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Modeling Glanzmann thrombasthenia using patient specific iPSCs and restoring platelet aggregation function by CD41 overexpression

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ABSTRACT

Glanzmann thrombasthenia (GT) is a rare monogenic hemorrhagic disorder involving aggregation defect of non-nuclear platelets. In this study we generated induced pluripotent stem cells (iPSCs) from skin fibroblasts of a GT patient with complex heterogeneous mutations of *ITGA2B* gene. GT-iPSCs could be successfully differentiated into platelets (GT-iPS-platelets). GT-iPS-platelets were CD41 −/CD42b +/CD61 − and were platelet activation marker (PAC-1) negative after adenosine diphosphate (ADP) activation. Furthermore, GT-iPS-platelets were defective in platelet aggregation tests *in vitro*. Moreover, exogenous expression of the wild-type *ITGA2B* gene in GT-iPS platelets restored CD41 expression and normal platelet aggregation. Our study suggested that patient-specific iPSCs could be a potential target of stem cell based gene therapy for platelet diseases.

1. Introduction

Generation of induced pluripotent stem cells (iPSCs) from differentiated adult cells like fibroblasts offered a promising means to investigate disease phenotypes in patient-derived cell model (Trounson et al., 2012). Moreover, iPSC technology opened a new era of for future cures of genetic disorders (Singh et al., 2015). Regular homologous recombination, by TALEN and CRISP/Cas9 techniques, offered the possibility to completely correct genetic defects (Hockemeyer et al., 2011; Kazuki et al., 2010; Li et al., 2015). To date, numerous disease-specific iPSCs were generated and iPSC-derived target cells already showed similar phenotype at cellular level, which indicate the potentials of iPSCs to study mechanism of genetic diseases.

Glanzmann thrombasthenia (GT) is an autosomal recessive genetic hemorrhagic disorder. The platelets in GT patients have deficiency or functional defect of platelet integrin αIIbβ3 (glycoprotein (GP) IIb/IIIa; CD41/CD61), which is essential to blood coagulation (Solh et al., 2015). Most GT is caused by mutations within the encoding gene *ITGA2B* and *ITGB3*, which impairs expression of αIIbβ3 or lead to expression of dysfunctional integrin (Nurden et al., 2015). To date, a major therapy of GT is platelet transfusion. However, platelet transfusion refractoriness may occur due to anti-platelet alloimmunization (Ishaqi et al., 2009). Thus, it is pivotal to find a better way of treating the disease. One possible and promising way is to generate genetically corrected pluripotent stem cells from GT patients. The iPS cell technology can produce patient-specific stem cells for therapeutic use and thus offer advantages over embryonic stem cell (ES cell) gene therapy in terms of immunorejection and ethical concerns (Maherali and Hochedlinger, 2008). After genetic corrections, patient-specific iPS cells can then be induced to produce autologous, normal platelets, which can be used to treat bleeding disorders in GT patients.

In our previous study, we reported a genetic diagnosis of a GT patient with two different mutations in the two *ITGA2B* alleles. Both mutations activated a same recessive intron splicing site and cause a 99-base deletion in IIb mRNA (Li et al., 2011). In the current study, we successfully established iPS cells from this GT patient (GT-iPSCs). Platelets (iGT-platelets) were generated from GT-iPSCs by directed differentiation. We found that iGT-platelets showed similar characteristics with platelets from GT patient. Furthermore, we restored the platelets' aggregation function by *ITGA2B* overexpression.

2. Materials and methods

2.1. Ethic issues

This study was approved by the Institutional Review Board (IRB) of Reproductive and Genetic Hospital of Citic-Xiangya (LL-SC-SG-2012-

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001). Written informed consent was obtained from parents of the Glanzmann thrombasthenia patient. The embryonic stem cell line chESC137 used in this study was generated with the approval of the IRB (2001—01) in previously published studies (Lin et al., 2009). This study was conducted in accordance with the Declaration of Helsinki.

2.2. Patient

A six-year-old boy was presented with recurrent nosebleed and diffused peliosis and petechia in his skin. He was diagnosed Glanzmann thrombasthenia in Xiangya hospital with the following clinical findings. aIIb and b3 were absent in the platelets of this patient analyzed by flow cytometry. There was no concentration aggregation action in response to ADP and adrenaline, but patient platelets could aggregate when treated by ristocetin (Li et al., 2011).

2.3. Generation of GT-iPSCs and culture

Skin fibroblasts of GT patients were obtained after approval by the competent authorities. Cells were grown in DMEM containing 10% fetal bovine serum (FBS) and 1 mM L-glutamine. Lentiviruses expressing OCT4, SOX2, KLF4, and cMyc were packaged using pMDG and pCMVΔR8.91.1 $\times$ 10^5 fibroblast cells from patients were infected using lentivirus in one well of a six-well dish. iPS cells were isolated as described previously (Papapetrou et al., 2009). All iPSCs were cultured in humidified chambers at 37 °C/20% CO_2/5% O_2. Human embryonic stem cells (hESCs) and iPSCs were cultured on irradiated mouse embryonic fibroblast (MEF) feeder cells in hESC medium consisting of DMEM/F12 supplemented with 15% knockout serum replacement (KOSR), 1% MEM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, and 4 ng/ml basic fibroblast growth factor (bFGF). All ingredients for hESC medium were obtained from Life Technologies (Life Technologies, Grand Island, NY, USA). The medium was changed every day and cells were mechanically passaged every 7 days.

2.4. Characterization of genetic defects in iPS cells

Genomic DNA was isolated from cells using DNeasy kit (QIAGEN, Germantown, MD, USA). Exon 4 of ITGA2B was amplified. PCR products were resolved via agarose gels, purified for sequencing. The PCR primers were described previously (Li et al., 2011).

2.5. Karyotyping analysis of GT-iPSCs

The GT-iPS cells were transferred to FBS-coated 60 mm-dishes for propagation and prevention of human embryonic fibroblasts (hEFs) contamination. After 2 passages, iPS cells were treated with 80 ng/ml Demecolcine solution (Sigma, St. Louis, MO, USA) for 4 h. Cells were fixed in methanol/glacial acetic acid (3:1) (Sigma). Metaphase spreads were made for standard G-banding karyotype analysis. The analysis was based on 5 to 10 metaphase cells for each sample.

2.6. AKP and immunofluorescence staining

Staining for alkaline phosphatase (AKP) was performed using a Fast Red Substrate Pack (Zymed Laboratories, South San Francisco, CA, USA) according to the manufacturer's protocol. For Immunofluorescence staining, Cells were fixed for 15 min in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) at room temperature, and then blocked with PBS containing 0.1% (wt/vol) Triton X-100 (Sigma), 10% normal goat serum, and 1% BSA at room temperature for 45 min. After blocking, the cells were incubated with primary antibody overnight at 4 °C, followed by incubation with secondary antibodies at room temperature in the dark for 1 h. The cells were washed between each step with PBS containing 0.1% BSA. The following antibodies were analyzed: mouse anti-SSEA-4 (1:50; R&D systems, Minneapolis, MN, USA), mouse anti-TRA-1-60, mouse anti-TRA-1-81 (1:50; Chemicon, Temecula, CA, USA), and mouse anti-OCT-3/4 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for undifferentiated iPSCs tests; mouse anti-AFP (1:500; Sigma), mouse anti-SMA (1:100; Chemicon, and mouse anti-β-tubulin (1:800; Sigma) were used for the spontaneous differentiation of iPS *in vitro* tests. Localization of antigens was visualized by using FITC conjugated second antibodies (1:400; Sigma) or Alexa Fluor 488 and 594 (1:1000; Life Technologies).

2.7. RT-PCR

Total RNA from GT-iPS or EBs was prepared from undifferentiated iPSCs using TRIzol (Life Technologies). cDNA was synthesized using 1 μg of total RNA in a 20-μl reaction. A RevertAid™ first strand cDNA synthesis kit (Fermentas Life Sciences, Hanover, MD, USA) was used according to the manufacturer's instructions. The absence of contaminating genomic DNA was confirmed for each RT-PCR experiment using controls. The specific primers for endogenous *OCT4*, endogenous *SOX2*, *NANOG*, *CRIPTO*, *LEFTA*, *THY1*, *REX1*, *FGF4*, *SOX17*, *AFP*, Brachury, *RUNX1*, *PAX6*, *SOX1*, *GATA2*, *FOG1*, *FLI-1*, *NF-E2*, GAPDH, beta-*ACTIN* were described in Table S1.

2.8. In vitro differentiation of GT-iPSCs

GT-iPSCs colonies were mechanically dissociated into small clumps and cultured as aggregates in suspension to form embryoid bodies (EBs). After 10–14 days the EBs were transferred to gelatin-coated 4-well plates for adherent culture. These cultures were stained immunohistochemically following 2 weeks incubation.

2.9. Teratoma formation and analysis

The GT-iPSCs were mechanically cut into small pieces (~50–100 cells per piece) and about 5 $\times$ 10^6 cells were injected subcutaneously into the backs of 4–6 weeks old nude mice. Seven weeks later, the mice were sacrificed and the exnografts were removed followed by fixation in 4% paraformaldehyde (Sigma) for 24 h. After paraffin embedment, the tumors were sectioned and stained by haematoxylin and eosin (H&E) (Sigma). In the experiment of the differentiation *in vivo*, the nude mice care was in accordance with guidelines for the Care and Use of Laboratory Animals, enunciated by the Ministry of Science and Technology of the People's Republic of China.

2.10. Platelet differentiation from ES and GT-iPSCs

Small clumps of chHESC-137 or GT-iPSCs were transferred onto C3H10T1/2 cells and cultured in differentiation medium, which was refreshed every 3 days according to Takayama's protocol (Takayama and Eto, 2012). In brief, on days 14 to 15 of culture with VEGF and PLGF (Stemcell Technologies, Vancouver, BC, Canada), hESCs or GT-iPSCs–derived sacs (hES-sacs or GT-sacs) were collected into a tube, gently crushed with a pipette. After passing through a 70-μm cell strainer, these cells were transferred onto fresh feeder cells in 6-well plates and maintained in differentiation medium supplemented with human TPO and SCF (Stemcell Technologies). The medium was replaced every 3 days; non-adherent cells were collected and analyzed after 20 to 24 days.

2.11. Flow cytometry analyses and Wright-Giemsa staining

Expression of cell surface molecules was analyzed by flow cytometry (FACS; BD). To detect the hemopoietic precursor cells, the cells collected from the hES-sacs or iPS-sacs were stained with antihuman CD34. To detect the megakaryocytes and platelets, the cells were stained with antihuman CD41a, antihuman CD61, antihuman CD42b. Binding of PAC-1 antibody was used to analyse IIb 3 activation in response to ADP