Neuronopathic Gaucher’s disease: induced pluripotent stem cells for disease modelling and testing chaperone activity of small compounds

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Gaucher’s disease (GD) is caused by mutations in the GBA1 gene, which encodes acid-β-glucosidase, an enzyme involved in the degradation of complex sphingolipids. While the non-neuronopathic aspects of the disease can be treated with enzyme replacement therapy (ERT), the early-onset neuronopathic form currently lacks therapeutic options and is lethal. We have developed an induced pluripotent stem cell (iPSc) model of neuronopathic GD. Dermal fibroblasts of a patient with a P.[LEU444PRO];[GLY202ARG] genotype were transfected with a loxP-flanked polycistronic reprogramming cassette consisting of Oct4, Sox2, Klf4 and c-Myc and iPSc lines derived. A non-integrative lentiviral vector expressing Cre recombinase was used to eliminate the reprogramming cassette from the reprogrammed cells. Our GD iPSc express pluripotent markers, differentiate into the three germ layers, form teratomas, have a normal karyotype and show the same mutations and low acid-β-glucosidase activity as the original fibroblasts they were derived from. We have differentiated them efficiently into neurons and also into macrophages without observing deleterious effects of the mutations on the differentiation process. Using our system as a platform to test chemical compounds capable of increasing acid-β-glucosidase activity, we confirm that two nojirimycin analogues can rescue protein levels and enzyme activity in the cells affected by the disease.

INTRODUCTION

Complex glycosphingolipids are degraded into glucosylceramide in the lysosome. Gaucher’s disease (GD) is an autosomal recessive lysosomal storage disorder caused by over 200 (1) different mutations in GBA1, the gene encoding acid-β-glucosidase, the lysosomal enzyme that further cleaves glucosylceramide into ceramide and glucose (2). Mutations in acid-β-glucosidase can result in decreased enzyme stability, increased retention and degradation in the endoplasmic reticulum (ER) and impaired trafficking to the lysosome (1,3). Lowered acid-β-glucosidase activity results in the accumulation of glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph) in the lysosome, leading to lysosomal dysfunction (4). Although the main cell types affected in GD are macrophages and neurons, recent evidence suggests that other cell types such as osteoblasts, T-cells and dendritic cells are also affected (5). The clinical presentation is highly variable and cannot be generally predicted from the genotype, but classically GD is divided into type 1 (non-neuronopathic), type 2 (acute neuronopathic) and type 3 (chronic...
neuronopathic) (6). GD type 1 is characterized by a systemic presentation, including skeletal defects, hematopoietic abnormalities and hepatosplenomegaly. GD type 2 is characterized by early onset of CNS damage in multiple brain structures and regions, including the basal ganglia, nuclei of the midbrain, hippocampus, cortex and putamen. Pathological hallmarks include gliosis, microglial proliferation, periventricular accumulation of macrophages and neuronal degeneration (4,7). In particular, treatment options for GD type 2 are completely lacking; patients develop the disease within 4–6 months of age and rarely survive more than 3 years (8). GD type 3 shows a clinical presentation that is intermediate between type 1 and type 2, showing both systemic involvement and CNS damage, although the onset is later than in GD type 2.

Strategies to treat the systemic aspects of the disease include enzyme replacement therapy (ERT) (9), substrate reduction therapy (SRT) (10) and stabilization of acid-β-glucosidase using chemical chaperones (11–13). However, as the recombinant enzyme cannot cross the blood brain barrier (14), no treatments are currently available for the acute neurodegeneration characteristic of GD type 2. Further efforts are clearly needed to identify alternative therapeutic options for all GD types and in particular for the neuronopathic form.

Our current knowledge of the pathogenic progression of GD is far from complete, due in part to the difficulty in obtaining the relevant cell types from patients. It is generally accepted that the accumulation of a substrate causes lysosomal dysfunction in macrophages; upon infiltration, glycolipid-laden macrophages (Gaucher cells) appear in multiple organs and tissues, eventually causing organ dysfunction. In the particular case of GD type 2, Gaucher cells are also found associated with brain capillaries, but the mechanisms underlying neurodegeneration are controversial (7). Most in vitro studies focusing on the basic mechanism and small compound screening efforts have been performed on patient fibroblasts, a cell type not primarily affected in patients. In addition, the existing mouse models, while valuable, suffer from the limitation of only partially reproducing the human phenotype (15–18) and fail to reflect the different phenotypes caused by the wide range of genotypes found in human patients. Therefore, alternative models based on human cells are required to further our understanding of the disease and develop novel therapies.

In the last few years, it has been shown that ectopic expression of a small number of transcription factors can reprogramme adult somatic cells to induced pluripotent stem cells (iPSc), which are similar to embryonic stem cells in many aspects (19–22). Furthermore, several groups have successfully demonstrated that iPSc can be derived from the cells obtained from human patients suffering from a range of conditions and used to model pathogenesis and test pharmacological compounds (23–33). In this paper, we describe the development of an iPSC model for the acute neuronopathic form of GD (GD type 2). GD iPSc were generated by transfection of a polycistronic reprogramming construct and characterized in terms of pluripotency and differentiation capacity. In particular, we differentiated them into macrophages and neurons (the two main disease relevant cell types), which showed markedly reduced acid-β-glucosidase protein and enzymatic activity levels. We use this system to test recently developed pharmacological compounds with acid-β-glucosidase chaperone activity. We found that chaperone treatment can rescue acid-β-glucosidase protein levels and activity, providing a novel human in vitro model for dissecting mechanisms of pathogenesis and for small molecule validation in the relevant cell types involved in GD.

RESULTS
iPSc derivation and characterization

Low passage (P4) GD P.[LEU444PRO];[GLY202ARG] fibroblasts showing low acid-β-glucosidase activity (Fig. 1A) were reprogrammed by nucleofection of a linear DNA fragment containing a polycistronic reprogramming cassette comprised of a CAG promoter driving expression of Oct4, Sox2, Klf4, c-Myc and GFP linked by 2A self-cleaving peptides. The polycistron was flanked by loxP sites, allowing the option of reprogramming cassette removal by Cre recombinase delivery if necessary. After 5 to 6 weeks, seven GFP+ colonies with ESc-like morphology were isolated, six of which had single insertions of the transgene as determined by southern blot (Fig. 1B). There was a noticeable delay in comparison to wildtype (wt) iPSc, which appeared around 4 weeks after nucleofection. Some iPSc lines silenced the reprogramming cassette spontaneously after 8–12 passages as judged by GFP expression, while others remained GFP positive, indicating persistence of transgene expression (Fig. 1C). In order to eliminate the recombination cassette from lines that had not silenced the transgene, we transduced them with a non-integrative lentiviral vector expressing Cre recombinase and cherry fluorescent protein. Three days after transduction, human pluripotent cells (Tra1-60) that had cherry+ were isolated by fluorescent activated cell sorting (FACS) and replated, giving rise to GFP-negative subclones (Fig. 1C). Southern blot analysis revealed that the GFP-negative subclones of the GFP+ GD iPSc line transduced with Cre recombinase had lost the reprogramming cassette (Fig. 1B). A line that had spontaneously silenced the transgene (iPSc-GD-A8) and a Cre recombinase deleted line (iPSc-GD-C21) were chosen for further study. These lines presented morphology similar to hESc, remained unchanged with long-term passaging (up to P40) and expressed pluripotency markers (alkaline phosphatase, Oct4, Sox2, Nanog, Tra1-81, Tra1-60, SSEA3 and SSEA4) (Fig. 1D). The pluripotency of the lines was further confirmed by microarray analysis, real-time PCR and FACS analysis (Fig. 2, Supplementary Material; Figs S1 and S2, Supplementary Material, Table S2). To evaluate the differentiation capacity of our iPSc, embryoid bodies were generated and induced to differentiate to ectoderm, mesoderm and endoderm. Differentiated cultures expressed Tuj1 and GFAP (ectoderm), GATA4 and ASA (mesoderm), and FOXA2 and AFP (endoderm) (Fig. 1E). Furthermore, pluripotent cells were injected into SCID-beige mice and formed teratomas (Fig. 1F) containing the three germ layers. Both the lines had a normal karyotype (Fig. 1G). Two iPSc lines (iPSc-wt-N17 & iPSc-wt-N22) were established from wt fibroblasts using the same strategy and characterized as described above. The characterization of the two wt and two GD iPSc
We genotyped the GD iPSc generated and confirmed that they carried the GBA1 mutations present in the original fibroblasts (Fig. 1H). We determined how acid-β-glucosidase expression behaved in wt versus GD iPSc lines in comparison to the original fibroblast population. Fibroblasts from the P.[LEU444PRO];[GLY202ARG] patient genotype were found to have around 2% of acid-β-glucosidase activity of wt fibroblasts (Fig. 1A). In wt iPSc acid-β-glucosidase activity was ~19% of the level found in wt fibroblasts. iPSc derived from the P.[LEU444PRO];[GLY202ARG] fibroblasts were found to have around 15% of the acid-β-glucosidase activity found in wt iPSc. Western blot analysis showed that lowered activity was due to decreased acid-β-glucosidase protein levels (Fig. 1I). Line iPSc-GD-C21 was transduced with a lentiviral vector over-expressing the wt GBA1 open reading frame and resulted in the establishment of four corrected subclones, three of which had acid-β-glucosidase enzymatic activity levels similar to or higher than that of wt lines (Supplementary Material, Fig. S3).

Differentiation of GD iPSc to macrophages

Having determined that GD iPSc recapitulated the low expression of acid-β-glucosidase found in the original patient’s fibroblasts, we set out to differentiate the iPSc to disease relevant cell types. Given their central role in GD, we sought to differentiate our iPSc to macrophages. We chose to focus on a GD line (iPSc-GD-C21) and a genetically rescued subclone of this line (L-GBA 3–15) that had acid-β-glucosidase activity levels similar to wt levels. In an initial attempt, we were unsuccessful
in differentiating various iPSc lines using the published protocol of Kambal et al. (34). Further attempts using two additional published protocols (Senju et al. (35), Karlsson et al. (36)) also failed. We then used a fourth published protocol (Choi et al. (37)) (with some modifications) to differentiate an iPSc line (CBiPS 4F5) originally derived from CD133+ cord blood progenitors (38) and succeeded in obtaining a population of cells with macrophage morphology, with a high proportion (86%) of cells expressing the monocyte-macrophage lineage marker CD11b (Supplementary Material, Fig. S4). However, the same protocol was unable to differentiate either wt or GD iPSc that had been reprogrammed from dermal fibroblasts. By increasing the culture times of the different steps of the protocol, we finally succeeded in differentiating fibroblast-derived iPSc into macrophages (Fig. 3A). Briefly, iPSc were cultured as embryoid bodies, differentiated to the haematopoietic lineage by co-culture on OP9 stromal cells and growth factor treatment for 14–17 days, and subjected to two successive macrophage inducing cytokine cocktail regimes for 2 and 10 days, respectively, as described in Materials and Methods section. This procedure resulted in a population containing cells with macrophage-specific morphology and CD profile (Fig. 3A). iPSc-GD-C21 derived macrophages were analysed by FACS and found to express markers for the monocyte-macrophage lineage: CD11b (18.3%), CD14 (35.6%), CD33 (35.5%) and CD163 (13.6%) (Fig. 3B). Further analysis showed populations expressing more than one marker: CD14 plus CD11b (17.1%), CD33 plus CD11b (20.1%) and CD14 plus CD163 (14.7%) (Fig. 3C). We then determined that these macrophages were capable of internalizing fluorescently labelled beads by phagocytosis (Fig. 3D and Supplementary Material, Movie S1). Macrophages differentiated from the corrected sub-clone L-GBA 3–15 showed an overall similar pattern of marker expression: CD11b (11.7%), CD14 (19.2%), CD33 (21%), CD163 (8.4%); CD14 plus CD11b (11%), CD33 plus CD11b (12%), and 9.6% of the cells expressing CD14 plus CD163 (9.6%) (Supplementary Material, Fig. S5). We then differentiated both the lines to macrophages and analysed the CD14 plus fraction for acid-β-glucosidase activity by FACS. The corrected cell line showed a 3-fold increase in GBA1-expressing cells and an overall higher average acid-β-glucosidase activity compared with the non-corrected line (Fig. 4). In sum, we obtained functional macrophages and concluded that diminished GBA expression levels do not affect macrophage differentiation in vitro.

**Differentiation of GD iPSc to dopaminergic neurons**

Given the severe neuronal involvement in GD type 2 patients, we sought to differentiate our iPSc to the neuronal lineage, particularly to dopaminergic neurons. We used a previously described protocol that involves embryoid body (EB) formation and culture of neural progenitors to form spherical neural masses (SNMs) that can be expanded and subsequently differentiated to dopaminergic neurons using a combination of small molecules and the mid-brain patterning factors fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) (39,40) (Fig. 5). This protocol has been shown to produce a high percentage of mature dopaminergic neurons capable of electrophysiological activity. Using this procedure, we differentiated iPSc-wt-N22, iPSc-GD-C21 and L-GBA 3–15 into SNMs that were positive for Pax6, Map2 and Tuj1, markers of neural precursors and the neural lineage, and subsequently to heterogeneous cultures with a high proportion of cells with neuron morphology that were positive for Tuj1. In addition, a significant percentage of Tuj1+ neurons were also positive for tyrosine hydroxylase (TH), a marker of dopaminergic neurons (Fig. 5A). While the majority of neurons were Map2+ and NeuN+ (data not shown), clusters of mature (neurofilament+, synapsin+ and NeuN+) neurons were also evident (Fig. 5B). We observed no significant differences in differentiation ability between the three lines, suggesting that GBA expression levels have little effect on differentiation; yield and proportion of mature neurons, however, were variable from experiment to experiment for all three lines.

**Testing activity of small compounds for chaperone activity**

At present, two therapeutic options are available for GD patients: enzyme replacement (9,41) and SRTs (42). The first strategy involves intravenous infusion of macrophage-targeted recombinant acid-β-glucosidase, three of which have been approved for the treatment of GD patients: alglucerase (Ceredase), imiglucerase (Cerezyme) and velaglucerase alfa (43–45), whereas substrate reduction can be achieved by oral administration of N-(n-butyl) deoxynojirimycin (NB-DNJ, Zavesca), which inhibits glucosyltransferase (46) and decreases substrate biosynthesis (47). Both the options have been shown to provide clinical benefit to patients for visceral, haematologic and skeletal aspects of the disease.
However, neither approach is effective for neurological symptoms of the condition, possibly due to low efficiency of delivery through the blood–brain barrier (3,10,52,53). A third strategy that has emerged in recent years is to use small compounds capable of reversible interaction with the acid-β-glucosidase enzyme as it transits through the ER (13). The interaction stabilizes the 3D structure of the enzyme, protecting it from premature degradation and facilitating its correct trafficking to the lysosomal compartment (11,12,54,55). In particular, if the compound interacts with the enzyme in the active site, its association constant must be low enough to allow its displacement by physiological levels of substrate and conditions found in the lysosome. An early candidate, the iminosugar-type glycomimetic \(N-(n\text{-nonyl})\)-deoxynojirimycin (NN-DNJ) increased enzyme activity of some acid-β-glucosidase-mutant forms (p.Asn370Ser and p.Gly202Arg) (56,57), but had the disadvantage of behaving as a broad range glucosidase inhibitor, simultaneously inhibiting both \(\alpha\)- and \(\beta\)-glucosidases, which could lead to unwanted secondary effects in a clinical setting (58).

Recently, a novel family of bicyclic nojirimycin analogue compounds with a sp\(^2\)-iminosugar structure was found to behave as highly selective competitive inhibitors of lysosomal \(\beta\)-glucosidase (59) and their chaperone effects partially characterized in GD fibroblasts. We sought to validate our model as a platform for small compound testing by further characterizing the effect of these inhibitors on macrophages and neurons derived from GD iPSc lines. Five compounds were

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**Figure 3.** Differentiation and characterization of macrophages derived from iPSc-GD-C21. (A) A brief outline of differentiation protocol; Giemsa stain of iPSc-derived macrophages (20 ×); (B) histograms showing percentage of cells positive for monocyte–macrophage lineage markers CD11b, CD163, Cd14 and CD33. (C) Scatter plots showing percentage of double positive cells for monocyte–macrophage lineage markers CD14/CD11b, CD33/CD11b and CD14/CD163. (D) Phagocytosis assay: micrographs showing morphology, fluorescent beads, DAPI stain and merge of iPSc-derived macrophages after internalization of fluorescent beads (40 ×).
chosen (Supplementary Material, Fig. S6) and initially tested on wt versus GD fibroblasts (P.[LEU444PRO];[GLY202ARG]). Acid-β-glucosidase activity was measured after treating the cells with a range of concentrations from 0 to 100 μM, as described in Materials and Methods section. Three compounds (6S-NOI-GJ, 6S-NOI-NJ and 6N-NOI-NJ) increased acid-β-glucosidase activity in GD fibroblasts 2- to 3-fold, depending on the compound and the concentration, but also depressed acid-β-glucosidase activity in wt fibroblasts to levels ranging from 40 to 90% of the values found in untreated fibroblasts. In contrast, compounds NOI-NJ and 6S-ADBI-NJ afforded 4- to 6-fold increases in acid-β-glucosidase activity of GD fibroblasts, while having little or no effect on enzyme activity in wt fibroblasts (Supplementary Material, Fig. S7). Therefore, we decided to focus on NOI-NJ and 6S-ADBI-NJ and test their ability to rescue acid-β-glucosidase activity in neurons.

SNMs derived from iPSc lines (iPSc-wt-N22, iPSc-GD-C21 and GD L-GBA 3–15) were further differentiated into neuron containing cultures using the Cho et al. protocol described above. During the last 4 days of differentiation, cultures were treated with 30 μM of either NOI-NJ or 6S-ADBI-NJ. Both the compounds resulted in significantly increased levels of both protein stability and enzyme activity (Fig. 6 and Supplementary Material, Fig. S8), suggesting that the compounds were capable of stabilizing acid-β-glucosidase protein levels and facilitating trafficking to the lysosome.

**DISCUSSION**

In this study, we describe the successful derivation of iPSc from dermal fibroblasts from a patient with GD type 2, the acute neuronopathic form of the disease. Our iPSc lines meet the criteria of quality standard in the field: they show ESc-like morphology, express a range of pluripotent markers (AP, Oct4, Sox2, Nanog, Tra1-60, Tra1-81 and SSEA4), clearly cluster with ESc by microarray analysis, are capable of differentiation to the three germ layers both in vitro and in vivo and have normal karyotype. We genotyped our GD iPSc and confirmed the presence of the original fibroblast genotype, P.[LEU444PRO];[GLY202ARG] genotype, a compound heterozygote mutation in exons 7 and 11 of the GBA1 gene. We differentiated the GD iPSc lines into the two main cell types affected by the disease: macrophages and neurons. We find that these differentiated cells reproduce the basic underlying acid-β-glucosidase expression deficiency both in protein levels and in enzymatic activity. Furthermore, we demonstrate that these differentiated cell types can be used...
to evaluate candidate chaperone compounds capable of rescuing enzyme activity, providing a novel human-based in vitro preclinical model.

Recently, a number of publications have reported the development of iPSc-based models of both monogenic and polygenic diseases (reviewed in 31), establishing a convenient source of human disease-specific cell types for dissecting mechanisms of pathogenesis and providing an intermediate level of testing of pharmacological compounds between animal models and clinical trials. The classic method of reprogramming involves using retroviral vectors expressing a small number of ESc-related transcription factors, most frequently Oct4, Sox2, Klf4 and c-Myc, either cloned individually or as a polycistron (60). In this study, we utilized a two-step strategy: reprogramming was achieved by nucleofection of a loxP-flanked polycistronic reprogramming cassette consisting of

![Diagram of iPSc differentiation](image)

Figure 5. (A) Differentiation of GD iPSc to dopaminergic neurons. Briefly, iPSc were differentiated to SNMs, differentiated to neurons and matured to the dopaminergic fate. Rows from top to bottom: iPSc, SNM, differentiating SNM (20×) and mature dopaminergic neurons (40×). Markers indicated in each panel. (B) Mature neurons derived from GD iPSc, 40×. Markers indicated in each panel.
Oct4, Sox2, Klf4 and c-Myc, and reprogramming cassette elimination was achieved by transient expression of Cre recombinase. Thus, genomic insertions are minimized and if the reprogramming cassette does not silence spontaneously, it can be removed with Cre recombinase. Ninety percent of the iPSc clones obtained had a single transgene insertion. Three of the seven GD iPSc clones isolated reduced transgene expression spontaneously after several passages. The rest did not and required transgene excision. Therefore, these lines have only one leftover genomic insertion (minimizing mutagenic effects) and possible reactivation of the reprogramming transgene during differentiation with its potential for biasing the developmental outcome of the culture is ruled out.

In attempting to derive iPSc from fibroblasts obtained from patients suffering from genetic disease, several publications have reported difficulty in iPSc derivation due to the effect of the gene mutations on the viability and physiology of the fibroblasts to be reprogrammed (61, 62), overcoming the problem by expressing the wt gene constitutively or conditionally ahead of the reprogramming protocol. We observed a noticeable delay in the appearance of GD iPSc colonies (5 to 6 weeks for GD fibroblasts versus 4 weeks for wt fibroblasts), suggesting diminished reprogramming ability possibly due to a slower rate of cell division observed in the original fibroblast population. Nevertheless, genetic rescue was not an absolute requirement for this particular GD genotype and we did not test whether such a rescue would have resulted in a higher efficiency of reprogramming. Furthermore, once obtained, the GD-iPSc lines were similar to wt iPSc by morphology, growth rate and expression of pluripotency markers as established by immunofluorescence for selected markers as well as microarray profiling, suggesting that acid-β-glucosidase has no role in the maintenance of the pluripotent state.

Realization of the potential of iPSc for disease modelling requires knowledge of which are the cell types affected and adequate differentiation protocols to these cell fates. The role of macrophages in GD type 1 and GD type 3 is firmly established (4), and therefore, we sought to differentiate our iPSc lines to this lineage. Differentiation to macrophages was initially problematic. Attempts to differentiate fibroblast-derived iPSc to macrophages using three different published protocols did not succeed. The protocols of Kambal et al. (34) and Karlsson et al. (36) were developed starting from CD34+ cord blood progenitors and hESc, respectively. Furthermore, we were unable to obtain macrophages using the protocol of Senju et al. (35), which was developed starting from fibroblast-derived iPSc. A fourth published protocol (Choi et al. (37)), developed with hESc as the starting cell type, (when modified as described in Materials and Methods) resulted in differentiated populations with high proportion (86%) of CD11b+ macrophages when differentiated from iPSc that had been derived from CD133+ cord blood progenitors, but did not yield macrophages when applied to iPSc derived from either wt or GD fibroblasts. However, extending culture times of the Choi et al. protocol allowed differentiation to macrophages in both wt and GD fibroblasts. Our procedure resulted in a population of differentiated cells with significant numbers of macrophages of high quality as judged by morphology, the presence of multiple monocyte–macrophage lineage markers and the ability to internalize fluorescent particles. The difficulty we encountered in achieving differentiation to the macrophage lineage suggests that the epigenetic state (or epigenetic memory) of the starting population could be playing a role in differentiation efficiency, as has been indicated by several reports (63–67), and that variation in subtle yet important parameters such as serum source and stromal cell line characteristics on differentiation efficiency should not be underestimated.

The matter of what neuronal subtypes are involved in neurodegeneration and CNS involvement in GD type 2 and type 3 is still unclear. A recent mouse model in which GBA1 was knocked in all tissues except skin presented severe neurodegeneration and apoptosis in the CNS (68). Interestingly, a conditional knockout limited to neural and glial progenitors presented a similar phenotype but with later onset, leading to the interpretation that the phenotype is mainly due to dysfunction in neurons and/or glial cells rather than microglia (68). The wide range of brain regions and structures affected suggests that the probability of cell-type specificity being restricted to a particular neuronal or glial type is low. One neuronal cell type known to be affected are pyramidal neurons of the hippocampus, where sensitivity to neurodegeneration correlates with functional regions: in the hippocampus, pyramidal SC2-4 neurons are affected while pyramidal SC1 neurons are relatively spared (7). Given that differentiation to pyramidal neurons is not well defined, we chose to focus on differentiating our iPSc lines to dopaminergic neurons, as this is one of the neuronal types with best characterized differentiation protocols and furthermore of potential interest because of a recently established pathogenic link between GD and Parkinson (69). The protocol used to differentiate iPSc to dopaminergic neurons resulted in heterogeneous cultures with a high proportion of TUJ1+ neurons. A majority
of neurons present in the culture were also Map2+, indicating an intermediate level of maturity, with clusters of more mature neurons positive for neurofilament, synapsin and NeuN. All our lines gave rise to TH+ dopaminergic neurons; however, there was some variability from line to line and between experiments. This variability is commonly experienced when doing in vitro differentiation and has been reported for neuronal lineages (70), haematopoietic cells (71) and cardiomyocytes (72). While variability did not correlate with the phenotype, some of the acid-β-glucosidase protein and activity measurements may reflect this heterogeneity.

While patients with GD type 1 and type 3 can treat the systemic aspect of their condition with ERT or SRT, these treatments result in no clinical benefit for GD type 2 patients. In addition, ERT therapy requires regular infusions in a hospital setting, carries the risk of side effects, immunological rejection of the recombinant enzyme and can impose a hard financial burden on patients, families and health care systems (13,73). Pharmacological chaperones are a potential alternative. Given the high cost of developing pharmaceutical compounds for disease, being able to test potential candidates early during the process on the relevant type of human cell is of great value. In this study, we have further characterized two members of the recently developed bicyclic nojirimycin analogue compounds with sp²-iminosugar structure (59). We find that relatively low concentrations (30 μM) of both compounds can increase protein and enzymatic activities several folds in differentiated neuronal cultures of GD type 2 patients; furthermore, this effect is also observed for wt cells, indicating that the rescue occurs over a wide range of concentrations of substrate and is not specific to the mutated form of the enzyme. Importantly, their small size and amphiphilic design enhance their ability to cross the blood–brain barrier. Recent studies have shown that the NOI-NJ has good properties regarding oral availability and ability to enhance acid-β-glucosidase activity in mouse tissues, including brain, as well as the lack of acute toxicity at high doses in normal mice (59). Furthermore, the use of a fluorescently labelled derivative recently demonstrated the ability to cross the cell membrane by diffusion and increase the levels of acid-β-glucosidase in mature and immature neuronal cells (74). Given estimates that only small increases in acid-β-glucosidase activity would be required to achieve a clinical effect (75), these results support further development of these compounds as therapeutic candidates.

To our knowledge, there have been two previous reports of derivation of GD iPSc in the literature. Park et al. (30) used individual retroviral vectors expressing Oct4, Sox2, Klf4 and c-Myc to generate a number of iPSc lines from fibroblasts of several monogenic diseases, including GD type 1. However, the study did not go beyond the generation and characterization of the pluripotency of the lines generated. In particular, no attempt was made to differentiate the GD iPSc to disease-specific cell types in vitro. A second publication (69) used the same iPSc line and differentiated it into dopaminergic neurons but not macrophages. Neither study attempted to use the system to evaluate therapeutic compounds. The present study is the first to report the development of iPSc from a GD type 2 patient, differentiate the line to both macrophages and dopaminergic neurons and test chaperone compound candidates on the differentiated cells. In differentiating our iPSc to neurons and macrophages, we provide a platform that fills this need for the particular case of the P[LEU444PRO]; [GLY202ARG] genotype. We envision that a panel of iPSc covering the most common genotypes of GD could be developed to pre-screen compounds for this condition. We believe that iPSc models of GD can offer a complementary approach to mouse modelling to advance our understanding of the disease and develop novel therapeutics.

**MATERIALS AND METHODS**

**Isolation of fibroblasts from GD patient fibroblasts**

Dermal fibroblasts from a patient with diagnosed GD type 2 were obtained following the protocol approved by the Hospital Clinic, Barcelona. The diagnosis was made based on the clinical features and low acid-β-glucosidase activity. Mutational analysis of the GBA1 gene confirmed the presence of a P[LEU444PRO];[GLY202ARG] compound heterozygote mutation.

**Cell culture**

Human GD fibroblasts, unaffected human fibroblasts, human foreskin fibroblasts (HFF) and 293T cells were maintained in Dulbecco’s minimal essential medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) (Life technologies) and penicillin/streptomycin at 37 °C with 5% CO₂. GD and wt iPSc lines were maintained on irradiated HFF feeder layers in HES medium (KO-DMEM, 20% knockout serum replacement, non-essential amino acids, L-glutamine, β-mercaptoethanol and basic fibroblast growth factor (bFGF) (5 ng/ml) (Life Technologies) and passaged mechanically. To obtain pure iPSc population for analysis, iPSc were cultured on matrigel (BD Biosciences) coated dishes, fed with HFF-conditioned HES medium and passaged by trypsinization. For seeding single iPSc for clone derivation, cultures were incubated with a Rock inhibitor (Y27632) 10 μM (Sigma) for 1 h, trypsinized and seeded on inactivated HFF in HES media (previously conditioned by culture on hESc grown on HFF) supplemented with 10 ng/ml neurotrophin 3 (NT-3) (Peprotech).

**IPSc derivation, reprogramming cassette elimination and rescue with wt GBA1**

The reprogramming DNA consisted of a linear 10 kb fragment containing an upstream fragment, a loxP site, a CAG promoter driving a polycistronic reprogramming construct and a second loxP site. The reprogramming construct carried the ORFs of mouse OCT4, SOX2, KLF4, c-MYC and EGFP linked by 2A self-cleaving peptides. Dermal fibroblasts were trypsinized and 10⁶ cells nucleofected with 2 μg of the DNA construct using the NHDF kit (Amaxa) according to the manufacturer’s instructions. The nucleofected fibroblasts were seeded on irradiated HFF feeder layers, and fed every other day with HES medium. After 1 week, conditioned HES medium was used and plates incubated until colonies were picked manually for expansion under standard hESc culture conditions. To
eliminate the reprogramming cassette, iPSc lines were trypsinized and transduced in suspension with a non-integrative lentiviral vector expressing Cre recombinase and cherry fluorescent protein. They were plated on matrigel-coated dishes and 72 h later cherry+ cells were isolated by FACS and plated for subclone isolation. Loss of the reprogramming cassette was confirmed by southern blot using probes both internal and external to the loxP-flanked segment of the construct. GD-iPSc lines were genetically rescued by transduction with a lentiviral vector constitutively expressing GBA1. The cells were trypsinized, transduced at low multiplicity of infection and subclones screened for lentiviral integration by PCR, followed by an acid-β-glucosidase activity assay.

**IPSc characterization**

IPSc lines were selected based on their hESC-like morphology and tested for alkaline phosphatase activity using the Blue Membrane Substrate solution kit (Sigma) following the manufacturer’s guidelines. Lines were further tested for pluripotency markers Oct4, Sox2, Nanog, Tra-1-60, Tra-1-81 and SSEA4 by immunofluorescence. The capability of differentiating *in vitro* into the three germ layers was tested as previously described (55). Briefly, embryoid bodies were induced to differentiate into endoderm by culturing in a differentiation medium containing FBS, to mesoderm by supplementing the differentiation medium with ascorbic acid and ectoderm by free floating culture of EBs in N2B27 medium (Life Technologies) supplemented with bFGF, SHH (R&D Systems) and FGF8 (Peprotech) followed by plating on PA-6 feeder cells in the absence of FGF2. *In vivo* differentiation ability was tested by teratoma formation as previously described (55). Briefly, 10⁶ iPSc were injected into the testis of SCID beige mice and 8–10 weeks later tumors processed by standard methods stained with anti-CD14 antibody (BD Biosciences). Differentiation of iPSc to macrophages

A four-step protocol was developed. Steps 1 and 2 were performed as previously described (37) with some modifications. Step 1: EBs from iPSc were prepared mechanically and cultured in ultra-low attachment dishes in growth media supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1x GlutaMAX (all from Life Technologies), 10ng/ml bFGF, 10 ng/ml Fli3l, 10 ng/ml VEGF, 10 ng/ml BMP-4, 20 ng/ml TPO and 25 ng/ml SCF (all from Peprotech). On the third day, cultures were transferred into differentiation medium. The cells were maintained by changing half of the medium volume every 3 days, and subsequently by replacing 25% of the medium volume every 3 to 4 days for a total of 14–16 days. Step 3: modified from Choi et al. (37): cultures were digested with 0.25% trypsin (Life Technologies), 0.1% collagenase type IV (Life Technologies) and DNase (Roche), washed with phosphate buffered saline (PBS) and cultured in ultra-low attachment dishes in α-minimum essential medium media (Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µm monothioglycerol solution (Sigma) and 200 ng/ml of granulocyte-macrophage colony-stimulating factor (Peprotech) for 2 days. Step 4: cells were subsequently washed in PBS and cultured in IMDM media, supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml macrophage colony-stimulating factor (both form Peprotech), 10 ng/ml IL-1β for an additional 10 days. At this time, the cells were collected, filtered through a 70 µm cell strainer and used for further experiments.

**CD profile analysis**

Cells were stained with monoclonal antibodies against human CD11b, CD14, CD33 (BD Biosciences), CD163 (R&D systems) conjugated with fluorescein, phycoerythrin or allophycocyanin according to the manufacturer’s instructions and analysed by using MoFlo high-performance cell sorter and flow cytometry analyzer Gallios (Beckman Coulter). Propidium iodide-stained dead cells were gated out. Human cell population was identified upon staining with antibodies against the pan-human marker TRA-1-85 (BD Biosciences).

**Phagocytosis assay**

For live cell imaging of phagocytosis, 50 000 cells were plated onto the glass surface of glass bottom dishes (Maltek) in 400 µl of media and were allowed to attach over night. Next day, the media was replaced with 200 µl of fresh media supplemented with opsonized fluorescein isothiocyanate (FITC)-labelled Zymosan A particles (Life Technologies) (20 particles per cell) for 100 min. Uninternalized particles were washed away, after which cells were stained with Hoechst vital dye and analysed with a confocal Leica SP5 AOBS microscope. For FACS analysis of phagocytic macrophages, the cells were cultured in 12-well cell culture dishes at a density of 200 000–500 000 per well, treated with opsonized FITC-labelled Zymosan A particles, trypsinized and in some experiments stained with anti-CD14 antibody (BD Biosciences).

**Differentiation to dopaminergic neurons**

IPSc were differentiated to dopaminergic neurons using the four-stage published protocol of Cho et al. (39,40). Briefly, iPSc were detached and cultured as EBs for 7 days in HES medium devoid of bFGF (step 1); EBs were plated on matrigel-coated dishes and neural precursors selected and expanded using 0.5% N₂ supplement and 20 ng/ml bFGF. SNMs consisting of neural progenitors were dissected and expanded as free floating spheres by mechanical passaging (step 2); SNMs were transferred to matrigel-coated dishes and cultured for 4 days with a neural induction medium containing a 2% B27 supplement (Life Technologies) and a 1% N₂ supplement (step 3). After 4 days, dopaminergic neuron fate was induced by adding 200 ng/ml SHH and 100 ng/ml...
FGF8 to the media and on day 8 of differentiation, 200 μM ascorbic acid was used for dopaminergic maturation (step 4).

**Chaperone treatment**

Compounds were synthesized and characterized by C. O. Mellet and J. M. García Fernández as described in (59,76). The cells were treated with chaperone compounds at 30 μM final concentration for 2 days, after which fresh medium and compound were added for another 2 days (total of 4 days of chaperone treatment) before assaying acid-β-glucosidase enzymatic activity.

**Microarray processing and analysis**

The RNA integrity was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples had high integrity (RNA integrity number (RIN) ≥8.7) and were subsequently used in microarray experiments.

Amplification, labelling and hybridizations were performed according to the protocols from Ambion and Affymetrix. Briefly, 200 ng of total RNA were amplified using the Ambion® wt expression kit (Ambion/Applied Biosystems, Foster City, CA, USA), labelled using the wt Terminal labeling kit (Affymetrix Inc., Santa Clara, CA, USA), and then hybridized to Human Gene 1.0 ST Array (Affymetrix, GEO Accession number GSE41243) in a GeneChip® Hybridization Oven 640. Washing and scanning were performed using the hybridization wash and stain kit and the GeneChip® system of Affymetrix (GeneChip® Fluidics Station 450 and Gene-Chip® Scanner 3000 7G).

Microarray data analysis was performed as follows: after quality control of raw data, it was background corrected, quantile-normalized and summarized to a gene level using the robust multi-chip average obtaining a total of 28 832 transcript clusters, excluding controls, which roughly correspond to genes. NetAffx annotations (version 32, human genome 19) were used to annotate analysed data.

Hierarchical cluster analysis was performed to see how data aggregate and a heat map was generated with pluriotency genes. All data analysis was performed in R (version 2.15) with packages aroma.affymetrix, Biobase, Affy, biomaRt and gplots. Ingenuity Pathway Analysis v 9.0, (Ingenuity® Systems, www.ingenuity.com) was used to perform functional analysis of the results.

**Acid-β-glucosidase enzymatic activity assay**

Acid-β-glucosidase activity in cell pellets was determined as previously described (77) with the fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (Sigma). Activities were measured in triplicate. Protein concentration was determined using the Lowry method. All measurements were derived in triplicate. The results were presented as mean ± SD. Student’s t-test was used to examine the significance of differences between-group means, and the differences in P-values <0.05 were considered significant. Acid-β-glucosidase activity was measured by FACS as described in (78).

**Western blot analysis**

Cells from differentiated and undifferentiated cultures were incubated in the presence or the absence of compounds 6S-ADBI-NJ or NOI-NJ (59). After indicated times, the cells were harvested and equal amounts of cell lysate (30 μg from Bradford-determined RIPA homogenates) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto Immobilon polyvinilidene difluoride (Millipore). For immunochemical detection, blots were incubated with the indicated primary antibodies (anti-GBA (ABCAM ab55080), anti-actin (Sigma A2172)). Subsequently incubated with secondary sheep anti-mouse IgG peroxidase-conjugated antibody (Amerham) and developed with the chemiluminescence ECL plus detection system (Amersham).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement:** The pharmacological chaperones assayed in this work are protected under patent laws: compounds promoting the activity of mutant glycosidases; priority number/date: ES20080002988/22-10-2008; application number/date: WO2009ES70449/21-10-2009.

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