Original Article

Differentiation of Human Adipose-Derived Mesenchymal Stromal/Stem Cells into Insulin-Producing Cells with A Single Tet-Off Lentiviral Vector System

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Abstract

Objective: Human adipose-derived mesenchymal stromal/stem cells (hASC) constitute an attractive source of stem cells for cell-based therapies in regenerative medicine and tissue engineering as they are easy to acquire from lipoaspirate, expansion, and genetic modification *ex vivo*. The combination of Pdx-1, MafA, and NeuroD1 has been indicated to possess the ability to reprogram various types of cells into insulin-producing cells. The aim of this study is to investigate whether MafA and NeuroD1 would cooperate with Pdx-1 in the differentiation of hASC into insulin-producing cells.

Materials and Methods: In this experimental study, we generated polycistronic expression vectors expressing Pdx1 and MafA/NeuroD1 with a reporter from a human EF-1α promoter using 2A peptides in a single tet-off lentiviral vector system. Briefly, hASC were transduced with the lentiviral vectors and allowed to differentiate into insulin-producing cells *in vitro* and *in vivo.* Thereafter, RNA expression, dithizone staining, and immunofluorescent analysis were conducted.

Results: Cleaved transcriptional factors from a single tet-off lentiviral vector were functionally equivalent to their native proteins and strictly regulated by doxycycline (Dox). Insulin gene expression in hASC transduced with Pdx1, Pdx1/MafA, and Pdx1/NeuroD1 in differentiation medium were successfully increased by 1.89 ± 0.39, 4.81 ± 0.98, 5.51 ± 0.63, respectively, compared to venus-transduced, control hASC. These cells could form dithizone-positive cell clusters *in vitro* and were found to express insulin *in vivo*.

Conclusion: Using our single tet-off lentiviral vector system, Pdx-1 and MafA/NeuroD1 could be simultaneously expressed in the absence of Dox. Further, this system allowed the differentiation of hASC into insulin-producing cells.

Keywords: Adipose-Derived Mesenchymal Stromal/Stem Cells, Doxycycline, Insulin-Producing Cells, Gene Expression Regulation, Genetic Vectors

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Introduction

Type 1 diabetes mellitus is characterized by the destruction of insulin-producing beta cells through autoimmune responses. Although insulin therapy has been used to treat diabetes, insulin administration via injection causes suffering and is inconvenient for patients with diabetes. Islet transplantation has been used to replace insulin therapy in patients with type 1 diabetes; however, donor shortage restricts the broad use of this therapy. Recent developments in stem cell biology have offered the exciting potential for generating islet β-cells from various cell types, including embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). However, ESCs and iPSCs may result in teratoma formation after transplantation. ESCs are also limited by ethical issues regarding the use of human embryos, as their retrieval requires the destruction of the embryo.

Mesenchymal stem/stromal cells (MSCs) can differentiate into various types of cells, including adipocytes, chondrocytes, osteoblasts (1), and possibly, though controversially, other non-mesodermal cells, such as keratinocytes (2), and neuronal cells (3). In addition, owing to their hypoimmunogenicity and immunomodulatory effects, MSCs are expected to be stem cell products for therapeutic purposes. Unlike other MSCs, human adipose-derived mesenchymal stromal/stem cells (hASC) can be easily and safely obtained in large quantities from a lipoaspirate from any person at any time and can be grown *ex vivo* under appropriate culture conditions. Furthermore, Strem et al. (4) reported that adipose tissue yields approximately 5 to 50 times more stem cells than bone marrow. In addition, compared to other types of stem cells, including hematopoietic stem cells, hASC can be readily transduced with all major clinically prevalent viral vector systems, including adenovirus (5),

murine retroviruses (6), lentiviruses (7), and adeno-associated viral vectors (8). Thus, hASC constitutes an attractive source of stem cells for autologous cell-based therapies in regenerative medicine and tissue engineering. Direct reprogramming of ASCs into pancreatic lineage cells has recently been reported (9-11). In addition, MSCs have been reported to express a variety of pancreas or islet progenitor genes by forced expression of pancreatic duodenal homeobox 1 (Pdx-1) (12, 13), a key transcription factor required for pancreatic development and insulin gene transcription (14). However, sequential expression of specific transcription factors, including Pdx-1, is also required for pancreatic endocrine differentiation. NeuroD1 is a class B basic helix-loop-helix transcription factor critical for pancreatic endocrine cell differentiation (15). In addition, MafA is a member of the basic leucine zipper family that is clearly expressed in the final stage of β cell differentiation and regulates insulin gene expression by binding to the insulin promoter in the C1box (16). Therefore, ectopic expression of Pdx-1, MafA, and/or NeuroD1 has been utilized to induce insulin production in mouse ESCs (17), mouse bone marrow MSCs (18), and mouse exocrine pancreatic cells *in vivo* (19). Although this approach is beneficial, there are two significant limitations to this approach. One is the inadequate efficiency of gene delivery when several genes are simultaneously transferred to the target cells and the other is the transfer of genes that stimulate differentiation to undifferentiated cells, which generally results in the inhibition of active cell proliferation, ultimately hindering the expansion of the cells required for transplantation. Solving these issues is thus inevitable for establishing the use of insulin-producing cells differentiated from autologous mesenchymal stem cells for cell regeneration therapy in the clinical.

To resolve these problems, we previously established a single tet-off lentiviral vector system, which allows for the tightly regulated and homogenous expression of genes of interest. This vector combines a modified tetracycline (tet)-response element composite promoter and a multi-cistronic strategy to express an improved version of the tet-controlled transactivator and the blasticidin resistance gene under the control of the elongation factor 1 a (EF-1a) promoter (7). In the present study, we generated polycistronic expression vectors that would express multiple transcription factors with a reporter from a single promoter using "self-cleaving" 2A peptides in our single tet-off lentiviral vector system. Using this revolutionary system, multiple transgene expression is strictly repressed in the presence of doxycycline (Dox) in undifferentiated cells during *in vitro* expansion, thereby overcoming the aforementioned limitation.

Here, we aimed to investigate whether MafA and NeuroD1 would cooperate with Pdx-1 in the differentiation of hASC into insulin-producing cells.

Materials and Methods

Animals

In this experimental study, a total of 60 male immunodeficient (C.B-17/IcrHsd-Prkdc^{scid}) mice (weight, 24-26 g; age, eight weeks) were purchased from Japan SLC, Inc. (Kyoto, Japan). All mice were housed in standard plastic cages and provided food and water ad libitum at Kindai University at 24˚C with a 12 hours light/dark cycle. The experimental procedures for the mice were approved by the Kindai University Animal Care and Use Committee (approval no. KAPR-2021-001), and the study was performed in compliance with the ARRIVE guidelines (20). Mice were anesthetized by inhalation of isoflurane (3% induction and 1% maintenance) using the SomnoSuite Small Animal Anesthesia System (Kent Scientific Corporation, Torrington, CT, USA). Before the experiment, the animals were acclimated to the facility for one week. After the completion of the experiment, the animals were euthanized using carbon dioxide at a flow rate of 30% of the chamber volume per minute in their home cages. Death was verified by the cessation of respiratory and cardiovascular movements by observation at room air for at least 10 minutes.

Adipose tissue samples

Subcutaneous adipose tissue samples (10-50 g each) were collected from a discarded tissue resected during plastic surgery for skin graft in ten subjects (four men and six women, 53 ± 5 years of age (mean ± SEM); range 30-76 years, non-obese, non-diabetic individuals). The study protocol was ethically coded and approved by the Review Board for Human Research of Osaka City University Graduate School of Medicine and the Kindai University Pharmaceutical Research and Technology Institute (reference number: 15-074). All participants provided written informed consent.

Cell culture

The hASC were isolated as previously reported (7, 21) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS, Nichirei Bioscience, Osaka, Japan), 1× GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA), and 10 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA). The cells were plated at a density of 4×10^3 cells/cm^2 on cell culture dishes, and the medium was replaced every two days.

Plasmid construction

The gene encoding Venus was a kind gift from Dr. Atsushi Miyawaki at the Brain Science Institute, RIKEN, Japan; this gene was subcloned into a pENTR11 vector (Thermo Fisher Scientific, USA), to create an entry vector, pENTR11-Venus. The pENTR11-PDX1-2A-Venus

was created according to the following outline: PDX1-T2A and Venus were amplified using polymerase chain reaction (PCR) using PDX1-2A F and R, or Venus F and R primers, respectively. PDX1-T2A and Venus were digested with EcoRI and ApaI, or ApaI and XbaI, respectively, and sub-cloned into the EcoRI and XbaI sites of the pENTR11 vector. To create pENTR11-PDX1-E2A-MAFA-T2A-Venus (hereafter PM-2A-Venus) and pENTR11-PDX1-P2A-NEUROD1-T2A-Venus (hereafter PN-2A-Venus), E2A-MAFA was amplified using PCR using 2A-MAFA F and R, or 2A-NEUROD1 F and R primers (Table S1, See Supplementary Online Information at www.celljournal.org), respectively. Thereafter, the E2A-MAFA or P2A-NEUROD1 fragment was digested with BamHI and subcloned into the BamHI site of the pENTR11-PDX1-2A-Venus vector. The entry vectors pENTR11-Venus, pENTR11-PDX1-2A-Venus, pENTR11-PM-2A-Venus, or pENTR11-PN-2A-Venus, and pTRE-RfA-EF-tTA-2A-Bsd (third-generation, self-inactivating lentiviral vector) (7) were incubated with LR clonase II enzyme mix (Thermo Fisher Scientific, USA) to generate pTRE-Venus-EF-tTA-2A-Bsd, pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd, pTRE-PM-2A-Venus-EF-tTA-2A-Bsd, or pTRE-PN-2A-Venus-EF-tTA-2A-Bsd, respectively. INS-Luc, a plasmid containing the luciferase (Luc) gene controlled by the human insulin gene promoter, was generated by subcloning the insulin promoter fragment (-342 to +6) into the pGV-B2 vector (TOYO B-net, Osaka, Japan).

Lentivirus production

Lentiviral vector particles were packaged as previously reported (7). Briefly, a 100 mm dish of non-confluent 2.5×10^6 HEK 293T cells (RIKEN BioResource Center, Tsukuba, Japan) was transfected with 10.2 µg of pCAG-HIVgp (kindly provided by Dr. Miyoshi, RIKEN BioResource Center), 10.2 µg of pCMV-VSVG-RSV-Rev (kindly provided by Dr. Miyoshi), and 18 µg of lentiviral vector plasmid (pTRE) mixed with 50 µL of 2.5M $CaCl_2$, and then mixed with 500 µL of 2×HBS (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH=7.05) and incubated at room temperature for 15 minutes. The calcium phosphate-DNA solution was then added to partially confluent HEK 293T cells for 12 hours at 37˚C in 5% CO_2. The supernatant medium, which contained lentiviral vectors, was collected two days after transduction and concentrated by centrifugation (6000 × g, 15 hours, 4˚C). Viral titers were determined using the Lenti-X qRT-PCR Titration Kit (Clontech, Mountain View, CA, USA). To obtain hASC with the tetracyclin-controlled expression of Venus, PDX1/Venus, PM-2A-Venus, or PN-2A-Venus, 1×10^5 cells of hASC at passage 3 were seeded into a 6-well plate in growth media. The next day, the lentiviral vector at a multiplicity of infection (MOI) of 250 (7) was added to each well. Then, the spinfection protocol (800 × g, 1 hour, 30˚C) was applied for efficient lentiviral vector transduction. After 24 hours, the medium was changed and cultured with 4 µg/mL blasticidin and 1 µg/mL Dox at 37˚C in 5% CO_2 for 14 days.

Western blot analysis

Whole-cell extracts were prepared by washing the cells with ice-cold phosphate-buffered saline (PBS) and lysing them with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Merck Millipore, Billerica, MA, USA), and probed with antibodies against GFP (1:1000, cat. no. A11122; Thermo Fisher Scientific, USA), and PDX1 (1:1000, cat. no. 2437, Cell Signaling Technology, Danvers, MA, USA), MAFA (1:2000, cat. no. A300-611A, BETHYL Laboratories, Montgomery, TX, USA) and NEUROD1 (1:2000, cat. no. ab109188, Abcam, Cambridge, UK), and actin (1:10000; cat. no. MAB1501, Merck Millipore). Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG antibodies (1:5000, cat. no. 7074, Cell Signaling Technology) were used as secondary antibodies, and immunoreactive bands were visualized using an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore). The band intensities were measured using the ImageJ software.

Luciferase assay

hASC transduced with INS-Luc and pGL4.74 (Promega, Madison, WI, USA) were further transfected with pTRE-Venus-EF-tTA-2A-Bsd, pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd, pTRE-PM-2A-Venus-EF-tTA-2A-Bsd, or pTRE-PN-2A-Venus-EF-tTA-2A-Bsd. Three days after transfection, according to the manufacturer's protocol, Firefly and Renilla luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega).

Induction of hASC differentiation into insulin-producing cells

Induction of hASC differentiation into insulin-producing cells was performed as previously described, with some modifications (22, 23). Before differentiation, hASC were cultured in a normal growth medium with 1 µg/mL Dox for five days. The cells were also cultured in serum-free low-glucose DMEM containing 1% dimethyl sulfoxide (DMSO) for three days and incubated with DMEM: F12(1:1) containing 1% B27 supplement (Thermo Fisher Scientific, USA), 0.5% N2 supplement (Thermo Fisher Scientific, USA), 10 mM nicotinamide (Calbiochem, San Diego, CA, USA), 10 nM exendin-4 (Sigma-Aldrich, St. Louis, MO, USA), and 10 nM Activin A (Peprotech) for 15 days. Finally, the cells were incubated with high-glucose DMEM containing 1% DMSO and 10% FBS for nine days. The medium was replaced every three days.

RNA extraction, complementary DNA generation, and quantitative polymerase chain reaction

Total RNA extraction, complementary DNA (cDNA) synthesis, and quantitative polymerase chain reaction (qPCR) analysis were performed as previously described (21) and were performed according to the Minimum

Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (24). Briefly, reactions were performed using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The relative expression of each gene was calculated using the ΔΔCt method, and the most reliable reference gene was identified from the eight genes (*ACTB, B2M, GAPDH, GUS, H6PD, UBC, UBE2D2*, and *UBE4A*) using the genormPLUS module in qbasePLUS software (Biogazelle, Zwijnaarde, Belgium). Details of the primers used in these experiments are listed in Table S2 (See Supplementary Online Information at www.celljournal.org).

Dithizone staining

Dithizone was purchased from Sigma-Aldrich. Staining was performed in a culture medium containing 0.01% dithizone at 37˚C for 15 minutes. After three rounds of rinsing with HBSS, the cells were examined under an inverted microscope.

Transplantation of hASC cells

Hyperglycemia was induced in adult male C.B-17/IcrHsd-Prkdc^{scid} mice via an intraperitoneal injection of 50 mg/kg of streptozotocin (STZ, Sigma-Aldrich) for five consecutive days. Blood glucose levels were determined using a blood glucose meter (Freestyle Freedom Lite; NIPRO, Osaka, Japan). Mice with blood glucose levels >300 mg/dl were determined as diabetic . Mice were randomly assigned to four study groups: Venus-hASC, PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, and PN-2A-Venus-hASC transplanted (n=15). Under general anesthesia, mice received a renal subcapsular transplant of 2.0×10^5 cells in the right subcapsular renal space. Blood glucose levels were monitored every two days after transplantation for five weeks. All experimental procedures were approved by the Kindai University Animal Care and Use Committee and were carried out according to institutional animal experimentation regulations.

Histology

Kidneys were fixed in 4% paraformaldehyde, embedded in an optimal cutting temperature compound, frozen, and cut into 10-µm-thick sections. The sections were then subjected to immunohistochemical analysis, as previously described (25). The sections were stained with guinea pig polyclonal antibody against insulin (1:100; cat. no. ab7842, Abcam). After the sections were washed with Tris-buffered saline, they were incubated at 4˚C for 3 hours with a Cy3 conjugated-donkey polyclonal antibody against guinea pig IgG (1:1000, cat. no. 706-165-148; Jackson ImmunoResearch). Images were obtained using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) and analyzed with the BZ Analyzer software (Keyence).

Statistical analysis

The values are expressed as mean ± SE. Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism software (GraphPad Software, Version 9, La Jolla, CA, USA). Differences were considered statistically significant at $P<0.05$. Data are presented as the mean ± SEM from three or four independent experiments.

Results

Generation and validation of the single tet-off lentiviral vectors carrying PDX1, MAFA, and NeuroD1

To increase the efficiency of hASC differentiation into insulin-producing cells, we first generated single tet-off lentiviral vectors carrying PDX1 (pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd), PDX1, and MAFA (pTRE-PM-2A-Venus-EF-tTA-2A-Bsd), and PDX1 and NeuroD1 (pTRE-PN-2A-Venus-EF-tTA-2A-Bsd) (Fig.1). EF-1α promoters drive the mRNA expression of advanced tTA linked to the *Bsd* gene by the Thosea asigna virus 2A (T2A) peptide sequence. This single transcript was then translated and cleaved into two proteins: tTA, which carries the 2A tag at the C-terminus (tTA-2A), and Bsd. In the absence of Dox, tTA-2A binds to TRE-tight and activates the transcription of the gene of interest to a very high level. However, tTA-2A cannot bind to TRE-Tight in a tet-responsive promoter in the presence of Dox. As a result, the system is inactive (7).

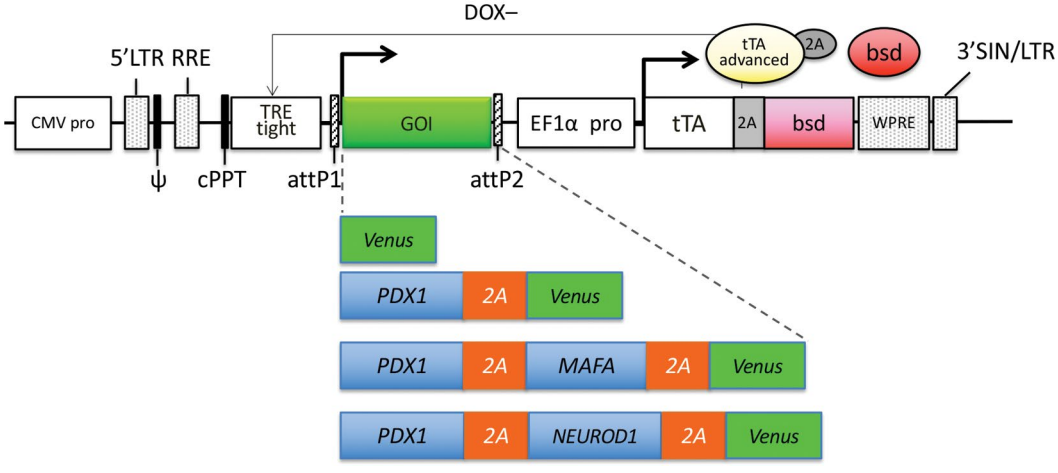

Fig.1: Schematic drawings of the single lentiviral vectors for the tet-off system used in this work.

The lentiviral vector, pTRE-EF-tTA-2A-Bsd, has an improved version of the tetracycline-controlled transactivator (tTA) linked to the blasticidin resistance (*Bsd*) gene by the Thosea asigna virus 2A (2A) peptide sequence, whose expression is regulated by the EF-1α promoter. In the absence of doxycycline (Dox), tTA-2A binds to the TRE-Tight promoter and activates the transcription of genes of interest (Venus, PDX1-2A-Venus, PDX1-2A-MAF1-2A-Venus, or PDX1-2A-NEUROD1-2A-Venus). CMV pro, CMV promoter; LTR, long terminal repeats; ψ, packaging signal; RRE, rev response elements; cPPT, central polypurine tract; TRE, tet-responsive element; GOI, gene of interest; tTA, tetracycline-controlled transactivator; Bsd, blasticidin resistance; WPRE, woodchuck hepatitis virus post-transcriptional control element; SIN, self-inactivating.

Various vectors were individually and transiently transduced into hASC to investigate the usefulness of these lentiviral vectors. As shown in Figure 2, we detected the cleaved immunoblot bands of Venus (~27 kDa), PDX1 (~46 kDa), MAFA (~40 kDa), and NeuroD1 (~50 kDa) at appropriate molecular sizes. Thereafter, we evaluated the functions of these cleaved transcription factors on the human insulin promoter. PDX1 (pCMV-PDX1-IresGFP) caused a ~2.3-fold higher enhancement of insulin promoter activity than the control (pTRE-Venus-EF-tTA-2A-Bsd) (Fig.2B). Moreover, the addition of MAFA (pCMV-MAFA-IresGFP) or NeuroD1 (pCMV-NeuroD1-IresGFP) to PDX1 (pCMV-PDX1-IresGFP) significantly upregulated the insulin promoter (i.e., ~8.5-fold) relative to the control, as previously reported (26) (Fig.2C, D). Intriguingly, transduction of pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd, pTRE-PM-2A-Venus-EF-tTA-2A-Bsd, or pTRE-PN-2A-Venus-EF-tTA-2A-Bsd resulted in the activation of the insulin gene promoter at the same levels as pCMV-PDX1-IresGFP, pCMV-PDX1-IresGFP, pCMV-MAFA-IresGFP, or pCMV-PDX1-IresGFP and pCMV-NeuroD1-IresGFP (Fig.2B-D), respectively. Such findings demonstrate that the activity of these cleaved transcriptional factors (carrying the 2A tag at the C-terminus) is functionally equivalent to that of their native protein.

Generation of hASC that express PDX1, MAFA, and NeuroD1 in a Dox-dependent manner

The single tet-off lentiviral vectors (pTRE-Venus-EF-tTA-2A-Bsd, pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd, pTRE-PM-2A-Venus-EF-tTA-2A-Bsd, and pTRE-PN-2A-Venus-EF-tTA-2A-Bsd) were transduced into hASC to generate PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, and PN-2A-Venus-hASC. As shown in Figure 3, the expression of Venus, PDX1, MAFA, and NeuroD1 in the absence of Dox could be confirmed through western blot analysis. As these expression levels were strictly suppressed in the presence of Dox, we confirmed that gene expression was strictly regulated by these vector constructs (Fig.3).

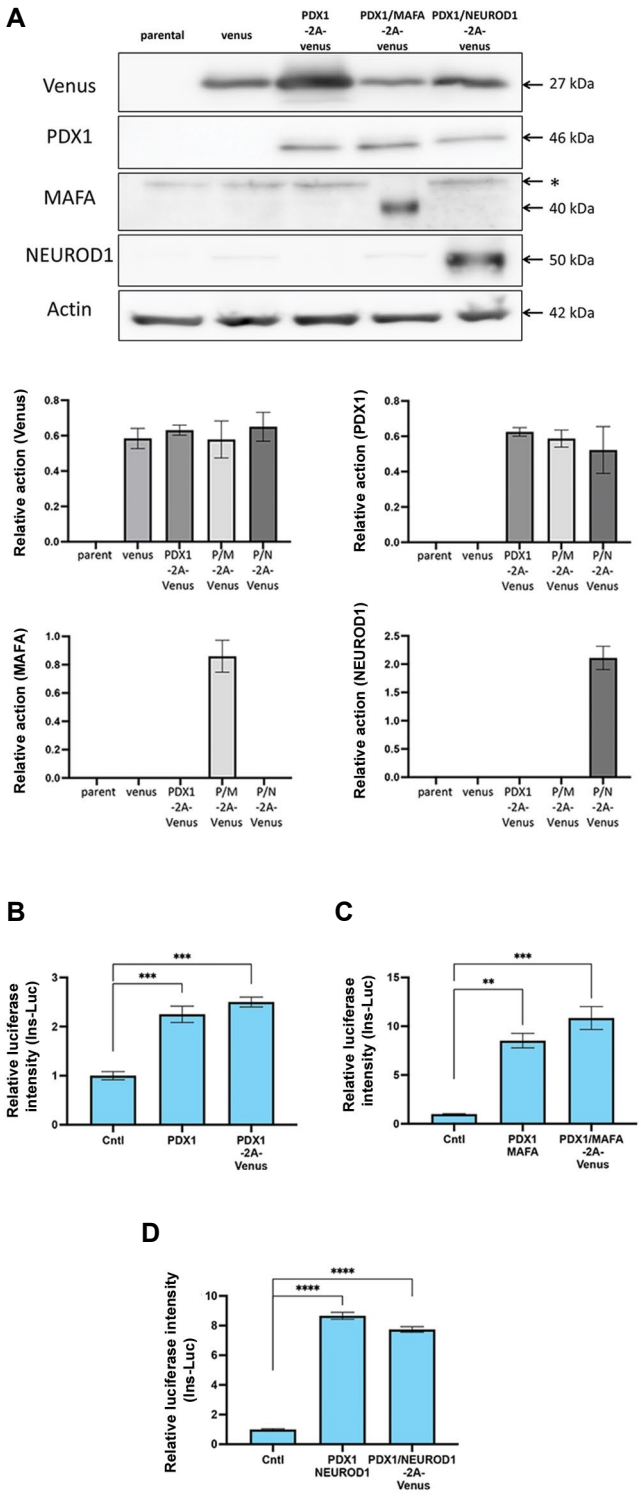

Fig.2: Evaluation of the functions of cleaved transcriptional factors. **A.** The plasmids pTRE-Venus-EF-tTA-2A-Bsd, pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd, pTRE-PM-2A-Venus-EF-tTA-2A-Bsd, or pTRE-PN-2A-Venus-EF-tTA-2A-Bsd were transiently transduced into hASC, and whole cell extract from the cells was subjected to western blot analysis. The asterisk indicates a non-specific band. The graphs indicate the mean ± SEM values from three independent experiments. **B.** The plasmids, pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd of pCMV-PDX1-IresGFP, **C.** pTRE-PM-2A-Venus-EF-tTA-2A-Bsd, or pCMV-PDX1-IresGFP and pCMV-MAFA-IresGFP, or **D.** pTRE-PN-2A-Venus-EF-tTA-2A-Bsd or pCMV-PDX1-IresGFP and pCMV-NeuroD1-IresGFP, were transiently transduced with plasmids insulin promoter-Luc and pGL4.74 into hASC. Thereafter, the effects of PDX1, MAFA, and/or NEUROD1 overexpression on INS(−342)-luc expression were evaluated. Data are presented as mean ± SEM from three independent experiments. ****; P<0.0001, ***; P<0.001, and **; P<0.01.

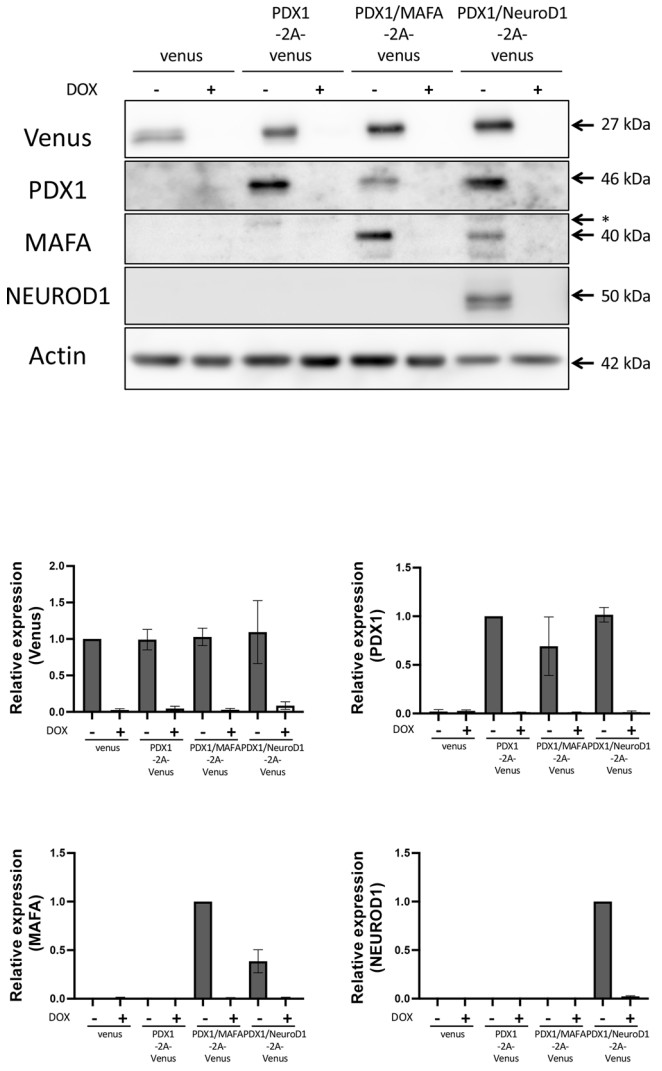

Fig.3: Evaluation of hASC that express PDX1, MAFA, and NeuroD1 in a Dox-dependent manner. hASC were transduced with pTRE-Venus-EF-tTA-2A-Bsd, pTRE-PDX1-2A-Venus-EF-tTA-2A-Bsd, pTRE-PM-2A-Venus-EF-tTA-2A-Bsd, or pTRE-PN-2A-Venus-EF-tTA-2A-Bsd at an MOI of 250. The cells were treated with 4 µg/mL blasticidin and 1 µg/mL Dox for two weeks. Thereafter, the cells were cultured in the absence (Dox –) or presence (Dox +) of 1 µg/mL Dox for four days and subjected to western blot analysis. The asterisk indicates a non-specific band. The graphs indicate mean ± SEM values from three independent experiments. hASC; Human adipose-derived mesenchymal stromal/stem cells and MOI; Multiplicity of infection.

In vitro differentiation of hASC into insulin-producing cells

Venus-hASC, PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, and PN-2A-Venus-hASC were differentiated into insulin-producing cells *in vitro* (Fig.4A). After 5–7 days in the differentiation medium, the cells became round, and cell clusters appeared; however, undifferentiated hASC remained adherent and displayed a normal fibroblast-like shape. After 12 days in the differentiation medium, a qPCR analysis was performed to determine whether the gene encoding insulin (INS) expression was upregulated in the differentiated cells. As shown in Figure 4B, *INS* expression in PDX1-2A-Venus-hASC was upregulated relative to that in Venus-hASC. Furthermore, *INS* expression levels in PM-2A-Venus-hASC and PN-2A-Venus-hASC were significantly higher than those in Venus-hASC and PDX1-2A-Venus-hASC (Fig.4B). At the end of differentiation, the extent of hASC differentiation into insulin-producing cells was evaluated via DTZ staining. DTZ specifically binds to zinc ions in insulin molecules, which allows for identifying clusters with insulin-producing cells. As shown in Figure 4C, cell aggregates in Venus-hASC were observed to be DTZ-positive, as previously reported (11); however, the number and size of the aggregates were small. The DTZ-positive cell clusters in PDX1-2A-Venus-hASC were larger than those in Venus-hASC. Moreover, markedly larger clusters that were positive for DTZ staining could be found in PM-2A-Venus-hASC and PN-2A-Venus-hASC. In contrast, none of these cells formed DTZ-positive aggregates without induction of differentiation (Fig.4D), demonstrating that transducing the transcription factors alone is insufficient for the differentiation of hASC into insulin-producing cells *in vitro*.

In vivo differentiation of hASC into insulin-producing cells

Despite failing to fully differentiate into insulin-producing cells without induction of differentiation, we noticed that PN-2A-Venus hASC spontaneously upregulated MAFA expression (Fig.3), suggesting that PM-2A-Venus and PN-2A-Venus hASC may spontaneously and partially differentiate into insulin-producing cells without inducing differentiation. Previous studies have reported that partially differentiated PDX1-MSC could differentiate into insulin-producing cells *in vivo* (12, 13, 27). We decided to transplant undifferentiated Venus-hASC, PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, and PN-2A-Venus-hASC into the renal subcapsular region of immunodeficient STZ-induced diabetic model mice to alter the external environment of the cells. Unfortunately, the blood glucose levels of the hyperglycemic mice that received Venus-hASC, PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, and PN-2A-Venus-hASC did not exhibit a significant reduction (data not shown). However, a small proportion of cells expressing insulin was observed in the renal tissue sections transplanted with PM-2A-Venus-hASC and PN-2A-Venus-hASC (Fig.5). These findings indicate that the transplanted PM-2A-Venus-hASC and PN-2A-Venus-hASC could differentiate into insulin-producing cells in the kidneys of STZ-treated mice. Thus, PM-2A-Venus-hASC and PN-2A-Venus-hASC, but Pdx1-ASCs, seemed competent to differentiate into insulin-producing cells *in vivo*.

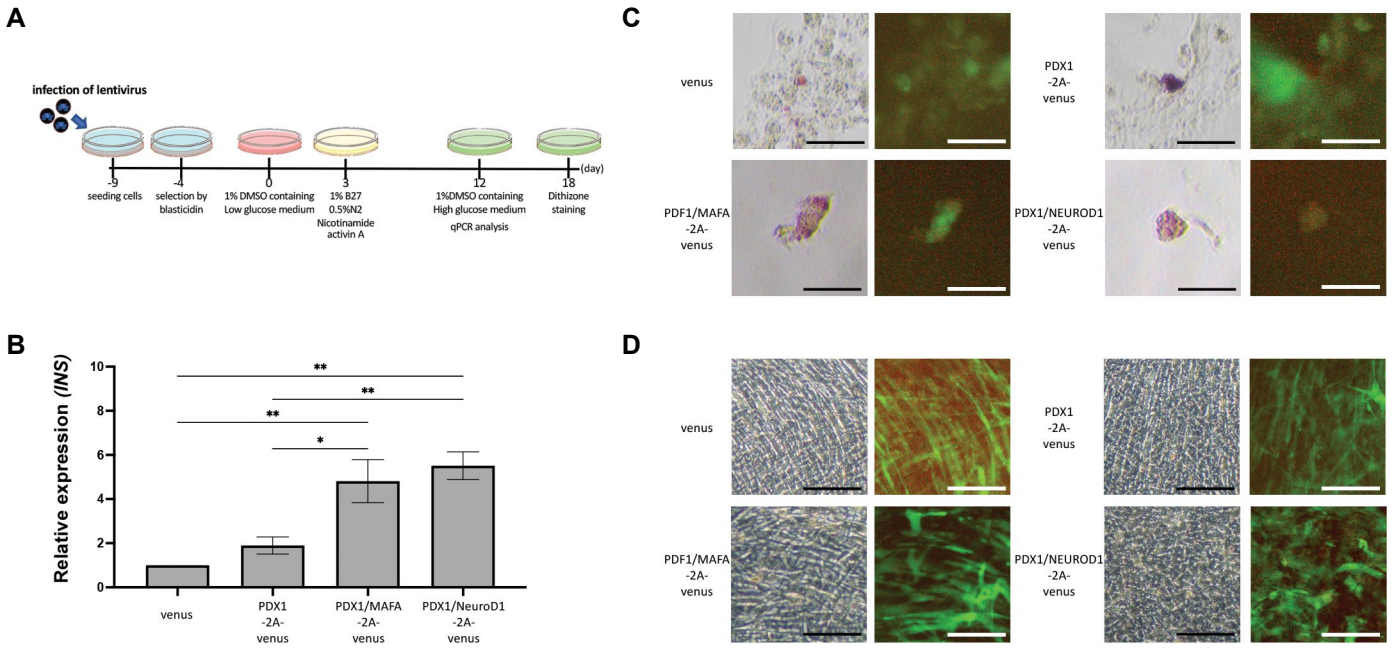

Fig.4: Evaluation of the capacities of hASC to differentiate into insulin-producing cells *in vitro*. Venus-hASC, PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, and PN-2A-Venus-hASC were differentiated into insulin-producing cells *in vitro*. **A.** Schematic illustration of the methods for differentiation of hASC into insulin-producing cells. **B.** qPCR analysis of insulin mRNA expression on day 12 of hASC differentiation was performed. Each expression value was calculated using the ΔΔCt method, with B2M as an internal control. Data represent mean ± SEM of four independent experiments. **C.** The cells were stained with dithizone (DTZ) after 18 days of differentiation. **D.** The cells were grown in a maintenance medium for 18 days (undifferentiated control) and stained with DTZ. An image of the same field of cells captured using brightfield (left) and fluorescence (right) microscopy. Venus expression was also observed to confirm the exogenous expression (right panel, scale bars: 200 µm). hASC; Human adipose-derived mesenchymal stromal/stem cells, qPCR; Quantitative polymerase chain reaction, **; P<0.01, and *; P<0.05.

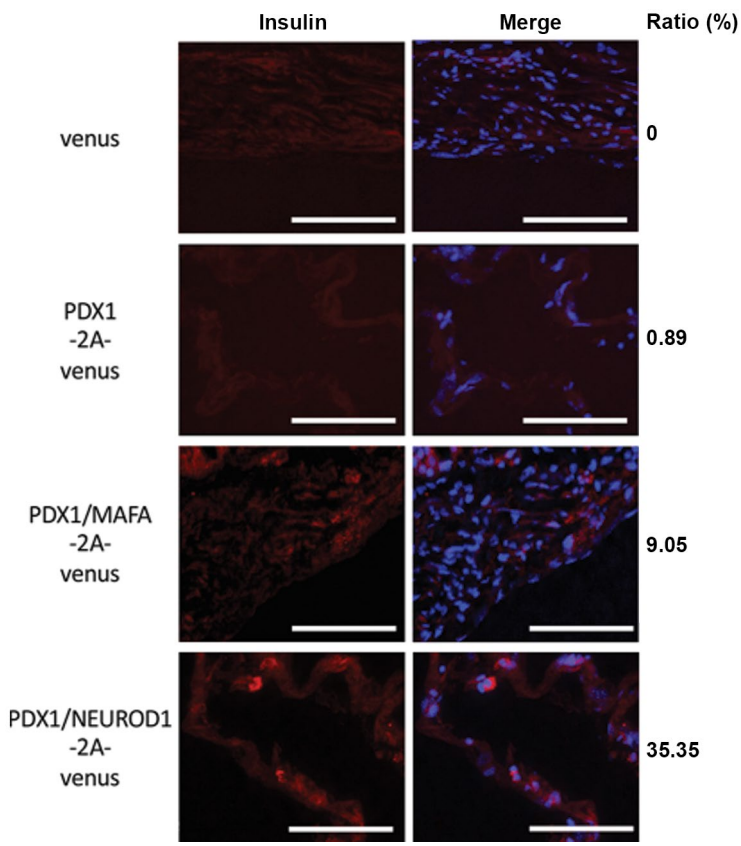

Fig.5: Evaluation of the capacities of hASC to differentiate into insulin-producing cells *in vivo*. Venus-hASC, PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, and PN-2A-Venus-hASC were transplanted into the right subcapsular renal space in STZ-treated diabetic mice. Three months after transplantation, the grafts were removed and subjected to immunofluorescence staining for insulin (red). The nuclei were stained with DAPI (scale bars: 100 µm). The numbers on the right side of the images indicate the percentage of insulin-positive cells. hASC; Human adipose-derived mesenchymal stromal/stem cells, STZ; Streptozotocin, and DAPI; 4',6-diamidino-2-phenylindole.

Discussion

Current attempts to generate functional insulin-producing cells include direct differentiation of MSCs, iPSCs, and fibroblasts (28). In this study, we demonstrated that the forced expression of PDX1 enhanced the ability of hASC to differentiate into insulin-producing cells *in vitro*. In addition, simultaneous expression of PDX1 in combination with MAFA or NEUROD1 in hASC markedly induced the differentiation of insulin-producing cells both *in vitro* and *in vivo*.

Recently, several researchers have attempted to induce the differentiation of MSCs into pancreatic lineage cells (9-11). One of the strategies is the forced expression of a single or a combination of transcription factors, including Pdx1, NeuroD1, and/or MafA, which play essential roles in promoting the development of pancreatic β cells (12-14). Kajiyama et al. (13) reported that human PDX1 gene-transduced murine ASCs acquire a functional β-cell phenotype *in vivo.* Other researchers have also demonstrated that PDX1 transduction by non-integrated lentivirus could successfully induce the differentiation of human ASCs into insulin-producing cells (12). However, our present study revealed that insulin-producing β cells were derived from PDX1-2A-Venus-hASC *in vitro* but not *in vivo*. Consistent with our present study, several researchers have found that single transduction of PDX1 could induce the expression of insulin; however, the effect elicited in MSCs was weak compared to that obtained with the combination of several factors (17, 29). PDX1 transduction has also been reported to cause severe hepatitis, as exocrine differentiation is also induced by the transduction of PDX1. Therefore, strategies using transcription factors that induce β cell differentiation without inducing the exocrine lineage should be assessed to derive an ideal system for pre-clinical applications. Kojima et al. (30) found that the combination of NeuroD1 and betacellulin induced islet neogenesis in the liver without producing hepatitis. MafA is another good candidate/therapeutic target for diabetes owing to its potent activator of insulin gene transcription (16). In the present study, NeuroD1 or MafA together with PDX1 resulted in increased insulin expression *in vitro* and *in vivo*, demonstrating that transduction of PDX1 with NeuroD1/MafA in hASC is crucial for differentiation into insulin-producing cells. To verify these results, some researchers have demonstrated that combining these three factors efficiently induces insulin gene promoter and pancreatic β cell differentiation from non-β cells (17, 18, 31, 32). As endogenous MAFA expression was observed in PN-2A-Venus-hASC, PDX1 and NEUROD1 might be a better combination than PDX1 and MAFA. However, the differentiation potential of PN-2A-Venus-hASC and PM-2A-Venus-hASC into β cells was almost identical in this study, which might be due to insufficient expression of MAFA in PN-2A-Venus-hASC. A lentiviral vector simultaneously expressing PDX1, NEUROD1, and MAFA was constructed for further experiments.

We transplanted undifferentiated hASC that were transduced with a lentiviral vector because several groups have previously demonstrated that partially differentiated PDX1-MSC [NeuroD1^{+}Insulin^{-} cells (27), Nkx2.2^{+}NeuroD1^{-}Insulin^{-} cells (13), and Ngn3^{+} glucagon^{-} cells (12)] could differentiate into insulin-producing cells *in vivo*. In addition, several researchers have shown further differentiation of MSCs into insulin-producing cells after cell transplantation under the renal capsule (33-35). Gabr et al. (33) reported that the proportion of insulin-producing cells at the end of the *in vitro* differentiation of human bone marrow-derived MSCs was relatively low (ranging between 0.12% and 3.4%) but had increased significantly (approximately 20 times) after transplantation under the kidney capsule. These data suggest that further differentiation of partially differentiated MSCs occurred after transplantation under the influence of favorable microenvironmental conditions. In the present study, neither PDX1-2A-Venus, PM-2A-Venus, or PN-2A-Venus hASC formed DTZ-positive aggregates without induction of differentiation. However, we shows that PN-2A-Venus hASC spontaneously upregulated MAFA expression, suggesting that PM-2A-Venus and PN-2A-Venus hASC may spontaneously and partially differentiate into insulin-producing cells without inducing differentiation. Consistent with our prediction, Limbert et al. (36) demonstrated that transduction of NGN3- and/or PDX1 into hMSC-TERT cell lines was sufficient to differentiate the cells into insulin-producing cells. Therefore, we assume that PM-2A-Venus and PN-2A-Venus hASC were partially differentiated and thus could differentiate into the insulin-producing cells *in vivo*.

In the present study, we did not observe a decline in the blood glucose levels of hyperglycemic mice administered either Venus-hASC, PDX1-2A-Venus-hASC, PM-2A-Venus-hASC, or PN-2A-Venus-hASC, despite their potential to differentiate into insulin-producing cells. A recent study by Gerace et al. (37) showed that *ex vivo* expansion of MSCs resulted in the impairment of differentiation into pancreatic β cells. In the present study, hASC were cultured for at least two weeks for selection with blasticidin before differentiation. The problem might be alleviated by using puromycin-resistant genes instead of blasticidin-resistant genes, as puromycin has a fast mode of action, which results in a rapid selection of transgene-positive cells compared to blasticidin. Adenovirus vectors might also aid in resolving this issue as adenoviral vectors enable high levels of protein expression and are easily amplified to high concentrations, which allows for the efficient preparation of large numbers of cells for transplantation. Notably, adenovirus vectors can contribute to a risk-benefit assessment of biosafety as they are rarely integrated into genomic DNA. However, our present lentivirus system was constructed to be compatible with the Gateway cloning technology; therefore, we can easily and rapidly transfer the insulin-inducing transcription factors to Gateway-compatible adenoviral vectors. Altogether, further experiments, including glucose-induced insulin secretion assay *in vitro* and proliferation assay of transplanted cells

in vivo, are required to address this issue. In addition, the limitation of the present study is the differentiation of hASC into only β cells because glucagon-producing α cells have been reported to be essential to maintain β cell proliferation and survival (38, 39). Therefore, the development of strategies for differentiation into not only β cells but also other pancreatic cell types might-be advantageous for the survival and proliferation of transplanted β cells. Recently, Xiao et al. (40) reported that transcription factors Pdx-1 and MafA were delivered *in vivo* with an adeno-associated virus to the mouse pancreas to reprogram α cells into functional β cells. Thus, our vector system could also be useful for this strategy. Our attractive vector system has shown some progress in increasing the efficiency of hASC differentiation into insulin-producing cells, which is an attractive cell source for clinical use. However, several improvements are required to achieve an ideal gene expression system for preclinical studies. Further improvement and fine-tuning of the present vector systems are needed to overcome the accompanying problems and reach our goals.

Conclusion

Using our single tet-off lentiviral vector system, Pdx-1 and MafA/NeuroD1 could be simultaneously expressed in the absence of Dox, and allowed the differentiation of hASC into insulin-producing cells. Although further solutions are required to overcome these limitations, the present study's findings might contribute to the development of novel cellular therapies for patients with type I diabetes.

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Authors' Contributions

H.M., M.M., T.O.; Performed the experiments. H.M., M.M., T.H.; Designed the study and interpreted the data. T.O., D.T.; Provided the samples. H.M., M.M.; Contributed to the funding for this study. H.M., M.M., T.H.; Wrote the manuscript. All authors read and approved the final manuscript.

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