

# In Vivo Delivery of Therapeutic Molecules by Transplantation of Genome-Edited Induced Pluripotent Stem Cells

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Ittetsu Nakajima<sup>1,2</sup>, Takahiro Tsukimura<sup>3</sup>, Terumi Ono<sup>1,2</sup>,  
Tomoko Shiga<sup>4</sup>, Hiroshi Shitara<sup>5</sup>, Tadayasu Togawa<sup>3</sup>,  
Hitoshi Sakuraba<sup>4</sup>, and Yuichiro Miyaoka<sup>1,2,6</sup> 

## Abstract

Human induced pluripotent stem cells (iPSCs) have already been used in transplantation therapies. Currently, cells from healthy people are transplanted into patients with diseases. With the rapid evolution of genome editing technology, genetic modification could be applied to enhance the therapeutic effects of iPSCs, such as the introduction of secreted molecules to make the cells a drug delivery system. Here, we addressed this possibility by utilizing a Fabry disease mouse model, as a proof of concept. Fabry disease is caused by the lack of  $\alpha$ -galactosidase A (GLA). We previously developed an immunotolerant therapeutic molecule, modified  $\alpha$ -N-acetylgalactosaminidase (mNAGA). We confirmed that secreted mNAGA from genome-edited iPSCs compensated for the GLA activity in GLA-deficient cells using an *in vitro* co-culture system. Moreover, iPSCs transplanted into Fabry model mice secreted mNAGA and supplied GLA activity to the liver. This study demonstrates the great potential of genome-edited iPSCs secreting therapeutic molecules.

## Keywords

induced pluripotent stem cells, genome editing, cell therapy, drug delivery system, Fabry disease

## Introduction

Induced pluripotent stem cells (iPSCs) have great potential as resources for cell therapy<sup>1</sup>. Since the first clinical trial of iPSC-based transplantation into an age-related macular degeneration patient in 2014<sup>2</sup>, several transplantation therapies using iPSC-derived platelets (jRCTa050190117), corneal epithelial cell sheets (jRCTa050190084), cardiomyocyte sheets (jRCT2053190081), and dopaminergic progenitors (UMIN000033564) have proceeded to clinical trials.

So far, these transplantations have utilized unmodified iPSCs from healthy subjects<sup>3</sup>. However, we can potentially improve the therapeutic effects of iPSCs by genome editing as long as the safety is guaranteed<sup>4–6</sup>. For example, disruption of some of the human leukocyte antigen (HLA) genes can expand the range of people who can receive the cells without immune responses<sup>3</sup>. Genomic insertion of chimeric antigen receptor has been utilized for T-cell immunotherapy<sup>7</sup>. Another prospect of genetic modification is to make iPSCs that secrete therapeutic molecules. Recently, the implantation of genome-engineered iPSCs that secrete interleukin-1 in rheumatoid arthritis mice demonstrated the potential of this strategy<sup>8</sup>. However, the transplantation of iPSCs to deliver therapeutic molecules *in vivo* has not been established as a

therapeutic option. Therefore, we decided to test whether iPSCs with genetic modifications can deliver therapeutic molecules *in vivo* in mice by focusing on Fabry disease as a proof of concept.

Fabry disease (OMIM 301500) is an X-linked genetic disorder<sup>9</sup> that is caused by a deficiency of  $\alpha$ -galactosidase

<sup>1</sup> Regenerative Medicine Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

<sup>2</sup> Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

<sup>3</sup> Department of Functional Bioanalysis, Meiji Pharmaceutical University, Tokyo, Japan

<sup>4</sup> Department of Clinical Genetics, Meiji Pharmaceutical University, Tokyo, Japan

<sup>5</sup> Laboratory for Transgenic Technology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

<sup>6</sup> Graduate School of Humanities and Sciences, Ochanomizu University, Tokyo, Japan

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## Corresponding Author:

Yuichiro Miyaoka, Regenerative Medicine Project, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan.

Email: miyaoka-yi@igakuken.or.jp



A (GLA, EC 3. 2. 1. 22). GLA is a lysosomal enzyme that is partially secreted, and incorporated by other cells. Therefore, the loss of GLA results in accumulation of its substrates such as globotriaosylceramide (Gb3) and globotriaosylsphingosine (Lyso-Gb3)<sup>10</sup>. The accumulation leads to various symptoms, including cardiomyopathy, renal failure, and stroke<sup>11</sup>.

Currently, there are two major treatments for Fabry disease: enzyme replacement therapy (ERT)<sup>12,13</sup> and pharmacological chaperone therapy (PCT)<sup>14</sup>. In ERT, the recombinant human GLA is intravenously infused every 2 weeks. However, this infusion can lead to the development of antibodies against GLA and hampers the therapeutic effect in some patients<sup>15</sup>. In PCT, small molecules that stabilize GLA are orally administered. However, PCT has no effect in patients with the complete loss of GLA<sup>16,17</sup>.

To solve these problems, we aimed to establish a new iPSC transplantation therapy for Fabry disease using a modified enzyme that we previously developed, modified  $\alpha$ -N-acetylgalactosaminidase (mNAGA). mNAGA was created by altering the substrate specificity of NAGA, which is a paralog of GLA, into that of GLA<sup>18</sup>. Because mNAGA maintains the original antigenicity, this modified enzyme has no immunological cross-reactivity with GLA, while having the GLA enzymatic activity. In this study, we tested if transplantation of iPSCs secreting mNAGA by genome editing could supply the GLA activity *in vivo*. Our strategy of cell therapy using genome-edited iPSCs can be applied to various diseases caused by loss of functional molecules.

## Materials and Methods

### Genome Editing of Human iPSCs

In this study, an iPSC line from a healthy subject, WTC11 (GM25256 from Coriell Institute), was used<sup>19</sup>. For disruption of GLA, we used the P3 Primary Cell Kit and Nucleofector 4D device (Lonza, Basel, Switzerland). Two hundred thousand WTC11 iPSCs were transfected with 500 ng of pX330 with a guide RNA (gRNA) targeting the first exon of the GLA gene (5'-GTTCCCTCAGCTGCATTGTCA-3') by the DS-138 program. Induced indels were detected by Droplet Digital PCR, and clones with indels were isolated by sib-selection (repeated limited dilutions), as described previously<sup>20</sup>.

For knock-in of mNAGA complementary DNA (cDNA) driven by the CAG promoter, we used the Human Stem Cell Nucleofector Kit-1 and Nucleofector 2b Device (Lonza). Two million iPSCs were transfected with 2  $\mu$ g of each AAVS1-TALEN-L/R (Addgene #59025 and #59026)<sup>21</sup> and 5  $\mu$ g of AAV-CAGGS-mNAGA donor plasmid (Addgene #196448). AAV-CAGGS-mNAGA was constructed by replacing the EGFP cDNA of AAV-CAGGS-EGFP (Addgene #22212)<sup>22</sup> with mNAGA cDNA. After transfection using Nucleofector 2b using the A-23 program, the treated cells were resuspended in mTeSR Plus with Y-27632 and plated

into a Matrigel-coated 96-well plate. Two days after transfection, the medium was exchanged with mTeSR Plus with Y-27632 and 0.5  $\mu$ g/ml Puromycin for selection of transfected cells. The cells were cloned by limiting dilution. We extracted genomic DNA from cloned cells and confirmed the knock-in by junction polymerase chain reaction (PCR) and then Sanger sequencing.

### Measurement of the GLA Activity Using an Artificial Fluorogenic Substrate

Hydrolysis of an artificial GLA substrate, 4-methylumbelliferyl- $\alpha$ -D-galactopyranoside (4MU-Gal), releases the fluorescent product, 4-MU<sup>23</sup>. Therefore, quantification of the amount of produced 4-MU can measure GLA activity. We mixed 40  $\mu$ l of 4MU-Gal with 10  $\mu$ l of tissue, culture supernatant, or cell lysate and incubated at 37°C for 30 min. The reaction was terminated by adding 950  $\mu$ l of 0.2 M Glycine buffer pH 10.7. We transferred 200  $\mu$ l of the solution into a well of a 96-well black microplate (Greiner Bio-One, Kremsmünster, Austria). The fluorescence at 415–445 nm of 4-MU was measured by excitation at 365 nm using GloMax (Promega, Madison, Wisconsin, United States). For the GLA activity of tissue or cell lysates, the GLA activity per volume (nmol/h/ml) was converted to the activity per protein amount (nmol/h/mg) using the protein concentration measured by a Micro BCA assay.

### The Mass Spectrometric Analysis of Gb3 and Lyso-Gb3

For measurement of Gb3 and Lys-Gb3 in organs and tissues of mice, a liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis was performed according to a previous report<sup>24</sup>. Briefly, the organs and tissues were homogenized in MES buffer, and a 10- $\mu$ l aliquot of the homogenate was mixed with a 70- $\mu$ l aliquot of chloroform:methanol (1:2). Then a 10- $\mu$ l aliquot of 5  $\mu$ g/ml Gb3 (C17:0; Matreya, LLC, State College, Pennsylvania, United States) and a 10- $\mu$ l aliquot of 500 nmol/l stable isotope-labeled (one <sup>13</sup>C and three deuteriums) Lyso-Gb3 (Nard Institute, Kobe, Japan) were added as internal standards. The mixture was centrifuged and the supernatant was transferred to an LC vial. For LC, a Union UK-C8 column (20  $\times$  3 mm ID., 3  $\mu$ m; Imtakt Co., Portland, United States) was used, and the column oven was set at 30°C. Chromatographic separation was performed with a binary gradient consisting of a mobile phase of water containing 0.1% acetic acid and 2 mmol/l ammonium acetate and methanol containing 0.1% acetic acid and 2 mmol/l ammonium acetate. The flow rate was 0.25 ml/min and the injection volume was 2  $\mu$ l. Then, Gb3 isoforms and Lyso-Gb3 in the samples were detected by MS/MS using a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray

ionization interface in positive-ion mode. The multiple reaction monitoring (MRM) conditions were optimized with an automatic MRM optimization function. The calculations for measurement of Gb3 isoforms and Lyso-Gb3 were performed using LabSolutions (Shimadzu), and the Gb3 contents of organs and tissues were calculated from the sums of those of the Gb3 isoforms.

### Co-culture of Two Types of Cells Using Transwells

We used the bottom dish and the insert for GLA-deficient cells and mNAGA-supplier cells, respectively (Fig. 2). These cells were plated in the bottom dish or the insert and separately cultured until the cells reached 100% confluence. Then, the insert was moved to the bottom dish for co-culture. For undifferentiated iPSCs, both the bottom dish and the insert were coated with Matrigel. We co-cultured for 3 days for mouse fibroblasts, and for 1 week for other cell types. We chose the medium for co-culture depending on the GLA-deficient cell types in the bottom dish.

### Transplantation of Human iPSCs into Mouse Testes

iPSCs were detached using 500  $\mu$ l of 0.5 mM ethylenediaminetetraacetic acid (EDTA) (Nacalai Tesque, Kyoto, Japan) in phosphate buffered saline (PBS) per well of a 6-well plate and resuspended in 1.5 ml of mTeSR Plus with 10  $\mu$ M Y-27632. We used a Cell Lifter (Greiner) to scrape the cells while keeping the cell clumps. One million cells were collected into a 1.5 ml microtube and centrifuged at  $200 \times g$  for 3 min at room temperature. We removed the supernatant and tapped the cell pellets. A mixture of 50  $\mu$ l of growth factor reduced (GFR) Matrigel Phenol Red-Free and 50  $\mu$ l of mTeSR Plus with 10  $\mu$ M Y-27632 was added and gently mixed with the cells. The cell suspension was aspirated by a 1-ml syringe (Terumo, Shibuya, Japan) with an 18G needle (Terumo) and stored on ice until transplantation. Mice were anesthetized by 2% isoflurane (Viatris, Canonsburg, Pennsylvania, United States). A 1-cm incision was made on the abdominal region, and the epididymal fat pad was carefully pulled out along with the testis. The cell suspension was injected using a syringe and held for 10 s to avoid backflow. The testis was pushed back to the origin location, and the incision was sutured.

### Statistical Analyses

Data are shown as the mean  $\pm$  standard error of the mean in all graphs generated using the Microsoft Excel software program (version 16.66 for Mac; Microsoft, Redmond, Washington, United States). For comparison of two samples, *P*-values were determined by an unpaired two-tailed

Student's *t*-test using the Microsoft Excel software program (version 16.66 for Mac; Microsoft).

## Results

### Generation of Human iPSCs Secreting mNAGA by Genome Editing

First, we established a human iPSC line that secretes mNAGA to test its therapeutic effects in Fabry model mice. To exclude the possible immunogenic reactions caused by the endogenous GLA of iPSCs in patients, we knocked down *GLA* by introducing a 6-bp deletion including the start codon by CRISPR-Cas9 (Fig. 1A; hereafter called GLA-KO iPSCs). We confirmed the loss of GLA activity in GLA-KO iPSCs (Fig. 1B). We also sequenced 22 most likely off-target sites predicted by Cas-OFFinder<sup>25</sup> in GLA-KO iPSCs and found no off-target effects (Supplemental Fig. S1).

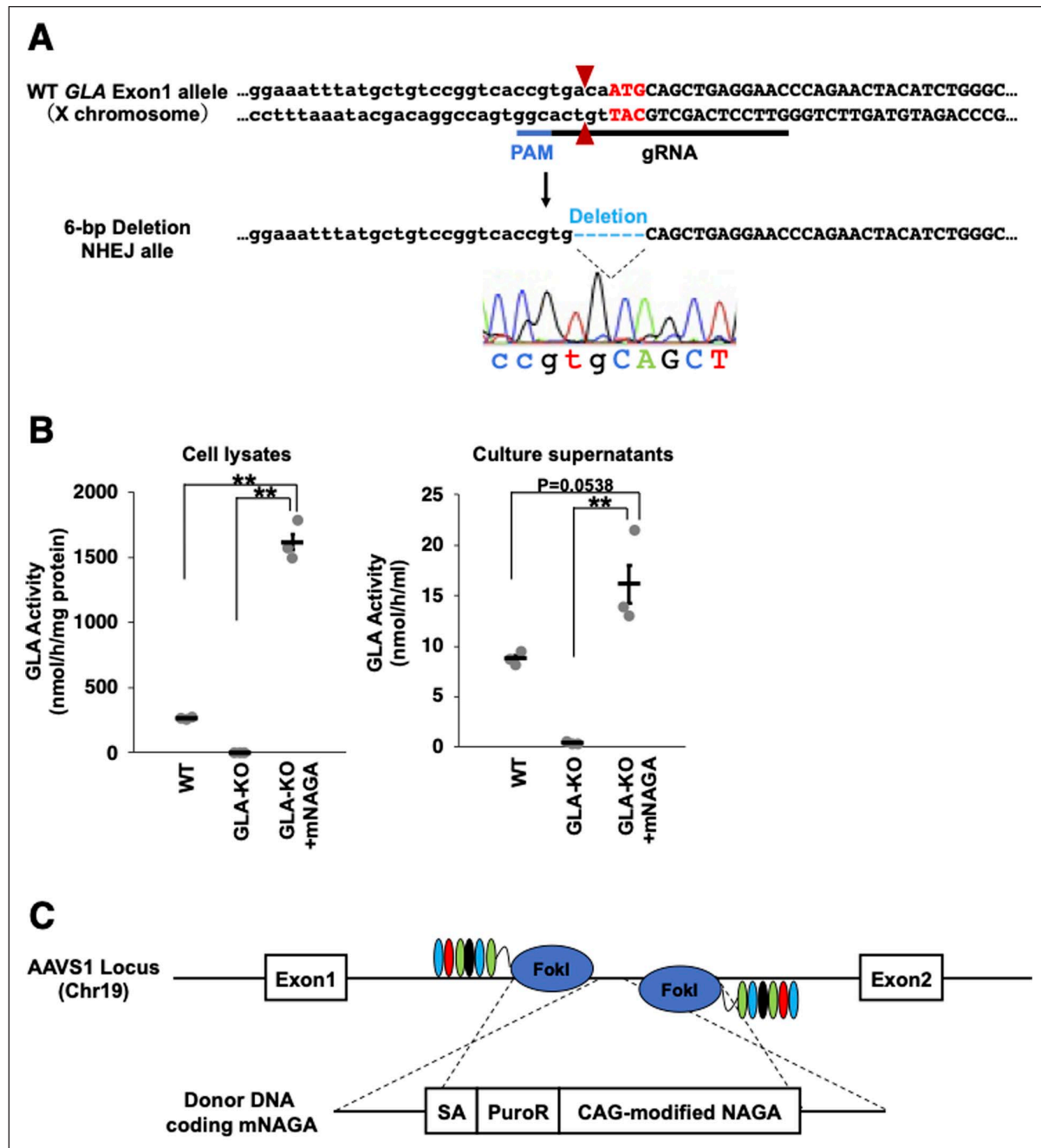
Next, to establish an iPSC line stably expressing mNAGA, we heterozygously knocked in mNAGA cDNA driven by the CAG promoter into adeno-associated virus integration site 1 (AAVS1), a safe harbor locus, by transcription activator-like effector nucleases (TALENs) previously reported to have no detectable off-target effects<sup>26</sup> (Fig. 1C; hereafter called GLA-KO+mNAGA iPSCs). This cell line exhibited higher GLA activity than wild type (WT) and GLA-KO iPSCs both in cell lysate and in culture supernatant (Fig. 1B). Thus, we successfully generated iPSCs for a potential cell therapy for Fabry disease by genome editing.

### mNAGA Secreted from iPSCs Was Taken Up by GLA-Deficient Cells in vitro

For cell therapy, mNAGA secreted from transplanted iPSCs must be taken up by patients' cells. Therefore, we investigated whether GLA-deficient cells could pick up mNAGA secreted from iPSCs by co-culture using transwells (Fig. 2A).

First, we investigated whether mNAGA secreted from iPSCs can be incorporated into fibroblasts derived from Fabry mice without functional GLA, as described previously<sup>27</sup>. We co-cultured Fabry mouse fibroblasts on the bottom of a transwell and undifferentiated GLA-KO or GLA-KO+mNAGA iPSCs on the insert for 3 days. We confirmed the secretion of mNAGA from the iPSCs by measuring the GLA activity in the culture medium (Fig. 2B). As expected, the GLA activity in the co-cultured Fabry mouse fibroblasts was higher than in the control. These findings indicated that secreted mNAGA restored the GLA activity of Fabry mouse fibroblasts (Fig. 2C).

Next, to simulate situations more relevant to actual cell therapy, we co-cultured GLA-KO iPSC-derived fibroblasts or cardiomyocytes with GLA-KO+mNAGA iPSCs. Compared with the GLA activity of WT cells (Fig. 2D), we observed the restoration levels of the GLA activity in GLA-KO iPSC-derived fibroblasts and cardiomyocytes by



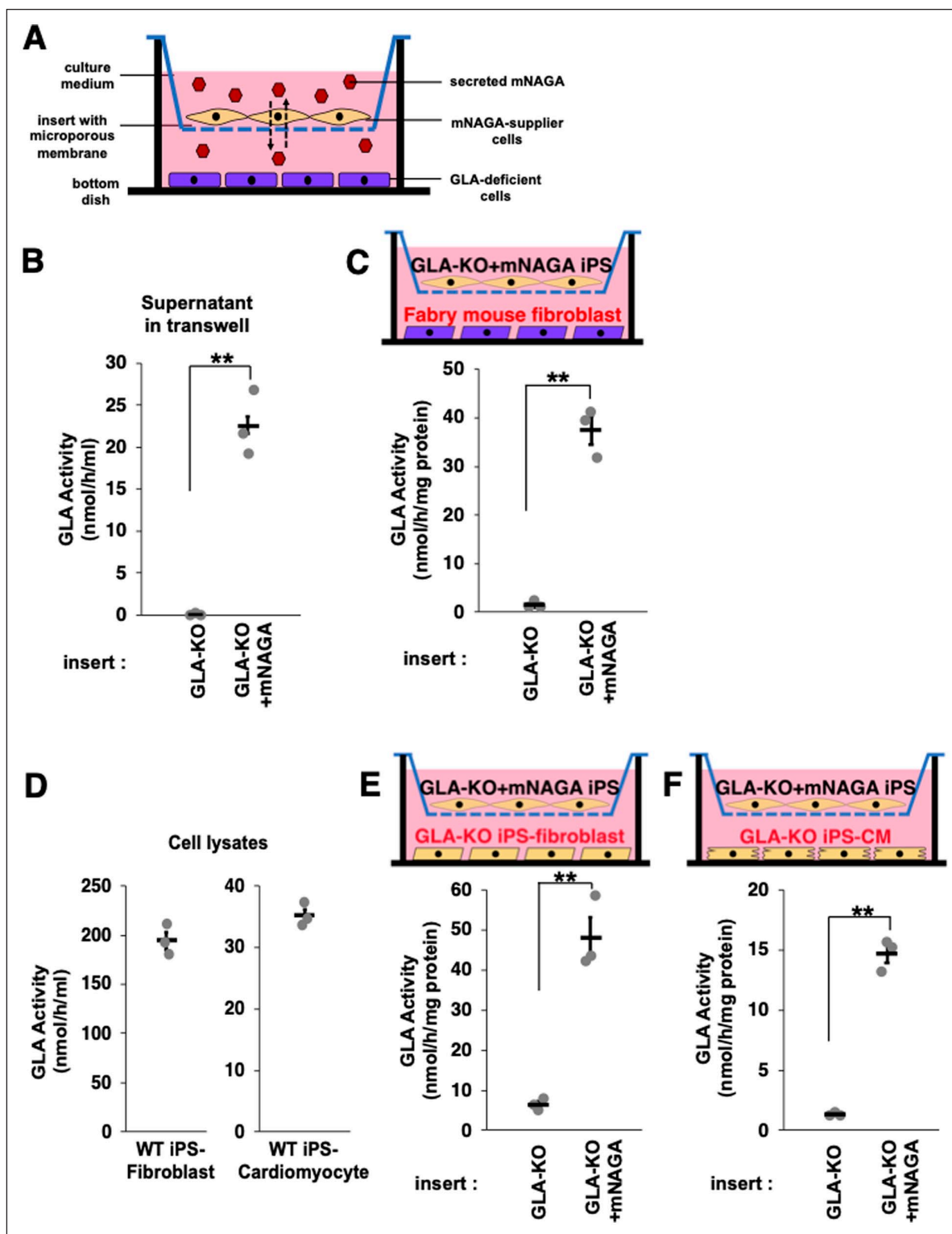
**Figure 1.** Establishment of mNAGA-secreting iPSCs. (A) Design of the disruption of the GLA gene in human iPSCs by CRISPR-Cas9. We introduced a 6-bp deletion containing the start codon. The cut site and start codon are shown by red triangles and red letters, respectively. Sanger sequencing data around the deletion are shown. (B) GLA activity in the lysates or culture supernatants of WT, GLA-KO, and GLA-KO+mNAGA iPSCs ( $n = 3$ ).  $**P < 0.01$ . (C) Design of knock-in of mNAGA cDNA driven by the CAG promoter into AAVS1 using TALENs. cDNA: complementary DNA; TALENs: transcription activator-like effector nucleases; PAM: protospacer adjacent motif; NHEJ: non-homologous end joining; gRNA: guide RNA; SA: splice acceptor; mNAGA: modified  $\alpha$ -N-acetylgalactosaminidase; iPSCs: induced pluripotent stem cells; GLA: galactosidase A; AAVS1: adeno-associated virus integration site 1; WT: wild type.

co-culturing were 24.7% and 41.8%, respectively (Fig. 2E, F). These results demonstrated that mNAGA secreted from GLA-KO+mNAGA iPSCs can be incorporated into mouse and human GLA-deficient cells and complement the lost GLA activity.

### Generation of an Immunodeficient Fabry Mouse Model

Next, we generated an immunodeficient Fabry mouse model in which human cells can be transplanted and engrafted. We





**Figure 2.** *In vitro* delivery of mNAGA secreted from iPSCs to GLA-deficient cells. (A) Schematic illustration of *in vitro* delivery of mNAGA using transwells. (B) GLA activity of the culture supernatants of GLA-KO or GLA-KO+mNAGA iPSCs ( $n = 3$ ).  $**P < 0.01$  (C) GLA activity of mouse fibroblast lysates co-cultured with GLA-KO or GLA-KO+mNAGA iPSCs ( $n = 3$ ).  $*P < 0.05$  and  $**P < 0.01$ . (D) GLA activity of WT iPSC-derived fibroblast and CM ( $n = 3$ ). (E, F) GLA activity of GLA-KO iPSC-derived fibroblasts (E) and GLA-KO iPSC-derived CMs (F) co-cultured with GLA-KO or GLA-KO+mNAGA iPSCs ( $n = 3$ ).  $*P < 0.05$  and  $**P < 0.01$ . mNAGA: modified  $\alpha$ -N-acetylgalactosaminidase; iPSCs: induced pluripotent stem cells; GLA: galactosidase A; WT: wild type; CM: cardiomyocyte.

disrupted the *Gla* gene in NOD.CB17-*Prkdc*<sup>scid</sup>/J (NOD SCID) mice, a mouse strain without functional T and B cells<sup>28</sup> by injecting Cas9 protein and single guide RNA (sgRNA) targeting exon1 of mouse *Gla* (Fig. 3A). We obtained 25 founder mice with various insertions and/or deletions out of 227 injected and transferred zygotes (Table 1). Among them, we established one mouse line with a 28-bp deletion (*Gla*<sup>Δ28</sup>) spanning the start codon of *Gla* (Fig. 3A). We confirmed that the GLA activity was lost in the liver, heart, kidney, and blood plasma of this *Gla*<sup>Δ28</sup> mouse strain (Fig. 3B).

Next, we measured the amounts of Gb3 and Lyso-Gb3, substrates of GLA, in the *Gla*<sup>Δ28</sup> mouse strain by MS/MS. As we expected, these substrates were accumulated in all analyzed tissues (Fig. 3C). These results confirmed that the NOD SCID *Gla*<sup>Δ28</sup> mouse strain we established could serve as an immunodeficient Fabry mouse model.

### Transplantation of GLA-KO+mNAGA iPSCs to NOD SCID Fabry Mice

Finally, we transplanted GLA-KO+mNAGA iPSCs into the NOD SCID Fabry mice. As the most efficient strategy to transplant the largest number of human cells, we formed teratomas in these mice.

We injected  $1.0 \times 10^6$  undifferentiated GLA-KO or GLA-KO+mNAGA iPSCs into the testes of 7- or 8-week-old male NOD SCID *Gla*<sup>Δ28</sup> mice. After 7 or 8 weeks, we observed teratomas in all treated mice (Fig. 4A and Supplemental Fig. S2). We confirmed that GLA-KO+mNAGA iPSC-derived teratoma maintained the GLA activity (Fig. 4B).

To investigate whether transplanted GLA-KO+mNAGA iPSCs improved the GLA activity in these mice, we measured the GLA activity in the liver, heart, kidney, and blood plasma. We observed no recovery of the GLA activity in the heart, kidney, or blood plasma. However, the GLA activity in the liver was significantly improved by the transplantation of GLA-KO+mNAGA iPSCs ( $P = 0.0213$ ; Fig. 4C). We also quantified the amounts of Gb3 and Lyso-Gb3 in the liver, but there was no detectable reduction of the substrates (Fig. 4D). Taken together, these data indicate that although further optimization is required, we delivered mNAGA from transplanted iPSCs to the liver *in vivo*.

## Discussion

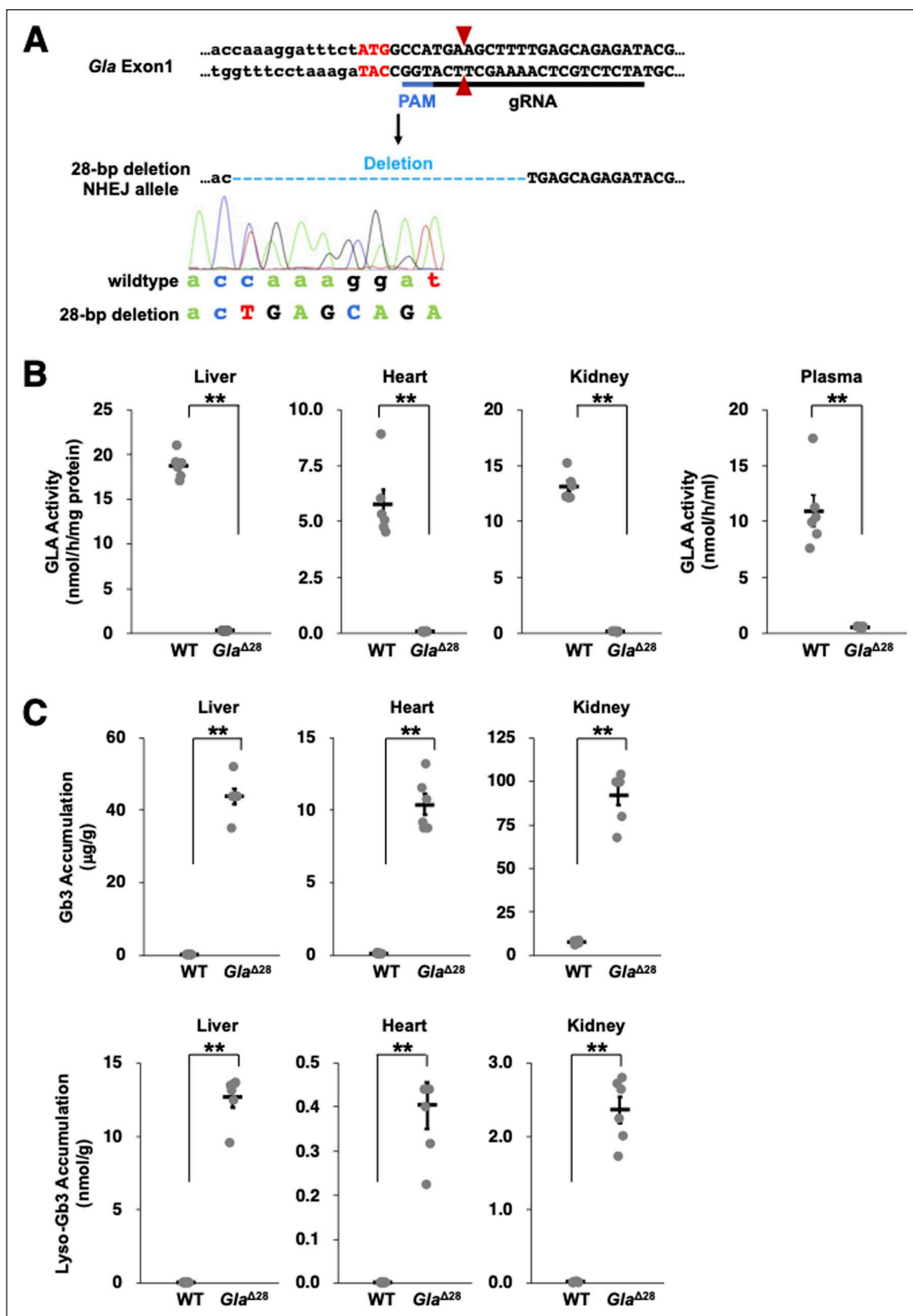
We generated human iPSCs expressing and secreting mNAGA by genome editing. Furthermore, the GLA gene was disrupted to exclude the potential immunogenicity of the endogenous GLA in iPSCs. We investigated the genomic sequences of 22 predicted off-target sites, and found no

change between before and after the GLA disruption (Supplemental Fig. S1). Although more thorough analysis such as whole-genome sequencing should be performed for clinical applications, our results support that similar genome editing strategies can be applied to other disorders associated with the loss of functional molecules.

Co-culture experiments demonstrated that genetically engineered iPSCs could secrete mNAGA and restore the GLA activity by 24.7% and 41.8% in GLA-deficient iPSC-fibroblasts and cardiomyocytes compared with WT cells, respectively. We also successfully established an immunodeficient Fabry mouse model. Then, we transplanted iPSCs into these mice to form teratomas. Although teratoma formation is not applicable as a treatment option, it was the best experimental approach, because teratoma formation can engraft a large number of cells and the transplanted cells can be easily identified in the mouse body. Multiple clinical studies to transplant iPSC-derived cells are ongoing, and transplantation methods are also rapidly evolving. We think that our results show potential benefits of replacing iPSCs currently used for transplantation by genome-edited iPSCs in the future. One of the organs most affected by Fabry disease is the heart. Therefore, one promising therapeutic option is to transplant cardiomyocyte sheets generated from iPSCs secreting mNAGA. Thus, one advantage of genetically modified iPSCs as a drug delivery system is its ability to provide therapeutic molecules directly to target organs and tissues by differentiating the cells into specific cell types.

GLA-KO+mNAGA iPSC-derived teratomas improved the GLA activity only in the liver of the treated Fabry model mice. We previously described that lysosomal enzymes such as GLA are incorporated into cells via cation-independent mannose 6-phosphate receptors and asialoglycoprotein receptors on the cell surface. These receptors are abundantly expressed in hepatic cells but not in heart or kidney cells<sup>29</sup>. Thus, the majority of lysosomal enzymes administered in ERT are incorporated into liver cells<sup>30,31</sup>. Our results are consistent with these previous reports (Fig. 4C).

Despite the improvement, the GLA activity in the liver of mice treated with GLA-KO+mNAGA iPSCs was still much lower (1.3 nmol/h/mg protein) than in the WT mouse liver (18.8 nmol/h/mg protein). Therefore, we should enhance the production and secretion of mNAGA from iPSCs. The GLA activity in the GLA-KO+mNAGA iPSCs lysate was six times higher than that of WT. However, the difference in the culture medium was only 1.8 times. These results suggest that most mNAGA was retained in the lysosomes, and not actively secreted<sup>32</sup>. Several genes are involved in regulating the transportation of lysosomal enzymes<sup>33–35</sup>. Thus, overexpression or



**Figure 3.** Generation of the immunodeficient Fabry mouse model. (A) Design of the disruption of *Gla* in NOD SCID mouse zygotes. The cut site and the start codon are shown by red triangles and red letters, respectively. The allele with a 28-bp deletion containing the start codon identified as a null allele, *Gla*<sup>Δ28</sup>, is shown. The Sanger sequencing data show the WT allele and the 28-bp deletion allele in a heterozygous mouse. (B) The GLA activity in the liver, heart, kidney, and blood plasma of male WT and *Gla*<sup>Δ28</sup> mice ( $n = 6$ ).  $^{***}P < 0.01$ . (C) Accumulation of Gb3 and Lyso-Gb3, substrates of GLA, in the liver, heart, and kidneys of male WT and *Gla*<sup>Δ28</sup> mice ( $n = 6$ ).  $^{***}P < 0.01$ . PAM: protospacer adjacent motif; NHEJ: non-homologous end joining; gRNA: guide RNA; GLA: galactosidase A; WT: wild type; Gb3: globotriaosylceramide; Lyso-Gb3: globotriaosylsphingosine.

**Table 1.** Edited *Gla* Alleles of NOD SCID Newborn Mice.

Genotype	<i>Gla</i> sequence
Wild type	acc <sup>a</sup> aaaggatttctATGGCCATGAAGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Male1: 15-bp del	acc <sup>a</sup> aaaggatttctATG-----AGCAGAGATACGCGGCT
Male2: 8-bp del	acc <sup>a</sup> aaaggatttctATGGCCATGA-----GCAGAGATACGCGGCT
Male3: 2-bp del	acc <sup>a</sup> aaaggatttctATGGCCAT--AGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Male4: 14-bp and 8-bp del (chimeric)	acc <sup>a</sup> aaaggatttct-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATGAAGC-----AGAGATACGCGGCT
Male5: 15-bp del	acc <sup>a</sup> aaaggatttctATG-----AGCAGAGATACGCGGCT
Male6: 1-bp ins	acc <sup>a</sup> aaaggatttctATGGCCATG <sup>a</sup> AAGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Male7: 98-bp del	gtcattaggagtag----- -----AGAGATACGCGGCT
Male8: 2-bp ins	acc <sup>a</sup> aaaggatttctATGGCCATGAAG <sup>a</sup> CGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Male9: 8-bp del	acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT
Male10: 8-bp del	acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT
Male11: 3-bp and 1-bp del (chimeric)	acc <sup>a</sup> aaaggatttctATGGCCATG---CTTTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATGA-GCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female1: 8-bp del homo	acc <sup>a</sup> aaaggatttctATGGCCATG---CTTTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATG---CTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female2: 28-bp del and WT	ac-----TGAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATGAAGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female3: 8-bp and 3-bp del	acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATG---CTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female4: 11-bp and 15-bp del	acc <sup>a</sup> aaaggatttctATGGC-----TGAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCA-----GAGATACGCGGCT
Female5: 9-bp del homo	acc <sup>a</sup> aaaggatttctATGGCCAT-----GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCAT-----GAGCAGAGATACGCGGCT
Female6: 1-bp del homo	acc <sup>a</sup> aaaggatttctATGGCCATG-AGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATG-AGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female7: 8-bp del and WT	acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATGAAGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female8: 8-bp and 1-bp del	acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATG-AGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female9: 8-bp del homo	acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female10: 3-bp del and WT	acc <sup>a</sup> aaaggatttctATGGCCATG---CTTTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATGAAGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female11: 19-bp and 8-bp del	acc <sup>a</sup> aaagga-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGC-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female12: 6-bp del homo	acc <sup>a</sup> aaaggatttctATGGCCAT-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCAT-----TTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female13: 1-bp del homo	acc <sup>a</sup> aaaggatttctATGGCCATG-AGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATG-AGCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT
Female14: 3-bp and 2-bp del	acc <sup>a</sup> aaaggatttctATGGCCATG---CTTTT <sup>a</sup> GAGCAGAGATACGCGGCT acc <sup>a</sup> aaaggatttctATGGCCATG--GCTTTT <sup>a</sup> GAGCAGAGATACGCGGCT

All sequences start from 5' side. Only the sequence of male7 with 98-bp deletion starts from 91-bp upstream of the open reading frame. Open reading frames are highlighted in capital letters. Deletions and insertions are shown in null bars and red letters, respectively. WT: wild type.

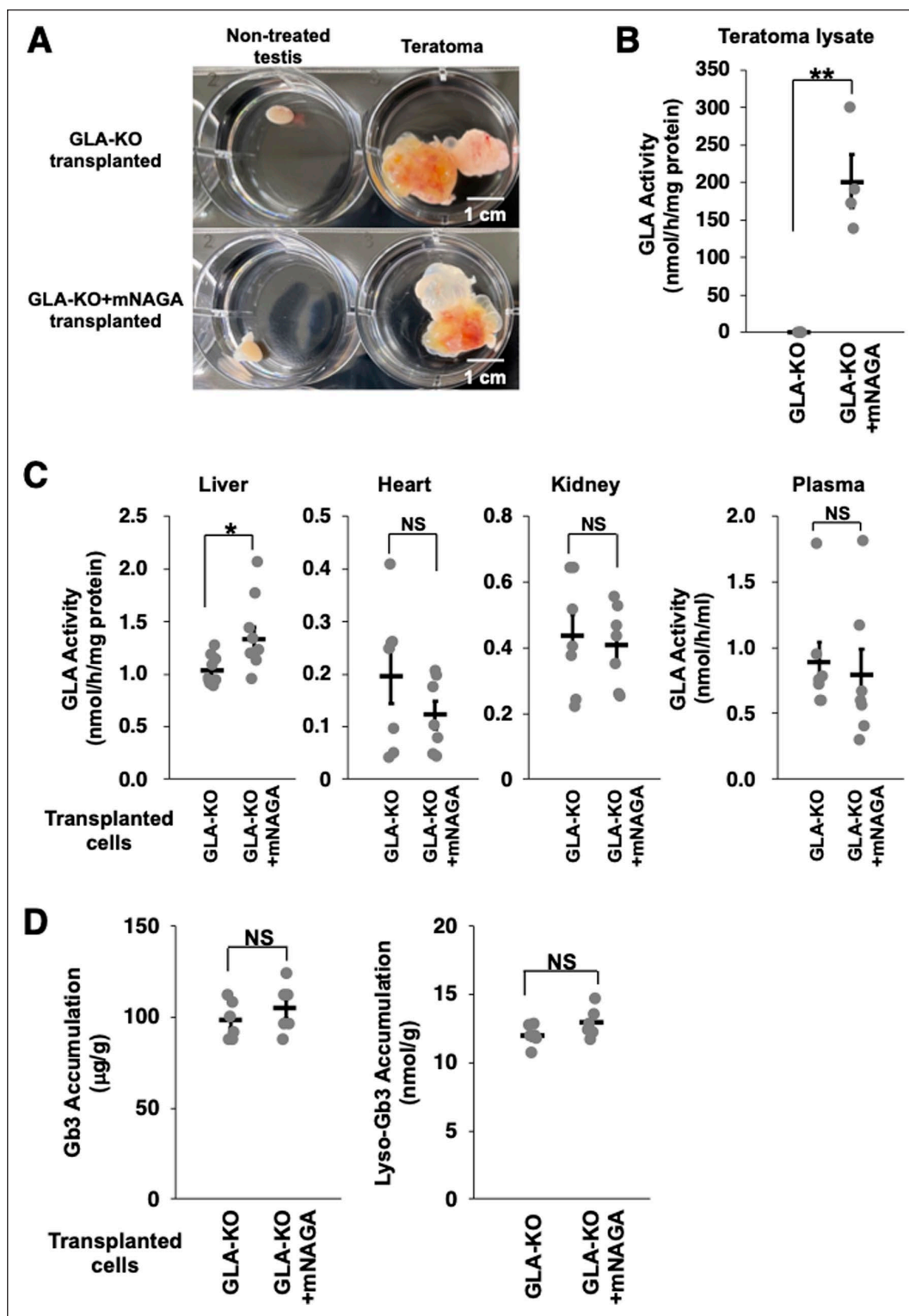
disruption of these genes potentially enhances the secretion or uptake of mNAGA. Recently, it was reported that transplantation of engineered spheroids composed of mouse embryonic fibroblasts reduced the amount of Lyso-Gb3 in Fabry model mice<sup>36</sup>. Therefore, the comparison of the two strategies would be beneficial.

We previously reported that the GLA activity of classical Fabry patients with severe symptoms and later-onset Fabry patients with milder symptoms was about 0.3% and 3.4% compared with control subjects, respectively<sup>37</sup>. The fact

that only about 3% difference in the GLA activity results in marked differences in the symptoms indicates that the required amount of mNAGA provided from iPSCs to have therapeutic effects might be small. Therefore, enhancement of mNAGA production and secretion is important for clinical purposes.

In summary, this study demonstrated that genome-edited iPSCs secreting therapeutic molecules could serve as not only a resource of cell transplantation but also a drug delivery system.





**Figure 4.** Transplantation of GLA-KO+mNAGA iPSCs to NOD SCID Fabry model mice. (A) Images of a non-treated testis and a testis with teratoma. Scale bars indicate 1 cm. (B) GLA activity in teratoma lysates derived from GLA-KO or GLA-KO+mNAGA iPSCs ( $n = 4$ ).  $*P < 0.05$  and  $**P < 0.01$ . (C) GLA activity in the liver, heart, kidney, and blood plasma of NOD SCID Fabry mice transplanted with GLA-KO or GLA-KO+mNAGA iPSCs ( $n = 9$  in only liver;  $n = 6$  in heart, kidney, and blood plasma).  $P < 0.05$ ,  $**P < 0.01$ , and NS ( $P > 0.1$ ). (D) Amounts of Gb3 and Lyso-Gb3 in the liver of Fabry mice transplanted with GLA-KO or GLA-KO+mNAGA iPSCs ( $n = 6$ ).  $*P < 0.05$ ,  $**P < 0.01$ , and NS ( $P > 0.1$ ). mNAGA: modified  $\alpha$ -N-acetylgalactosaminidase; iPSCs: induced pluripotent stem cells; GLA: galactosidase A; NS: not significantly different; Gb3: globotriaosylceramide; Lyso-Gb3: globotriaosylsphingosine.

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## Author Contributions

I.N. and Y.M. designed the experiments. Y.M. and H. Shitara generated genome-edited iPSCs and mouse zygotes, respectively. T.O. conducted the co-culture experiments. I.N. and T.O. transplanted iPSCs to mice. T. Tsukimura, T.S., and T. Togawa performed the mass spectrometry. I.N. conducted all other experiments. I.N. and Y.M. wrote the manuscript with help from other authors. Y.M. and H. Sakuraba supervised the projects.

## Availability of Data and Materials

All data and materials described in this study are available upon request. The donor plasmid, AAV-CAGGS-mNAGA, is available from Addgene (#196448).

## Ethical Approval

This study was approved by the Recombinant DNA Experiments Committee of Tokyo Metropolitan Institute of Medical Science. The approval number: #22-003.

## Statement of Human and Animal Rights

This study was approved by the Ethics and Animal Experimentation Committees of Tokyo Metropolitan Institute of Medical Science. The approval numbers: #21-49, #22-016.

## Statement of Informed Consent

Informed consent was obtained for the use of WTC11 iPSC line.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## ORCID iD

Yuichiro Miyaoka  <https://orcid.org/0000-0002-8874-4738>

## Supplemental Material

Supplemental material for this article is available online.

## References

1. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131(5):861–72.
2. Mandai M, Watanabe A, Kurimoto Y, Hirami Y, Morinaga C, Daimon T, Fujihara M, Akimaru H, Sakai N, Shibata Y, Terada M, et al. Autologous induced stem-cell-derived retinal cells for macular degeneration. *N Engl J Med*. 2017;376(11):1038–46.
3. Xu H, Wang B, Ono M, Kagita A, Fujii K, Sasakawa N, Ueda T, Gee P, Nishikawa M, Nomura M, Kitaoka F, et al. Targeted disruption of HLA genes via CRISPR-Cas9 generates iPSCs with enhanced immune compatibility. *Cell Stem Cell*. 2019;24(4):566–578.
4. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*. 2010;11(9):636–46.
5. Joung JK, Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol*. 2013;14(1):49–55.
6. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337(6096):816–21.
7. Iriguchi S, Yasui Y, Kawai Y, Arima S, Kunitomo M, Sato T, Ueda T, Minagawa A, Mishima Y, Yanagawa N, Baba Y, et al. A clinically applicable and scalable method to regenerate T-cells from iPSCs for off-the-shelf T-cell immunotherapy. *Nat Commun*. 2021;12(1):430.
8. Choi Y-R, Collins KH, Springer LE, Pferdehirt L, Ross AK, Wu C-L, Moutos FT, Harasymowicz NS, Brunger JM, Pham CTN, Guilak F. A genome-engineered bioartificial implant for autoregulated anticytokine drug delivery. *Sci Adv*. 2021;7(36):eabj1414.
9. Fabry J. Ein Beitrag zur Kenntniss der Purpura haemorrhagica nodularis (Purpura papulosa haemorrhagica Hebrae). *Arch F Dermat*. 1898;43(1):187–200.
10. Sweeley CC, Klionsky B. Fabry's disease: classification as a sphingolipidosis and partial characterization of a novel glycolipid. *J Biol Chem*. 1963;238:3148–50.
11. Germain DP. Fabry disease. *Orphanet J Rare Dis*. 2010;5:30.
12. Eng CM, Guffon N, Wilcox WR, Germain DP, Lee P, Waldek S, Caplan L, Linthorst GE, Desnick RJ; International Collaborative Fabry Disease Study Group. Safety and efficacy of recombinant human alpha-galactosidase A replacement therapy in Fabry's disease. *N Engl J Med*. 2001;345(1):9–16.
13. Schiffmann R, Kopp JB, Austin HA, Sabnis S, Moore DF, Weibel T, Balow JE, Brady RO. Enzyme replacement therapy in Fabry disease: a randomized controlled trial. *JAMA*. 2001;285(21):2743–49.
14. Germain DP, Hughes DA, Nicholls K, Bichet DG, Giugliani R, Wilcox WR, Feliciani C, Shankar SP, Ezgu F, Amantino H, Bratkovic D, et al. Treatment of Fabry's disease with the pharmacologic chaperone migalastat. *N Engl J Med*. 2016;375(6):545–55.
15. Concolino D, Deodato F, Parini R. Enzyme replacement therapy: efficacy and limitations. *Ital J Pediatr*. 2018;44(suppl 2):120.
16. Benjamin ER, Della Valle MC, Wu X, Katz E, Pruthi F, Bond S, Bronfin B, Williams H, Yu J, Bichet DG, Germain DP, et

- al. The validation of pharmacogenetics for the identification of Fabry patients to be treated with migalastat. *Genet Med*. 2017;19(4):430–38.
17. Kobayashi M, Ohashi T, Kaneshiro E, Higuchi T, Ida H. Mutation spectrum of  $\alpha$ -Galactosidase gene in Japanese patients with Fabry disease. *J Hum Genet*. 2019;64(7):695–99.
  18. Tajima Y, Kawashima I, Tsukimura T, Sugawara K, Kuroda M, Suzuki T, Togawa T, Chiba Y, Jigami Y, Ohno K, Fukushima T, et al. Use of a modified alpha-N-acetylgalactosaminidase in the development of enzyme replacement therapy for Fabry disease. *Am J Hum Genet*. 2009;85(5):569–80.
  19. Miyaoka Y, Chan AH, Judge LM, Yoo J, Huang M, Nguyen TD, Lizarraga PP, So PL, Conklin BR. Isolation of single-base genome-edited human iPS cells without antibiotic selection. *Nat Methods*. 2014;11(3):291–93.
  20. Miyaoka Y, Berman JR, Cooper SB, Mayerl SJ, Chan AH, Zhang B, Karlin-Neumann GA, Conklin BR. Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Sci Rep*. 2016;6:23549.
  21. González F, Zhu Z, Shi Z-D, Lelli K, Verma N, Li QV, Huangfu D. An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. *Cell Stem Cell*. 2014;15(2):215–26.
  22. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKolver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol*. 2009;27(9):851–57.
  23. Mayes JS, Scheerer JB, Sifers RN, Donaldson ML. Differential assay for lysosomal alpha-galactosidases in human tissues and its application to Fabry's disease. *Clin Chim Acta*. 1981;112(2):247–51.
  24. Tsukimura T, Shiga T, Saito K, Ogawa Y, Sakuraba H, Togawa T. Does administration of hydroxychloroquine/amiodarone accelerate accumulation of globotriaosylceramide and globotriaosylsphingosine in Fabry mice? *Mol Genet Metab Rep*. 2021;28:100773.
  25. Bae S, Park J, Kim J-S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics*. 2014;30(10):1473–75.
  26. Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, Cost GJ, Zhang L, Santiago Y, Miller JC, Zeitler B, et al. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol*. 2011;29(8):731–34.
  27. Ohshima T, Murray GJ, Swaim WD, Longenecker G, Quirk JM, Cardarelli CO, Sugimoto Y, Pastan I, Gottesman MM, Brady RO, Kulkarni AB. Alpha-Galactosidase A deficient mice: a model of Fabry disease. *Proc Natl Acad Sci USA*. 1997;94(6):2540–44.
  28. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol*. 2007;7(2):118–30.
  29. Sakuraba H, Murata-Ohsawa M, Kawashima I, Tajima Y, Kotani M, Ohshima T, Chiba Y, Takashiba M, Jigami Y, Fukushima T, Kanzaki T, et al. Comparison of the effects of agalsidase alfa and agalsidase beta on cultured human Fabry fibroblasts and Fabry mice. *J Hum Genet*. 2006;51(3):180–88.
  30. Lee K, Jin X, Zhang K, Copertino L, Andrews L, Baker-Malcolm J, Geagan L, Qiu H, Seiger K, Barngrover D, McPherson JM, et al. A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. *Glycobiology*. 2003;13(4):305–13.
  31. Ioannou YA, Zeidner KM, Gordon RE, Desnick RJ. Fabry disease: preclinical studies demonstrate the effectiveness of alpha-galactosidase A replacement in enzyme-deficient mice. *Am J Hum Genet*. 2001;68(1):14–25.
  32. Medin JA, Tudor M, Simovitch R, Quirk JM, Jacobson S, Murray GJ, Brady RO. Correction in trans for Fabry disease: expression, secretion and uptake of alpha-galactosidase A in patient-derived cells driven by a high-titer recombinant retroviral vector. *Proc Natl Acad Sci USA*. 1996;93(15):7917–22.
  33. Yogalingam G, Bonten EJ, van de Vlekkert D, Hu H, Moshiah S, Connell SA, d'Azzo A. Neuraminidase 1 is a negative regulator of lysosomal exocytosis. *Dev Cell*. 2008;15(1):74–86.
  34. Medina DL, Fraldi A, Bouche V, Annunziata F, Mansueto G, Spampanato C, Puri C, Pignata A, Martina JA, Sardiello M, Palmieri M, et al. Transcriptional activation of lysosomal exocytosis promotes cellular clearance. *Dev Cell*. 2011;21(3):421–30.
  35. van Meel E, Lee W-S, Liu L, Qian Y, Doray B, Kornfeld S. Multiple Domains of GlcNAc-1-phosphotransferase Mediate Recognition of Lysosomal Enzymes. *J Biol Chem*. 2016;291(15):8295–307.
  36. Kami D, Yamanami M, Tsukimura T, Maeda H, Togawa T, Sakuraba H, Gojo S. Cell Transplantation combined with recombinant collagen peptides for the treatment of Fabry disease. *Cell Transplant*. 2020;29:963689720976362.
  37. Sakuraba H, Tsukimura T, Togawa T, Tanaka T, Ohtsuka T, Sato A, Shiga T, Saito S, Ohno K. Fabry disease in a Japanese population-molecular and biochemical characteristics. *Mol Genet Metab Rep*. 2018;17:73–79.