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REVIEW ARTICLE

Mitochondrial diseases and mtDNA editing

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Abstract Mitochondrial diseases are a heterogeneous group of inherited disorders characterized by mitochondrial dysfunction, and these diseases are often severe or even fatal. Mitochondrial diseases are often caused by mitochondrial DNA mutations. Currently, there is no curative treatment for patients with pathogenic mitochondrial DNA mutations. With the rapid development of traditional gene editing technologies, such as zinc finger nucleases and transcription activator-like effector nucleases methods, there has been a search for a mitochondrial gene editing technology that can edit mutated mitochondrial DNA; however, there are still some problems hindering the application of these methods. The discovery of the DddA-derived cytosine base editor has provided hope for mitochondrial gene editing. In this paper, we will review the progress in the research on several mitochondrial gene editing technologies with the hope that this review will be useful for further research on mitochondrial gene editing technologies to optimize the treatment of mitochondrial diseases in the future.

Introduction

Serving as energy factories, mitochondria carry out oxidative phosphorylation to generate ATP for energy, which is essential for the proper homeostasis and function of individuals.[1] Mitochondria are semiautonomous organelles with their own genetic material and genetic system, in which 1500 mitochondria-associated proteins are encoded

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by nuclear genes, while a few rRNAs, tRNAs, and 13 protein subunits related to oxidative phosphorylation are self-encoded by mitochondrial genes[2]; thus, mitochondrial function is dependent on the interaction of nuclear and mitochondrial genes. Mitochondrial diseases are a group of genetic disorders that are characterized by defects in oxidative phosphorylation. Mutations in both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA), which are related to mitochondrial structure and function, may cause mitochondrial diseases. Mitochondrial diseases caused by mtDNA mutations are maternally inherited and permeable, and mtDNA mutations are prognostic for mitochondrial diseases. Mitochondrial diseases have gained much attention in recent years due to their unique pathogenesis and inheritance. In mitochondrial diseases, mitochondria contain both mutant and wild-type mtDNA, a condition known as heteroplasmy, and the ratio of mutant to wild-type mtDNA can result in a wide spectrum of clinical symptoms, with inherited metabolic-related disorders (*e.g.*, Leigh's disease) and inherited neurological disorders (*e.g.*, Leber hereditary optic neuropathy, LHON) being the main manifestations of mitochondrial defects.[3] For the random assortment of mitochondria during cell division, the offspring of mothers with mitochondrial diseases do not necessarily have the same disease phenotype because of the different number of mutant mitochondria acquired, which makes the treatment of mitochondrial diseases difficult. In the United States, an estimated 1000 affected children are born each year, while in the United Kingdom, the prevalence is estimated at 1 in 5000.[4] Mitochondrial replacement therapy (MRT) and mtDNA gene editing are two main potential treatments for mitochondrial diseases. For MRT, the nucleus is transferred from an oocyte at risk for mitochondrial disease to another cell with normal mitochondria. The disadvantages of this method are the inability to remove residual mtDNA, the long cycle time, the complexity of the operation, and ethical issues.[5] In mtDNA gene editing, mutated mtDNA is edited by mitochondrially targeted engineered nucleases, including mitochondrial-targeted transcription activator-like effector nucleases (mitoTALENs) and mitochondrial-targeted zinc-finger nucleases (mitoZFNs). With the development of third-generation gene editing, the clustered regularly interspaced short palindromic repeats (CRISPR) system emerged as an effective gene editing tool that can play the role of "universal scissors" in the nucleus. However, whether the CRISPR-Cas9 system can be used to edit mtRNA is controversial.[6] Recently, a new tool called DddA-derived cytosine base editor (DdCBE) has shown promise for mitochondrial gene editing.[7] In this paper, we briefly introduce mitochondrial diseases and provide an overview of mtDNA editing approaches to address mtDNA mutations and the problems that still exist to lay some foundation for the study of mitochondrial diseases, the elimination of mitochondrial mutations, and the development of potential treatments for mitochondrial diseases (Table 1).

Mitochondrial diseases

Mitochondria are essential for all cells to produce ATP through a process called oxidative phosphorylation, and as such, they are especially important for the more energy-consuming organs (brain, nerves, and heart). Mitochondrial diseases are a group of genetic disorders that are manifested by defects in the respiratory chain and caused by mutations in mtDNA and/or nDNA. The prevalence of mtDNA mutations was estimated to be 20 in 100,000, which is relatively higher than that for nDNA mutations (9 in 100,000).[8,9] Mitochondrial diseases affect approximately 1000 births per year in the US and 1 in 5000 in the UK.[9] With the analysis of large patient populations and advances in gene sequencing technologies, defects in more than 350 mitochondrial and nuclear-derived genes that may lead to mitochondrial diseases have been recognized, among which mitochondrial genes are the defective genes in approximately 77%.[10] As depicted in Figure 1, mitochondrial diseases caused by pathogenetic mtDNA mutations are maternally inherited. During cell division, mitochondria are mixed and randomly distributed to daughter cells. As a result, mtDNA mutations can be heterogeneous, and the offspring of the same patient genetically acquire widely different numbers of mutant mitochondria. Mitochondrial diseases occur only when the number of heterogeneous mutations exceeds a certain threshold level. Mitochondria diseases manifest mainly in the form of neurological and muscular pathologies, such as LHON, mitochondrial

Table 1 Mitochondrial genome editing technologies.

Types	ZFNs	TALENs	CRISPR/Cas9	DdCBE
Advantages	small specific	relatively easy to design highly specific	easy to design	highly specific low off-target edit mutation introduce targeted mutations
Limitations	mtDNA copy varation long execution time difficult to design high off-target	mtDNA copy varation large high off-target	gRNA import	only C→T
Recognition	ZFP	TALE	sgRNA	TALE
Effector	FokI	FokI	Cas9	Split-DddA halves

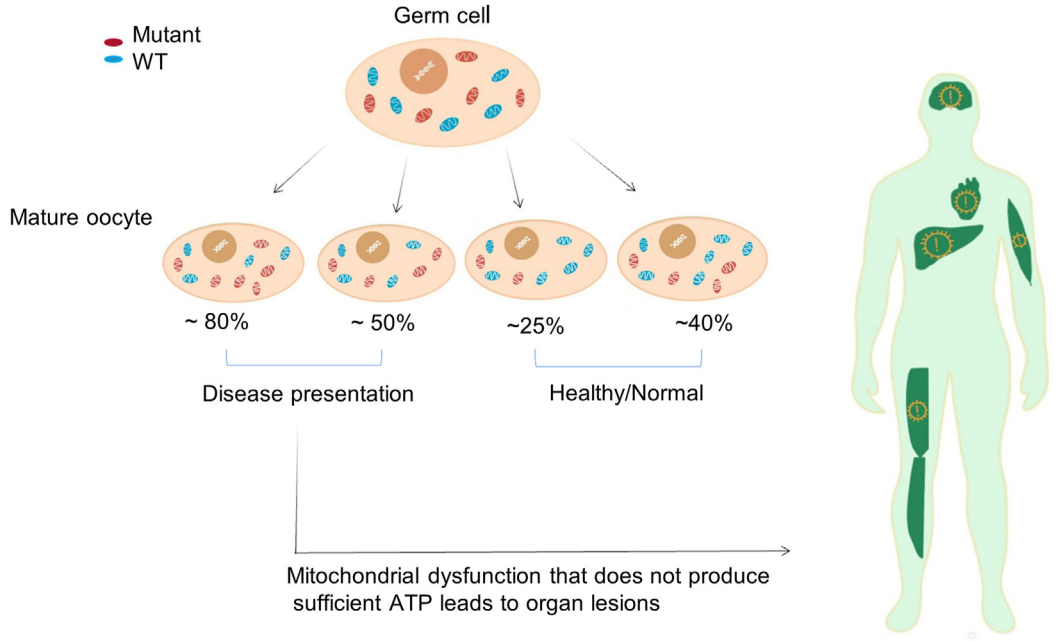

Figure 1 Mitochondria are heterogeneous, and germ cells carrying mutant mitochondria are inherited in such a way that the number of mutant mitochondria acquired by each oocyte is different; only when the number of mutant mitochondria reaches a certain threshold the mutation leads to mitochondrial disease.

encephalopathy lactic acidosis stroke-like episodes (MELAS), maternally inherited diabetes and deafness (MIDD), and myoclonic epilepsy with ragged red fibers (MERRF). Most patients with LHON are male, with a peak incidence in young adulthood, and the main symptom is painless acute vision loss in both eyes. Approximately 95% of patients with LHON are affected by mutations at the mG11778A, mT4484C, and mG3460A loci resulting in mitochondrial dysfunction and retinal neuronal damage leading to optic nerve atrophy.[11,12] The majority of MELAS patients are children and exhibit multisystem involvement, including encephalomyopathy, stroke-like episodes, heart disease, dementia, epilepsy, recurrent headache, hearing impairment, and diabetes mellitus. Approximately 80% of MELAS cases are caused by mutations in the A3243G locus of the mtDNA tRNA Leu (UUR).[13] mtDNA A3243G point mutations are also found in patients with MIDD. This locus mutation is a rare cause of diabetes mellitus and is often associated with deafness and some other complications similar to those of MELAS. Mitochondrial dysfunction in the pancreatic β-cells of patients with MIDD renders these cells unable to produce sufficient ATP, and their resulting insufficient insulin secretion causes abnormal glucose metabolism.[14] The typical clinical features of MERRF include myoclonus, epilepsy, ataxia, and myopathy.[15] MERRF syndrome can be caused by at least 18 mutaions, and it is mainly due to mutations in the A83344G locus of the mtDNA-encoded tRNA Lys (UUR) gene, resulting in reduced synthesis of complexes I and IV in the electron transport chain and the disruption of mitochondrial respiratory function.[16] Due to the lack of corresponding disease models, it is difficult to predict the inheritance and pathogenesis of mitochondrial diseases, and there is an urgent need to find an effective research method to obtain an in-depth understanding of mitochondrial diseases to find an effective therapeutic approach.

Currently, mitochondrial transplantation has been attempted to conquer mtDNA mutations, and MRT has been used to treat mitochondrial diseases in the clinic. Mitochondrial transplantation was proposed over 25 years ago and has been validated for protoplast transfer and spindle—chromatin complex transfer. In brief, the nucleus of an oocyte with mutated mtDNA is removed and subsequently transplanted into an oocyte with normal mitochondria, which is then fertilized. Mitochondrial transplantation is currently approved in the UK, and babies have been born following the implementation of this method. However, several studies have concluded that mitochondrial transplantation is associated with immune reactions and that multiple transplants are required to maintain the therapeutic effect.[17] Moreover, there are some ethical issues. As such, this approach has a narrow audience. With the in-depth development of traditional gene editing, gene editing targeting mitochondria has shown great promise for mitochondrial disease treatment. In contrast to mitochondrial transplantation, mitochondrial gene editing technologies do not have the abovementioned concerns. Mitochondrial gene editing technologies are targeted to edit the mutated mtDNA that causes mitochondrial diseases and to reduce the proportion of mutated mtDNA to alleviate symptoms. Mitochondrial gene editing technologies have broader application prospects than mitochondrial transplantation. In addition, the use of gene editing technology to develop relevant disease models will facilitate our understanding of the pathogenesis of mitochondrial diseases and mitochondrial functional pathways. Despite the rapid development of nuclear gene editing technology in recent years, no effective mitochondrial gene editing

technology has been found for the treatment of mitochondrial diseases due to the specificity of mtDNA, but great progress has been made since the introduction of mitochondrial gene editing, and it will be a powerful tool for the treatment of mitochondrial diseases in the future as research continues to progress.

mtDNA editing technology

As mentioned earlier, mitochondrial diseases often affect various organs throughout the body, and the most important cause of mitochondrial diseases is mtDNA mutations. With the development of gene editing technologies employing tools including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR) systems, and several other emerging genome editing tools, mtDNA editing technology has emerged and made it possible to manipulate mtDNA mutations and alleviate mitochondrial dysfunction.[18]

As shown in Figure 2, mitoZFNs and mitoTALENs work based on similar principles. Due to the permeability of the mitochondrial outer membrane, an efficient delivery system is needed to introduce exogenous DNA into the mitochondria. Mitochondria targeting sequence (MTS) is one of the commonly used mitochondrial targeted delivery tools, which can be recognized by translocation enzymes in the mitochondrial membrane and delivered to the mitochondrial matrix; mitoZFNs and mitoTALENs utilize proteins as guides that can cross the mitochondrial membrane. By fusing with MTS, ZFNs and TALENs enter the mitochondria and therein edit mutant mtDNA. ZFNs and TALENs bind to specific nucleic acid sequences based on their DNA binding sequence and exert nucleic acid endonuclease activity to break the mutant double-stranded mtDNA. Inefficient or nonexistent nonhomologous end jointing and homologous recombination prevent the rapid repair of DNA double-strand breaks.[19] The broken mitochondrial DNA is extremely unstable and prone to degradation, thus allowing the mutant mtDNA to be destroyed and reducing the effects of the mutation. However, ZFNs and TALENs cannot correct the mutated bases. CRISPR-Cas9 is a powerful tool for editing nuclear genes, but the mitochondrial membrane does not allow the passage of gRNA, making it unable to work on mitochondrial genes. A new RNA-free method DdCBE, which is capable of correcting mutated cytosines to uracils in mitochondria, has recently emerged. This technique is promising owing to its high target specificity and high product purity, enabling us to study the pathogenesis of mitochondrial diseases in greater depth and giving hope to some patients with mitochondrial diseases caused by C—U mutations.

ZFNs

ZFNs consist of two components, zinc finger proteins and the restriction nuclease cleavage domain FokI. The former is responsible for recognizing specific DNA sequences, while the restriction nuclease FokI domain mediates DNA cleavage and causes fragment insertion or code-shift mutations in target genes through DNA damage repair mechanisms.[20] The specificity of ZFNs is determined by the zinc finger protein region, which contains three to six Cys_2/His_2 fingers, each of which recognizes a triplet of encoded nucleotides. Two zinc finger proteins bind in close proximity to opposite strands of DNA, allowing the fused FokI

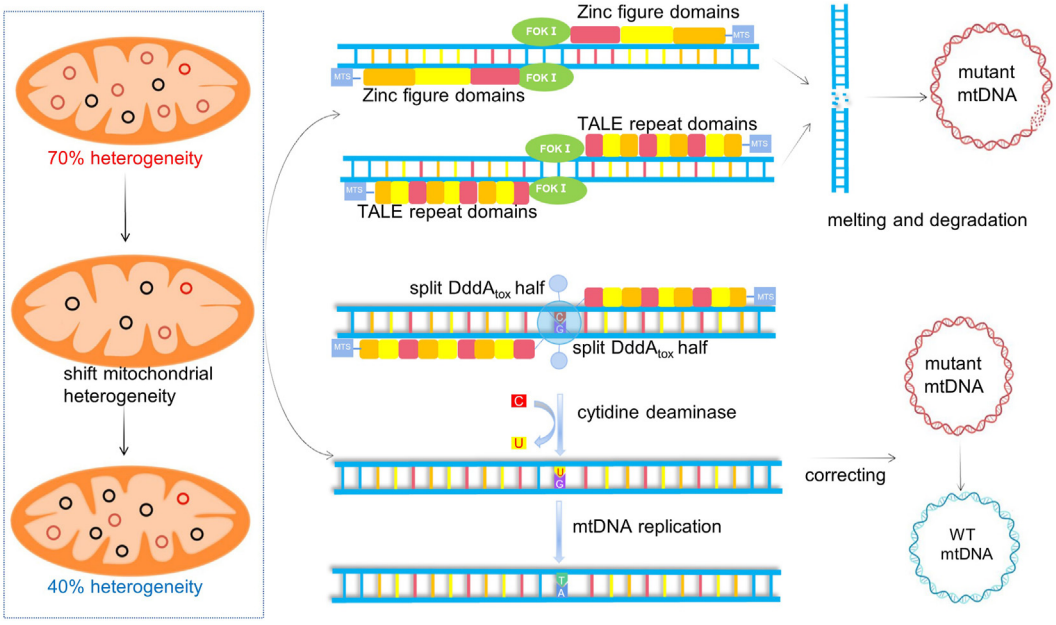

Figure 2 Several mitochondrial editing tools. By fusing with mitochondria targeting sequence (MTS), mitochondria editing machinery can enter the mitochondria. In the mitochondria, ZFNs and TALENs bind to specific mtDNA sites according to their DNA binding sequences and exert nucleic acid endonuclease activity to break the mutated double-stranded mtDNA, thus allowing the mutated mtDNA to be cleaved. Cytosine base editing DdCBE is capable of single base editing of mitochondrial genes, converting C-G base pairs to T-A base pairs and correcting mutated mtDNA.

endonuclease to form a functional dimer that cleaves DNA at the target site.[21] The FokI endonuclease cleavage domain is nonspecific and functions only when it is present as a dimer, which requires the design of 5′ to 3′ and 3′ to 5′ structural domains. ZFNs have been continuously improved to make it possible to edit mitochondrial genes more precisely. From the original pair of monomeric ZFNs targeting mitochondrial DNA to single-stranded ZFNs proteins with double FokI to dimeric ZFNs that have been further refined to use ZFNs with heterodimeric FokI for editing, ZFNs are constantly being updated for better application in mitochondrial gene editing.

By precisely targeting the mtDNA T8993G mutation and controlling the expression of mitoZFNs in the cells of patients carrying this mutation, the metabolic and mitochondrial respiratory functions of the mutant cells could be improved, and the phenotype of mitochondrial disease caused by this mutation could be alleviated.[22,23] Gammage et al developed the m.5024C > T tRNA (Ala) mouse model, which recapitulates the common molecular features of heterogeneous mtDNA diseases in cardiac tissue. In an experiment with these model mice, mitoZFNs targeting mtDNA C5024T were delivered to mutant mice and used to disrupt the mutation. A significant reduction in the level of mutated mtDNA was detected in the hearts of the mice, and a reversal of the disease phenotype caused by the mutation of this locus was observed. Mitochondrial function was restored in the heart tissue. These results suggest that mitoZFNs can be applied in mouse mitochondrial disease models to reduce specific mutant mtDNA copies and thus alleviate the corresponding mitochondrial disease.[24] These results predicted that the programmable ZFNs could be applied in the treatment of mitochondrial diseases. The programmable ZFNs are effective in alleviating the molecular biochemical characterization of heterogeneous mitochondrial diseases.[25]

As a traditional gene editing technique, the ZFNs method has its disadvantages, such as low editing efficiency and long execution time. In addition, each zinc finger proteome (including approximately 30 amino acids linked to a single zinc atom) binds 3 bp of DNA, which may also lead to potential inaccuracies. Moreover, the ZFNs shearing process does not depend on the formation of homodimers, so there are also off-target effects prone to cytotoxicity. These issues also need to be studied and optimized for better application of ZFNs to human mitochondrial gene editing.

TALENs

Transcription activator-like effector (TALE) was originally discovered in bacterial infections of plants due to the ability of TALE to target the promoter of transcriptional activation effectors to regulate transcription. TALENs are designed by fusing the DNA binding domain from TALE and the catalytic domain of FokI endonucleases.[26] Unlike ZFNs, TALENs have a stronger targeting specificity, and the DNA recognition domain in TALENs consists of approximately 20 TALE repeats, each of which recognizes the corresponding base, and the FokI endonuclease performs the corresponding cleavage. The DNA binding specificity of TALENs is higher than that of ZFNs, so TALENs can be more widely used in life sciences than ZFNs.[27]

mitoTALENs have been applied to mitochondrial gene editing. mitoTALENs can cure male sterility by knocking out genes (orf79 and orf125) associated with cytoplasmic male sterility in rice and oilseed rape varieties.[28] Redy et al demonstrated that targeted elimination of mutant mitochondrial DNA by mitoTALENs can correct heteroplasmic transfer to prevent germline transmission of mitochondrial disease.[29] Yang et al demonstrated that mitoTALENs successfully eliminated the m.3243A > G mutation in mitochondrial disease patient-specific induced pluripotent stem cells. After editing, these cells showed normal mitochondrial respiration and energy production, and differentiated neuronal progenitor cells also exhibited normal metabolism. In addition, the injection of mitoTALENs mRNA resulted in a significant reduction in human mutant mtDNA in reconstituted porcine oocytes. The potential of using mitoTALENs to specifically target mutant mtDNA in iPSCs and mammalian oocytes has been shown to be high, for example, for MELAS caused by m.3243A > G mtDNA mutations.[30] mitoTALENs research has also provided new insights to stop the intergenerational transmission of mitochondrial diseases and to avoid the operational complexities and ethical issues associated with "three-parent babies".[31]

TALENs are one of the most widely used mitochondrial gene editing technologies, but they are still associated with some problems. TALENs have a very high molecular weight, which creates a strong barrier to viral packaging and cellular and mitochondrial import. In addition, TALENs are often unable to repair mitochondrial genes when specifically cleaving mutant mtDNA and are rapidly cleaved, making conventional TALENs incapable of base editing and limiting their application for altering specific bases of mitochondrial DNA.

CRISPR-Cas9

The CRISPR-Cas9 system is an immune defense mechanism developed by archaea and bacteria over a long period to fight against foreign viruses and invasive exogenous DNA. Now, the CRISPR-Cas9 system has been extensively exploited to manipulate genes in mammals and is a Nobel Prize-winning technology.[32] Unlike the abovementioned nucleases that recognize target sequences, CRISPR-Cas9 performs nuclease cleavage of target sequences through RNA–DNA base pairing.[33] A single guide RNA (sgRNA) targets the gene of interest, and the Cas9 protease cleaves the target gene to form a double-stranded DNA break. The repair of double-stranded DNA break leads to small insertions or deletions, large deletions, and chromosomal translocations, achieving the purpose of gene editing. Currently, CRISPR-Cas9 can edit the nuclear genome effectively, but for the smaller and more delicate structure of mitochondrial CRISPR-Cas9 (mito-CRISPR-Cas9) to work, there are some apparent challenges.

In a study published by Jo and colleagues, CRISPR-Cas9 was used to degrade mtDNA using sgRNAs that recognize mitochondrial-specific sites. A decrease in mtDNA and its transcription was observed, which led to a disruption of the

mitochondrial membrane potential and a change in the inhibition of cell growth.[34] In addition, Bian et al applied the mito-CRISPR-Cas9 system to modify the Cas9 protein *in vitro* with MTS and transcripts. They transferred the mito-CRISPR-Cas9 protein into cells by microinjection. The mito-Cas9 protein was found to specifically target mtDNA and reduce the mtDNA copy number in human cells and zebrafish. The inserted exogenous single-stranded DNA was accurately knocked into the targeting site, and this mutagenesis was stably transmitted to the F1 generation of zebrafish.[35] The results of this experiment are consistent with a recent demonstration of CRISPR-mediated genome editing in yeast mitochondria and Chlamydomonas chloroplasts,[36] where simultaneous transfer of Cas9/sgRNAs and exogenous DNA homologous to the target site into cells revealed that the donor DNA could be integrated into the target site and was heritable. Although the results of these experiments suggest that CRISPR-Cas9 appears to be able to produce some integration and disruption of mitochondrial genes, these experiments do not explain the effective passage of sgRNAs across mitochondrial membranes. Controversy remains as to whether the CRISPR-Cas system can be used for mitochondrial gene editing and the mechanism of sgRNA import into mammalian mitochondria,[37] mainly because the inability of mitochondria to mediate sgRNA hinders the recognition of target gene sequences. In addition, an accepted molecular mechanism for RNA import into mammalian mitochondria is lacking, and existing studies have not effectively addressed this issue. For the application of CRISPR-Cas9 in mitochondrial gene editing, hindering the effect of the mitochondrial membrane on gRNA, how to accurately localize gRNA to mtDNA-specific sites, the relative size of CRISPR-Cas9, its operability in mitochondria, and the off-target effects are all issues that should be considered. Therefore, finding a safe and reliable method to efficiently introduce nucleic acids into mammalian mitochondria is a key prerequisite for performing mitochondrial genome editing with CRISPR-Cas9. The potential for the use of nanoparticles and Leishmania protozoan RNA introduction complexes to be used for mitochondrial delivery of exogenous DNA or RNA components has recently been demonstrated,[38] but both methods are currently still associated with multiple problems, and further research is needed to establish a complete system. At present, the application of CRISPR-Cas9 to mitochondrial gene editing is a challenging task with many problems to be solved.

DdCBE

To date, gene editing of mitochondrial DNA using nucleases can reduce mutations only by inducing double-strand breaks and degradation; it cannot correct mtDNA single-base mutations, which limits the application of ZFNs and TALENs for mitochondrial gene editing. Recently, scientists have developed a CRISPR-free mitochondrial base editing technique. The core of this technique is an interbacterial toxin called cytidine deaminase toxin (DddA), which catalyzes the deamination of cytidine and then enables the direct conversion of cytosine to uracil.[31] DddA per se is toxic to human cells and will damage DNA indiscriminately at various sites if left unchecked. To solve this problem, researchers split the active site of deaminase (G1333, G1397) into two parts ($DddA_{tox}$-N and $DddA_{tox}$-C). Only when the two parts are near each other will the deaminase work. DdCBE is mainly composed of two DddA halves, the mitochondrial targeting signal sequence MTS, TALE array protein, and the uracil glycosylase inhibitor UGI. Under the guidance of MTS, the abovementioned constituents enter mitochondria smoothly; therein, programmable TALE localizes to the customized DNA sequence, the adjacent split DddA are brought together, and cytosine is converted to uracil at the desired site.[39] UGI is adopted to protect uracil from the action of glycosylases; when the C-G conversion to U-G continues with the next round of replication, the guanine on the complementary strand is replaced by adenine, eventually converting the C-G base pair to a T-A base pair.[40]

In human cells, the editing ability of the DdCBE system has been demonstrated with efficiencies ranging from 4.6% to 49%.[31] The factors affecting the editing efficiency mainly lie in the location of the DddA break, the spacing between the two DddA hemisomes, the design of the TALE, and the binding location of the TALE to the target site. Researchers have used DdCBE to study pathogenic mutant mtDNA in many animals and human embryos. Mutant zebrafish and mice carrying base-modified mtDNA were generated by injecting DdCBE mRNA into the offspring. These mutations were heritable, with heterogeneity levels up to 26%.[41,42] In addition, Chen et al demonstrated for the first time the feasibility of DdCBE-mediated mitochondrial base editing in human 3 PN embryos.[43] Silva Pinheiro et al tested the *in vivo* use of this emerging tool by delivering DdCBE into mouse hearts using an AAV vector, and they showed that the DdCBE could perform the desired mtDNA editing in both adult and neonatal mice.[44]

From the previous cleavage of mutated mtDNA to the reduction in the number of disease-causing mtDNA copies to the correction of specific mutated sites in mtDNA to single-base editing in mitochondrial genes, the development of the DdCBE system can be deemed one of the important milestones in gene editing technology.[25] The DdCBE system has various advantages, such as high target specificity, high product concentration, the targeting of double-stranded DNA without generating DNA strand breaks, and the ability to introduce targeted mutations into mitochondria. However, the DdCBE system can effectively edit only cytidines next to thymines in the genome and can be used only for mitochondrial diseases related to C—U mutations in mitochondria, which greatly restricts its application. Researchers are also trying to find more deaminases to act on different bases so that multiple mtDNA mutations can be repaired. Furthermore, the current therapeutic efficacy of the DdCBE system is limited, as it does not eliminate all accumulated mutant mtDNA. The off-target nature of DdCBE, the production of nonspecific transcriptional products, and the safety of using DdCBE during long-term treatment need to be further optimized to improve the DdCBE system mitochondrial gene editing. Further validation of the DdCBE system in additional cellular and animal disease models is needed before it is implemented as a potential treatment for mtDNA mutation-related diseases.[20,21] In conclusion, the DdCBE system is a

promising tool for mitochondrial mtDNA editing, and manufacturing mtDNA-targeted mutations to establish corresponding mitochondrial disease models is of great significance for studying the pathogenesis of mitochondrial diseases, screening for relevant pathway drugs, and correcting gene mutations as therapy. It is believed that with further research and optimization, the application of the DdCBE system can meet clinical needs.

Conclusion

Mitochondrial diseases, as heterogeneous maternally inherited diseases, are often caused by mtDNA mutations. The pathogenesis of mitochondrial diseases often involves multiple organ lesions throughout the body, and the causative mutations are passed on to offspring in a non-Mendelian manner. Currently, we know little about the pathogenesis, heterogeneous dynamic control mechanisms, and genetic inheritance of mitochondrial diseases. An effective research method to obtain an in-depth understanding of mitochondrial diseases needs to be found. With the rapid development of gene editing technologies, the study of mitochondrial diseases to try to find potential treatment methods has become a hot topic in research. In this paper, we summarized several mitochondrial gene editing approaches, and we discussed recent advances and breakthroughs in mitochondrial gene editing to provide potential new therapies for mitochondrial diseases caused by mtDNA mutations. Traditional gene editing modalities, such as those employing ZFNs and TALENs, have been designed, and these RNA-free programmable nucleases can be applied in mitochondrial gene editing to induce mtDNA double-strand breaks and reduce the number of mutant mtDNAs. The use of ZFNs and TALENs has led to some progress in mitochondrial genome editing engineering, with TALENs being the most common and widely used editing tools for mitochondrial gene editing. However, neither approach is effective in correcting mtDNA pathogenic single-base mutations. The application of CRISPR-Cas9, which is omnipotent in the nuclear genome, to mitochondrial genome gene editing is still controversial, mainly because no suitable strategy has been found to achieve mitochondrial transport of gRNAs; therefore, effective gRNA transport means need to be found for CRISPR-Cas9 to edit mtDNA. The discovery of DdCBE not only shed light on the study of mitochondrial genome function but also bridged the gap between mitochondrial gene editing and the clinical treatment of mitochondrial diseases, providing hope to patients with diseases caused by mtDNA mutations. However, much work is needed to promote the maturation of DdCBE technology, ensuring its safety and long-term effectiveness.

Mitochondrial gene editing technology is in its infancy, and there are still many challenges. The current issues that need to be addressed in mitochondrial gene editing are hindering the effect of the mitochondrial bilayer membrane, the lack of repair pathways in the mitochondrial genome to degrade mtDNA rapidly after breakage, the traditional gene editing techniques that can base edit only single strands of DNA, the optimal gene editing system, the off-target effects of mitochondrial gene editing methods, and safety issues such as whether nonspecific binding will cause cytotoxicity in the long term. It is believed that mitochondrial gene editing technology will play an important role in several aspects of mitochondrial diseases after these challenges are overcome. As a powerful potential therapeutic approach, mitochondrial gene therapy will be applied in the treatment of mitochondrial diseases one day, and we look forward to the early arrival of that day.

Author contributions

MS, LY, and YY prepared the initial manuscript and contributed equally to the work. MS, XL, and XH drafted the figures. SH and MY designed and oversighted the work.

Conflict of interests

All authors disclosed no relevant conflict of interests.

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References

1. Fang T, Wang M, Xiao H, Wei X. Mitochondrial dysfunction and chronic lung disease. *Cell Biol Toxicol*. 2019;35(6):493—502.
2. Suomalainen A, Battersby BJ. Mitochondrial diseases: the contribution of organelle stress responses to pathology. *Nat Rev Mol Cell Biol*. 2018;19(2):77—92.
3. Pereira CV, Gitschlag BL, Patel MR. Cellular mechanisms of mtDNA heteroplasmy dynamics. *Crit Rev Biochem Mol Biol*. 2021;56(5):510—525.
4. Ghaoui R, Sue CM. Movement disorders in mitochondrial disease. *J Neurol*. 2018;265(5):1230—1240.
5. Adashi EY, Rubenstein DS, Mossman JA, Schon EA, Cohen IG. Mitochondrial disease: replace or edit? *Science*. 2021; 373(6560):1200—1201.
6. Wang G, Shimada E, Zhang J, et al. Correcting human mitochondrial mutations with targeted RNA import. *Proc Natl Acad Sci U S A*. 2012;109(13):4840—4845.
7. Guo J, Chen X, Liu Z, et al. DdCBE mediates efficient and inheritable modifications in mouse mitochondrial genome. *Mol Ther Nucleic Acids*. 2022;27:73—80.
8. Ng YS, Bindoff LA, Gorman GS, et al. Mitochondrial disease in adults: recent advances and future promise. *Lancet Neurol*. 2021;20(7):573—584.
9. Gorman GS, Schaefer AM, Ng Y, et al. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. *Ann Neurol*. 2015;77(5):753—759.
10. Craven L, Alston CL, Taylor RW, Turnbull DM. Recent advances in mitochondrial disease. *Annu Rev Genom Hum Genet*. 2017; 18:257—275.

11. Zeng B, Huang Y, Jin S, et al. Development and validation of a mitochondrial metabolism-associated nomogram for prediction of prognosis in clear cell renal cell carcinoma. *Clin Transl Med*. 2020;10(3):e120.
12. Stenton SL, Prokisch H. Genetics of mitochondrial diseases: identifying mutations to help diagnosis. *EBioMedicine*. 2020; 56:102784.
13. Gorman GS, Chinnery PF, DiMauro S, et al. Mitochondrial diseases. *Nat Rev Dis Prim*. 2016;2:16080.
14. Yang M, Xu L, Xu C, et al. The mutations and clinical variability in maternally inherited diabetes and deafness: an analysis of 161 patients. *Front Endocrinol*. 2021;12:728043.
15. Finsterer J. Pharmacotherapeutic management of epilepsy in MERRF syndrome. *Expet Opin Pharmacother*. 2019;20(10): 1289—1297.
16. Villanueva-Paz M, Povea-Cabello S, Villalón-García I, et al. Pathophysiological characterization of MERRF patient-specific induced neurons generated by direct reprogramming. *Biochim Biophys Acta Mol Cell Res*. 2019;1866(5):861—881.
17. Yamada Y, Ito M, Arai M, Hibino M, Tsujioka T, Harashima H. Challenges in promoting mitochondrial transplantation therapy. *Int J Mol Sci*. 2020;21(17):E6365.
18. Zhong G, Madry H, Cucchiarini M. Mitochondrial genome editing to treat human osteoarthritis-a narrative review. *Int J Mol Sci*. 2022;23(3):1467.
19. Moretton A, Morel F, Macao B, et al. Selective mitochondrial DNA degradation following double-strand breaks. *PLoS One*. 2017;12(4):e0176795.
20. Yang X, Jiang J, Li Z, Liang J, Xiang Y. Strategies for mitochondrial gene editing. *Comput Struct Biotechnol J*. 2021;19: 3319—3329.
21. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*. 2010;11(9):636—646.
22. Gammage PA, Gaude E, Van Haute L, et al. Near-complete elimination of mutant mtDNA by iterative or dynamic dose-controlled treatment with mtZFNs. *Nucleic Acids Res*. 2016; 44(16):7804—7816.
23. Gammage PA, Rorbach J, Vincent AI, Rebar EJ, Minczuk M. Mitochondrially targeted ZFNs for selective degradation of pathogenic mitochondrial genomes bearing large-scale deletions or point mutations. *EMBO Mol Med*. 2014;6(4):458—466.
24. Gammage PA, Viscomi C, Simard ML, et al. Genome editing in mitochondria corrects a pathogenic mtDNA mutation *in vivo*. *Nat Med*. 2018;24(11):1691—1695.
25. Wang W, Wang X. New potentials of mitochondrial DNA editing. *Cell Biol Toxicol*. 2020;36(5):391—393.
26. Miller JC, Tan S, Qiao G, et al. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol*. 2011;29(2):143—148.
27. Li T, Liu B, Spalding MH, Weeks DP, Yang B. High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat Biotechnol*. 2012;30(5):390—392.
28. Kazama T, Okuno M, Watari Y, et al. Curing cytoplasmic male sterility via TALEN-mediated mitochondrial genome editing. *Nat Plants*. 2019;5(7):722—730.
29. Reddy P, Ocampo A, Suzuki K, et al. Selective elimination of mitochondrial mutations in the germline by genome editing. *Cell*. 2015;161(3):459—469.
30. Yang Y, Wu H, Kang X, et al. Targeted elimination of mutant mitochondrial DNA in MELAS-iPSCs by mitoTALENs. *Protein Cell*. 2018;9(3):283—297.
31. Mok BY, de Moraes MH, Zeng J, et al. A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. *Nature*. 2020;583(7817):631—637.
32. Graham DB, Root DE. Resources for the design of CRISPR gene editing experiments. *Genome Biol*. 2015;16:260.
33. Gaj T, Gersbach CA, Barbas 3rd CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013;31(7):397—405.
34. Jo A, Ham S, Lee GH, et al. Efficient mitochondrial genome editing by CRISPR/Cas9. *BioMed Res Int*. 2015;2015: 305716.
35. Bian WP, Chen YL, Luo JJ, Wang C, Xie SL, Pei DS. Knock-in strategy for editing human and zebrafish mitochondrial DNA using Mito-CRISPR/Cas9 system. *ACS Synth Biol*. 2019;8(4): 621—632.
36. Yoo BC, Yadav NS, Orozco Jr EM, Sakai H. Cas9/gRNA-mediated genome editing of yeast mitochondria and *Chlamydomonas* chloroplasts. *PeerJ*. 2020;8:e8362.
37. Silva-Pinheiro P, Minczuk M. The potential of mitochondrial genome engineering. *Nat Rev Genet*. 2022;23(4): 199—214.
38. Haddad S, Abánades Lázaro I, Fantham M, et al. Design of a functionalized metal-organic framework system for enhanced targeted delivery to mitochondria. *J Am Chem Soc*. 2020; 142(14):6661—6674.
39. Aushev M, Herbert M. Mitochondrial genome editing gets precise. *Nature*. 2020;583(7817):521—522.
40. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*. 2016;533(7603): 420—424.
41. Guo J, Zhang X, Chen X, et al. Precision modeling of mitochondrial diseases in zebrafish via DdCBE-mediated mtDNA base editing. *Cell Discov*. 2021;7(1):78.
42. Lee H, Lee S, Baek G, et al. Mitochondrial DNA editing in mice with DddA-TALE fusion deaminases. *Nat Commun*. 2021;12: 1190.
43. Chen X, Liang D, Guo J, et al. DdCBE-mediated mitochondrial base editing in human 3PN embryos. *Cell Discov*. 2022;8:8.
44. Silva-Pinheiro P, Nash PA, Van Haute L, Mutti CD, Turner K, Minczuk M. *In vivo* mitochondrial base editing via adeno-associated viral delivery to mouse post-mitotic tissue. *Nat Commun*. 2022;13(1):750.