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Original article

Generation of Fabry cardiomyopathy model for drug screening using induced pluripotent stem cell-derived cardiomyocytes from a female Fabry patient

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ABSTRACT

Background: Fabry disease is an X-linked disease caused by mutations in α-galactosidase A (GLA); these mutations result in the accumulation of its substrates, mainly globotriaosylceramide (Gb3). The accumulation of glycosphingolipids induces pathogenic changes in various organs, including the heart, and Fabry cardiomyopathy is the most frequent cause of death in patients with Fabry disease. Existing therapies to treat Fabry disease have limited efficacy, and new approaches to improve the prognosis of patients with Fabry cardiomyopathy are required.

Methods and results: We generated induced pluripotent stem cell (iPSC) lines from a female patient and her son. Each iPSC clone from the female patient showed either deficient or normal GLA activity, which could be used as a Fabry disease model or its isogenic control, respectively. Erosion of the inactivated X chromosome developed heterogeneously among clones, and mono-allelic expression of the *GLA* gene was maintained for a substantial period in a subset of iPSC clones. Gb3 accumulation was observed in iPSC-derived cardiomyocytes (iPS-CMs) from GLA activity-deficient iPSCs by mass-spectrometry and immunofluorescent staining. The expression of ANP was increased, but the cell surface area was decreased in iPS-CMs from the Fabry model, suggesting that cardiomyopathic change is ongoing at the molecular level in Fabry iPS-CMs. We also established an algorithm for selecting proper Gb3 staining that could be used for high-content analysis-based drug screening.

Conclusions: We generated a Fabry cardiomyopathy model and a drug screening system by using iPS-CMs from a female Fabry patient. Drug screening using our system may help discover new drugs that would improve the prognosis of patients with Fabry cardiomyopathy.

1. Introduction

Fabry disease is an X-linked lysosomal storage disorder caused by mutations in α-galactosidase A (GLA). The mutation impairs the activity of the GLA enzyme, which results in the accumulation of glycosphingolipids containing a terminal α-1,4 galactosidic linkage, mainly globotriaosylceramide (Gb3) [1, 2]. The accumulation of glycosphingolipids impairs the morphology and function of various cell types, causing progressive cardiac, renal and cerebrovascular diseases. Approximately 60% of Fabry disease patients exhibit cardiac manifestations called Fabry cardiomyopathy. Fabry cardiomyopathy usually emerges as left ventricular hypertrophy (LVH) and develops into heart failure, myocardial infarction and life-threatening arrhythmias [1–3]. Importantly, these cardiovascular complications are the most frequent

Abbreviation: RFLP, restriction fragment length polymorphism; TUNEL, TdT-mediated dUTP nick end labeling; PBMC, peripheral blood mononuclear cell; MEF, mouse embryonic fibroblast; BSA, bovine serum albumin

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causes of death among patients with Fabry disease [4–6]. Supplementation of recombinant GLA enzyme, which is called enzyme replacement therapy (ERT), is now widely used for causal treatment for Fabry disease. ERT prevents LVH progression, reduces cardiovascular events and improves the mortality of Fabry disease patients; however, its effect is still limited, and further approaches to improve the prognosis of Fabry cardiomyopathy patients are required [7–10].

Human induced pluripotent stem cells (iPSCs) established from patients with genetic disorders are a promising cell source for investigating disease mechanisms and drug discovery. Patient-derived iPSCs harbor the genomic mutation that is identical to that of the patient, and disease-causing cells differentiated from those patient-derived iPSCs are expected to show the same cellular dysfunctions observed in the patient [11]. The establishment of iPSCs and the characterization of iPSC-derived cardiomyocytes (iPS-CMs) from Fabry disease patients were reported from several groups [12–14]. Itier et al. showed that Gb3 accumulation was observed in iPS-CMs from Fabry disease patients using mass spectrometry and showed that ERT decreases Gb3 accumulation, suggesting the usefulness of Fabry iPS-CMs for drug discovery [13].

In the present study, we generated iPSC lines from two Fabry disease patients: a heterozygous female and her son. We found that the GLA activity of each iPSC clone from the heterozygous female was either normal or deficient and that the iPSC line with normal GLA activity can be used as a non-engineered isogenic control. Gb3 accumulation in iPS-CMs from GLA activity-deficient iPSC lines could be quantified by immunofluorescent staining coupled with a high-content analysis system and by mass spectrometry. Based on the findings, we established a high-content analysis protocol that can be used for high-throughput screening of the new drug for Fabry cardiomyopathy.

2. Materials and methods

2.1. Patients

We generated iPSCs from a female Fabry disease patient and her son. Both patients were clinically suspected to have Fabry disease and were diagnosed by the lack of GLA activity approximately 30 years ago (Supplementary Fig. 1A). Both of them had a c.779G > C (p.G260A) mutation in exon 5 of the *GLA* gene, which is reported to confer classical phenotypes of Fabry disease [15]. The female patient exhibited LVH and angina in her 40s and developed heart failure and sick sinus syndrome with atrial fibrillation during her clinical course, but did not show severe dysfunctions in other organs. The blood sampling for iPSC generation was performed in her 70s. Her son exhibited LVH and end stage renal disease and underwent kidney transplantation in his 30s. The blood sampling for iPSC generation was performed in his 40s. Written informed consent was obtained from the patients according to the protocol approved by the Institutional Review Board of Osaka University (accession number: 13254(829-1)-3).

2.2. Generation of human iPSCs

iPSCs were generated from peripheral blood mononuclear cells (PBMCs) using the Sendai virus vector as previously reported [16, 17]. Reprogramming factors were introduced to the PBMCs using the Sendai virus-based CytoTune-iPS Reprogramming Kit (Life Technologies) according to the manufacturer's instructions. PBMCs were then plated on mitomycin C-treated mouse embryonic fibroblasts (MEFs). Approximately 2 weeks later, colonies with iPSC-like morphologies were picked up and propagated. iPSCs were cultured on mitomycin C-treated MEFs in KnockOut D-MEM/F-12 medium (Thermo Fisher Scientific) supplemented with 20% KnockOut Serum Replacement (Thermo Fisher Scientific), 1× MEM non-essential amino acids (Thermo Fisher Scientific), 1× GlutaMAX (Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific), 0.5× penicillin/streptomycin (Thermo Fisher Scientific) and 20 ng/mL basic FGF (Wako Pure Chemical Industries).

2.3. Cardiac differentiation of iPSCs and purification of differentiated cardiomyocytes

iPSCs were differentiated into cardiomyocytes using an embryoid body formation protocol as described previously with slight modification [11, 18] using StemPro-34 medium (Thermo Fisher Scientific). Differentiated cardiomyocytes were purified by metabolic selection [19] using no glucose DMEM (Nacalai Tesque) supplemented with 4 mM L-lactate (Wako Pure Chemical Industries) and 0.5% bovine serum albumin (BSA, Wako). After the purification, cells were cryopreserved in STEM-CELLBANKER (ZENOAQ) by the PDF-2000 program deep freezer (Strex), cooling at −1 °C per minute until reaching −80 °C.

2.4. Preparation of iPS-CMs for experiments

Cryopreserved iPS-CMs were thawed in a 37 °C water bath and slowly diluted with DMEM with 20% FBS. After centrifugation, cells were resuspended in DMEM with 20% FBS and plated at 1.0 to 1.8×10^5 cells/cm^2. At day 3, cells were replated at the density of 5.9×10^4 cells/cm^2 for RNA, lipid and GLA activity analysis, 5000 cells/coverslip for immunostaining and 2000 cells/well in 96-well plates for high-content analysis. The medium was changed every 3 days, and the analysis was performed 9 days later. All the experiments were performed approximately 50 days of culture after the start of the differentiation.

2.5. GLA activity assay

GLA activity was measured as previously reported [20]. Cell pellets were sonicated and incubated with 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (Sigma-Aldrich) and 40 mM N-acetyl-D-galactosamine (Wako Pure Chemical Industries) in a citrate-phosphate buffer (pH 4.4) for 30 min at 37 °C, and the reaction was terminated by the addition of 0.2 M glycine-NaOH buffer (pH 10.7). The fluorescence of 4-methylumbelliferone was measured at an excitation wavelength of 360 nm and at an emission wavelength of 448 nm. The activity was expressed as nmol/mg-protein/h.

2.6. RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was extracted using TRIzol reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA samples were reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed using the Universal Probe Library (UPL, Roche, Supplementary Table 1) and THUNDERBIRD Probe qPCR Mix (TOYOBO). Relative expression levels of the target genes were normalized to the expression of an internal control gene (*RPL30*) using the comparative Ct method.

2.7. Restriction fragment length polymorphism (RFLP) assay

RNA extraction and reverse transcription was performed as described above. The region of cDNA containing the mutation site was amplified by PCR using Blend Taq -Plus- (TOYOBO) according to the manufacturer's instructions. Primer sequences are listed in Supplementary Table 1. The PCR product was then incubated with BsaJ1 (New England Biolabs), which can digest the wild-type allele but not the mutant allele (Supplementary Fig. 1B). The size of the digested fragments was analyzed by 8% acrylamide gel electrophoresis.