generate mtDNA mutant mice has been to manipulate the mitochondrial replication machinery. By disrupting the catalytic subunit of the nuclear-encoded PolG, it has been possible to obtain mice known as "mtDNA mutator mice," which accumulate high levels of multiple mtDNA mutations that are transmissible in the germline (Trifunovic *et al*, 2004; Kujoth *et al*, 2005). These mice did not show typical signs of mtDNA diseases, but rather severe premature aging and early death. In a similar manner, the disruption of the nuclear-encoded mtDNA replication-associated helicase TWINKLE led to mice with multiple mtDNA deletions, known as "mtDNA deletor mice" (Tyynismaa *et al*, 2005). These mice developed features reminiscent of CPEO.

The first heteroplasmic mice harboring a pathogenic mtDNA mutation were the "mito-mice" (Inoue *et al*, 2000; Nakada *et al*, 2001). They were generated by fusing cytoplasts carrying a pathogenic 4,696 bp mtDNA deletion with mouse embryos. The mice had a severe phenotype and died early because of renal failure. By microinjecting cybrid embryonic stem cells (ESCs) carrying a single mtDNA mutation in the gene *MT-ND1* into mouse embryos, it was then possible to generate mice carrying a homoplasmic pathogenic mtDNA mutation (Kasahara *et al*, 2006). These mice displayed a milder phenotype with no change in life span. A similar approach was used to generate mice carrying a mutation in gene *MT-ND6* associated with LS and LHON phenotypes (Yokota *et al*, 2010).

These latter mice showed Complex I deficiency and lactate overproduction, but no other phenotype reminiscent of LS patients. Another mouse model of this mutation developed visual problems and degeneration of optic nerve cells (Lin *et al*, 2012). Heteroplasmic mice were derived using cybrid ESCs carrying a pathogenic mutation of a mtDNA-encoded tRNA known to cause MELAS-like clinical phenotype (Shimizu *et al*, 2014). These animals showed skeletal muscle defects and renal failure, thereby recapitulating some features of the human disease (Shimizu *et al*, 2015). Recently, it was possible to obtain mice showing metabolic defects carrying high heteroplasmy level of the pathogenic tRNA mutation m.2748A > G, which is orthologous to the human mutation m.3302A > G associated with MELAS (Tani *et al*, 2022).

As an alternative strategy, allotopic expression of mtDNA mutations has been attempted. This led to the development of mice with nuclear expression of the mitochondrial gene *MT-ATP6* harboring a mutation associated with NARP/LS syndromes (Dunn & Pinkert, 2012). These mice showed some behavioral defects. However, this approach remains controversial, as other works *in vitro* demonstrated that proteins expressed allotopically from the nucleus are not properly imported into the mitochondria (Perales-Clemente *et al*, 2011).

Induced pluripotent stem cells (iPSCs) and mtDNA modifications

Modeling mtDNA diseases can benefit from the development of cellular models that not only carry specific pathogenic mtDNA mutations but also exhibit the functional and metabolic properties of the cells and tissues affected in the patients. A technology that allowed establishing models with such features came in 2006–2007 with the discovery of induced pluripotent stem cells (iPSCs; Takahashi & Yamanaka, 2006; Takahashi *et al*, 2007).

iPSCs are obtained from somatic cells through forced expression of transcription factors that are specific for ESCs. Like ESCs, iPSCs acquire the ability to proliferate indefinitely (i.e., self-renewal) and to generate cell progenies belonging to all three germ layers (i.e., pluripotency). Unlike ESCs, however, iPSCs do not require embryos, and can be derived from the somatic cells of virtually every individual using a robust and relatively straightforward process known as cellular reprogramming (Takahashi *et al*, 2007; Shi *et al*, 2017). Effective reprogramming has been demonstrated using a number of different techniques and diverse parental somatic cells (Shi *et al*, 2017). Nowadays, iPSCs are considered a remarkable platform not only for investigating disease-related mechanisms but also for the establishment of drug discovery pipelines (Shi *et al*, 2017; Rowe & Daley, 2019).

One relevant issue to take into consideration before applying the iPSC system for modeling mtDNA diseases is to determine whether the mtDNA profile can be effectively retained during the reprogramming process. In 2011, we investigated this aspect and found that human iPSCs carried mtDNA variants that were not observed in the parental fibroblasts (Prigione *et al*, 2011). Numerous works later confirmed these initial observations (Perales-Clemente *et al*, 2016; Kang *et al*, 2016a; Zambelli *et al*, 2018; Deuse *et al*, 2019; Palombo *et al*, 2021; Wei *et al*, 2021). Detected mtDNA mutations affected various mtDNA genes (encoding for mRNAs, tRNAs, or rRNAs) as well as mtDNA haplogroup-specific regions, and encompassed both synonymous and non-synonymous modifications. Overall, it was estimated that in human iPSCs, the mutation rate for mtDNA is about 8.62×10^{-5} per base pair (Wei *et al*, 2021), while the mutation rate for the nuclear genome is about 1×10^{-9} per base pair (Kuijk *et al*, 2020). Importantly, the heteroplasmy of these mtDNA mutations could differ greatly among iPSC lines generated from the same donor cells. mtDNA mutations in iPSCs may have consequences on bioenergetics, redox balance, and overall cellular functions, and may also encode neoantigens potentially responsible for eliciting immune responses (Chen *et al*, 2018; Deuse *et al*, 2019). Hence, differences in mtDNA profiles might contribute to behavioral heterogeneity among iPSC clones (Kelly *et al*, 2013; Carelli *et al*, 2022). Several groups have, therefore, argued that screening for mtDNA modifications should become part of the routine quality control of all newly generated iPSC lines, in a similar manner as nuclear chromosomal rearrangements are currently monitored (Hämäläinen, 2016; Lorenz & Prigione, 2016; Carelli *et al*, 2022; Rossi *et al*, 2022).

The mechanisms underlying mtDNA changes upon reprogramming of somatic cells into iPSCs are not known (Sercel *et al*, 2021). During the generation of iPSCs, there is a reduction in the mtDNA copy number that can in turn increase upon differentiation (Facucho-Oliveira & St John, 2009; Prigione *et al*, 2010; St John, 2016). For this reason, we initially interpreted changes in mtDNA mutation profile in iPSCs as a "genetic bottleneck" (Prigione *et al*, 2011), a phenomenon reminiscent of the events occurring during germ line development where a restriction in the mtDNA amount can cause a different distribution of mutated mtDNA to the daughter cells (Stewart & Chinnery, 2015). This interpretation still holds true, but it remains to be determined whether it is a pure random phenomenon or whether there are mechanistic drivers (Sercel *et al*, 2021; Carelli *et al*, 2022).

To simplify this phenomenon, we could imagine two potential scenarios underlying the mtDNA heteroplasmy shift in iPSCs reprogrammed from fibroblasts (Fig 2). The first possibility is that the mtDNA changes seen in iPSCs are a consequence of the mtDNA heterogeneity of the starting somatic cell population (Young *et al*, 2012). Since iPSCs are clonally derived from reprogramming one fibroblast cell, it is possible that this particular cell may be different from the rest of the fibroblast population and could carry rare mtDNA variants (Fig 2 left, yellow mitochondria). The derived iPSC lines will thus carry these rare mtDNA variants and not variants expressed by other fibroblast cells that were not reprogrammed (Fig 2 left, blue and red mitochondria). The rare mtDNA variants present in iPSC lines could also vary in heteroplasmy due to changes in mtDNA content that may lead to different ratios of mtDNA mutation load. The result will be that some of the mtDNA variants contained in the original fibroblast population are lost, while some are retained with similar or increased levels of heteroplasmy. The second possibility is that mtDNA mutations may occur *de novo* during iPSC reprogramming (Fig 2). Hence, in addition to rare mtDNA variants present in the original parental cell, iPSCs could harbor mtDNA mutations that were not present in the parental samples (Fig 2 right, purple mitochondria). These *de novo* mtDNA modifications could also show various heteroplasmy levels in different iPSC lines due the mentioned bottleneck effect.

Overall, it remains to be determined whether the mtDNA bottleneck effect and related heteroplasmy shift in iPSCs are simply stochastic or rather driven by biological mechanisms, for example, due

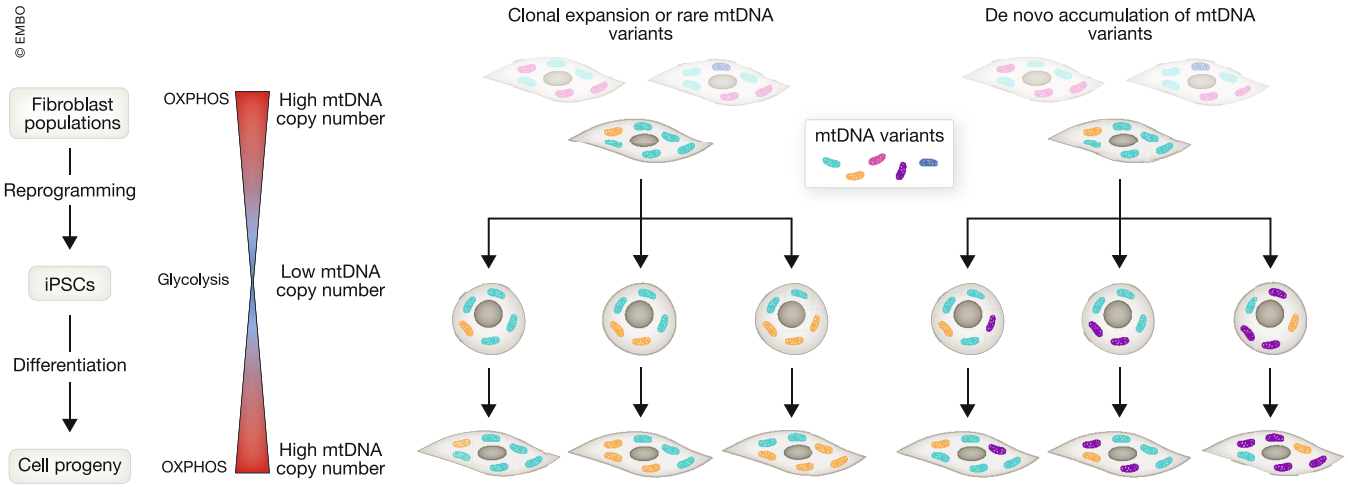

Figure 2. mtDNA bottleneck and heteroplasmy shift in human iPSCs.

During reprogramming of somatic fibroblasts to induced pluripotent stem cells (iPSCs), the cells acquire a glycolytic metabolism and reduce their number of mtDNA copies. Upon differentiation of iPSCs into energy-demanding progenies, the metabolism shifts toward oxidative phosphorylation (OXPHOPS) and the mtDNA copy number increases. These processes are accompanied by changes in the mtDNA profile that are not fully understood. mtDNA profile changes could be due to clonal expansion of rare mtDNA variants (left, yellow mitochondria) or to *de novo* introduction of mtDNA alterations (right, purple mitochondria). In the first case, the heterogeneity of the somatic cell population may play a role. Since iPSCs are clonally derived, it could be that the fibroblast cell from which iPSCs are generated would carry mtDNA variants that are different from the rest of the fibroblast cell population. In the second case, there may be mtDNA alterations arising as a consequence of reprogramming (e.g., because of errors in mtDNA polymerase gamma). In all cases, the rare variants derived from the parental cells (left, yellow mitochondria) or introduced during reprogramming (right, purple mitochondria) could acquire different heteroplasmy in various iPSC clones. The mtDNA profile of each iPSC line appears to be overall maintained during differentiation, despite changes in metabolism and mtDNA copy number (Perales-Clemente *et al*, 2016; Kang *et al*, 2016a; Palombo *et al*, 2021; Wei *et al*, 2021).

to altered PolG activity or redox imbalance. Some mtDNA variants might have a selective advantage (Yoneda *et al*, 1992; Russell *et al*, 2018) or may promote the metabolic–epigenetic reconfiguration associated with cellular reprogramming (Shakiba *et al*, 2019; Sercel *et al*, 2021). During the generation of iPSCs, in parallel to epigenetic changes leading to the establishment of an embryonic-like gene expression program, there are modifications occurring at the mitochondrial and metabolic levels (Prigione *et al*, 2010; Folmes *et al*, 2011; Agathocleous & Harris, 2013; Lisowski *et al*, 2018). iPSCs acquire a glycolytic-driven metabolic state that is then converted to OXPHOS upon differentiation. Hence, specific mtDNA modifications might potentially be needed to support this metabolic reconfiguration. However, multiple groups demonstrated that mtDNA mutations were retained without significant heteroplasmic changes during subsequent cultivation and differentiation of the iPSC lines (Perales-Clemente *et al*, 2016; Kang *et al*, 2016a; Palombo *et al*, 2021; Wei *et al*, 2021). Furthermore, mtDNA modifications including point mutations and large-scale deletions have been observed also in human ESCs (Maitra *et al*, 2005; Van Haute *et al*, 2013). Thus, it remains unclear how reprogramming-associated and differentiation-associated metabolic reconfigurations could influence the heteroplasmy shift in iPSCs. Understanding these processes will likely have important implications for the application of iPSCs in biomedical research (Box 1).

iPSCs and 3D organoids as next-generation models for mtDNA diseases

iPSCs hold great potential for modeling mtDNA diseases given their ability to generate virtually any tissue of the human body. mtDNA diseases particularly affect tissues and cells that are highly dependent on mitochondrial energy production, such as neuronal cells, muscle, and cardiac cells (Pfeffer *et al*, 2013; Carelli & Chan, 2014). Differentiated cells from iPSCs do in fact show reliance on OXPHOS metabolism (Chung *et al*, 2007; Zheng *et al*, 2016b; Cliff & Dalton, 2017; Tanosaki *et al*, 2021). Hence, by using differentiated cells from patient-derived iPSCs, it may be possible to obtain model systems that carry patient-specific mtDNA mutations and exhibit appropriate metabolic and functional properties. Such models could shed light on the mechanisms underlying mtDNA diseases and could help identify targets of intervention (Inak *et al*, 2017; McKnight *et al*, 2021).

In addition to two-dimensional (2D) differentiation of iPSCs, it is now possible to obtain three-dimensional (3D) organized tissues known as organoids. Several organoid models have been obtained from human iPSCs, including brain, gut, liver, blood vessels, kidney, and retina (Nakano *et al*, 2012; Lancaster *et al*, 2013; Takebe *et al*, 2013; Taguchi *et al*, 2014; Workman *et al*, 2017; Wimmer *et al*, 2019; Cowan *et al*, 2020; Lewis-Israeli *et al*, 2021). Organoids recapitulate features of human organs with respect to cellular organization and architectures. Their formation *in vitro* is accomplished by modulating signaling pathways that are known to guide and govern patterning and tissue morphogenesis *in vivo* (McCauley & Wells, 2017). In the context of mtDNA diseases, organoids might thus help address the impact of mtDNA mutations in different human tissues and organs.

Given the strong impact that mtDNA defects typically have on cells of the nervous system, for investigating mtDNA diseases it may be particularly interesting to employ 3D structures recapitulating features of the human brain (Chiaradia & Lancaster, 2020; Le *et al*, 2021). These organoids, defined as brain organoids or neural organoids, can be obtained to reproduce features of different brain

Box 1. In need of answers

(i) What are the mechanisms underlying the heteroplasmy shift and bottleneck effect of mtDNA occurring upon reprogramming somatic cells to pluripotency? Can we modulate such phenomena?
(ii) If we cannot prevent the occurrence of mtDNA modifications in human iPSCs, can we design pipelines to effectively monitor the mtDNA profile and remove unwanted mutations for all iPSC lines used in medical applications and translational studies?
(iii) If we apply mtDNA base editing to introduce mtDNA mutations in healthy iPSCs, can we reach a level of mutation load high enough to cause functional defects and recapitulate disease-related phenotypes? Similarly, if we apply mtDNA base editing to correct mtDNA mutations in patient-derived iPSCs, can we decrease the mutation load significantly to the point that the functional defects are corrected, and the disease phenotypes ameliorated?
(iv) Is it possible to reliably predict which mtDNA base editing constructs and configurations are best suited to target each specific mtDNA mutation?
(v) How can we further reduce unwanted off-target effects in nuclear or mitochondrial genomes that might be associated with mtDNA base editing?
(vi) Will it be possible to develop novel mtDNA editing approaches to target pathogenic mtDNA mutations in sites that are currently not editable? And can there be editing approaches effective also for large-scale deletions?
(vii) Can we obtain complex organoids and assembloid systems including vasculature and immune cells? And could such models be able to show the impact of mtDNA mutations and their segregation in different human tissues?

regions, including spinal cord, midbrain, cortex, striatum, or cerebellum (Paşca *et al*, 2022). Such brain region-specific organoids can then be mixed into so-called "assembloids" that can be used to address the communication between different brain regions (Miura *et al*, 2022). Brain organoids typically consist of cells belonging to the neuro-ectodermal lineage. For this reason, several groups are working on strategies to incorporate into brain organoids also endodermal-derived blood vessel cells and mesodermal-derived microglia. The presence of vasculature structure is necessary *in vivo* to enable effective neuronal commitment and maturation (Lange *et al*, 2016) and would be crucial for distributing oxygen and nutrients throughout the organoids. The incorporation of microglia into cerebral organoids would not only provide support for neuronal development but may also help unveil the impact of immune-mediated mechanisms in the modeled diseases.

In the past few years, several groups employed iPSCs derived from patients affected by mtDNA diseases to generate 2D and 3D models (McKnight *et al*, 2021; Table 2). The iPSC-based modeling works have so far mainly focused on diseases caused by single mtDNA mutations.

Several studies focused on MELAS, particularly, on the common mtDNA mutation m.3243A > G in the tRNA-encoding *MT-TL1* gene. In agreement with the heteroplasmy shift and bottleneck effect of iPSC generation, MELAS iPSC lines were found to carry the mutation at variable heteroplasmy (Folmes *et al*, 2013; Hämäläinen *et al*, 2013; Kodaira *et al*, 2015; Gunnewiek *et al*, 2020). Mutation-low or mutation-free iPSC lines could be obtained and used as isogenic controls (i.e., carrying the same nuclear DNA background). Differentiated neurons with high m.3243A > G mutation load showed defective mitochondrial complex activity and mitophagy (Hämäläinen *et al*, 2013), and aberrant neuronal network functionality and synchronicity (Gunnewiek *et al*, 2020). The MELAS mutation m.5541C > T in *MT-TW* gene caused defective neuronal differentiation without apparent impairment of neural progenitors or skeletal muscle cells (Hatakeyama *et al*, 2015). Other cell types were investigated as MELAS models, including endothelial cells that exhibited pro-atherogenic properties (Pek *et al*, 2019) and retinal pigment epithelium cells that showed dysfunctional phagocytosis (Chichagova *et al*, 2017). Lastly, by using 3D spinal cord organoids carrying the mutation m.3243A > G at high heteroplasmy, it was possible to uncover neurogenesis delays and impaired neurite outgrowth as mechanistic disease features of MELAS (Winanto *et al*, 2020).

LS iPSCs have been obtained from patients harboring homoplasmic mutations in gene *MT-ATP6*. Neural cells differentiated from these LS iPSCs exhibited defects in bioenergetics (Ma *et al*, 2015) and calcium homeostasis (Lorenz *et al*, 2017), and increased susceptibility to glutamate toxicity (Zheng *et al*, 2016a). Neurons derived from an LS individual carrying relatively low heteroplasmy level of a mutation in gene *MT-ND5* also showed defects in calcium signaling (Galera-Monge *et al*, 2020). In addition, 3D models of LS have been generated. Brain organoids carrying the mutation m.8993T > G in gene *MT-ATP6* at high heteroplasmy developed abnormally, showing reduced size and altered cortical architecture (Romero-Morales *et al*, 2022). Interestingly, similar defects in size and cellular organization were also observed in brain organoids derived from LS patients carrying mutations in the nuclear gene *SURF1* (Inak *et al*, 2021). These works collectively suggest that mitochondrial diseases such as LS could affect the physiological process of human neurogenesis (Brunetti *et al*, 2021).

To model LHON, iPSCs carrying mutations in the gene *MT-ND4* were differentiated into neurons (Danese *et al*, 2022) and retinal ganglion cells (Yang *et al*, 2020). These latter cells showed defective neurite outgrowth and increased mitochondrial biogenesis (Wu *et al*, 2018), disrupted action potentials (Yang *et al*, 2019), and increased oxidative stress and apoptosis with aberrant mitochondrial transport (Yang *et al*, 2019).

MERFF has also been modeled with iPSCs derived from patients carrying the mutation m.8344A > G in the gene *MT-TK* (Chou *et al*, 2016). There, differentiated cardiomyocytes and neural progenitors showed oxidative stress and defective mitochondrial dynamics. Neurons directly derived from fibroblasts of MERFF patients exhibited oxidative stress with reduced bioenergetics and increased mitophagy (Villanueva-Paz *et al*, 2019).

Human iPSCs carrying large-scale mtDNA deletions have been generated from patients affected by KSS and PMPS (Cherry *et al*, 2013; Russell *et al*, 2018; Lester Sequiera *et al*, 2021; Peron *et al*, 2021; Hernández-Ainsa *et al*, 2022). Initial studies showed defects in hematopoietic progenitors (Cherry *et al*, 2013), but the contribution of mtDNA deletions to the disease pathogenesis in differentiated progenies has not been addressed in detail.

Besides mechanistic studies, some iPSC studies focused on using the derived models to test therapeutic strategies. The drug Sonlicromanol, currently employed in a stage IIB clinical trial for MELAS (Janssen *et al*, 2019), was found beneficial on the activity and transcriptional signature of neuronal cells derived from MELAS patients

Table 2. iPSC models of mtDNA diseases.

Disease	Genes and mutations studied	Mutation levels in iPSC lines	Differentiated cell models investigated	Publications
Leigh Syndrome (LS)	*MT-ND5* (m.13513G > A) *MT-ATP6* (m.9185T > C; m.8993T > G)	Homoplasmy or high heteroplasmy for *MT-ATP6*. Low heteroplasmy for *MT-ND5*	Neural progenitors, neurons, and brain organoids	Ma *et al* (2015), Lorenz *et al* (2017), Zheng *et al* (2016a), Galera-Monge *et al* (2020), Romero-Morales *et al* (2022)
Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS)	*MT-TL1* (m.3243A > G) *MT-ND1* (m.5541C > T)	Variable heteroplasmy, including mutation-free iPSCs	Neurons, skeletal muscles, endothelial cells, retinal pigment epithelium, and spinal cord organoids	Hämäläinen *et al* (2013), Hatakeyama *et al* (2015), Winanto *et al* (2020), Pek *et al* (2019), Chichagova *et al* (2017), Gunnewiek *et al* (2020)
Myoclonic Epilepsy and Ragged-Red Fibers (MERRF)	*MT-TK* (m.8344A > G)	Variable heteroplasmy, including mutation-free iPSCs	Cardiomyocytes and neural progenitors	Chou *et al* (2016)
Leber's Hereditary Optic Neuropathy (LHON)	*MT-ND4* (m.11778G > A)	Homoplasmy	Retinal ganglion cells	Wu *et al* (2018), Yang *et al* (2019), Yang *et al* (2020)
Kearns–Sayre Syndrome (KSS)	Common deletion	Variable heteroplasmy	Neural progenitors, fibroblast-like cells, and cardiomyocytes	Lester Sequiera *et al* (2021)
Pearson Marrow–Pancreas syndrome (PMPS)	Common deletion	Variable heteroplasmy	Hematopoietic progenitors	Cherry *et al* (2013)

(Gunnewiek *et al*, 2021). Rapamycin improved the bioenergetics of neurons carrying homoplasmic *MT-ATP6* mutations and enhanced their resistance to glutamate toxicity (Zheng *et al*, 2016a). Somatic cell nuclear transfer (SCNT) was employed in iPSCs as a means to replace mutant mitochondria carrying a homoplasmic mutation in the gene *MT-ATP6* with healthy donor oocyte mitochondria (Ma *et al*, 2015). iPSC-derived neural cells from patients with mtDNA diseases can also be used to perform drug screenings to identify new treatments. In a proof-of-concept work, iPSC-derived neural cells harboring *MT-ATP6* mutations associated with LS were used to screen selected FDA-approved drugs, leading to the identification of potential compounds to be repositioned for LS (Lorenz *et al*, 2017).

The base editing revolution for mtDNA

A groundbreaking finding for the mtDNA disease field came in 2020 with the development by Mok *et al* (2020) of a genome engineering method that allows targeted C-to-T base conversion in the mtDNA. This approach is reminiscent of the CRISPR/Cas base editor system since it does not generate DSB and does not require a DNA template. In contrast to CRISPR/Cas, however, this new method does not depend on gRNAs for guidance to the target site. Instead, it modifies the mitochondrial genome through a deaminase enzyme guided by proteins termed transcription activator-like effectors (TALEs).

Mok *et al* (2020) identified a cytidine deaminase from *Burkholderia cenocepacia* that can act on double-stranded DNA and dubbed this enzyme "double-stranded DNA deaminase toxin A" (DddA). The discovery was remarkable as other deaminases known to date could only manipulate single-stranded DNA and were therefore dependent on enzymes capable of unwinding DNA double strands, such as Cas proteins. This limited the application of such base editing systems to nuclear DNA. Conversely, DddA can act directly on double-stranded DNA and enables transitions of C·G to T·A bp through deamination of cytidines at TC sites in the mtDNA. In the first step, cytidine is deaminated to uridine. In a second step during DNA replication, uridine is read as thymidine by the DNA polymerase and adenine is incorporated on the opposite strand. Uridine is then replaced by thymidine, thus completing the base editing process (Fig 3A). The DddA enzyme is split into two halves to avoid widespread deamination in unwanted locations, and each half is fused to TALEs designed to bind left and right of the desired target site. In order to reconstitute the deaminase at the target locus, two constructs are required for each manipulation, bringing both halves in close proximity. The constructs are imported into mitochondria via mitochondrial targeting sequences (MTS) fused to each N-terminus. Editing efficiency is improved through the fusion of one copy of uracil glycosylase inhibitor (UGI) to each C-terminus, which should prevent uracil excision during BER (Nilsen *et al*, 1997). This novel genome editing tool was coined "DddA-derived cytosine base editor" (DdCBE; Mok *et al*, 2020; Fig 3A).

Since the original publication in 2020, multiple groups have demonstrated the feasibility of this new DdCBE technique. Employed models included mice (Lee *et al*, 2021, 2022b; Guo *et al*, 2022; Silva-Pinheiro *et al*, 2022b; Wei *et al*, 2022b), rats (Qi *et al*, 2021), zebrafish (Guo *et al*, 2021; Sabharwal *et al*, 2021), and human zygotes (Chen *et al*, 2022; Wei *et al*, 2022c). Most of the works used mRNA microinjection as delivery system (Lee *et al*, 2021, 2022b; Qi *et al*, 2021; Sabharwal *et al*, 2021; Chen *et al*, 2022; Guo *et al*, 2022; Wei *et al*, 2022b, 2022c). Additionally, it was also possible to deliver DdCBE constructs into mouse heart via AAV, providing a proof of concept of *in vivo* mtDNA editing of post-mitotic tissues (Silva-Pinheiro *et al*, 2022b).

Besides being applied in a variety of organisms, the DdCBE technique is already evolving (Fig 3B). The tool has been adapted for use with zinc fingers as targeting proteins, a platform dubbed "zinc finger deaminases" (ZFD; Lim *et al*, 2022b). Besides achieving base editing with up to 30% efficiency in mtDNA, ZFD/DdCBE

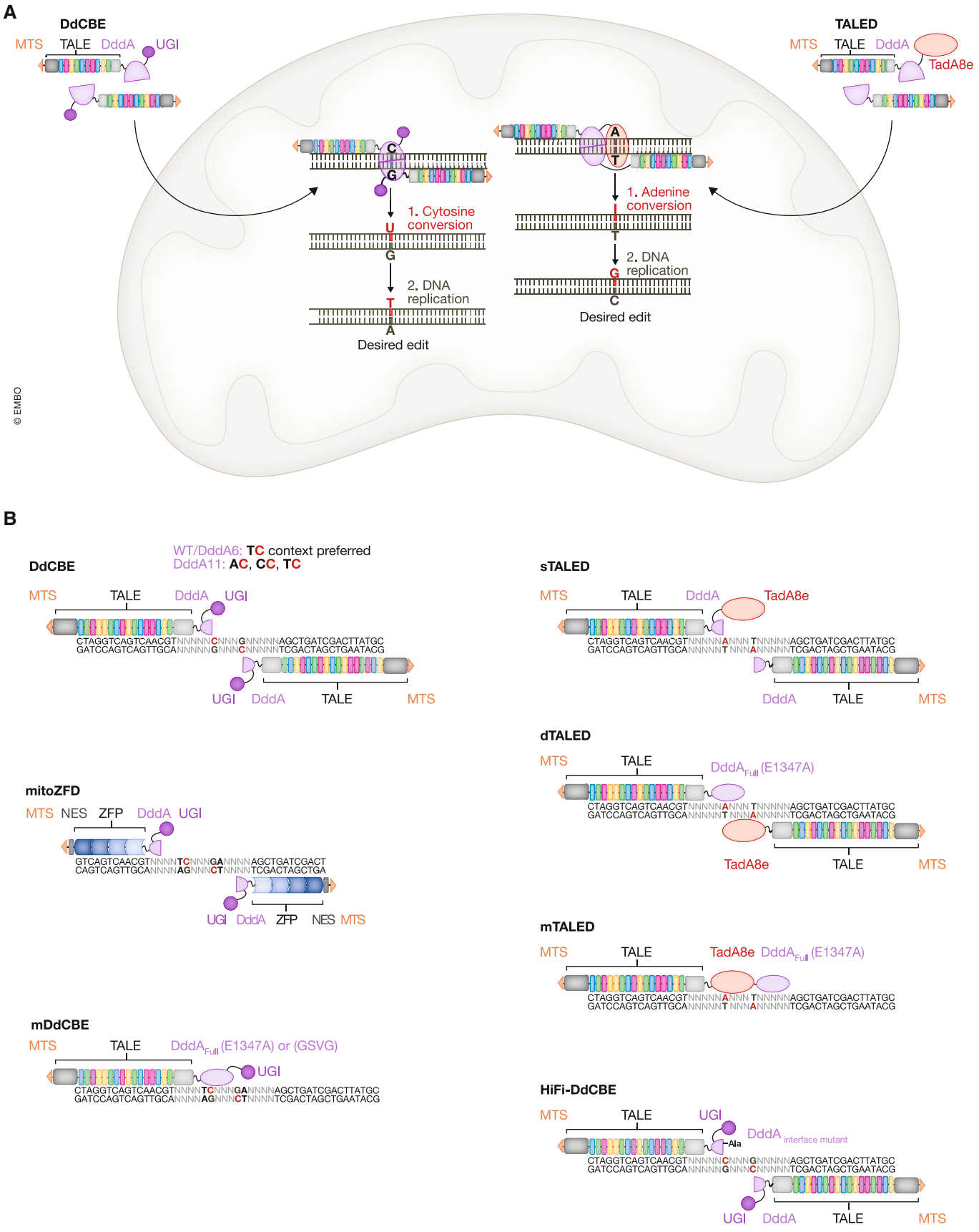
A
DdCBE
MTS TALE DddA UGI
TALED
MTS TALE DddA TadA8e
1. Cytosine conversion
2. DNA replication
Desired edit
1. Adenine conversion
2. DNA replication
Desired edit
© EMBO
B
DdCBE
WT/DddA6: TC context preferred
DddA11: AC, CC, TC
MTS TALE DddA UGI
UGI DddA TALE MTS
sTALED
MTS TALE DddA TadA8e
DddA TALE MTS
dTALED
MTS TALE DddA$_{Full}$ (E1347A)
TadA8e TALE MTS
mitoZFD
MTS NES ZFP DddA UGI
UGI DddA ZFP NES MTS
mTALED
MTS TALE TadA8e DddA$_{Full}$ (E1347A)
mDdCBE
MTS TALE DddA$_{Full}$ (E1347A) or (GSVG)
UGI
HiFi-DdCBE
MTS TALE UGI
DddA interface mutant
Ala
UGI DddA TALE MTS

Figure 3.

Figure 3. Base editing of mtDNA.

(A) Using double-stranded DNA deaminase (DddA)-derived cytosine base editors (DdCBE) or transcription activator-like effector (TALE)-linked deaminases (TALEDs), it is possible to introduce precise single gene modifications of the mtDNA. Nucleotides that are directly targeted are shown in red. The constructs are imported into the mitochondria via their mitochondrial targeting sequence (MTS) and are guided to the target site by their TALEs. In mitochondria, DddA halves are brought into close proximity and the enzyme is reconstituted. For DdCBE, in the first editing step, the nucleobase is deaminated, resulting in cytidine conversion to uridine (C > U). During DNA replication (second step), uridine is read as thymidine by the DNA polymerase and adenosine is incorporated on the opposite strand. In the next replication cycle, uridine is replaced by thymidine, ultimately resulting in the conversion of the mismatched U·G to a T·A bp. Analogously, in case of TALEDs, adenosine is first deaminated to inosine (A > I) and then the I·T bp is converted to a G·C bp during replication. Here, inosine is read as guanosine, and cytidine is incorporated on the opposite strand. In the next replication cycle, inosine is replaced by guanosine, thus completing the editing round. (B) Schematic representation of different mtDNA editing platforms. Both DNA strands can be edited. Targeted nucleotides are shown in red. DdCBE: DddA is split into two halves and thus two constructs per target site are required. Wild-type (WT) DddA and DddA6 prefer TC contexts, while DddA11 can additionally deaminate cytidines in AC and CC contexts. Zinc finger deaminases (ZFD): DddA is also split into two halves and editing occurs preferentially at TC sites. The constructs are guided to the target site via zinc finger proteins (ZFP), while nuclear export signals (NES) reduce nuclear localization. Monomeric DdCBE (mDdCBE): a full-length DddA mutant, either catalytically attenuated (E1347A) or with reduced cytotoxicity (GSVG), is fused to one single TALE protein with MTS and uracil glycosylase inhibitor (UGI). Only one construct per target site is required. TALEDs exist in three different configurations where adenine in any sequence context is converted to guanine. Split TALED (sTALED): DddA is split into two halves and a full-length adenine deaminase (TadA8e) is fused to the C-terminus of one construct. Dimeric TALED (dTALED): catalytically attenuated full-length DddA (E1347A) is fused to one construct and the full-length adenine deaminase is fused to another construct. Monomeric TALED (mTALED): only one construct per target site is required, and catalytically attenuated full-length DddA (E1347A) and full-length adenine deaminase TadA8e are fused to one single TALE protein with MTS. High-fidelity DdCBE (HiFi-DdCBE): DddA is split into two halves. An interface mutant with reduced spontaneous self-assembly due to an alanine mutation (−Ala) is fused to one construct; cytidines at TC, AC, and CC sites can be targeted with reduced off-target editing. Additional abbreviations: TadA8e: adenine deaminase; $DddA_{Full}$(E1347A): catalytically impaired full-length DddA; $DddA_{Full}$(GSVG): full-length DddA mutant with reduced cytotoxicity. C, cytidine; G, guanosine; U, uridine; A, adenosine; T, thymidine; I, inosine.

hybrids were shown to modify mtDNA in unique mutation patterns, sometimes even avoiding unwanted bystander editing (Lim *et al*, 2022b). Another improvement was the development of two mutant enzymes engineered from wild-type DddA: DddA6 for enhanced editing at TC sites, and DddA11 for editing in AC and CC sequence contexts in addition to TC sites (Mok *et al*, 2022a). Both DddA6 and DddA11 exhibited increased off-target editing of mtDNA, but enabled greater editing efficiency for difficult-to-edit loci and broaden the substrate scope (Mok *et al*, 2022a). Additionally, it has been possible to engineer a monomeric DdCBE (mDdCBE), a non-toxic full-length variant of DddA that does not need to be split into two halves (Mok *et al*, 2022b). The small size of mDdCBE facilitates the packaging into AAV, and the presence of a single TALE-binding site increases the number of targetable sites, adding more versatility to the mtDNA base editing method (Mok *et al*, 2022b).

Another important milestone for mtDNA base editing has been the development of TALE-linked deaminases (TALEDs; Cho *et al*, 2022). This technology allows A-to-G base conversion in the mtDNA through adenine deamination. This is achieved by fusing a known adenine deaminase (TadA8e) to a DdCBE construct. The possibility of modifying A·T to G·C bp significantly expands the number of potentially targeted disease-associated mtDNA mutations. Different TALED configurations have been reported, thereby increasing the options available for mtDNA base editing design with respect to the choice of delivery vehicle (e.g., AAV) and accessibility of target sites.

DdCBE has been already used to generate a mouse model carrying the pathogenic mutation m.13513G > A in *MT-ND5* gene (m.12918G > A in mice) that is associated in humans with LS, MELAS, and LHON syndromes (Lee *et al*, 2022b). The authors demonstrated improved editing efficiency at the target site by co-injecting DdCBE with mito-TALENs into mouse embryos. While DdCBE introduced the desired mutation, mito-TALENs were designed to specifically cut unedited copies. The resulting mice exhibited damaged mitochondria in tissues, as well as abnormal brain structures resulting in premature death (Lee *et al*, 2022b). Moreover, the development of a library of DdCBEs to knock out mouse mtDNA protein-coding genes led the Minczuk group to obtain a mouse model carrying the mutation m.8069G > A in gene *mt-Apt6* at high heteroplasmy (Silva-Pinheiro *et al*, 2022a).

One major concern for mtDNA base editing remains safety. Whereas off-target editing in mtDNA was shown to be minimal in the original work (Mok *et al*, 2020), increasing evidence suggests that it can be more substantial (Guo *et al*, 2021, 2022; Qi *et al*, 2021; Chen *et al*, 2022; Lee *et al*, 2022a; Silva-Pinheiro *et al*, 2022b; Wei *et al*, 2022b, 2022c). No off-target editing was observed in nuclear pseudogenes (Mok *et al*, 2020; Chen *et al*, 2022; Guo *et al*, 2022; Lee *et al*, 2022a; Silva-Pinheiro *et al*, 2022b; Wei *et al*, 2022c). However, the studies that analyzed genome-wide specificity on the nuclear genome detected significant off-target editing (Lei *et al*, 2022; Lee *et al*, 2022b; Wei *et al*, 2022b). It has been suggested that the split halves may spontaneously reassemble rather than depending on both TALE arrays for guidance (Lei *et al*, 2022; Lee *et al*, 2022b). To avoid this spontaneous reassembly, high-fidelity DdCBE (HiFi-DdCBE) was developed (Lee *et al*, 2022a). HiFi-DdCBE was obtained by mutating key residues at the split interface of DddA, thereby leading to decreased off-target assembly of both halves, while still enabling guided reconstitution at target site. Off-target editing could also be markedly reduced by including nuclear export signals (NES) on the editing constructs (Lei *et al*, 2022; Lee *et al*, 2022b), or by co-transfection of the DddA inhibitor $DddI_A$ (Lei *et al*, 2022; Lee *et al*, 2022b).

In less than 3 years since the original discovery of DdCBE, much has happened in the field of precise mtDNA manipulation (Table 3). Nevertheless, more work is needed to reliably predict which constructs and configurations are best suited for any given target site. More safety studies are also required before translating mtDNA base editing technology into human applications. Furthermore, not all types of mutations are targetable. With base editors, mtDNA manipulations are restricted to transitions (i.e., C<->T and G<->A), whereas transversions (i.e., interchanges of purines with pyrimidines), insertions, deletions, or mutations encompassing more than one nucleotide are not achievable (Box 1).

Table 3. mtDNA base editing platforms.

Platform	Type of mutation targetable	Pros	Cons	Publications
DdCBE	C > T and G > A in TC motifs	• Precise editing with minimal bystander editing • Large amount of data available • Tested in multiple organisms	• Editing restricted to TC motifs ➜ targetable pathogenic mutations are limited • Two constructs per target site required ➜ large-size, two TALE binding sites required	Mok *et al* (2020), Chen *et al* (2022), Wei *et al* (2022c), Lei *et al* (2022), Qi *et al* (2021), Lee *et al* (2021), Silva-Pinheiro *et al* (2022a), Silva-Pinheiro *et al* (2022b), Guo *et al* (2022), Wei *et al* (2022b), Lee *et al* (2022b), Guo *et al* (2021), Sabharwal *et al* (2021)
DddA6	C > T and G > A in TC motifs	• Enables editing of difficult-to-edit loci at TC sites with reduced bystander editing	• Editing restricted to TC motifs ➜ targetable pathogenic mutations are limited • Two constructs per target site required ➜ large size, two TALE binding sites required • Higher off-target editing	Mok *et al* (2022a)
DddA11	C > T and G > A in TC, AC, and CC motifs	• Broadens substrate scope to editing at AC, CC, and TC sites ➜ more mutations targetable	• Higher bystander editing ➜ less precise • Two constructs per target site required ➜ large size, two TALE binding sites required • High off-target editing	Mok *et al* (2022a)
ZFD	C > T and G > A in TC motifs	• Not limited to available TALE binding sites • ZFD/DdCBE hybrids exhibit distinct editing patterns ➜ alternative to DdCBE for difficult-to-edit loci	• Editing restricted to TC motifs ➜ targetable pathogenic mutations are limited • Two constructs per target site required ➜ large size	Lim *et al* (2022b)
TALED	A > G and T > C	• Adenine deamination significantly increases number of targetable mutations • No sequence context limitations • Three configurations for different target sites and experimental goals	• Precise targeting of distinct A or T in editing window difficult • Two constructs per target site required ➜ large size, two TALE binding sites required	Cho *et al* (2022)
mDdCBE	C > T and G > A in TC motifs	• Smaller size facilitates packaging into AAV • Only one TALE binding site required ➜ design more flexible and larger substrate scope	• Little data available	Mok *et al* (2022b)
HiFi-DdCBE	C > T and G > A in TC, AC, and CC motifs	• Decreased off-target editing	• Little data available	Lee *et al* (2022a)

Combining mtDNA editing with iPSCs and organoid models

To properly dissect the contribution of specific gene defects, it is important to use cell models that harbor the same genetic background in order to reduce variations that are not due to gene-specific changes. In the iPSC field, the gold standard for modeling diseases caused by nuclear mutations is to employ CRISPR/Cas systems to produce isogenic iPSC lines. Isogenic iPSCs carry the same genetic background and differ only at the site of a specific gene mutation. Isogenic iPSCs can be obtained either by introducing the disease mutation into control healthy iPSCs or by correcting the mutation in patient-derived iPSCs. By comparing 2D and 3D models derived from the unedited iPSCs and their engineered isogenic counterparts, it is then possible to identify defects that are caused specifically by the studied nuclear gene mutation (Ben Jehuda *et al*, 2018).

As mentioned above, such approaches are unfortunately more challenging for mtDNA defects. However, some strategies can be envisioned. In the investigation of pathogenic heteroplasmic mtDNA mutations, it is possible to take advantage of the spontaneous heteroplasmy shift occurring upon reprogramming to iPSCs. This can be achieved by screening for iPSC clones carrying very low levels of heteroplasmy without the need for external mtDNA manipulation (Fujikura *et al*, 2012; Hämäläinen *et al*, 2013; Ma *et al*, 2015; Lin *et al*, 2019; Gunnewiek *et al*, 2020; Fig 4A). However, given that the iPSC-associated heteroplasmy shift cannot be effectively

controlled or predicted, the identification of isogenic iPSC lines may require extensive screening of numerous iPSC clones. In order to more precisely manipulate the heteroplasmy level of patient-derived iPSCs, it is possible to employ mitoREs, mito-TALENS, or mtZFNs (Yahata *et al*, 2017, 2021; Yang *et al*, 2018, 2020). These approaches cause heteroplasmy shifts that have the potential to lead to the generation of isogenic iPSC lines containing low levels of mtDNA mutations. Lastly, mtDNA base editing strategies such as DdCBE might also be applied to directly insert precise site-specific modifications into the mitochondrial genome.

The ability to modify mtDNA via base editing opens the way to the generation of effective disease models also for homoplasmic mtDNA mutations (Fig 4B). Homoplasmic mtDNA mutations have so far not been possible to introduce or correct, neither through cellular reprogramming-associated modifications nor with conventional heteroplasmy shift approaches. DdCBE and other mtDNA base editing platforms may thus enable the derivation of isogenic iPSC lines, for example, by targeting a homoplasmic mtDNA mutation to significantly reduce its mutation load in order to revert the disease defects.

It is important to notice that the possibility to engineer mtDNA mutations in iPSCs could also enable the generation of mtDNA disease models without the need for obtaining somatic cells from patients (Fig 4C). This approach, already commonly accepted for nuclear gene defects, would allow investigating of cells and organoids for mutants and controls with identical nuclear genetic backgrounds. This strategy is particularly convenient for rare and pediatric conditions, as in the case of mtDNA diseases. Nevertheless, this approach would not allow for investigating the nuclear–mitochondrial genomes interplay. mtDNA alterations will only be

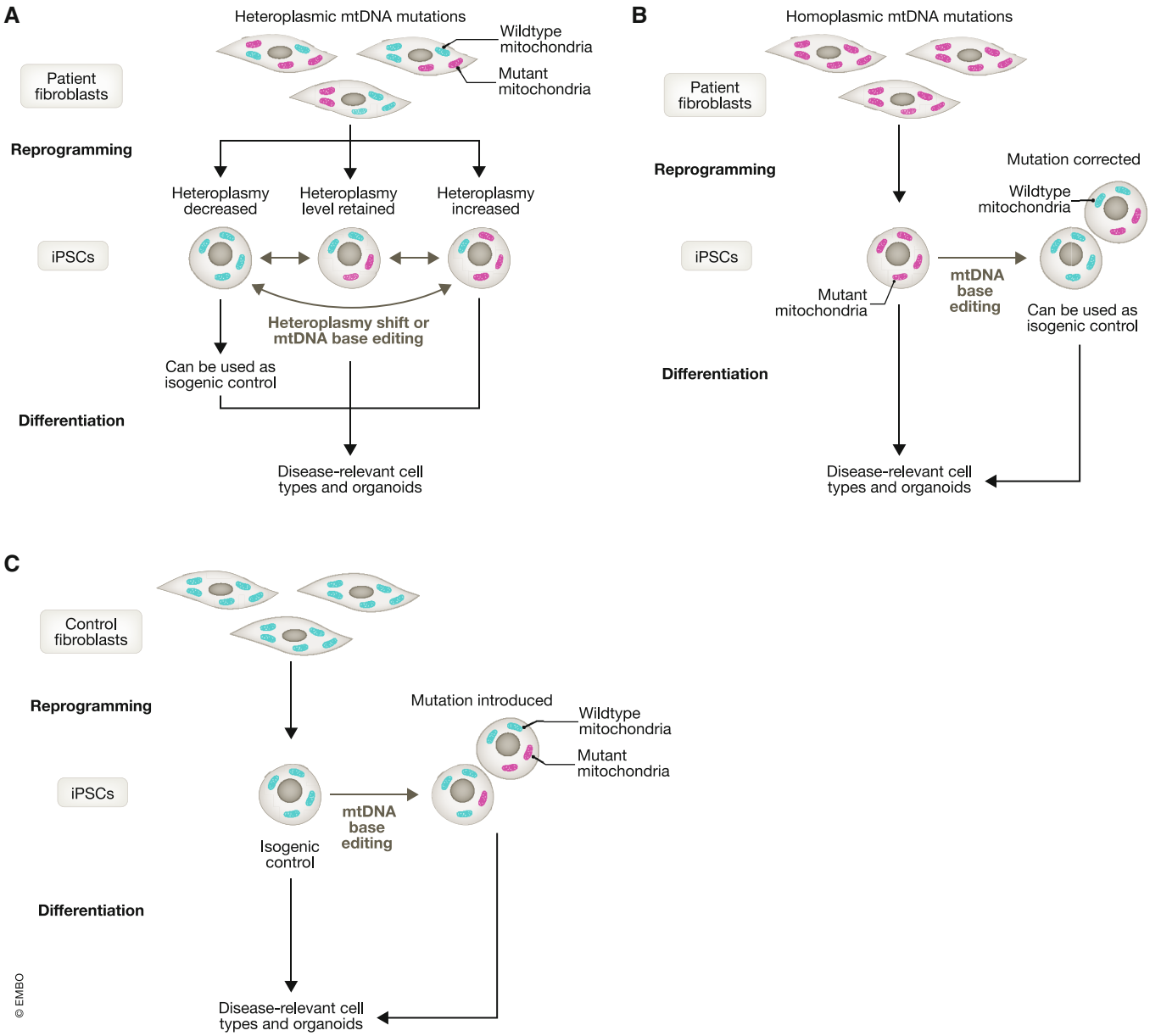

Figure 4.

Figure 4. Modeling mtDNA diseases combining iPSCs and mtDNA editing.
(A) Reprogramming to pluripotency of somatic cells carrying heteroplasmic mtDNA mutations may result in three different outcomes: (i) maintenance of the same heteroplasmy level, (ii) increase in heteroplasmy, and (iii) reduction in heteroplasmy. In case of decreased heteroplasmy, the obtained iPSC lines may be used as isogenic control without the need for further genetic manipulation. Mutation load might also be altered using heteroplasmy shift approaches, such as mitoREs, mito-TALENS, or mtZFNs. Lastly, mtDNA base editing like DdCBE could be employed to target the mutation and thereby modify its heteroplasmy. The resulting iPSCs carrying a pathogenic mtDNA mutation at various heteroplasmy levels in the same patient-specific nuclear and mitochondrial genetic background can then be differentiated into disease-relevant cell types or organoids for disease modeling. (B) During reprogramming to pluripotency of somatic cells carrying homoplasmic mtDNA mutations, homoplasmy is retained at the same level as the parental cells. Hence, heteroplasmy shift approaches are not effective in modulating the mutation load. In order to edit homoplasmic mtDNA mutations in iPSCs, mtDNA base editing would be the only option available. The edited iPSCs can be further differentiated into disease-relevant cell types or organoids for disease modeling. (C) mtDNA base editing might also be applied to modify the mtDNA profile of iPSCs derived from healthy control individuals. By introducing pathogenic mtDNA mutations, it may be possible to obtain models of mtDNA diseases that do not require the use of patient-derived cells. However, this approach will not allow for addressing the potential interplay between the nuclear and mitochondrial genomes in a patient-specific manner.

related to a control nuclear background, thereby limiting the possibility to unveil the interconnection between patient-specific nuclear DNA and the presence or absence of defined mtDNA alterations.

Lastly, mtDNA base editing in iPSCs may have potential utility also for modeling diseases caused by nuclear mutations. In fact, given that during cellular reprogramming changes in mtDNA profile can occur, one could envision the use of mtDNA editing to remove these unwanted mtDNA mutations from newly generated iPSC lines, before proceeding to use the iPSC lines in disease modeling and therapeutic applications (Box 1).

Concluding remarks and future outlook

The recent establishment of key remarkable technologies has brought important advances for mtDNA diseases. The multi-copy nature of mtDNA, its lack of efficient double-strand break repair mechanisms, and its cellular localization within a double-membrane organelle have made editing mtDNA a major challenge. As a result, only a limited number of cellular and animal models for mtDNA diseases could be created. The discovery of mtDNA base editing combined with the possibility to build 3D organoid models from human iPSCs have the potential to lead to the development of new effective model platforms for mtDNA diseases. These technologies may also help establish therapies in a personalized medicine manner, which could be potentially highly beneficial for highly heterogeneous conditions such as mtDNA diseases.

In order to move forward using these technologies, crucial questions and technical aspects remain to be addressed (Box 1). For example, we still do not know the mechanisms underlying the heteroplasmy shift occurring in human iPSCs. Moreover, current mtDNA base editing approaches only work for point mutations and not for large-scale deletions, and even for point mutations, they can only target few specific sites and cannot edit or correct all known pathogenic mtDNA mutations. Nevertheless, we expect that mtDNA-engineered iPSCs could represent a decisive advance in the study of mtDNA diseases. The heterogeneous and complex nature of these diseases makes it inherently difficult to decipher the contribution of a mutation to the pathophysiology of the disease. Comparing both mutant and control cells under the same nuclear background conditions could allow elucidating the contribution of specific mtDNA defects to disease phenotypes in different human tissues. Moreover, the use of complex organoid models could shed light on how mtDNA heteroplasmy segregates during human tissue organization. This knowledge could in turn lead to a better understanding of genotype–phenotype correlation and heterogeneous organ specificity of mtDNA mutations in patients with mtDNA diseases.

Overall, by using novel mtDNA base editors and applying them to iPSC-derived organoids, it may be possible to develop complex human models of mtDNA diseases. These new models could lead to the discovery of relevant pathological mechanisms and might pave the way to interventional strategies for these challenging diseases with high unmet medical needs.

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Author contributions

Isabella Tolle: Writing – original draft. **Valeria Tiranti:** Writing – review and editing. **Alessandro Prigione:** Conceptualization; supervision; funding acquisition; writing – review and editing.

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The authors declare that they have no conflict of interest.

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