

A dysfunction in mitochondrial energy metabolism and/or toxicity of accumulated long-chain fatty acids in the parathyroid glands may have contributed to the pathogenesis of hypoparathyroidism in the patients with MTP deficiency [Saudubray et al., 1999]. In fact, hypoparathyroidism has also been reported in other disorders of mitochondrial fatty acid oxidation, including LCHAD deficiency [Tyni et al., 1997] and MCAD deficiency [Baruteau et al., 2009]. However, this hypothesis cannot explain why hypoparathyroidism has not been reported in patients with the most common inborn mitochondrial fatty acid β -oxidation disorders of carnitine palmitoyl transferase II and very-long-chain acyl-CoA dehydrogenase deficiencies or the severe form of MTP deficiency (a lethal phenotype). Thus, the mechanisms underlying the pathogenesis of the disease presented here remains to be elucidated. Tyni et al. [1997] reported the autopsy findings of a patient with hypoparathyroidism and LCHAD deficiency where the parathyroid glands were severely hypoplastic. These findings suggest that mutations of the proteins associated with β -oxidation cause hypoparathyroidism by congenital malformations of parathyroid glands.

Our studies demonstrate decreased expression of the mutant β -subunits (N389D and A392V) and a failure of those subunits to associate with the wild-type α -subunit (Fig. 2). Moreover, N389D and A392V are located close to the active site and are likely have an immediate impact on the structure and function of the catalytic core of human MTP (Fig. 3C,D). A recent study demonstrated that the β -subunit of MTP interacts and colocalizes with the estrogen receptor α or β in the mitochondria and suggested an important role of the β -subunit in estrogen-mediated lipid metabolism [Zhou et al., 2012a,b]. From this perspective, mutant N389D and A392V β -subunits may cause mitochondria dysfunction, including MTP deficiency due to a failure to associate with the α -subunit of MTP and other proteins that result in dysfunction of parathyroid glands. Further case studies are required to determine whether the specific mutations located in the proximity of the active site of the β -subunit are associated with hypoparathyroidism and MTP deficiency.

Early diagnosis and treatment are important for patients with the neuromyopathic phenotype of MTP deficiency, since peripheral polyneuropathy is progressive [Spiekerkoetter et al., 2004; Yamaguchi et al., 2012]. Our study has demonstrated that MTP deficiency should be considered when patients have hypoparathyroidism as the initial presenting feature in infancy.

ACKNOWLEDGMENTS

We would like to thank the patients and their family members who participated in this study. This work was supported by the Takeda Science Foundation, a Health Labor Sciences Research Grant, and a grant (#21390319) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to N.W.

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Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Functional analysis of iPSC-derived myocytes from a patient with carnitine palmitoyltransferase II deficiency



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ARTICLE INFO

Article history:

Received 13 April 2014

Available online 26 April 2014

Keywords:

Carnitine palmitoyltransferase II deficiency
iPSC
Disease modeling
Rhabdomyolysis
Bezafibrate

ABSTRACT

Introduction: Carnitine palmitoyltransferase II (CPT II) deficiency is an inherited disorder involving β -oxidation of long-chain fatty acids (FAO), which leads to rhabdomyolysis and subsequent acute renal failure. The detailed mechanisms of disease pathogenesis remain unknown; however, the availability of relevant human cell types for investigation, such as skeletal muscle cells, is limited, and the development of novel disease models is required.

Methods: We generated human induced pluripotent stem cells (hiPSCs) from skin fibroblasts of a Japanese patient with CPT II deficiency. Mature myocytes were differentiated from the patient-derived hiPSCs by introducing myogenic differentiation 1 (*MYOD1*), the master transcriptional regulator of myocyte differentiation. Using an *in vitro* acylcarnitine profiling assay, we investigated the effects of a hypolipidemic drug, bezafibrate, and heat stress on mitochondrial FAO in CPT II-deficient myocytes and controls.

Results: CPT II-deficient myocytes accumulated more palmitoylcarnitine (C16) than did control myocytes. Heat stress, induced by incubation at 38 °C, leads to a robust increase of C16 in CPT II-deficient myocytes, but not in controls. Bezafibrate reduced the amount of C16 in control and CPT II-deficient myocytes.

Discussion: In this study, we induced differentiation of CPT II-deficient hiPSCs into mature myocytes in a highly efficient and reproducible manner and recapitulated some aspects of the disease phenotypes of CPT II deficiency in the myocyte disease models. This approach addresses the challenges of modeling the abnormality of FAO in CPT II deficiency using iPSC technology and has the potential to revolutionize translational research in this field.

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1. Introduction

β -Oxidation of long-chain fatty acids (LCFA) occurs in the mitochondria with the activity of carnitine palmitoyltransferase II (CPT II; EC2.3.1.21), carnitine-acylcarnitine translocase (CACT), CPT I, and acyl-coenzyme A (CoA) synthetase. These enzymes mediate LCFA transport from the cytosol into the mitochondria.

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In response to conditions with a high-energy demand, such as intensive exercise, severe infection, and fasting, LCFA transfer is promptly activated [1–4]. *CPT2* maps to chromosome 1p32, spans 20 kb, contains five exons, and encodes the CPT II enzyme. Defects in CPT II enzymatic activity are classified into three clinical categories in humans: lethal neonatal (MIM #608836), severe infantile (MIM #600649), and mild adult-onset (MIM #255110) types. Due to the low enzymatic activity of CPT II, the neonatal and infantile forms result in liver failure, hypoketotic hypoglycemia, and cardiomegaly. The neonatal form causes death within several months. The infantile form has been implicated in cases of sudden infant death syndrome. On the other hand, the adult-onset type

<http://dx.doi.org/10.1016/j.bbrc.2014.04.084>

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manifests as recurrent myalgia (muscle pain), rhabdomyolysis, and myoglobinuria, which can cause acute renal failure. CPT II deficiency is generally considered an autosomal recessive disease; however, many cases of symptomatic carriers have been reported [5]. Individuals who carry a *CPT2* mutation [6–9] may develop the clinical features of CPT II deficiency when treated with medications that affect the activity of the remaining wild-type CPT II enzyme.

In this study, we successfully derived human induced pluripotent stem cells (hiPSCs) from a patient with CPT II deficiency, differentiated them into a mature myocyte lineage within 2 weeks in a highly efficient and reproducible manner, and recapitulated some of the disease phenotypes associated with CPT II deficiency. We discuss the opportunities to use iPSC technology for modeling defects in FAO and for evaluating therapeutic regimens for CPT II deficiency.

2. Patient and methods

2.1. Patient

The subject of the current study was a 24-year-old Japanese man whose genetic and clinical presentation has already been described [10]. The patient suffered from acute renal failure induced by rhabdomyolysis and was diagnosed as having adult-onset CPT II deficiency. Skin biopsy samples were obtained from the patient with his written informed consent. This study was approved by the Ethics Committee on hereditary disease, Research of the Graduate School of Medical Sciences, Fukuoka University, and by the Ethics Committee of Kyoto University. The dermal fibroblasts were expanded from skin biopsy explants in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Japan Bioserum, Hiroshima, Japan). Control iPSCs (201B7) were previously established from the facial dermis of a 36-year-old Caucasian woman at the Center for iPSC Cell Research and Application (CiRA), Kyoto University [11].

2.2. Methods

2.2.1. Generation of hiPSCs from the patient

CPT II deficiency-specific hiPSCs were derived from the patient by transducing the four reprogramming factors (OCT4, SOX2, KLF4, and c-MYC) or three factors (excluding c-MYC) into skin fibroblasts with retrovirus vectors as previously described [11,12]. In brief, fibroblasts derived from the CPT II-deficient patient were maintained and expanded in DMEM containing 10% fetal bovine serum. The patient fibroblasts were seeded in 6-well plates at 1.0×10^5 cells/well. The next day, the cells were infected with Slc7a1 lentiviruses with 4 μ g/mL polybrene (Nacalai Tesque). Fibroblasts expressing the mouse Slc7a1 were seeded in 6-well plates at 1.0×10^5 cells/well 1 day before transduction. Equal amounts of four retrovirus-containing supernatants were mixed and supplemented with 4 μ g/mL polybrene. Six days after transduction, the fibroblasts were replated onto mitomycin C-treated SNL feeder cells. Thirty days after transduction, iPSC colonies were selected for expansion.

2.2.2. Cell culture

CPT II-deficient hiPSCs were cultured as previously described [11]. The hiPSCs were grown on mitomycin C-treated SNL feeder cells in Primate ES medium (ReproCELL, Kanagawa, Japan) supplemented with 500 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 4 ng/mL recombinant human basic fibroblast growth factor (bFGF, Wako, Osaka, Japan). For routine passaging,

hiPSC colonies were dissociated by an enzymatic method with CTK dissociation solution consisting of 0.25% trypsin (Invitrogen), 0.1% collagenase IV (Invitrogen), 20% knockout serum replacement (KSR, Invitrogen), and 1 mM CaCl_2 in PBS (Nacalai Tesque) and split at a ratio between 1:3 and 1:6.

2.2.3. Embryoid body (EB) formation

For EB formation, a 10-cm plate containing hiPSCs was rinsed with PBS and treated with 1 mg/mL type IV collagenase (Invitrogen) in DMEM for 10 min at 37 °C. The collagenase was rinsed away with PBS and replaced with undifferentiation medium. The cells were then scraped off with a cell scraper (IWAKI, Tokyo, Japan), dissociated by pipetting, and distributed into a low attachment 6-well plate (Corning, Tokyo, Japan) containing knockout-DMEM (Invitrogen) supplemented with 20% KSR, 0.1 mM non-essential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 500 U/mL penicillin/streptomycin, and 0.55 mM 2-mercaptoethanol (Invitrogen). After 8 days as a floating culture, the EBs were transferred to gelatin-coated plates and cultured in the same medium for another 8 days.

2.2.4. Teratoma formation

The undifferentiated iPSCs were harvested using CTK dissociation solution, collected, and centrifuged, and the pellets were resuspended in DMEM/F12 (Invitrogen). A quarter of the iPSCs from a confluent 10-cm plate was injected into the testes of a non-obese diabetic/severe combined immunodeficient (NOD-SCID mouse, CLEA, Tokyo, Japan). Nine to 12 weeks after injection, the tumors were dissected and fixed with PBS containing 4% paraformaldehyde (PFA). Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin.

2.2.5. Mutational analysis of the *CPT2* in patient-derived iPSCs

Overlapping PCR primers that targeted *CPT2* exons were designed to cover the entire coding region (Table 1; GenBank accession No. M58581). The PCR protocol was as follows: 30 cycles of 1 min at 94 °C for denaturation, 1 min at 60 °C for annealing, and 1 min at 72 °C for extension, followed by 1 cycle of 10 min at 60 °C for completion. Each PCR product was sequenced on an automated DNA sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems Hitachi, Tokyo, Japan) by using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Foster City, CA) and the sequencing primers listed in Table 1.

2.2.6. Induction of hiPSCs into skeletal muscle cells

We used our previously reported method in which *MYOD1* overexpression in undifferentiated hiPSCs efficiently and reproducibly induces differentiation into mature skeletal muscle cells within 10 days [13]. Briefly, we transduced a self-contained Tet-inducible *MYOD1* expressing *piggyBac* vector (Tet-*MYOD1* vector) and transposase into CPT II-deficient iPSCs by lipofection. This system allows the indirect monitoring of induced *MYOD1* expression in response to doxycycline (Dox) by co-expression of a red fluorescent protein (mCherry). It was also reported that low glucose culture conditions purified the cardiomyocytes from mouse and human iPSC differentiation cultures by selecting only cardiomyocytes, based on the findings of the substantial biochemical differences in glucose and lactate metabolism between cardiomyocytes and undifferentiated iPSCs [14]. We used a similar strategy to increase the purity of generated myocytes and cultured the hiPSC-derived differentiated cells with low glucose media (1.0 g/L) for an additional day after 10 days of myocyte induction by *MYOD1* overexpression. The low-glucose medium was composed of MEM (Sigma, St. Louis, MO) containing 0.4% bovine serum albumin (Sigma), 0.4 mM L-carnitine (Sigma), 0.2 mM unlabeled palmitic acid (Nacalai Tesque), and 500 U/mL penicillin/streptomycin. For

Table 1
Oligonucleotide sequences, related to Fig. 1. Sequences of primers used in this study.

Gene	Forward primer: 5' to 3'	Reverse primer: 5' to 3'
hOCT4Tg	GCTCTCCCATGCATTCAAACCTGA	CCCTTTTCTGGAGACTAAATAAA
hSOX2 Tg	TTCACATGTCCAGCACTACCAGA	GACATGGCCTGCCCGTTATTATT
hKLF4 Tg	CCACCTCGCCTTACACATGAAGA	GACATGGCCTGCCCGTTATTATT
hcMYC Tg	ATACATCCTGTCCGTCCAAGCAGA	GACATGGCCTGCCCGTTATTATT
hOCT4 Total	CCCCAGGGCCCCATTTTGGTACC	ACCTCAGTTTGAATGCATGGGAGAGC
hSOX2 Total	TTCACATGTCCAGCACTACCAGA	TCACATGTGTGAGAGGGCAGTGTGC
hKLF4 Total	GATTACGCGGGTCCGCGCAAACCTACACA	TTAAAAATGTCTTTCATGTGTAAGGCGAG
hcMYC Total	ATACATCCTGTCCGTCCAAGCAGA	TCACGCACAAGAGTTCCTGTAGCTGTTCAAG
CPT2 exon1	CGGCCTGTGTTTAGACTCC	CTTCCAGATTAGGGGCTGTG
CPT2 exon2	GCCTTACACTGACCTGCTT	AGGTTCTGGGTTCTGGAGA
CPT2 exon3	TTCCAGGTTTATAGGCTATG	GGAGGATGAGACGTTACTTC
CPT2 exon4	TAGGGACAGCATTAAACATTT	TGGCCTTGTCTCAGTGAAG
CPT2 exon4	GTCCAGTATTTTCGGCTTT	TGTGGGACAAGTGAGACAAGG
CPT2 exon4	GAGTTTCCCTGGCATACT	GCCTCTCTCTGAAACTGGA
CPT2 exon4	ACAGCTGTAAGGAAAAGTT	CAAGACCAAGGGCATGTCT
CPT2 exon5	CTGAGACGCTGGTTTCCA	GGTAGCTTTTCATCTGCCCA

immunostaining analyses of hiPSC-derived myocytes, human myosin heavy chain (MHC) antibody (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions. Samples were observed under an inverted type fluorescence phase-contrast microscope (BZ-9000E; Keyence, Osaka, Japan).

2.2.7. *In vitro* probe assay of AC profiles

hiPSC-derived myocytes were cultured in a 6-well plate for 96 h with 1 mL medium A composed of MEM, 0.4% bovine serum albumin, 0.4 mM L-carnitine, 0.2 mM unlabeled palmitic acid, and 1% penicillin/streptomycin without L-glutamine, or medium B composed of medium A supplemented with 0.4 mM bezafibrate (Sigma) [15]. Cultured cells were incubated with medium A or B at 38 °C for 96 h to determine the effects of heat stress on mitochondrial FAO.

2.2.8. Quantitative acylcarnitine analysis

Acylcarnitine in the culture supernatant was analyzed by MS/MS (API 3000; Applied Biosystems).

2.2.9. Heat stimulation

Differentiated myocytes on culture day 9 were subjected to heat stress at 38 °C on a hot plate.

2.2.10. Regulated PCR array for skeletal muscle-related genes

To analyze the expression of skeletal muscle-related genes, we performed a regulated PCR array. For first-strand cDNA synthesis, 1 µg total RNA was reverse-transcribed in a 20-µl reaction mix and the RT² First Strand Kit (RT² Profiler PCR Array, SuperArray Bioscience, Frederick, MD) according to the manufacturer's instructions. qRT-PCR was performed with a CFX96 (Bio-Rad, Hercules, CA) and universal cycling conditions (10 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C for 40 cycles). The fold change in gene expression was determined by the comparative cycle Ct ($\Delta\Delta C_t$) method. Statistical calculations were based on the web-based RT² Profiler PCR Array Data Analysis (SuperArray Bioscience).

2.2.11. Microarray analysis

aRNA preparation, fragmentation, hybridization, and scanning of the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) were performed according to manufacturer's protocols. Labeled aRNA was prepared with the GeneChip 3'IVT Express Kit (Affymetrix). Briefly, cDNA was generated from total RNA (100 ng), using reverse transcriptase and a T7-oligo (dT) primer. After second-strand cDNA synthesis, the cDNA was converted to aRNA by an *in vitro* transcription reaction with

biotin-labeled ribonucleotides and T7 RNA polymerase. After synthesis, the aRNA was purified to remove enzymes, salts, and unincorporated nucleotides. The concentration of cRNA was determined from the absorbance at 260 nm in a UV spectrophotometer. The aRNA was fragmented at 94 °C in fragmentation buffer (Affymetrix). The samples were hybridized to the GeneChip(R) Human Genome U133 Plus 2.0 Arrays at 45 °C for 16 h with rotation (60 rpm) in an oven. The arrays were automatically washed and stained with the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The Probe Array was scanned using a GeneChip Scanner 3000 7G (Affymetrix). Intensity data and the CHP files were generated by Affymetrix GeneChip Command Console Software and Affymetrix Expression Console Software.

3. Results

3.1. Generation of CPT II-deficient iPSCs (CPTIID-iPSCs) from patient fibroblasts

The skin fibroblasts were converted into iPSCs after transduction with four retroviral vectors encoding OCT4, SOX2, KLF4, and c-MYC, or with three vectors (excluding c-MYC). Quantitative reverse-transcription PCR was used to evaluate the CPTIID-iPSC clones with repression of the exogenously introduced genes analyzed as the ratio of transgene (Tg) expression to total (endogenous and transgene) expression (Table 1). Based on these analyses, the iPSC clone with the highest level of repression was selected for further experiments. This clone exhibited characteristic human embryonic stem cell (ESC) morphology (Fig. 1A), expressed pluripotency markers, including OCT4, NANOG, SOX2, SSEA4, TRA-1-60, TRA-1-81, and alkaline phosphatase (AP) activity (Fig. 1B), and had a normal karyotype (Fig. 1C). The pluripotent properties of CPTIID-iPSCs were also assessed using embryoid body (EB) and teratoma formation upon intratesticular injection of undifferentiated CPTIID-iPSCs into NOD-SCID mice (Fig. 2A and B). Genetic identity was confirmed by STR analyses of the patient fibroblasts and iPSCs (data not shown). Mutation analysis of the causative gene revealed that the patient had compound heterozygous mutations in the CPT2 [10]. Genomic analysis showed that both CPTIID-iPSCs and their parental fibroblasts possessed mutant CPT2 alleles (Fig. 1D). Sequencing from the 5' and 3' ends showed a CT deletion in the TCT at codon 408 (1223delCT), resulting in a stop signal at codon 420, and a sense mutation of arginine to cysteine at codon 631 (1891C→T; R631C). These results suggest that disease-specific iPSCs can be generated from the skin fibroblasts of a CPT II-deficient patient.

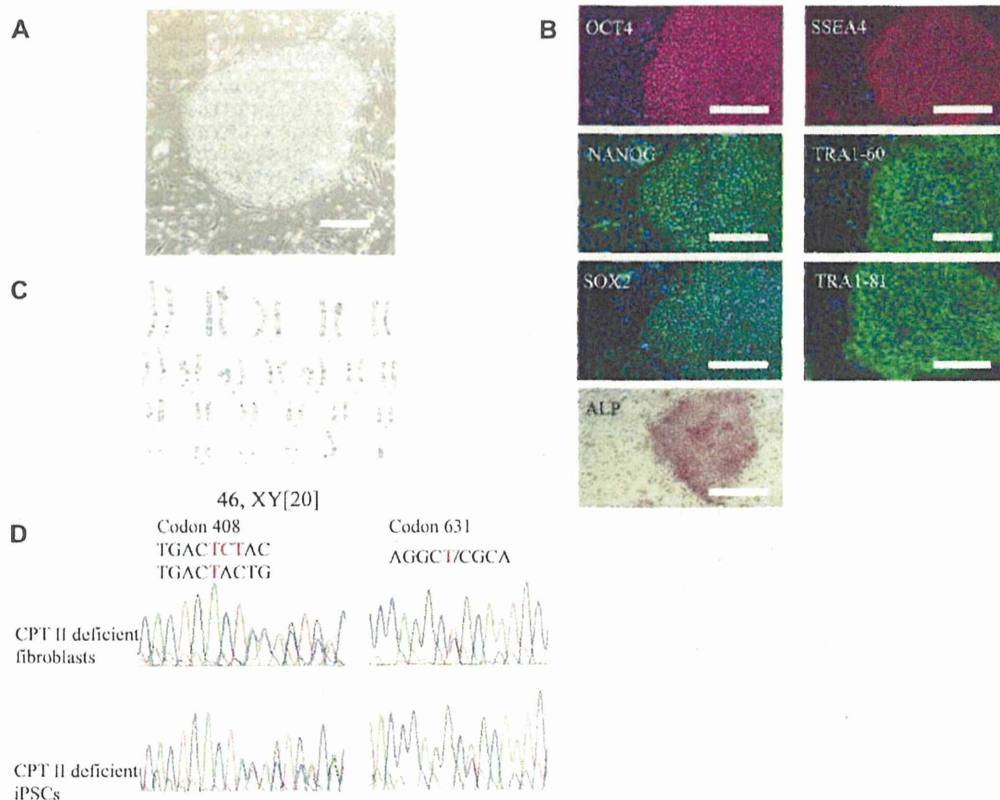


Fig. 1. Generation of iPSCs from a patient with CPT II deficiency. (A) Typical image of human embryonic stem cell (ESC)-like colony. Scale bars: 50 μ m. (B) Immunocytochemistry for OCT4, NANOG, SOX2, SSEA4, TRA1-60 and TRA1-81, and the examination of alkaline phosphatase (AP) enzyme activity. Nuclei were stained with Hoechst 33342 (blue). Scale bars: 100 μ m. (C) Karyotype analyses of CPT II-deficient iPSCs. (D) Mutational analyses of CPT II-deficient iPSCs and their parental fibroblasts. Sequencing from the 5' and 3' ends reveals a CT deletion from the TCT at codon 408, and an arginine to cysteine substitution at codon 631.

3.2. Differentiation of CPTIID-iPSCs into mature myocytes

We next examined whether the patient-derived iPSCs could be differentiated into myocytes, the target cell type of CPT II deficiency. We recently reported a highly efficient myocyte differentiation method based on overexpression of the *MYOD1* gene, a master regulator of myocyte lineage differentiation, in undifferentiated hiPSCs [13]. Tohyama et al. reported a non-genetic method for purifying cardiomyocytes in mouse and human iPSC differentiation cultures [14]. Their strategy is based on the substantial biochemical differences in glucose and lactate metabolism between cardiomyocytes and non-cardiomyocytes, including undifferentiated iPSCs. We used a combination of these strategies to generate myocytes from CPTIID-iPSCs. We transduced a Tet-MYOD1 vector and transposase into CPTIID-iPSCs by lipofection. We forced expression of *MYOD1* with Dox in undifferentiated CPTIID-iPSCs for 10 days. We then used low glucose medium for an additional day to select myocytes. After culture in low glucose medium, the remaining undifferentiated cells disappeared, and the differentiated cells survived, yielding myocyte generation at 50%–60% induction efficiency.

We confirmed the presence of mature myocytes by staining with anti-human MHC antibody (Fig. 3A). Electron microscopy revealed that differentiated myocytes derived from CPTIID-iPSCs had myofibrils containing mature myosin fibers and Z line-like structures (Fig. 3B). We also performed a PCR array and unsupervised clustering to generate myogenic gene profiles for myocytes differentiated from CPTIID-iPSCs, myocytes from control iPSCs (201B7), undifferentiated CPTIID-iPSCs, and undifferentiated 201B7 cells (Fig. 3C, Table 2). We confirmed the upregulation of

markers of skeletal muscle contractility, skeletal myogenesis, and skeletal muscle autocrine signaling in myocytes differentiated from CPTIID-iPSCs compared to undifferentiated CPTIID-iPSCs. The expression patterns of muscle-related genes also differed between myocytes derived from CPTIID-iPSCs and control myocytes. These results suggest that mature myocytes can be efficiently generated from CPTIID-iPSCs by introducing a master transcriptional regulator of myocyte differentiation, *MYOD1*, and culturing in low glucose conditions.

3.3. Acylcarnitine (AC) profiles of the CPT II-deficient myocytes

An acylcarnitine profile determined by tandem mass spectrometry is essential for the definitive diagnosis of CPT II deficiency [15]. We thus examined the profile in myocytes differentiated from CPTIID-iPSCs. CPT II-deficient myocytes accumulated more C16 (palmitoylcarnitine) than did control myocytes (Fig. 4). Results were similar in myocytes differentiated from other iPSC clones from the same patient in this study (data not shown). These data indicated that patient-derived iPSCs recapitulated one of the clinical features of CPT II deficiency.

We previously reported that fibroblasts from patients with LCFA β -oxidation disorders, including CPT II deficiency, were more susceptible to heat stress in comparison to the fibroblasts of patients with medium-chain fatty acid β -oxidation disorders or healthy controls [15]. We thus investigated the effects of heat stress on myocytes derived from CPTIID-iPSCs and found that this treatment significantly increased C16 in CPT II-deficient cells, but not in controls (Fig. 4). We also demonstrated that bezafibrate, an agonist of peroxisome proliferator-activated receptor (PPAR), restores FAO activity in fibroblasts

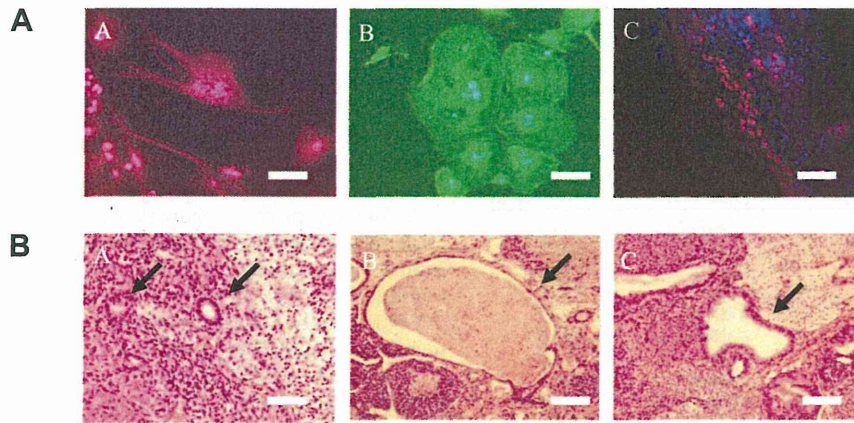


Fig. 2. Embryoid body (EB)- and teratoma-mediated differentiation of CPT II-deficient iPSCs. (A) Immunostaining of EBs generated from CPT II-deficient iPSCs for TUJ1 (ectoderm, A), α -SMA (mesoderm, B), and SOX17 (endoderm, C). Nuclei were stained with Hoechst 33342 (blue). Scale bars: 100 μ m. (B) Hematoxylin and eosin staining of histological sections of teratomas derived from CPT II-deficient iPSCs. Neural tissues (ectoderm, A), cartilage (mesoderm, B), gut-like epithelia (endoderm, C). Scale bars: 100 μ m.

with CPT II deficiency [15]. We investigated the effects of bezafibrate on myocytes derived from CPTIIID-iPSCs. At 37 °C and 38 °C, bezafibrate decreased C16 levels in controls and CPT II-deficient myocytes. In the CPT II-deficient myocytes, bezafibrate at 37 °C reduced C16 levels to those observed in controls (Fig. 4). These results suggest that mature myocytes derived from CPTIIID-iPSCs recapitulate some of the phenotypes associated with CPT II deficiency.

4. Discussion

Rhabdomyolysis occurs after exhaustive exercise or severe infection without trauma or drugs in CPT II deficiency. The adult-onset form presents with myoglobinuria and myalgia, frequently

leading to acute renal failure. Rhabdomyolysis may repeatedly develop within the same families. Hereditary rhabdomyolysis is often caused by compromised enzymatic activity associated with LCFA metabolism.

Accurate experimental modeling of the disease and its response to clinical intervention are hampered due to a lack of appropriate animal models. To address this issue, we sought to derive iPSCs from a patient with CPT II deficiency, who had a history of repetitive rhabdomyolysis and acute renal failure. There have been no reports of derivation of iPSCs from patients with FAO disorders, including CPT II deficiency, and to the best of our knowledge, we are the first to report the successful generation of iPSCs from a CPT II-deficient patient.

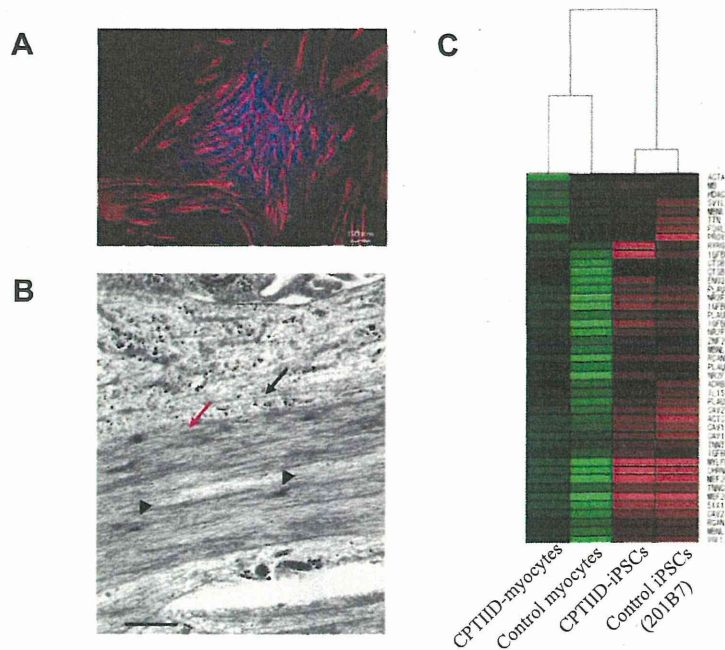


Fig. 3. Directed differentiation of CPT II-deficient iPSCs into skeletal muscle cells. (A) Immunocytochemistry of the myocytes derived from CPT II-deficient iPSCs with anti-human Myosin Heavy Chain (MHC) antibody. Nuclei were stained with Hoechst 33342 (blue). Scale bar: 100 μ m. (B) Structural analysis of myocytes differentiated from CPT II-deficient iPSCs by electron microscopy. A red arrow indicates myofibrils. Black arrowheads indicate immature Z lines. A black arrow indicates myosin fibers. Scale bar: 500 nm. (C) Characterization of iPSC-derived myocytes. Myogenic gene profiles and unsupervised clustering based on markers associated with myocytes in undifferentiated iPSCs and differentiated myocytes. CPTIIID-myocytes, control myocytes, CPTIIID-iPSCs, and control iPSCs. Green indicates up-regulated genes and red indicates down-regulated genes. Up-regulated genes were identified by changes of at least 2-fold. CPTIIID-myocytes; Myocytes differentiated from CPTIIID-iPSCs.

Table 2

The expression of markers for skeletal muscle contractility, skeletal myogenesis, and skeletal muscle autocrine signaling in the myocytes differentiated from CPT II-deficient iPSCs. Gene expression was evaluated using quantitative real time RT-PCR as described in Materials and Methods; GAPDH was the internal control. Results are shown as fold change relative to control samples of undifferentiated CPT II-deficient iPSCs.

	Symbol	Fold change
Skeletal muscle contractility	ATP2A1	4.6
	CAV3	22.0
	DES	33.5
	DMPK	2.1
	DYSF	2.2
	LMNA	3.9
	MB	8.3
	MYH1	86.5
	MYOT	4.0
	NEB	4.3
	SGCA	4.1
	TNNC1	4.1
	TNNI2	8.4
	TNNT1	2.2
	TNNT3	35.7
	TTN	6.4
	Skeletal myogenesis	ACTA1
CAPN2		3.1
CAV1		3.8
IGF1		5.7
IGFBP3		5.9
IGFBP5		15.1
MEF2C		43.3
MSTN		74.8
MUSK		11.0
MYOG		477.3
PAX3		2.7
Skeletal muscle autocrine signaling	IGF1	5.7
	IGF2	59.7
	IL6	3.2

development and function in CPT II-deficient myocytes. Culture in low glucose caused the death of undifferentiated iPSCs, which require large quantities of glucose, while the differentiated cells require limited glucose and produce lactic and pyruvic acids to more effectively obtain energy by mitochondrial oxidative phosphorylation. Thus, by using low-glucose medium, we increased the differentiation efficiency of iPSC-derived myocytes.

Yamaguchi et al. showed that an *in vitro* AC profiling assay could reliably detect various FAO disorders, consistent with reports from other groups [16–18]. In particular, C16 accumulation was found to be a reliable biomarker that could be used to diagnose CPT II deficiency. Consistent with these findings, our hiPSC-derived myocytes mimicked the metabolic characteristics of the disease.

The effects of PPAR agonists on mitochondrial FAO have been extensively examined [19]. Bezafibrate has proven efficacy in the treatment of long-chain FAO disorder [20–24]. We found that bezafibrate reduced long-chain ACs more effectively in myocytes from patient-derived iPSCs than in those from control hiPSCs. These findings indicate that bezafibrate could be a therapeutic drug for CPT II deficiency and other mitochondrial FAO disorders.

In conclusion, we successfully derived disease-specific iPSCs from a patient with CPT II deficiency and differentiated them into myocytes. Our results suggest that cellular models using patient-derived iPSCs will be of significant benefit for research groups studying CPT II deficiency-related diseases, and that these iPSC disease models may be a valuable resource for testing novel therapeutic strategies for these disorders.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We thank Ms. Y Sasaguri for technical and secretarial assistance and Dr. S Mae for technical support and helpful discussion. This work was supported in part by research funds no. 101001 (H.K.) and no. 122502 (T.Y.) from the Central Research Institute of Fukuoka University, a Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science (23791200, 25870995).

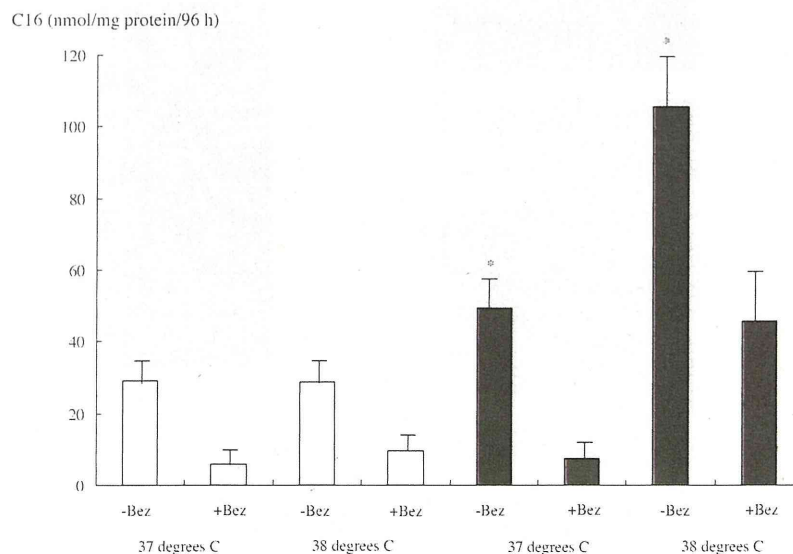


Fig. 4. Acylcarnitine (AC) profiles in culture medium of iPSC-derived myocytes loaded with palmitic acid after bezafibrate (Bez) treatment. Data are shown as mean \pm SD (nmol/mg protein/96 h) ($n = 3$). White bars: Myocytes from control iPSCs; Black bars: Myocytes from CPT II-deficient iPSCs. Statistically significant differences between 37 °C Bez (-) of control and 37 °C or 38 °C Bez (-) of patients shown as * $p < 0.05$.

to T.Y.), Takahashi Industrial and Economic Research Foundation, and Kaibara Morikazu Medical Science Promotion Foundation. This work was also supported by the Japan Society for the Promotion of Science (JSPS) through its "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)" to K.O. and by the Japan Science and Technology Agency (JST) through its research grant "Core Center for iPS Cell Research, Research Center Network for Realization of Regenerative Medicine" to K.O.

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Original article

Carnitine–acylcarnitine translocase deficiency: Two neonatal cases with common splicing mutation and *in vitro* bezafibrate response

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Received 28 July 2014; received in revised form 7 October 2014; accepted 8 October 2014

Abstract

Background: Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of acute encephalopathy- or myopathy-like illness. Carnitine–acylcarnitine translocase (CACT) deficiency is a rare FAO disorder, which represent an energy production insufficiency during prolonged fasting, febrile illness, or increased muscular activity. CACT deficiency is caused by mutations of the *SLC25A20* gene. Most patients developed severe metabolic decompensation in the neonatal period and died in infancy despite aggressive treatment.

Patients and methods: We herein report the clinical findings of two unrelated cases of CACT deficiency with mutation confirmation, and *in vitro* bezafibrate responses using *in vitro* probe acylcarnitine (IVP) assay. Patients 1 and 2 are products of nonconsanguineous parents. Both patients developed cardiac arrest at day 3 of life but survived the initial events. Their blood chemistry revealed hypoglycemia and metabolic acidosis. The acylcarnitine profiles in both patients demonstrated increased long-chain acylcarnitines, suggesting CACT or carnitine palmitoyltransferase-2 (CPT2) deficiency.

Results: The mutation analysis identified homozygous IVS2-10T>G in the *SLC25A20* gene in both patients, confirming the diagnosis of CACT deficiency. The IVP assay revealed increased C16, C16:1, but decreased C2 with improvement by bezafibrate in the cultured fibroblasts. The short-term clinical trial of bezafibrate in Patient 1 did not show clinical improvement, and died after starting the trial for 6 months.

Conclusion: This splicing mutation has been identified in other Asian populations indicating a possible founder effect. IVP assay of cultured fibroblasts could determine a response to bezafibrate treatment. A long-term clinical trial of more enrolled patients is required for evaluation of this therapy.

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Keywords: CACT deficiency; *SLC25A20* mutation; IVP assay; Bezafibrate

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<http://dx.doi.org/10.1016/j.braindev.2014.10.005>

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