Human Pompe disease-induced pluripotent stem cells for pathogenesis modeling, drug testing and disease marker identification

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Pompe disease is caused by autosomal recessive mutations in the acid alpha-glucosidase (GAA) gene, which encodes GAA. Although enzyme replacement therapy has recently improved patient survival greatly, the results in skeletal muscles and for advanced disease are still not satisfactory. Here, we report the derivation of Pompe disease-induced pluripotent stem cells (PomD-iPSCs) from two patients with different GAA mutations and their potential for pathogenesis modeling, drug testing and disease marker identification. PomD-iPSCs maintained pluripotent features and had low GAA activity and high glycogen content. Cardiomyocyte-like cells (CMLCs) differentiated from PomD-iPSCs recapitulated the hallmark Pompe disease pathophysiological phenotypes, including high levels of glycogen and multiple ultrastructural aberrances. Drug rescue assessment showed that exposure of PomD-iPSC-derived CMLCs to recombinant human GAA reversed the major pathologic phenotypes. Furthermore, L-carnitine treatment reduced defective cellular respiration in the diseased cells. By comparative transcriptome analysis, we identified glycogen metabolism, lysosome and mitochondria-related marker genes whose expression robustly correlated with the therapeutic effect of drug treatment in PomD-iPSC-derived CMLCs. Collectively, these results demonstrate that PomD-iPSCs are a promising in vitro disease model for the development of novel therapeutic strategies for Pompe disease.

INTRODUCTION

Pompe disease (1) (also known as glycogen storage disease type II) is an autosomal recessive disorder caused by mutations in the gene encoding the lysosomal glycogen-degrading enzyme, acid alpha-glucosidase (GAA) (EC 3.2.1.20) (2,3). Pompe disease can be classified into infantile-onset (1) and late-onset forms (4) according to the time of onset, which may be related to the residual activities of mutated enzymes (5). Patients with the infantile-onset disease typically manifest hypotonia and signs of heart failure between 3 and 5 months of age. Without treatment, most patients die by the age of 18 months (6). The cellular and ultrastructural pathology of Pompe disease has been recently characterized in myocytes (7,8). According to the characterization of Thurberg et al. (7), several consecutive pathophysiological events, such as
the accumulation of membrane-bound glycogen at an early stage, increasing cytoplasmic glycogen particles resulting from the rupture of lysosomes, mitochondrial aberrance and progressive autophagic build-ups, occur in Pompe disease muscle cells.

After several successful pilot studies (9–11), recombinant human GAA (rhGAA), named alglucosidase alfa (Lumizyme and Myozyme), has been approved for use for Pompe disease. The rhGAA enzyme replacement treatment (ERT) has provided a dramatic improvement in survival, respiratory function and cardiomyopathy in patients with the infantile-onset form of disease (12–14), and also improved the walking distance and stabilized the pulmonary function in patients with late-onset Pompe disease (15,16). Although rhGAA ERT is currently the only clinically effective therapy for Pompe disease, the therapeutic effect of rhGAA is variable in skeletal muscles, partially owing to significant autophagy dysfunction and the subsequent trapping of rhGAA in autophagic areas in muscle cells (17). Additionally, the immunogenic reaction caused by the frequent repeat administration of a high amount of rhGAA also limits the effectiveness of ERT (12,14,18). Therefore, further efforts are needed to identify novel therapeutic options for Pompe disease.

Our current understanding of the pathophysiological progression of Pompe disease during development is still limited, partly due to the difficulty in obtaining culture specimens from patients. To develop efficient therapies, a more thorough understanding of the pathophysiological development of Pompe disease at the cellular level is essential. Although several types of GAA knockout mice (19,20) have been created to study the pathophysiological progression and drug effects of the disease, these mice may be restricted in their use because none of them are able to mimic the whole spectrum of conditions of human Pompe disease that are caused by many different mutations. Thus, other models that can better mimic Pompe disease in humans are needed to address the problem of improving treatment.

Recently, it has been demonstrated that the generation of induced pluripotent stem cells (iPSCs) from both mouse (21,22) and human (23,24) somatic cells by defined factors is possible and these cells resemble human embryonic stem cells (hESCs) in many aspects. Furthermore, several studies have successfully demonstrated that iPSCs generated from human patients with different genetic diseases (25–35) can be used to model the pathogenesis and treatment of human diseases. Nevertheless, the generation of iPSCs from diseased somatic cells (such as those found in the infantile form of Pompe disease) is challenging, as reprogramming efficiency may be compromised by their severe pathophysiological defects.

In this study, we successfully generated Pompe disease-specific human iPSCs (PomD-iPSCs) and show that these cells possess hESC characteristics and pluripotent developmental propensity. Furthermore, we show that PomD-iPSCs are able to give rise to cardiomyocyte-like cells (CMLCs), which exhibit multiple pathophysiological phenotypes of Pompe disease in vitro. We also attempted to identify new disease markers and test whether other drugs, when used in combination with rhGAA, can reverse the in vitro disease phenotypes. Our results suggest that PomD-iPSCs are a useful in vitro disease model of human Pompe disease that can be used for multiple diagnostic and therapeutic purposes.

RESULTS

Derivation and characterization of PomD-iPSCs

To generate iPSCs, dermal fibroblasts were obtained from four patients with Pompe disease and transduced with retroviral vectors encoding for OCT4, SOX2, KLF4 and c-MYC (OSKM) as described previously (23). In total, we carried out seven consecutive trials in which 17 fibroblast samples (6, 3, 4 and 4 samples from patients A, B, C and D, respectively) were used for reprogramming experiments (Supplementary Material, Table S1). However, none of these experiments yielded iPSC clones (Supplementary Material, Table S1). We excluded the possibility that this failure resulted from technical problems as we were able to generate human iPSC (hiPSC) lines from unaffected control fibroblasts using the same reprogramming procedures at the same time (36). Since massive glycogen accumulation in lysosomes and cytoplasm often result in a significant compromise of cellular functions (37), we reasoned that it might be the cellular physiological defects resulting from the GAA mutation that were responsible for the failure to generate iPSCs. We, thus, rescued the Pompe fibroblasts by conditional over-expression of GAA with an inducible lentiviral vector prior to the transduction with retroviral vectors encoding for OSKM (Fig. 1A). By GAA rescue, we were able to generate PomD-iPSCs from fibroblasts of two patients (20 and 4 lines from patients A and B, respectively; Supplementary Material, Table S1). Genomic PCR analysis indicated that all the tested PomD-iPSC lines contained viral OSKM or OSK integrations but only 7 out of 24 clones contained viral GAA integrations (Supplementary Material, Table S4). We next examined the expression of exogenous and endogenous hESC marker genes and mutant and exogenous wild-type GAA in the PomD-iPSC lines (Supplementary Material, Table S5 and Fig. 1B). Reverse-transcription–polymerase chain reaction (RT–PCR) analysis showed that they all expressed endogenous hESC marker genes (Supplementary Material, Table S5 and Fig. 1B), and most of them (18 out of 24 clones) did not express exogenous GAA (Supplementary Material, Fig. S1A). To further determine the cellular and functional characteristics of the PomD-iPSCs, we selected four optimal iPSC lines (cell lines A10, A17, A25 and B03), which expressed endogenous OSKM (Supplementary Material, Fig. S1A) and mutant GAA (Fig. 1B), silenced exogenous OSKM (Supplementary Material, Fig. S1B) and did not have the viral GAA integration (Supplementary Material, Fig. S1C). These PomD-iPSCs expressed alkaline phosphatase and hESC markers homogenously, including NANOG, OCT4, SSEA3 and SSEA4 (Fig. 1C). Genomic sequencing analysis confirmed that these lines still contained the Pompe disease-specific mutations (Fig. 1D). Furthermore, comparative hierarchical clustering analysis of genome-wide gene expression profiles of three PomD-iPSC cell lines, two hESC lines and parental fibroblasts showed that PomD-iPSCs were more similar to hESCs than to their parental fibroblasts.
To assess the pluripotent properties of PomD-iPSCs, we generated embryoid bodies (EBs) from PomD-iPSCs. After replating EBs onto gelatinized culture dishes, we observed that they spontaneously differentiated into cells with varied morphology. Furthermore, RT–PCR and immunofluorescence (IF) staining showed that the differentiated PomD-iPSCs expressed marker genes and proteins of all three embryonic germ layers (Fig. 2A and B). To examine their in vivo developmental propensity, we injected undifferentiated PomD-iPSCs (lines A10 and B03) intramuscularly into NOD-SCID mice and the formation of teratomas that contain cells representing all three embryonic germ layers was confirmed (Fig. 2C). Collectively, these results demonstrate that fully reprogrammed PomD-iPSCs with in vivo and in vitro pluripotent propensity can be effectively derived from Pompe disease fibroblasts.

PomD-iPSCs manifest partial pathophysiological features of Pompe disease

To investigate whether PomD-iPSCs exhibited the distinct pathophysiological features of Pompe disease, we compared GAA enzyme activity, glycogen content, mitochondrial function and ultrastructural changes among hESCs, unaffected hiPSCs and PomD-iPSCs. All PomD-iPSC lines had remarkably depressed GAA activities compared with hESCs, as did
their parental fibroblasts (Fig. 3A). The glycogen assay also showed that the glycogen content in PomD-iPSCs was significantly higher than that in hESCs and unaffected hiPSCs (Fig. 3B). With the same genetic background, the PomD-iPSCs with the integrated GAA transgene can act as an autologous control to the PomD-iPSCs. We, thus, further examined the GAA activity and the glycogen content of the PomD-iPSC clones with GAA transgene rescue (A11, A13 and A20 from patient A and B04 from patient B), although some of the viral OSKM genes had not been silenced completely in these four clones. The GAA activity in the GAA transgene-rescued PomD-iPSC clones increased remarkably to a normal level after doxycycline induction (Supplementary Material, Fig. S3A). And again, the glycogen content in all four GAA-rescued PomD-iPSC clones was also significantly reduced after GAA induction by doxycycline (Supplementary Material, Fig. S3B). The above results thus demonstrated that the observed phenotypes are indeed due to the GAA mutation and that can be alleviated with the wild-type GAA transgene in the same genetic background.

As mitochondrial dysfunction is one of the pathophysiological features of Pompe disease cells, we thus examined...
the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of PomD-iPSCs. The intact cellular respiration assay revealed that PomD-iPSCs had significantly lower OCR and ECAR than hESCs and unaffected hiPSCs (Fig. 3C), suggesting mitochondrial dysfunction in the PomD-iPSCs. Electron microscopy analysis (Supplementary Material, Fig. S4A–E) showed that PomD-iPSCs exhibited some Pompe-disease ultrastructural abnormalities, such as freely dispersed glycogen (Supplementary Material, Fig. S4E), in comparison with hESCs, hiPSCs and their parental fibroblasts. In addition to the common cellular pathophysiological phenotypes of Pompe disease, we also noted that the growth rate of PomD-iPSC was slower than that of hESCs, as demonstrated by the growth curve analysis (Fig. 3D). Together, these results demonstrated that PomD-iPSCs have already manifested partial Pompe disease-specific pathophysiology even at the pluripotent stage.

**PomD-iPSCs differentiate into CMLCs that recapitulate Pompe disease-specific cellular and ultrastructural abnormalities**

Since the heart is one of the most important organs targeted by Pompe disease, we examined whether cardiomyocytes derived from PomD-iPSCs also exhibit the typical pathophysiological features of Pompe disease. To test whether PomD-iPSCs were able to generate cardiomyocytes, we induced *in vitro* differentiation via EB formation in PomD-iPSCs, as well as in
unaffected hiPSCs and hESCs. Spontaneously contractile loci (Supplementary Material, Video S1) could be readily observed at day 20 after EBs were replated and cultured in a serum-containing differentiation medium. No obvious differences in the ability to generate CMLCs were observed between PomD-iPSCs and unaffected hiPSCs or hESCs (data not shown). RT–PCR and IF staining showed that CMLCs manually isolated from beating loci of the differentiated PomD-iPSC-expressed genes enriched in cardiomyocytes (Fig. 4A) and stained strongly positive for mature cardiomyocyte markers, such as myosin heavy chain (Fig. 4B), cTnl (also known as TNNI3; Fig. 4B) and α-actinin (Fig. 4B). To test whether the PomD-iPSC-derived CMLCs recapitulated pathophysiological features of Pompe disease, CMLCs derived from PomD-iPSCs and unaffected hiPSCs were analyzed by histochemistry and IF staining. Notably, CMLCs derived from PomD-iPSCs had intense periodic acid-Schiff (PAS) staining, coarse LAMP-1-positive granules and coarse LC3-positive granules in comparison with their counterparts derived from hESCs or unaffected hiPSCs (Fig. 4C and D; LAMP-1, a lysosomal marker; LC3, an autophagosome marker). The glycogen assay also showed that the glycogen content in the PomD-iPSC-derived CMLCs was significantly higher than that in hESCs and unaffected hiPSCs (Fig. 4E). Furthermore, an approximately 2-fold increase in glycogen content was found in PomD-iPSC-derived CMLCs compared with their undifferentiated PomD-iPSC counterparts (compare Figs 3B and 4E). In addition to the PomD-iPSC clones without the GAA transgene, we also examined the glycogen content of CMLCs derived from four PomD-iPSC clones with GAA transgene rescue and found that their glycogen content was significantly reduced after doxycycline induction (Supplementary Material, Fig. S3C). However, the number of available beating foci derived from these clones was lower and their size was smaller than that from the PomD-iPSC clones without the GAA transgene, suggesting interference with CM differentiation by non-silenced viral OSKM genes.

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Figure 4. Characterization of PomD-iPSC-derived CMLCs. (A) RT–PCR analysis of mature cardiomyocyte marker gene expression in PomD-iPSC-derived CMLCs. (B) IF staining of cardiomyocyte markers in PomD-iPSC-derived CMLCs. Nuclei were stained with DAPI (blue). Scale bar, 5 μm. (C) Confocal microscopy showed that CMLCs derived from PomD-iPSCs (A10 as the representative clone) contained cells strongly positive for PAS, LAMP-1 or LC3. Inset, higher-magnification views showing coarse cytoplasmic granules positive for LAMP-1 or LC3 (arrows). CMLCs from hESCs were used as a control. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. (D) CMLCs derived from any of the PomD-iPSCs lines had significantly higher glycogen content than that derived from hESC and control (WT) iPSC lines (N = 3, *P < 0.05).
Furthermore, we also subjected the PomD-iPSCs, unaffected hiPSCs and hESC-derived CMLCs to electron microscopy analysis and compared the ultrastructural changes (Fig. 5 and Supplementary Material, Fig. S4F–H). Unlike their counterparts derived from hESCs (Fig. 5A and Supplementary Material, Fig. S4F) and unaffected hiPSCs (Supplementary Material, Fig. S4G), the PomD-iPSC-derived CMLCs exhibited obvious and full-blown ultrastructural abnormalities of Pompe disease (Fig. 5B–D and Supplementary Material, Fig. S4H), including the formation of large glycogen-containing vacuoles (Fig. 5C and D), deteriorated mitochondria with swollen cristae (Fig. 5B and C) and the formation of autophagosome-like structures (Supplementary Material, Fig. S4H). Importantly, the scope and level of the ultrastructural abnormalities in PomD-iPSCs were increased after cardiomyocyte differentiation (Supplementary Material, Fig. S4B, E and H), demonstrating that the ultrastructural pathological changes in PomD-iPSCs progressively increased after differentiation. As expected, the PomD-iPSC-derived CMLCs could be maintained only for a shorter period of time (normally less than 30 days) than their counterparts derived from hESCs or unaffected hiPSCs (>50 days) in vitro. Nevertheless, the survival of CMLCs could be significantly improved as the number of the beating loci was significantly increased after supplementing rhGAA in the differentiation medium (Supplementary Material, Fig. S5). Together, these results demonstrate that PomD-iPSC-derived CMLCs recapitulate multiple hallmark pathophysiological features of Pompe disease.

**Drug treatment rescues pathophysiological phenotypes of differentiated PomD-iPSCs**

Given that PomD-iPSCs and their CMLC derivatives recapitulated the pathophysiological features of Pompe disease, it was thus of interest to know whether they were able to respond to drug stimulation in vitro. To this end, we examined the effects of three drugs: rhGAA, which has been regularly used for Pompe disease treatment; 3-methyladenine (3-MA), which is an autophagy inhibitor; and L-carnitine, which is an endogenous substance required for the transport of fatty acids from the cytosol into the mitochondria. The PomD-iPSCs that underwent CMLC differentiation were exposed to these drugs and assayed for glycogen content and OCR at days 0, 7, 14 and 21 after drug treatment. Our results at day 21 (Fig. 6; see Supplementary Material, Figs S6 and S7 for data at days 0, 7 and 14) showed that the glycogen content of PomD-iPSC-derived CMLCs was significantly reduced ($P < 10^{-4}$) to a level indistinguishable from that of CMLCs derived from hESCs or unaffected hiPSCs after rhGAA or rhGAA plus 3-MA treatment.
(Fig. 6A), whereas treatment of 3-MA or l-carnitine alone did not significantly reduce glycogen content in PomD-iPSC-derived CMLCs. Moreover, OCR analysis demonstrated that treatment with l-carnitine resulted in a significant increase in OCR (\(P<0.05\)) in PomD-iPSC-derived CMLCs (Fig. 6B), whereas 3-MA or rhGAA plus 3-MA had less effect on OCR improvement (Fig. 6B). Collectively, our data confirmed that differentiated derivatives of PomD-iPSCs can functionally respond to drug treatment in vitro and suggest the potential of PomD-iPSCs for use in new drug screening for Pompe disease therapies.

Identification of molecular markers for the evaluation of drug effect in PomD-iPSC-derived CMLCs

To search for markers that may be useful in monitoring the therapeutic effects of drugs in Pompe disease, we performed a comparative genome-wide gene expression analysis on hESCs- and PomD-iPSC-derived CMLCs. We focussed on those genes related to glycogen metabolism (Fig. 7Aa), lysosomes (Fig. 7Ab) or mitochondria (Fig. 7Ac) according to their roles in primary or secondary defects in PomD-iPSCs or their differentiated derivatives. The analysis revealed that the expression levels of 16 genes (data not shown and Fig. 7A) were significantly (by at least 2-fold) down- or up-regulated in CMLCs derived from PomD-iPSCs (in all three lines) in comparison with hESCs. Quantitative RT–PCR (Q-RT–PCR) analysis (Supplementary Material, Fig. S8; eight genes labeled in Fig. 7A) further confirmed that the expression levels of eight genes were significantly different in PomD-iPSCs- and hESC-derived CMLCs. Next, we compared the expression levels of all the eight selected genes in PomD-iPSC-derived CMLCs in drug-treated and untreated groups. We found that the expression level of six out of the eight genes, three down-regulated genes (\(FNBP1, NAAA\) and \(CYB5R3\)) and three up-regulated genes (\(DRAM, IL6ST\) and \(LAMP2\)), strongly correlated with rhGAA or rhGAA plus 3-MA treatment (Fig. 7B), suggesting that these genes may be useful as in vitro molecular markers for monitoring the effects of drugs on PomD-iPSC-derived CMLCs. Among the six genes, we also noted that the expression level of \(CYB5R3\) was significantly associated with the treatment of l-carnitine, but no genes were associated with 3-MA treatment (Fig. 7B). Interestingly, the expression of \(DRAM\) only correlated with the treatment of PomD-iPSC-derived CMLCs from patient A, but not from patient B (Fig. 7B). Finally, to understand whether the expression changes of these six marker genes were cell-type (CMLC)-specific, we examined the expression levels of the above genes in the Pompe-diseased (parental cells of the PomD-iPSCs) and wild-type (parental cells of the wild-type iPSCs) fibroblasts after various drug treatments. Q-RT–PCR analysis showed no significant correlation between expression changes in any of the tested genes and treatment with any of the drugs in the Pompe-diseased fibroblasts with the exception of the expression level of the \(LAMP2\) gene, which was significantly decreased after rhGAA treatment in comparison to the untreated group (Fig. 7C). This suggests that the specificity of these genes as markers for drug treatment is largely cell-type-dependent. Together, our results suggest that these genes may be used as molecular markers to evaluate the efficacy of drug treatment on PomD-iPSC-derived CMLCs.

DISCUSSION

Here we demonstrated the successful derivation of PomD-iPSCs from the fibroblasts of two patients using a rescue-based strategy. We provide evidence that PomD-iPSCs can differentiate into cells representing all three embryonic germ layers, including CMLCs. Pompe disease-specific
biochemical, cellular and ultrastructural abnormalities can be reproduced readily in these cells. Moreover, we found that PomD-iPSC-derived CMLCs can be used as a cellular model to evaluate the efficacy of drug treatment and screen potential markers that can reflect the effects of drugs for Pompe disease.

Recently, several reports that detail promising patient-specific iPSCs for disease modeling have been published, especially for monogenic diseases; however, technical challenges, such as difficulty in reproducing disease-specific pathology in late-onset diseases, have been highlighted (38). Very few studies, if any, have considered the possibility that, in addition to other challenges, somatic cells derived from patients with early-onset or severe diseases (such as infantile Pompe disease) may be compromised by the pathophysiological defects that result from genetic mutation(s), and thus may not be suitable for conversion into iPSCs using a conventional reprogramming strategy. After several failed attempts using a conventional retroviral-based OSKM method to reprogram Pompe-disease fibroblasts, here we adopted a rescue strategy using a lentiviral doxycycline inducible vector to conditionally over-express wild-type GAA based on the rationale that the expression of GAA may improve the cellular function of somatic Pompe disease cells and therefore improve the reprogramming efficiency. This strategy did indeed result in the successful derivation of PomD-iPSC lines exhibiting cellular phenotypes of Pompe disease. Importantly, we found that some of the derived PomD-iPSC lines did not have GAA-expressing lentivirus integration. Since GAA is a secretable enzyme (39), it is tempting to suggest that the correct GAA made from cells rescued by lentiviral GAA infection may supply surrounding cells via a so-called secretion-recapture mechanism (40) and contributes to the improvement of iPSC generation. Thus, our results suggest that such a rescue-based strategy using a wild-type cDNA-containing inducible vector or the recombinant wild-type protein to rescue diseased fibroblasts may be useful to derive patient-specific iPSCs from fibroblasts of early-onset and severe genetic diseases, especially lysosomal storage diseases, if regular derivation methods fail.

The ultrastructural abnormalities of CMLCs differentiated from PomD-iPSCs faithfully recapitulated those seen in patients with Pompe disease (7). Therefore, this in vitro disease model provides an excellent opportunity to observe the very early ultrastructural changes of human Pompe disease at the stage of pluripotent stem cells and early lineage differentiation. Our results showed that obvious mitochondrial abnormalities, such as swollen cristae, are widespread in PomD-iPSC-derived CMLCs. Similar morphological mitochondrial abnormalities have been observed previously in Pompe-disease cells (7,8,41) and were considered as a consequence secondary to lysosomal dysfunction. Thurberg et al. (7) classified the ultrastructural disease progression of Pompe disease into five stages and concluded that apparent abnormalities in mitochondria occur at Stage 3, when rupture of some lysosomes is seen. Furthermore, biochemical and enzymatic abnormalities also exist in Pompe-disease mitochondria, as partial carnitine deficiency has been found to be associated with infantile Pompe disease (41) and most Pompe-disease muscle respiratory enzyme activities are decreased in comparison with control subjects (41). Consistent with these reports, our results showed that PomD-iPSCs and their CMLC derivatives also exhibit the functional abnormalities of Pompe-disease mitochondria. Although we have detected abundant punctate LC3 signals and occurrence of autophagosome-like ultrastructures in the PomD-iPSC-derived CMLCs, we have not been able to identify extensive and florid autophagic abnormalities at the ultrastructural level in our in vitro model as reported in vivo previously (17,42). Therefore, whether our PomD-iPSCs can be used as an in vitro differentiation model to dissect autophagy dysfunction and test new drugs for inhibiting abnormal autophagic events remains to be determined in future studies.

The ability to derive PomD-iPSCs provides an excellent opportunity for us to conduct proof-of-principle studies on drug testing with these cells. Our results demonstrated that supplement of L-carnitine may partially rescue some mitochondrial phenotypes of PomD-iPSC-derived CMLCs. However, our data indicated that rhGAA rescue has no significant effect on the mitochondrial function (OCR). The failure of rhGAA rescue was probably because the duration of rhGAA treatment was not long enough to overcome the downstream mitochondrial abnormalities. Alternatively, lysosomal impairment might have caused intracellular structural destruction that was too severe to be rescued by rhGAA. As suggested by Thurberg et al. (7), the integrity of mitochondria may play an important role in the efficient delivery of rhGAA to the lysosome as an effective receptor uptake of the enzyme is an energy-dependent process. Therefore, our proof of the efficacy of adjunct therapy with L-carnitine in rescuing the mitochondrial function in PomD-iPSC-derived CMLCs may have valuable clinical implications. Furthermore, previously reported phagolysosome build-ups in cells of Pompe disease (42) prompted us to test the effect of 3-MA, an autophagy inhibitor, in suppressing the pathophysiological phenotypes in our PomD-iPSC system. So far, we only found a significant decrease in glycogen storage in the CMLCs derived from the PomD-iPSC line of patient B when 3-MA was used in combination with rhGAA, suggesting either that 3-MA alone had no effect on clearing glycogen or that the dosage (2 mM) of 3-MA used in this study was not sufficient to have a significant effect. Exploration of the effect of other autophagy inhibitors on suppressing autophagic or other pathophysiological phenotypes of Pompe disease in a PomD-iPSC-derived CMLC system will be an important task for identifying novel treatments for Pompe disease.

Taking advantage of the PomD-iPSC system, we identified six molecular biomarkers for monitoring the in vitro therapeutic effect of drugs in PomD-iPSCs. The proteins encoded by FNBP1, NAAA, DRAM and LAMP2 are mainly localized in lysosomes and act as enzymes (43) or regulators of endocytosis (44) or autophagy (45) or play a role in the maintenance of lysosomes (39). Since rhGAA rescue improves the function of lysosomes and may prevent the build-up of autophagy (7), it is conceivable that their expression returned to a level close to normal in response to rhGAA rescue. CYB5R3 is a membrane-bound protein involved in cholesterol synthesis and drug metabolism and also plays an essential role in mitochondrial metabolism due to its contribution to cellular redox homeostasis (46). Therefore, it is unsurprising that CYB5R3...
expression correlated with L-carnitine treatment, which is known to improve mitochondrial energy metabolism. Elevation of the level of IL6ST transcripts in PomD-iPSC-derived CMLCs and their response to rhGAA is an intriguing finding. IL6ST encodes gp130, a well-known signal transducer shared by several cytokines (47); previous studies showed that glycogen availability may affect gp130 expression in human skeletal muscles (48) and gp130 activation signals the cardiomyocyte survival pathway during heart failure (49). Therefore, we reason that increased IL6ST expression possibly reflects a signal of energy restriction and cell distress, which may be reversed after glycogen metabolism is improved by rhGAA rescue.

Our unique method of generating PomD-iPSCs by using a virus-mediated GAA rescue strategy may have further clinical implications. One of the most exciting long-term prospects of iPSCs is their possible application in isogenic cell-based therapy, a promising topic that has been recently advocated and discussed by Byrne (50). Using a combination of virus-mediated GAA rescue, researchers may develop PomD-iPSCs with a near-normal GAA function, which can be differentiated into different cell types and re-introduced into the same patients with Pompe disease to repair dysfunctional tissues.

In summary, we have successfully generated patient-specific iPSCs from patients with Pompe disease using a rescue strategy to circumvent the underlying difficulty in re-programming these severely diseased fibroblasts. Such PomD-iPSCs are capable of recapitulating the biochemical, cellular and ultrastructural abnormalities of Pompe disease after they are differentiated into CMLCs. We have also demonstrated that PomD-iPSC-derived CMLCs can be used as an in vitro disease model to evaluate the efficacy of several drugs in reducing the primary or secondary pathologic-al abnormalities caused by GAA mutations. They can also be used to identify novel genes whose expression faithfully monitors the in vitro effect of drug therapy for Pompe disease. These PomD-iPSCs may, thus, contribute greatly to the improvement of therapy for Pompe disease in the future.

**MATERIALS AND METHODS**

**Isolation of fibroblasts from patients with Pompe disease**

Dermal fibroblasts from four patients (A, B, C and D) with infantile Pompe disease were obtained through skin biopsy with the written informed consent of their parents and used for this study according to the protocol approved by the Internal Research Board of National Taiwan University Hospital. The diagnosis of Pompe disease in these patients was made based on clinical features, enzyme activity assay and the evidence of mutational analysis of the GAA gene by geneticists at the National Taiwan University Hospital. The GAA mutations of these patients are: patient A: c.1935C>A (p.D645E)/c.1935C>A (p.D645E); patient B: c.1935C>A/ c.2040+1G>T; patient C: c.1935C>A (p.D645E)/ c.1935C>A (p.D645E); patient D: c.1062C>G (p.Y354X)/ c.1935C>A (p.D645E).

**Cell culture**

Human Pompe disease fibroblasts, unaffected human fibroblasts (36), mouse embryonic fibroblast cells (MEFs) and 293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), non-essential amino acid, l-glutamine and penicillin/streptomycin at 37°C with 5% CO₂. hESCs, human foreskin-derived hiPSCs (36) and PomD-iPSCs were maintained on mitomycin-C-inactivated MEFs with DMEM/F12 supplemented with 20% serum replacement, non-essential amino acid, l-glutamine, β-mercaptoethanol and 5 ng/ml of basic fibroblast growth factor.

**PomD-iPSC derivation and wild-type GAA rescue**

Derivation of PomD-iPSCs was performed as described previously (24). Briefly, dermal fibroblasts were plated at a density of 8×10^5 cells/10 cm dish in DMEM with 10% FBS. On the next day, a combination of VSV-G-coated retroviruses expressing human OCT3/4, SOX2, KLF4 or MYC was added into target cells twice at a 24 h interval. Four days later, the infected fibroblasts were replated onto mitomycin-C-inactivated MEFs. On the next day, the fibroblast medium was replaced by the hESC medium, as described above. For GAA rescue-based PomD-iPSC derivation, 4 days before retrovirus infection, dural fibroblasts were first infected with lentiviruses containing wild-type human GAA cDNA controlled by a doxycycline-inducible promoter (FUW-TeTO-GAA; Addgene, Cambridge, MA, USA). ESC-like cell colonies emerged approximately 20 days after cells were replated on the feeder layer. They were picked up manually and cultured in conditions for hESC propagation. All experiments involving recombinant DNA were performed according to the US National Institutes of Health guidelines.

**Characterization of PomD-iPSC cells**

Genomic DNA and RNA were extracted from PomD-iPSC clones with TRI Reagent kit (Molecular Research Center, Inc., Cincinnati, OH, USA) and PCR analysis was performed with specific primers as described previously (21,23) to confirm the integration of retroviral transgenes. RT–PCR

**Figure 7.** Identification of novel markers for monitoring the in vitro therapeutic effect of drug treatment on PomD-iPSCs. (A) Gene ontology analysis of cDNA microarray data focussing on genes related to: (a) glycogen metabolism, (b) lysosomes or (c) mitochondria. Sixteen genes with at least 2-fold average expression changes between all three lines of PomD-iPSC-derived CMLCs and hESC2-derived CMLCs (B9) were selected using fold change analysis in GeneSpring software; eight of these genes, whose expression changes were confirmed by Q-RT–PCR (Supplementary Material, Fig. S8), are marked as indicated. (B) Q-RT–PCR analysis verified that the expression changes of six out of eight genes correlated with the therapeutic effect of various drug treatments (based on the data in Fig. 6A and B) on PomD-iPSCs (three lines from patient A and one line from patient B). Data are represented as the fold change relative to the WT-HF control (without drug treatment) and correspond to the mean and SDs of triplicate experiments (A∗P < 0.05). (C) Q-RT–PCR analysis indicated that the expression of five out of six genes (except LAMP2) in (B) did not change significantly with various drug treatments either in normal human fibroblasts (WT-HF) or in Pompe fibroblasts (Pompe-F). In other words, these five genes were more specific for monitoring therapeutic effects in cardiomyocytes. Data are represented as the fold change relative to the WT-HF control (without drug treatment) and correspond to the mean and SDs of triplicate experiments (A∗P < 0.05).
was performed with specific primers (21,23) to assay the expression of viral transgenes of OCT4, SOX2, KLF4 and MYC, their endogenous counterparts and the pluripotency-associated genes. Alkaline phosphatase staining, IF staining, confocal microscopy, RT–PCR, Q-RT–PCR and PAS staining can be found in Supplementary Material, Methods.

**In vitro differentiation of PomD-iPSCs**

For in vitro differentiation, PomD-iPSC colonies were dispersed into small clumps using dispase (Sigma-Aldrich, MO, USA; 1 mg/ml for 30 min) and transferred onto ultra-low attachment plates (Corning, NY, USA) for EB formation. The medium was changed daily for 4 days using the same medium as for routine hESC culture. EBs were then transferred onto 0.1% gelatin-coated culture dishes with the FBS-containing medium. The medium was changed every 2 days for 20 days.

**Teratoma formation assay**

Approximately 1–1.5 × 10^7 PomD-iPSC cells were injected subcutaneously into the back of 6–8-week-old NOD-SCID mice (National Laboratory Animal Center, Taipei). Teratomas were allowed to develop for up to 12 weeks. All the animal experiments were approved by the Animal Care and Use Committee of Academia Sinica and performed in accordance with the Institutional Animal Care and Use Committee guidelines of Academia Sinica.

**Growth curve determination of hESCs/PomD-iPSCs**

The growth curves of hESCs and PomD-iPSCs were analyzed using Real Time Cell Analyzer SP (xCelligence, Roche Applied Science, Germany) according to the manufacturer’s instructions.

**Cardiac differentiation of hESCs/hiPSCs/PomD-iPSCs**

hESCs/hiPSCs/PomD-iPSCs were first dispersed to free-floating colonies using dispase (Sigma-Aldrich; 1 mg/ml for 30 min). The free-floating colonies were then transferred into ultra low attachment six-well plates (Corning) and cultured in suspension for 3 days with cardiac differentiation medium (DMEM/F12 supplemented with 20% FBS, 1% non-essential amino acid, 2 mM l-glutamine, 0.1 mM β-mercaptoethanol and 0.3 mM ascorbic acid) for EB formation. At day 4, EBs were replated onto 0.1% gelatin-coated plastic culture dishes and the cardiac differentiation medium was changed daily. Spontaneously contractile loci could be observed from day 20 onward and observed by stereomicroscope daily. The beating loci were manually isolated under stereomicroscope for other experiments or reseeded onto Matrigel (BD)-coated culture dishes and cultured in the cardiac differentiation medium.

**Cellular glycogen content**

Cellular glycogen content was analyzed using a Glycogen Assay Kit (Biovision, Mountain View, CA, USA). The assay was performed in accordance with the manufacturer’s protocol. All results were analyzed in triplicate and presented as the mean ± standard deviation (SD).

**GAA enzyme activity measurement**

GAA enzyme activity was measured as described previously (51). All results were analyzed in triplicate and presented as the mean ± SD.

**Measurement of intact cellular respiration**

Intact cellular respiration was measured using the Seahorse XF24 Extracellular Flux Analyzers (Seahorse Bioscience, North Billerica, MA, USA) according to the manufacturer’s protocol. hESCs/hiPSCs/PomD-iPSCs were plated at a density of 40 000 cells/well on Matrigel (BD Biosciences, Franklin Lakes, NJ, USA)-coated XF24 tissue culture plates. OCR was measured under basal conditions to assess maximal oxidative capacity. ECAR was measured by determining the change in extracellular pH over time. OCR and EACR measurements were normalized to cell numbers for hESCs/hiPSCs/PomD-iPSCs.

**Electron microscopy**

Fibroblasts, hESCs/hiPSCs/PomD-iPSCs or hESC/hiPSC/PomD-iPSC-derived CMLCs were detached from culture plates, pelleted by centrifugation and fixed in 4% paraformaldehyde and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 4°C. After post-fixing in 1% osmium tetroxide (Electron Microscopy Sciences) for 1 h at room temperature, samples were dehydrated in a gradient alcohol series and immersed in acetone, infiltrated with the increasing ratio of resin to acetone, then embedded in pure Spurr’s resin (Electron Microscopy Sciences). Ultrathin sectioning was performed on a Reichert Jung Ultracut E ultramicrotome. Ultrathin sections (70 nm thickness) were collected on copper grids and stained with uranyl acetate and lead citrate. The sections were examined under a Hitachi 7000 electron microscope (Hitachi, Tokyo, Japan).

**Drug treatment**

rhGAA was made by Genzyme; 3-MA and l-carnitine were purchased from Sigma-Aldrich. Drugs were added into the culture medium and changed every 2 days during the cardiac differentiation procedure. The doses of drugs used followed previous studies (52–54).

**Statistical analysis**

All in vitro results were derived from triplicate experiments. Results were presented as mean ± SD. Student’s t-test was used to examine the significance of differences between-group...
means, and differences with P-values less than 0.05 were considered significant.

**Microarray analysis**

All gene expression data were obtained from the Affymetrix microarray platform by the Affymetrix Gene Expression Service Laboratory at Academia Sinica, Taiwan. Chips were scanned with an Affymetrix GeneChip Scanner 7G and data were analyzed by GeneSpring X software (Agilent, Santa Clara, CA, USA). Raw data were normalized independently for each experiment using Robust Multichip Average and weakly expressed signals (means < 20% of total samples) were excluded. Global clustering of the filtered genes was performed using Cluster 3.0 software and presented using Java TreeView 1.1.5r2. The raw microarray data are available through the Gene Expression Omnibus (GEO, GSE27280; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27280).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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