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Energy utilization of induced pluripotent stem cell-derived cardiomyocyte in Fabry disease

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ABSTRACT

Background: Fabry disease (FD) is a lysosomal storage disease in which glycosphingolipids (GB3) accumulate in organs of the human body, leading to idiopathic hypertrophic cardiomyopathy and target organ damage. Its pathophysiology is still poorly understood.
Objectives: We aimed to generate patient-specific induced pluripotent stem cells (iPSC) from FD patients presenting cardiomyopathy to determine whether the model could recapitulate key features of the disease phenotype and to investigate the energy metabolism in Fabry disease.
Methods: Peripheral blood mononuclear cells from a 30-year-old Chinese man with a diagnosis of Fabry disease, GLA gene (IVS4 + 919G > A) mutation were reprogrammed into iPSCs and differentiated into iPSC-CMs and energy metabolism was analyzed in iPSC-CMs.
Results: The FD-iPSC-CMs recapitulated numerous aspects of the FD phenotype including reduced GLA activity, cellular hypertrophy, GB3 accumulation and impaired contractility. Decreased energy metabolism with energy utilization shift to glycolysis was observed, but the decreased energy metabolism was not modified by enzyme rescue replacement (ERT) in FD-iPSCs-CMs.
Conclusion: This model provided a promising *in vitro* model for the investigation of the underlying disease mechanism and development of novel therapeutic strategies for FD. This potential remedy for enhancing the energetic network and utility efficiency warrants further study to identify novel therapies for the disease.

1. Introduction

Fabry disease (FD) is an X-linked recessive lysosomal storage disorder resulting from mutations in the a-galactosidase A gene (*GLA*) that cause deficient α-galactosidase A (α-Gal A) activity [1–5]. This defect of α-Gal A causes accumulation of glycosphingolipids, mainly globotriaosylceramide (GB3) in lysosomes within blood vessels, tissue and organs such as skin, brain, kidney and heart, leading to proper function impairment. Various symptoms such as acroparaesthesias (neuropathic pain), corneal opacities (cornea verticillata), cardiomyopathy, and impaired renal function may present depending on which organs are involved [6]. Recent newborn screening studies based on identification of abnormalities in the *GLA* gene or deficiency in α-Gal A activity reported a birth prevalence of at least 1 in 4000 in European populations [3,7] while a higher prevalence, especially of cardiac variant FD, has been found in Taiwan [8]. Cardiovascular manifestations in FD include hypertension, coronary disease, arrhythmias, valvular abnormalities, heart failure and sudden death [8].

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Cardiac involvement in FD usually presents as concentric left ventricular hypertrophy (LVH), and myocardial fibrosis is a typical feature of advanced stage Fabry cardiomyopathy. Although FD is an inherited disease of lysosomal storage disorder with enzyme activity deficiency, the detailed mechanism responsible for LVH remained undetermined. In addition, the enzyme replacement therapy (ERT) is the treatment of choice for FD, but consistent benefits have not always been achieved, and it is usually less effective among those with advanced disease status. Recently, the technique of induced pluripotent stem cells (iPSCs) established by Yamanaka with introduced reprogramming with Yamanaka factors (*e.g.*, Klf4, Oct4, Sox2, c-Myc) to generate patient- or disease-specific cells has provided a novel model for disease targeted stem cell research in living patients [9]. To our interest, the heart demands more energy demand than any other organs in the human body and balanced bioenergetics is crucial for maintaining the normal function of the heart [10]. The cardiac energy metabolism involves the transition of substrate utilization such as fatty acid and glucose, oxidative phosphorylation and ATP transfer [10]. Because cardiomyocytes are sensitive to energy utilization and metabolism, but little is known about the bioenergetics in cardiomyocytes in FD. Therefore, we would like to generate a model by using patient-specific iPSCs-derived cardiomyocytes (iPSCs-CM), recapitulating characteristic abnormalities of FD to investigate the substrate utilization in cardiomyocyte of FD.

2. Methods

2.1. Ethics statement

All experimental procedures and protocols involving animals were approved by the institutional animal care committee of Taipei Veterans General Hospital and complied with the Guide for the Care and Use of Laboratory Animals. This research followed the tenets of the Declaration of Helsinki and protocols for this study were approved by the Internal Research Board of Taipei Veterans General Hospital. All samples were obtained after receiving informed consent from the patients.

2.2. Patient clinical details and genetic profile

The patient in this study was a 30-year-old Chinese man who presented at the hospital with palpitations and exertional dyspnea. The results of comprehensive testing showed atrial fibrillation arrhythmia, marked left ventricular hypertrophy with prominent thickening of the anterior septum and posterior wall, and decreased plasma α-Gal A activity, as checked by enzyme assay. Genetic testing revealed the mutation of IVS4 + 919G > A in the *GLA* gene. Endomyocardial biopsy showed a mosaic of normal and hypertrophied cardiomyocytes containing perinuclear and cytoplasmic vacuoles consisting of membrane bound myelin bodies. In addition, electron microscopy (EM) revealed vacuoles consisting of myelin bodies denoting glycosphyngolipid infiltration, confirming the diagnosis of FD with cardiac involvement (Supplement Fig. 1).

2.3. PBMC isolation and expansion

Peripheral blood mononuclear cells (PBMC) were isolated from patients with FD and one unaffected control using Ficoll-Plaque Plus (Amersham Biosciences) according to the manufacturer's protocol as reported previously [11]. In brief, the blood sample was layered on one ratio of Ficoll-Plaque Plus, pellet (400 × g, 30 min at 20 °C) and the buffy coat is collected, washed twice with PBS and cultured in PBMC medium (StemPro-34 SFM (Life Technologies), 100 ng/ml SCF, 100 ng/ml FLT-3, 20 ng/ml IL-3, 20 ng/ml IL-6).

2.4. Human iPSCs generation and culture

Human iPSCs were generated from human PBMC derived from subjects with FD and a 25-year-old unrelated healthy donor. To generate integration-free iPSCs, cells were transduced with four reprogramming factors using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Life Technologies) according to the manufacturer's protocol. In brief, PBMC were transduced using the CytoTune 2.0 Sendai reprogramming vectors at MOI 5 and incubated overnight. Subsequently, the medium was replaced with fresh complete PBMC medium to remove the reprogramming virus. At day 3, the transduced cells were placed on mouse embryonic fibroblasts (MEFs) culture dishes in StemPro-34 medium (Life Technologies) without cytokines. At day 7, the cells were transitioned into iPSCs medium (DMEM/F12 (Gibco) supplemented with 20% KnockOut serum replacer (KSR; Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 10 ng/ml recombinant human basic fibroblast growth factor (bFGF), and antibiotics (Gibco) and changed every day. After 21 to 28 days of re-plating, ES-like colonies appeared and were placed onto freshly thawed inactivated MEFs culture dishes for expansion and characterization. To prevent cell contamination by MEFs, these iPSCs were transferred to feeder-free/serum-free culture in mTeSR1 medium (StemCell Technologies) without KSR supplementation.

2.5. In vitro differentiation of iPSCs

iPSCs were dispersed into small clumps using dispase (Sigma-Aldrich, MO, USA; 1 mg/ml for 1 min) and transferred onto ultra-low attachment plates (Corning, NY, USA) for embryoid body formation. After 3 days, the aggregated cells were plated onto 0.1% gelatin-coated culture dishes with the FBS-containing medium. The medium was changed every 2 days. The cells were stained with an *anti*-α-smooth muscle actin monoclonal antibody (1004-01, IBSC), an anti-MAP2 antibody (4542, Cell Signaling), and an anti-alpha-fetoprotein monoclonal antibody (3903, Cell Signaling).

2.6. Teratoma formation and histological analysis

Undifferentiated iPSCs (1×10^6) were suspended in phosphate-buffered saline (PBS) and delivered by a 26-gauge syringe (BD Biosciences) to the subrenal capsule of 10- week old NOD SCID mice (BioLASCO). Eight weeks after the injection, tumors were dissected from the mice. The samples were weighed, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. The sections were stained with hematoxylin and eosin.

2.7. Quantitative PCR and RT-PCR for the marker genes

Reverse transcription reactions were performed using SuperScript III reverse transcription (Invitrogen). The resulting cDNA was used for quantitative PCR (qPCR) and RT-PCR. qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The signals were detected with a 7900HT Fast Real-Time PCR system (Applied Biosystems). The primer sequences are listed in Supplementary Tables 1 and 2.

2.8. Cardiac differentiation from iPSCs

The iPSCs were differentiated into cardiomyocytes (CMs) according to a previously established protocol [12]. The iPSCs were cultured on Geltrex-coated plates in mTeSR1 medium, and Versene solution (Life Technologies) was used to detach the iPSCs from the plates. Then, the iPSCs were resuspended in mTeSR1 with 5 μM of Y27632 (Tocris Bioscience), a ROCK inhibitor, and were plated on Geltrex-coated plates. The culture medium was initially mTeSR1 and RPMI/1640 (Life Technologies), with B-27 without insulin (Life Technologies) with CHIR99021 (Selleckchem), a GSK3 inhibitor. After 24 h, the medium was replaced with RPMI/B-27 without insulin. On day 3 of differentiation, combined medium was prepared by mixing the old medium with fresh RPMI/B-27 without insulin at a 1:1 ratio. The medium was replaced with combined medium containing 5 μM of IWP2 (Tocris Bioscience), a Wnt signaling inhibitor. On day 5 of differentiation, the medium was replaced with fresh RPMI/B-27 without insulin. RPMI with B-27 (Life Technologies) was added on day 8 of differentiation and changed every three days.

2.9. Western blot assay

The extraction of proteins from cells and western blot analysis were performed as described previously [13]. Whole cell lysates were separated by electrophoresis on 10% SDS–PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat milk at room temperature for 1 h. The blots were incubated with primary antibodies in TBST buffer containing 5% nonfat milk at 4 °C overnight and subsequently with secondary antibodies conjugated with peroxidase at 25 °C for 1 h. The immunoblots were developed using an enhanced chemiluminescence system, and the luminescence was visualized on X-ray film. The antibodies for western blotting are shown in Supplementary Table 3.

2.10. Immunofluorescence staining and measurement of CM size

The living cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 5% normal bovine serum albumin (BSA) in PBS. The cells were incubated with cTnT antibody, and the antibody and conditions are shown in Supplementary Table 3. After being washed three times with PBS, the cells were incubated with secondary antibodies conjugated with FITC (green). DAPI (blue) was used as the nuclear stain. Labeled cells were imaged with a laser-scanning confocal microscope (Olympus). The total amount of retained immunofluorescent material was determined in the red (546) and the green (488 nm) channels. The cellular area contents of the normal CMs and the F-CMs were quantified using the area measurement tool of ImageJ software package (National Institutes of Health, Bethesda, MD).

2.11. Transmission electron microscopy (TEM)

Cells (1×10^7) were suspended in 1.2% agarose and fixed in 0.1 M phosphate buffer (PB), pH 7.4, containing 4% paraformaldehyde and 2.5% glutaraldehyde at 4 °C overnight. The samples were washed with 0.1 M PB before post-fixation with 1% OsO4 in 0.05 M PB for 1 h. After washing with distilled water, the samples were rinsed in block-stain with 0.2% uranyl acetate at 4 °C overnight. The samples were dehydrated in a serial dilution of ethanol for 10 min each (from 50% to 100% ethanol) and further infiltrated with a 100% ethanol/acetone (1:1) mixture and 100% acetone for 15 min each. Then, they were infiltrated with 100% acetone/Spurr resin (1:1) and (1:3) mixture for 1 h each. The samples were changed to Spurr resin for continuous infiltration for 24 h before being

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