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ARTICLE

Disease modeling and lentiviral gene transfer in patient-specific induced pluripotent stem cells from late-onset Pompe disease patient

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Pompe disease is an autosomal recessive inherited metabolic disease caused by deficiency of acid α-glucosidase (GAA). Glycogen accumulation is seen in the affected organ such as skeletal muscle, heart, and liver. Hypertrophic cardiomyopathy is frequently seen in the infantile onset Pompe disease. On the other hand, cardiovascular complication of the late-onset Pompe disease is considered as less frequent and severe than that of infantile onset. There are few investigations which show cardiovascular complication of late onset Pompe disease due to the shortage of appropriate disease model. We have generated late-onset Pompe disease-specific induced pluripotent stem cell (iPSC) and differentiated them into cardiomyocytes. Differentiated cardiomyocyte shows glycogen accumulation and lysosomal enlargement. Lentiviral GAA rescue improves GAA enzyme activity and glycogen accumulation in iPSC. The efficacy of gene therapy is maintained following the cardiomyocyte differentiation. Lentiviral GAA transfer ameliorates the disease-specific change in cardiomyocyote. It is suggested that Pompe disease iPSC-derived cardiomyocyte is replicating disease-specific changes in the context of disease modeling, drug screening, and cell therapy.

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INTRODUCTION

Pompe disease (PD) is an autosomal recessive, lysosomal storage disease caused by deficiency of acid-α-glucosidase (GAA), which is located in lysosome and degrades glycogen.[1] Based on clinical manifestation, PD is divided into infantile form, which is remarkable for neonatal onset progressive weakness of skeletal muscle and cardiac hypertrophy, and late-onset form, which is known for later and slower progressive weakness of skeletal muscle.[2]

Enzyme replacement therapy (Myozyme) was initiated in 2006; however clinical response was known to be variable.[3,4] *Ex vivo* lentiviral gene therapy is promising alternative treatment modality. Several human trials of lentiviral gene therapy have been conducted and efficacy is reported in some monogenic diseases.[5,6] Other treatment strategy, such as chemical chaperon, which enhances the residual enzyme activity by stabilizing mutated enzyme, is still under development.[7] There are few available treatment modalities at this point and novel therapeutic strategy is warranted.

In 2011, Huang *et al*.[8] reported the generation of iPS cells from infantile form PD patient and cardiomyocyte differentiation. Transient expression of GAA was required to generate PD-specific iPS cells. Induced cardiomyocyte contains higher amount of glycogen and manifests disarrayed cardiomyocyte fiber.

Although, it has been considered that cardiovascular complication of PD is limited to infantile onset PD. Several researches showed that cardiac complication was sometimes seen in late-onset Pompe disease (LOPD), even though it is less severe and frequent.[9,10] However, shortage of bio-resource made it difficult to investigate the disease mechanism.

iPS cells are excellent tools for screening drugs. However, some concerns exist if disease phenotypes are maintained after differentiation to specific cells, such as cardiomyocyte. In addition, differentiated pluripotent stem cell replacement might be useful in terms of autologous pluripotent stem cell therapy if combined with gene correction method such as viral transfer and homologous recombination by TALEN or CRISPR/Cas9.[11]

iPS cells from LOPD patient have been successfully generated and even after cardiomyocyte differentiation disease hallmarks were observed in induced cardiomyocyte. We additionally conducted gene transfer to PD iPS cells and rescued the phenotype both biochemically and pathologically.

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RESULTS

Generation of Pompe disease pluripotent stem cell and its characterization

Three iPSC clones from one late-onset PD patient and one clone from one normal control were analyzed. Pluripotency markers, Oct3/4, Sox2, Klf4, Myc, Nanog, Gdf3, Rex1, DPPA2, and DPPA4, were analyzed by reverse transcription polymerase chain reaction (RT-PCR). All of the pluripotency markers were expressed in both PD and control iPSC almost equally except relatively low expression of GDF3 in control iPSC (Figure 1a).

Pluripotency markers such as SSEA-4, Tra-1–60, and Tra-1–81 were also analyzed by immunofluorescence and all of the markers were positively stained equally both control and PD iPSC without difference between both cell lines (Figure 1b).

Next we have conducted embryoid body formation and checked pluripotency. Ectoderm (PAX6 and MAP2), mesoderm (Brachury and MSX1), and endoderm (FOXA2 and AFP) markers were analyzed by RT-PCR. In all cell lines, at least one of ectoderm, mesoderm, and endoderm markers expression was confirmed (Figure 1c). Pluripotency was also evaluated by directed differentiation to three germ layers confirmed by immunofluorescence. The expression of Otx2 (Ectoderm), Brachury (Mesoderm), and Sox17 (Endoderm) were observed in all patient and control cell lines (Figure 1d).

These results showed that both control and Pompe disease iPSCs have similar characteristics as pluripotent stem cell.

Pompe disease pluripotent stem cells have disease-specific phenotypes

Gene analysis showed compound heterozygote mutation, c.796C>T and c.1316T>A, which is corresponding to late onset Pompe disease (Figure 2a).

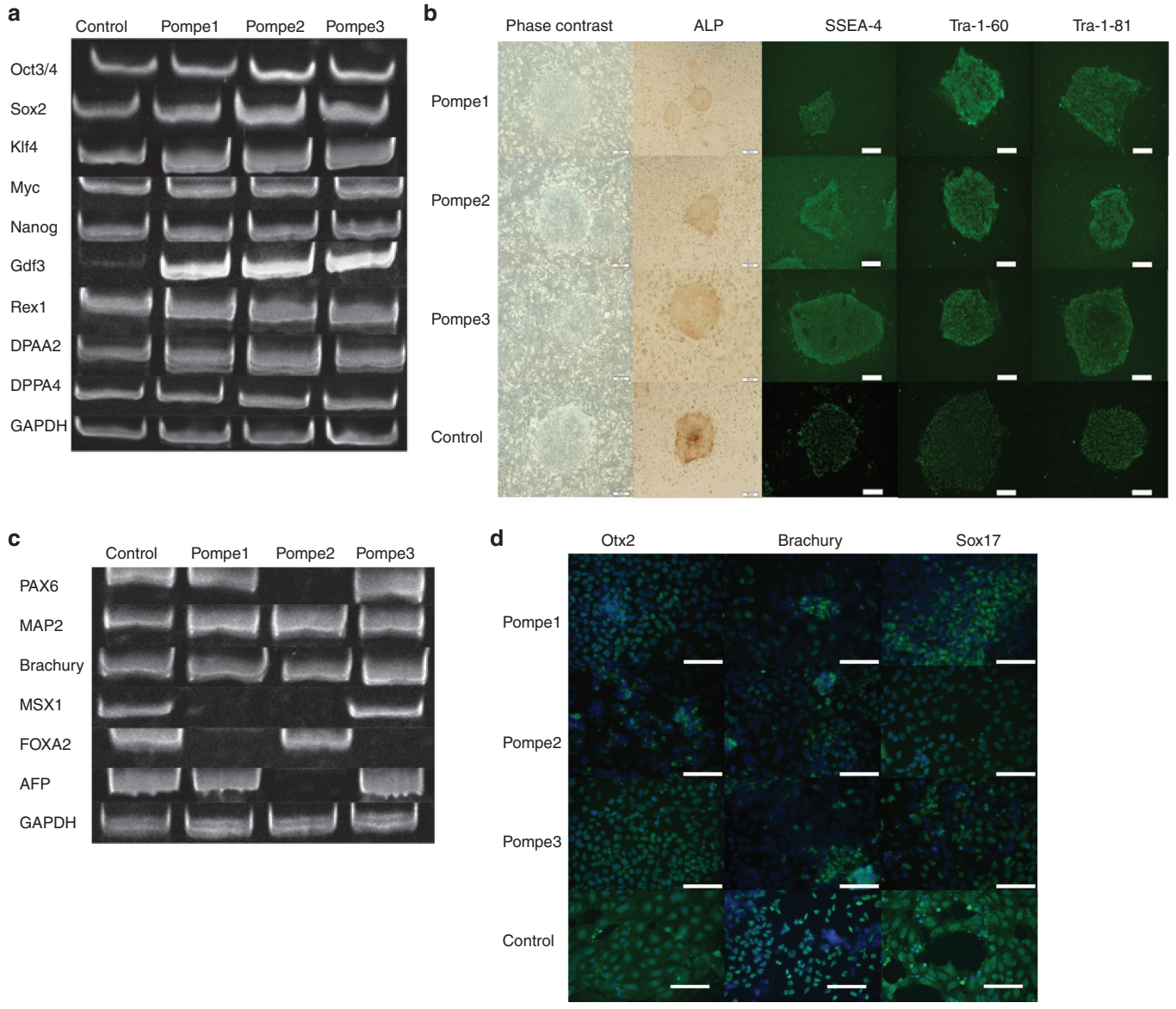

Figure 1 Characterization of iPS cell lines. (**a**) Reverse transcription polymerase chain reaction of iPS cell lines (Control, Pompe1, Pompe2, and Pompe3). Oct3/4, Sox2, Klf4, Myc, Nanog, Gdf3, Rex1, DPPA2, DPPA4, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were analyzed. (**b**) Immunofluoroscence of iPS cell lines (Pompe-1, Pompe2, Pompe3, and control). ALP stain, SSEA-4 (Alexa488), Tra-1–60 (Alexa488), and Tra-1–81 (Alexa488) were analyzed. Scale bar, 100 µm. (**c**) RT-PCR of embryoid body from iPS cell lines (Control, Pompe1, Pompe2, and Pompe3). PAX6, MAP2, Brachury, MSX1, FOXA2, AFP, and GAPDH were analyzed. (**d**) Immunofluoroscence of directed differentiated three germ layers. Otx2 (Alexa488), Brachury (Alexa488), and Sox17 (Alexa488) were analyzed. Scale bar, 100 µm.

Electron microscopy showed lysosomal accumulation of glycogen in Pompe disease iPSCs compared to control (Figure 2b). Patient-specific iPSC lines show the weak expression of GAA compared to the healthy control iPSC (Figure 2c). Pompe disease iPSCs have increased glycogen content compared to the healthy control iPSC (Figure 2d). Pompe disease iPSCs have disease-specific characteristics both pathologically and biochemically.

Pathological hallmarks of cardiomyocyte derived from Pompe disease iPSC

We have differentiated iPSCs into cardiomyocyte according to the differentiation protocol shown in Figure 3a. Robust differentiation was also possible in healthy control and Pompe disease iPSCs (Figure 3b). Beating cardiomyocyte was observed around 10 days after the differentiation in healthy control and patient specific iPSCs (Supplementary Movies S1 and S2).

RT-PCR showed the expression of cardiomyocyte markers including MLC2A, MLC2V, NKX2.5, MYH6, MYH7, and cardiac troponin I (cTnI) in control and Pompe disease iPSC-derived cardiomyocytes (Figure 3c).

Cardiac troponin T (cTnT) positive, mature cardiomyocyte derived from control and Pompe disease iPSCs were observed by immunofluorescence (Figure 3d). From these observations, we successfully differentiated cardiomyocyte form PD and control iPSCs and characteristics of both cardiomyocyte were similar.

Electron microscopy showed control and Pompe disease iPSC-derived cardiomyocyte has sarcomeric cardiac fibers with gap junction. Massive accumulation of glycogen in the lysosome of the cardiomyocyte derived from Pompe disease iPSCs, not from control, were also observed (Figure 3e). Interestingly, there is no remarkable change in the structure of the cardiomyocyte fiber, such as disarray and hypertrophy.

Cellular pathology of late-onset Pompe disease is reflected in patient-specific iPSC-derived cardiomyocyte.

GAA rescue ameliorates the disease phenotype of Pompe disease iPSCs

Third-generation lentiviral vector which express GAA has been generated (Figure 4a). Then we have infected lentiviral vector to Pompe disease iPSCs at the multiplicity of infection of 0, 10, 50, and 100. GAA enzyme activity was increased in dose-dependent manners (Figure 4b). Glycogen contents were significantly decreased by GAA transduction by lentiviral vector only in the highest multiplicity of infection ($P < 0.01$). Glycogen contents were not normalized in treated iPSCs within 48 hours (Figure 4c).

Immunofluorescence using anti-GAA antibody reveals that GAA expression of patent specific iPSC was increased by lentiviral gene therapy according to the strength of the transfection (Figure 4d). Immunofluorescence using anti-GAA antibody and anti-LAMP-2 antibody also reveals that rescued GAA in Pompe disease iPSC was co-localized with lysosomal protein, LAMP-2, which is indicating expressed GAA was localized in lysosome (Figure 4e).

Electron microscopy showed amelioration of disease hallmarks in iPSC morphology such as glycogen accumulation and lysosomal enlargement (Figure 4f).

Lentiviral GAA transfer ameliorates pathological and biochemical abnormality seen in patient-specific iPSC.

The efficacy of GAA rescue remains after cardiomycote differentiation

The efficacy of lentiviral gene transfer after the cardiomyocyte differentiation has been assessed. Dose-dependent expression of GAA was observed in enzyme assay in every iPSC-derived cardiomyocyte (Figure 5a). Immunofluorescence shows GAA positive-cTnT positive

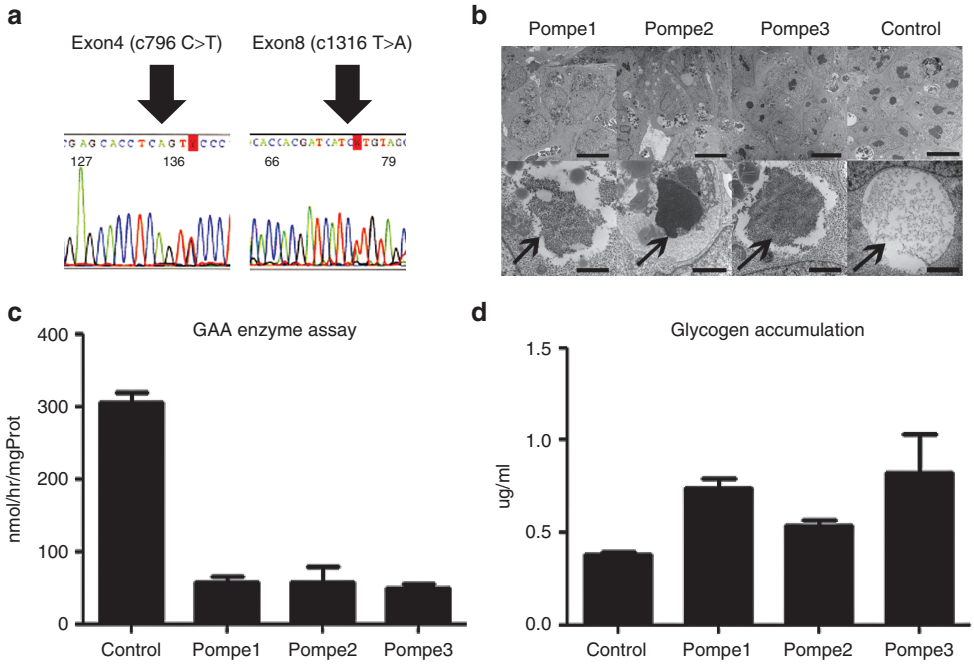

Figure 2 Disease-specific change of Pompe disease iPSCs. (**a**) Mutation analysis shows compound heterozygote mutation (c.796 C>A and c.1316 T>A). (**b**) Electron microscopy of iPS cell lines (Pompe-1, Pompe2, Pompe3, and control). Arrow is demonstrating accumulated glycogen. Upper scale bar, 5 µm; lower scale bar, 1 µm. (**c**) GAA enzyme assay of iPS cell lines (Control, Pompe1, Pompe2, and Pompe3). Data were expressed as means ± SEM. (**d**) Glycogen assay of iPS cell lines (Control, Pompe1, Pompe2, and Pompe3). Data were expressed as means ± SEM.

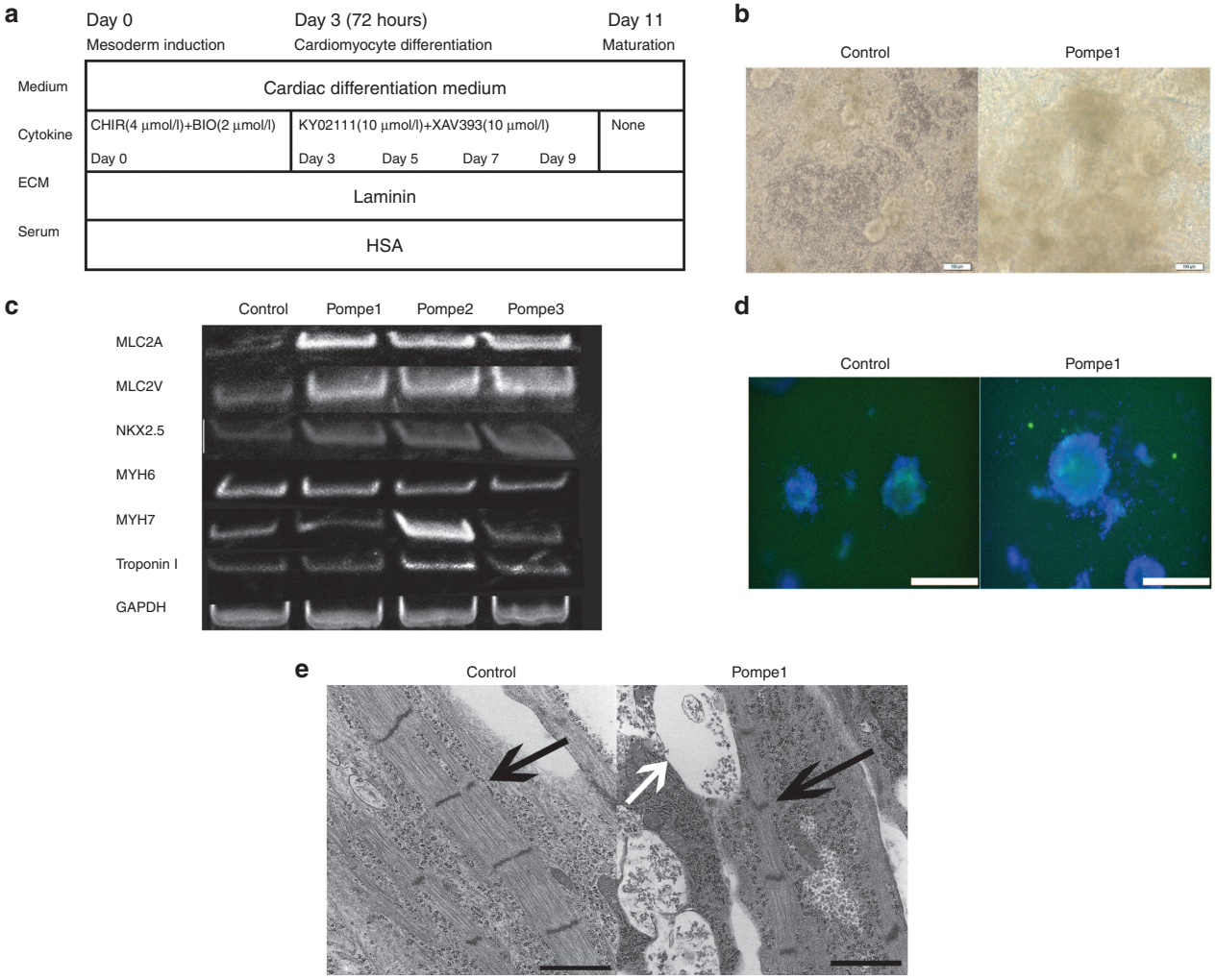

Figure 3 Cardiomyocyte differentiation of Pompe disease iPSC. (**a**) Differentiation protocol. (**b**) Phase contrast microscopy of derived cardiomyocyte from Control and Pompe-1 iPSC. Scale bar, 100 μm. (**c**) RT-PCR of differentiated cardiomyocyte. MLC-2A, MLC-2V, NKX2.5, MYH6, MYH7, Troponin I, and GAPDH were analyzed in cardiomyocyte (Control, Pompe1, Pompe2, and Pompe3). (**d**) Immunofluoroscence of differentiated cardiomyocte (Control and Pompe1). Cardiac troponin T (Alexa488) and DAPI were stained after 4% PFA fixation. Scale bar, 100 μm. (**e**) Electron microscopy of cardiomyocyte (Control and Pompe1). Black arrow is indicating sarcomeric cardiac fiber connected with gap junction and white arrow is indicating enlarged lysosome. Scale bar, 1 μm.

cardiomyocyte in each Pompe disease derived cardiomyocyte after gene transfer (Figure 5b). Electron microscopy showed the amelioration of cellular pathology, such as glycogen accumulation and lysosomal enlargement after the GAA gene transfer (Figure 5c).

The efficacy of lentiviral gene transfer is maintained after the cardiac differentiation and cellular pathology is improved by GAA rescue in dose-dependent manner.

DISCUSSION

Pompe disease is classified into the infantile type and the late-onset type according to the clinical phenotype. One of the remarkable differences between infantile and late-onset type is the disease onset and clinical phenotype is not identical. Cardiovascular complication is another important phenotype and major clinical finding particularly in the infantile onset disease. However, the cardiovascular complication is now seeking attention even in the LOPD.

Some report showed that cardiovascular complication such as cardiac hypertrophy and arrhythmia are sometime seen in the LOPD, usually less frequent and severe than infantile onset.[9,10] In recent, Lee *et al.*[12] showed five LOPD patients complicated with hypertrophic cardiomyopathy. It is difficult to investigate the disease mechanism of cardiac complication of LOPD because it is difficult to obtain human sample and there is no appropriate disease model which simulates cardiac complication of LOPD.

To solve this problem, we have generated iPSC from LOPD patient and attempted to conduct disease modeling. Our results are compatible with the previous clinical observations because pathological change in the cardiomyocyte is reproduced *in vitro*. Our model is useful for the investigation of pathological mechanism occurred in the cardiomyocyte of LOPD. Since the discovery of human pluripotent stem cell, a lot of research about patient-specific iPSC has been carried out. Disease modeling, drug screening, and cell therapy are main topics of the stem cell research.[13,14] Our investigation shows that disease modeling of LOPD and gene correction with lentiviral gene transfer.

Most of the Pompe disease patients are currently receiving enzyme replacement therapy (ERT). In infantile form, the drastic improvement of survival is achieved according to the ERT.[3] Similarly, in late-onset form, clinical improvement is reported.[4] However, the refractory mechanism to enzyme replacement therapy (ERT)

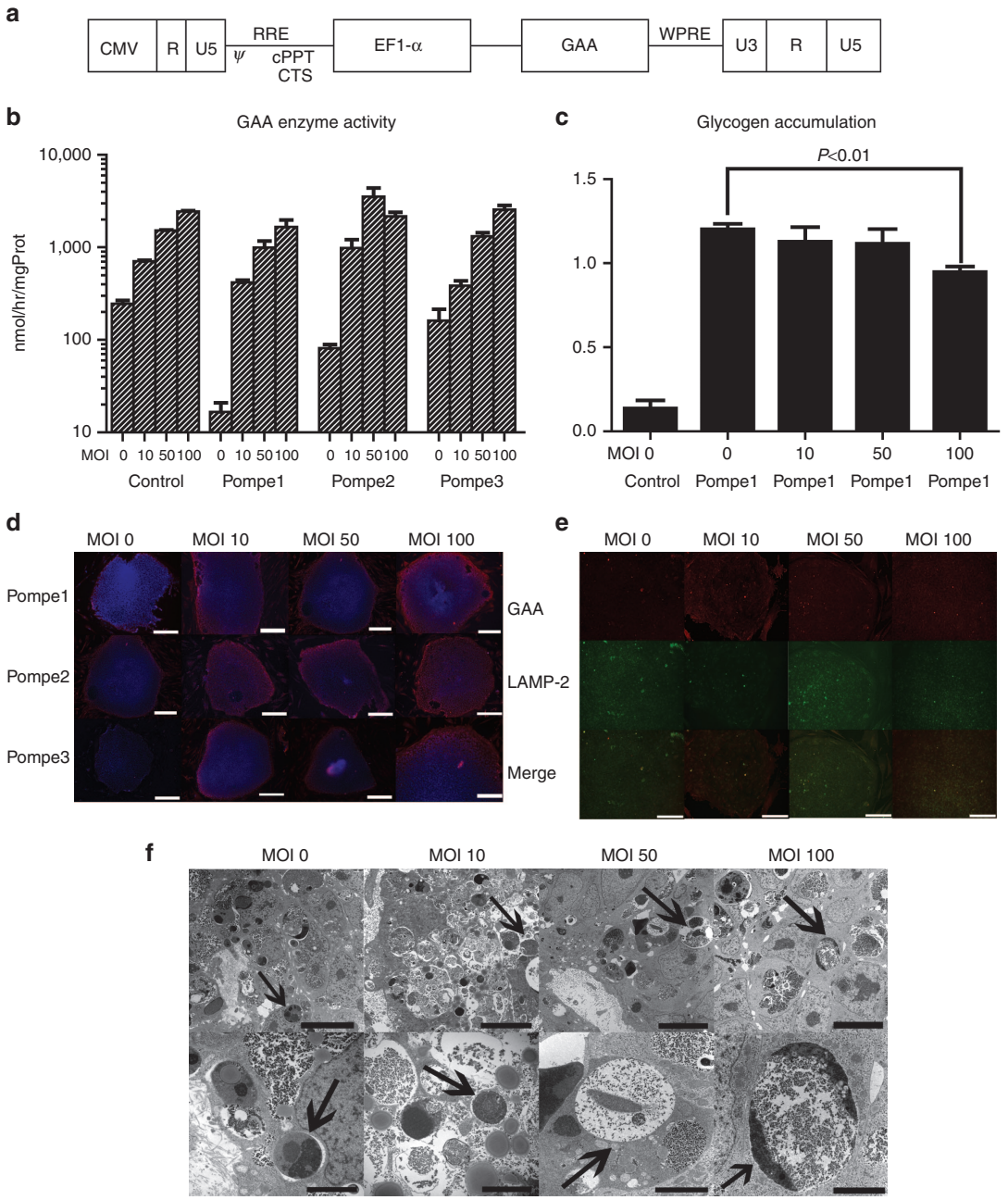

Figure 4 Gene transfer to Pompe disease iPSC. (**a**) Vector construct. GAA was cloned into downstream of EF-1-α promoter. (**b**) GAA enzyme assay of transfected iPS cell lines (Control, Pompe1, Pompe2, and Pompe3). Transfection is conducted at the multiplicity of infection of 0, 10, 50, and 100. Data were expressed as means ± SEM. (**c**) Glycogen assay was conducted in iPSCs (Control, Pompe1, Pompe2, and Pompe3). Data were expressed as means ± SEM. (**d**) Immunofluoroscence of transfected iPSCs (Pompe1, Pompe2, and Pompe3). GAA (Alexa568) were stained with DAPI. Scale bar, 100 µm. (**e**) Immunofluoroscence of transfected iPSCs (Pompe1) GAA (Alexa568) and LAMP-2 (Alexa488) were stained to confirm colocalization. Scale bar, 100 µm. (**f**) Electron microscopy of iPSCs after lentiviral GAA transfer. Upper scale bar, 5 µm, lower scale bar, 1 µm.

such as neutralizing antibody formation and autophagic buildup is considered.[15,16] Lentiviral gene therapy is one of the alternative treatment modality to PD patient.[17] Stem cell therapy such as differentiated pluriptent stem cell replacement is another strategy to treat monogenic disorder and can be combined with the viral gene therapy and homologous recombination.

In our model, gene dosage effect in pluripotent stem cell was evaluated in the context of lentiviral gene therapy to monogenic disease. Our data show that high-intensity gene therapy could rescue the disease phenotype of PD. Disease biochemical phenotype is recovered in terms of enzyme activity, glycogen accumulation, and colocalization of enzyme. Glycogen reduction is statically significant compared to nontreated iPSC but the total content of glycogen was not normalized in 48 hours after transduction. There are two possible explanations why glycogen level was not normalized even if enzyme activity increased over wild-type iPSC. One of the explanations is that very few cells were transduced and glycogen was not reduced in untransduced cells. The other explanation is that 48 hours is not enough to reduce glycogen profoundly. Using same vector construct expressing fluorescent protein, almost 60% wild type were transducted (data not shown). Thus, the former possibility is unlikely. It is speculated that it takes more time to normalize

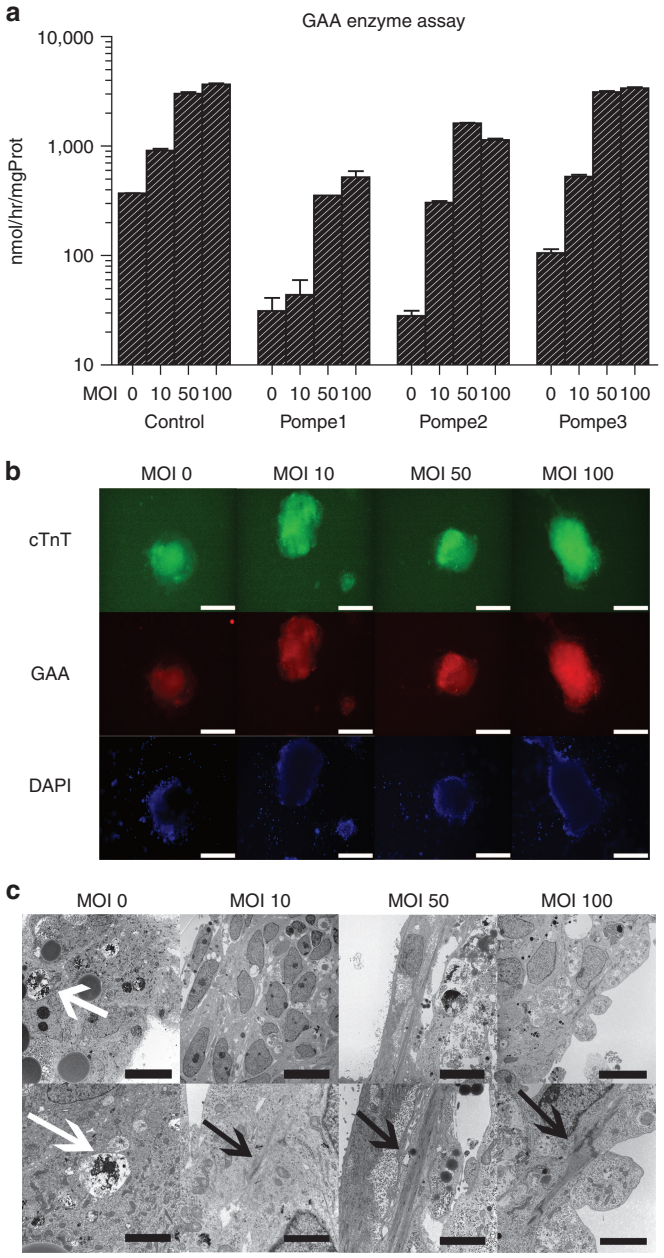

Figure 5 Cardiac differentiation after gene transfers. (**a**) Acid α-glucosidase (GAA) enzyme assay of differentiated cardiomyocyte (Control, Pompe1, Pompe2, and Pompe3). Data were expressed as means ± SEM. (**b**) Immunofluoroscence of differentiated cardiomyocyte (Pompe1). Cardiac troponin T (Alexa488) and GAA (Alexa568) were stained with DAPI. Scale bar, 100 μm. (**c**) Electron microscopy of cardiomyocyte (Pompe1) after gene transfer. Black arrow is indicating sarcomeric cardiac fiber and white arrow is indicating enlarged lysosome. Upper scale bar, 5 μm, lower scale bar, 1 μm.

glycogen content in cells even if very high level of enzyme is exist in cells.

Cell therapy based on iPSC technology is a promising therapeutic modality in the context of the treatment of genetic diseases. In fact, clinical trial of iPSC-based therapy is ongoing in the retinal disease. Gene addition such as lentiviral gene transfer will be combined with stem cell differentiation and differentiated stem cell might be transplanted. In terms of safety, homologous recombination is much more promising because we should concern tumorigenicity if we use integrating viral vector. On the other hand, higher expression will be achieved in lentiviral gene transfer and the amount of transplanted cell to be required to therapeutic effect can be reduced compared to homologous recombination. Thus lysosomal storage disorder is the ideal target of gene transfer based iPS cell therapy because the cross correction of therapeutic protein might improve untransduced cells.

Tedesco *et al*.[18] successfully treated limb-gurdle muscular dystrophy model mouse by human patient derived pluripotent stem cell. In this study, they differentiated patient-derived pluripontent stem cell into meso-angioblast and then infected lentiviral vector targeting for α-sarcoglycan. Transplanted immunodeficient model mice lacking α-sarcoglycan showed phenotypical correction and it is proof of principle study for differentiated pluripotent stem cell replacement combined with lentiviral gene transfer.

Other strategy is differentiated pluripotent stem cell combined with gene correction without chromosomal integration such as *sleeping beauty* transposone system. Filareto *et al*.[19] showed that they have generated pluripotent stem cell from tip tail fibroblast of mice lacking both dystrophin and utrophin and replaced therapeutic gene by *sleeping beauty* transposone system targeting for micro-utrophin. They transplanted differentiated gene corrected pluripotent stem cell and disease phenotype of model mouse is recovered. Our study also suggests that it is possible to investigate differentiated pluripotent stem cell replacement therapy also to the late-onset Pompe disease.

Limitation of our study is the number of cell lines of iPSC. We could not obtain iPSC from the infantile onset and did not compare the phenotypes. Three different clones from one patient were examined in this study. Previous reports have used infantile-onset PD iPSC, so our model is unique in the context of the phenotypic variation of monogenic disease. Our PD iPSCs have residual enzyme activity which might cause more modest disease-specific change and treatment efficacy might be easy to be obtained. Comparison between infantile- and late-onset Pompe disease might be important because underlying mechanism could not be identical and the difference among patients could be evaluated by pluripotent stem cell research. Inclusion of infantile/early-onset PD cardiomyocyte would have also added valuable information to our study. Further study including multiple patient cell lines should be conducted to investigate disease mechanism and variation of infantile and late onset Pompe disease. It is suggested that our investigation could bring the possibility of expansion of the pluripotent stem cell research in the field of Pompe disease research.

MATERIALS AND METHODS

iPSC generation

Patient-specific iPSC (HSP0175, 0176, 0177; Pompe1, 2, 3) were kindly provided from RIKEN BRC. Healthy control iPSC (HPS0223; Control) was also provided from RIKEN BRC.[20] Both iPS cell lines were generated by introducing four reprogramming factors, Klf-4, Oct 3/4, Sox-2, and c-Myc, by Sendai virus.[21]

iPSC culture

iPS cell lines were cultured by Dulbecco's Modified Eagle's Medium/F12 (Sigma Aldrich, St Louis, MO), 20%KSR (Life Tech, Carlsbad, CA), 1%glutamine (Life Tech), 1%Penicillin-streptomycine (Life Tech), 0.01% β-melcaptoethanol (Life Tech), 5 ng/ml bFGF (Wako Pure Chemical, Japan). Radiation inactivated SNL cells were used as feeder cells and collagen I coated dish (AGC TECHNO GLASS, Japan) was used. In feeder-free culture, Corning Synthemax (Corning, Tewksbury, MA) was used as extracellular matrix and mTeSR1 (StemCell Technologies, Vancouver, Canada) was used as culture medium.

In vitro differentiation

To form embryoid body, 1*10^6 feeder depleted iPSC was transferred to petri dish and maintained by floating culture for 28 days.[22] iPSC medium containing bFGF was changed every 2 days. Directed differentiation was conducted by Human iPSC characterization kit (R&D systems, Minneapolis, MN).

Cardiac differentiation

Confluent iPSC was transferred to petri dish (BD Biosciences, San Jose, CA) for aggregation, after 8–24 hours cells were allowed to attach Laminin coated six-well plate(BD) at the density of 3–10*10^6 cells in each well. Cardiomyocyte was induced by the cardiac differentiation protocol using Wnt inhibitor (KY02111) as described before.[23] To briefly describe, CHIR99021 and BIO were added to the medium until 72 hours after the differentiation and then KY02111 and XAV393 were added to the medium until 10 days after the differentiation.

Mutation analysis

Genomic DNA was extracted from iPSCs. For gene analysis, intron-franking primer was designed to detect genetic mutation (Supplementary Table S1).

RT-PCR

Total RNA was collected and RT-PCR was done by Primescript One-step RT-PCR kit (TAKARA BIO, Japan). RT-PCR primers are shown in (Supplementary Table S2).

Immunofluorescence

iPSC was reacted with primary antibody, SSEA-4, Tra-1–60 and Tra-1–81 using ES cell characterization kit (Milltenyi Biotec, Germany).

Differentiated three germ layers were stained with anti-Otx2, Brachury and Sox17 antibodies (R&D) and nuclei were stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (Dojindo, Japan).

Patient-specific iPSCs were stained with anti-GAA antibody (Sigma) and anti-LAMP2 antibody (Santa Cruz Biotechnologies, Dallas, TX) and nuclei were stained with DAPI (Dojindo) .

Cardiomyocyte was stained by cardiac troponin T antibody (Thermo Fisher Scientific, Waltham, MA), anti-GAA antibody and nuclei were stained with DAPI (Dojindo).

Electron microscopy

Electron microscopy analysis was done after 2%glutalaldehyde fixation and followed by 1% osmium tetraoxide fixation. Ethanol dehydration followed by epon embedding was done.

Lentivirus generation and infection

Third-generation lentivirus vector plasmid (CS2-EF1α-MCS) was kindly provided form Dr. Miyoshi (RIKEN BRC). GAA was cut out using EcoR1 site and then ligated into CS2-EF1α-MCS digested by EcoR1.

In viral production, vector, gag/pol, REV and VSVG plasmid was cotransfected into HEK293T cell (1,200, 780, 300, and 420 μg). Collected viral containing supernatant was concentrated by centricon (EMD Millipore, Germany) and ultra-centrifuge, then viral titer was measured by p24 ELISA using Quick Titer HIV Lentivirus quantitation kit (Cell Biolabs, San Diego, CA).

Transfection was done by multiplicity of infection 0, 10, 50, and 100 to the 5*10^5 iPSC including Pompe1, 2, 3 and Control. A final concentration of 8 μg/ml polybrane (Sigma) was added to the medium.

Enzyme assay

Collected cells were homogenized into sterile water and protein concentration was measured by BCA protein assay kit (Thermo Scientific). GAA enzyme assay was done by 4-MU-a-glucopyronside (Sigma) assay as previously described.

Glycogen content analysis

Collected cells were homogenized into sterile water. Glycogen was measured by the Glycogen Colorimetric/ Fluorometric assay kit (BioVision, Milpitas, CA).

CONFLICT OF INTEREST

T.O., H.I., and Y.E. have active research support from Genzyme Japan Co., Ltd. and Shire Japan Co., Ltd. These activities have been fully disclosed and are managed under a Memorandum of Understanding with the Conflict of Interest Resolution Board of The Jikei University School of Medicine.

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