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Direct effects of mitochondrial dysfunction on poor bone health in Leigh syndrome

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ABSTRACT

Mitochondrial diseases are the result of aberrant mitochondrial function caused by mutations in either nuclear or mitochondrial DNA. Poor bone health has recently been suggested as a symptom of mitochondrial diseases; however, a direct link between decreased mitochondrial function and poor bone health in mitochondrial disease has not been demonstrated. In this study, stem cells from human exfoliated deciduous teeth (SHED) were isolated from a child with Leigh syndrome (LS), a mitochondrial disease, and the effects of decreased mitochondrial function on poor bone health were analyzed. Compared with control SHED, LS SHED displayed decreased osteoblastic differentiation and calcium mineralization. The intracellular and mitochondrial calcium levels were lower in LS SHED than in control SHED. Furthermore, the mitochondrial activity of LS SHED was decreased compared with control SHED both with and without osteoblastic differentiation. Our results indicate that decreased osteoblast differentiation potential and osteoblast function contribute to poor bone health in mitochondrial diseases.

1. Introduction

Mitochondria are organelles that produce ATP by oxidative phosphorylation. Almost all mitochondrial proteins are encoded by nuclear DNA, and are imported into the mitochondria after synthesis in the cytosol [1]. However, mitochondria are unique among organelles in that they also contain their own genome, with 13 respiratory chain subunits encoded by mitochondrial DNA (mtDNA) and synthesized in the mitochondria in humans [2]. Mitochondrial diseases such as Leigh syndrome (LS); mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS); and myoclonic epilepsy with ragged red fibers (MERRF) are caused by reduced mitochondrial function caused by mutations in mitochondrial proteins encoded by either nuclear DNA or mtDNA [3–5].

The main symptoms of mitochondrial diseases manifest in the brain, skeletal muscle, and liver, which are highly dependent on their mitochondria for energy production [3–5]. In addition, manifestations of poor bone health, such as osteoporosis and osteopenia, have been suggested to be symptoms of mitochondrial diseases [5,6]. Mice with mutated mtDNA polymerase are used a mouse model of mitochondrial disease, and display symptoms of osteoporosis [7]. “Mito-mice”, another mouse model of mitochondrial disease caused by large deletions in the mtDNA genome, have decreased cortical bone thickness [8]. A recent epidemiological study also suggested that mitochondrial diseases pose a risk to bone health, with bone fractures and reduced bone density reported in 73% of patients with mitochondrial diseases [6]. It has been speculated that poor bone health associated with mitochondrial diseases is caused by secondary factors, such as endocrine dysfunction and loss of motor function due to encephalomyopathy, which is also a symptom of mitochondrial diseases [5,6]. However, whether reduced mitochondrial function has direct effects on bone health remains unknown.

LS is a progressive neurodegenerative disease that causes symmetrical necrotizing lesions in the basal ganglia and brainstem, and manifests as psychomotor retardation, muscle weakness and

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hypotonia, growth impairment, and myopathy [3,9]. The pathogenesis of LS involves reduced mitochondrial function caused by mutations in either the mtDNA or in both the nuclear DNA and mtDNA [3]. In this study, using stem cells from human exfoliated deciduous teeth (SHED), a mesenchymal stem cell [10], isolated from a patient with LS, we have analyzed the effects of reduced mitochondrial function associated with mitochondrial disease on bone formation ability, and demonstrate that optimal mitochondrial function is required for both osteoblastic differentiation and osteoblast function in these cells.

2. Materials and methods

2.1. Isolation and culture of SHED

Experiments using human samples were reviewed and approved by the Kyushu University Institutional Review Board for Human Genome/Gene Research (permission number: 678-00) and were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from the patient's guardians. Deciduous teeth were collected from a healthy control and a patient with LS at 4 and 6 years of age, respectively. SHED were isolated from dental pulp tissues as previously described [10], and grown in a culture medium consisting of Minimum Essential Medium Eagle Alpha Modification (Sigma-Aldrich, MO, USA) with 15% fetal bovine serum (Sigma-Aldrich), 100 μM L-ascorbic 2-phosphate (Wako Pure Chemical Industries, Osaka, Japan), 2 mM L-glutamine (Life Technologies, NY, USA), 100 U/mL penicillin (Life Technologies), 100 μg/mL streptomycin (Life Technologies) and 25 μg/mL Fungizone (Life Technologies) at 37 °C in 5% CO_2. SHED were used at passage 3–10. To evaluate their mesenchymal stem cell characteristics, SHED were immunostained with anti-STRO1 antibody (MAB1038; R&D systems, MN, USA), counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan), and observed by fluorescent microscopy as previous described [10].

2.2. Measurement of bone mineral density

The bone mineral density of the patient with LS was measured by dual energy x-ray absorptiometry (DEXA) scanning of the lumbar spine from L2 to L4 using a Discovery A (Hologic, MA, USA) scanner.

2.3. Osteogenic differentiation of SHED

$4.5 \times 10^4/cm^2$ cells were cultured in 6-well plates to confluence. To differentiate SHED into osteoblasts, the cells were cultured in differentiation medium, which was the culture medium described above supplemented with 1.8 mM potassium dihydrogen phosphate (Nakarai tesque, Kyoto, Japan) and 10 nM dexamethasone (Sigma-Aldrich). The medium was changed twice in a week.

2.4. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA extraction and RT-qPCR were performed as previously described [10]. The sequences of the primer sets used in this study were as follows: alkaline phosphatase (ALP), 5′-ACGTGGCTAAGAATGTCATC-3' (ALP forward) and 5′-CTGGTAGGCGATGTCCTTA-3' (ALP reverse); and 18S rRNA, 5′-CGGCTACCACATCCAAGGAA-3' (18S rRNA forward) and 5′-GCTGGAATTACCGCGGCT-3' (18S rRNA reverse). The relative expression levels of the target genes were analyzed using the comparative threshold cycle method by normalizing to 18S rRNA.

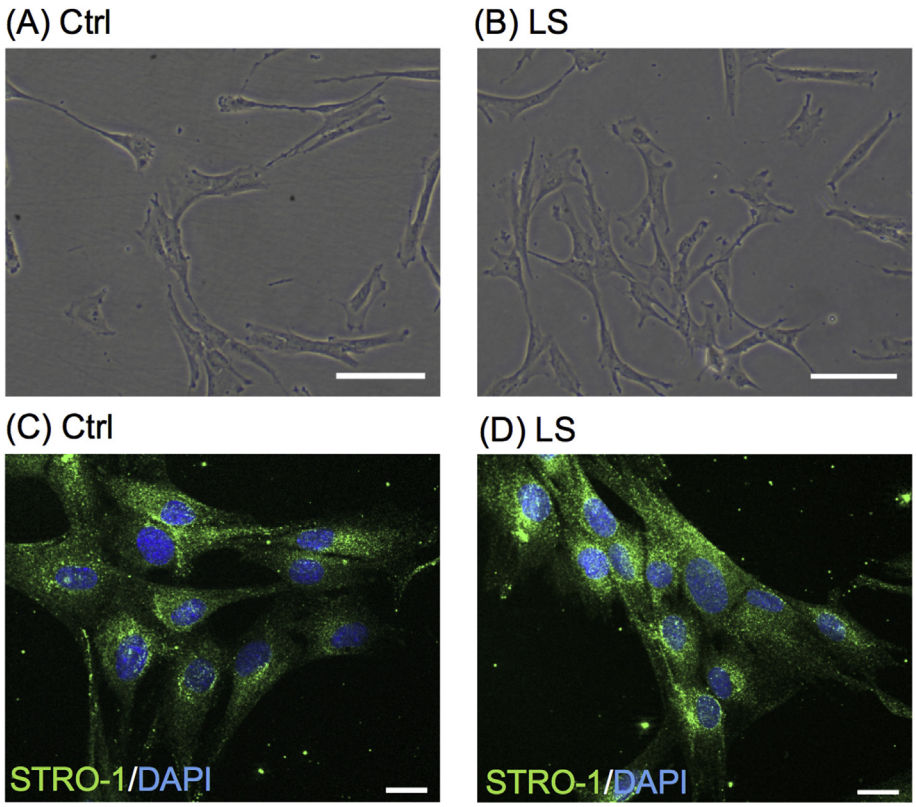

Fig. 1. Isolation and characterization of SHED from a patient with LS.
(A, B) Isolated SHED were observed with a phase-contrast microscope. Scale bar = 100 μm. (C, D) SHED were stained with an anti-STRO1 antibody and DAPI. Scale bar = 20 μm.

2.5. Alizarin Red-S staining

SHED were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min at room temperature, and washed three times with phosphate-buffered saline. The cells were then rinsed with dH_2O and stained with 1% Alizarin Red-S (pH 4.2; Sigma-Aldrich) for 30 s. Cells were imaged using an ES-2200 scanner (EPSON, Nagano, Japan). To quantify Alizarin Red-S staining, 10% (v/w) cetylpyridinium chloride (Nakarai tesque) in 10 mM sodium phosphate buffer (pH 7.0) was used to extract Alizarin Red-S, the absorbance of which was measured at 570 nm using an Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland). To measure the Alizarin Red-S stained area, three images were randomly taken from three experiments using an IX41 microscope (Olympus, Tokyo, Japan) equipped with a digital camera NEX-5T (Sony, Tokyo, Japan) with an NY-1S adaptor (MeCan Imaging, Saitama, Japan), and were analyzed using MetaMorph software version 7.8.2.0 (Molecular Devices, CA, USA).

2.6. Intracellular and mitochondrial calcium measurements

To measure intracellular calcium, the cells were cultured in 96-well plates and stained with 2 μM Fluo-4 AM (Life Technologies), 0.05% (w/v) Pluronic F-127 (Sigma-Aldrich), and 500 μM probenecid (Sigma-Aldrich) in Hanks' Balanced Salt Solution (HBSS) for 1 h at 37 °C. The fluorescence signals were measured by excitation at 485 nm and emission at 525 nm using an Infinite 200 PRO plate reader (Tecan). To measure mitochondrial calcium, the cells were cultured in 96-well plates and stained with 10 μM Rhod-2 AM (Life Technologies) and 0.05% (w/v) Pluronic F-127 in HBSS for 45 min at 37 °C. The fluorescence signals were measured by excitation at 552 nm and emission at 581 nm using an Infinite 200 PRO plate reader (Tecan).

To observe intracellular and mitochondrial calcium fluorescence signals, the cells were cultured in μ-dishes (ibidi, Munich, Germany) and stained with Fluo-4 AM and Rhod-2 AM. Fluorescent images were captured using a Nikon C2 confocal microscope (Nikon, Tokyo, Japan). The images were analyzed using NIS-Elements software version 4.00.06 (Nikon).

2.7. Measurement of mitochondrial membrane potential

Mitochondrial membrane potentials were measured by staining with JC-1 (Life Technologies) as previously described [10]. Populations of 10,000 cells were analyzed for each sample, and green and red JC-1 signals were detected using the FL1 and FL2 channels, respectively, of a FACSCalibur (BD Bioscience, CA, USA) flow cytometer. The geometric means of FL1 and FL2 were measured using CellQuest software (BD Bioscience), and the FL2/FL1 ratio was calculated.

2.8. Analysis of ATP levels

To measure ATP levels, CellTiter-Glo Luminescent Cell Viability Assay (Promega, WI, USA) was used with undifferentiated SHED, and ATP Assay Kit (Colorimetric/Fluorometric; abcam, Cambridge, UK) was used with osteogenically differentiated SHED.

2.9. Statistical analysis

Statistical analyses were performed by Student's t-test using JMP software version 12 (SAS Institute, NC, USA). P values < 0.05 were considered significant.

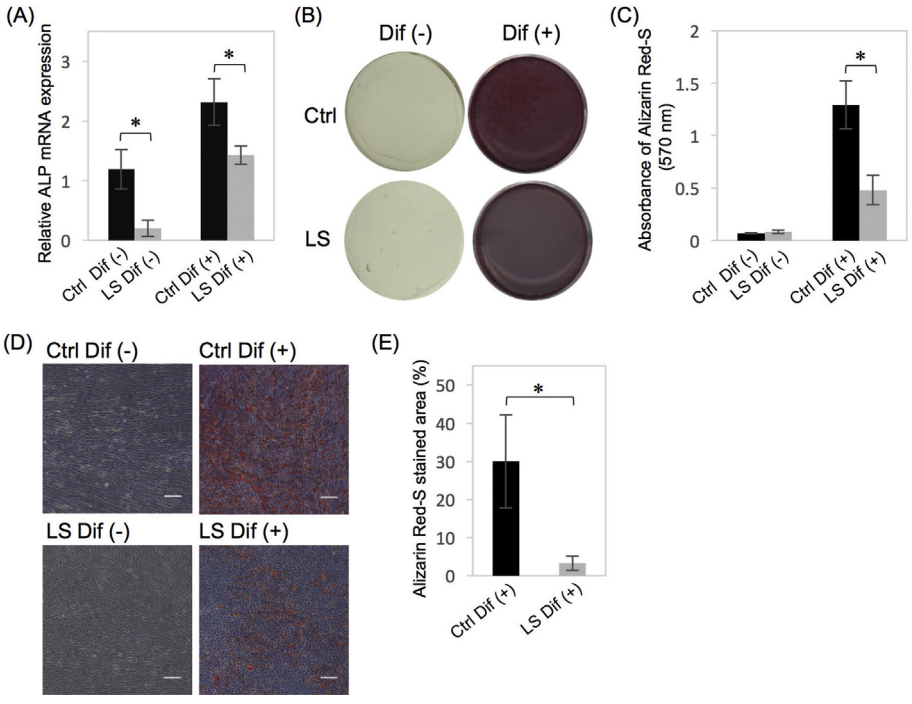

Fig. 2. Reduction of osteoblastic differentiation and calcium mineralization in LS- SHED.
(A) SHED were cultured in culture medium, Dif (−), or differentiation medium, Dif (+), for one week, and the expression of ALP mRNA was measured by RT-qPCR. (B, C) SHED were cultured in Dif (−) or Dif (+) medium for 4 weeks. The cells were stained with Alizarin Red-S (B), which was extracted and measured by absorbance at 570 nm (C). (D, E) Alizarin Red-S stained cells were observed (D). Alizarin Red-S stained areas were measured, and the percentage of Alizarin Red-S stained areas were calculated (E). Data represent the mean ± standard deviation (SD) from three experiments. *$P < 0.05$. Scale bars = 100 μm.

3. Results

3.1. Isolation of SHED from a patient with LS

To examine the impact of mitochondrial dysfunction on osteogenesis, SHED were isolated from a primary tooth of a patient with LS carrying a 13513G > A heteroplasmic mutation in mtDNA, affecting the ND5 subunit of respiratory chain complex I. Notably, the bone density of the patient was 0.381 g/cm^2, 40% lower than that of the same age (0.630 g/cm^2) [11]. SHED prepared from the patient with LS (LS-SHED) had fibroblast-like morphology (Fig. 1A and B) and expressed STRO-1, a mesenchymal stem cell marker, as did SHED isolated from a healthy child (Ctrl-SHED; Fig. 1C and D).

3.2. Reduction of osteoblastic differentiation and calcium mineralization in LS-SHED

The ability of LS-SHED and Ctrl-SHED to differentiate into osteoblasts and mineralize calcium were analyzed. The expression of the osteoblast marker ALP was analyzed, and was significantly lower in LS-SHED than in Ctrl-SHED both with and without osteoblastic differentiation (Fig. 2A). Using Alizarin Red-S staining to analyze calcium mineralization, we observed lower staining intensity in LS-SHED than in Ctrl-SHED (Fig. 2B and C). The area of Alizarin Red-S staining was also significantly smaller with LS-SHED than with Ctrl-SHED (Fig. 2D and E). These results demonstrate that LS-SHED have reduced ability to differentiate into osteoblasts and to mineralize calcium compared with Ctrl-SHED.

3.3. Reduction of intracellular and mitochondrial calcium levels in LS-SHED

Intracellular calcium levels are important for the activation of factors involved in osteoblastic differentiation [12], and osteoblast mitochondrial calcium is important for the calcification of new bone [13]. Compared with Ctrl-SHED, we observed reductions in both intracellular (Fig. 3A and B) and mitochondrial calcium levels (Fig. 3C and D) in LS-SHED, with and without osteoblastic differentiation.

3.4. Decreased mitochondrial activity in LS-SHED

To measure mitochondrial activity, we analyzed the mitochondrial membrane potential (Fig. 4A) and ATP levels (Fig. 4B), and found that both were significantly lower in LS-SHED compared to Ctrl-SHED, with and without osteoblastic differentiation.

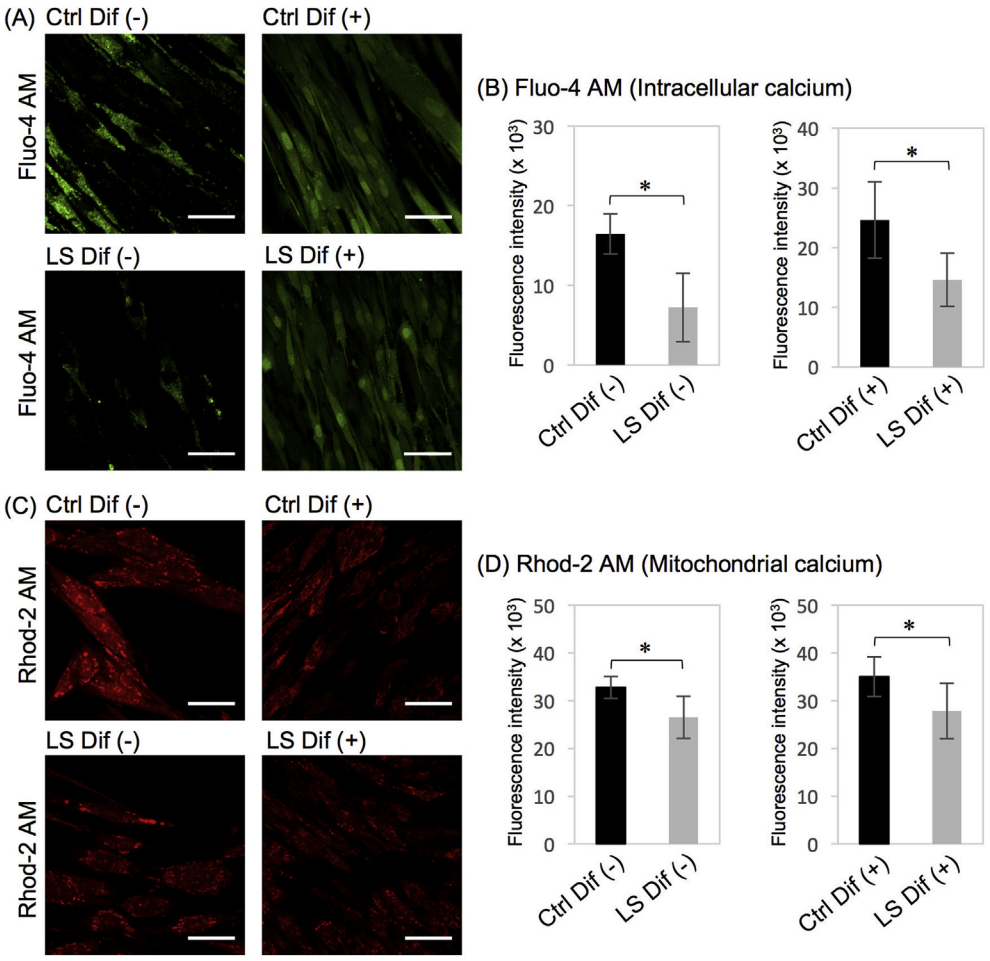

Fig. 3. Reduction of intracellular and mitochondrial calcium levels in LS-SHED.
(A) The cells were stained with Fluo-4 AM and observed with confocal microscopy, and (B) the fluorescence intensity of Fluo-4 AM was measured using a plate reader. (C) The cells were stained with Rhod-2 AM and observed with confocal microscopy, and (D) the fluorescence intensity of Rhod-2 AM was measured using a plate reader. Scale bars = 50 μm. Data represent the mean ± SD from three (B) or four (D) experiments. *$P < 0.05$. Dif (−), SHED cultured for 24 h in culture medium. Dif (+), SHED cultured for one week in differentiation medium.

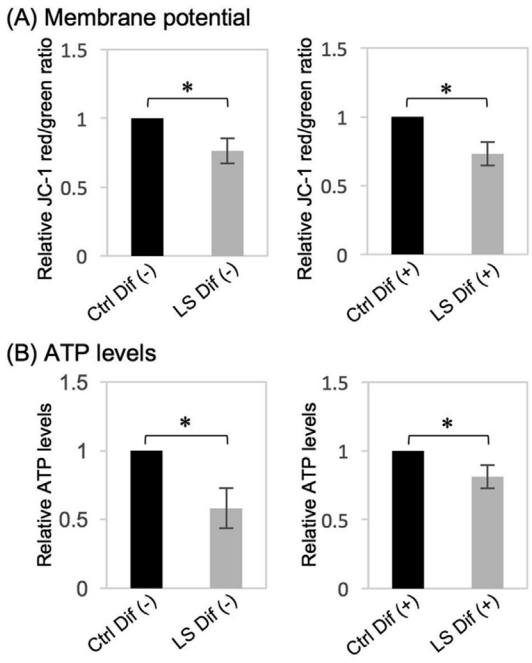

Fig. 4. Reduction of mitochondrial activity in LS-SHED.
(A) The cells were stained with JC-1, red and green fluorescent signals were analyzed by flow cytometry. The relative differences in the ratio of red/green fluorescence were calculated, and the ratio of red/green fluorescence in the Ctrl-SHED was set as 1. (B) Relative differences in the ATP levels of the SHED were calculated, and the Ctrl-SHED level was set to 1. Data represent the mean ± SD from three experiments. $^{*}P < 0.05$. Dif (−), SHED cultured for 24 h in culture medium. Dif (+), SHED cultured for one week in differentiation medium.

4. Discussion

In the present study, we have shown for the first time that reduced mitochondrial activity in LS-SHED is associated with reduced osteoblastic differentiation ability. Mitochondria are important for the differentiation of stem cells, including embryonic, induced pluripotent, and mesenchymal stem cells [14,15]. Chen et al. demonstrated that during the differentiation of mesenchymal stem cells into osteoblasts, mitochondrial activity is enhanced, with increases in the expression of respiratory chain complex enzymes and in ATP production [15]. They also showed that modulation of mitochondrial activity using a respiratory chain complex inhibitor resulted in decreased osteoblastic differentiation [15]. The patient-derived SHED used in this study contained a mtDNA 13513G > A variant that caused a mutation in the ND5 subunit of respiratory chain complex I [16]. This mutation reduced the mitochondrial activity in the LS-SHED, reducing their ability to differentiate into osteoblasts.

We observed that LS-SHED contained lower intracellular calcium levels than Ctrl-SHED, both with and without osteoblastic differentiation. Intracellular uptake of calcium is regulated by ATP-sensitive potassium (K_{ATP}) channels [17], which inhibit the flow of potassium into the cell in an ATP-dependent manner. K_{ATP} channels are comprised of the pore-forming subunits Kir6.1 or Kir6.2, and sulfonylurea receptors. Flow of potassium into cells is inhibited when ATP binds to Kir6x, causing cell membrane depolarization and opening potential-dependent Ca^{2+} channels, resulting in an influx of calcium into the cell. Kir6.2 levels increase during the differentiation of mesenchymal stem cells into osteoblasts, and ATP-dependent regulation of intracellular calcium via K_{ATP} channels is considered important for osteoblastic differentiation [17]. In LS-SHED, the lower levels of intracellular ATP may have resulted from decreased cell membrane depolarization by K_{ATP} channels and reduced intracellular calcium influx.

Intracellular calcium is reported to activate the Wnt/β-catenin signaling pathway, which promotes osteoblastic differentiation [18–20]. Therefore, the lower levels of intracellular calcium in LS-SHED may have resulted in reduced activation of this signaling pathway. In future research, we will elucidate the molecular mechanisms of abnormal osteoblastic differentiation in LS-SHED by analyzing the activation of Wnt/β-catenin signaling, as well as other pathways involved in osteoblastic differentiation.

Mitochondrial calcium levels were lower in LS-SHED than in Ctrl-SHED. Calcium uptake by mitochondria depends on the mitochondrial membrane potential [21], and the lower mitochondrial calcium observed in LS-SHED may have been caused by the decreased mitochondrial membrane potential observed in these cells. Mitochondrial calcium is important for mineralization during bone formation [13]. Osteoblasts secrete matrix proteins (mainly type I collagen) that form the osteoid tissue, which is then calcified to form bone [22]. Mineralization of osteoid tissue is initiated by small vesicles, called matrix vesicles, which are secreted by osteoblasts and contain calcium, phosphoric acid, and hydroxyapatite, among other materials [22]. The calcium inside these vesicles is supplied by the mitochondria [13,23]. In LS-SHED, the lower mitochondrial membrane potential resulted in lower mitochondrial calcium levels, which may have impaired mineralization by reducing the calcium supplied to matrix vesicles. It is therefore possible that LS results in not only decreased osteoblastic differentiation, but also decreased osteoblast function.

Poor bone health has been suggested as a symptom of mitochondrial diseases, and secondary factors such as endocrine dysfunction and loss of motor function due to encephalomyopathy have been cited as possible causes [5,6]. In the present study, we have used SHED to demonstrate that osteoblastic differentiation ability and osteoblast function are both reduced in LS. Although these effects should also be investigated in other mitochondrial diseases, the results of our study suggest that reduced osteoblastic differentiation ability and reduced osteoblast function are directly associated with decreased mitochondrial function in LS.

Conflicts of interest

The authors have no conflicts of interest to declare.

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