# GENE CORRECTION TO TREAT MITOCHONDRIAL ASSOCIATED DISEASES

## BACKGROUND

Mitochondria are unique organelles in the cell that play a critical role in energy production, among other functions. They are responsible for creating a vast majority of the energy needed by the body to sustain life and organ function. Thus, when they fail, as in the case of mitochondrial diseases, a cell is not capable of generating sufficient energy and cell death follows. This leads to dysfunction in organs with the highest energy demands, such as the heart, brain and muscles. Despite decades of efforts, optimal treatments have not yet been established.

Mitochondrial proteins are encoded by both nuclear (nDNA) and mitochondrial (mtDNA) genomes. Mitochondria have their own translation system to synthesize mtDNA-encoded proteins essential for mtDNA replication, transcription, translation, and assembly of the oxidative phosphorylation system complexes. Mutations in either the nDNA or mtDNA as well as defects in the translation machinery can cause protein abnormalities that result in mitochondrial disease. Recent studies have identified homozygous mutations in the *gfm1* gene, which encodes for the mitochondrial translation factor EFG1, in mitochondrial disease patients. EFG1 catalyzes the translocation of peptidyl tRNA from the ribosomal acceptor aminoacyl site to the peptidyl site following peptide bond formation, with the concomitant removal of the deacylated tRNA, advancement of the mRNA by one codon and exposure of the next codon. Thus, mutations in EFG1 may cause a deficiency in mitochondrial translation and function, thereby resulting in mitochondrial disease. Recent advances in genome editing technologies provide the possibility to target and correct the underlying genetic mutation in monogenic diseases, such as this one. These technologies do have limitations in that they have semi-random integration of the vectors, incomplete control over transgene copy number and expression level, a risk of insertional mutagenesis, as well as low efficiency. Recently, we have developed a gene editing strategy termed Homology-Independent Targeted Insertion (HITI) that is based on the CRISPR/Cas9 system, which harnesses elements of the NHEJ pathway to achieve efficient targeted knock-in in both proliferating and non-dividing cells. Our HITI method can specifically target the genetic locus associated with the disease with minimal insertion/deletion frequency. In addition, our HITI technology can be applied to gene correction in post-mitotic cells *in vivo*.

## SPECIFIC AIMS

We aim to develop new strategies for the treatment of mitochondrial related diseases using HITI technology for efficient gene targeting and mutation correction of the *gfm1* gene.

## EXPERIMENTAL APPROACH

### 1. Establishment of a HITI system to correct a mutation in *gfm1* gene

1-a. Construction of genome targeting HITI vectors: the HITI method allows for high efficient DNA knock-in in to a targeted locus in both dividing and non-dividing cells, even *in vivo*. For HITI, the donor plasmids lack homology arms. Thus, the Cas9-induced-DSB repair cannot occur thorough the HDR pathway. The donor DNA is instead associated with a Cas9 cleavage site, which is cleaved together with the genomic target sequence. The DSBs associated with the linearized donor DNA plasmid thus represent substrates for repair by NHEJ.

If the genomic DSB is resealed by error-free NHEJ, it will be cut again by Cas9. This cut-repair cycle will continue until NHEJ-mediated insertion of the Cas9-linearized donor DNA (in the correct orientation) destroys the Cas9 target sequence. To restore EFG1, we will construct the donor DNA vector to include the wild type *gfm1* cDNA sequence from Exon 16 to 18 and then induce knock-in of the donor DNA into intron 15 upstream of the mutation site (FIG.1).

1-b. Evaluation of gene targeting efficiency in primary cells: first, to evaluate gene targeting efficiency *in vitro*, we will introduce the donor vector with the expression vector for Cas9 and sgRNA into either primary mouse brain neurons or liver cells. Next, we will test gene targeting efficiency and then investigate whether the correction restores wild type EFG1 expression, mitochondrial translation, as well as mitochondrial function *in vitro*.

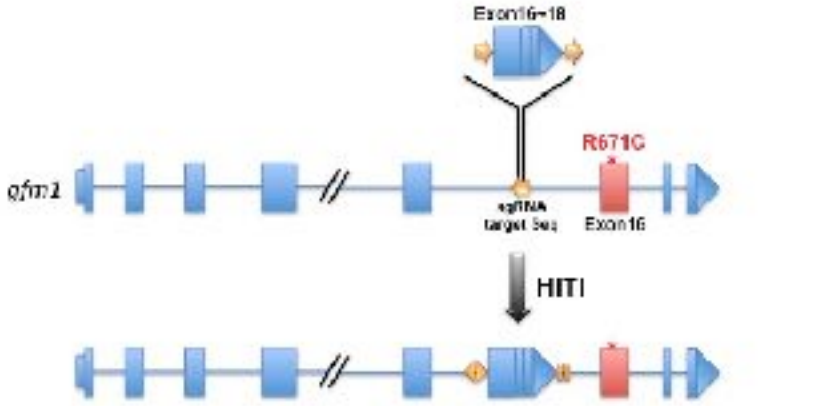



**FIGURE 1. Schematic representation of gfm1 correction through HITI**
Yellow arrows represent sgRNA target sequence. Blue boxes, exons of *gfm1*. Red box, exon 16 including mutation.

**2. Gene correction in human iPSCs derived from patients**

2-a. Gene targeting in human iPSCs: we will introduce HITI vectors in to human iPSCs derived from gmf1 mutations mitochondrial disease patient and isolate clones where the knock-in correctly occurred.

2-b. Investigation of whether the correction restores function of differentiated cells: first, we will induce differentiation of patient and corrected iPSCs into either mature neurons or hepatocyte-like cells. Next, we will investigate whether the gene correction can restore EFG1 expression, mitochondrial translation, as well as mitochondrial function in the differentiated cells. In addition, we will test if the function of the differentiated cell can be rescued. For neurons, we will analyze cell stability, differentiation rate, and level of activity. For hepatocyte-like cells, we will test lactate levels, creatine kinase and alanine aminotransferase activity within cells or in the culture medium.

**3. Gene correction in human organoids with mitochondrial disease**

To develop in vivo gene targeting strategies for the treatment of mitochondrial related diseases, we will investigate whether HITI technology allows for efficient gene targeting in human organoids.

3-a. Mitochondrial disease modeling using an organoid system: we will produce brain and liver organoids using iPSCs derived from mitochondrial disease gfm1 patient. An organoid is a simplified organ produced in vitro in 3D culture conditions that offers the opportunity to model human diseases. Once we establish organoids, we will perform histological analyses of the organoids. Further, we will analyze cell stability, differentiation rate, and level of mitochondrial activity in the organoids.

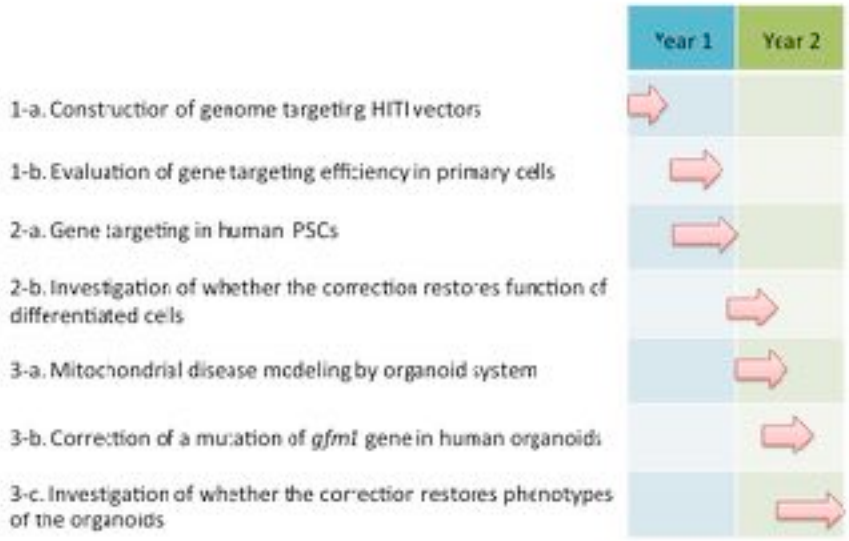
3-b. Correction of a mutation in the *gfm1* gene in human organoids: for gene correction in organoids, we will sub-clone HITI constructs (either Cas9 and sgRNA or donor DNA) into adeno-associated virus (AAV) vectors and then package them into AAV. To knock-in the donor DNA, we will inject the HITI AAVs directly in to the organoids. By using the genomic sequence, we will test gene targeting efficiency, wild type EFG1 expression, mitochondrial translation, as well as mitochondrial function in the organoids.

3-c. Investigation of whether the correction alleviates the phenotypes of the organoids: it has been reported that some patients show hypoplasia of the cerebellar vermis and severe pontine atrophy of the brainstem. In order to investigate whether the correction by HITI restores brain development and function in brain organoids, we will perform histological analysis and electro-physiological analysis after HITI AAVs injection. To test liver organoid function, we will monitor different physiological and biochemical parameters.

**SIGNIFICANCE AND IMPACT**

Our preliminary data show that HITI is effective for genome editing of *in vivo* liver and even non-dividing brain cells. These findings strongly support the hypothesis that HITI can be used to correct mitochondrial diseases This technology will be beneficial to different cell ptypes like stem cell populations, such as NSCs, which depend on their niches for self-renewal. Success of this study will constitute a first proof of concept towards the treatment of mitochondrial-associated diseases in general, and of Gfm1 disease in particular.

**TIME LINE**




**BUDGET**
We request salary support for 1 postdoctoral associate and supplies, $100,000 per year for 2 years.

## EQUIPO LIDERADO POR EL INVESTIGADOR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