Mitochondrion-targeted RNA therapies as a potential treatment strategy for mitochondrial diseases

Timofei Chernega,1,5 Jaehyoung Choi,1,5 Leonardo Salmena,1,2 and Ana Cristina Andreazza1,3,4

Mitochondrial diseases are one of the largest groups of neurological genetic disorders. Despite continuous efforts of the scientific community, no cure has been developed, and most treatment strategies rely on managing the symptoms. After the success of coronavirus disease 2019 (COVID-19) mRNA vaccines and accelerated US Food and Drug Administration (FDA) approval of four new RNAi drugs, we sought to investigate the potential of mitochondrion-targeting RNA-based therapeutic agents for treatment of mitochondrial diseases. Here we describe the causes and existing therapies for mitochondrial diseases. We then detail potential RNA-based therapeutic strategies for treatment of mitochondrial diseases, including use of antisense oligonucleotides (ASOs) and RNAi drugs, allotopic therapies, and RNA-based antigenomic therapies that aim to decrease the level of deleterious heteroplasmy in affected tissues. Finally, we review different mechanisms by which RNA-based therapeutic agents can be delivered to the mitochondrial matrix, including mitochondrion-targeted nanocarriers and endogenous mitochondrial RNA import pathways.

INTRODUCTION
Mitochondrial diseases are a group of genetic disorders caused by direct and indirect defects of mitochondrial respiratory chain function. Clinical symptoms of mitochondrial diseases are notably heterogeneous and often associated with tissues of high energy demand; they can be present in any stage of life at the multi-organ or organ-specific level.1 The human mitochondrial proteome is under dual genomic control, encoded by more than 1,000 genes from nuclear DNA (nDNA) and 37 genes from mitochondrial DNA (mtDNA).2 To date, mutations in more than 350 genes across both genomes have been suggested to cause different mitochondrial diseases.3

The diagnosis of mitochondrial disease is challenging because of the heterogeneity of clinical, biochemical, and molecular features. Most clinical phenotypes of mitochondrial diseases overlap with different diagnoses, and molecular testing lacks clinical validation.4,5 The diagnostic complexities often result in flawed diagnoses, delayed diagnoses, or the so-called “diagnosis odyssey,” where patients consult with multiple clinicians and can undergo invasive screenings before receiving a correct diagnosis.6,7 Next-generation sequencing (NGS) is currently employed for detection of causative genetic origins of mitochondrial diseases in nDNA and mtDNA, but timely access to precise molecular-based diagnostics remains a challenge.8

Strategies to treat mitochondrial diseases currently consist of case-specific symptom management and pharmacological supplementation; cofactors, coenzyme Q10, antioxidants, or vitamins are typically recommended.1 The most common regimen for treatment of mitochondrial disease is a drug cocktail consisting of supplemental antioxidants, vitamins, and xenobiotics to partially alleviate the symptoms.9 Although a few mutation-specific targeted therapies have resulted in clinical trials, development of treatments appropriately targeted to specific genetic causes is needed.10–12 Recently, dramatic advances have been made in development of RNA-based therapeutic agents. Here we review the potential of mitochondrion-targeted RNA therapies as a treatment strategy for mitochondrial diseases. We begin by reviewing the genetics of mitochondrial disease, and then we present an overview of current mitochondrial disease therapeutic agents and the current state of knowledge regarding mitochondrial and RNA therapeutic agents. We conclude this review by discussing RNA delivery to mitochondria as a potential strategy for precision mitochondrial disease therapy development.

MITOCHONDRIAL DISEASES
Genetics of mitochondrial diseases
Genetic characterization of mitochondrial diseases is a complex process. Human mitochondria are under the control of about 1,200 nuclear protein-encoding genes, 300 of which are associated with various mitochondrial disease pathogeneses.4,11 Human cells contain hundreds to thousands of mitochondria per cell, and each mitochondrion contains 2–10 copies of the mitochondrial genome.13

https://doi.org/10.1016/j.omtn.2022.10.012.
5These authors contributed equally
Correspondence: Ana Cristina Andreazza, University of Toronto, Medical Science Building, Room 4206, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada. E-mail: ana.andreazza@utoronto.ca
multi-copy nature of mtDNA may give rise to heteroplasmy, where an individual, and even a single cell, can harbor distinct mtDNA genomes. It is estimated that even heteroplasmy exists in up to 61% of the population. Typically, when the heteroplasmic load surpasses 70%–80% of total mtDNA genomes, clinical symptoms may manifest. The mitochondrial haplogroup, characterized by ancestry-linked combinations of single nucleotide polymorphisms, can also be associated with clinical symptoms and may affect disease risk and pathology.

The global prevalence of clinically diagnosed mitochondrial disease was recently estimated to be between 10 and 20 per 100,000 across different studies in England, the United States, Canada, Sweden, Portugal, and Japan. Epidemiological studies of clinically manifested mitochondrial diseases are considerably in agreement across regions, although there are limited numbers and powers of studies to date. Further characterization of the genetic prevalence of disease-causing mutations and genotype-to-phenotype penetrance is necessary to understand mitochondrial disease expressed in variable forms and the true burden of mitochondrial diseases.

The prevalence of disease-causing mutations in adult clinical cohort studies was best estimated by Gorman et al. to be 1 in 5,000 for mtDNA mutations (20 per 100,000) and 2.9 per 100,000 for nDNA mutations among adults in northern England. The m.3243A>G point mutation was found at 7.9 per 100,000, which makes up ~40% of the total prevalence. The total mutation prevalence of mt-tRNA regions sums up to 10.6 per 100,000 (52% of the total), and combined Leber hereditary optic neuropathy (LHON)-associated mutations in ND1, 4 and 6 (m.3460A>G, m.11778G>A, and m.14484T>C) summed up to be 8.1 per 100,000. These results replicated a prior study by Schafer et al. that made similar estimates.

An attempt to further characterize the genetics of mitochondrial disease and the genotype-phenotype correlations was made by a recent analysis of the NAMDC (North American Mitochondrial Disease Consortium) registry. Patients with mitochondrial disease had a higher frequency of mtDNA pathogenic mutations (62%) than nDNA (38%), and showed a trend where mtDNA mutations were prevalent in patients older than 2, and nDNA mutations were more frequently found in the infancy-onset disease group. mtDNA mutations were highly associated with endocrinopathies and diabetes mellitus compared with nDNA mutations, suggesting a possible association of mtDNA mutations with age-related, non-mitochondrial diseases. Similar to previous reports, MT-TL1 M.3243A>G was most frequent, followed by single large-scale mtDNA deletion and then MT-TK m.8344A>G.

Across different clinical cohort studies, seems that mt-tRNA mutations represent the largest group of mitochondrial disease-causing mutations, and m.3243A>G is also the single most frequent pathogenic mutation replicated almost uniformly across different studies. LHON-associated mtDNA mutations in ND-coding mtDNA regions, identified by m.3460A>G, m.11778G>A, and m.14484T>C, represent the other major group of mutations.

A few other population-based studies identified a higher prevalence of pathogenic mtDNA mutation carriers without clinical diagnosis of primary mitochondrial disease. Blue Mountains Eye and Hearing Studies (BMEHS), an Australian population study of about 3,000 White participants, estimated the prevalence of m.3243A>G as 0.24% and m.1555A>G as 0.21% in mtDNA extracted from hair follicles, and these mutations were not identifiable in blood. A very similar frequency (0.19%) of the m.1555A>G mutation was reported in children in a British cohort study. Elliot et al. reported that the frequency of pathogenic mtDNA mutations of neonatal cord blood samples from live births in North Cumbria, England, was estimated to be 0.54% (1555A→G, 3243A→G, 3460G→A, 11778G→A, 14484T→C), and the de novo pathogenic mtDNA mutation rate (not found in the mother) was estimated to be 107 per 100,000. Evidence from these non-mitochondrial disease patient cohort studies suggests that causative mutations may be more prevalent than its current estimate and underly different unforeseen pathologies.

Several limitations for characterizing the genetic epidemiology of mtDNA mutations in mitochondrial disease and their contribution to various pathologies are suggested throughout the literature. Maintenance of mtDNA is thought to be controlled at three large stages: oogenesis bottleneck, embryonic bottleneck, and somatic quality control. Mitochondrial inheritance patterns and bottlenecks involve stochastic processes, thus it is difficult to predict the inheritance of mitochondrial disease mutations. The resulting genetic drift may introduce heteroplasmic variation and altered risk of mtDNA mutation-derived pathologies. It was observed that mtDNA mutation in mt-tRNA and mt-rRNA regions are less likely to be removed through purifying selection compared with mt-mRNA regions during bottle-necks. Some mutant mtDNA copies, including m.3243A>G, confer a replicative advantage.

It is generally believed that mitochondrial heteroplasmy of inherited or de novo mutations increases with age because of low-fidelity polymerase γ and cumulative stress. Through quality control, a decrease in pathogenic mtDNA blood heteroplasmic levels may be achieved, as exemplified by reports of heteroplasmic deleterious mutations found in other somatic tissues not being found in the blood. However, this decreasing trend is only apparent in fast proliferative cell/tissue types, leading to difficulty in detection of the variants. In contrast, post-mitotic tissues in heart, muscle, and brain in the geriatric population carry an elevated heteroplasmic load, indicating that tissues with lower proliferative capacity may be more affected by carrying heteroplasmic levels of deleterious mtDNA mutations contributing to aging and pathologies.

Finally, numerous pathogenic mtDNA mutations are reported as causative in mitochondrial diseases. Careful and thorough examination of possibly disease-associated mtDNA variants and
heteroplasmic load is needed for identification of genetic perturbations in patients with possible mitochondrial diseases.

MITOCHONDRIAL DNA POINT MUTATIONS

The human mitochondrial genome is composed of 16,569-bp circular DNA encoding 13 proteins, 2 rRNAs, and 22 tRNAs. mtDNA is composed of heavy and light chains, where a non-coding displacement loop (D-loop; position 16,024 to 576) forms a DNA triple-complex loop structure, and the remaining genome efficiently encodes genes with no introns. The light chain encodes ND6 and 6 tRNAs, and the heavy strand encodes the remaining 12 mRNAs, 2 rRNAs, and 16 tRNAs. To date, nearly 100 confirmed disease-causing mtDNA point mutations have been characterized (MitoMap.org).44 Figure 1 illustrates a brief abstract of mitochondrial DNA and disease.

mRNA region

The mtDNA encodes for seven subunits of electron transport chain (ETC) complex I, one subunit of complex III, three subunits of complex IV, and two subunits of complex V. Point mutations in the coding region of mtDNA may have direct functional effects on subunits involved in the ETC. Of 42 confirmed point mutations in the mRNA-encoding regions, the LHON-associated m.3460G>A mutation exemplifies the pathogenic penetrance of ETC subunit deficiency.46 As a consequence of the maternally inherited m.3460G>A mutation, a specific mitochondrial ETC complex I deficiency was observed in heteroplasmic and homoplasmic carriers, while there were functional differences in complex II activity that was absent and complex IV activity was increased.47 However, depending on the site of the mutation, one point mutation may cause a global decline in ETC function through disruption of ETC supercomplex formation.48

tRNA region

The human mitochondrion encodes tRNAs that are distinct from nuclear tRNAs. Like nuclear tRNAs, mitochondrial tRNAs are extensively modified. Genetic perturbations in the mitochondrial tRNA coding regions can lead to several different biological alterations in transcription and translation.49 For instance, the mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)-associated tRNA mutation m.3243A>G in the D-arm leads to failure in the post-transcriptional modification at the distal wobble position of the anti-codon, leading to mitochondrial translational defects.50 Mitochondrial tRNAs also play a crucial role in the processing of polycistronic mitochondrial gene transcripts, which act as sites of segregating different mRNA regions in the compactly encoded mt genome.51 In this process, RNase P and Z excise the 5’ and 3’ ends of tRNA and modify adjacent flanking genes on both ends, where near-cleavage site mutations or even subtle structural alterations induced by distal mutations can result in altered recognition of modifying enzymes.52–54 Hence, tRNA regions are hotspots for pathogenicity and may affect not only translational efficiency but global mitochondrial function at multiple points of the mitochondrial transcriptional machinery.50,55 A brief overview of the mitochondrial transcriptome and processing is shown in the supplemental information and Table S1.

rRNA region

Two pathogenic mutations in the rRNA encoding region have been reported: m.1555A>G and m.1494C>T.56 The mutations in the mt-RNR1 region have been associated with age-related hearing loss, aminoglycoside-induced adverse effects, and autism spectrum disorder.57 The mutation leads to a reduction of global mitochondrial protein expression and may also be influenced by the presence of other mtDNA polymorphisms.58
Mutations in mtDNA may lead to different manifestations of mitochondrial diseases and other pathologies because of mitochondrial dysfunction. Although advances in our understanding of the mt genome, mt transcriptome, and molecular mechanisms are being made, more research is required to study therapeutic stratification. In the next section, we review the therapeutic options in development of treatments for mitochondrial diseases.

**CURRENT MITOCHONDRIAL DISEASE THERAPEUTIC AGENTS**

There is no cure for mitochondrial diseases. The standard guideline for treatment of mitochondrial diseases involves supplemental cocktails (including coenzyme Q10, vitamin B, amino acids, vitamins, and antioxidants) and case-specific symptomatic management. Examples of prevalent symptoms in patients with mitochondrial disease include myopathy, neurological and metabolic manifestations, stroke-like episodes, and epileptic and myoclonic seizures. Because it is possible to utilize existing therapeutic strategies for management and alleviation of symptoms bearing analogous pathology, a wide range of studies, including pre-clinical and clinical trials, is being conducted on potential candidate compounds for treatment of mitochondrial disease. Drugs used for treatment of mitochondrial dysfunction-associated diseases, such as type II diabetes mellitus and neurodegenerative disorders, can alleviate mitochondrial dysfunction by modulating ETC complex activity, increasing substrate availability, or inducing mitochondrial biogenesis.

On the other hand, drugs for symptomatic management of mitochondrial diseases should be selected, administered, and monitored with caution. For instance, administration of metformin for treatment of diabetes mellitus can induce lactic acidosis and result in multisystemic adverse effects. For epileptic and myoclonic seizures in mitochondrial disease patients, combined administration of antiepileptic and antimyoclonic medications is often used. Although several antiepileptic drugs are available, clinical use of these drugs may have detrimental effects in patients with mitochondrial disease. For example, administration of sodium valproate may result in drug-induced mitochondrial damage, exacerbating mitochondrial dysfunction in the underlying pathology of epileptic seizures. Valproate is administered in patients with mitochondrial disease as a broad-spectrum antiepileptic medication, but it requires careful monitoring and possible combination with other medications with mitochondrion-protective effects. In-clinic treatment of myoclonic epilepsy with ragged red fibers (MERRF)-induced myoclonic seizures with the antiepileptic drug levetiracetam in combination with the antimyoclonic drug clonazepam demonstrated high effectiveness with decreased adverse effects compared with previously used antimyoclonic medications.

Extensive effort is being made to develop therapeutic options for treatment of mitochondria, including small-molecule drugs, biological analogs, and gene therapy (Table 1). However, a successful transition of precision medicine targeting specific disease-causing mutations is not yet reflected. Mitochondrial medicine is an imperative challenge for modern precision medicine aiming to shatter heterogeneous and currently incurable genetic disorders. The successes of allotopic gene therapy of LHON via local adeno-associated virus (AAV) mitochondrial gene delivery have demonstrated the potential of precision medicine in mitochondrial diseases. Likewise, several RNA-based approaches have been explored at the cellular level to specifically target mitochondrially encoded transcripts. RNA therapeutic agents abate the risk of genomic integration and can be pharmacologically controlled because of their transient mechanisms of action, providing an advantage over DNA therapies. Unlike small-molecule drugs, which are primarily used for symptom management, RNA-based therapeutic agents are able to directly target the cause of disease, such as replacing missing proteins because of mtDNA deletions.

With the accelerating research in RNA therapeutic agents, development of mitochondrial RNA therapeutic agents deserves more attention to target the dynamic nature of mitochondrial physiology in a specific and tractable mode with the prospect of treating diseases of mitochondrial genetic origin at different causal (inherited, de novo/age-related) and physiological (systemic, organ-specific, heteroplasmic) levels. In the next section, we review RNA-based therapeutic agents for treatment of mitochondrial diseases as a potential therapeutic strategy, biological mechanisms of RNA species import into mitochondria, and delivery strategies of directing RNA to dysfunctional mitochondria.

**RNA-based therapeutic agents**

RNA-based therapeutic agents are designed to target RNA transcripts with a goal of modulating gene expression through mechanisms including altering transcript processing, adjusting transcript stability, or modifying translation of a given transcript. The great promise of RNA therapeutic agents includes unparalleled specificity, an ability to target traditionally undruggable genes, and the possibility of rapid and efficient production of RNA drugs in situ. RNA-based therapeutic agents have already delivered astonishing results in the treatment of some genetically well-defined diseases. Classes of current RNA-based therapeutic agents include antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and mRNA therapeutic agents, which have been used very effectively as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines. ASOs are single-stranded oligonucleotides that exert their action primarily through complementary binding to a target mRNA or premature mRNA inducing one or more of degradation, altered splicing, or inhibited translation. The first ASO drug, fomivirsen, was approved by the US Food and Drug Administration (FDA) in 1998 for treatment of cytomegalovirus (CMV) retinitis. siRNAs are small double-stranded oligonucleotides that inhibit gene expression predominantly through RNAi, which is mediated by the RNA-induced silencing complex (RISC) in the cytoplasm. To date, four siRNA drugs have been FDA approved. In 2018, the first-in-class siRNA drug patisiran, which targets transthyretin messenger RNA for degradation, was approved for treatment of hereditary transthyretin-mediated amyloidosis.

This was followed by an acceleration of siRNA drug approvals, including givosiran (approved in 2019 for treatment of...
| Intervention | MOA | Target disease/symptoms | Primary outcome measures | Status | Registry number | Updated date | Reference |
|-------------|-----|-------------------------|--------------------------|--------|-----------------|--------------|-----------|
| Idebenone   | synthetic analog of coenzyme Q10; antioxidant | MELAS | mean change in cerebral and venous lactate concentration | clinical trial phase 2 | NCT08887562 | October 2016 | Ahmed et al.65 |
|             |      | LON                    | best recovery of logMAR visual acuity | clinical trial phase 2 | NCT0747487 | May 2013 | Rudolph et al.66 |
|             |      | N/A                    | follow-up (NCT0747487) | clinical trial phase 3 | NCT0142138 | May 2013 | Klopopst et al.67 |
| L-arginine  | nitric oxide donor | MELAS | muscle function investigation | clinical trial phase 2 | NCT01603446 | December 2013 | Rodan et al.69 |
| L-arginine and L-citrulline | nitric oxide donor | MELAS | change in nitric oxide production | clinical trial early phase 1 | NCT01339494 | April 2016 | El-Hattab et al.69 |
| KL1333      | novel NAD+ modulator; oxidation of NADPH | MELAS | number of reported adverse events | clinical trial phase 1 | NCT0356209 | April 2018 | Seo et al.71 |
| CY6463 (I6W6463) | CNS-penetrating sGC stimulator; amplification of NO-sGC-cGMP | MELAS | treatment-emergent adverse event | clinical trial phase 2 (recruiting) | NCT04475549 | July 2021 | Correa et al.72 |
| Cysteamine bitrate | Enhancement of glutathione biosynthesis | MELAS | change in Newcastle Pediatric Mitochondrial Disease Scale score | clinical trial phase 2 | NCT02473445 | May 2018 | Guha et al.73 |
| Cysteamine bitrate (RP103, delayed-release capsules) | enhancement of glutathione biosynthesis | KSS | change from baseline in Newcastle Pediatric Mitochondrial Disease Scale sections I-IV | clinical trial phase 2 | NCT02023866 | November 2017 | Guha et al.73 |
| KH176       | ROS redox modulator; protection against redox perturbation; enhances peroxidase activity | MELAS | change from baseline in ECG results and hematology laboratory test results | clinical trial phase 1 | NCT02544217 | November 2020 | Beyrath et al.74 Koene et al.75 |
|             |      | LON                    | clinical trial phase 3 (rescue) | NCT02652767 | January 2020 | Koene et al.75 |
|             |      | LON                    | clinical trial phase 3 (reverse) | NCT02652780 | January 2020 | Newman et al.76 |
|             |      | LON                    | clinical trial phase 3 (follow-up) | NCT03406104 | February 2021 | |
| GS010       | AAV2-MT-ND4 gene therapy | MELAS | change from baseline in EDRS visual acuity | clinical trial phase 2 | NCT01721733 | August 2020 | Martinelli et al.77 |
| EPI-743     | NADP dehydrogenase modulator | Leight syndrome | Plasma level of follistatin, myostatin, nitrates, BNP, creatine kinase, MMP-9, TNF-α, TGF-β, follistatin to myostatin ratio | clinical trial phase 2 | NCT02964377 | January 2019 | Silva Santos et al.78 |
| (+)-Epicatechin | stimulation of mitochondrial respiration and biogenesis | DMD | change in plasma follistatin to myostatin ratio; percent change in cardiac ejection fraction and shortening fraction by MRI | clinical trial phase 2 | NCT02652767 | January 2020 | Silva Santos et al.78 |
| (-)-Epicatechin | stimulation of mitochondrial respiration and biogenesis | Leight muscular dystrophy | Plasma level of follistatin, myostatin, nitrates, BNP, creatine kinase, MMP-9, TNF-α, TGF-β, follistatin to myostatin ratio | clinical trial phase 2 | NCT03236662 | July 2021 | McDonald et al.79 |
| Vamorolone  | Stabilization of myofiber membrane; anti-inflammatory | DMD | overall summary of adverse events | clinical trial phase 2 | NCT02760264 | January 2019 | Smith et al.80 |

(Continued on next page)
acute intermittent porphyria), lumasiran (approved in 2020 for treatment of primary hyperoxaluria type 1), and inclisiran (approved in 2021 for treatment of heterozygous familial hypercholesterolemia). Success of mRNA vaccines pioneered the application of large (more than 4,000 nt in length) coding mRNA delivery in vivo, and on-going clinical trials of mRNA replacement therapy illustrate the foreseeable transition of mRNA drugs to the clinic. The great promise of RNA therapeutic agents has finally become a reality, and successful clinical translation of these drugs has illustrated the great potential of RNA-based drugs in precision medicine. mRNA delivery is being actively explored as a mode of delivering gene editing enzymes, such as clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 and small guiding RNA (sgRNA). Many new RNA-targeted therapeutic agents are currently in development or in clinical trials, and soon, any remaining questions on whether RNAs can be effectively utilized for gain-of-function or gene replacement strategies can be addressed.

**RNA-based therapeutic agents for mitochondrial diseases**

Despite the current need, RNA-based therapeutic agents have not been approved for treatment of mitochondrial diseases. Based on our knowledge of mitochondrial biology and diseases, effectively targeting mitochondrial RNAs could be of therapeutic value. One can imagine use of ASOs and siRNA to reduce levels of mutant mitochondrial proteins, mRNA-based drugs and wild-type (WT) mtRNAs as allosteric therapies to substitute defective mitochondrial RNAs and proteins, or antireplicative RNAs as antigenic therapies to reduce the levels of mutant mtDNA in a cell (heteroplasm) (Figure 2).

Delivery of siRNA, mitochondrial microRNA (mitomiRNA) mimics, or ASOs to mitochondria may be used therapeutically to increase or decrease mitochondrial gene expression for specific therapeutic outcomes (Figure 2A). For instance, nanocarrier-mediated delivery of let-7b to mitochondria has been shown to decrease expression of the mitochondrial genes COX1 and COX2 in the non-small cell lung cancer (NSCLC) cell line A542. Others have utilized the RNA import complex (RIC) and D-loop of tRNA from *Leishmania tropica* to direct small antisense RNAs to mitochondria, where they suppressed expression of *CYB*, *ND1*, *COX1*, *COX2*, and *ND5* mRNAs. ASOs have also been delivered to the mitochondrial matrix using a liposome-based nanocarrier, MITO-porter, where they were able to decrease expression of COX2 mRNA.

The existence of an endogenous RNAi-like mechanism in mitochondria remains controversial. Argonaute RISC catalytic component 2 (AGO2), a key component of the RISC, has been found to colocalize with mitochondria. However, the presence of a functional RNAi
A mechanism in mitochondria was never shown. MitomiRNAs have been found to regulate mitochondrial translation and even transcription.\textsuperscript{108–111} ASO and siRNA therapeutic agents have been shown to suppress translation of their target mitochondrial mRNAs.\textsuperscript{98,112} However, whether the siRNA-induced mitochondrial gene silencing follows an RNAi-like, antisense-based, or different mechanism is unknown. Gao et al.\textsuperscript{112} have shown that AGO2 slicing activity is required for mitochondrial siRNA-mediated gene silencing, which may suggest the existence of a mitochondrial RNAi-like mechanism.

Another way in which RNA-based therapeutic agents can be used for treatment of mitochondrial diseases is as a replacement for defective RNAs and proteins in the mitochondrial matrix (Figure 2B). Specifically, functional tRNAs, rRNAs, and mRNAs have been delivered to the mitochondrial matrix \textit{in vitro} to substitute their defective or missing counterparts. Several reports show that delivering WT or \textit{Saccharomyces cerevisiae}-derived mimics of tRNA\textsubscript{Lys}\textsuperscript{UUU} and tRNA\textsubscript{Leu}\textsuperscript{UUR} to mitochondria leads to rescue of protein expression in MERRF and MELAS cybrid cell lines and patient-derived fibroblasts, respectively.\textsuperscript{104,113–115} Similarly, pre-tRNA\textsuperscript{Phe} and 12s rRNA have been delivered to the mitochondrial matrix using the MITO-porter system, resulting in rescue of the mitochondrial respiration rate in patient-derived fibroblasts containing the m.G625A deleterious mutation in the RNA\textsuperscript{Phe} gene and the m.A1555G deleterious mutation in the mitochondrial 12s rRNA gene, respectively.\textsuperscript{116,117} Coding RNAs that are translated to express one or several proteins have been successfully delivered to mitochondria. For example, Mahato et al.\textsuperscript{118} delivered a polycistronic RNA (pcRNA), using 8 \textit{in vitro} assembled subunits of RIC, that rescued mitochondrial respiration in models of Kearns-Sayre syndrome (KSS). Wang et al.\textsuperscript{104} delivered mRNA containing the human \textit{COX2} transcript into the mitochondrial matrix of mouse embryonic fibroblasts, where it was translated into a functional \textit{COX2} protein. Yamada et al.\textsuperscript{100} demonstrated that mitochondrial delivery of mRNA encoding the ND3 gene rescues the mitochondrial respiration rate in Leigh syndrome patient-derived fibroblasts harboring the m.T10158C mutation in ND3.

![Figure 2. Potential RNA-based strategies for treatment of mitochondrial diseases](https://example.com/figure2.png)
Mitochondrial RNA delivery strategies

Mitochondrial RNA delivery strategies is an emerging field that requires further studies prior to widespread use. Currently, there are two major ways in which therapeutic RNAs can be delivered to the mitochondrial matrix (Figure 3). First is direct delivery of RNAs into the mitochondria, where a mitochondrion-targeted carrier releases the RNAs directly into the mitochondrial matrix. Second is indirect delivery, where RNA molecules are modified with endogenous mitochondrial RNA import determinants and delivered to the cytosol of the target cell by means of a conjugate or carrier. These modified RNA molecules are released in the cytosol and directed to mitochondrial import by the endogenous RNA import pathway. Both delivery strategies are described and evaluated below.

Direct import of RNA-based therapeutic agents using a mitochondrion-targeted carrier

Exogenous RNA molecules can be assembled into a mitochondrion-targeted carrier that will release the RNAs directly into the mitochondrial matrix. This approach has been made available by development of RNA nanocarrier systems able to enter the cell and actively target mitochondria. Over the past two decades, liposomal, inorganic, polymer-based, or DQAsome nanocarriers have been developed to target mitochondria on their own or using targeting molecules. Mitochondrial targeting is often achieved with delocalized lipophilic cations (DLCs), such as triphenylphosphonium cations (TPPs), mitochondrion-penetrating peptides, mitochondrion-targeting signal peptides, RNA aptamers, and DQAsomes. Most mitochondrion-targeted nanocarriers were designed to carry small-molecule drugs for anticancer therapy, but some were adapted to carry RNAs. A polymer-based nanocarrier system made up of an amine-terminated, generation 5, poly(amideamine) dendrimer conjugated to TPP (PAMAM(G5)-TPP) for mitochondrion targeting, carrying a miRNA, let-7b, has been shown to colocalize with mitochondria of A549 cells and significantly decrease expression of the mt-mRNAs COX1 and COX2. Expression of the cytoplasmic mRNAs KRAS and p21 has also been affected, corresponding to at least a partial release of let-7b in the cytosol.

Liposome-based nanocarriers were more successful for delivery of RNA to mitochondria. A research group from Hokkaido University has advanced this field by developing a non-cationic lipidosome-based nanocarrier called MITO-porter. MITO-porter is a mitochondria fusogenic liposome that enters the cell by macropinocytosis, mediated by high-density octa-arginine (R8) present on the surface of the nanocarrier. In the cell, MITO-porter escapes the endosome and binds mitochondria through electrostatic interactions between the
MITO-porter because various RNA molecules can be delivered directly to the mitochondria of target cells.

A disadvantage of MITO-porter is that its cellular uptake efficiency varies between different cell types. To target cell types where MITO-porter internalization is particularly low, such as cardiomyocytes, other liposome-based nucleic acid nanocarriers are able to target mitochondria. For example, Yamada et al. demonstrated successful delivery of mRNA via β-MEND particles modified with the RNA aptamer (RP/β-MEND) to the mitochondria of H9c2 myoblasts. Nanocarriers for active targeting of therapeutic RNA molecules to the mitochondria of specific tissues are very promising. Unlike other methods, the RNAs do not need to be modified beyond the modifications necessary for their therapeutic efficacy.
The nanocarrier can protect the RNA therapeutic agent from degradation in blood serum and cytoplasm, and it can be engineered to target specific tissues or cells in the body, limiting off-target cell effects.\textsuperscript{143,149}

**Indirect import of RNA-based therapeutic agents via the mitochondrial RNA import pathway**

Another strategy for delivering therapeutic RNAs to the mitochondrial matrix is to modify the RNAs in such way that, upon cytoplasmic delivery, they are directed for import into the mitochondrial matrix via the endogenous mitochondrial RNA import machinery. This strategy is advantageous over mitochondrion-targeted carriers because cytosol-targeted nanocarrier and conjugate systems for cytosolic delivery of RNAs to the cell, such as LNPs or GalNAc conjugates, have already been tested for use in humans in preparations including COVID mRNA vaccines, siRNAs, and ASOs.\textsuperscript{199} There is much more flexibility in the design of such particles and, therefore, their cell-targeting abilities because they are not restricted by the requirement of mitochondrial targeting. A comprehensive review of non-viral RNA delivery by Paunovska et al.\textsuperscript{199} highlighted the potential for development and use of lipid- and polymer-based nanocarriers and RNA conjugates. The review also addressed the need for further investigation of the clinical implications of adding the targeting ligands to the nanocarriers, interactions between the carrier and the RNA payload, as well as the need for better animal models before non-viral RNA delivery systems can reach their full potential. Viral delivery of RNA therapeutic agents is possible, but it poses clinical translation challenges because of the presence of a human homolog, but it was included in the transfected vectors to increase the efficiency of tRK mitochondrial import. Karicheva et al.\textsuperscript{115} were able to change the amino acid identity of tRK1, tRK3, and tRK93 by introducing a discriminator base A73, recognizable by human mt-LeuRS, and by adding the Leu anticodons UAA or CAA. One such recombinant tRK version, tRK1\textsuperscript{CAA}, was able to partially rescue general mitochondrial translation and improve respiration rates. When they tried to transfect the T7 transcripts of the recombinant tRKs, despite import to mitochondria, the recombinant tRKs were mainly present in a deacetylated state.\textsuperscript{115}

This was hypothesized to be due to the lack of post-transcriptional modifications in the T7 transcripts.\textsuperscript{115} This might mean that, if this strategy is to be used for therapeutic purposes, then the RNA mimics would have to be post-transcriptionally modified before administration, which may complicate their manufacturing. This strategy is also limited by the low efficiency of import and specific structural requirement of the therapeutic agent, but the import determinants of tRK1 can be used separately to target other exogenous RNA molecules to the mitochondria.\textsuperscript{105,115}

Comte et al.\textsuperscript{105} have first shown that \textit{in vitro}-synthesized RNA carrying the import determinants of yeast tRK1 (F- and D-loops) or human 5S rRNA (its \(\alpha\) and \(\gamma\) domains) attached to a 20-nt sequence complementary to the mutant mtDNA sequence formed as a result of the fusion of the deletion boundaries can decrease heteroplasmy in a KSS cybrid cell line (carrying an 8,363- to 15,438-nt deletion). The antigenomic RNAs were successfully imported into human mitochondria and partial rescue of the respiration rate and mitochondrial translation.\textsuperscript{105} However, One such strategy involves modified \textit{S. cerevisiae} tRNAs to mimic WT human mitochondrial tRNAs for treatment of MERRF and MELAS. \textit{S. cerevisiae} encodes three tRNA\textsuperscript{\textit{S}}: tRNA\textsuperscript{\textit{Sj}}\textsuperscript{UUU} (tRK1; partially imported into mitochondria), tRNA\textsuperscript{\textit{Sj}}\textsuperscript{UUU} (tRK2; not imported into mitochondria), and tRNA\textsuperscript{\textit{Sj}}\textsuperscript{UUU} (tRK3; encoded by mtDNA and localized to mitochondria).\textsuperscript{146,173} tRK1, tRK3, and a mutant version of tRK2, tRK93 (tRK2 with the following modifications: \(\Psi\)1G, A72C, and G73U), have been shown to be imported into human mitochondria \textit{in vivo}.\textsuperscript{114} Kolesnikova et al.\textsuperscript{114} were able to stably express yeast tRK3 and tRK93 together with the MSK1 gene in a human MERRF (m.A8344G) \textit{trans}-mitochondrial cybrid line and patient-derived MERRF (m.A8344G) fibroblasts, which led to their partial import into human mitochondria and partial rescue of the respiration rate and mitochondrial translation. MSK1 encodes pre-MSK1p, a protein carrier that facilitates mitochondrial import of tRKs. Pre-MSK1p is not essential for import of tRKs into human mitochondria because of the presence of a human homolog, but it was included in the transfected vectors to increase the efficiency of tRK mitochondrial import. Karicheva et al.\textsuperscript{115} were able to change the amino acid identity of tRK1, tRK3, and tRK93 by introducing a discriminator base A73, recognizable by human mt-LeuRS, and by adding the Leu anticodons UAA or CAA. One such recombinant tRK version, tRK1\textsuperscript{CAA}, was able to partially rescue general mitochondrial translation and improve respiration rates. When they tried to transfec...
Some nucleus-encoded RNAs are actively imported into the mitochondrial matrix. They play a variety of roles in mitochondria, ranging from cytosol-mitochondrion signaling to participation in mitochondrial essential processes such as translation or mtRNA metabolism.^{143,144} All of the known imported RNA species are non-coding, and no mRNA has been reported to be naturally targeted for import into human mitochondria.^{145} Most of the mitochondrial functions of the imported RNAs remain undefined and can be different from the functions of these RNAs in the cytosol. At the top, the types of imported RNA species, their mechanism of import, and their function in mitochondria are shown. From left to right, these are as follows. tRNA (tRNA\textsubscript{Gln}, tRNA\textsubscript{UUG}, and tRNA\textsubscript{Aua}),^145,146 Cytosolic function: translation. Mitochondrial function: mitochondrial translation; could play a role in conditional adaptation in mitochondrial protein synthesis.^{147} Import mechanism: not well defined in humans; ATP dependent; may require cytosolic factors and be similar to that in S. cerevisiae (reviewed here: Kamenski et al.\textsuperscript{148}). SS rRNA. Cytosolic function: structural component of a large subunit of cytosolic ribosomes. Mitochondrial functions: still to be defined. Does not act as a scaffold for mitochondrial ribosomes.\textsuperscript{150} Disruption of SS rRNA import leads to a decrease in mitochondrial translation.\textsuperscript{151} Import mechanism: pre-MRP-L18 binds the y'-domain of SS rRNA in the cytosol; this exposes the x'-domain of SS rRNA, which binds nascent rhodanese, displacing pre-MRP-L18 in the process. The rhodanese-SS rRNA complex is then imported into mitochondria. PNPase may be involved in transfer of SS rRNA because its knockdown leads to decreased import of SS rRNA.\textsuperscript{151} Import determinants: y'-domain (specifically loop E and helix IV) and x'-domain (helix I).\textsuperscript{152} RPPH1 (RNA component of RNAse P; H1 RNA). Cytosolic function: component of RNAse P, 5' RNA maturation.\textsuperscript{153} Mitochondrial function: mitochondrial localization induces replicative cellular senescence.\textsuperscript{154} May play a role in mt-tRNA maturation as a part of mitochondrial RNAse P.\textsuperscript{153} Import mechanism: mediated by PNPase.\textsuperscript{153,155} Import determinants: 20-nt stem-loop, nucleotides 115–134 (NR_002312.1). RMRP (RNA component of RNAse MRP). Cytosolic function: component of the nuclear RNAse MRP; 5' 5.8S rRNA maturation; participates in generation of double-stranded RNA (dsRNA) precursors of siRNA; cell cycle progression.\textsuperscript{155,156} Mitochondrial function: not firmly defined. May participate in mitochondrial RNA metabolism in a complex with GRSF1.\textsuperscript{156,157} Import mechanism: exported from the nucleus in a complex with GRSF1.\textsuperscript{156,157} The precise mechanism of RMRP transport through the mitochondrial membrane is unknown, although it has been hypothesized that it may cross the OMM via the TOMM40-complex and intramembrane space (IMS) in a PNPase-dependent manner.\textsuperscript{156} It has also been shown that levels of GRSF1 (associated with RMRP in the mitochondrial matrix) in mitochondria can affect RMRP localization.\textsuperscript{156,157} Import determinants: 20-nt stem-loop, Nucleotides 151–170 (NR_003051.3).\textsuperscript{156,157} GAS5 (growth arrest-specific 5). Cytosolic function: RNA sponge to buffer miRNAs; regulates mRNA transcription in adipocytes; acts as a decay hormone response element for gluocorticoid receptor (GR).\textsuperscript{151,153} Mitochondrial function: modulates mitochondrial tricarboxylic acid (TCA) cycle flux.\textsuperscript{153} Import mechanism: Unknown. Import determinants: GAS5-loop 2, hTERC (human telomerase RNA gene). Cytosolic function: telomerase-related functions; Cellular senescence and cognitive decline in mice as hTERC-S3.\textsuperscript{156,157} Mitochondrial function: mitochondrial-cytosol crosstalk (specifically loop E and helix IV). Translation? Transcription? Translation? Cellular senescence? mt-tRNA maturation? mtRNA metabolism? Modulation of tricarboxylic acid cycle flux mitochondrion-cytosol crosstalk \( \downarrow \downarrow \) translation

Figure 4. The RNA importome of human mitochondria

Some nucleus-encoded RNAs are actively imported into the mitochondrial matrix. They play a variety of roles in mitochondria, ranging from cytosol-mitochondrion signaling to participation in mitochondrial essential processes such as translation or mtRNA metabolism.\textsuperscript{143,144} All of the known imported RNA species are non-coding, and no mRNA has been reported to be naturally targeted for import into human mitochondria.\textsuperscript{145} Most of the mitochondrial functions of the imported RNAs remain undefined and can be different from the functions of these RNAs in the cytosol. At the top, the types of imported RNA species, their mechanism of import, and their function in mitochondria are shown. From left to right, these are as follows. tRNA (tRNA\textsubscript{Gln}, tRNA\textsubscript{UUG}, and tRNA\textsubscript{Aua}),^145,146 Cytosolic function: translation. Mitochondrial function: mitochondrial translation; could play a role in conditional adaptation in mitochondrial protein synthesis.\textsuperscript{147} Import mechanism: not well defined in humans; ATP dependent; may require cytosolic factors and be similar to that in S. cerevisiae (reviewed here: Kamenski et al.\textsuperscript{148}). SS rRNA. Cytosolic function: structural component of a large subunit of cytosolic ribosomes. Mitochondrial functions: still to be defined. Does not act as a scaffold for mitochondrial ribosomes.\textsuperscript{150} Disruption of SS rRNA import leads to a decrease in mitochondrial translation.\textsuperscript{151} Import mechanism: pre-MRP-L18 binds the y'-domain of SS rRNA in the cytosol; this exposes the x'-domain of SS rRNA, which binds nascent rhodanese, displacing pre-MRP-L18 in the process. The rhodanese-SS rRNA complex is then imported into mitochondria. PNPase may be involved in transfer of SS rRNA because its knockdown leads to decreased import of SS rRNA.\textsuperscript{151} Import determinants: y'-domain (specifically loop E and helix IV) and x'-domain (helix I).\textsuperscript{152} RPPH1 (RNA component of RNAse P; H1 RNA). Cytosolic function: component of RNAse P, 5' RNA maturation.\textsuperscript{153} Mitochondrial function: mitochondrial localization induces replicative cellular senescence.\textsuperscript{154} May play a role in mt-tRNA maturation as a part of mitochondrial RNAse P.\textsuperscript{153} Import mechanism: mediated by PNPase.\textsuperscript{153,155} Import determinants: 20-nt stem-loop, nucleotides 115–134 (NR_002312.1). RMRP (RNA component of RNAse MRP). Cytosolic function: component of the nuclear RNAse MRP; 5' 5.8S rRNA maturation; participates in generation of double-stranded RNA (dsRNA) precursors of siRNA; cell cycle progression.\textsuperscript{155,156} Mitochondrial function: not firmly defined. May participate in mitochondrial RNA metabolism in a complex with GRSF1.\textsuperscript{156,157} Import mechanism: exported from the nucleus in a complex with GRSF1.\textsuperscript{156,157} The precise mechanism of RMRP transport through the mitochondrial membrane is unknown, although it has been hypothesized that it may cross the OMM via the TOMM40-complex and intramembrane space (IMS) in a PNPase-dependent manner.\textsuperscript{156} It has also been shown that levels of GRSF1 (associated with RMRP in the mitochondrial matrix) in mitochondria can affect RMRP localization.\textsuperscript{156,157} Import determinants: 20-nt stem-loop, Nucleotides 151–170 (NR_003051.3).\textsuperscript{156,157} GAS5 (growth arrest-specific 5). Cytosolic function: RNA sponge to buffer miRNAs; regulates mRNA transcription in adipocytes; acts as a decay hormone response element for gluocorticoid receptor (GR).\textsuperscript{151,153} Mitochondrial function: modulates mitochondrial tricarboxylic acid (TCA) cycle flux.\textsuperscript{153} Import mechanism: Unknown. Import determinants: GAS5-loop 2, hTERC (human telomerase RNA gene). Cytosolic function: telomerase-related functions; Cellular senescence and cognitive decline in mice as hTERC-S3.\textsuperscript{156,157} Mitochondrial function: mitochondrial-cytosol crosstalk (specifically loop E and helix IV). Translation? Transcription? Translation? Cellular senescence? mt-tRNA maturation? mtRNA metabolism? Modulation of tricarboxylic acid cycle flux mitochondrion-cytosol crosstalk \( \downarrow \downarrow \) translation

when transiently transfected, the decrease in heteroplasmy was observed 4–6 days after transfection and was reversed a few days later, most likely when the antigenomic RNAs were degraded.\textsuperscript{152} Loutre et al.\textsuperscript{120} were able to replicate the results of stable 5S rRNA-based anti-replicative RNA transfection in a KSS cybrid line and showed that the decrease in heteroplasmy depends on the number of
anti-replicative RNAs in the mitochondrial matrix. Tonin et al. designed similar anti-replicative TRK1-based molecules targeting a single point mutation, m.A13514G, in the NDS gene. Transfection of a transmitochondrial cybrid cell line with this anti-replicative RNA resulted in an ∼22% stable decrease in heteroplasmy. A similar shift in heteroplasmy was obtained upon transfection of patient fibroblasts. Although this shift in heteroplasmy stabilized 6 days after transfection and remained stable until 8 days after transfection, whether it remains stable for longer remains to be investigated. The reduction in mutant mtDNA copy number in all of these studies was only partial, but it can still be therapeutic because usually only a high proportion of mutant mtDNA causes pathological effects, whereas clinical severity is proportionate to the level of heteroplasmy.

A more versatile RNA import determinant was discovered by Wang et al. They identified a 20-nt stem-loop region in RPPH1 and RMRP (supplemental information) that allowed them to be targeted for polynucleotide phosphorylase (PNPase)-dependent mitochondrial import (supplemental information). Two years later, Wang et al. attached this stem-loop sequence to the non-imported RNA species, which allowed them to be imported into the mitochondrial matrix. They were able to stably transfect mouse embryonic fibroblast with the hCOX2 gene and either a 5′-20 nt stem-loop sequence of RPPH1. hCOX2 mRNA was found to be targeted to the mitochondrial matrix, where it was translated into a functional hCOX2 protein. This proves the ability of this approach to import large (at least 683-nt) protein-coding mtRNAs into the mitochondrial matrix to compensate for mutant mitochondrial genes.

They were able to target allotopically expressed WT mt-tRNAs and mt-tRNA(UUR) into mitochondria of MERRF and MELAS cybrid lines, respectively. Genes encoding the precursors of these tRNAs were modified in the following ways: (1) the 20-nt stem-loop sequence from RPPH1 was added to the 5′ of the genes to target the tRNAs for PNPase-dependent import; the 3′ UTR from the MRPS12 gene was added to the 3′ of the tRNA precursor genes to localize them to the mitochondrial surface; (2) ribonucleotides adjacent to the aminocyl stem of the tRNAs were modified to prevent cleavage of 5′ and 3′ terms of the tRNAs in the nucleus (may not be necessary upon nanocarrier delivery). These tRNA constructs were stably translocated into mitochondrial matrix across the mitochondrial surface; (3) mitochondrial import targeted by more than 80%. They have conjugated a cholesterol residue via a cleavable pH-triggered hydrazone bond to the anti-replicative RNA targeting NDS m.13514A>G, demonstrating that it can be taken up by patient fibroblasts, where, in the acidic endosome environment, the cholesterol residue is cleaved. Upon release from the endosome, the antireplicative RNA was localized to the mitochondrial matrix, where it induced a partial decrease in the level of mutant mtDNA. There is potential for mitochondrial RNA therapies to become a breakthrough treatments of mitochondrial diseases.

CHALLENGES AND FUTURE PERSPECTIVES
Mitochondrial diseases are one of the largest group of neurological genetic disorders, and despite continued efforts of the scientific community, there is a need to improve therapeutic strategies. In particular, novel strategies should follow the general principles of personalized therapies, including individual variability in genetics, heteroplasmy, and environmental exposure, bringing precision medicine approaches to mitochondrial disease.

RNA-based therapeutic agents that alter mitochondrial function on the RNA or protein level offer a better alternative to mitochondrial
gene therapies with low pharmacological control and small-molecule drugs, which are often unable to address mitochondrial diseases directly. However, the behavior of some therapeutic RNAs in mitochondria and their delivery to the mitochondrial matrix warrant further investigation.

Although mRNA therapeutic agents that act as a gene replacement therapy and ASO and siRNA drugs that affect cytoplasmic transcripts are well studied and have been approved for use in humans or are now going through clinical trials, their mechanism of action and stability in the mitochondrial matrix are not fully understood. For example, mitochondrial proteins are often more stable than the cytoplasmic ones, which may result in different durations of action of mRNA-based drugs that aim to replace missing or dysfunctional mitochondrial proteins. Although it is evident that siRNA drugs are able to suppress translation of select mitochondrial transcripts, it is still unknown whether RNAi-like mechanisms are present in mitochondria or whether siRNA drugs have a different mode of action in the mitochondrial matrix. Finally, the stability of the heteroplasmic shift induced by antireplicative RNAs needs to be further investigated. Based on current reports, the stability of the heteroplasmic shift by antireplicative RNA depends on the nature of the pathogenic mutation in mtDNA. So far, most of the studies done with RNA-based therapeutic agents are proof-of-concept studies that require more fundamental knowledge to understand and optimize their mechanism of action, and more in vivo studies have to be conducted to determine whether the therapeutic effects seen at a cellular level will translate to rescue of a healthy phenotype at a tissue and organismal level.

A greater challenge lies in delivery of RNA-based therapeutic agents to the mitochondrial matrix. To the best of our knowledge, strategies that use endogenous RNA import determinants have not been tested in vivo. However, in vivo testing of mitochondrial-targeted nanocarriers has been performed for the purpose of delivering anticancer medicine rather than RNA therapeutic agents. Specifically, the issue of RNA delivery to the mitochondrial matrix can be broken down into two parts. First is delivery of the nanocarrier to the target tissues, and second is delivery of the RNA therapeutic agent to the mitochondrial matrix in the cell. The issue of target tissue delivery is shared by other nanocarrier systems that aim to fix nDNA defects. Now more than ever, new targeting ligands and new particle compositions are tested for different tissue specificities and body distribution, but ligand-targeted nanocarriers have not yet been approved for use in the clinic. In fact, one of the more developed mitochondrial-targeted nanocarriers, MITO-porter, has low uptake efficiency in cardiomyocytes, which can be overcome by development of specific nanocarrier systems, such as RPβ-MEND, which have different cellular uptake efficiency profiles.

RNA import into mitochondria is a largely understudied process. It seems that the efficiency of RNA import varies with tissue type and developmental stage of the tissue. However, more studies need to be done to understand the fundamental principles of RNA import into mitochondria. This will allow optimization of specific mitochondrial RNA import determinants that can be used as a signal sequence on therapeutic RNAs themselves or as a targeting ligand on a mitochondrial-targeted nanocarrier. On a positive note, there is general excitement in the RNA field about the success achieved with mRNA vaccines and by the accelerated FDA approval of four new RNAi drugs. This success is the culmination of decades of research and discovery, not to mention many failed and disappointing biotech ventures. RNA therapies have finally entered the limelight. These are really exciting times for RNA therapies, and targeting mitochondria with RNA drugs is just one of the possibilities.

Finally, if mitochondrial RNA therapies can benefit patients with mitochondrial disease, then they may also be explored for broader applications in common diseases and health conditions that stem from or result in mitochondrial dysfunction, including CVD, cancer, and diabetes.

DATA AVAILABILITY STATEMENT
No new data were generated or analyzed in this study; thus, data sharing is not applicable.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.10.012.

ACKNOWLEDGMENTS
We acknowledge funding support from the Canadian Institutes of Health Research (CIHR; ACA 505546) for supporting the scholarship for J.C., the Canada Research Chair (CIHR; 501090) for supporting the research program of A.C.A., and the Department of Pharmacology, University of Toronto, for supporting the research project of T.C. We thank Taewon Kim for preparation of Table 1.

AUTHOR CONTRIBUTIONS
J.C. and T.C. performed literature reviews and preparation of the manuscript. L.S. reviewed the manuscript and contributed knowledge of T.C.. We thank Taewon Kim for preparation of Table 1.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
1. Vafai, S.B., and Mootha, V.K. (2012). Mitochondrial disorders as windows into an ancient organelle. Nature 491, 374–383. https://doi.org/10.1038/nature11707.
2. Rath, S., Sharma, R., Gupta, R., Ast, T., Chan, C., Durham, T.J., Goodman, R.P., Grabarek, Z., Haas, M.E., Hung, W.H.W., et al. (2021). MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations. Nucleic Acids Res. 49, D1541–D1547. https://doi.org/10.1093/nar/gkaa1011.
3. Rahman, J., and Rahman, S. (2018). Mitochondrial medicine in the omics era. Lancet 391, 2560–2574. https://doi.org/10.1016/S0140-6736(18)30727-X.
4. Parihi, S., Goldstein, A., Koenig, M.K., Scaglia, F., Enns, G.M., Saneto, R., Anselm, I., Cohen, B.H., Falk, M.J., Greene, C., et al. (2015). Diagnosis and management of
guanylate cyclase stimulator CY4643 reveals its therapeutic potential in neurodegenerative diseases. Front. Pharmacol. 12, 65561. https://doi.org/10.3389/fphar.2021.65561.

73. Guha, S., Kenkwo, C., Lavorato, M., Mathew, N.D., Peng, M., Ostrovsky, J., Kwon, Y.-J., Polyk, E., Lightfoot, R., Seiler, C., et al. (2019). Pre-clinical evaluation of cysteine bitartrate as a therapeutic agent for mitochondrial respiratory chain disease. Hum. Mol. Genet. 28, 1837–1852. https://doi.org/10.1093/hmg/ddz023.

74. Beyrath, J., Pellegrini, M., Renkema, H., Houben, L., Pecheritsyna, S., van Zandvoort, P., van den Broek, P., Bekel, A., Effekhari, P., and Smeitink, J.A.M. (2018). KH176 safeguards mitochondrial diseased cells from redox stress–induced cell death by interacting with the thiolredoxin system/peroxiredoxin enzyme machinery. Sci. Rep. 8, 6577. https://doi.org/10.1038/s41598-018-24900-3.

75. Koene, S., Spaans, E., Van Bortel, L., Van Lancker, G., Delafontaine, B., Badilini, F., Newman, N.J., Yu-Wai-Man, P., Carelli, V., Moster, M.L., Biousse, V., Vignal-Porter: a liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. Biochim. Biophys. Acta 177, 239–249. https://doi.org/10.1016/j.bbamem.2007.11.002.

76. Newman, N.J., Yu-Wai-Man, P., Carelli, V., Moster, M.L., Biousse, V., Vignal-Porter: a liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. Biochim. Biophys. Acta 177, 239–249. https://doi.org/10.1016/j.bbamem.2007.11.002.

77. Martinelli, D., Catteruccia, M., Piemonte, F., Pastore, A., Tozzi, G., Dionisi-Vici, C., Balwani, M., Sardh, E., Ventura, P., Rees, D.C., Stolzel, U., Bissell, D.M., Abu-Shaar, M., and Reddel, R.R. (2013). Apoptosis and cell death by interacting with the thiolredoxin system/peroxiredoxin enzyme machinery. Sci. Rep. 3, 3355. https://doi.org/10.1038/srep03355.

78. Sahel, J.-A., Newman, N.I., Yu-Wai-Man, P., Vignal-Clermont, C., Carelli, V., Biousse, V., Moster, M.L., Sergott, R., Klopstock, T., Sadun, A.A., et al. (2021). Gene therapies for the treatment of leber hereditary optic neuropathy. Int. Ophthalmol. Clin. 61, 195–208.

88. Damase, T.R., Suhkovershin, R., Booda, C., Taraballi, F., Pettigrew, R.L., and Cooke, J.P. (2021). The limitless future of RNA therapeutics. Front. Bioeng. Biotechnol. 9, 628317.
103. Tonin, Y., Heckel, A.M., Vysokikh, M., Dovydenko, I., Meschinnova, M., Röting, A., Munnich, A., Venynimina, A., Tarassov, I., and Entelis, N. (2014). Modeling of antigenic therapy of mitochondrial diseases by mitochondrially addressed RNA targeting a pathogenic point mutation in mitochondrial DNA. J. Biol. Chem. 289, 13323–13334. https://doi.org/10.1074/jbc.M113.528968.

104. Wang, G., Shimada, E., Zhang, J., Hong, J.S., Smith, G.M., Teitel, M.A., and Koehler, C.M. (2012). Correcting human mitochondrial mutations with targeted RNA import. Proc. Natl. Acad. Sci. USA 109, 4840–4845. https://doi.org/10.1073/pnas.1116792109.

105. Comte, C., Tonin, Y., Heckel-Mager, A.M., Boucheham, A., Smirnov, A., Auré, K., Lombris, A., Martin, R.P., Entelis, N., and Tarassov, I. (2013). Mitochondrial targeting of recombinant RNAs modulates the level of a heteroplasmic mutation in human mitochondrial DNA associated with Kearns Sayre Syndrome. Nucleic Acids Res. 41, 418–433. https://doi.org/10.1093/nar/gks685.

106. Mukherjee, S., Mahata, B., Mahato, B., and Adhya, S. (2008). Targeted mRNA degrada
tion by complex-mediated delivery of antisense RNAs to intracellular human mitochondria. Hum. Mol. Genet. 17, 1292–1298. https://doi.org/10.1093/hmg/ddn017.

107. Bandiera, S., Rüberg, S., Girard, M., Cagnard, N., Hanein, S., Chrétien, D., Munnich, A., Mukherjee, S., Mahata, B., Mahato, B., and Adhya, S. (2008). Targeted mRNA degradation by complex-mediated delivery of antisense RNAs to intracellular human mitochondria. Hum. Mol. Genet. 17, 1292–1298. https://doi.org/10.1093/hmg/ddn017.

108. Zhang, X. (2021). Active RNA interference in mitochondria. Cell Res. 31, 607–619. https://doi.org/10.1038/s41422-020-0394-5.

109. Yang, J., Park, J.-S., Yin, L., Laureano, R., Jacquinet, E., Yang, J., Liang, S., Frassetto, A., Zhou, J., Yan, X., et al. (2020). A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. Nature 583, 631–637. https://doi.org/10.1038/s41586-020-2477-4.

110. Yang, Y., Wu, H., Kang, X., Liang, Y., Lan, T., Li, T., Tan, T., Peng, J., Zhang, Q., An, G., et al. (2018). Targeted elimination of mutant mitochondrial DNA in MELAS iPSCs by mitoTALENs. Protein Cell 9, 283–297. https://doi.org/10.1007/s13238-017-0499-y.

111. Hussain, S.-R.A., yalvar, M.E., Khoo, B.E., Eckardt, S., and McLaughlin, K.J. (2021). Adapting CRISPR/Cas9 system for targeting mitochondrial genome. Front. Genet. 12, 627050. https://doi.org/10.3389/fgene.2021.627050.

112. Uchida, S., and Dimmeler, S. (2015). Long noncoding RNAs in cardiovascular dis
eres. Circ. Res. 116, 737–750. https://doi.org/10.1161/CIRCRESAHA.116.302521.

113. Grunevald, A., Kumar, K.R., and Sue, C.M. (2019). New insights into the complex role of mitochondria in Parkinson’s disease. Prog. Neurobiol. 177, 73–93. https://doi.org/10.1016/j.pneurobio.2018.09.003.

114. Yamada, Y., Furukawa, R., Yasuzaki, Y., and Harashima, H. (2011). Dual function MITO-porter, a nano carrier integrating both efficient cytoplasmic delivery and mitochondrial macromolecule delivery. Mol. Ther. 19, 1449–1456. https://doi.org/10.1038/mt.2011.99.

115. Yamada, Y., Fujishita, N., and Harashima, H. (2020). A nanocarrier for the mitochondrial delivery of nucleic acids to cardiomyocytes. Nucleosides Nucleotides Nucleic Acids 39, 141–155. https://doi.org/10.1080/07328398.2019.1675167.

116. Kolesnikova, O.A., Entelis, N.S., Mireau, H., Fox, T.D., Martin, R.P., and Tarassov, I.A. (2000). Suppression of mutations in mitochondrial DNA by tRNAs imported from the cytoplasm. Science 289, 1931–1933. https://doi.org/10.1126/science.289.5486.1931.

117. Wang, G., Shimada, E., Koehler, C.M., and Teitel, M.A. (2012). NPNASe and RNA trafficking into mitochondria. Biochim. Biophys. Acta 1819. https://doi.org/10.1016/j.bbadgr.2011.10.001.

118. Wang, Z., Guo, W., Kuang, X., Hou, S., and Liu, H. (2017). Nano preoperations for mitochondria targeting drug delivery system: current strategies and future prospective. Asian J. Pharm. Sci. 12, 498–508. https://doi.org/10.1016/j.ajps.2017.05.006.

119. Hu, T., Qin, Z., Shen, C., Gong, H.-L., and He, Z.-Y. (2021). Multifunctional mitochondria-targeting nanosystems for enhanced anticancer efficacy. Front. Bioeng. Biotechnol. 9, 786621. https://doi.org/10.3389/fbioe.2021.786621.

120. Qin, J., Gong, N., Liao, Z., Zhang, S., Timashev, P., Hua, S., and Liang, X.-J. (2021). Recent progress in mitochondria-targeting based nanotechnology for cancer treatment. Nanoscale 13, 7108–7118. https://doi.org/10.1039/D1NR01068A.

121. Yamada, Y., Furukawa, R., and Harashima, H. (2016). A dual-ligand liposomal sys
tem composed of a cell-penetrating peptide and a mitochondrial RNA aptamer synergistically facilitates cellular uptake and mitochondrial targeting. J. Pharm. Sci. 105, 1705–1713. https://doi.org/10.1016/j.xphs.2016.03.002.

122. Yamada, Y., Maruyama, M., Kitagawa, K., Uchida, S., and Harashima, H. (2020). The use of a MITO-Porter to deliver exogenous therapeutic RNA to a mitochondrial disease’s cell with a A1555G mutation in the mitochondrial 12S rRNA gene results in an increase in mitochondrial respiratory activity. Mitochondrion 55, 134–144. https://doi.org/10.1016/j.mito.2020.09.008.
172. Sato, S., Ishikawa, H., Yoshikawa, H., Inumikawa, K., Simpson, R.J., and Takahashi, N. (2015). Collaborator of alternative reading frame protein (CARF) regulates early processing of pre-ribosomal RNA by retaining XRN2 (5'--3' exoribonuclease) in the nucleoplasm. Nucleic Acids Res. 43, 10397–10410. https://doi.org/10.1093/nar/gkv1069.

173. Tarassov, I.A., and Martin, R.P. (1996). Mechanisms of tRNA import into yeast mitochondria: an overview. Biochimie 78, 502–510. https://doi.org/10.1016/0300-9084(96)84756-0.

174. Adhya, S. (2008). Leishmania mitochondrial tRNA importers. Int. J. Biochem. Cell Biol. 40, 2681–2685. https://doi.org/10.1016/j.biocel.2007.10.025.

175. Koley, S., and Adhya, S. (2013). A voltage-gated pore for translocation of tRNA. Biochem. Biophys. Res. Commun. 439, 23–29. https://doi.org/10.1016/j.bbrc.2013.08.036.

176. Goswami, S., Dhar, G., Mukherjee, S., Mahata, B., Chatterjee, S., Home, P., and Adhya, S. (2006). A bifunctional tRNA import receptor from Leishmania mitochondria. Proc. Natl. Acad. Sci. USA 103, 8354–8359. https://doi.org/10.1073/pnas.0510869103.

177. Dovydenko, I., Tarassov, I., Venyaminova, A., and Entelis, N. (2016). Method of carrier-free delivery of therapeutic RNA importable into human mitochondria: lipophilic conjugates with cleavable bonds. Biomaterials 76, 408–417. https://doi.org/10.1016/j.biomaterials.2015.10.075.

178. Weng, Y., Xiao, H., Zhang, J., Liang, X.-J., and Huang, Y. (2019). RNAi therapeutic and its innovative biotechnological evolution. Biotechnol. Adv. 37, 801–825. https://doi.org/10.1016/j.biotechadv.2019.04.012.

179. Kowalski, P.S., Rudra, A., Miao, L., and Anderson, D.G. (2019). Delivering the messenger: advances in technologies for therapeutic mRNA delivery. Mol. Ther. 27, 710–728. https://doi.org/10.1016/j.ymthe.2019.02.012.

180. Krishna, S., Arrojo e Drigo, R., Capitanio, J.S., Ramachandra, R., Ellisman, M., and Hetzer, M.W. (2021). Identification of long-lived proteins in the mitochondria reveals increased stability of the electron transport chain. Dev. Cell 56, 2952–2965.e9. https://doi.org/10.1016/j.devcel.2021.10.008.