Trends in Molecular Medicine

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Review

Therapeutic Manipulation of mtDNA Heteroplasmy: A Shifting Perspective

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Mutations of mitochondrial DNA (mtDNA) often underlie mitochondrial disease, one of the most common inherited metabolic disorders. Since the sequencing of the human mitochondrial genome and the discovery of pathogenic mutations in mtDNA more than 30 years ago, a movement towards generating methods for robust manipulation of mtDNA has ensued, although with relatively few advances and some controversy. While developments in the transformation of mammalian mtDNA have stood still for some time, recent demonstrations of programmable nuclease-based technology suggest that clinical manipulation of mtDNA heteroplasmy may be on the horizon for these largely untreatable disorders. Here we review historical and recent developments in mitochondrially targeted nuclease technology and the clinical outlook for treatment of hereditary mitochondrial disease.

Highlights

Organelle-targeted nucleases allow specific destruction of mutant mtDNA.

In combination with adeno-associated viruses, these nucleases allow organ-specific changes in mtDNA heteroplasmy.

Recent data have provided early proof of principle for gene therapy of incurable mtDNA disorders using these engineered enzymes.

Mitochondrial Genetics and Human Disease

In the past 30 years the medical relevance of **mtDNA** (see Glossary) has brought this unusual genome to the fore [1]. This is, in the most part, owing to the increasing recognition of mitochondria as a major biochemical hub, the confirmation of mitochondrial dysfunction and mtDNA mutations in common diseases [1,2], and the role of mtDNA in disease-relevant cell signalling pathways [3,4], in concert with a number of high-profile attempts at preventing untreatable, hereditary mitochondrial disease using *in vitro* fertilisation techniques [known as **mitochondrial replacement therapy** or mitochondrial donation] [5]. With such an upsurge of interest in mtDNA, effort towards generating methods for robust manipulation of mtDNA, for both clinical and experimental means, has been galvanised [6,7].

Mitochondrial diseases are one of the most common forms of inherited metabolic disease, with asymptomatic carriers of pathogenic mtDNA variants estimated at one in 200 adults, and approximately one in 5000 adults affected by resultant mitochondrial disease [8]. The presentation of patients bearing identical mtDNA mutations is often highly variable, with mostly unpredictable penetrance and severity. When combined with the current lack of reliable biomarkers of mitochondrial disease, diagnosis of these conditions is a major challenge [4].

Despite being clinically and genetically described for several decades, there are still no curative therapeutics for patients with mtDNA disease. As a branch of medicine still lacking such treatment, the clinical potential of gene therapy for mitochondrial disease is considerable [1].

Fundamentals of Human Mitochondrial Genetics and Heteroplasmy Manipulation

Human mtDNA is a small, multicopy genome encoding 13 protein subunits of the respiratory chain complexes I, III, and IV and ATP synthase, and all RNA molecules necessary for their translation by mitochondrial ribosomes [9]. As with any genetic material, mtDNA is susceptible to the introduction of point mutations and rearrangements by a combination of endogenous and

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exogenous factors, and these genetic lesions are observed in human mitochondrial disease [8,10]. However, as mtDNA is multicopy, mutated genomes can coexist with wild-type genomes, a state termed **heteroplasmy** (Figure 1A).

For the impact of heteroplasmic mtDNA mutations to biochemically and clinically manifest, the ratio of mutated to nonmutated mtDNA has to exceed a certain upper limit, a phenomenon known as the **threshold effect** (Figure 1B) [10]. This effect is mutation specific, with large deletions of mtDNA that encompass several genes generally manifesting at lower heteroplasmic levels (~60%) than single point mutations. These thresholds also vary between individuals with the same mutation as well as between tissues in a single patient (Figure 1A,B).

While there is general agreement on the maternal inheritance of human mtDNA, the exact mechanisms of the inheritance and segregation of mtDNA, from tissues to populations, remain a matter of debate. A genetic bottleneck during female gamete development has been identified as the major source of variance between generations, often resulting in unaffected mothers bearing children with severe mitochondrial disease (Figure 1A) [11]. Further complexity is added through mutation-specific variance, where the same mutation can be of variable distribution and pathogenicity within an individual as well as between individuals [10].

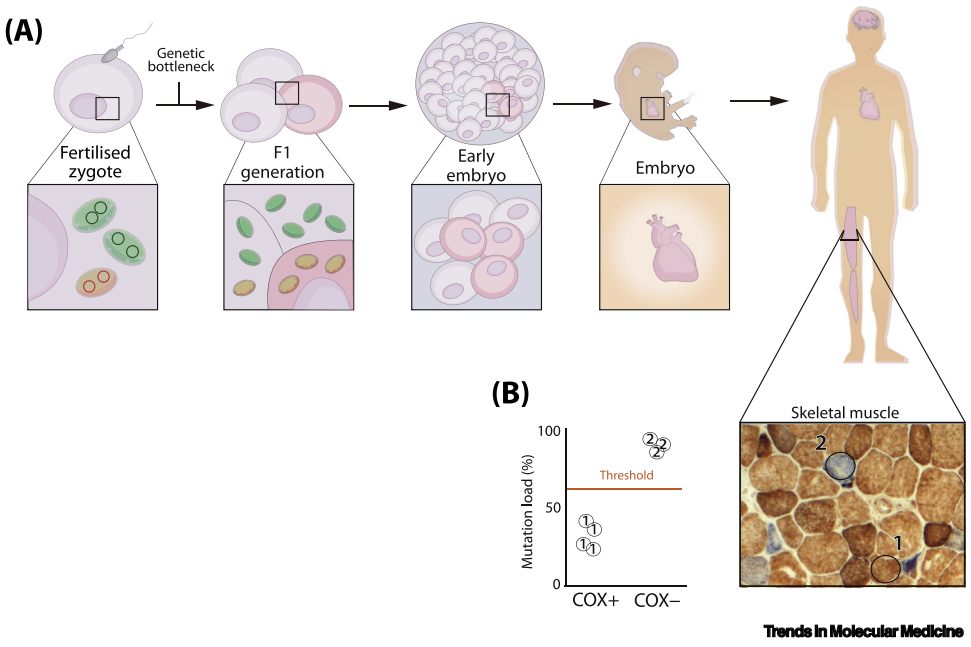

Figure 1. Heredity of Mitochondrial DNA (mtDNA) Heteroplasmy and Clinical Mosaicism. (A) mtDNA heteroplasmy, a state in which wild-type (green circle) and mutant (yellow circle) mtDNA molecules coexist in a certain proportion with one another, is initially determined in primordial germ cells in the maternal lineage during embryogenesis, indicated by 'genetic bottleneck'. Once fertilised, the mutation load of the F1 generation can be unevenly distributed throughout the developing embryo, leading to mosaicism within and between the tissues of an individual. Mutations can also have tissue-specific impacts, leading to a complex clinical picture. (B) An example of within-tissue mosaicism of a single mutation of mtDNA from patient skeletal muscle. A cross-section stained for cytochrome *c* oxidase (COX) (brown) and succinate dehydrogenase (SDH) (blue) activity. This assessment indicates the contributions of the mitochondrial and nuclear genomes to mitochondrial dysfunction, as COX requires mtDNA-encoded subunits whereas SDH does not. When separate regions of such a tissue preparation are analysed (1, 2), the degree of COX deficiency is proportional to the mutation heteroplasmy.

Glossary

Adeno-associated virus (AAV): non-enveloped, non-integrating virus used to deliver DNA to target cell or tissue.
Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9): Cas is guided by short RNA sequences – CRISPR – to specific DNA regions, which evolved as a specific prokaryotic defence strategy against phage infection. In contrast to ZFNs and TALENs, the Cas9 enzyme needs guide RNAs to target any given DNA sequence.
Cybrid: cybrid cells are generated by the fusion of a cytoplast (enucleated cell) derived from patients bearing mtDNA mutations with an immortalised cell lacking mtDNA (commonly referred to as ρ0). Through this process, cell lines with varying mutation heteroplasmy on similar nuclear genetic backgrounds can be created.
Double-strand break (DSB): through the activity of restriction endonucleases, DSBs are induced. In mitochondria, this results in the rapid degradation of linear mtDNA molecules.
Heteroplasmy: the co-occurrence of mtDNA molecules of different sequences in the same cell, whether natural polymorphisms or mutations.
Mitochondrial DNA (mtDNA): a circular 16.5-kb molecule encoding 37 genes (two rRNAs, 22 tRNAs, and 11 mRNAs) in Mammalia.
Mitochondrial replacement therapy: an approach to the prevention of mitochondrial disease inheritance by swapping mitochondria with mutated mtDNA molecules with healthy mtDNA-containing mitochondria in oocytes.
mtDNA copy number: the number of mtDNA molecules per cell.
Repeat variable diresidue (RVD): the two amino acid positions in each repeating TALE DNA-binding module that determine the binding specificity of that 33–35-aa module.
Threshold effect: a term used to describe the extent of mtDNA mutation burden that must be exceeded for a biochemical or clinical phenotype to manifest.
Transcription activator-like effector nucleases (TALENs): a TALE DNA-binding domain fused to a DNA cleavage domain, engineered to target specific DNA sequences.

While the stochastic transmission of mtDNA continues to be a major clinical complication, the threshold effect has been the source of inspiration for numerous therapeutic strategies, aiming to reduce the heteroplasmy of deleterious variants to alleviate disease through both direct and indirect targeting of pathogenic mitochondrial genomes [12], an approach often referred to as heteroplasmy shifting. This approach takes advantage of the multicopy nature of mtDNA and the functionally recessive nature of virtually all pathogenic mutations in mtDNA [13]. As heteroplasmic mtDNA mutations coexist with wild-type mtDNA, shifting mtDNA heteroplasmy below the pathogenic threshold in somatic tissues offers a rational approach to the treatment of primary mitochondrial disease [13] (Figure 2A).

Zinc-finger nuclease (ZFN): a zinc-finger DNA-binding domain fused to a DNA cleavage domain, engineered to target specific DNA sequences.

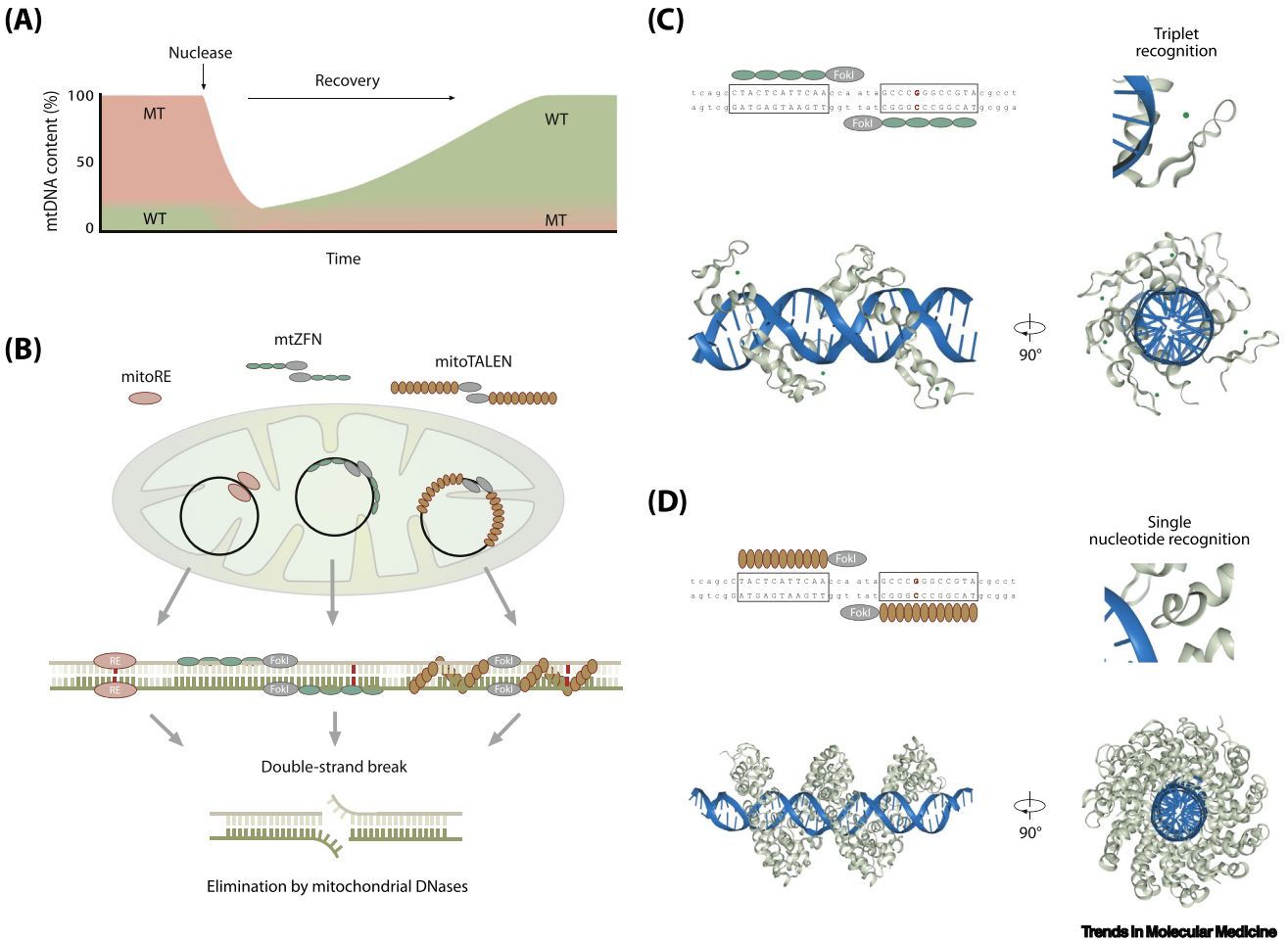

Figure 2. Manipulating Mitochondrial DNA (mtDNA) Heteroplasmy with Engineered Mitochondrial Nucleases. (A) A schematic of therapeutic mtDNA heteroplasmy manipulation based on *in vitro* observations. An initial heteroplasmic ratio with a corresponding mitochondrial defect is rapidly altered by treatment with mitochondrially targeted nucleases specific to the mutation in question. A brief, temporary depletion of total mtDNA copy number may follow, with ensuing rounds of DNA replication resulting in shifted mtDNA heteroplasmy away from the mutant, with concomitant improvements in mitochondrial function. (B) Mitochondrially targeted nucleases currently used for heteroplasmy manipulation. mitoRE, mitochondrially targeted restriction endonuclease; mtZFN, mitochondrially targeted zinc-finger nuclease; mitoTALEN, mitochondrially targeted transcription activator-like effector nuclease. All classes of nuclease are expressed in the cytoplasm and imported into mitochondria via the TIM/TOM machinery (not depicted). Once within the mitochondrial matrix, the mutant haplotype is specifically bound. Proximal *FokI* domains dimerise and introduce a double-strand break, which results in rapid turnover of the cleaved mtDNA by, possibly among others, the known mitochondrial exonuclease activity of Pol γ and MGME1. (C) Putative mtZFN target site for m.8993T>G and the structure of zinc-finger protein (ZFP) (white) bound to a cognate DNA target (blue). Green spheres indicate zinc atoms. Zoom panel exemplifies protein–DNA interaction (PDB 1P47 [67]). (D) Putative mitoTALEN target site for m.8993T>G and the structure of TALE (white) bound to a cognate DNA target (blue). Zoom panel exemplifies protein–DNA interaction (PDB 3UGM [68]).

Many avenues to the manipulation of mtDNA heteroplasmy have been explored, including: upregulation of mitochondrial turnover [14,15]; the use of antisense oligonucleotides to block mutant mtDNA replication [16]; xenotopic expression of DNA recombination enzymes [17]; the use of mitochondrial toxins [18]; general toxins [19]; G-quadruplex-binding agents [20]; and the application of programmable nucleases [21,22]. It is the lattermost of these approaches that we discuss in detail (Figure 2B).

Experimental Origins of Direct Heteroplasmy Manipulation

The first reported heteroplasmy shifting approaches made use of peptide nucleic acid (PNA) oligomers [16,23]. PNAs are synthetic polymers comprising canonical DNA bases linked in series through a repeating *N*-(2-aminoethyl)-glycine backbone by peptide bonds. The absence of negatively charged phosphates in PNA, compared with DNA, was seen as advantageous with regard to mitochondrial import competence, as the electrochemical potential across the inner mitochondrial membrane (IMM) sits at ~−180 mV, effectively precluding the import of DNA and RNA molecules. Once within mitochondria, the binding of a PNA molecule to a specific mutant mtDNA sequence, but not wild type, was predicted to preferentially block the replication of mutant mtDNA, thereby shifting mtDNA heteroplasmy in a replication-dependent fashion. Initial work reported encouraging results, with specific stalling of DNA replication through PNA binding to mutant mtDNA templates *in vitro* [16]. However, later efforts that conjugated PNA oligomers to mitochondrial targeting sequences (MTS) or positively charged triphenylphosphonium moieties that facilitate the mitochondrial uptake of small molecules [23–25] failed to demonstrate mtDNA heteroplasmy shifts in cultured cells. More recently, MTS-mediated delivery of PNA 4-mers to the mitochondrial matrix of living cells has been achieved [26]. However, PNAs of at least 7-mer length would be required for a unique site in mtDNA to be targeted, and whether single-stranded mtDNA at the replication fork is readily bound by PNA remains to be determined. A similar approach has also emerged attempting to achieve mtDNA mutation-specific antireplicative effects using imported RNA molecules [27]. However, in addition to the question of single-stranded mtDNA accessibility at the replication fork, the import of RNA into mammalian mitochondria is contentious, with strong evidence against the existence of any efficiently operating pathway [7].

The earliest experiments in the field of nuclease-mediated heteroplasmy shifting used mitochondrially targeted restriction endonucleases (mitoREs), highly specific bacterial endonucleases that target and cleave specific foreign sequences in the 'innate immunity' restriction-modification system of bacteria [28]. This approach was first explored using a mitochondrially targeted form of the restriction endonuclease *Pst*I, which was capable of depleting **mtDNA copy number** when expressed in mouse cells containing mtDNA with a *Pst*I recognition site [28]. Heteroplasmy shifting activity was also demonstrated when a hybrid cell line containing both rat mtDNA (bearing two *Pst*I sites) and an alternative mouse mtDNA (without any *Pst*I sites) was transfected with the same mito*Pst*I construct, resulting in a specific shift towards the mouse haplotype [28]. In later work, a mitochondrially targeted form of *Sma*I was used in patient-derived **cybrid** cells, exploiting a unique *Sma*I recognition site introduced by the pathogenic mtDNA variant m.8993T>G, associated with neuropathy, ataxia, retinitis pigmentosa (NARP) syndrome. This resulted in complete elimination of the m.8993T>G allele within 48 h, accompanied by improvement in measures of mitochondrial physiology [29,30].

Besides an unidentified form of copy number control, which both maintains stable cellular mtDNA copy number over time and ensures the recovery of copy number following nucleolytic cleavage of mutant mtDNAs [31], the precise mechanism underlying heteroplasmy shifts in these experiments was unclear. Initially, a lack of DNA repair in mitochondria was the speculated mechanism underpinning heteroplasmic shifts after the introduction of DNA **double-strand breaks (DSBs)** [31].

However, it has since been determined that, while not only lacking DSB repair, mammalian mitochondria possess a highly efficient mechanism for degradation of linear DNA molecules involving the exonucleolytic activity of the replicative mtDNA polymerase Pol γ, exonuclease MGME1 and the DNA helicase Twinkle [32–34], underpinning the heteroplasmy-shifting capacity of DSBs in mtDNA. Nevertheless, while the mechanism underlying heteroplasmy shifts remained unclear, these early studies offered proof of principle for nuclease-mediated heteroplasmy shifting approaches, tempered by the reality that m.8993T>G is one of very few pathogenic mutations known to introduce a unique mitoRE site into mtDNA. Limited clinical application notwithstanding, mitoRE approaches developed further [35–37], culminating in the demonstration of large heteroplasmic shifts of a nonpathogenic mtDNA variant in the NZB/BALBc mouse model by means of the mitoRE *ApaL*I [36] and later across multiple mouse tissues when delivered by **adeno-associated virus (AAV)** [38,39].

The use of mitoREs in heteroplasmy manipulation was the first significant step in nuclease-mediated mammalian mitochondrial genome engineering. However, mitoRE approaches are profoundly limited by the availability of REs for any given site produced by mtDNA mutations, and the refractory nature of REs to the engineering of alternate DNA binding specificity rendered this avenue unsuitable for further development.

Heteroplasmy Shifting in the Era of Programmable Mitochondrial Nucleases

With longstanding efforts in nuclear genome manipulation yielding zinc-finger protein (ZFP) and transcription activator-like effector (TALE) technologies [40], early forms of both mitochondrially targeted **zinc-finger nucleases** (mtZFNs) and mitochondrially targeted **TALE nucleases** (mitoTALENs) were soon to follow [21,22,41]. The principle of both technologies lies in sequence specific DNA-binding domains, delivered to mitochondria using MTS peptides, coupled to the functionally dimeric, sequence nonspecific endonuclease domain from the RE *Fok*I (Figure 2B). This approach creates a DSB only when both monomers are bound to the target DNA, limiting off-target activity as the nucleolytic reagent is assembled and active only at the target site [40]. For both mtZFNs and mitoTALENs, sequence specificity is conveyed via protein–DNA interactions through tandemly linked repetitive modules, varying at key nucleic acid-contacting residues that are derived from either eukaryotic (ZFP) or bacterial (TALE) DNA-binding proteins.

Two studies describing the use of **clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)** in mitochondria have been published in recent years [42,43]. However, the major hurdle to this approach – RNA import of guide RNA-containing molecules into mitochondria, an essential component of this programmable nuclease system – is not adequately addressed by the authors and these reports are replete with unconvincing data [7]. Further, Cas9 endonucleases are subject to target site limitations in terms of the length of site that may be efficiently targeted (typically ~15–22 bp) and the proscribed protospacer-adjacent motif (PAM). PAM sites are 2–6 bp sequences that are essential for the introduction of a DSB into DNA, varying between the Cas9 species used. The limitations in target site preference presented by even the most permissive PAM-bearing Cas9 variants would render a mitochondrial CRISPR/Cas9 approach unable to target the majority of pathogenic mtDNA mutations. Therefore, even if an efficient mitochondrial CRISPR/Cas9 platform were to be created, it would be of limited use in the context of therapeutic heteroplasmy shifting (see Table 1 for a summary).

Development and *In Vitro* Use of mtZFN Technology

Zinc-finger nucleic acid-binding motifs are among the most abundant protein folds in eukaryotic biology. The capacity for DNA sequence-specific binding of Cys_2His_2 ZFPs is mediated by repeating ~25-amino-acid (aa) ZFP modules, or fingers, that each bind a 3-bp sequence. This binding is coordinated 5′–3′ in a C-terminal to N-terminal orientation through hydrogen bonding in the major groove,

Table 1. Comparison of Engineered Mitochondrial Nucleases

Feature	mtZFN	mitoTALEN	CRISPR/Cas9
Motif	ββα	RVD	Cas9/gRNA
Interaction	Protein–DNA	Protein–DNA	RNA–DNA
Binding	1 ZFP:3 (+1) bp	1 TALE:1 bp	1 gRNA:18–22 bp
Specificity	1 bp	1 bp	Unknown
Off target			
mtDNA	At high expression	At high expression	Unknown
Nuclear	Not detected	Unknown	Unknown
AAV compatible	Yes	Two capsids needed	Yes
Pros	Small, highly specific, several in-body clinical trials	Easy to design	Easy to design
Cons	Difficult to design	Large, limited target sites	Mitochondrial RNA import, limited target sites

with additional cross-strand contacts to the final nucleotide of the preceding triplet and nonspecific phosphate interactions [44] (Figure 2B,C). These zinc-finger domains, originally characterised in the murine transcription factor zif268, comprise antiparallel β-strands and a zinc ion-stabilised α-helix formed through the invariant cysteine and histidine residues for which the fold is named, arranged in a ββα configuration. DNA binding is conveyed by the α-helical residues 1, 2, 3, and 6 of the ZFP [44] (Figure 2C), which when arranged in tandem using short flexible linkers, form an engineered, sequence-specific DNA-binding ZFP. In the case of engineered ZFPs, typically four to six fingers (12–18 bp target site) are used per monomer, providing a total binding site of 24–36 bp.

The rational construction of ZFPs from preselected libraries is challenging, as DNA binding of consecutive fingers is highly DNA/ZFP context dependent. As such, substantial libraries of candidate ZFPs and linkers were created using phage display [44], which have been further iterated to provide targeting depth that allows the generation of ZFPs to virtually any DNA sequence [45].

The potential of zinc-finger-based approaches to mtDNA manipulation was first seen in the demonstration of site-specific methylase activity by a three-finger, monomeric, mitochondrially targeted ZFP specific to m.8993T>G, fused to DNA methyltransferase 3a [46]. The use of a nuclear export signal (NES) was essential for mitochondrial localisation of ZFPs in these experiments, as cryptic internal nuclear localisation signals form an intrinsic part of the ZFP motif. This work was further built on with a rudimentary mtZFN design for heteroplasmy shifting, where two *Fok*I domains separated by a long, flexible linker are attached to a single ZFP monomer specific to m.8993T>G [41]. This early mtZFN design was capable of shifting heteroplasmy in cybrid cells but was limited to a single ZFP binding site (four fingers, 12 bp) and was a constitutively active nuclease, leading to undesired mtDNA copy number depletions that limited further use.

Early attempts at generating a dimeric mtZFN failed to produce efficacious reagents [41,47] until an optimised architecture was designed capable of shifting m.8993T>G and 'common deletion' heteroplasmy in cybrid cells, demonstrating recovery of mitochondrial function in the latter [21]. While incorporating rational protein design alterations, these dimeric constructs also exploited an engineered form of *Fok*I bearing mutations in the protein–protein dimerisation interface required for DSB induction [48,49]. These paired interface mutations in (+) and (–) versions of the endogenous *Fok*I C-terminal domain result in electrostatic repulsion between (+)/(+) and (–)/(–) domains, demonstrating significantly diminished formation of homodimers and commensurate improvement of heteroplasmy shifting coupled to decreased mtDNA copy number depletion.

Later studies have detailed the variation in heteroplasmy shifting efficiency seen with these chimeric enzymes based on the rate of catalysis and associated off-target activity, resulting in near-complete elimination of mutant mtDNA with a single, transient treatment of mtZFNs when expression levels are fine-tuned [50]. This approach was subsequently used as an experimental tool to generate isogenic cell lines with variable mtDNA heteroplasmy, allowing assessment of metabolic rewiring in the presence of mtDNA mutation-derived metabolic dysfunction [51].

Development and *In Vitro* Use of mitoTALENs

The TALE DNA-binding domains used in mitoTALENs are derived from *Xanthomonas* bacteria, which deliver TALEs into plant cells via type III secretion mechanisms to activate host promoters that enable infection and propagation [52]. The DNA sequence-specific binding of mitoTALENs is mediated by 33–35-aa modules, formed of two α-helices linked by a loop, that each bind a 1-bp site in the major groove through two hypervariable or **repeat variable diresidues (RVDs)** contained in the loop region, with additional nonspecific phosphate interactions mediated by the helices [52] (Figure 2B,D). The structural basis of TALE DNA-binding specificity, in stark comparison with ZFPs, is simple and mostly reproducible, with a minimal library of RVD modules encoding all base specificities [40]. However, this simplicity is coupled to limitations in DNA target site preference, in terms of sequence composition, length, and a strong preference for T in position 0 at the 5′ end of the binding site [40,52] (Figure 2D).

The development of mitoTALENs was first reported with data describing the manipulation of 'common deletion' and m.14459G>A point mutation heteroplasmy in cybrid cells, with recovery of mitochondrial function observed in the latter [22]. Bacman and colleagues exploited the requirement for T at position 0 in the TALE binding site, which is produced in the complementary H-strand by the m.14459G>A mutation, resulting in a highly specific reagent to this site; an approach that is termed the T_0 strategy. However, two major challenges faced the further development of mitoTALENs: first, achieving single-nucleotide binding specificity at mutation sites that are not N>A or N>T and cannot be targeted using a T_0 strategy, and second, creating specific mitoTALENs with unconventionally short target sites that will be compatible with downstream gene therapy approaches such as AAV, which cannot easily deliver the length of DNA-encoding sequence required for full-length mitoTALENs as described in the original reports. Although mitoTALEN activity at target sites that do not permit the T_0 strategy appears to be variable [22,53], some success was had in producing truncated TALE domains to allow AAV compatibility, at the cost of diminished binding specificity [54].

mitoTALENs, or close variants thereof, have also been applied to: (i) manipulate the heteroplasmy of induced pluripotent stem cells (iPSCs) bearing the m.3243A>G and m.13513G>A mutations [55,56]; (ii) induce single-strand breaks in mtDNA [57]; and (iii) shift the heteroplasmy of oocytes and oocyte–cytoplast fusions [53]. A further variant of TALE-based mitochondrial nuclease, where TALE DNA-binding domains are fused to the linker and catalytic domain of the monomeric homing endonuclease I-*Tev*I from T4 phage, known as mitoTev-TALE, has also been reported [58]. While this architecture appears capable of modest heteroplasmy shifting activity, inherent specificities associated with the *Tev*I nuclease domain and linker, in addition to the difficulties associated with use of a constitutively active nuclease, are likely to limit further applications.

Manipulating mtDNA Heteroplasmy *In Vivo*

The transition from cell cultures to autochthonous animal models of disease biology is a challenging process for any gene therapy. Translation of potential gene therapies for heteroplasmic mitochondrial disease are particularly vexed in this regard, as state-of-the-art methods for manipulating mtDNA do not currently allow directed generation of disease models to test emerging approaches. Presently, the only available model of pathogenic heteroplasmic mtDNA mutation

is the m.5024C>T mouse, generated through laborious random mutagenesis and extensive breeding/screening. This animal bears a heteroplasmic mutation in the acceptor stem of mitochondrial tRNA alanine (mt-tRNA^{Ala}) and recapitulates molecular features of mitochondrial disease, such as tRNA instability, diminished mitochondrial translation, and metabolic indications of heart failure, at high levels of heteroplasmy [59]. As such, the m.5024C>T model has been essential to proof-of-principle experiments for the *in vivo* manipulation of pathogenic mtDNA heteroplasmy by mtZFNs and mitoTALENs.

While the target organ and delivery site are major determinants of the medium used to convey therapeutic genetic cargo across gene therapies, the current gold-standard modality for somatic gene therapy is AAV (Box 1).

Several serotypes of AAV demonstrate specificity for skeletal and heart muscle (e.g., AAV6, AAV9, and engineered serotypes thereof) and despite limited DNA-packaging capacity, have remained the vector of choice for *in vivo* experiments using mitochondrially targeted nucleases. The first application of mtZFNs *in vivo* used the engineered AAV9-derived serotype AAV9.45, a liver-detargeted form of the wild-type AAV9 that exhibits approximately tenfold-lower transduction in the liver, a major off-target of the wild-type AAV [60]. By *in vitro* screening for heteroplasmy shifting activity in m.5024C>T mouse embryonic fibroblasts (MEFs), a pair of mtZFNs specific to a 28 bp binding site, including the m.5024C>T mutation, were selected. While the mtZFN-encoding sequences are, theoretically, of proportions amenable to packaging within a single AAV capsid (1.2–1.4 kb per monomer), initial proof-of-concept experiments were performed using co-injected AAVs, with the two mtZFN monomers encoded in two separate AAV genomes. Using this approach, efficient viral transduction of the heart was achieved, and large shifts of mtDNA heteroplasmy (from ~70% to ~35% m.5024C>T) were detected 10 weeks after intravenous administration of AAVs in adult animals. These shifts were accompanied by commensurately increased steady-state levels of mt-tRNA^{Ala} and, at optimised doses, improved mitochondrial metabolism in heart tissue with no detectable depletions of mtDNA copy number. Importantly, irrespective of dose employed, no evidence of off-target effects in the nuclear genome were observed [61].

Proof of concept for the use of mitoTALENs *in vivo* utilised wild-type AAV9, also employing the co-administration of two AAV genomes, each encoding one mitoTALEN monomer. Following screening of mitoTALEN candidate pairs in MEFs, a pair of mitoTALENs specific to a 21-bp binding site, including the m.5024C>T mutation, exploiting the previously mentioned T_0 strategy were selected. Focal injection of AAVs encoding these mitoTALENs into the tibialis anterior muscle led to variable shifts in heteroplasmy that were stable over time and linked to an increased mt-tRNA^{Ala} steady-state level in animals demonstrating large shifts from a high-mutant-load baseline at 24 weeks post-injection [62]. Following systemic administration of AAV in neonates, modest shifts in

Box 1. Adeno-Associated Viruses.

Naturally occurring AAVs of the *Dependoparvovirus* genus comprise a protein-only, unenveloped capsid formed of three structural proteins and do not encode a full complement of replicative or expression-associated viral genes required for a complete lytic cycle within their compact (~4.2 kb), single-stranded DNA genome [65]. Once within a given human cell, the AAV genome begins the lysogenic cycle, integrating into the AAVS1 locus on chromosome 21 through an incompletely understood mechanism mediated by inverted terminal repeats (ITRs). The replication and release of AAV capsids from the cell, completing the lysogenic/lytic cycle, is dependent on polymerases, helicases, and other transcription-effecting genes expressed by either adenovirus or herpesvirus. AAV infection is widespread among mammals and is not known to cause disease or significant immunogenicity, with numerous serotypes of AAV demonstrating significant tropism for individual tissues. Given their capacity to infect both mitotic and post-mitotic cells, to be maintained episomally, and the broad range of highly tissue-specific serotypes available, AAVs have outstripped previous gene therapy vehicles, such as adenoviruses or retroviruses, in all aspects but DNA packaging capacity.

heteroplasmy (≤15% change) were also detected in heart and quadriceps muscles. Shifts of heteroplasmy by mitoTALENs were not associated with depletions of mtDNA copy number or the generation of deleted mtDNAs, as had previously been reported in connection with mitoREs; however, the effects of mitoTALENs on nuclear DNA were not assessed.

Clinical Outlook

At present, pre-clinical data for mtZFN and mitoTALEN approaches appear encouraging. Both platforms permit re-engineering to target multiple mtDNA mutations *in vitro* [21,22,53–56] and can be successfully translated to induce broadly similar shifts of mutation heteroplasmy across entire organs and muscle groups with rescue of molecular disease phenotypes *in vivo* [60,61]. However, the following challenges still face both approaches in the transition to clinical trials.

Immunogenicity

While the immunogenicity of AAV capsids is a broadly discussed issue of relevance to most gene therapies [63], the immunogenicity of the encoded transgene is often overlooked. In the case of engineered nucleases, an obvious source of potential immune activation is the *Fok*I catalytic domain derived from bacteria. It is likely that, through the turnover of mitochondria by autophagy, peptides from the *Fok*I domains of mitochondrial nucleases will be presented to T cells at the plasma membrane. In the case of mitoTALENs, the same issue is of relevance with regard to the TALE DNA-binding domain itself, which is of bacterial origin. Although it is currently unclear whether peptides from the *Fok*I or TALE domains will mediate an immune response, this is an open question that remains to be addressed.

Off-Target Effects in Nuclear DNA and mtDNA

Assessing the safety of gene therapy is of paramount importance, and unexpected off-target effects in the genome are an ongoing concern. However, given the mitochondrial localisation of mtZFNs and mitoTALENs, these concerns are somewhat muted compared with nuclear DNA gene editing. This is because any rare off-target events in mtDNA, due to poor reagent specificity or nuclease overdose, do not result in the introduction of mutations into mtDNA but in the degradation of the genome containing the erroneous DNA DSB [32–34]. Thus, in a worst-case scenario, partial mtDNA copy number depletions could be observed. It is possible to attempt to circumvent this issue by predicting the optimal nuclease dosage and heteroplasmy shift efficiencies using mathematical modelling [64]. However, any pathological effect of such partial depletions remains unclear, and in the context of newly engineered *Fok*I domains, with up to 3000-fold-lower off-target activity [65], the issues of nuclease dosage seen with earlier obligatory heterodimeric iterations of this domain used in mitochondria [50] seem unlikely to present a problem.

Owing to the mitochondrial localisation of these nucleases, nuclear DNA off-targets are unlikely to present an issue with the clinical translation of these technologies. While this possibility was investigated for mtZFNs, with no off-target activity in nuclear DNA identified [61], the same has not been demonstrated for mitoTALENs [62]. Nonetheless, further robust analysis of both the nuclear and the mitochondrial off-target milieu will be needed to allow the transition of these promising technologies from the laboratory to the clinic.

Reliable Generation of Specific Reagents

Both mtZFNs and mitoTALENs have shown activity at multiple mtDNA targets; however, the generation of effective reagents to a given target site is not guaranteed. ZFP engineering entails significant design redundancy, with recent developments allowing targeting depths of >100 pairs for a given 25-bp sequence [45] and near-complete modification of target loci with undetectable off-target activity in nuclear DNA [65]. By contrast, the engineering of TALE:DNA binding

Clinician's Corner

No curative therapies for mitochondrial disorders are available and treatment options are very limited. Typically, symptoms arise when mutated mtDNA molecules exceed a ~60% threshold, where the remaining healthy fraction can no longer compensate for the loss of function. Direct correction of mtDNA mutations (>300 known) *in situ* is not possible due to technical and, as is likely, biological reasons. The development of engineered nucleases targeted to mitochondria allows cleavage of the mutant mtDNA in a mixed or heteroplasmic, multicopy population. Subsequently, owing to robust mtDNA copy number control, the remaining normal copies of mtDNA replicate and repopulate treated cells. This 'shifting' results in a diminished proportion of mutated mtDNA molecules, potentially to subsymptomatic levels, which allows recovery of mitochondrial function.

The design of these nucleases is generally not limited by the sequence targeted. However, to date, these approaches have been exclusively limited to DNA-interacting protein modules such as mtZFNs and mitoTALENs. Owing to issues with targeting guide RNA to mitochondria and limited targetable sites in mtDNA, CRISPR/Cas9 is unlikely to present a viable route to heteroplasmy shifting.

Combining tissue-specific AAVs with these nucleases has been proven to be effective in preclinical mouse models of a mitochondrial tRNA mutation that presents with cardiomyopathy. These first reports of *in vivo* mitochondrial gene therapy demonstrate effective alteration of the mutation load in affected tissues of adult animals, a major conceptual advance towards gene therapy for heteroplasmic mitochondrial disease.

specificity is of far more limited scope, with significant preference for particular lengths of target site and DNA sequence composition [52]. Although this may sometimes be advantageous (e.g., T_0 strategy), it also represents a significant limitation, and it is likely that the collection of effective mitoTALENs will be less extensive than that of mtZFNs.

Efficient AAV Packaging and Dosage

Developing the capability for the delivery of either mtZFNs or mitoTALENs by AAV at safe levels, compatible with current recombinant AAV manufacturing capacity and clinical guidelines, remains a major challenge [66]. The tactics employed in preclinical experiments thus far are unlikely to be of broader use, as the delivery of significant amounts of two separate AAVs into mice was required to detect heteroplasmy shifting activity by either programmable nuclease platform [61,62]. If scaled to human dosage, these quantities of AAV would be impractically high and potentially unsafe. Thus, methods that allow a reduction in the administered titres of mitochondrial nuclease-encoding AAV will be key to clinical application. Improved AAV capsid specificity, temporary immunosuppression regimens, focal injection/delivery into the organ of interest, and/or packaging of mtZFN or mitoTALEN monomers in a single AAV capsid are potential ways in which this issue could be addressed. The clinical development of programmable mitochondrial nucleases will be likely to hinge on advances in these areas.

Concluding Remarks

Numerous approaches to shifting mtDNA heteroplasmy have been developed in parallel over the past few decades. During this time, nuclease-mediated heteroplasmy shifting in particular has matured from humble beginnings to robust technical platforms capable of efficiently shifting various mtDNA heteroplasmies at will, both *in vitro* and *in vivo* [53,61,62] (see Table 2).

Outstanding Questions

What are the long-term effects of mitochondrially targeted nuclease treatments?

Is there potential to engineer DNA protein binding motifs with reduced catalytic rate while maintaining high specificity?

Are some tissues, indications, or modes of delivery better suited to treatment with mitochondrially targeted nucleases?

Are incomplete or heterogeneous shifts of heteroplasmy across organs sufficient to rescue clinical phenotypes?

Table 2. mtDNA Sequences and Models Successfully Targeted with Engineered Mitochondrial Nucleases[a]

Genotype	Gene	Phenotype	Model/tissue	Method	Refs
Human					
m.3243A>G	*MT-TL1*	MELAS	iPSC	mitoTALEN	[55,56]
m.8344A>G	*MT-TK*	MERRF	Cybrid	mitoTev – TALE	[58]
m.8483_13459del4977	Multiple	CPEO, KSS, Pearson	Cybrid	mtZFN, mitoTALEN	[21,22]
m.8993T>G	*MT-ATP6*	NARP, Leigh	Cybrid	mtZFN	[21,50]
m.9176T>C	*MT-ATP6*	NARP	Murine oocyte/ cybrid fusion	mitoTALEN	[53]
m.13513G>A	*MT-ND5*	MELAS, Leigh	iPSC	mitoTALEN	[54,56]
m.14459G>A	*MT-ND6*	LHON	Cybrid	mitoTALEN	[22]
Mouse					
m.5024C>T	*MT-TA*	CM	Heart, skeletal muscles	mtZFN/mitoTALEN	[61,62]
BALB/NZB	–	–	Skeletal muscles, brain, embryo, oocyte (mitoTALEN)	mitoRE/mitoTALEN	[36,53]

[a]Abbreviations: CM, cardiomyopathy; CPEO, chronic external ophthalmoplegia; Leigh, Leigh syndrome; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy lactic acidosis with stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; NARP, neuropathy, ataxia, retinitis pigementosa; Pearson, Pearson marrow–pancreas syndrome.

While the clinical potential of mitochondrially targeted programmable nucleases is clear, the means of transition to clinical use requires further work. Although the application of these nucleases for somatic manipulation of heteroplasmy in patients with mitochondrial disease appears likely, they could similarly be used to eliminate carryover of mutant mtDNA, a potential complication of mitochondrial donation/mitochondrial replacement therapy [5]. Beyond the realms of mitochondrial disease, the mtDNA heteroplasmy of human cancers is also likely to represent a meaningful therapeutic target [2]. However, as potential first-in-class gene therapy approaches, these methods currently face an uncertain regulatory environment in addition to the requirement for necessarily robust preclinical evaluation and assessment.

Obvious challenges and strategic considerations notwithstanding (see Outstanding Questions), the creation of powerful genetic tools, such as mtZFNs and mitoTALENs, has fired the starting gun in the race for heteroplasmic mitochondrial disease gene therapy. The era of mitochondrial genome medicine, while certainly still on the horizon, has never been closer.

Disclaimer Statement

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