# 学 位 論 文 の 要 旨

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| 学 位 論 文 名 | MELAS-Derived Neurons Functionally Improve by Mitochondrial Transfer From Highly Purified Mesenchymal Stem Cells (REC) |
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## 論 文 内 容 の 要 旨

### INTRODUCTION

Mitochondrial diseases (MD) are a group of multisystem disorders that occur when mutations in mitochondria-associated nuclear or mitochondrial DNA (mtDNA) lead to defective oxidative phosphorylation and impaired energy metabolism. The adenine-to-guanine transition (m.3243A>G) at nucleotide 3243 of the mtDNA in the *MT-TL1* gene coding for *tRNAleu (UUR)* is one of the most common pathogenic mtDNA mutations that can cause mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS).

MELAS is the most common progressive form of MD and is accompanied by epilepsy, psychopathology, cortical sensory defects, and myopathy. In the general population, the majority of asymptomatic carriers have a rate as high as 1:400. Symptom onset, expression, and clinical severity typically increase with the copy percentage of the m.3243A>G mutation (heteroplasmy). There is no specific treatment for patients with MELAS. Therefore, a possible strategy for the treatment or palliation of MELAS is imperative.

The peculiarities of mitochondrial genetics (heteroplasmy phenomena, spontaneous mutations over time) have led to difficulties in modeling mtDNA-associated MD diseases in vivo

and in vitro. However, human induced pluripotent stem cells (iPSCs) of specific origin overcome these limitations. Numerous studies have shown that disease-specific iPSCs can be differentiated into disease-associated functional cells. This specific disease cellular model is helpful for understanding the genotypic and phenotypic characteristics of the disease. Thus, disease-specific iPSC-derived neurons have significant potential for modeling progressive MDs. Furthermore, Mesenchymal stem cell (MSC)-based regenerative medicine represents a promising therapeutic strategy. our previously reported RECs (high-purity MSCs) showed superior homogeneity and mitochondrial quality. Therefore, this may be a better source of exogenous mitochondria. As an exogenous mitochondrial donor, RECs have a significant restorative effect on bioenergetics and mitochondrial function in mitochondria-deficient cells. However, it is unclear whether RECs donate mitochondria to MELAS neurons and whether these mitochondria are functional or not.

Therefore, this study aimed to clarify the possible mitochondrial transfer pathway between RECs and neurons and to explore the effect of exogenous mitochondria on mitochondrial function in MELAS neurons, providing a possible strategy for the treatment of MELAS.

## MATERIALS AND METHODS

MELAS neurons differentiated from MELAS patient-derived induced pluripotent stem cells (iPSCs) and RECs/MSCs were cultured under contact or noncontact conditions. To reveal the role of exogenous mitochondria, we examined relevant mitochondrial functions, including mitochondrial membrane potential (MMP), reactive oxygen species (ROS), intracellular calcium content, cellular bioenergetics, and oxygen consumption rate (OCR). Possible mitochondrial transfer pathways, tunneling nanotubes (TNTs), connexin 43 (Cx43)-gap junctions (GJs) and extracellular vesicles (EVs) were also explored using the corresponding inhibition assay.

Data were analyzed, and statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Results with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ were considered significant. The study protocol was approved by the Research Ethics Committee of Shimane University (20131116-1).

## RESULTS AND DISCUSSION

1. Establishing the MELAS neuron model

Human mtDNA encodes many key proteins involved in the assembly and activity of the mitochondrial respiratory complex and is closely associated with MD. The percentage of mutated copies of mtDNA (heteroplasmy) plays a role in the development of symptoms, as well as the severity of the disease, with high heteroplasmy (>60%) usually accompanying disease pathogenicity and phenotypic manifestations. Due to the uniqueness and complexity of mtDNA, the establishment of a stable cellular model of MD is fundamental to this study. Our study

showed that MELAS neurons carrying a high level of heteroplasmy (m.3243A>G mutation) faithfully replicated the features of respiratory complex deficiency, including high levels of ROS, OXPHOS defects, respiratory dysfunction, and decreased cell proliferation. In addition, the dependence on anaerobic glycolysis and energy deficiency exhibited by MELAS neurons provides clues for understanding the pathological mechanisms associ-ated with abnormal energy metabolism in the brain.

2. Mitochondrial transfer pathways

In this study, we focused on three pathways of mitochondrial transfer from RECs to neurons compared to that by regular MSCs (TNTs, Cx43-GJs and EVs). Both MSCs and RECs were capable of donating mitochondria to neuron. However, compared to MSCs, we found that the content of TNTs generated by RECs was significantly lower, consistent with previous reports. Therefore, TNTs may not be the primary method by which RECs donate mitochondria. Our study found high expression of Cx43 at cell junctions, suggesting the possible formation of GJC plaques. However, even if both mitochondrial transfer pathways, TNTs and GJCs, are blocked, donor cell mitochondria still exist in MELAS neurons. Recently, abundant evidence has shown that mitochondria-derived vesicles (MDVs) or intact mitochondrial translocation is mainly mediated by multivesicular bodies (MVBs). We used dynasore to inhibit the formation and release of MVBs and MDVs, thereby inhibiting mitochondrial transfer and reception. The mitochondrial transfer rate of RECs was significantly reduced compared to that of MSCs, showing the strongest inhibition efficiency among multiple inhibitions. This revealed the importance of EVs in the mitochondrial transfer mechanism of RECs.

3. Advantages of high-purity MSCs (REC)

Because MSCs can donate mitochondria to recipient cells, they offer potential possibilities for the treatment of MD. However, there are limitations to the treatment with MSCs. MSCs isolated from human BM using traditional methods proliferate and differentiate inconsistently, the cell populations obtained are highly heterogeneous, and autologous BM-derived MSCs are expensive. In addition, the collection of adult BM-derived MSCs is highly invasive and carries a high risk of infection. In contrast, RECs have low batch-to-batch variability, uniform cell size, high proliferation rate and mitochondrial content, and no ethical issues. Our results also showed that REC is advantageous in restoring mitochondrial functions, such as elevated MMP, ATP, OCR, and the regulation of ROS homeostasis. These findings highlight the advantages of functional mitochondria (RECs) in restoring neuronal mitochondrial function.

## CONCLUSION

These data suggest that REC-donated mitochondria (highly efficient, homogenized) may offer a potential therapeutic strategy for treating neurological dysfunction in MELAS.