Isolation of hADMPC and spermine-treatment

Human adipose tissue-derived multi-lineage progenitor cells (hADMPC) were prepared as described previously[1-5] Briefly, the resected excess adipose tissue was minced and then digested at 37°C for 1 h in Hank's balanced salt solution (HBSS, GIBCO Invitrogen, Grand Island, NY) containing containing Liberase MNP-S (Roche Diagnostics, Basel, Schweiz). Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 x *g* for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep (d=1.077; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) with 10% defined fetal bovine serum (FBS, GIBCO Invitrogen) for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/l ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin (FN)-coated dishes (AGC, Tokyo, Japan) in Stem Cell Medium (Nipro, Osaka, Japan), 1 x insulin-transferring selenium (ITS, GIBCO Invitrogen), 1 nM dexamethasone (Sigma Aldrich), 100 μM ascorbic acid 2-phosphate (Sigma Aldrich), 10 ng/ml epidermal growth factor (EGF, PeproTec, Rocky Hill, NJ), and 5% FBS (FBS, GIBCO Invitrogen., Carlsbad, CA.). After passaging 5 to 6 times in the same medium, the hADMPC were culturedwith spermine (Wako Purechemicals, Osaka, Japan.) for 24 h.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs of sperimine-pre-treated and post-treated hADMPC were isolated using an RNAeasy kit (Qiagen, Valencia, CA). After treatment with DNase, the cDNA was synthesized using Superscript III RNase H-minus Reverse Transcriptase (Invitrogen). Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). 20X Assays-on-Demand™ Gene Expression Assay Mix for human *islet-1* (Hs00158126_m1), *GATA-4* (Hs00171403_m1), *Nkx2.5* (Hs00231763_m1), *alpha-cardiac actin* (Hs01109515_m1), *myosin light chain* (*MLC*) (Hs00166405_m1), *cardiac troponin I* (Hs00165957_m1), *myosin heavy chain* (*MHC*) (Hs00411908_m1) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Hs99999905_m1) were obtained from Applied Biosystems. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2X), was also purchased from Applied Biosystems. Reactions were performed in quadruplicate for each trial and the mRNA levels were normalized relative to human GAPDH expression.

Animal model of myocardial infarction and cell transplantation

Chronic myocardial infarction swine models were prepared as described previously.[1] From 5 days before cell transplantation to the end of the experiment, the swine received CyA as an immunosuppressant (6.0 mg/kg/day intramuscularly). The CyA-immunosuppressed chronic MI swine (ejection fraction <40% by echocardiography) were randomly assigned to receive intracoronary transplantation of spermine-treated hADMPC ($1x10^5$, $3x10^5$, $1x10^6$ and $1x10^7$ cells/mL concentration of cell suspension respectively, 1mL/kg cell suspension was transplanted.) (each group n=3), or placebo lactic Ringer's solution with heparin (n=3), at 4 weeks after the second occlusion/reperfusion. Transplantation procedure was performed as following, the hockey stick type catheter was placed in the left coronary artery, and then the cell-suspensions or placebo control solution were transplanted via straight type 5Fr catheter which was introduced by the guide wire into LAD (#6).

Assessment of swine cardiac function and histological analysis

Cardiac ultrasound studies were performed before cell-transplantation and at 4, 8 and 12 weeks after transplantation using a VIVID 7 system (GE Healthcare Biosciences, Uppsala, Sweden). The studies were shown as M-mode with short axis view observed from left 5th intracostal space.

For histological analysis, the swine hearts were dissected out at the end of the experiment and immediately fixed overnight in 4% paraformaldehyde, and applied for embedding in paraffin wax. The sections were cut at 3-μm thickness, and then incubated with mouse monoclonal antibodies to human alpha-cardiac actin (American Research Products., Belmont, MA.), human actinin (Abcam, Cambridge, UK) diluted in blocking solution, followed by Alexa Fluor 488-labeled donkey anti-mouse IgG (Molecular Probes, Eugene, OR) with counter

DAPI-staining. The stained all slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka, Japan).

Results

Cardiac commitment of hADMPC into cardiac linage by spermine-treatment

The potential of Spermine committed hADMPC into cardiac-lineage was evaluated from the mRNA expression of several cardiac differentiation markers by RT-PCR before and after spermine-induction, as follows: *islet-1 is* a cardiac stem cell marker; *Nkx2.5* and *GATA-4* are transcription factors required for subsequent cardiac differentiation; and *alpha-cardiac actin (CA)*, *myosin light chain* (*MLC*), cardiac troponin I, and *myosin heavy chain* (*MHC*) are markers of cardiac differentiation (Fig 1). In each marker, after induction hADMPC expression rate was divided by pre-induction hADMPC expressed rate. In *islet-1*, after 1 day induction hADMPC expression rate is about 10.1 times higher than that of pre-induction hADMPC. And also, in *Nkx2.5*, after 1 day induction hADMPC expression rate is about 660 times higher. *GATA-4*, after 1 day induction hADMPC expression rate is about 21 times higher. *Alpha-CA*, after 1 day induction hADMPC expression rate is about 95 times higher. *MLC*, after 1 day induction hADMPC expression rate is about 11 times higher. *Cardiac Troponin I*, after 1 day induction hADMPC expression rate is about 13 times higher. *MHC*, after 1 day induction hADMPC expression rate is about5.1 times higher.

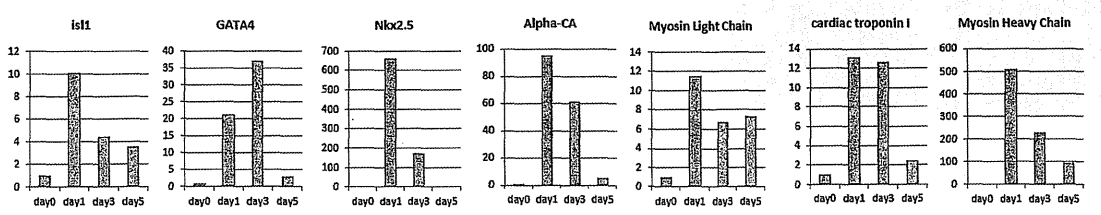

Figure 1. Spermine committed ADMPC into cardiac-lineage.

The mRNA expressions of islet-1, GATA-4, Nkx2.5, alha-cardiac actin (alpha-CA), myosin light chain (MLC), cardiac troponin I and MHC were analyzed by quantitative–polymerase chain reaction with TaqMan Probe®. After incubation with spermine for indicated time, the cells showed the increment of cardiocytic marker-expressions.

Sperimine-treated hADMPC transplantation improved cardiac function

Cardiac function was assessed by echocardiography at pretransplantation, and every 4 weeks after transplantation. The left ventricular ejection fraction (EF) and ΔEF improved improved in the implanted group, but not in control swine. This indicate that intracoronary transplantation of spermine-treated hADMPC resulted in recovery of cardiac function. The most effective dose was $3x10^5$ cells/kg.

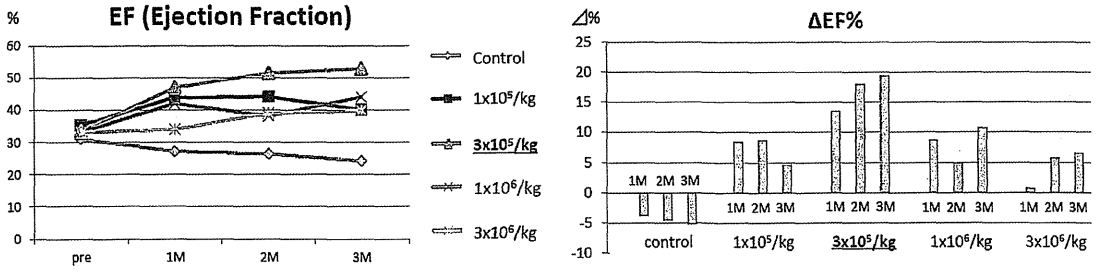

Figure 2. Sperimine-treated hADMPC transplantation improved cardiac function

Cardiac function was assessed by echocardiography at pretransplantation, and every 4 weeks after transplantation. The left ventricular ejection fraction (EF) and ΔEF improved in the implanted group, but not in control swine. The most effective dose was $3x10^5$ cells/kg.

spermine-treated hADMPC integrated in situ with the cardiac milieu

The *in situ* differentiation capacity of the implanted spermine-treated hADMPC into cardiomyocytes after grafting onto the scarred myocardium was assessed by immunohistochemical staining for human alpha-CA, human alpha actinin and HLA. Human alpha-CA and alpha actinin-positive cells were identified on the scarred myocardium indicating that at least in part of spermine-treated hADMPC integrated *in situ* with the cardiac milieu (Figure 3, left panel). Co-localizatuion of actinin (Green) and HLA (Red) indicated that spermine-treated ADMPC showed *in situ* reprogrammming into cardiomyocyte (Figure 3, right panel).

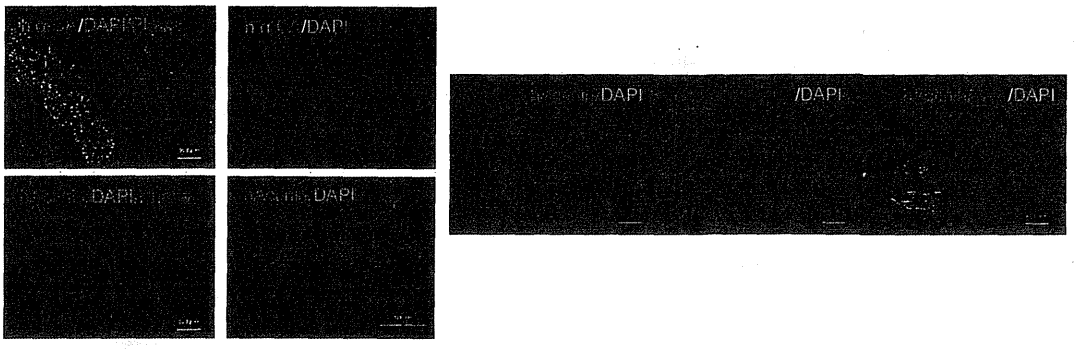

Figure 3. *in situ* reprogramming of spermine-treated ADMPC into cardiomyocyte

(Left) Spermine-treated hADMPC showed reprogramming in situ at 3-month- after transplantation. Note the presence of human alpha-CA positive or human actinin cardiac muscle bundles or cells. (Right) Co-localizatuion of actinin (Green) and HLA (Red), indicating spermine-treated ADMPC showed in situ reprogrammming into cardiomyocyte.

Discussion

There are several advantages to intracoronay transplantation of spermine-treated hADMPC for regeneration therapy. First, the source of adipose-derived cells is easily and safely accessible and large quantities of the cells can be obtained without serious ethical issues. Second, spermine-treated hADMPC can survive *in vivo* within the myocardial milieu. Finally, the reconstruction of a thick myocardial wall rescued cardiac dysfunction after chronic myocardial infarction. In conclusion, we showed that the spermine -treated hADMPCs were successfully engrafted into the scarred myocardium. The spermine-treated hADMPC-transplantation via the coronary artery resulted in recovery of cardiac function. Thus, transplantation of spermine-treated hADMPC in heart patients is probably a potentially effective therapeutic strategy for cardiac tissue regeneration within a few years.

Source of funding

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TRANSPLANTATION OF HUMAN ADIPOSE TISSUE-DERIVED MULTI-LINEAGE PROGENITOR CELLS BUT NOT ADIPOSE TISSUE-DERIVED STROMAL/STEM CELLS REDUCES SERUM CHOLESTEROL IN HYPERLIPIDEMIC WATANABE RABBITS.

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Abstract

Familial hypercholesterolemia (FH) is an autosomal codominant disease characterized by high concentrations of proatherogenic lipoproteins and premature atherosclerosis secondary to low-density lipoprotein (LDL) receptor deficiency. We have supposed that human adipose tissuederived multilineage progenitor cells (hADMPC, which were reported by Okura et al.) localized in the portal triad after transplantation via portal vein, subsequently integrated into the hepatic parenchyma and showed hepatocytic differentiation in vivo and lowered serum cholesterol in the WHHL rabbit, an animal model for homozygous FH. Here we showed that transplantation of hADMPC but not human adipose tissue-derived stromal/stem cells (hADSC, which were reported by Zuk et al.), could correct the metabolic defects of WHHL rabbit. Transplantation of hADMPC via portal vein resulted in significant reductions in total cholesterol, and the reductions maintained for 12 weeks. On the other hand, the total cholesterol levels of hADSC-transplanted group showed no significant difference to those of saline control group. To confirm transplantation of hADMPC but not hADSC reduces serum cholesterol in hyperlipidemic Watanabe rabbits, we examined LDL turnover studies using ^{125}I-labelled LDL. ^{125}I-LDL turnover study showed that the 24 hour clearance rate of LDL was significantly higher and LDL half-life was significantly shorter in the hADMPC transplanted-WHHL rabbits than those of saline control group. There was no significant difference on the ^{125}I-LDL turnover study between hADSC-transplanted group and saline control one. These results indicated that ransplantation of hADMPC but not hADSC could correct the metabolic defect of the WHHL rabbit and be a novel therapy for inherited liver diseases.

Keywords: adipose tissue, Familial hypercholesterolemia (FH), LDL

Introduction

Familial hypercholesterolemia (FH) is an autosomal co-dominant disease characterized by high concentrations of proatherogenic lipoproteins and premature atherosclerosis secondary to low-density lipoprotein (LDL) receptor deficiency. We have supposed that human adipose tissue-derived multi-lineage progenitor cells (hADMPC) localized in the portal triad after transplantation via portal vein, subsequently integrated into the hepatic parenchyma and showed hepatocytic differentiation *in vivo* and lowered serum cholesterol in the WHHL rabbits, indicating that hADMPC-transplantation via portal vein might be a novel cell therapy strategy for the treatment of FH in the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH. [1-3] However, there was no report whether hADMPC but not hADSC could lower the serum cholesterol level in WHHL rabbit after transplantation.

Materials and Methods

Adipose tissue

Adipose tissue samples were resected from 5 human subjects during plastic surgery (all females, age, 20-60 years) as excess discards. Ten to 50 grams of subcutaneous adipose tissue were collected from each subject. All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine and Foundation for Biomedical Research and Innovation.

Isolation of hADMPC and hADSC

Human adipose tissue-derived multi-lineage progenitor cells (ADMPC) were prepared as described previously.[1-5] Briefly, the resected excess adipose tissue was minced and then digested at 37°C for 1 h in Hank's balanced salt solution (HBSS, GIBCO Invitrogen, Grand Island, NY) containing Liberase MNP-S (Roche Diagnostics, Basel, Schweiz). Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 x *g* for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep (d=1.077; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) with 10% defined fetal bovine serum (FBS, GIBCO Invitrogen) for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/l ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated on human fibronectin (FN)-coated dishes (AGC, Tokyo, Japan) in Stem Cell Medium (Nipro, Osaka, Japan), 1 x insulin-transferring selenium (ITS, GIBCO Invitrogen), 1 nM dexamethasone (Sigma Aldrich), 100 μM ascorbic acid 2-phosphate (Sigma Aldrich), 10 ng/ml epidermal growth factor (EGF, PeproTec, Rocky Hill, NJ), and 5% FBS (FBS, GIBCO Invitrogen., Carlsbad, CA.). After 5 to 6 passages, the ADMPC were used for transplantation. Human adipose-tissue derived stromal/stem cells (hADSC) were obtained according to Zuk et al.[6]

Adipogenic, osteogenic and chondrogenic differentiation procedure.

For adipogenic differentiation, cells were cultured in Differentiation Medium (Zen-Bio, Inc., Research Triangle Park, NC). After three days, half of the medium was changed with Adipocyte Medium (Zen-Bio) every two days. Five days after differentiation, adipocytes were characterized by microscopic observation of intracellular lipid droplets by Oil Red O staining. Osteogenic differentiation was induced by culturing the cells in DMEM containing 10 nM dexamethasone, 50 mg/dl ascorbic acid 2-phosphate, 10 mM beta-glycerophosphate (Sigma), and 10% FBS. Differentiation was examined by Alizarin red staining. For chondrogenic differentiation, hADMPCs were first trypsinized and 2 x 10^5 cells were centrifuged at 400 x g for 10 minutes. The resulting pellets were cultured in chondrogenic medium (alpha-MEM supplemented with 10 ng/ml TGF-beta, 10 nM dexamethasone, 100 μM ascorbate, and ITS Solution) for 14 days. For Alcian Blue staining, nuclear counter-staining with Weigert's hematoxylin was followed by 0.5% Alcian Blue 8GX for proteoglycan-rich cartilage matrix.

hADMPC/ hADSC transplantation and immunosuppression regimen.

WHHL rabbits (8-week-old. Purchased from Kitayama Labes, Inc., Japan) were anesthetized with pentobarbital (50 mg/kg). An incision distal and parallel to the lower end of the ribcage was made. The peritoneum was incised and hADMPC or hADSC (3 x 10^7 cells/body) suspended in 3 mL of lactic Ringer's solution with heparin, or 3 mL of control (n=6) were infused in 5 minutes into the portal vein via a 18-gauge AngiocathTM (BD, UT). The immunosuppression regimen consisted of the following: i) intramuscular injection of cyclosporin A (6 mg/kg/day) daily from the day before surgery to sacrifice, ii) intramuscular injection of rapamycin (0.05 mg/kg/day) daily from the day before surgery to sacrifice, iii) methylprednisolone at 3 mg/kg/day (day -1 to 7), followed by tapering to 2 mg/kg/day (day 8 to 14), 1 mg/kg/day (day 15 to 21) and 0.5 mg/kg/day (day 22 to the time at sacrifice), iv) intravenous injection of cyclophosphamide (20 mg/kg/day) at day 0, 2, 5 and 7, v) ganciclovir (2.5 mg/kg/day i.m.) was also administrated to avoid viral infection in the immunocompromised host.

Assay for lipid profiling.

Serum samples were obtained from nonfasting rabbits before and after transplantation. Serum total cholesterol was measured in each sample using assay kits from Wako Pure Chemical Industries (Osaka, Japan).

Clearance of ^{125}I-LDL from rabbit serum.

Eight weeks after transplantation, the animals were tested by the LDL turnover assay. ^{125}I-LDL (BT-913R, Biomedical Technologies Inc., Inc. Stoughton, MA) was delivered via the marginal ear vein of the WHHL rabbits and normal control rabbits in physiological saline containing 2 mg/mL bovine serum albumin. Blood was collected from the opposite ear after injection at 5 min, 1 h, 2 h, 4 h, 6 h and 24 h. ^{125}I-labeled apolipoprotein B-containing LDL was precipitated with 20% of trichloroacetic acid (Wako Pure Chemical Industries) (serum; 320 μL, 100% w/v TCA 80 μL), and then the precipitants were applied for counting.

Results

Characteristic differences between hADMPC and hADSC

Adipogenic, osteogenic and chondrogenic differentiation potential of hADMPC and hADSC were examined. As shown in Fig. 1, adipocytic, osteocytic and chondrocytic differentiation potentials of ADMPC were higher than those of ADSC.

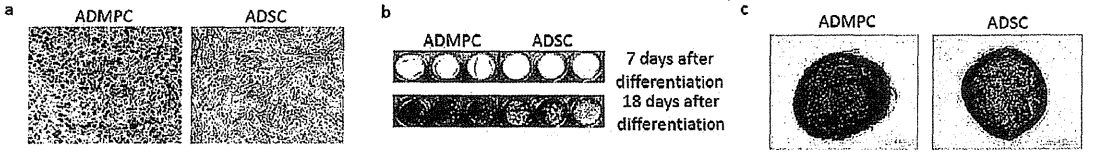

Figure 1. Adipocytic, osteocytic and chondrocytic differentiation of hADMPC and hADSC

Adipocytic (a), osteocytic (b) and chondrocytic (c) differentiation potentials of ADMPC were higher than those of ADSC.

Serum cholesterol level and LDL clearance in WHHL rabbit with transplants

To reveal the effects of hADMPC or hADSC transplantation onto the lipid profiles of the WHHL rabbit, serum cholesterol levels were monitored over 14 weeks after transplantation. Significant reductions in total serum cholesterol were observed within 4 weeks of the transplantation, and the reductions were maintained for the entire period. The reduction in serum cholesterol in the hADMPC-transplanted animals was significantly greater than that of the control or hADSC-transplantation group (Fig. 2 left panel). As shown in the right panel of Fig2, the 24 hour clearance rate of ^{125}I-LDL was significantly higher in the hADMPC-transplanted-WHHL rabbits than those of hADSC-transplanted and control group. There was no significant difference on the ^{125}I-LDL turnover study between hADSC-transplanted group and control one. These results indicated that hADMPC but not hADSC could lower the serum cholrsterol level of the WHHL rabbit, as model animal of FH.

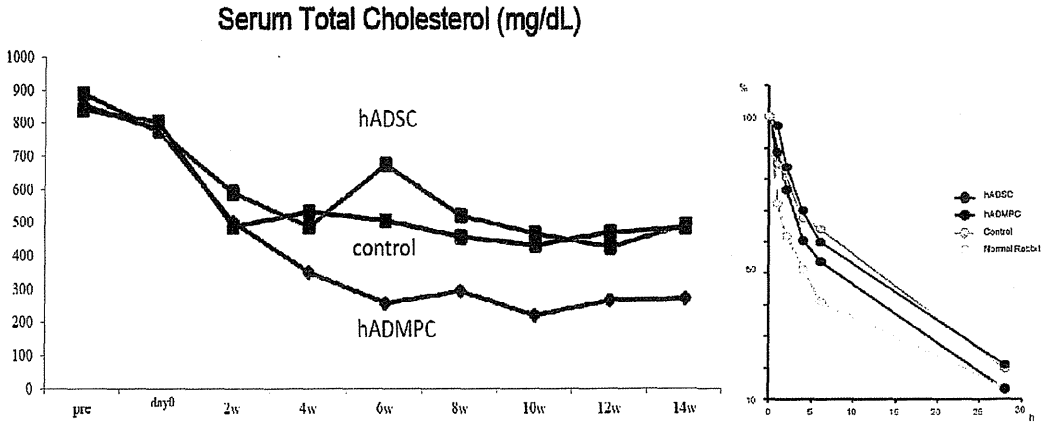

Figure 2. Serum cholesterol level and LDL clearance in WHHL rabbit with transplants.

(Left panel) Transplantation of hADMPC via portal vein resulted in significant reductions in total cholesterol, and the reductions maintained for 14 weeks. On the other hand, the total cholesterol levels of hADSC-transplanted group showed no significant difference to those of control group. (Right Panel) ^{125}*I-LDL turnover study showed that the 24 hour clearance rate of LDL was significantly higher in the hADMPC-transplanted-WHHL rabbits than those of hADSC-transplanted and control group.*

Discussion

We have used the WHHL rabbit to study the differences between hADMPC and hADSC, and the results indicated that hADMPC but not hADSC could lower the serum cholrsterol level of the WHHL rabbit, as model animal of FH.

A successful regenerative medical therapy relies on the type of cells used. The source of stem cells for such therapy should be easily and safely accessible, free of any ethical issue, and when possible, available in large amounts. Adipose tissue is considered a suitable cell source as mentioned above; because it is abundant and readily accessible. In this context, Zuk et al.[6] reported ADSC. The procedure used for obtaining cells from lipoaspirates is somewhat similar to that described by Bjorntorp et al.[7] and used for obtaining preadipocytes. Therefore, adipose tissues could be potentially suitable for use in regenerative therapy as resource of stem cells.

Next, the successful regenerative medical therapy relies on the stemness of cells used. As mesenchymal stem cells, hADMPC showed the higher differentiation ability, i.e. stemness than hADSC in adipocytic, osteocytic and chondrocytic differentiation. It has been under research why hADMPC show higher stemness than hADSC. hADMPC could only be detached by treatment with EDTA solution 24-48h-after reseeding of stromal vascular fraction and showed self-aggregation properties and EDTA-sensitiveness.[1-5] With regard to the gene expression profiling, hADMPC expressed islet-1, a marker of undifferentiated cells.[1-5] We supposed that the self-aggregation properties, EDTA-sensitiveness an silet-1 expression might lead the stemness.

We reported in the present study that hADMPC showed the therapeutical effect on the FH model WHHL rabbits, which could be obtained by EDTA treatment as a new procedure for the preparation of adipose tissue-derived cells, giving us an idea that ADMPC-transplantation might be a novel cell therapy for hemophilia, alpha-1 antitrypsin deficiency, mucolipidosis and other diseases caused by genetic defects for liver function. In near future, the therapy will be a novel therapy for kinds of inherited liver diseases.

Source of funding

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TRANSPLANTATION OF ADIPOSE TISSUE-DERIVED MULTI-LINEAGE PROGENITOR CELLS REDUCES SERUM CHOLESTEROL IN HYPERLIPIDEMIC WATANABE RABBITS.

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Abstract

Background: Familial hypercholesterolemia (FH) is an autosomal co-dominant disease characterized by high concentrations of pro-atherogenic lipoproteins and premature atherosclerosis. We have examined the response to *in situ* stem cell therapy using human adipose tissue-derived multi-lineage progenitor stem cells (hADMPC) in the LDL-receptor deficient Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH.

Methods: WHHL rabbits received either normal control rabbit-derived GFP-rabbit-derived or WHHL rabbit-derived ADMPC (normal-ADMPC, GFP-ADMPC and diseased-ADMPC, respectively) via the portal vein. This was followed by 12-week immunosuppressive therapy to avoid allogenic rejection. In situ survival and differentiation of the ADMPC into hepatocytes was examined by immunohistochemical analysis, respectively. Lipid profile was examined before-, and 4-, 8- and 12 weeks after transplantation. LDL clearance was examined at the end of the study by ^{125}I-LDL turnover.

Results: In situ survival of GFP-ADMPC was confirmed after transplantation. The cells integrated into the hepatic parenchyma and co-expressed GFP and hepatocyte markers such as albumin, indicating that the cells were reprogrammed into hepatocytes-like cells *in situ*. Transplantation of normal-ADMPC but not diseased-ADMPC resulted in a significant reduction of serum total- and LDL- cholesterol after transplantation. ^{125}I-LDL turnover study showed significant improvement in the rate of LDL clearance in the WHHL rabbits with transplanted normal-ADMPC but not in those transplanted with diseased-ADMPC.

Conclusion: Transplantation of ADMPC but not diseased ones corrected the metabolic defects in WHHL rabbits, suggesting that ADMPC transplantation is a potentially useful therapy for FH.

Keywords: adipose tissue, Familial hypercholesterolemia (FH), LDL

Introduction

Familial hypercholesterolemia (FH) is an autosomal codominant disease characterized by high concentrations of proatherogenic lipoproteins and premature atherosclerosis secondary to low-density lipoprotein (LDL) receptor deficiency. We have reported that human adipose tissue derived multi-lineage progenitor cells (ADMPC) localized in the portal triad after transplantation via portal vein, subsequently integrated into the hepatic parenchyma and showed hepatocytic differentiation *in vivo* and lowered serum cholesterol in the WHHL rabbits[1-3], indicating that ADMPC-transplantation might be *in situ* stem cell therapy strategy for the treatment of FH in the LDL-receptor deficient Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH.

Materials and Methods

Cells

Rabbit adipose tissue-derived multi-lineage progenitor cells (ADMPC) were prepared as described previously.[1-5] In brief, the resected rabbit adipose tissue was minced and then digested at 37°C for 1 h in Hank's

balanced salt solution (HBSS, GIBCO Invitrogen, Grand Island, NY) containing Liberase MNP-S (Roche Diagnostics, Basel, Schweiz). Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 x *g* for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep (d=1.077; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) with 10% defined fetal bovine serum (FBS, GIBCO Invitrogen) for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/l ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin (FN)-coated dishes (AGC, Tokyo, Japan) in Stem Cell Medium (Nipro, Osaka, Japan), 1 x insulin-transferring selenium (ITS, GIBCO Invitrogen), 1 nM dexamethasone (Sigma Aldrich), 100 μM ascorbic acid 2-phosphate (Sigma Aldrich), 10 ng/ml epidermal growth factor (EGF, PeproTec, Rocky Hill, NJ), and 5% FBS (FBS, GIBCO Invitrogen., Carlsbad, CA.). Ten to 50 grams of subcutaneous adipose tissue were collected from each rabbit. The protocol was approved by the Review Board for Rabbit Research of Foundation for Biomedical Research and Innovation. After 5 to 6 passages, the ADMPC were used for transplantation.

Rabbit ADMPC transplantation and immunosuppression regimen.

WHHL rabbits (8-week-old. Purchased from Kitayamalabes, Inc., Japan) were anesthetized with pentobarbital (50 mg/kg). An incision distal and parallel to the lower end of the ribcage was made. The peritoneum was incised and healthy GFP rabbit-derived ADMPC (n=3) or WHHL rabbit-derived ADMPC (n=3) (3 x 10^7 cells/ body) suspended in 3 mL lactic Ringer's solution with heparin, or 3 mL of control (n=3) were infused in 5 minutes into the portal vein via a 18-gauge AngiocathTM (BD, UT). The immunosuppression regimen consisted of the following: i) intramuscular injection of cyclosporin A (6 mg/kg/day) from the day before surgery to sacrifice, ii) intramuscular injection of rapamycin (0.05 mg/kg/day) from the day before surgery to sacrifice, iii) methylprednisolone at 3 mg/kg/day (day -1 to 7), followed by tapering to 2 mg/kg/day (day 8 to 14), 1 mg/kg/day (day 15 to 21) and tapered off, iv) intravenous injection of cyclophosphamide (20 mg/kg/day) at day 0, 2, 5 and 7, v) ganciclovir (2.5 mg/kg/day i.m.) was also administrated to avoid viral infection in the immunocompromised host.

Assay for lipid profiling.

Serum samples were obtained before and after transplantation. Serum total- and LDL- cholesterol were measured in each sample using assay kits from Wako Pure Chemical Industries (Osaka, Japan).

Immunohistochemical staining of WHHL rabbit liver sections.

The WHHL livers were harvested and fixed immediately with 10% formalin. They were placed into optimal cutting temperature (OCT) compound (Sakura Finetechnical Co.), frozen immediately, and then sectioned at 7 μm-thickness. The sections were then incubated with blocking solution (Blocking one; Nacalai Tesque) for 1 h. The samples were incubated with rabbit anti-albumin mouse monoclonal antibody (MBL, Nagoya, Japan) followed by Alexa Fluor 546-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR). The treated sample was examined with a BioZero laser scanning microscope (Keyence, Osaka, Japan).

Clearance of ^{125}I-LDL from rabbit serum.

The rabbits were immunosuppressed. Eight weeks later, the animals were tested by the LDL turnover assay. [I-^{125}] rabbit LDL (BT-913R, Biomedical Technologies Inc., Inc. Stoughton, MA) was delivered via the marginal ear vein of the WHHL rabbits and normal control rabbits in physiological saline containing 2 mg/mL bovine serum albumin. Blood was collected from the opposite ear after injection at 5 min, 1 h, 2 h, 4 h, 6 h and 24 h. ^{125}I-labeled apolipoprotein B-containing LDL was precipitated with 20% of trichloroacetic acid (Wako Pure Chemical Industries) (serum; 320 μL, 100% w/v TCA 80 μL), and then the precipitants were applied for counting.

Results

Serum cholesterol in WHHL rabbit with transplants

Normal- and diseased-ADMPC were separated from subcutaneous adipose tissues of healthy rabbits and WHHL rabbits respectively, cultured for 5 to 7 passages and then applied for transplantation into WHHL rabbits. WHHL were transplanted 3 x 10^7 normal- or diseased-ADMPC by portal vein infusion. To reveal the beneficial effects of normal-ADMPC but not diseased-one transplantation onto the lipid profiles of the WHHL rabbit, serum total- and LDL-cholesterol levels were monitored at the previous day and 8 week after transplantation. Significant reductions in total- and LDL- cholesterol were observed 8 week after transplantation but not in diseased one, compared to the controls.

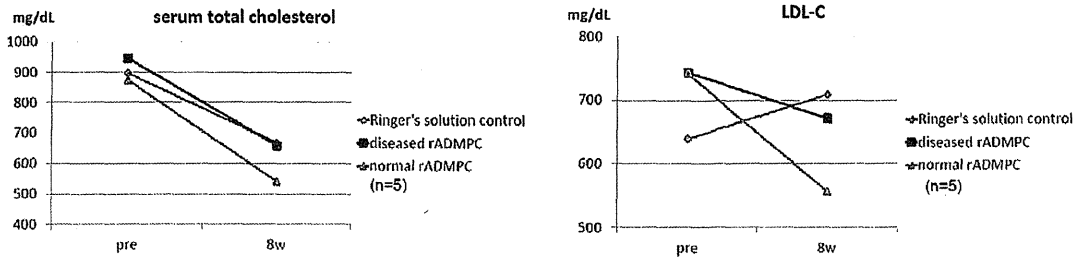

Figure 1. Serum cholesterol reduction effect of rADMPC transplantation.

Total serum cholesterol and LDL cholesterol levels. Normal /diseased rADMPC-transplantation, and Ringer's solution control in WHHL rabbits was followed for 8 weeks. Total cholesterol and LDL cholesterol levels of normal rADMPC transplanted WHHL rabbits were significantly lower than those of placebo-treated and diseased rADMPC transplanted ones.

Next, clearance experiments were performed with human LDL in order to confirm that the transplanted normal-ADMPC but not in diseased-one contributed the fall in serum cholesterol through uptake of LDL via LDL receptors. The rate of LDL clearance was significantly higher in the WHHL rabbits with transplanted normal-ADMPC than that of the control and diseased-ADMPC transplantation group.

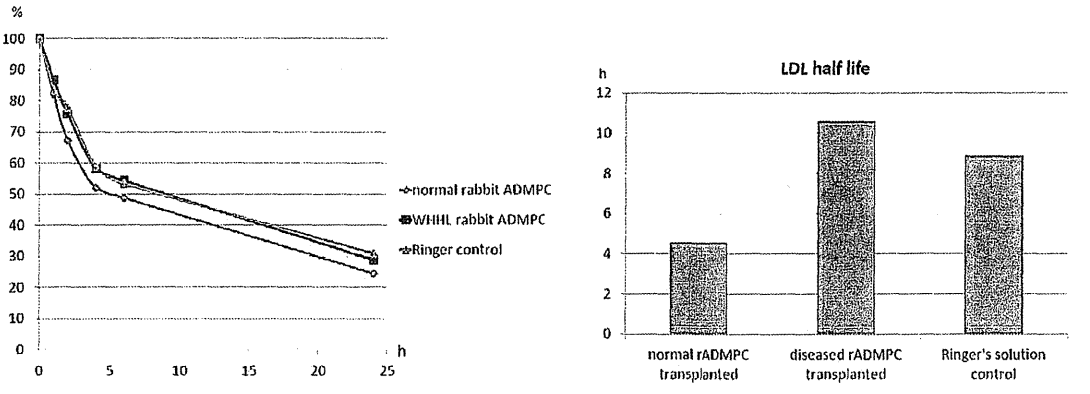

Figure 2. LDL turn over study.

^{125}I-LDL turnover study showed that the 24 hour clearance rate of LDL was significantly higher and LDL half life was significantly shorter in the normal-ADMPC transplanted-WHHL rabbits than those of diseased-ADMPC transplanted ones and Ringer's solution control.

in situ reprogramming of rabbit ADMPC into Hepatpcyte-like cells

To examine whether transplanted ADMPC should survive into hepatic parenchyma and exert function like hepatocytes, immunohistochamical analysis were performed.Eight weeks after transplantation of ADMPC

derived from healthy GFP rabbit, co-presence of albumin (red) and GFP-fluorescence (green) on the same cells was observed, indicating the transplanted ADMPC might differentiate into hepatocyte-like cells *in situ*.

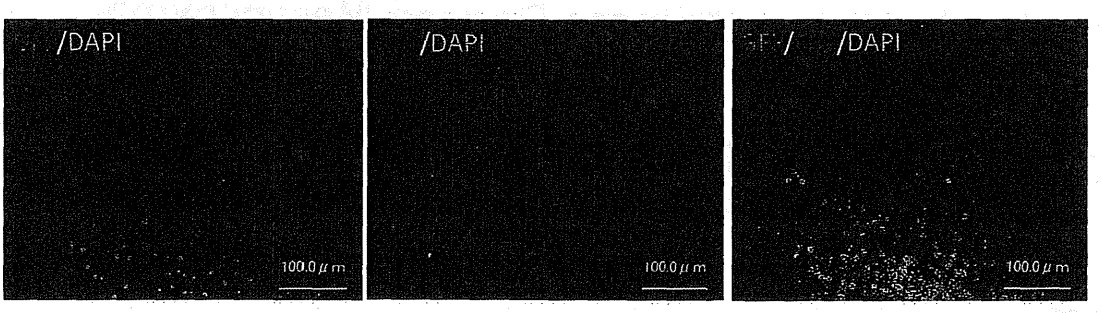

Figure 3. *in situ* reprogramming of rabbit ADMPC into Hepatpcyte-like cells

Immunohistochemical identification of albumin-positive cells in liver sections of WHHL rabbits after GFP-ADMPC- transplantation. Eight weeks after transplantation, the cells integrated into the hepatic parenchyma and almost GFP positive cells expressed albumin, indicating that the cells were reprogrammed into hepatocytes-like cells in situ.

Discussion

We have used the WHHL rabbit to study the ability of ADMPC-derived hepatocytes to lower serum cholesterol in an animal model of FH. The reductions in cholesterol brought about by the engrafted ADMPC-derived hepatocytes suggest that LDL receptors of normal-ADMPC can act as replacement for the mutant LDL receptors in the WHHL rabbit. The substantial decrease in serum cholesterol achieved suggests that the ADMPC-derived hepatocytes both internalize LDL and metabolize the cholesterol to bile for excretion. The correlation between cholesterol and coronary heart disease has been well documented, and decreases in serum cholesterol of the magnitude that we have demonstrated would be expected to decrease morbidity and mortality in the patients with severe FH[5]. These results suggested that ADMPCs transplantation via portal vein could correct the metabolic defects of FH patients and that ADMPC-derived hepatocytes could function as supplier with plasma proteins derived from liver, giving us an idea that ADMPC-transplantation might be a novel cell therapy for hemophilia, alpha-1 antitrypsin deficiency, mucolipidosis and other diseases caused by genetic defects for liver function. In near future, the therapy will be a novel therapy for kinds of inherited liver diseases.

Source of funding

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PLOS ONE

Tightly Regulated and Homogeneous Transgene Expression in Human Adipose-Derived Mesenchymal Stem Cells by Lentivirus with Tet-Off System

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Abstract

Genetic modification of human adipose tissue–derived multilineage progenitor cells (hADMPCs) is highly valuable for their exploitation in therapeutic applications. Here, we have developed a novel single tet-off lentiviral vector platform. This vector combines (1) a modified tetracycline (tet)-response element composite promoter, (2) a multi-cistronic strategy to express an improved version of the tet-controlled transactivator and the blasticidin resistance gene under the control of a ubiquitous promoter, and (3) acceptor sites for easy recombination cloning of the gene of interest. In the present study, we used the cytomegalovirus (CMV) or the elongation factor 1 α (EF-1α) promoter as the ubiquitous promoter, and EGFP was introduced as the gene of interest. hADMPCs transduced with a lentiviral vector carrying either the CMV promoter or the EF-1α promoter were effectively selected by blasticidin without affecting their stem cell properties, and EGFP expression was strictly regulated by doxycycline (Dox) treatment in these cells. However, the single tet-off lentiviral vector carrying the EF-1α promoter provided more homogenous expression of EGFP in hADMPCs. Intriguingly, differentiated cells from these Dox-responsive cell lines constitutively expressed EGFP only in the absence of Dox. This single tet-off lentiviral vector thus provides an important tool for applied research on hADMPCs.

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Introduction

Human adipose tissue–derived mesenchymal stem cells (MSCs), also referred to as human adipose tissue–derived multilineage progenitor cells (hADMPCs), are multipotent stem cells that can differentiate into various types of cells, including hepatocytes [1], cardiomyoblasts [2], pancreatic cells [3], and neuronal cells [4–6]. They can be easily and safely obtained from lipoaspirates without posing serious ethical issues and can also be expanded ex vivo under appropriate culture conditions. Moreover, MSCs, including hADMPCs, have the ability to migrate to injured areas and secrete a wide variety of cytokines and growth factors necessary for tissue regeneration [7–11]. Because of their hypoimmunogenicity and immune modulatory effects, hADMPCs are good candidates for gene delivery vehicles for therapeutic purposes [12]. Thus, hADMPCs are an attractive material for cell therapy and tissue engineering, making the development of technologies for permanent and highly controlled genetic modification of hADMPCs quite valuable.

Lentiviral vectors are powerful tools for gene transfer in primary human cells, as they integrate into the host cell genome, resulting in stable long-term transgene expression. Lentiviral vectors are less prone to transcriptional silencing than oncoretroviral vectors [13,14]; however, researchers have reported that transgene silencing occurs when a strong promoter, such as the cytomegalovirus (CMV) promoter, is used in certain cell types, especially embryonic stem cells [15–17]. Recently, it has been reported that the CMV promoter is also silenced in rat bone marrow–derived MSCs [18,19], suggesting that consideration of promoter used in the lentiviral vector is one of the most critical issues.

In addition to the choice of promoters, the specific gene expression system can have a great impact on the properties and functions of the infected hADMPCs. In order to express therapeutic genes, master regulatory genes, or microRNAs, the development of a tightly regulated, inducible gene expression system is required. The tetracycline (tet)-regulated transgene expression (tet-off) system is the most advanced system being used in gene therapy trials [20]. Two expression cassettes need to be delivered for use of the tet-off system: the regulatory unit for the constitutive expression of the transactivator (tTA), and the tet-controlled responsive unit for the expression of the gene of interest. Traditionally, these 2 cassettes should be transduced separately to establish tet-inducible cell lines. This time-consuming process

significantly limits the number of cell lines that can be generated for target gene expression. Recently, several researchers attempted to develop single-vector–based tet-inducible lentiviral systems [21–24]. However, the large plasmid size and lack of antibiotic selectable markers in these systems made the generation of plasmid constructs, high titer lentiviral particles, and stably expressing transgenic cell lines difficult.

To overcome the limitations of the current single vector-based tet-inducible lentiviral systems, we generated a robust system that incorporates all the necessary components for tet-off gene expression, restriction enzyme treatment/ligation independent cloning system, and antibiotic selectable markers in a single lentiviral vector. This vector consists of a modified tet-response element composite promoter (TRE-Tight) followed by a Gateway cassette containing *att*R recombination sites flanking a *ccd*B gene and a chloramphenicol resistant gene, which allows for easy and rapid shuttling of the gene of interest into the vector. This vector also carries an improved version of the tet-controlled transactivator (tTA-advanced) and the blasticidin resistance gene, linked by the self-cleaving viral T2A peptide, under a ubiquitous promoter. In the present study, we examined 2 ubiquitous promoters commonly used in mammalian systems: the CMV promoter and the human polypeptide chain elongation factor 1 α (EF-1α) promoter, to determine which promoter is more efficient in hADMPCs. In addition, we also confirmed whether genetically modified hADMPCs maintained their stem cell properties following transduction with this single tet-off lentiviral vector. We examined the expression pattern of cell surface markers, as well as the cells' differentiation potential into adipocytes, chondrocytes, osteocytes, and neuronal cells. Our data demonstrated that hADMPCs transduced with our all-in-one lentiviral vector were effectively selected by blasticidin without affecting their stem cell properties, and transgene expression was strictly regulated by doxycycline (Dox) not only in undifferentiated cells but also in differentiated cells. A single tet-off lentiviral vector system thus provides a powerful tool for applied research on hADMPCs.

Materials and Methods

Adipose Tissue Samples

Subcutaneous adipose tissue samples (10–50 g each) were resected during plastic surgery in 5 women (age, 20–60 years) as excess discards. The study protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Foundation for Biomedical Research and Innovation, and Kinki University Pharmaceutical Research and Technology Institute (reference number: 10-005). Each subject provided signed informed consent.

Cell Culture

hADMPCs were isolated as previously reported [1,11,25,26] and maintained in a medium containing 60% DMEM-low glucose, 40% MCDB-201 medium (Sigma Aldrich, St. Louis, MO, USA), 1× insulin-transferrin-selenium (Life technologies, Carlsbad, CA, USA), 1 nM dexamethasone (Sigma Aldrich, St. Louis, MO, USA), 100 mM ascorbic acid 2-phosphate (Wako, Osaka, Japan), 10 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), and 5% fetal bovine serum. The cells were plated to a density of 5×10^3 cells/cm^2 on fibronectin-coated dishes, and the medium was replaced every 2 days.

Plasmid Construction and Lentivirus Production

EGFP was cloned into a pENTR11 vector (Invitrogen) to create an entry vector, pENTR11-EGFP. To generate pTRE-RfA, the tet-responsive element (TRE) of the pTRE-Tight vector (Clontech, Mountain Veiw, CA, USA) and the Reading frame A (RfA), a Gateway cassette containing *att*R recombination sites flanking a *ccd*B gene and a chloramphenicol-resistance gene (Invitrogen) were introduced into *Xba*I-*Xho*I sites of pSico (Addgene plasmid 11578). An improved version of the tet-controlled transactivator (tTA-advanced: pTet-off-advanced Clontech) was linked to the blasticidin resistance (Bsd) gene by the viral T2A peptide to generate tTA-2A-Bsd. Briefly, 2A-Bsd was amplified by PCR using the following primers:

2A-Bsd F: GGGGGATCCGGCGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAAAATCCCGGGCCCATGAAGACCTTCAACATCTCTCAG, Bsd R: GCGAGATCTTTAGTTCCTGGTGTACTTG. The resultant product was confirmed by sequencing and ligation with the SmaI site of tTA. EF promoter/CMV promoter and tTA-2A-Bsd was introduced into pTRE-RfA to produce pTRE-RfA-EF-tTA-2A-Bsd or pTRE-RfA-CMV-tTA-2A-Bsd. The entry vector pENTR11-EGFP and pTRE-RfA-EF-tTA-2A-Bsd, pTRE-RfA-CMV-tTA-2A-Bsd, CSII-EF-RfA, or CSII-CMV-RfA (kindly provided by Dr. Miyoshi, RIKEN BioResource Center, Tsukuba, Japan) were incubated with LR clonase II enzyme mix (Invitrogen) to generate pTRE-EGFP-EF-tTA-2A-Bsd, pTRE-EGFP-CMV-tTA-2A-Bsd, CSII-EF-EGFP or CSII-CMV-EGFP. The resultant plasmid was mixed with packaging plasmids (pCAG-HIVg/p and pCMV-VSVG-RSV-Rev, kindly provided by Dr. Miyoshi) and transfected into 293T cells. The supernatant medium, which contained lentiviral vectors, was collected 2 days after transduction and concentrated by centrifugation (6000× *g*, 15 h, 4°C). Viral titers (transduction unit: TU) were determined by serial dilution on 293T cells and the percentage of EGFP positive cells was measured by Guava easyCyte 8HT flow cytometer (Merck-Millipore, Billerica, MA, USA).

Plasmid Propagation in *E. coli*

DH5α (F-, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK-, mK+), phoA, supE44, λ-, thi-1, gyrA96, relA1) were used for general purpose. To propagate plasmids containing the ccdB gene, One Shot® ccdB Survival™ 2 T1 Phage-Resistant (T1R) chemically competent *E. coli* (Invitrogen) were used.

Western Blot Analysis

Cells were washed with ice-cold phosphate-buffered saline and lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific Pierce, Rockford, IL, USA). Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (Immobilon-P; Merck-Millipore), and probed with antibody against TetR (from Clontech). Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Cell Signaling Technology, Danvers, MA, USA) was used as a probe, and immunoreactive bands were visualized with the Immobilon Western Chemiluminescent HRP substrate (Millipore). The band intensity was measured using ImageJ software.

Flow Cytometry Analysis

hADMPCs were seeded at a density of 2×10^4 cells per well in 12-well culture plates and were transduced with CSII-EF-EGFP or CSII-CMV-EGFP at a multiplicity of infection (m.o.i.) of 25, 50,

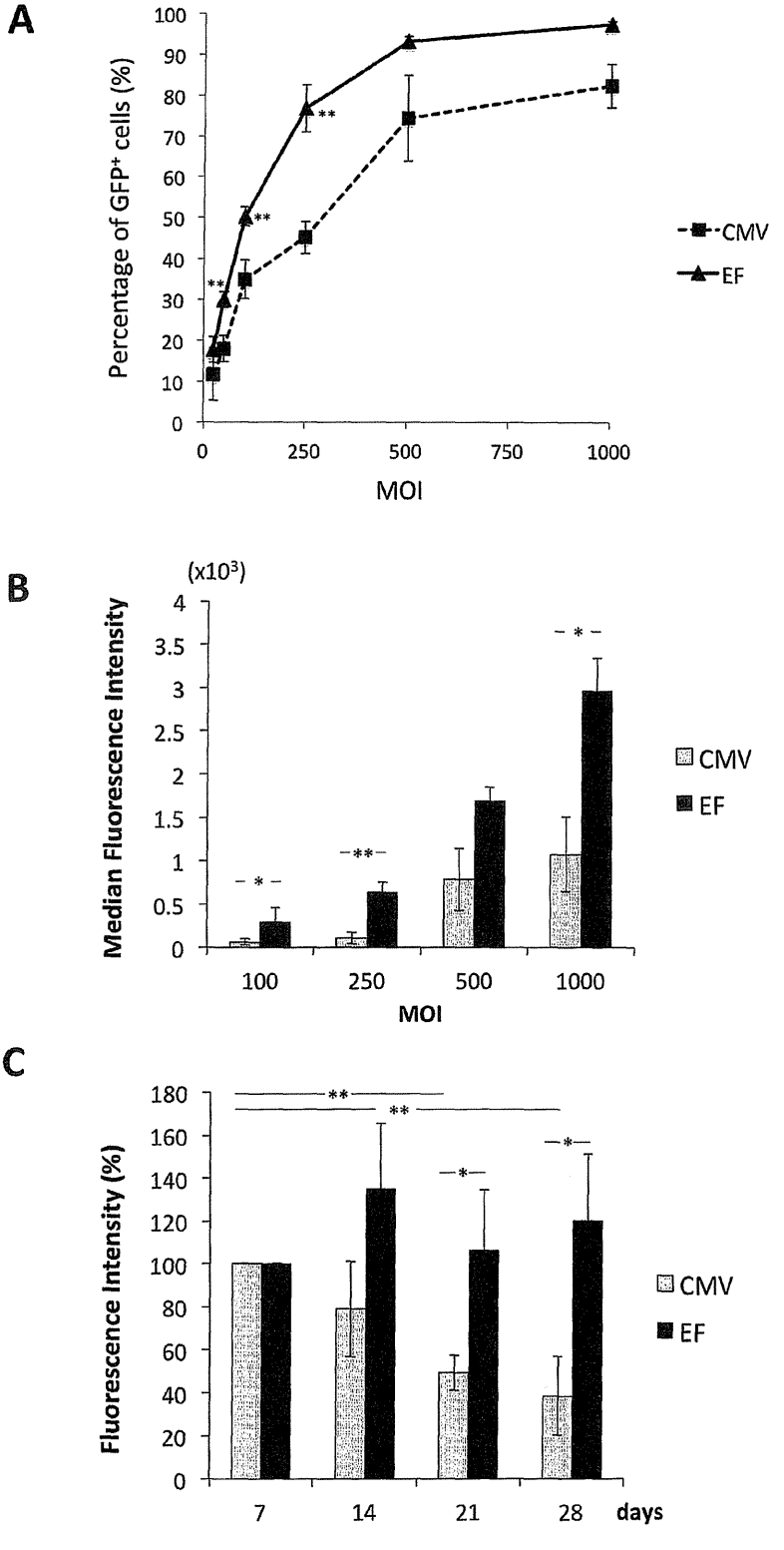

Figure 1. The efficiency of CMV or EF-1α promoter in hADMPCs. Lentiviral vectors encoding EGFP under the control of CMV or EF-1α promoter were transduced with hADMPCs at m.o.i. of 25, 50, 100, 250, 500, and 1000, and the cells were analyzed by flow cytometry. (A) The percentage of EGFP-positive hADMPCs transduced with CSII-CMV-EGFP (CMV) or CSII-EF-EGFP (EF). (B) (C) The median fluorescence intensities of the

EGFP-expressing populations. (B) hADMPCs transduced with CSII-CMV-EGFP or CSII-EF-EGFP at m.o.i. of 100, 250, 500, and 1000 were analyzed. (C) hADMPCs transduced with CSII-CMV-EGFP or CSII-EF-EGFP at m.o.i. of 1000 were analyzed over a 28 day period. Error bars represent the standard error of 3 independent analyses. **, $P<0.01$; *, $P<0.05$ (Student's t test).
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100, 250, 500, and 1000. Four days later, the cells were analyzed with a Guava easyCyte 8HT flow cytometer (Merck-Millipore) using an argon laser at 488 nm. Dead cells were excluded with the LIVE/DEAD fixable far red dead cell stain kit (Invitrogen). For analysis of hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd or pTRE-EGFP-CMV-tTA-2A-Bsd, hADMPCs were transduced with the lentiviral vector at a m.o.i. of 250 and were cultured with or without 1 μg/mL Dox. Four days later, a part of the cells were analyzed with a Guava easyCyte 8HT flow cytometer. The rest of the cells were cultured with 4 μg/mL blasticidin and 1 μg/mL Dox for 3 weeks. Then, the cells were seeded in 6-well plates and cultured with or without Dox for 4 days. The cells were harvested and re-suspended in staining buffer (PBS containing 1% BSA, 2 mM EDTA, and 0.01% sodium azide) at a density of 1×10^6 cells/mL and incubated with phycoerythrin (PE)-conjugated antibody against CD13, CD29, CD34, CD44, CD73, CD90, CD105, or CD166 for 20 min. Non-specific staining was assessed using relevant isotype controls. 525/30 nm and 583/26 nm band pass filters were used for the detection of EGFP and PE, respectively. Dead cells were excluded with the LIVE/DEAD fixable far red dead cell stain kit (Invitrogen). FlowJo software (TreeStar Inc., Ashland, OR, USA) was used for quantitation analysis. The threshold for gating was determined as the fluorescence value above which less than 1% of the control cells were considered as positive events.

Fluorescence Microscopy

Phase contrast and fluorescence images were obtained using Fluorescence Microscope (BZ-9000; Keyence, Osaka, Japan) using BZ Analyzer Software (Keyence).

Adipogenic, Osteogenic, Chondrogenic, and Neurogenic Differentiation Procedures

For adipogenic differentiation, cells were cultured in differentiation medium (Zen-Bio, Durham, NC, USA). After 3 days, half of the medium was changed to adipocyte medium (Zen-Bio), and this was repeated every 3 days. Three weeks after differentiation, characterization of adipocytes was confirmed by microscopic observation of intracellular lipid droplets by oil red O staining. Osteogenic differentiation was induced by culturing the cells in DMEM containing 10 nM dexamethasone, 50 mg/dL ascorbic acid 2-phosphate, 10 mM β-glycerophosphate (Sigma), and 10% FBS. Differentiation was examined by alizarin red staining. For chondrogenic differentiation, 2×10^5 hADMPCs were centrifuged at $400\times g$ for 10 min. The resulting pellets were cultured in chondrogenic medium (α-MEM supplemented with 10 ng/mL transforming growth factor-β, 10 nM dexamethasone, 100 mM ascorbate, and 1× insulin–transferrin–selenium solution) for 14 days, as described previously [27]. The pellets were fixed with 4% paraformaldehyde in PBS, embedded in OCT, frozen, and sectioned at 8 μm. The sections were incubated with PBSMT (PBS containing 0.1% Triton X-100, 2% skim milk) for 1 h at room temperature, and then incubated with mouse monoclonal antibody against type II collagen (Abcam, Cambridge, MA, USA) and rabbit polyclonal antibody against GFP (Invitrogen) for 1 h. After washing with PBS, cells were incubated with Alexa 546 conjugated anti-mouse IgG and Alexa 488 conjugated anti-rabbit IgG for chondrocytes (Invitrogen) or Alexa 546 conjugated anti-rabbit IgG and Alexa 488 conjugated anti-rat IgG (Invitrogen) for neuronal cells. The cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen) to identify cellular nuclei. For neurogenic differentiation, cells were cultured in Hyclone AdvanceSTEM neural differentiation medium (Thermo Scientific, South Logan, UT, USA) for 2 days. Differentiation was examined by immunofluorescent staining against β3-tubulin. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4°C and then washed 3 times in PBS. Blocking was performed with PBSMT for 1 h at room temperature. The differentiated cells were incubated with rabbit monoclonal antibody against β3-tubulin (Cell Signaling Technologies, Danvers, MA, USA) and rat monoclonal antibody against GFP (Nacalai, Kyoto, Japan). After washing with PBS, cells were incubated with Alexa 546 conjugated anti-rabbit IgG and Alexa 488 conjugated anti-rat IgG (Invitrogen). The cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen) to identify cellular nuclei.

Results

The Efficiency of the EF-1α Promoter was Higher than that of the CMV Promoter in hADMPCs

To determine the efficiency of the EF-1α promoter and the CMV promoter, hADMPCs were transduced with CSII-EF-EGFP or CSII-CMV-EGFP at a m.o.i. of 25, 50, 100, 250, 500, and 1000 and analyzed by flow cytometry. As shown in Figure 1A, percentage of GFP-positive cells increased in a dose-dependent manner. Intriguingly, transduction efficiency of CSII-EF-EGFP was significantly higher than that of CSII-CMV-EGFP in hADMPCs (Figure 1A). Moreover, a higher induction level of GFP was observed under the EF-1α promoter than under the CMV promoter, based on the median fluorescent intensity (Figure 1B). Furthermore, GFP fluorescent intensities driven from the CMV promoter were significantly decreased (from 100% on day 7 to 49.3% on day 21 and 38.4% on day 28; Figure 1C), indicating that promoter silencing occurred as previously reported [19]. In contrast, hADMPCs transduced with CSII-EF-EGFP sustained GFP expression levels with no significant reduction throughout the 28-day experimental period (Figure 1C).

Construction and Characterization of Dual-promoter Lentiviral Vectors in hADMPCs

Next, we constructed dual-promoter lentiviral vectors, which contain TRE-Tight followed by an improved version of tet-controlled transactivator (tTA advanced) induced under the CMV or EF-1α promoter (Figure 2A). In this "single tet-off lentiviral vector platform", the regulator and response elements are combined in a single lentiviral genome, along with a Gateway cassette containing *att*R recombination sites flanking a *ccd*B gene and a chloramphenicol-resistance gene, which allows an easy and rapid shuttling of the gene of interest into the vectors using the Gateway LR recombination reaction (Figure 2A). Using this system, we constructed pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd (Figure 2B). Both the CMV and the EF-1α promoters drive the mRNA expression of tTA advanced linked to the Bsd gene by the Thosea asigna virus 2A (T2A) peptide sequence. This single transcript is then translated and cleaved into 2 proteins; tTA advanced carrying 2A tag at the

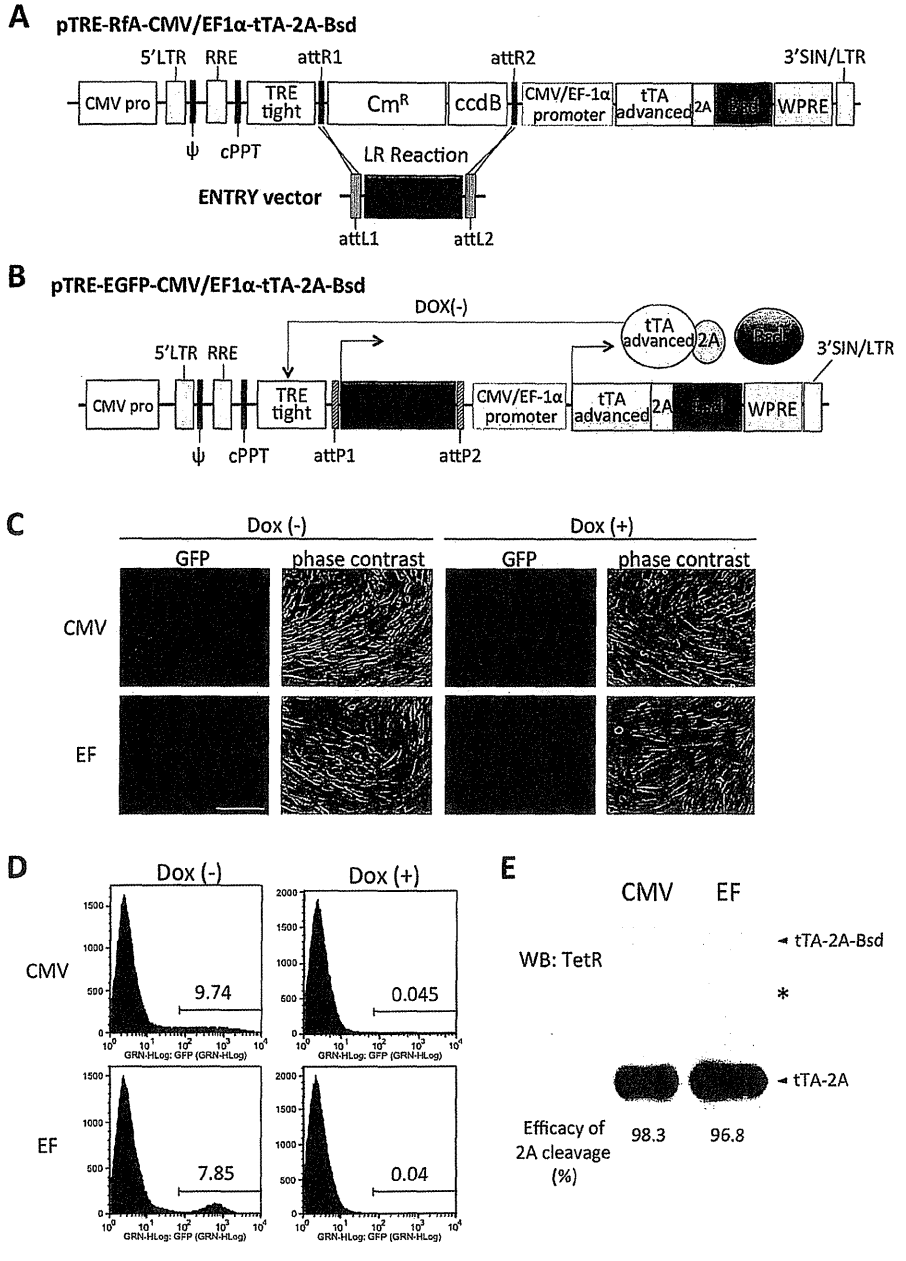

Figure 2. Schematic drawings of the single lentiviral vectors for tet-off system used in this work. (A) Gateway-compatible destination vectors containing *attR* recombination sites flanking a *ccdB* gene and a chloramphenicol-resistance gene, which allows an easy and rapid shuttling of gene of interest flanked by attL sites into the destination vectors using the Gateway LR recombination reaction. They also have an improved version of tetracycline-controlled transactivator (tTA) linked to the blasticidin resistant (Bsd) gene by the Thosea asigna virus 2A (2A) peptide sequence, whose expression is regulated by the CMV or EF-1α promoter. In the present study, we constructed an entry vector encoding EGFP flanked by attL, resulting in a destination clone, pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd (B). In the absence of doxycycline (Dox), tTA-2A binds to the TRE-Tight promoter and activates EGFP transcription. For more details, see the Results section. CMV pro, CMV promoter; LTR, long terminal repeats; ψ, packaging signal; RRE, rev response elements; cPPT, central polypurine tract; TRE, tet-responsive element; Cm^R, chloramphenicol resistance; tTA, tetracycline-controlled transactivator; Bsd, blasticidin resistance; WPRE, woodchuck hepatitis virus posttranscriptional control element; SIN, self-inactivating. (C) (D) hADMPCs were transduced with pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd at m.o.i. of 250. Four days after transduction, the cells were divided into 2 populations; with 1 μg/mL of Dox (Dox (+)) and without Dox (Dox (-)). (C) Fluorescent and phase contrast images. Scale bar, 200 μm. (D) Log fluorescence histograms of EGFP by flow cytometry analysis. (E) The whole cell lysates from hADMPCs transduced with pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd were subjected to western blotting to monitor the cleavage efficiency of tTA-2A-Bsd proteins. A primary antibody against TetR was used to detect either tTA-2A-Bsd (non-cleaved form) or tTA-2A (cleaved form). Asterisk indicates a nonspecific band.

doi:10.1371/journal.pone.0066274.g002

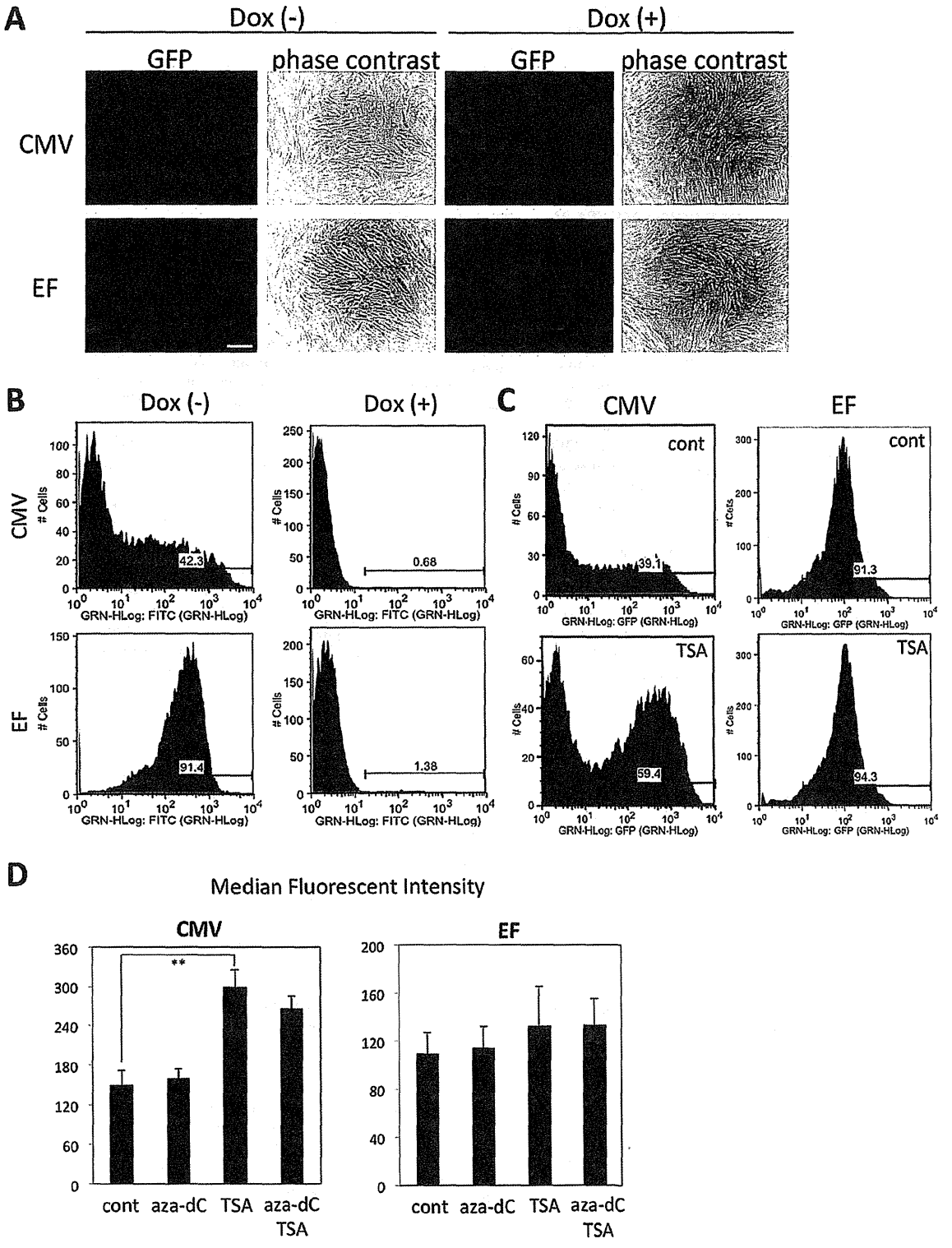

Figure 3. Blasticidin selection of hADMPCs transduced with single tet-off lentiviral vector platform. hADMPCs were transduced with pTRE-EGFP-CMV-tTA-2A-Bsd (CMV) or pTRE-EGFP-EF-tTA-2A-Bsd (EF) at m.o.i. of 250. The cells were treated with 4 μg/mL blasticidin and 1 μg/mL Dox for 2 weeks. Then, the cells were cultured in the absence (Dox (-)) or presence (Dox (+)) of 1 μg/mL Dox for 4 days, and analyzed under a microscope (A) and flow cytometer (B). The cells were treated with 100 nM TSA (TSA), 5 μM 5-aza-dC (aza-dC), or both for 48 h before analyzed by flow

cytometer. (C) A representative fluorescence histogram of EGFP. (D) The median fluorescence intensities of the EGFP-expressing populations. Error bars represent the standard error of 3 independent analyses. **, P<0.01 (Student's t test). Scale bar, 200 μm.
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C-terminus (tTA-2A) and Bsd. tTA-2A binds to the TRE-tight in the absence of Dox, a tet derivative, and activates transcription of EGFP to a very high level. In the presence of Dox, tTA-2A is unable to bind the TRE-Tight in a tet-responsive promoter, and the system is inactive.

To investigate the usefulness of these lentiviral vectors, hADMPCs were transduced with pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd at a m.o.i. of 250. As shown in Figure 2C, expression of EGFP was observed in the absence of Dox, whereas addition of Dox (1 μg/mL) was enough to suppress the expression. Flow cytometry analysis revealed that the transduction efficiency was relatively low (EGFP-positive cells were 7.5~10%) compared with that of CSII-CMV-EGFP or CSII-EF-EGFP (EGFP-positive cells were 45% or 77% at a m.o.i. of 250, respectively; Figure 1A), and the tet-off system completely abolished gene expression in the presence of Dox (Figure 2D). Flow cytometry analysis also revealed that fluorescent intensity was relatively uniform in hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd, but a wide range of fluorescent intensities was observed in hADMPCs infected with pTRE-EGFP-CMV-tTA-2A-Bsd. These data suggest that tTA-2A functions properly in this system. Moreover, western blot analysis against tTA showed the efficient cleavage (>95%) of tTA-2A-Bsd proteins into tTA-2A and Bsd (Figure 2E).

To further determine that Bsd cleaved from tTA-2A-Bsd was effective in this system, 4 μg/mL blasticidin was administered to hADMPCs. Within 1 week after the selection, control hADMPCs were completely killed (data not shown), whereas hADMPCs that were successfully transduced with either pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd could survive and proliferate, demonstrating that Bsd from tTA-2A-Bsd is sufficient to confer blasticidin resistance to the cells. The surviving cells were kept in culture medium with blasticidin and then divided into 2 populations, either with Dox (1 μg/mL) or without Dox. As shown in Figure 3A and 3B, almost all (>90%) the cells transduced with pTRE-EGFP-EF-tTA-2A-Bsd strongly expressed EGFP in the absence of Dox. In hADMPCs transduced with pTRE-EGFP-CMV-tTA-2A-Bsd, however, >50% of the cells were EGFP negative regardless of their blasticidin resistance. Moreover, fluorescent intensities were quite variable; some cells expressed very high levels of EGFP, while others expressed very low levels (Figure 3A and 3B). This might be due to "promoter suppression," transcript repression of an upstream transcriptional unit by a downstream unit when 2 transcriptional units lie adjacent in head-to-tail tandem on a chromosome [28,29]. Studies have revealed that the suppression by adjacent units is epigenetic and involves modification of the chromatin structure, including DNA methylation at CpG sites within the promoter, histone deacetylation, histone methylation at specific residues (e.g., H3K9, H3K27), and densely packed nucleosomes that create a closed chromatin structure. In order to determine if inhibiting histone deacetylases or DNA methylation would re-induce EGFP expression, pTRE-EGFP-CMV-tTA-2A-Bsd cells were treated with histone deacetylase inhibitor trichostatin A (TSA) and/or DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC). TSA treatment significantly increased the number of EGFP-positive cells and strengthened the fluorescent intensities of EGFP, whereas 5-aza-dC had no effect, suggesting that EGFP expression was repressed by histone deacetylation when stably transduced with pTRE-EGFP-CMV-tTA-2A-Bsd (Figure 3C and 3D). These inhibitors had no effect on hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd. These data suggest that the dual-promoter lentiviral vector using the EF promoter is more resistant to gene silencing than that using the CMV promoter.

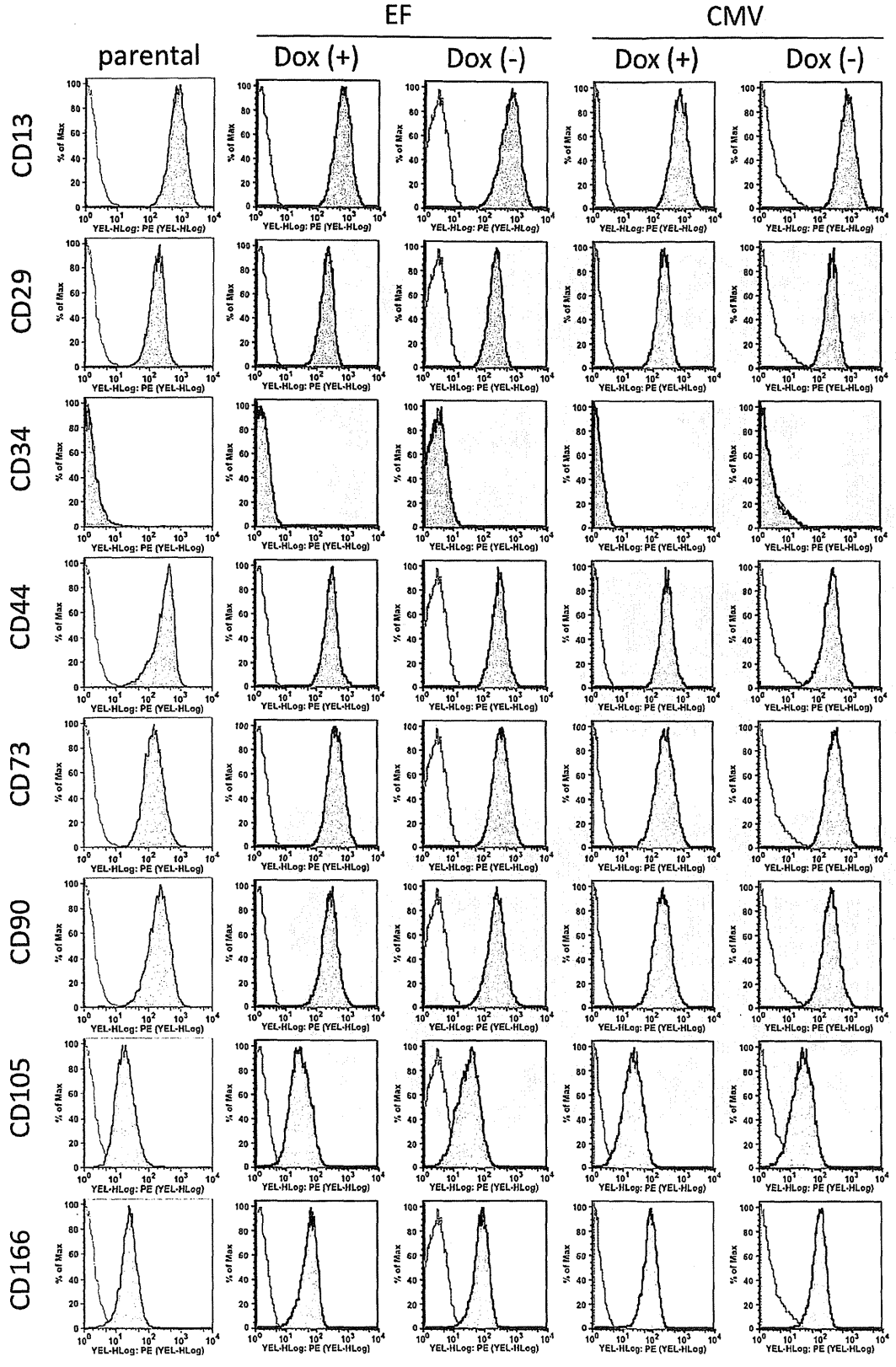
Blasticidin-selected hADMPCs Maintain the Properties of Their Parental hADMPCs

hADMPCs are an attractive material for cell therapy because of their ability to secrete various cytokines and growth factors. These cells also have the ability to differentiate into various types of cells, including adipocytes, chondrocytes, osteocytes, hepatocytes, cardiomyoblasts, and neuronal cells. Gene manipulation of hADMPCs may thus generate great possibilities for cell therapy and tissue engineering. From this point of view, the development of an efficient and stable Dox-responsive gene transfer system to achieve high levels of transgene expression in hADMPCs, without affecting the phenotype, is of special interest for the field. We therefore studied the cell properties of hADMPCs transduced with the single tet-off lentiviral vector after blasticidin selection. Flow cytometry analysis revealed no changes in the expression of the main surface markers (positive for CD13, CD29, CD44, CD73, CD90, CD105, and CD166, and negative for CD34) either in the absence or presence of Dox (Figure 4). To further confirm the properties of hADMPCs, the cells were differentiated into adipocytes, osteocytes, chondrocytes, and neuronal cells. As shown in Figure 5, blasticidin-selected hADMPCs maintained their ability to differentiate into adipocytes, osteocytes, chondrocytes, and neuronal cells. Moreover, EGFP was stably expressed in the differentiated cells only in the absence of Dox (Figure 5).

Discussion

In recent years, there is growing interest in the use of MSCs for cell therapy and tissue engineering because of their differentiation potential and ability to secrete growth factors [7–11]. Furthermore, because of their hypo-immunogenicity and immune modulatory effects, MSCs are good candidates for gene delivery vehicles for therapeutic purposes [12,14]. In addition to primary MSCs, genetically modified MSCs have been applied to bone regeneration, muscle repair, diabetes, Parkinson's disease, and myocardial infarction recovery [14,30–35]. Duan et al. reported that the angiogenic effect of MSCs could be enhanced by adenovirus-mediated HGF overexpression in the treatment of cardiac ischemia injury [14]. Karnieli et al. and Li et al. both reported the reversal of hyperglycemia in streptozotocin-induced diabetic mice after transplantation of insulin-producing cells originating from genetically modified Pdx-1 expressing MSCs [32,33].

While significant progress has been made in the use of genetically modified MSCs for basic and applied research, the current methods for gene manipulation are still insufficient for some applications. Adenoviral vectors are commonly used for transient expression because they remain epichromosomal in the host cells, and their ability to transiently infect target cells minimizes the risk of insertional mutagenesis [36]. However, relatively brief transgene expression may limit the utility of this approach to tissue repair applications. On the other hand, lentiviral vectors, which are promising vectors for gene delivery in primary human cells, integrate into the host cell genome, which may be an appropriate strategy for tissue repair applications


EF
CMV
parental
Dox (+)
Dox (-)
Dox (+)
Dox (-)
CD13
CD29
CD34
CD44
CD73
CD90
CD105
CD166
% of Max
YEL-HLog: PE (YEL-HLog)