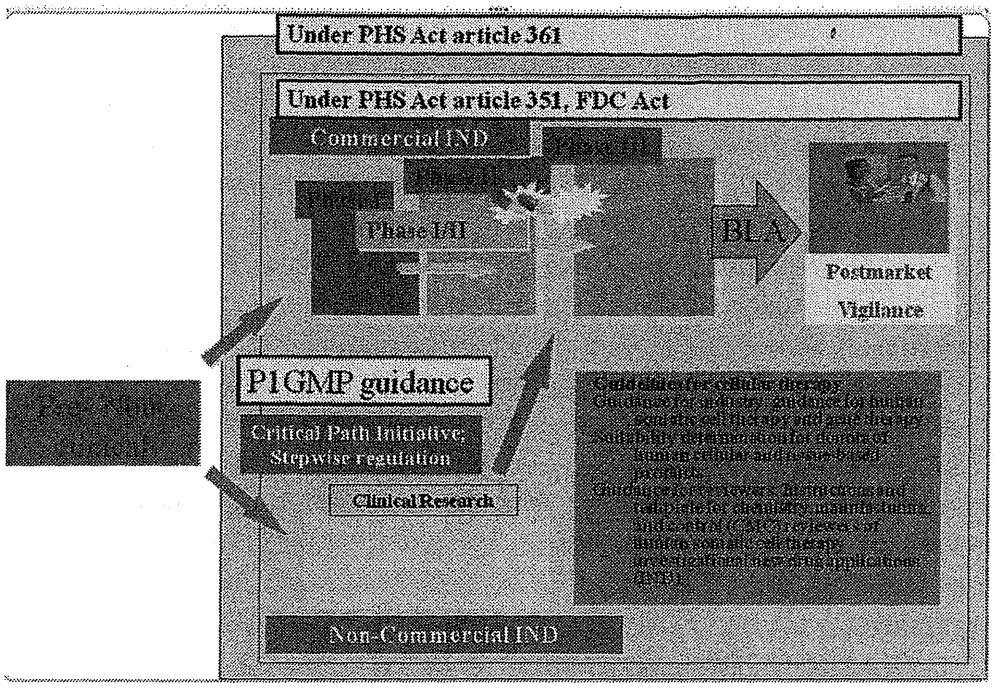

Fig. 1. Regulation of cell-based medical/medicinal products in US. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/PPL-130368)

studies using stem cells. In fact, the Ministry of Health, Labor and Welfare, of the Japanese Government has implemented "The guideline for clinical research using human stem cells", which was launched on September 1, 2006 (Notification No. 425. 2006. MHLW, Japan). Since the launch of that guideline, the Ministry of MHLW, Japan has approved more than 40 protocols that involve the use of human somatic stem/progenitor or precursor cells in the treatment of patients. The majority of the approved protocols involve the use of bone marrow-derived mesenchymal stem cells in clinical investigation. The main purpose of the guideline is for the clinical studies implemented under the Medical Service Law and the Medical Practitioner's Law to use human somatic stem cells to maintain health, and prevent, diagnose and treat illness. The "guideline for clinical research using human stem cells" encourages researchers to respect human dignity and human rights and promote social understanding based on scientific knowledge. The major goal of the guideline is to reinforce and ensure safety of any investigational new regenerative medical products. The guideline is also intended for clinical studies that implement the administration or transplantation of human somatic stem cells and their derivatives. Consequently, future research and the development of new tissue-engineered medical products shall fulfill in the long run the unmet medical needs of patients with chronic diseases.

On December 3, 2008, the ISSCR released the Guidelines for the Clinical Translation of Stem Cells. The guideline urged the Japanese Government to revise the guideline Notification No. 425. 2006 (MHLW, Japan.). Since then, the government has invited academic experts in the field of regenerative medicine, and the established committee proposed the permission of clinical studies using iPS cells based

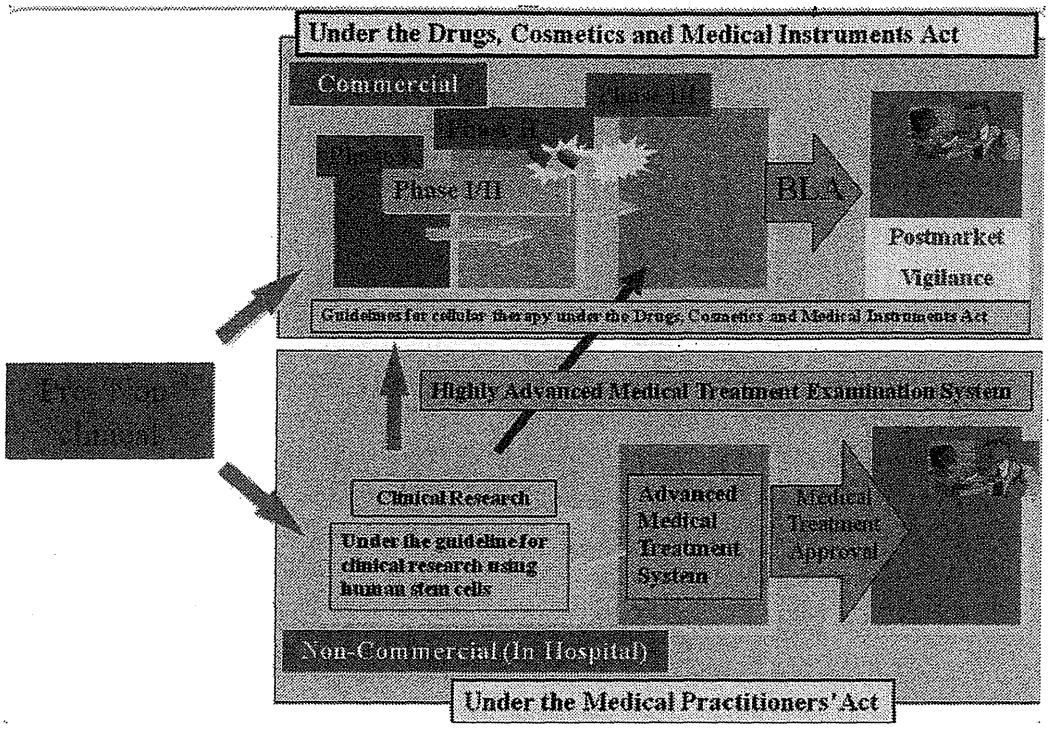

Fig. 2. Regulation of cell-based medical/medicinal products in Japan. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/PPL-130368)

on recent advances in the field of stem cell research. On November 1, 2010, "The guideline for clinical research using human stem cells" was revised as Notification No. 380. 2010. MHLW, Japan. The revised guideline legalized the use of autologous and/or allogenic iPS cells, and their products, but not hES cells, in basic and clinical research. It also stipulated that the Japanese biomedical researchers will be allowed to the end of 2011 to use ES cells only in basic research but not in clinical studies and/or trials. The FDA has already approved hES cells as IND for patients with subacute severe spinal injury and for patients with various forms of retinopathies. The US approval has prompted the Japanese Government to again revise "The guideline for clinical research using human stem cells" (Notification No. 380. 2010) and thus academic experts in the field of regenerative medicine were recently (May 7, 2011) invited for second revision of the guideline.

The development of ES/iPS-like cells-based medical/medicinal products requires the concerted efforts and expertise of various professionals to tackle scientific, ethical, public, economic, international, collaborative and translational challenges. To integrate these elements rationally, thereby leading to productive and efficient outcome, we need to establish relevant regulatory environment. In this regard, several processes need to be considered carefully in any plan designed for the production and evaluation of ES/iPS-like cells-based medical products. These include; 1) Gene transfer or other relevant methods for reprogramming. 2) Cultivation employing various growth factors for mass production. 3) Establishment of various cell lines, cell

banks, databases, quality control systems, maintenance, constant and ample supply, and storage, 4) Characterization of ES/iPS-like cells including their identity, purity, potency, stability, and safety (nontumorigenicity and/or teratogenicity). 5) Differentiation to the desired cells (type of inducers, selection of optimal culture conditions for consistent differentiation). 6) Characterization of the desired differentiated cells (e.g., survival, multiplication, heterogeniety, tumorigenicity). 7) Formulation of the desired cells, in combination with non-cellular materials. 8) Characterization of the final product, including its stability, safety and efficacy employing basic and clinical studies. 9) Safety concerns may include ectopic tissue expression, inappropriate differentiation/tumorigenicity, undesired phenotype expression, immunorejection and/or other unanticipated immunoresponses. 10) Quality control, e.g., after MAA and PMS. Based on the recent achievements in the field, we believe that a variety of ES/iPS-like cells-based medical products will be produced in the near future. However, we will also need to challenge the scientific validity, ethical validity, social understanding and recognition, and economic validity of these products, but there will be no readily available answers. Although the difficulties tackling these challenges will be enormous, our endeavor should not weaken, in order to better serve the public interest and health.

8. Linking the pharmaceutical affairs law track to the medical service law and medical practitioners law track

To accelerate the realization of translational research in regenerative medicine, the Japanese researchers are allowed to apply for "Evaluation of highly advanced medicine" (in Japanese, Kodo-iryo-hyoka). According to the system, a governmental committee estimates the efficacy, safety and quality of the clinical protocol, recommends to the Minister of Health, Labor and Welfare, Japan, an estimate of the funds required to execute the research and requests approval of the applications. This system should bind the Medical practitioner's Law and Pharmaceutical Affair's Law and is useful for realizing cell-based regenerative medicine. The major goal of these guidelines and the system is to reinforce and ensure the safety of any new investigative work related to the production of new regenerative medicines.

9. Conclusion

All clinical studies/trials carries an associated risk due to its investigative nature. However, it is in everybody's interest to ensure that the field avoids any major setbacks arising from novel cell-based medical/medicinal products used in clinical studies/trials. Translational researchers and clinicians are encouraged to contact the regulators at the initial stage of research and development on cell-based regenerative medical/medicinal products to use various regulatory mechanisms such as orphan

drug designation, if pertinent, and to help them reduce the high costs associated with research. We believe that the translational gap between stem cell biology and therapies is bridgeable although it seems very complicated. However, only by applying the highest quality research, safety, and efficacy standards, can we anticipate step-by-step forward movement of the stem cell field.

Needless to say, the common goal of the interested parties of academia, industry and regulatory bodies should be to design and manufacture more useful cell/tissue-based medical/medicinal drugs and technology to serve the needs of the patients and improve quality of life. Various hurdles including scientific, legal, ethical, public, economic, international, collaborative and translational issues, need to be overcome for successful development of highly effective cell/tissue-based medical/medicinal products. To integrate these issues rationally and thereby lead to efficient and effective development of cell/tissue-based medical/medicinal products, establishment of appropriate regulatory climate including development of appropriate guideline is important in this process.

We anticipate further development of new cell-based medical/medicinal products in the near future, including new medical technologies requiring new clinical applications. Their scientific validity, ethical validity social understanding and recognition, and economic validity need to be scrutinized to ensure widespread use in the medical community. Therefore, it will be very important for all concerned to concentrate their knowledge and efforts on the development of new medical therapies, including dealing with problems arising during such work, with a unanimous goal of supplying excellent products and effective medical technologies for patients. Though the difficulties will be enormous when challenging these issues, our endeavor should not falter in order to better serve the public interest and health.

Acknowledgments

This work was supported in part by the "Highway Program for Realization of Regenerative Medicine (JST)".

in situ reprogrammed spermine treated-adipose tissue-derived multi-lineage progenitor cells improve left ventricular dysfunction in a swine chronic myocardial infarction model

Okura H.[1,2], Soeda M.[1], Miyagawa S.[3], Sawa Y.[3], Ichinose A.[4], Matsuyama A.[1,2,5]

[1] *Platform for Realization of Regenerative Medicine, Foundation for Biomedical Research and Innovation, (Japan)*
[2] *The Center for Medical Engineering and Informatics, Osaka University,(Japan)*
[3] *Department of Surgery, Osaka University Graduate School of Medicine, (Japan)*
[4] *Department of Plastic Surgery, Kobe University Hospital, (Japan)*
[5] *RIKEN Program for Drug Discovery and Medical Technology Platforms. (Japan)*
e-mail: akifumi-matsuyama@umin.ac.jp

Abstract

Background: Spermine, known as one of polyamines, has been reported to make embryonic stem cells differentiate into cardiac lineage. In this study, we examined whether spermine could commit human adipose tissue–derived multi-lineage progenitor cells (hADMPC) into cardiac lineage and whether the spermine treated-hADMPCs would differentiate into cardiomyocytes-like cells and improve left ventricular dysfunction in a swine chronic myocardial infarction model. ***Methods and Results:*** After 24h-treatment with spermine, hADMPC showed the augmentation of cardiac marker-expressions; nkx2.5, islet-1, alpha-cardiac actin and cardiac troponin I (11.2-, 27.5-, 43.6- and 19.1-fold to hADMPC *per se*, respectively). To examine the effect of spermine treated-hADMPC on left ventricular dysfunction, swine chronic MI model were built up by first ballooning and reperfusion to first diagonal branch and second one to left ascending coronary artery (#6) 1 week-later. Four week-later second one, the swine (immunization with CyA 0.6mg i.m./kg/day) received transplantation of spermine treated-hADMPC ($1x10^5$, $3x10^5$, $1x10^6$ and $3x10^6$ cells/kg) or lactic Ringer's solution via intracoronary (#6), and echocardiogram was examined at 0, 4, 8 and 12 weeks after transplantation. Follow-up showed rescue of function in the transplanted, and the most effective dose was $3x10^5$ cells/kg (EF; 33.4%, 47.0%, 51.5% and 52.9% at 0, 4, 8 and 12 week-after transplantation, respectively). Histologically, the spermine treated-hADMPC were engrafted into the scarred myocardium and reprogrammed into human specific troponin I and alpha-cardiac actin positive cells *in situ* 12 week-after transplantation. ***Conclusion:*** The transplantation of spermine treated-hADMPC is a potentially effective therapeutic strategy for future cardiac tissue regeneration.

Keywords: adipose tissue, spermine, cardiocytoc differentiation, myocardial infarction

Introduction

Spermine, known as one of polyamines, has been reported to make embryonic stem cells differentiate into cardiac lineage. In this study, we examined whether spermine could commit human adipose tissue–derived multi-lineage progenitor cells (hADMPC) into cardiac lineage and whether the spermine treated-hADMPC would differentiate into cardiomyocytes-like cells and improve left ventricular dysfunction in a swine chronic myocardial infarction model.

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 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. A*lpha-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.

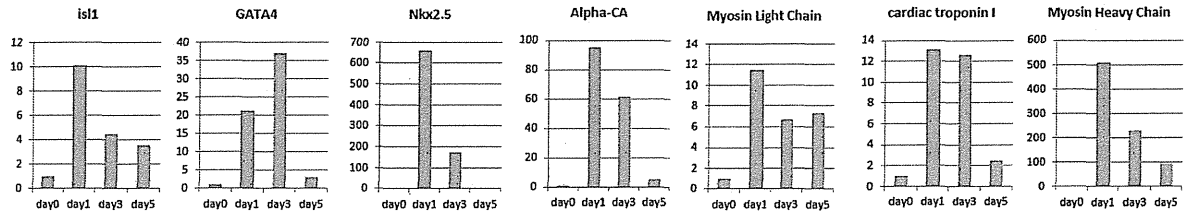

Figure 1. Spermine committed ADMPC into cardiac-lineage.

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.

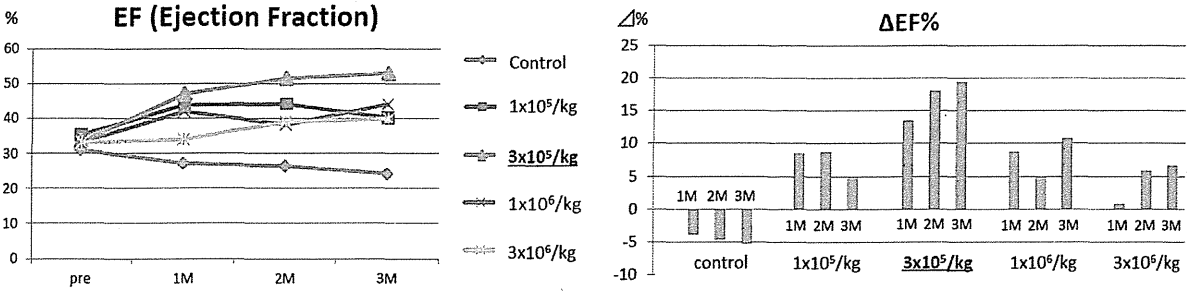

Figure 2. Sperimine-treated hADMPC transplantation improved cardiac function

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).

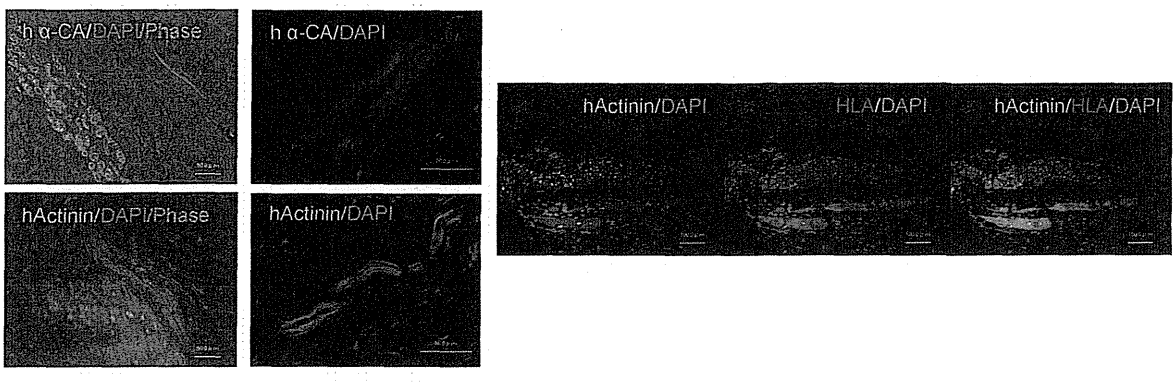

Figure 3. *in situ* reprogramming of spermine-treated ADMPC into cardiomyocyte

(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.

Okura H.[1,2], Soeda M.[1], Morita M.[1], Ichinose A.[3], Matsuyama A.[1,2,4]

[1] *Platform for Realization of Regenerative Medicine, Foundation for Biomedical Research and Innovation, (Japan)*
[2] *The Center for Medical Engineering and Informatics, Osaka University,(Japan)*
[3] *Department of Plastic Surgery, Kobe University Hospital, (Japan)*
[4] *RIKEN Program for Drug Discovery and Medical Technology Platforms. (Japan)*
e-mail: akifumi-matsuyama@umin.ac.jp

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.

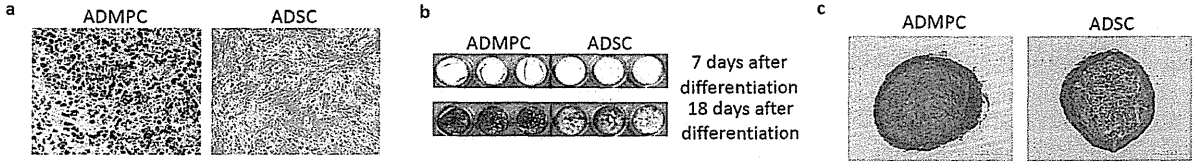

Figure 1. Adipocytic, osteocytic and chondrocytic differentiation of hADMPC and hADSC

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.

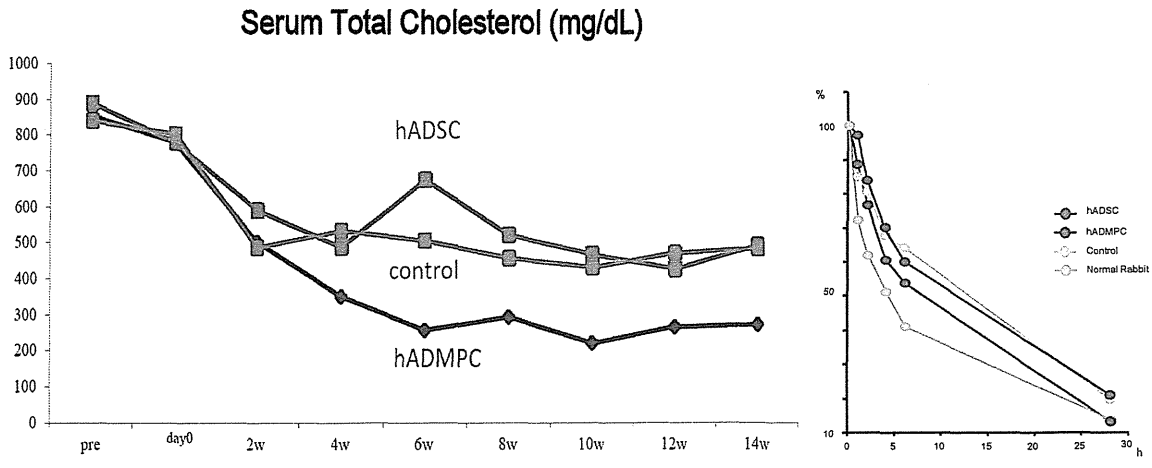

Figure 2. Serum cholesterol level and LDL clearance in WHHL rabbit with transplants.

(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

This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan.

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

Okura H.[1,2], Soeda M.[1], Morita M.[1], Ichinose A.[3], Matsuyama A.[1,2,4]

[1] *Platform for Realization of Regenerative Medicine, Foundation for Biomedical Research and Innovation, (Japan)*
[2] *The Center for Medical Engineering and Informatics, Osaka University,(Japan)*
[3] *Department of Plastic Surgery, Kobe University Hospital, (Japan)*
[4] *RIKEN Program for Drug Discovery and Medical Technology Platforms. (Japan)*
e-mail: akifumi-matsuyama@umin.ac.jp

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.

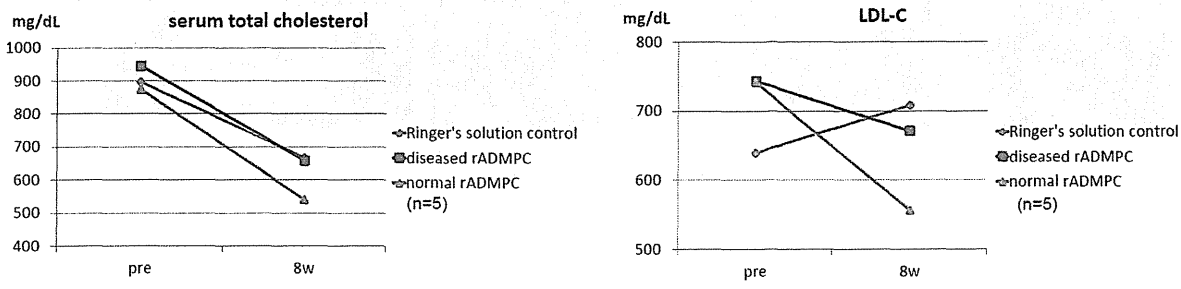

Figure 1. Serum cholesterol reduction effect of rADMPC transplantation.

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.

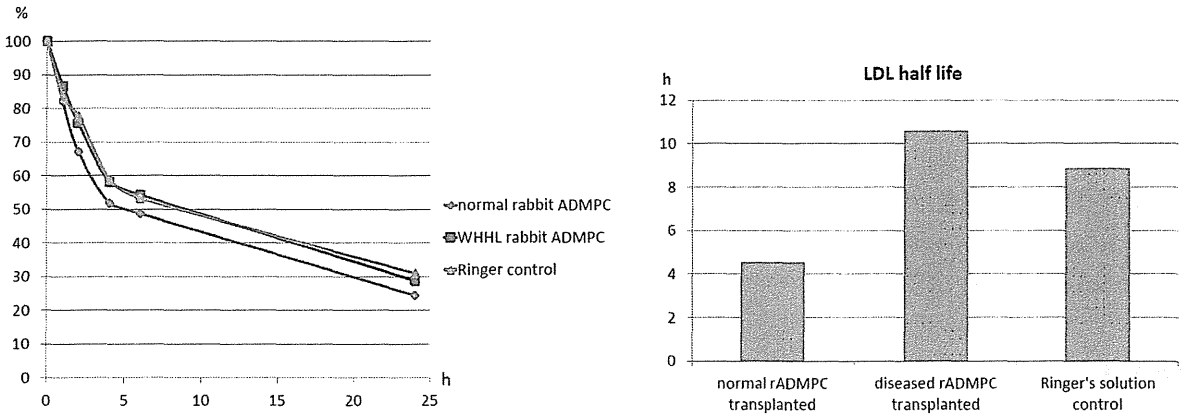

Figure 2. LDL turn over study.

^{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*.

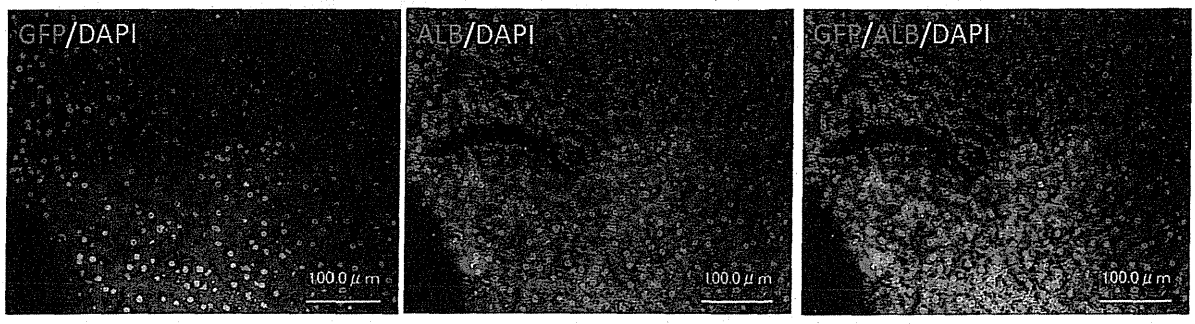

Figure 3. *in situ* reprogramming of rabbit ADMPC into Hepatpcyte-like cells

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

This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan.

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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

Hiroyuki Moriyama[1*☯], Mariko Moriyama[1,2☯], Kei Sawaragi[1], Hanayuki Okura[2], Akihiro Ichinose[3], Akifumi Matsuyama[2], Takao Hayakawa[1]

1 Pharmaceutical Research and Technology Institute, Kinki University, Higashi-Osaka, Osaka, Japan, **2** Platform for Realization of Regenerative Medicine, Foundation for Biomedical Research and Innovation, Chuo-ku, Kobe, Hyogo, Japan, **3** Department of Plastic Surgery, Kobe University Hospital, Chuo-ku, Kobe, Hyogo, Japan

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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Competing Interests: The authors have declared that no competing interests exist.

* E-mail: moriyama@phar.kindai.ac.jp

☯ These authors contributed equally to this work.

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,

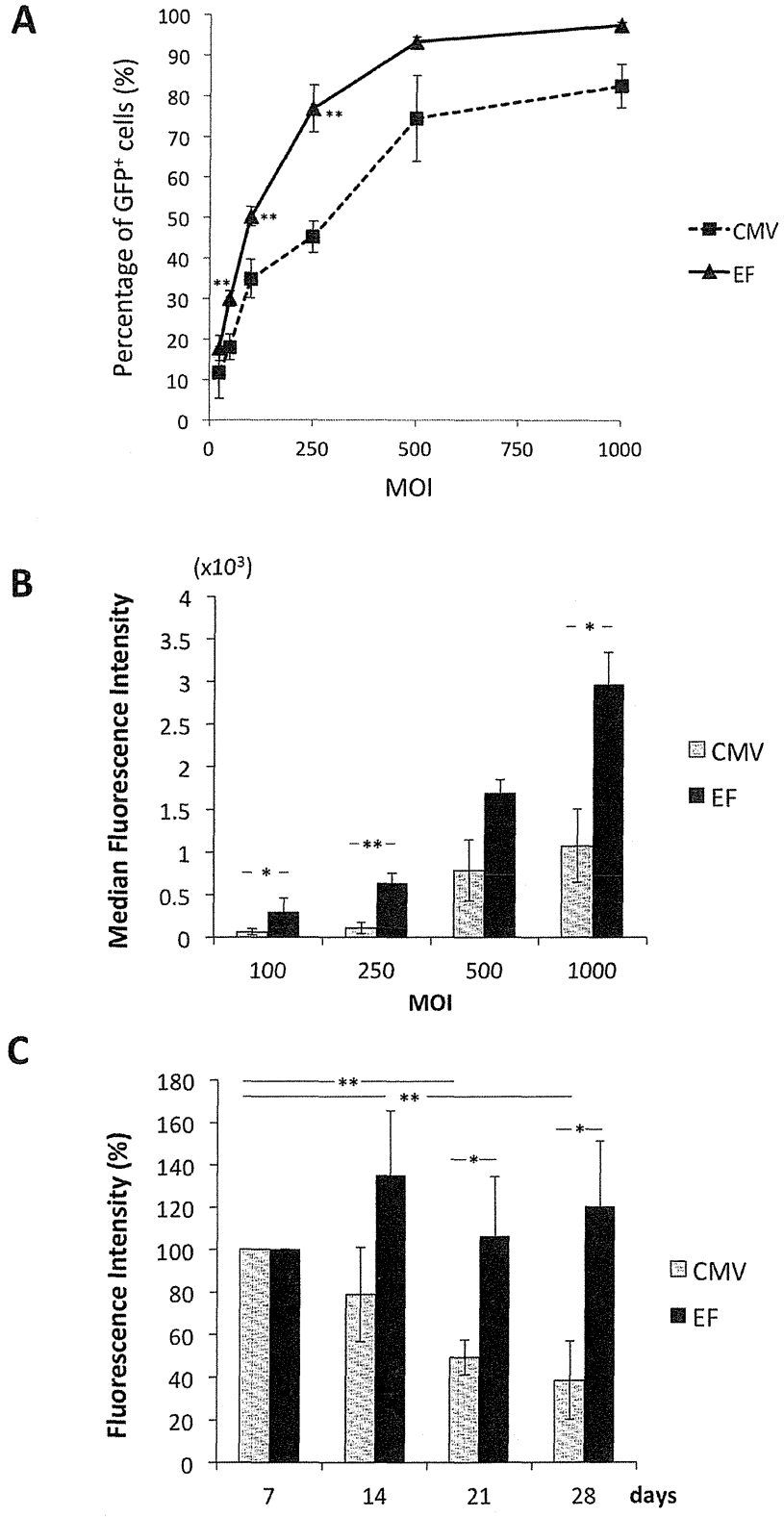

Figure 1. The efficiency of CMV or EF-1α promoter in hADMPCs. Lentiviral vectors encoding EGFP under the control of CMV or EF-1α promoter were transduced with hADMPCs at m.o.i. of 25, 50, 100, 250, 500, and 1000, and the cells were analyzed by flow cytometry. (A) The percentage of EGFP-positive hADMPCs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