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Successful Induction of Pluripotent Stem Cells From a Fabry Disease Mouse Model: Toward the Development of Safe Lentiviral Gene Therapy

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Abstract

Induced pluripotent stem (iPS) cells are now recognized as a valuable tool for cell repair through autologous transplantation. These cells are obtainable from somatic cells through the induction of the transcriptional factors Oct-3/4, Klf4, and Sox2. In this study, we successfully established iPS cells from the tail-tip fibroblasts of a α-galactosidase A-knockout mouse, a well-known Fabry disease mouse model. These Fabry-iPS cells exhibited an embryonic stem (ES) cell phenotype, characterized by the expression of SSEA-1, increased alkaline phosphatase activity, silencing of the retroviral-transgene, and embryoid body (EB) formation. Subcutaneous inoculation of Fabry-iPS cells into nude mice resulted in teratoma formation. At day 6, EBs in differentiation media showed hematopoietic lineage-specific gene expression. In addition, we observed spontaneous contraction of EBs cultured on OP9 stroma cells for 5-7 days. RT-PCR demonstrated that various cardiac marker genes, such as *Nkx2.5*, *Gata4*, *Tnnt2* (cardiac troponin T), and *Mlc2a* were more highly expressed in differentiation cultures of Fabry iPS cells than of control feeder cells. To assess their potential use for gene therapy, lentiviral transduction of Fabry-iPS cells with α-galactosidase-A cDNA, the therapeutic gene for Fabry disease, was performed. This transduction resulted in elevated intracellular and secreted α-galactosidase A activity. The ES cell-specific gene expression profile remained unaltered by lentiviral therapeutic gene transfer for more than 30 days post-transduction. These findings demonstrate that Fabry-iPS cells are readily obtainable and amenable for use in gene therapy.

Keywords: α-galactosidase A; Cardiac marker genes; Embryonic stem cell; Fabry disease; Gene expression profile; Induced pluripotent stem cell; Mouse model

Introduction

Fabry disease is a lysosomal storage disorder resulting from a deficiency in α-galactosidase A (α-gal A; EC 3.2.1.22) [1] and is characterized by the systemic accumulation of sphingolipids [2]. Enzyme replacement therapy is currently available for Fabry disease, and improvement in some clinical and pathological manifestations has been reported [3,4]; however, frequent infusions are required and the long-term outcome in terms of vital organ function remains unclear. Treatment of storage disorders using allogeneic cell sources has been reported [5-7]. Although overall outcomes have improved, the risks of treatment-related mortality due to engraftment failure or severe graft-versus-host disease (GvHD) continue to be the major limitations of this approach. In addition, unmodified hematopoietic stem cells (HSCs) and their progenies fail to secrete therapeutic levels of enzyme, which can also be taken up by uncorrected yet affected organs. Cells derived from autologous sources circumvent the risk of GvHD. Additionally, the transplantation of target cells that have been genetically modified to over-express and secrete the corrective enzyme promises to be more effective in maintaining therapeutic levels. We have previously shown efficient therapeutic outcomes in Fabry disease model mice after transplantation of syngeneic HSCs that had been transduced with retroviral vectors carrying a gene encoding the human α-gal A [8-10]. However, the increased incidence of leukemias occurring in retroviral gene therapy clinical trials for X-SCID patients, due to the random integration of vectors near oncogenes, remains a major hurdle to the clinical application of this approach [11,12].

Because HSCs are less capable of maintaining their stem cell phenotype during *ex vivo* gene-manipulation, selecting clones with safer vector-integration sites is an arduous task. In contrast, embryonic stem (ES) cells maintain pluripotency during gene manipulation, retaining the capacity to differentiate into any cell type *in vitro* [13]. ES cell-based therapy, however, is complicated by immune rejection. In several recent studies, somatic cells were reprogrammed into pluripotent stem cells (termed "induced pluripotent stem cells" or iPS cells) by transduction of various transcription factors [14,15]. iPS cells are highly similar to ES cells in terms of their genetics, epigenetics, differentiation profiles, and ability to maintain pluripotency during genetic manipulation. Meng et al. has reported generation of iPS cells from a mouse model of Fabry disease [16]. They successfully demonstrated *in vitro* cardiac differentiation and *in vivo* multiple lineage differentiation of Fabry-iPS cells; however, *in vitro* hematopoietic differentiation or gene correction of iPS cells for production of the therapeutic enzyme α-gal A has not been demonstrated.

In the present study, we established Fabry mouse model-derived iPS cells, differentiated these cells *in vitro* into cells of a hematopoietic lineage, as well as *in vivo*, and transduced the cells with therapeutic lentiviral vectors to gain insights into their therapeutic potential.

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Materials and Methods

α-Gal A-knockout mouse (Fabry mouse)

α-Gal A-deficient Fabry disease model mice [17] were bred at the Kagoshima University, Kagoshima, Japan. Animal experimentation followed protocols approved by the Kagoshima University Animal Care Committee.

Cell culture and establishment of Fabry mouse-derived tail-tip fibroblasts

RF8 ES cells (kindly provided by Dr. Robert Farese of the Gladstone Institutes, San Francisco, CA) and Fabry-iPS cells were maintained on feeder layers of mitomycin C-treated mouse embryonic fibroblasts (ReproCELL, Tokyo, Japan) or mitomycin C-treated SNL 76/7 cells (European Collection of Cell Culture, Salisbury, UK) [18] in ES medium (DMEM [Invitrogen, Carlsbad, CA], containing 15% fetal bovine serum [FBS, Invitrogen], 2 mM L-glutamine [Invitrogen], 0.1 μM nonessential amino acids [Invitrogen], 0.1 μM 2-mercaptoethanol [Invitrogen], and 50 units/ml each of penicillin and streptomycin [Invitrogen]). As a source of leukemia inhibitory factor (LIF), we used human recombinant LIF (1:1000 dilution, Wako, Tokyo, Japan) or SNL feeder cells that had been clonally derived from the STO cell line transduced with LIF cDNA [18].

Plat-E packaging cells (kindly provided by Dr. Toshio Kitamura, University of Tokyo, Tokyo, Japan) [19], which were used to produce retroviruses, were maintained in DMEM containing 10% FBS, 50 units/ml each of penicillin and streptomycin, 1 μg/ml puromycin (Sigma–Aldrich, St Louis, MO), and 100 μg/ml blasticidin S (Funakoshi, Tokyo, Japan). OP9 cells (RIKEN BRC Cell Bank, Ibaraki, Japan) were maintained in alpha-MEM (Invitrogen) containing 20% FBS, 100 units/ml each of penicillin and streptomycin. Tail-tip fibroblasts (TTFs) were established from Fabry mice as previously described [20].

Retroviral vector production and infection

One day prior to transduction, Plat-E cells [19] were seeded at 3.5 × 10^6 cells per 100-mm tissue culture dish. On the next day, pMXs-based retroviral vectors (pMXs-Oct3/4, pMXs-Klf4, and pMXs-Sox2, obtained from Addgene, Cambridge, MA) [14] were introduced into these cells using Fugene 6 transfection reagent (Roche, Mannheim, Germany) as per the manufacturer's instructions. Twenty-seven microliters of Fugene 6 was diluted in 300 μl of Opti-MEM (Invitrogen) and incubated for 5 min at room temperature. Nine micrograms of each plasmid was added to the mixture, which was incubated for another 15 min at room temperature. Thereafter, the respective DNA/Fugene 6 mixtures were added dropwise to the Plat-E cells. Cells were then incubated overnight at 37°C under 5% CO_2. Twenty-four hours after transfection, the medium was replaced.

Fabry-TTFs were seeded at 4 × 10^5 cells per 100-mm dish on the day before transduction. Forty-eight hours after transfection, viral vector-containing supernatants from transfected Plat-E cells were collected and filtered through 0.45 μm filters (Millipore, Billerica, MA) and supplemented with 4 μg/ml polybrene (Nacalai Tesque, Tokyo, Japan). Target cells were incubated in the virus/polybrene-containing supernatants overnight. Three days after infection, 4 × 10^4 to 4 × 10^5 transduced cells were plated onto mitomycin C-treated SNL feeder cells in ES medium. Clones with ES cell-like morphology were selected after 2–3 weeks.

Flow cytometric analysis and alkaline phosphatase staining

Fabry-iPS cells were stained using phycoerythrin (PE)-conjugated anti-mouse SSEA-1 antibody (clone MC-480, BioLegend, San Diego, CA) or PE-conjugated mouse IgM isotype control (clone MM-30, BioLegend). Cells were then analyzed by flow cytometry using a FACScan (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo (Tree Star Inc., Ashland, OR). Alkaline phosphatase staining was performed using the Alkaline Phosphatase Detection Kit (Millipore) as per the manufacturer's instructions.

Reverse transcriptional PCR and Real time PCR for marker genes

Total RNA was extracted using RNAqueous (Applied Biosystems, Foster City, CA). We performed reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was done with KOD-plus (TOYOBO, Osaka, Japan). Primer sequences and PCR conditions were as previously described [21,22].

Real-time PCR was performed with Taqman Universal PCR Master Mix (Applied Biosystems) and Taqman Gene Expression Assays (Applied Biosystems). The Taqman Gene Expression Assays used were as follows: *Nanog* (Assay ID: Mm02384862_g1), *Zfp42* (Mm01194090_g1), *Utf1* (Mm00447703_g1), *Eras* (Mm03053919_s1), *Gata1* (Mm01352636_m1), *Gata2* (Mm00492299_g1), *Runx1* (Mm01213405_m1), *Scl/Tal1* (Mm01187033_m1), *Sfpi1* (Mm01213405_m1), *Mesp1* (Mm00801883_g1), *Brachyury* (Mm01318252_m1), *Gata4* (Mm00484689_m1), *Tnnt2* (Mm00441922_m1), *Mlc2a* (Mm00440384_m1), and *Gapdh* (Mm99999915_g1). The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles each consisting of 95°C for 15 s and 60°C for 1 min. Signals were detected with an ABI7300 Real-Time PCR System (Applied Biosystems).

Teratoma formation

Fabry-iPS cells were suspended at 5 × 10^7 cells/ml in phosphate-buffered saline (PBS; Invitrogen). Nude mice were anesthetized with 100 mg/kg bodyweight of ketamine and 10 mg/ml of xylazine. We injected 20 μl of the cell suspension (1 × 10^6 cells) subcutaneously into both inguinal regions. Five weeks after the injection, tumors were surgically dissected from the mice. Samples were fixed in PBS containing 4% formaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

In vitro differentiation of iPS cells into hematopoietic lineage cells

For EB differentiation, cells were harvested by trypsinization and transferred to gelatin-coated dishes in ES media lacking LIF for 1 h to allow feeder cells to adhere. Non-adherent cells were collected, resuspended in differentiation media (IMDM [Invitrogen] containing 15% differentiation serum [StemCell Technologies, Vancouver, Canada], 200 μg/ml holo-transferrin [Sigma], 4.5 mM monothioglycerol [Sigma], 50 μg/ml ascorbic acid [Sigma], 2 mM L-glutamine, and 50 U and 50 mg/ml of penicillin and streptomycin, respectively), in hanging drops at a concentration of 100 cells per 15 μl drop, in an inverted petri dish. EBs were collected from the hanging drops at day 2 and transferred into 10 ml differentiation media in slowly rotating 10-cm petri dishes. At day 4, EBs was fed by exchanging half of the spent medium for fresh differentiation media. Cells were harvested at day 6 by collagenase treatment and the expression of hematopoietic-specific genes was then analyzed [23].

In vitro differentiation of iPS cells into cardiac lineage cells

Five days after EB differentiation in ES media lacking LIF, EBs were plated onto OP9 cells for another 5-7 days for cardiac lineage

differentiation. Spontaneously contracting cells were harvested between days 10 and 12 by collagenase treatment and the expression of cardiac lineage-specific genes was then analyzed.

Lentiviral vector production and transduction of Fabry-iPS cells

The lentiviral vector plasmid, pHR′-EF1α-human α-gal A-WPRE-SIN (LV/human α-gal A), expressing human α-gal A, was used as previously described [10]. Vesicular stomatitis virus glycoprotein-pseudotyped lentiviral vectors were generated by transient transfection of 293T cells (ATCC, Manassas, VA) using the three-plasmid system (lentiviral vector plasmid constructs, the packaging plasmid pCMVΔR8.91, and the vesicular stomatitis virus glycoprotein envelope-coding plasmid, pMD.G) [24]. Transfections were performed with FuGENE6 (Roche). Supernatants containing viral particles were collected 48 h later for use in transduction.

α-Gal A activity assay

Alpha-Gal A activity was measured by a fluorometric assay as previously described [25]. Samples were added to a microtiter-plate reader (Twinkle LB970, Berthold Technology), and the 4-methylumbelliferone product was determined quantitatively by comparison with known standards (Sigma). Cell pellets were sonicated for 10 s in assay buffer (28 mM citric acid/44 mM disodium phosphate, pH 4.4) with 5 mg/ml sodium taurocholate.

Results

Establishment of Fabry-iPS cells

Fabry-TTFs were transduced simultaneously with pMXs retroviral vectors encoding three transcriptional factors (mouse Oct3/4, mouse Sox2, and mouse Klf4), along with the enGFP marker gene. Transduction efficiency was determined by enGFP expression: more than 95% of Fabry-TTFs expressed the marker gene as assessed by flow cytometric analysis (Figure 1A). An ES cell-like morphology was discernible 14 days after transduction (Figure 1B). Between day 14 and day 21 after transduction, 20 colonies with ES cell-like morphology were picked for further expansion. To determine whether those cells expressed ES cell-specific markers, stage-specific embryonic antigen 1 (SSEA-1) expression was analyzed by flow cytometry. As shown in Figure 1C, more than 90% of those cells expressed SSEA-1. In addition, enGFP expression was almost completely silenced, indicating that the retrovirally transduced cells were in an ES cell state (Figure 1C).

Alkaline phosphatase activity, another major phenotype of the ES cell state, was assessed by alkaline phosphatase staining. SSEA-1 (+)/enGFP (-) clones stained for this enzyme (Figure 1D). Expression of

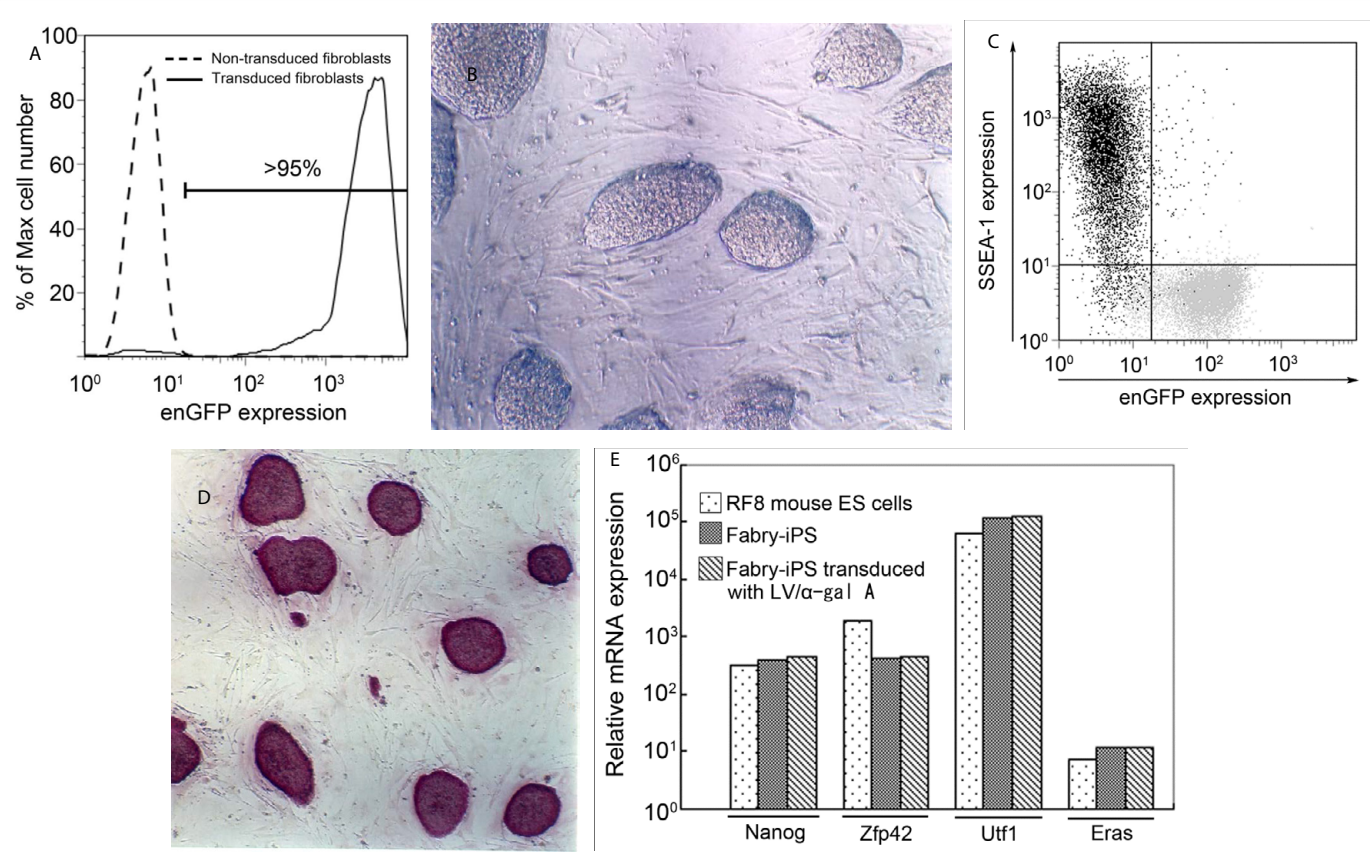

Figure 1: Embryonic stem (ES) cell-like colonies produced from Fabry mouse-derived induced pluripotent stem (iPS) cells
(A) Transduction of Fabry-TTFs with pMXs-enGFP retrovirus generated in Plat-E packaging cells.
(B) Morphology of ES cell-like colonies.
(C) SSEA-1 expression, with enGFP-silencing, in ES cell-like colonies. Mouse Oct3/4-, Klf4-, Sox2- and enGFP-transduced Fabry-TTFs were used as a control (gray dots).
(D) Alkaline phosphatase staining of ES cell-like colonies
(E) Relative ES cell-specific gene expression in Fabry-iPS cells. Real-time PCR analysis of ES cell marker genes in ES cells (RF8), Fabry-iPS cells, Fabry-TTFs, and LV/α-gal A-transduced Fabry-iPS cells. Values indicate the relative mRNA levels normalized to Fabry-TTFs. *Gapdh* was used as an internal control.

other ES cell markers was determined by real-time PCR. As shown in Figure 1E, *Nanog*, *Zfp42*, *Utf1*, and *Eras* mRNAs were induced in the cells to levels comparable to those in a control mouse ES cell line (RF8). Non-transduced Fabry-TTFs were used as negative controls.

In vivo differentiation of Fabry-iPS cells

Next, we examined the pluripotency of iPS cells by assessing their ability to induce teratoma formation. We observed tumors after subcutaneous inguinal injections of these cells into nude mice (Figure 2A). Histological examination revealed that the injected iPS cells differentiated into all three germ layers (Figure 2B).

In vitro differentiation of Fabry-iPS cells

The above data demonstrated that the iPS clones exhibited an ES cell phenotype. We then analyzed the *in vitro* differentiation capacity of Fabry-iPS cells. iPS cells formed EBs in non-coated plastic dishes (Figure 3A). Six days after inducing EB formation in differentiation cultures (EB6), *Gata1*, *Gata2*, *Runx1*, *Scl/Tal1*, and *Sfpi1* mRNA expression was up-regulated when compared to the levels in Fabry-iPS cells, as determined by real-time PCR, while ES cell-specific genes (*Nanog*, *Zfp42*, *Utf1*) were markedly down-regulated (Figure 3B). Thus, we confirmed that iPS cells were capable of differentiation into cells of a hematopoietic lineage, indicating a target for a gene therapy approach in future.

For cardiac lineage differentiation of iPS cells, spontaneously contracting EBs could be induced from EBs cultured for 5–7 days on OP9 stroma cells (data not shown). Real-time PCR demonstrated that various cardiac marker genes, such as *Nkx2.5*, *Gata4*, *Tnnt2*, and *Mlc2a*, were highly expressed in differentiation cultures of Fabry-iPS cells as compared to control feeder cells (Figure 4A and 4B).

Lentiviral transduction of Fabry-iPS cells

Fabry-iPS cells were transduced with lentiviral vector/enGFP at a multiplicity of infection (MOI) of 84 (57.8 ng p24 antigen for 1 × 10^5 Fabry-iPS cells), and enGFP expression was measured by flow cytometry with SSEA-1 co-staining at 4 days after transduction. As shown in Figure 4C, Fabry-iPS cells were efficiently transduced with lentiviral vector/enGFP vectors (up to 60%) and still maintained the expression of SSEA-1.

Next, we examined therapeutic gene transfer into Fabry-iPS cells using a human α-gal A lentiviral vector (LV/α-gal A). Fabry-iPS cells (1 × 10^5) were transduced with 55.6 ng p24 antigen of LV/α-gal A. Thirty days after transduction; intracellular α-gal A activity was measured. α-Gal A activity was elevated in LV/α-gal A-transduced Fabry-iPS cells by more than 1.7-fold higher than that of normal RF8 ES cells (Figure 4D). In addition, α-gal A activity in the culture media from the LV/α-gal A-transduced Fabry-iPS cells was assessed to measure the activity of

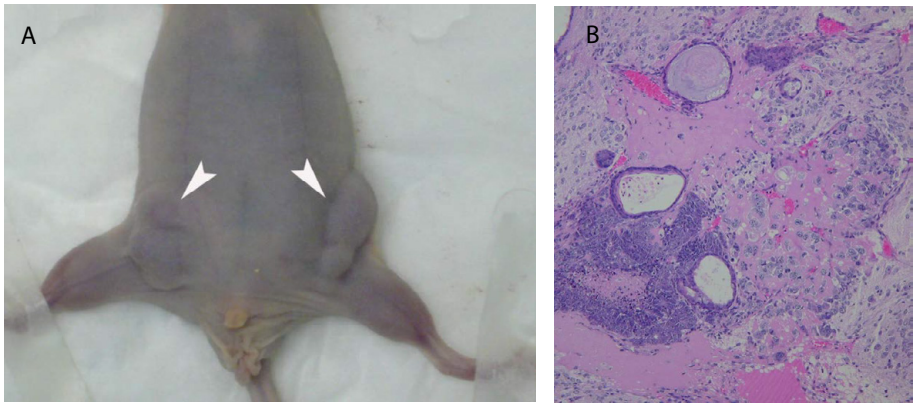

Figure 2: Teratoma formation by Fabry-iPS cells. Fabry-iPS cells were subcutaneously transplanted into nude mice. After 5 weeks, teratomas were photographed.
(A) Analyzed histologically with hematoxylin.
(B) Eosin staining.

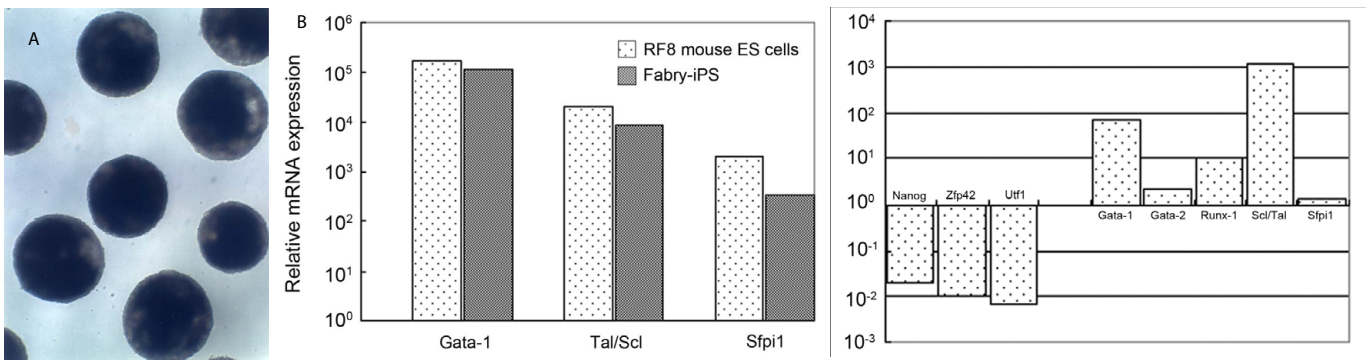

Figure 3: Differentiation of Fabry-iPS cells into cells of a hematopoietic lineage
(A) *In vitro* embryoid body formation. Embryoid bodies were photographed 5 days after induction
(B) *In vitro* differentiation of Fabry-iPS cells into cells of a hematopoietic lineage. Gene expression is shown relative to that in Fabry-iPS cells. Quantitative RT-PCR analysis of embryonic stem (ES) cell-specific and hematopoietic lineage marker genes in day 6 embryoid bodies. *Gapdh* was used as an internal control

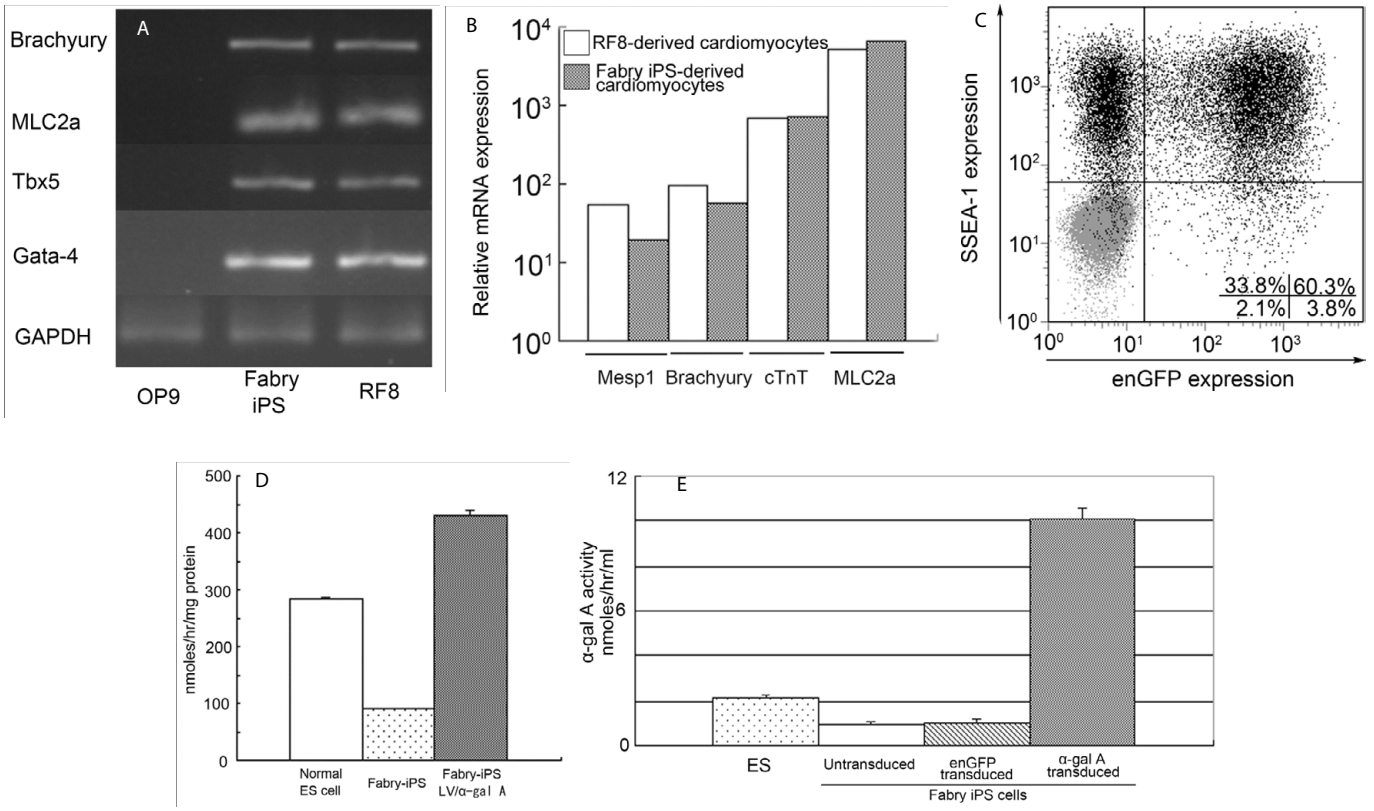

Figure 4: Differentiation of Fabry-iPS cells into cells of a cardiac lineage and transduction of Fabry-iPS cells with lentiviral vectors
(A) *In vitro* differentiation of Fabry-iPS cells into cardiac lineage. RT-PCR analysis of cardiac lineage-specific genes in contracting cells, OP9 stroma cells, and mouse embryonic stem cells (RF8). *Gapdh* was used as an internal control.
(B) *In vitro* differentiation of Fabry-iPS cells into cells of a cardiac lineage. Real-time PCR analysis of cardiac lineage marker genes in Fabry-iPS-derived contracting cells and mouse ES cell (RF8)-derived contracting cells. *Gapdh* was used as an internal control.
(C) Transduction of Fabry-iPS cells with a lentiviral vector (LV)/enGFP vector at a multiplicity of infection of 84. Transduced Fabry-iPS cells maintained SSEA-1 expression (upper right panel).
(D) Intracellular α-galactosidase A (α-gal A) activity in LV/α-gal A-transduced Fabry-iPS cells.
(E) Secreted α-gal A activity in LV/α-gal A-transduced Fabry-iPS cells.

the secreted form of α-gal A. α-Gal A was secreted in culture media of the transduced Fabry-iPS cells at levels 5-fold higher than those in the media from RF8 cells (Figure 4E).

Discussion

In the present study, we established Fabry mouse-derived iPS cells with the transduction of defined factors and confirmed their pluripotency *in vitro* and *in vivo*. Fabry-iPS cells were also efficiently and functionally transduced with therapeutic lentiviral vectors. Identification of the vector integration sites in transduced Fabry-iPS cells by inverse PCR is currently under investigation. One of the most important applications of disease-specific iPS cells is to create relevant *in vitro* disease models. In this study, Fabry-iPS cell derived cardiomyocytes were differentiated from Fabry mouse model derived cells. This *in vitro* model could be used to explore the pathological mechanism of cardiac Fabry disease. Kawagoe et al. recently reported generation of cells with the morphological features of iPS cells from Fabry patient-derived skin fibroblasts [26]. They also demonstrated expression of high levels of alkaline phosphatase, SSEA-4, TRA-1-60, and TRA-1-81; however, they did not demonstrate teratoma formation or differentiation of several cell type lineages. Those authors claimed that the difficulty of differentiation of these iPS cells into cardiomyocytes was due to accumulation of membranous cytoplasmic bodies in lysosomes. However, these findings need to be confirmed using other Fabry patient-derived fibroblasts, since the induction efficacy varies in fibroblasts from independent origins. In addition, further *in vivo* studies are warranted for investigating i) whether therapeutically corrected iPS cells, employing safer vector integration approaches, are indeed safe, ii) the development of efficient and stringent methods for differentiation of these cells into tissue stem cells, including HSCs, avoiding formation of teratomas, and iii) the long-term outcome of therapeutic intervention using therapeutically manipulated and differentiated iPS in relevant animal models.

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