IN VIVO GENE CORRECTION TO TREAT MITOCHONDRIAL ASSOCIATED DISEASES

BACKGROUND

Mitochondria are unique organelles that play a critical role on creating energy within cells. Thus, when they fail, 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 mitochondria 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 until October 2019

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).

Figure 1

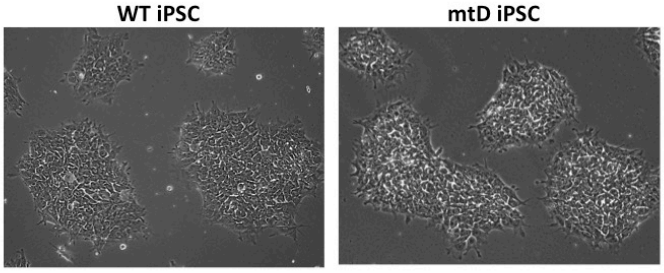
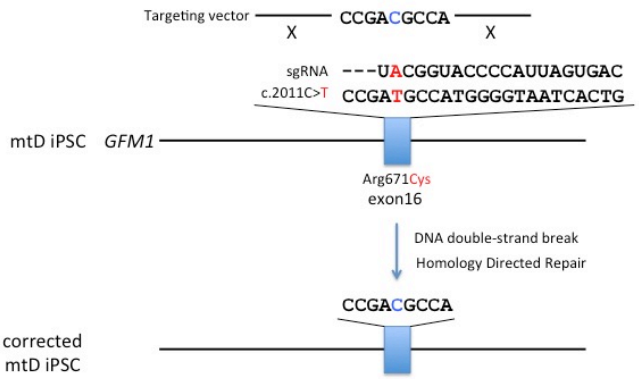


Figure 2

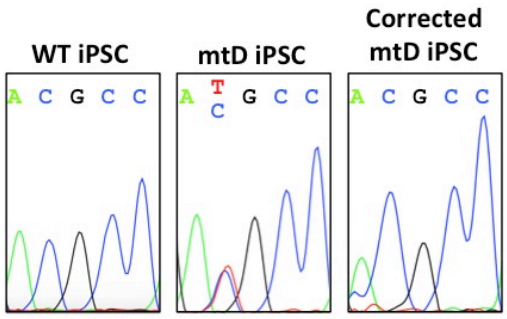


2. Correct GFM1 mutation in the patient-specific iPSC

An isogenic control cell line can be defined as a cell line that shares an identical genotype with the parental cell line. Isogeneic 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).

Figure 3



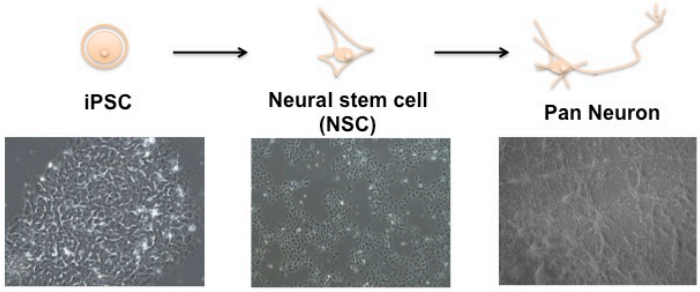
RESULTS since November 2019

As described above, we have successfully 1) established patient-specific iPSC lines and 2) corrected mutations on *GFM1* gene in the patient-specific iPSCs.

Next, to investigate whether gene correction can restore the protein EFG1 expression in the corrected iPSCs, we performed Western Blotting analysis with anti-mtEFG1 antibody. We found that the level of EFG1, which is depleted in mtD iPSCs, was recovered in the corrected mtDiPSCs. In addition, mitochondrial DNA analysis using quantitative PCR revealed that the diminished copy number of mitochondria in patient-specific iPSCs was also recovered to WT level in the corrected mtDiPSCs.

Further, we investigated whether the gene correction can restore mitochondrial functions. Based on reports of symptoms or phenotypes observed in patients with mutations on *GFM1* gene, dysfunction of EFG1 causes neurological disorder. Therefore, we have decided to examine mitochondrial function in either neurons or hepatic cells derived from the iPSC lines. First, we established methods to induce differentiation of iPSCs to neurons. To induce neural induction, iPSCs were cultured with N2B27, CHIR99021, SB431542, and Dorsomorphine. Through differentiation protocol, we have successfully generated matured neurons from the iPSC lines (Figure 4).

Figure 4



Next, we examined mitochondrial function in the neurons derived from WT, mtD, and corrected mtD iPSCs using Seahorse Cell Mito Stress Tests, including basal respiration, ATP-linked respiration, and maximal and reserve capacities. As a result, we found that mitochondrial function is decreased in mtD neurons, while its abnormality was rescued in corrected neurons. Thus, the gene correction restores number of mitochondria and its function.

After this functional analysis, unfortunately, our experiments had been completely suspended for two months because of Salk Institute closure due to prevent the spread of COVID-19. After the closure period, Salk was reopened but access is still restricted. We are currently in Phase I of reopening activities. Although it's kind of tough situation for research, we are doing all that we can on this research.

FUTURE PLAN

We will determine if the rescue of mitochondrial function restore neuronal functions. For neurons, we will analyze cell stability, differentiation rate, and neural activity. In addition to neuronal disorder, abnormality of hepatic cells is observed in patients with mutations of *GFM1.* Therefore, we will induce differentiation of iPSCs to hepatic cells and then examine lactate levels, creatine kinase and alanine aminotransferase activity within cells or in culture medium.