**BACKGROUND**

Mitochondria are unique organelles that play a critical role in creating energy within cells. Thus, when they fail, the amount of energy is severely diminished within cells. Mitochondrial disease is a chronic or genetic disorder that is due to cell and organ dysfunction caused by deficiencies in mitochondrial energy production. A variety of symptoms are observed, e.g. muscle weakness, poor growth, learning disability, neuronal problems, liver diseases and heart disease, among others. Despite decades of efforts, optimum treatment has not been established yet. Mitochondria-associated genes encoded in mitochondrial own genome (mtDNA) or nuclear genome are essential on maintenance of functional mitochondria. The mitochondria genes encode essential factors for mtDNA replication, transcription, translation, and assembly of the oxidative phosphorylation system complexes. Mitochondria have their own translation system to synthesize mtDNA-encoded proteins. Therefore, defects of the translation machinery can cause mitochondrial abnormality thereby, resulting in mitochondrial diseases. Recent studies have identified the homozygous mutations in GFM1 gene, encoding 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, mutation of EFG1 may cause deficiency of mitochondrial translation and mitochondrial function, thereby resulting in mitochondrial disease. However, it remains unknown how depletion of EFG1 affect function of specific cells, how it leads to organ-specific dysfunctions, and how it leads to the different phenotypes seen in mitochondrial disease.

**SPECIFIC AIMS**

To investigate the mechanisms by which depletion of EFG1 results in mitochondrial disease, we have established a cellular model of mitochondrial disease using patient-specific iPSCs.

1. Establish patient-specific iPSC lines
2. Correct GFM1 mutation in the patient-specific iPSC
3. Examine the function of neurons derived from the patient-specific iPSCs

**RESULTS AND EXPERIMENTAL APPROACH**

1. **Establish patient-specific iPSC lines**

To better understand the mechanism of mitochondrial disease, we first established iPSC lines derived from a mitochondrial disease patient. We obtained primary fibroblast cells established from healthy control and a patient with mitochondrial disease that have mutations in GFM1. The EBNA-1/OriP-based episomal vectors encoding OCT4, SOX2, KLF4, c-MYC, and p53-shRNA were transfected into the cells using nucleofection. After transfection, the cells were placed with medium for culturing human embryonic stem cell on a matrigel plate for 10 days. They were then placed into MEF culture. iPSC-like colonies were handpicked and passaged on MEF plates. We confirmed pluripotent gene expression and loss of the episomal vectors in these iPSC lines. No obvious differences were observed between healthy control (WT) and mitochondrial disease (mtD) iPSC on cell morphology and growth rate (Figure 1).

2. **Correct GFM1 mutation in the patient-specific iPSC**
An isogenic control cell line can be defined as a cell line that shares an identical genotype with the parental cell line. Isogenic controls are typically established by the targeted correction of a mutation associated with the parental cells. Taking advantage of isogenic controls would allow us to precisely investigate the effect of mutations on the parental cells, a clear advantage over comparing cell lines derived from individuals with different genomic backgrounds. To investigate how depletion of EFG1 affect function of specific cells, we generated an isogenic control using CRISPR-mediated gene correction. The mtD iPSCs has a heterozygous mutation, c. 2011C>T, p.(Arg671Cys), in exon16 of the GFM1 gene. We designed the sgRNA targeting only mutated sequence (Figure 2). We transfected the targeting vector, the expression vectors of sgRNA and Cas9 into mtD iPSCs and then picked colonies up. Through homology directed repair process, we obtained the corrected mtD iPSC clones (Figure 3).

In the next phase of this project we will investigate whether gene correction can restore EFG1 expression, mitochondrial translation, as well as mitochondrial function in the differentiated cells.

3. Examine the function of neurons derived from the patient-specific iPSCs

In addition, we will test if the mitochondrial functions and differentiated cell functions can be rescued. For neurons, we will analyze cell stability, differentiation rate, and mitochondrial oxidative phosphorylation system (OXPHOS). For hepatocyte-like cells generated in vitro, we will test lactate levels, creatine kinase and alanineaminotransferase activity within cells or in culture medium.

**FUTURE PLAN**

Recent advances in genome editing technologies provide hope about how to target and correct the underlying genetic mutation in monogenic diseases. However, those technologies imply semi-random integration of the vectors, with an incomplete control over transgene copy number, expression level, as well as risk of insertional mutagenesis, as well as low efficiency. Recently, we have developed a strategy termed Homology-Independent Targeted Insertion (HITI) based in the CRISPR/Cas9 system, which harnesses elements of NHEJ pathway to achieve efficient targeted knock-in in both proliferating and non-dividing cells. The HITI method can target specifically the genetic locus associated to the disease with minimal insertion/deletion frequency. In addition, HITI technology can be applied to gene correction in post-mitotic cells in vivo. To develop new strategies for the treatment of mitochondrial related diseases we will apply HITI technology better for efficient in vivo gene targeting and mutation correction of the gfm1 gene in human iPS model, organoid-derived iPSC model, and mouse model.

**SIGNIFICANCE AND IMPACT**

Our preliminary data have showed 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 in vivo. The feasibility to use HITI technology in vivo will be beneficial to stem cell populations, such as NSCs, that depend on their niches for self-renewal. Success of this study will enable the use of HITI to treat different mitochondrial-associated diseases.