Shifting m.3243A>G heteroplasmy with PBGENE-PMM: Gene editing therapy for primary mitochondrial myopathy

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INTRODUCTION

- Mitochondria contain a multi-copy circular genome that encodes 37 genes critical for oxidative phosphorylation.
- Mitochondrial DNA (mtDNA) mutations are commonly heteroplasmic, where both wild-type (WT) and mutant genomes co-exist in the same cell. Clinical symptoms manifest once the percentage of mutant mtDNA exceeds a particular threshold.
- A point mutation in mt-tRNA$^{Leu(UUR)}$ (m.3243A>G) is one of the most common heritable pathogenic mtDNA mutations and impairs the enzymatic activity of the mitochondrial respiratory chain. This mutation is associated with primary mitochondrial myopathy (PMM), among other diseases.
- Mitochondria lack an efficient double-strand break repair mechanism and instead rapidly degrade any linearized mtDNA molecules. There is a tightly controlled mechanism for maintaining mtDNA copy number that results in the replication of any genomes remaining following mtDNA degradation.
- ARCUS is derived from I-*Cre*I, a homing endonuclease that recognizes and cleaves a 22 base pair DNA target sequence in a species of green algae.
- Amino acid modifications to I-*Cre*I allow for novel target sequence recognition.
- ARCUS nucleases are small, monomeric proteins that do not require a guide RNA to edit DNA and can be engineered to optimize activity and specificity.
- ARCUS can be targeted to mitochondria to selectively eliminate mutant mtDNA.

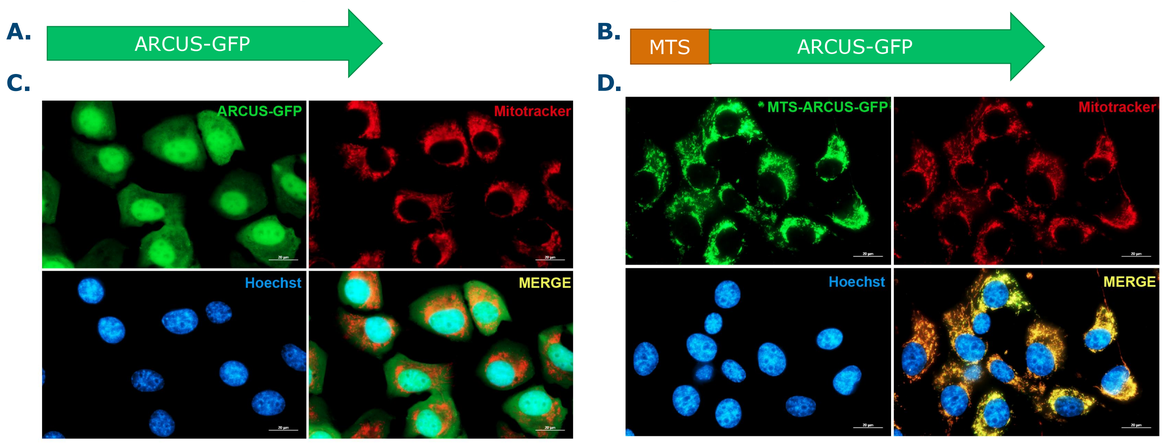
PBGENE-PMM

- PBGENE-PMM comprises an ARCUS nuclease specific for m.3243G mutant mtDNA, a mitochondrial targeting sequence (MTS), and a nuclear export signal (NES).
- Our objective is to deliver PBGENE-PMM to the skeletal muscle of patients afflicted with m.3243-associated PMM by utilizing a single dose of an AAV administered systemically.
- Upon delivery to target tissues, PBGENE-PMM is designed to specifically eliminate mutant m.3243G mtDNA, leaving WT mtDNA to repopulate the cell. This shift in heteroplasmy should result in an improvement in mitochondrial function and alleviation of clinical symptoms.

Delivery	Tissue-Specific Expression	Subcellular Localization	Specificity	Activity
Single AAV administration drives gradual nuclease expression	Muscle-specific promoter limits nuclease expression to target tissues	MTS traffics nuclease to mitochondria, NES excludes enzyme from nucleus	PBGENE-PMM specifically cuts mutant m.3243G mtDNA	Cells treated with PBGENE-PMM contained reduced levels of mutant mtDNA and improved mitochondrial function

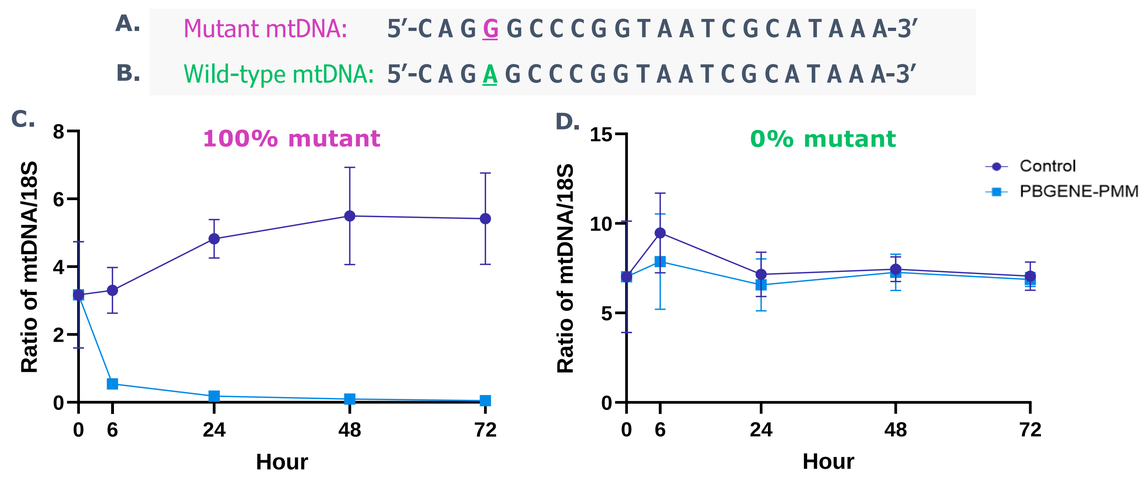
RESULTS

FIGURE 1. ARCUS is efficiently trafficked to the mitochondria using an N-terminal MTS



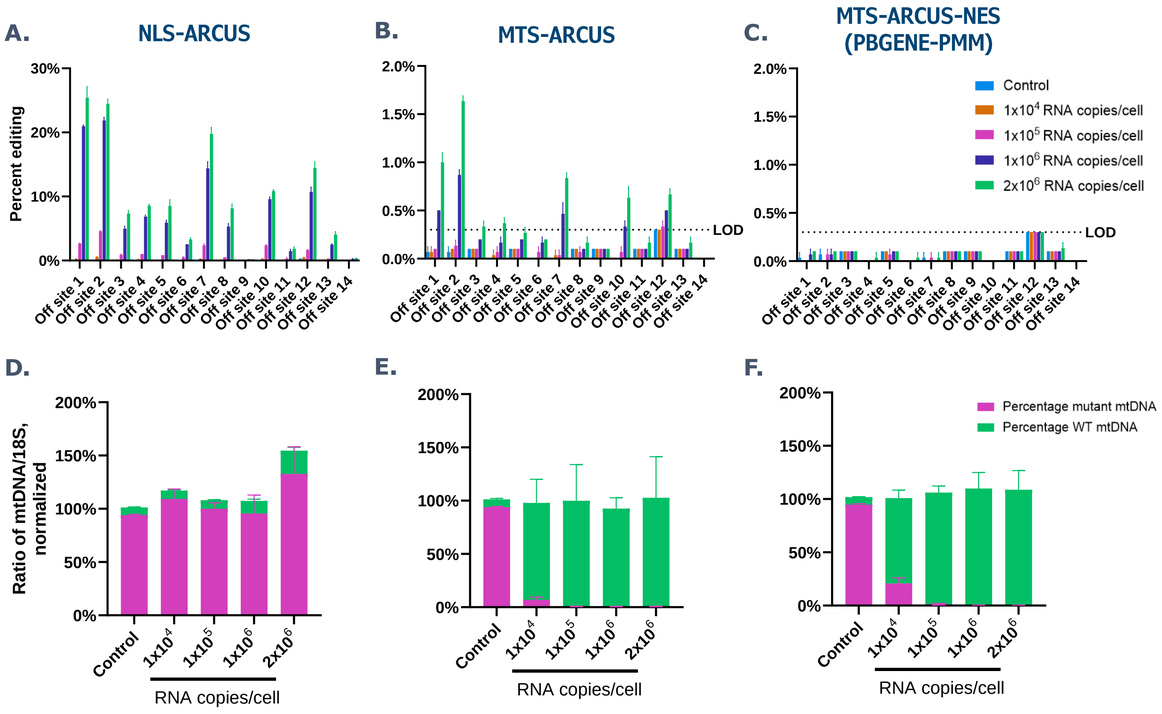
- Two ARCUS constructs were transfected into cells: ARCUS lacking a subcellular targeting sequence (**1A**) or ARCUS with an MTS fused at the N-terminus (**1B**). The cells were stained at 24 hours for Hoechst 33342 (blue) and Mitotracker (red). ARCUS appears in green.
- Without a targeting sequence, ARCUS exhibits diffuse, cytoplasmic/nuclear localization (**1C**). With an MTS, ARCUS co-localizes with Mitotracker showing efficient mitochondrial delivery (**1D**).

FIGURE 2. PBGENE-PMM efficiently eliminates mutant m.3243G mtDNA without significant cleavage of WT mtDNA



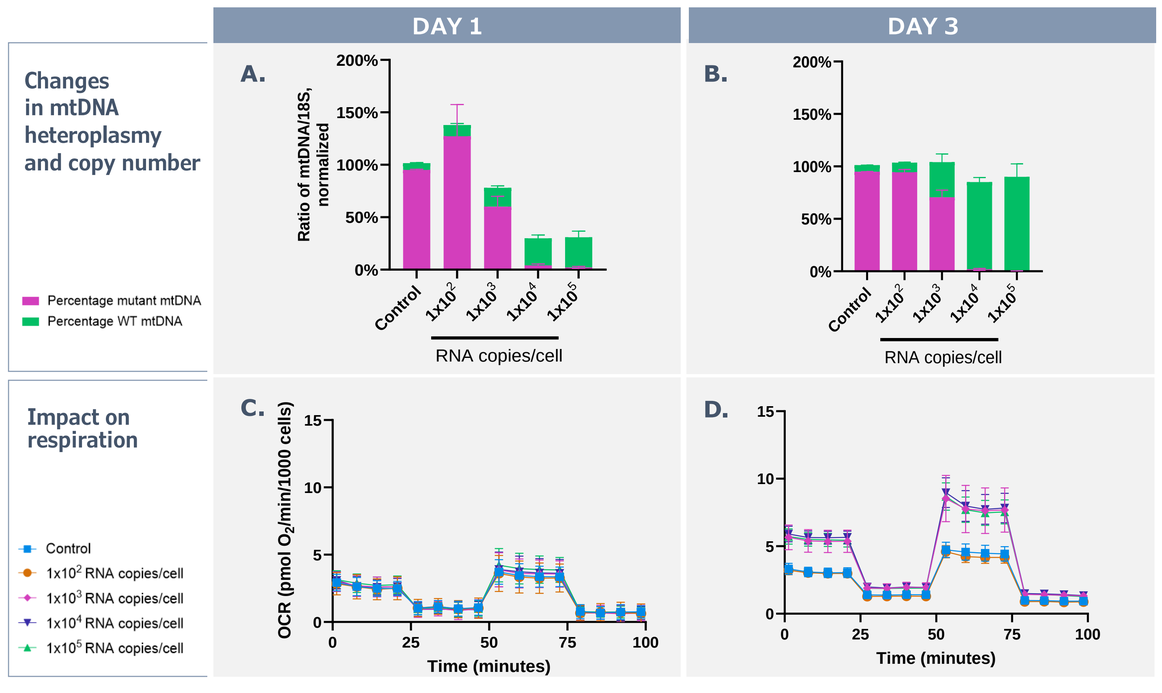
- Nuclease specificity is paramount, as the 22bp target DNA sequence (**2A**) and corresponding WT sequence (**2B**) only differ by a single nucleotide.
- Two homoplasmic cell lines were used to evaluate PBGENE-PMM for on-target (100% mutant) and off-target (0% mutant) activity. Activity was measured in terms of mtDNA depletion at a dose of 1.5x10^5 RNA copies/cell.
- PBGENE-PMM exhibited robust elimination of mutant m.3243G mtDNA (**2C**) without significant cleavage of WT m.3243A mtDNA (**2D**).

FIGURE 3. Incorporation of an MTS and NES to ARCUS eliminates nuclear off-target editing



- In addition to specificity in the context of the mtDNA, we characterized the nuclease for its potential to cleave unintended sequences within the nuclear DNA. 14 potential nuclear off-target sites were identified in an unbiased, genome-wide cell-based assay (data not shown).
- 96% mutant m.3243G cells were transfected with different versions of m.3243G-targeting ARCUS – either containing a nuclear localization signal (NLS), MTS, or MTS and NES (PBGENE-PMM). gDNA was isolated and analyzed for editing at each of the identified off-target sites (**3A**, **3B**, **3C**) as well as heteroplasmy and mtDNA copy number changes (**3D**, **3E**, **3F**) at day 6 post-transfection.
- Nuclear off-target editing was observed with NLS-ARCUS (**3A**), reduced with MTS-ARCUS (**3B**), and was undetectable with PBGENE-PMM (**3C**). The on-target mtDNA heteroplasmy shift was not impacted by the addition to the NES (**3F**).

FIGURE 4. PBGENE-PMM shifts heteroplasmy and improves mitochondrial function in 96% mutant m.3243G cybrid cells without deleterious impacts to respiration during transient mtDNA depletion



- 96% mutant m.3243G cells were transfected with nuclease mRNA at 10-fold dilutions. At days 1 and 3 post-transfection, gDNA was isolated and analyzed for heteroplasmy and mtDNA copy number (**4A**, **4B**) and the live cells were evaluated for respiratory changes using the Seahorse Cell Mito Stress Test (**4C**, **4D**).
- At day 1, a dose-dependent mtDNA depletion was observed that corresponded to the selective cleavage of mutant mtDNA (**4A**). Despite the mtDNA depletion, respiration was unaffected (**4C**).
- At day 3, mtDNA copy number was restored to control levels. The cells treated with the three highest mRNA doses exhibited a significant decrease in mutant mtDNA and a significant increase in WT mtDNA (**4B**). The dose-dependent shifts in heteroplasmy resulted in concomitant improvements in basal and maximal respiration (**4D**). Notably, small shifts in heteroplasmy (66.5% mutant ± 3.4%, achieved with a dose of 1x10^3 RNA copies/cell) conferred a similar functional benefit to a complete shift.

Source: Shoop *et al.* Efficient elimination of MELAS-associated m.3243G mutant mitochondrial DNA by an engineered mitoARCUS nuclease. *Nat Metab* **5**, 2169-2183 (2023).

CONCLUSIONS

1. PBGENE-PMM is successfully trafficked to mitochondria where it can specifically eliminate m.3243G mutant mtDNA, resulting in a shift in heteroplasmy and improvement in mitochondrial function.
2. Nuclear off-target editing is prevented through the incorporation of an NES.
3. Together, these data support the development of PBGENE-PMM as an *in vivo* gene editing therapeutic for the treatment of m.3243-associated PMM.

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