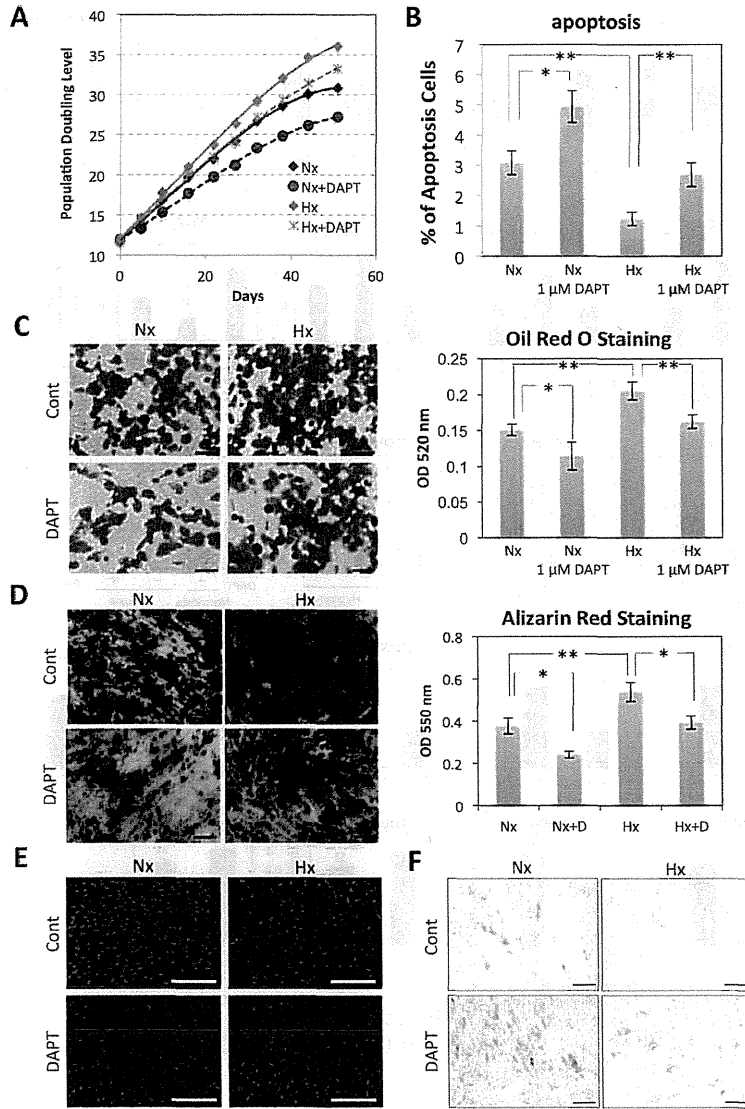
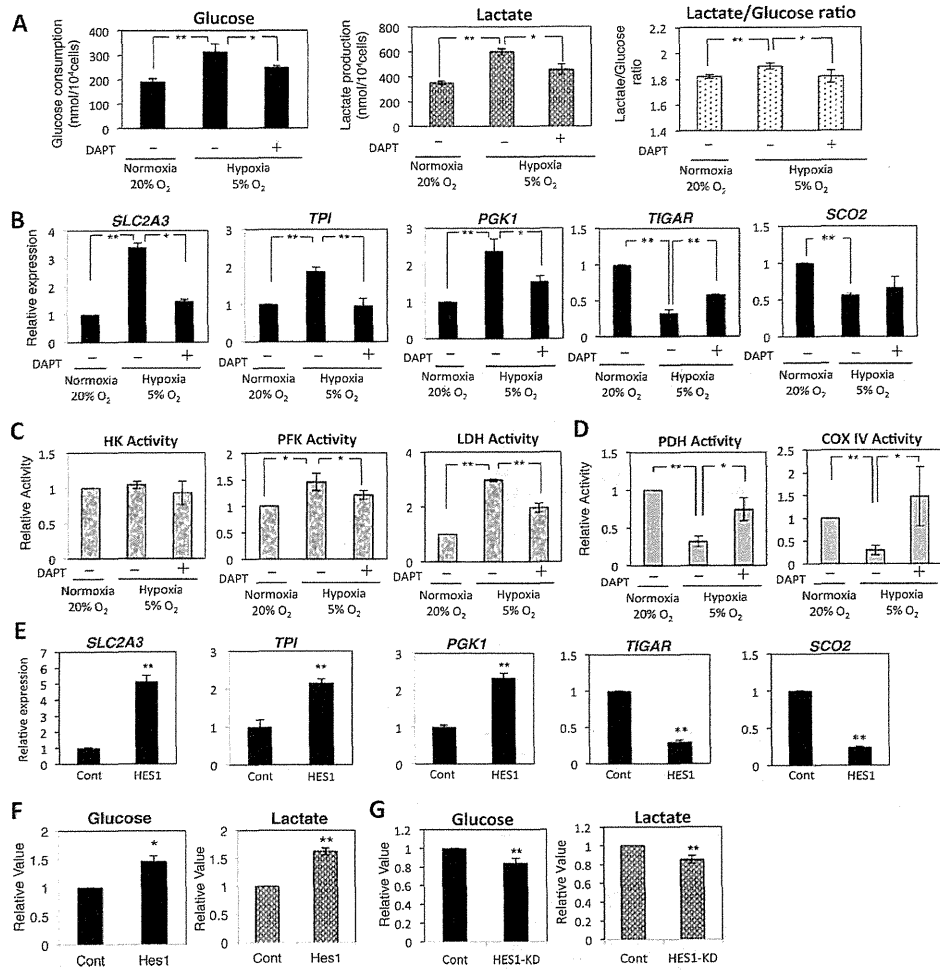


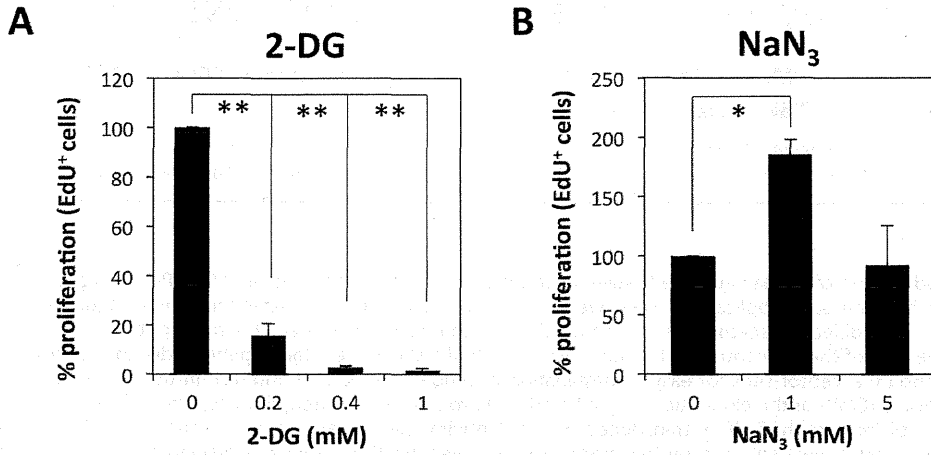
1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



MoriyamaFig5  
171x247mm (300 x 300 DPI)



MoriyamaFig6  
169x176mm (300 x 300 DPI)



MoriyamaFig7  
151x72mm (300 x 300 DPI)

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

Hiroyuki Moriyama<sup>1\*</sup>, Mariko Moriyama<sup>1,2,3</sup>, Kei Sawaragi<sup>1</sup>, Hanayuki Okura<sup>2</sup>, Akihiro Ichinose<sup>3</sup>, Akifumi Matsuyama<sup>2</sup>, Takao Hayakawa<sup>1</sup>

**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 (hADMPs) 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  $\alpha$  (EF-1 $\alpha$ ) promoter as the ubiquitous promoter, and EGFP was introduced as the gene of interest. hADMPs transduced with a lentiviral vector carrying either the CMV promoter or the EF-1 $\alpha$  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-1 $\alpha$  promoter provided more homogenous expression of EGFP in hADMPs. 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 hADMPs.

**Citation:** Moriyama H, Moriyama M, Sawaragi K, Okura H, Ichinose A, et al. (2013) Tightly Regulated and Homogeneous Transgene Expression in Human Adipose-Derived Mesenchymal Stem Cells by Lentivirus with Tet-Off System. PLoS ONE 8(6): e66274. doi:10.1371/journal.pone.0066274

**Editor:** Niels Olsen Saraiva Câmara, Universidade de Sao Paulo, Brazil

**Received:** December 20, 2012; **Accepted:** May 2, 2013; **Published:** June 12, 2013

**Copyright:** © 2013 Moriyama et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported in part by MEXT KAKENHI Grant Number 23791304 to M.M. and 24791927 to H.M. This work was also supported in part by grants from the Ministry of Health, Labor, and Welfare of Japan and a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**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 (hADMPs), 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 hADMPs, 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, hADMPs are good candidates for gene delivery vehicles for therapeutic purposes [12]. Thus, hADMPs are an attractive material for cell therapy and tissue engineering, making the development of technologies for permanent and highly controlled genetic modification of hADMPs 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 hADMPs. 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 *attR* recombination sites flanking a *ccdB* 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  $\alpha$  (EF-1 $\alpha$ ) 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  $\times$  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  $5 \times 10^3$  cells/cm<sup>2</sup> 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 View, CA, USA) and the Reading frame A (RfA), a Gateway cassette containing *attR* recombination sites flanking a *ccdB* gene and a chloramphenicol-resistance gene (Invitrogen) were introduced into *XbaI-XhoI* 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: GCGA-GATCTTTAGTTCCTGGTGTACTTG. 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 ( $6000 \times g$ , 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*

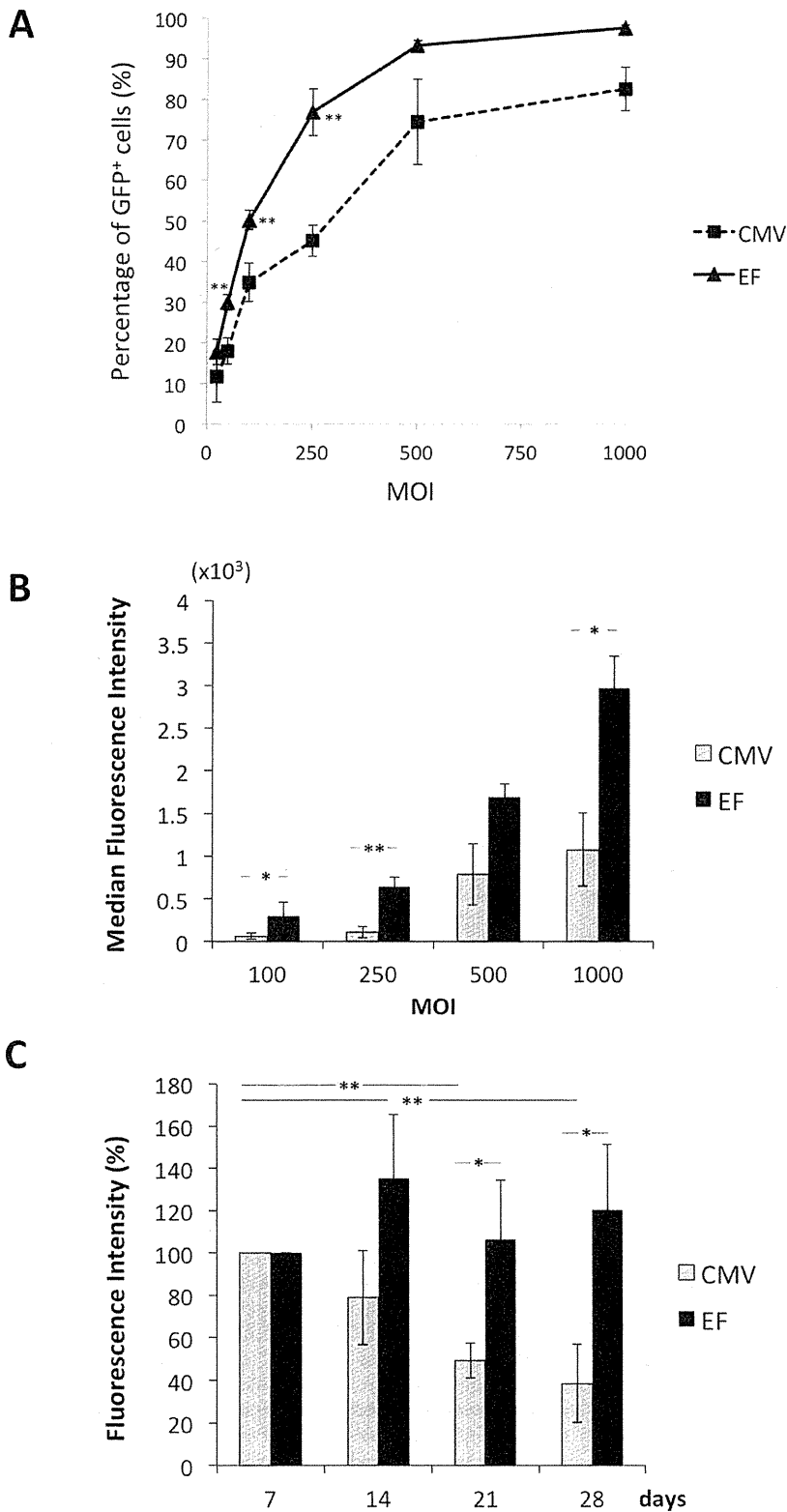
DH5 $\alpha$  (F-,  $\Phi$ 80dlacZAM15,  $\Delta$ (lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK-, mK+), phoA, supE44,  $\lambda$ -, thi-1, gyrA96, relA1) were used for general purpose. To propagate plasmids containing the *ccdB* gene, One Shot<sup>TM</sup> *ccdB* Survival<sup>TM</sup> 2 T1 Phage-Resistant (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  $2 \times 10^4$  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-1 $\alpha$  promoter in hADMPs.** Lentiviral vectors encoding EGFP under the control of CMV or EF-1 $\alpha$  promoter were transduced with hADMPs 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 hADMPs 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). doi:10.1371/journal.pone.0066274.g001

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  $\mu\text{g}/\text{mL}$  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  $\mu\text{g}/\text{mL}$  blasticidin and 1  $\mu\text{g}/\text{mL}$  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  $1 \times 10^6$  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  $\beta$ -glycerophosphate (Sigma), and 10% FBS. Differentiation was examined by alizarin red staining. For chondrogenic differentiation,  $2 \times 10^5$  hADMPCs were centrifuged at  $400 \times g$  for 10 min. The resulting pellets were cultured in chondrogenic medium ( $\alpha$ -MEM supplemented with 10 ng/mL transforming growth factor- $\beta$ , 10 nM dexamethasone, 100 mM ascorbate, and  $1 \times$  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  $\mu\text{m}$ . 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  $\beta$ 3-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  $\beta$ 3-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.

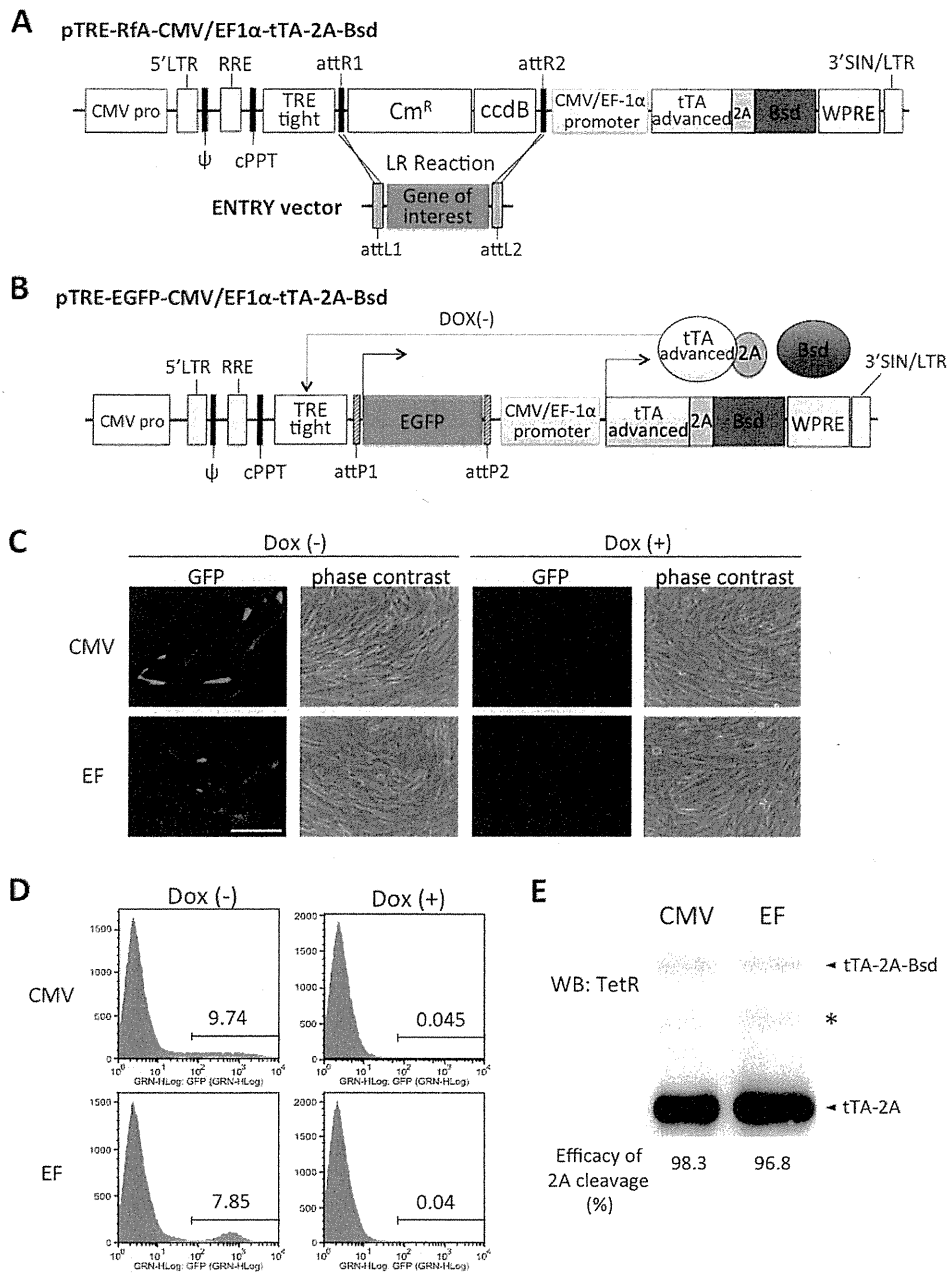
## Results

### The Efficiency of the EF-1 $\alpha$ Promoter was Higher than that of the CMV Promoter in hADMPCs

To determine the efficiency of the EF-1 $\alpha$  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-1 $\alpha$  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).

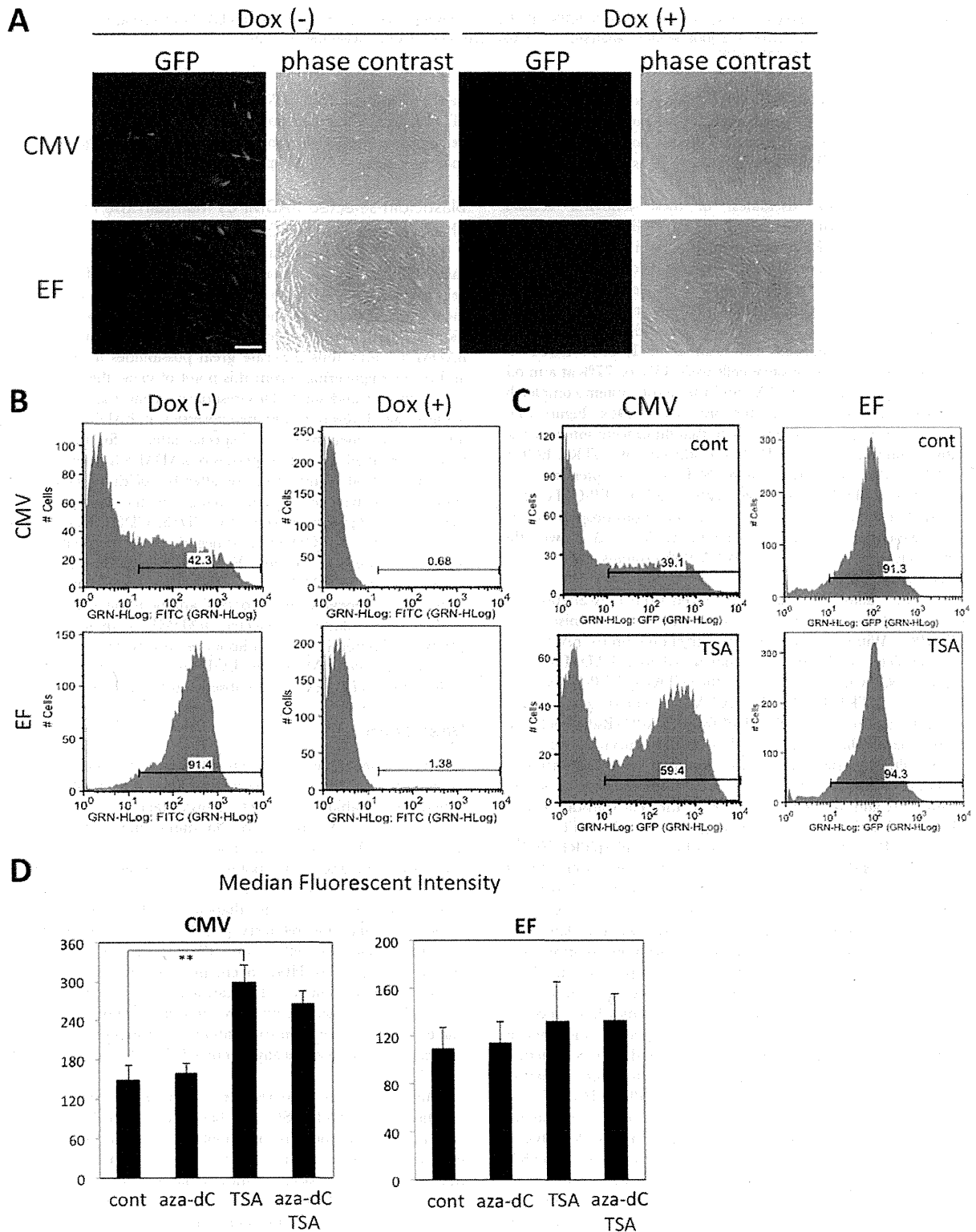
### Construction and Characterization of Dual-promoter Lentiviral Vectors in hADMPCs

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-1 $\alpha$  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 *attR* recombination sites flanking a *ccdB* 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-1 $\alpha$  promoters drive the mRNA expression of tTA advanced linked to the Bsd gene by the Thoesa 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 Thosa asigna virus 2A (2A) peptide sequence, whose expression is regulated by the CMV or EF-1 $\alpha$  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;  $\psi$ , packaging signal; RRE, rev response elements; cPPT, central polypurine tract; TRE, tet-responsive element; Cm<sup>R</sup>, chloramphenicol resistance; tTA, tetracycline-controlled transactivator; Bsd, blasticidin resistance; WPRE, woodchuck hepatitis virus posttranscriptional control element; SIN, self-inactivating. (C) 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  $\mu$ g/mL of Dox (Dox (+)) and without Dox (Dox (-)). (C) Fluorescent and phase contrast images. Scale bar, 200  $\mu$ m. (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 hADMPs transduced with single tet-off lentiviral vector platform.** hADMPs 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  $\mu\text{g}/\text{mL}$  blasticidin and 1  $\mu\text{g}/\text{mL}$  Dox for 2 weeks. Then, the cells were cultured in the absence (Dox (-)) or presence (Dox (+)) of 1  $\mu\text{g}/\text{mL}$  Dox for 4 days, and analyzed under a microscope (A) and flow cytometer (B). The cells were treated with 100 nM TSA (TSA), 5  $\mu\text{M}$  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  $\mu\text{m}$ . doi:10.1371/journal.pone.0066274.g003

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, hADMPs 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  $\mu\text{g}/\text{mL}$ ) 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 hADMPs transduced with pTRE-EGFP-EF-tTA-2A-Bsd, but a wide range of fluorescent intensities was observed in hADMPs 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  $\mu\text{g}/\text{mL}$  blasticidin was administered to hADMPs. Within 1 week after the selection, control hADMPs were completely killed (data not shown), whereas hADMPs 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  $\mu\text{g}/\text{mL}$ ) 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 hADMPs 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 hADMPs 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.

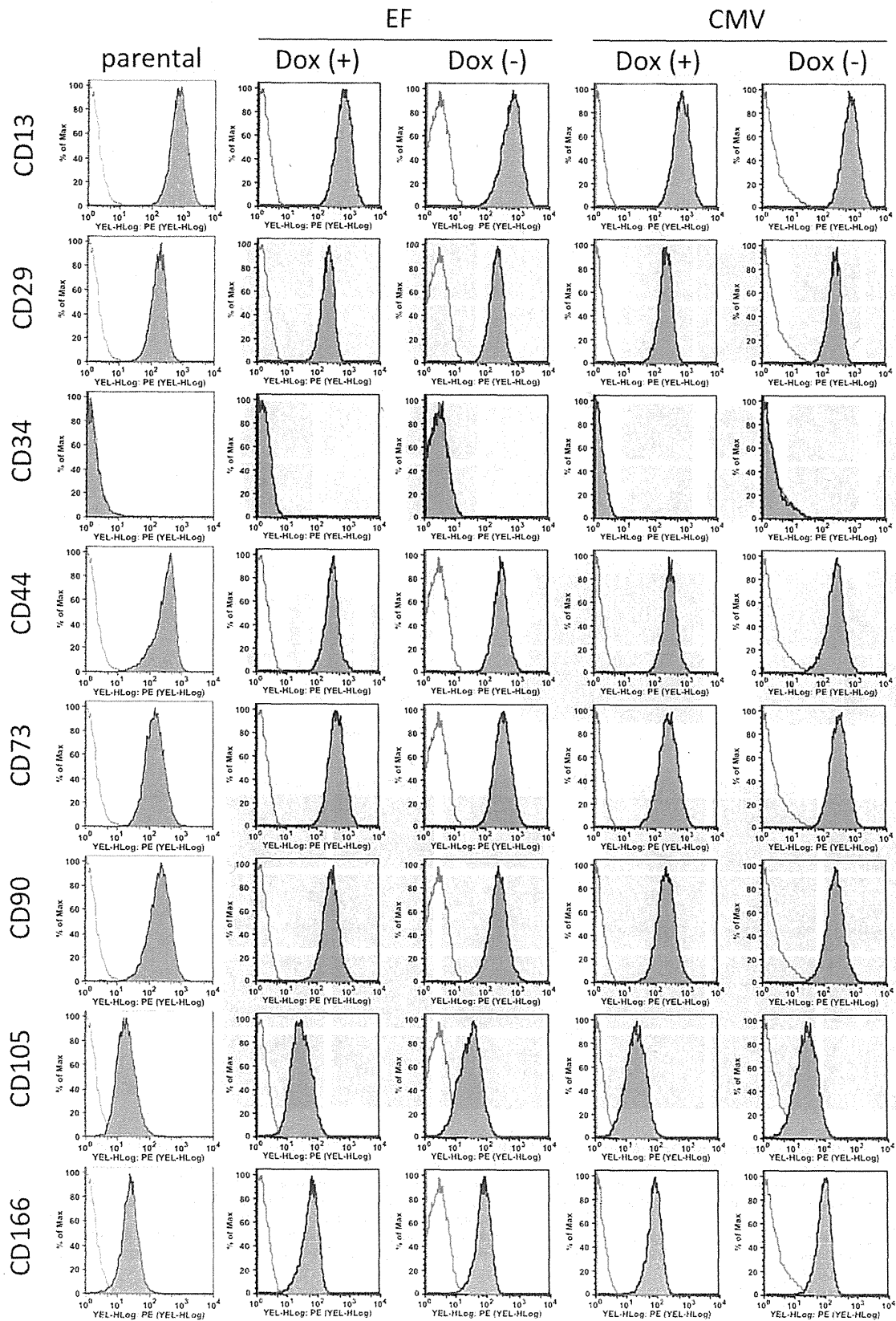
### Blasticidin-selected hADMPs Maintain the Properties of Their Parental hADMPs

hADMPs 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 hADMPs 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 hADMPs, without affecting the phenotype, is of special interest for the field. We therefore studied the cell properties of hADMPs 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 hADMPs, the cells were differentiated into adipocytes, osteocytes, chondrocytes, and neuronal cells. As shown in Figure 5, blasticidin-selected hADMPs 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).

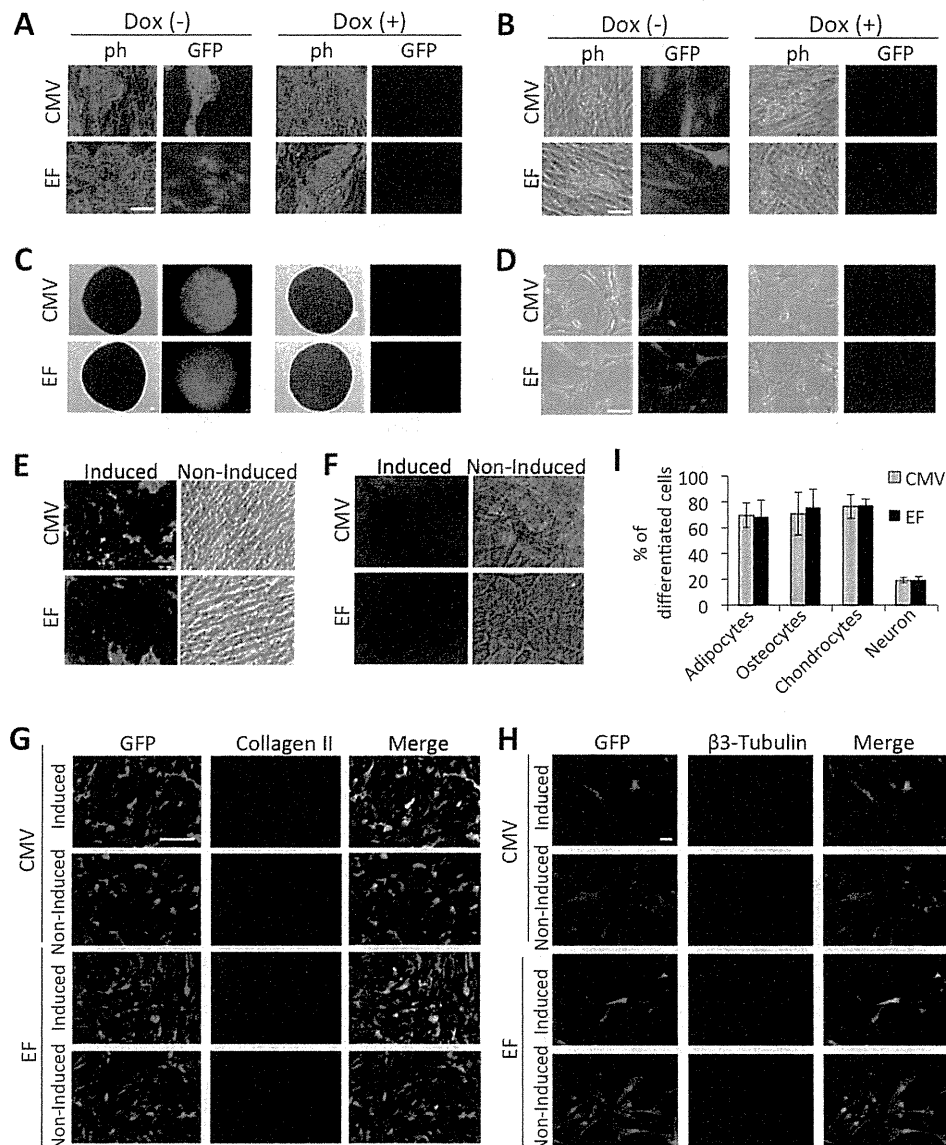
### Discussion

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



**Figure 4. Expression pattern of surface cell markers on Dox-responsive hADMPCs.** Dox-responsive hADMPCs after selection by blasticidin were cultured in the absence (Dox(-)) or presence (Dox(+)) of 1  $\mu$ g/mL Dox for 4 days. Expression of the different surface markers were analyzed by flow cytometry and compared to the expression by a parental hADMPCs. They were stained with PE-coupled antibodies against CD13, CD29, CD34, CD44, CD73, CD90, CD105, and CD166. Histogram of a PE-coupled mouse IgG1  $\kappa$  isotype control is shown in gray. CMV; hADMPCs transduced with pTRE-EGFP-CMV-tTA-2A-Bsd, EF; hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd. doi:10.1371/journal.pone.0066274.g004



**Figure 5. Differentiation potential of Dox-responsive hADMPCs.** Dox-responsive hADMPCs were differentiated into adipocytes (A, E), osteocytes (B, F), chondrocytes (C, G), and neuronal cells (D, H). (A–D) Phase contrast (ph) and fluorescent (GFP) images. Dox-responsive hADMPCs were differentiated in the absence of Dox (Dox(-)) or in the presence of 1  $\mu$ g/mL Dox (Dox(+)) as described in the material and methods section. (E–I) Confirmation of differentiated cells by oil red O staining for adipocytes (E), alizarin red staining for osteocytes (F), immunohistochemical staining against collagen II for chondrocytes (G), and immunohistochemical staining against  $\beta$ 3-tubulin for neuronal cells (H). The percentages of differentiated cells to each cell type were calculated by the computerized image analysis (I). Cells that were not induced to differentiate (non-induced) were used as a negative control. CMV; hADMPCs transduced with pTRE-EGFP-CMV-tTA-2A-Bsd, EF; hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd. Scale bar, 50  $\mu$ m. doi:10.1371/journal.pone.0066274.g005

requiring sustained, long-term expression of therapeutic proteins. In this study, we generated novel lentiviral vectors with a tet-off system, and demonstrated that our lentiviral vector systems were significantly effective and strictly regulated in hADMPCs, without affecting their stem cell properties.

Gene silencing is of considerable importance where stable, long-term expression is required. Researchers have reported that transgene silencing occurred when the CMV promoter was used in some cell types, especially in embryonic stem cells [15–17]. Since Kawabata et al. also demonstrated that virus-derived promoters inefficiently functioned in embryonic stem cells in gene transfer experiments [37], down-regulation and unsuitability of promoters in stem cells should be considered. Therefore, transduction efficacy and durability of transgene expression in hADMPCs is also an important issue to be determined. Qin et al. reported that the human EF-1 $\alpha$  promoter and the TRE promoter are more efficient than the CMV promoter to drive lentiviral mediated transgene expression in rat bone marrow-derived MSCs [18]. McGinley et al. also showed that EF-1 $\alpha$  and human phosphoglycerate kinase-1 (PGK) promoters have a clear advantage over the CMV promoter in transducing rat bone marrow-derived MSC transduction with lentivirus [19]. Consistent with their findings, our data also demonstrated that the EF-1 $\alpha$  promoter was more efficient than the CMV promoter to drive EGFP expression in hADMPCs (Figure 1A, B). Moreover, a significant decrease in fluorescent intensity was observed by 28 days after transduction with lentiviral vector CSII-CMV-EGFP (Figure 1C), suggesting that the CMV promoter might be silenced in hADMPCs. We also demonstrated the intriguing finding that most (>90%) of the hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd strongly expressed EGFP in the absence of Dox, whereas >50% of the cells transduced with pTRE-EGFP-CMV-tTA-2A-Bsd were EGFP negative, regardless of their blasticidin resistance (Figure 3A, B). Our data demonstrated that the inhibitor of histone deacetylation trichostatin A (TSA) re-induced the expression of EGFP (Figure 3C, D), suggesting that “promoter suppression” might occur by histone deacetylation, not by DNA methylation of CpG sites within the TRE tight promoter. “Promoter suppression” is a transcript repression of a 5' transcriptional unit by a 3' unit when 2 transcriptional units lie adjacent in head-to-tail tandem on a chromosome [28,29]. In this study, it is possible that the downstream unit of CMV-tTA-2A-Bsd repressed the upstream unit of TRE-EGFP because (1) resistance to blasticidin implies the transcriptional unit of CMV-tTA-2A-Bsd is active, and (2) reactivation of EGFP expression by TSA implies the transcriptional unit of TRE-EGFP is epigenetically silenced. In order to eliminate the promoter suppression or transcriptional interference between 2 transcriptional units, some researchers have been trying to separate the 2 units by polyadenylation, terminator, and insulator sequences [28,38]. However, these sequences extend the lentiviral vector size, which may affect the lentiviral titers produced from the vector. From this point of view, our finding that the transcriptional unit driven from the TRE tight promoter is resistant to gene silencing when arranged in tandem with the EF-tTA-2A-Bsd transcriptional unit (Figure 3) is of interest in the fields of both basic and clinical research, although the underlying mechanism remains elusive.

In general, large numbers of cells displaying the appropriate phenotypes are required for tissue engineering. Moreover, fully differentiated cells do not proliferate [39]. Therefore, in order to obtain enough cells to perform a transplant from genetically modified MSCs, it is important to develop a system in which the gene of interest is tightly regulated and inducible, and in which stably expressing transgenic cell lines can be obtained without

affecting their stem cell properties. Using the system, MSCs transduced with lentiviral vectors can be selected and increased in numbers from a limited number of MSCs, before the target genes are induced. After obtaining an adequate number of gene-manipulated MSCs, the target genes could be induced in order to start differentiation. According to our data, hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd were successfully selected by blasticidin, could proliferate, maintain their stem cell properties, and regulate EGFP expression tightly by Dox (Figure 4, 5), demonstrating that this all-in-one lentiviral vector is a promising gene delivery system for generating the material for artificial organs.

A major advantage of using the 2A cleavage factor in the construction of multi-cistronic vectors is its small size compared to internal promoter entry site (IRES) sequences. Because the titer of the lentivirus decreases with increasing size of the lentiviral vector, it is important to minimize the length of the sequences. In addition, linkage of 2 genes by 2A peptide resulted in efficient co-expression of the genes, whereas a gene placed downstream of an IRES is expressed at 2- to 3-fold lower levels than a gene placed upstream [40,41]. In this study, tTA-2A-Bsd cassette driven from CMV or EF-1 $\alpha$  promoter showed ~90% cleavage (Figure 3). However, the point that should be considered is the effect of residual 2A peptide on the protein. As the processing occurred at the end of the 2A peptide, the 2A tag remains attached at the tTA C-terminus. Our data demonstrated that the presence of this extra 2A peptide did not seem to interfere with the activity of tTA since Dox strictly regulated the expression of EGFP under the control of TRE-tight promoter (Figure 2D, 3A, 3B and 5). Moreover, when Bsd is cleaved, an additional proline is attached at the N-terminus. We demonstrated that this did not affect a function of Bsd because hADMPCs transduced with either pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd could survive and proliferate in medium containing blasticidin at a concentration at which all of the parental hADMPCs died.

Another advantage of our lentiviral system is the availability of a restriction enzyme treatment/ligation independent cloning system, called the Gateway system (Invitrogen). In general, the construction of lentiviral vectors using a conventional restriction enzyme/ligation cloning method has poor efficiency due to the large sizes and the lack of proper cloning sites. In our hands, cloning efficiency into our new lentiviral vectors pTRE-RfA-CMV-tTA-2A-Bsd or pTRE-RfA-EF-tTA-2A-Bsd using LR recombination reaches nearly 100%, saving time and effort in construction of the vectors. In addition, there are several resources available that take advantage of the Gateway vector. For example, CCSB Human ORFeome Collection (Dana-Farber Cancer Institute, Center for Cancer Systems Biology) represents almost 12,000 fully-sequenced cloned human ORFs which can be readily transferred to Gateway compatible destination vectors for various functional proteomics studies [42]. Block-iT pol II miR RNAi system from Invitrogen, which is designed to express artificial miRNAs, also enables compatibility with Gateway destination vectors for gene knock-down experiments [43].

In conclusion, our new single tet-off lentiviral vector system provides powerful tools not only for applied research on hADMPCs and other stem cells, but also basic research on a variety of cell lines and primary cells.

## Acknowledgments

We thank J. Uda, S. Tamura, C. Sone, K. Nakagita, and H. Isshi for technical support. We thank Dr. Tyler Jacks for providing the pSico plasmid and Dr. Hiroyuki Miyoshi for the CSII-EF-RfA, CSII-CMV-RfA, pCMV-VSVG-RSV-Rev, and pCAG-HIVg/p plasmids.

## Author Contributions

Conceived and designed the experiments: HM MM. Performed the experiments: HM MM KS HO AM. Analyzed the data: HM MM KS.

Contributed reagents/materials/analysis tools: HM MM HO AI AM. Wrote the paper: HM MM TH.

## References

- Okura H, Komoda H, Saga A, Kakuta-Yamamoto A, Hamada Y, et al. (2010) Properties of hepatocyte-like cell clusters from human adipose tissue-derived mesenchymal stem cells. *Tissue engineering Part C, Methods* 16: 761–770.
- Okura H, Matsuyama A, Lee CM, Saga A, Kakuta-Yamamoto A, et al. (2010) Cardiomyoblast-like cells differentiated from human adipose tissue-derived mesenchymal stem cells improve left ventricular dysfunction and survival in a rat myocardial infarction model. *Tissue engineering Part C, Methods* 16: 417–425.
- Okura H, Komoda H, Fumimoto Y, Lee CM, Nishida T, et al. (2009) Transdifferentiation of human adipose tissue-derived stromal cells into insulin-producing clusters. *Journal of artificial organs : the official journal of the Japanese Society for Artificial Organs* 12: 123–130.
- Safford KM, Safford SD, Gimble JM, Shetty AK, Rice HE (2004) Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells. *Experimental neurology* 187: 319–328.
- Leu S, Lin YC, Yuen CM, Yen CH, Kao YH, et al. (2010) Adipose-derived mesenchymal stem cells markedly attenuate brain infarct size and improve neurological function in rats. *Journal of translational medicine* 8: 63.
- Ikegame Y, Yamashita K, Hayashi S, Mizuno H, Tawada M, et al. (2011) Comparison of mesenchymal stem cells from adipose tissue and bone marrow for ischemic stroke therapy. *Cytotherapy* 13: 675–685.
- Tan B, Luan Z, Wei X, He Y, Wei G, et al. (2011) AMP-activated kinase mediates adipose stem cell-stimulated neurogenesis of PC12 cells. *Neuroscience* 181: 40–47.
- Reid AJ, Sun M, Wiberg M, Downes S, Terenghi G, et al. (2011) Nerve repair with adipose-derived stem cells protects dorsal root ganglia neurons from apoptosis. *Neuroscience*.
- Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, et al. (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 109: 1292–1298.
- Lee EY, Xia Y, Kim WS, Kim MH, Kim TH, et al. (2009) Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 17: 540–547.
- Moriyama M, Moriyama H, Ueda A, Nishibata Y, Okura H, et al. (2012) Human adipose tissue-derived multilineage progenitor cells exposed to oxidative stress induce neurite outgrowth in PC12 cells through p38 MAPK signaling. *BMC Cell Biol* 13: 21.
- Wu H, Ye Z, Mahato RI (2011) Genetically modified mesenchymal stem cells for improved islet transplantation. *Mol Pharm* 8: 1458–1470.
- Pfeifer A, Ikawa M, Dayn Y, Verma IM (2002) Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci U S A* 99: 2140–2145.
- Duan HF, Wu CT, Wu DL, Lu Y, Liu HJ, et al. (2003) Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor. *Mol Ther* 8: 467–474.
- Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, et al. (2004) Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med* 6: 395–404.
- Kim S, Kim GJ, Miyoshi H, Moon SH, Ahn SE, et al. (2007) Efficiency of the elongation factor-1 $\alpha$  promoter in mammalian embryonic stem cells using lentiviral gene delivery systems. *Stem Cells Dev* 16: 537–545.
- Meilinger D, Fellingner K, Bultmann S, Rothbauer U, Bonapace IM, et al. (2009) Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. *EMBO Rep* 10: 1259–1264.
- Qin JY, Zhang L, Clift KL, Huler I, Xiang AP, et al. (2010) Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One* 5: e10611.
- McGinley L, McMahon J, Strappe P, Barry F, Murphy M, et al. (2011) Lentiviral vector mediated modification of mesenchymal stem cells & enhanced survival in an in vitro model of ischaemia. *Stem Cell Res Ther* 2: 12.
- Weber W, Fussnegger M (2006) Pharmacologic transgene control systems for gene therapy. *J Gene Med* 8: 535–556.
- Shi Q, Tian X, Zhao Y, Luo H, Tian Y, et al. (2011) Anti-arthritic effects of FasL gene transferred intra-articularly by an inducible lentiviral vector containing improved tet-on system. *Rheumatol Int*.
- Wiederschain D, Wee S, Chen L, Loo A, Yang G, et al. (2009) Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle* 8: 498–504.
- Hioki H, Kuramoto E, Konno M, Kameda H, Takahashi Y, et al. (2009) High-level transgene expression in neurons by lentivirus with Tet-Off system. *Neurosci Res* 63: 149–154.
- Benabdellah K, Cobo M, Munoz P, Toscano MG, Martin F (2011) Development of an all-in-one lentiviral vector system based on the original TetR for the easy generation of Tet-ON cell lines. *PLoS One* 6: e23734.
- Okura H, Saga A, Fumimoto Y, Soeda M, Moriyama M, et al. (2011) Transplantation of human adipose tissue-derived multilineage progenitor cells reduces serum cholesterol in hyperlipidemic Watanabe rabbits. *Tissue engineering Part C, Methods* 17: 145–154.
- Saga A, Okura H, Soeda M, Tani J, Fumimoto Y, et al. (2011) HMG-CoA reductase inhibitor augments the serum total cholesterol-lowering effect of human adipose tissue-derived multilineage progenitor cells in hyperlipidemic homozygous Watanabe rabbits. *Biochemical and biophysical research communications* 412: 50–54.
- Okura H, Saga A, Fumimoto Y, Soeda M, Moriyama M, et al. (2011) Transplantation of human adipose tissue-derived multilineage progenitor cells reduces serum cholesterol in hyperlipidemic Watanabe rabbits. *Tissue Eng Part C Methods* 17: 145–154.
- Villemure JF, Savard N, Belmaaza A (2001) Promoter suppression in cultured mammalian cells can be blocked by the chicken beta-globin chromatin insulator 5'HS4 and matrix/scaffold attachment regions. *J Mol Biol* 312: 963–974.
- Emerman M, Temin HM (1986) Comparison of promoter suppression in avian and murine retrovirus vectors. *Nucleic Acids Res* 14: 9381–9396.
- Tai K, Pelled G, Sheyn D, Bershteyn A, Han L, et al. (2008) Nanobiomechanics of repair bone regenerated by genetically modified mesenchymal stem cells. *Tissue Eng Part A* 14: 1709–1720.
- Goudeyne S, Pisani DF, Wdzickowski B, Di Santo JP, Bagnis C, et al. (2009) Enhancement of myogenic and muscle repair capacities of human adipose-derived stem cells with forced expression of MyoD. *Mol Ther* 17: 1064–1072.
- Li Y, Zhang R, Qiao H, Zhang H, Wang Y, et al. (2007) Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells. *J Cell Physiol* 211: 36–44.
- Karnieli O, Izhar-Prato Y, Bulvik S, Efrat S (2007) Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation. *Stem Cells* 25: 2837–2844.
- Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, et al. (2004) Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 113: 1701–1710.
- Pan L, Lin C, Zhuo S, Chen L, Liu N, et al. (2009) Transplantation with survivin-engineered mesenchymal stem cells results in better prognosis in a rat model of myocardial infarction. *Eur J Heart Fail* 11: 1023–1030.
- Ghosh SS, Gopinath P, Ramesh A (2006) Adenoviral vectors: a promising tool for gene therapy. *Appl Biochem Biotechnol* 133: 9–29.
- Kawabata K, Sakurai F, Yamaguchi T, Hayakawa T, Mizuguchi H (2005) Efficient gene transfer into mouse embryonic stem cells with adenovirus vectors. *Mol Ther* 12: 547–554.
- Tian J, Andreadis ST (2009) Independent and high-level dual-gene expression in adult stem-progenitor cells from a single lentiviral vector. *Gene Ther* 16: 874–884.
- Clarke MF, Fuller M (2006) Stem cells and cancer: two faces of eve. *Cell* 124: 1111–1115.
- Chinnasamy D, Milsom MD, Shaffer J, Neuenfeldt J, Shaaban AF, et al. (2006) Multicistronic lentiviral vectors containing the FMDV 2A cleavage factor demonstrate robust expression of encoded genes at limiting MOI. *Virology* 3: 14.
- Ibrahimi A, Vande Velde G, Reumers V, Toelen J, Thiry I, et al. (2009) Highly efficient multicistronic lentiviral vectors with peptide 2A sequences. *Hum Gene Ther* 20: 845–860.
- Temple G, Gerhard DS, Rasooly R, Feingold EA, Good PJ, et al. (2009) The completion of the Mammalian Gene Collection (MGC). *Genome Res* 19: 2324–2333.
- Liang Z, Wu H, Reddy S, Zhu A, Wang S, et al. (2007) Blockade of invasion and metastasis of breast cancer cells via targeting CXCR4 with an artificial microRNA. *Biochem Biophys Res Commun* 363: 542–546.

## Long-Term Self-Renewal of Human ES/iPS-Derived Hepatoblast-like Cells on Human Laminin III-Coated Dishes

Kazuo Takayama,<sup>1,2,3</sup> Yasuhito Nagamoto,<sup>1,2</sup> Natsumi Mimura,<sup>2</sup> Katsuhisa Tashiro,<sup>4</sup> Fuminori Sakurai,<sup>1</sup> Masashi Tachibana,<sup>1</sup> Takao Hayakawa,<sup>5</sup> Kenji Kawabata,<sup>4</sup> and Hiroyuki Mizuguchi<sup>1,2,3,6,\*</sup>

<sup>1</sup>Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan

<sup>2</sup>Laboratory of Hepatocyte Differentiation, National Institute of Biomedical Innovation, Osaka 567-0085, Japan

<sup>3</sup>iPS Cell-Based Research Project on Hepatic Toxicity and Metabolism, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan

<sup>4</sup>Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Osaka 567-0085, Japan

<sup>5</sup>Pharmaceutical Research and Technology Institute, Kinki University, Osaka 577-8502, Japan

<sup>6</sup>The Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka 565-0871, Japan

\*Correspondence: mizuguch@phs.osaka-u.ac.jp

<http://dx.doi.org/10.1016/j.stemcr.2013.08.006>

This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

### SUMMARY

The establishment of self-renewing hepatoblast-like cells (HBCs) from human pluripotent stem cells (PSCs) would realize a stable supply of hepatocyte-like cells for medical applications. However, the functional characterization of human PSC-derived HBCs was not enough. To purify and expand human PSC-derived HBCs, human PSC-derived HBCs were cultured on dishes coated with various types of human recombinant laminins (LN). Human PSC-derived HBCs attached to human laminin-111 (LN111)-coated dish via integrin alpha 6 and beta 1 and were purified and expanded by culturing on the LN111-coated dish, but not by culturing on dishes coated with other laminin isoforms. By culturing on the LN111-coated dish, human PSC-derived HBCs were maintained for more than 3 months and had the ability to differentiate into both hepatocyte-like cells and cholangiocyte-like cells. These expandable human PSC-derived HBCs would be manageable tools for drug screening, experimental platforms to elucidate mechanisms of hepatoblasts, and cell sources for hepatic regenerative therapy.

### INTRODUCTION

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have the ability to self-replicate and to differentiate into all types of body cells including hepatoblasts and hepatocytes. Although cryopreserved primary human hepatocytes are useful in drug screening and liver cell transplantation, they rapidly lose their functions (such as drug metabolism capacity) and hardly proliferate in *in vitro* culture systems. On the other hand, human hepatic stem cells from fetal and postnatal human liver are able to self-replicate and able to differentiate into hepatocytes (Schmelzer et al., 2007; Zhang et al., 2008). However, the source of human hepatic stem cells is limited, and these cells are not available commercially. Therefore, the human pluripotent stem cell (hPSC)-derived hepatoblast-like cells (HBCs), which have potential to differentiate into the hepatocyte-like cells, would be an attractive cell source to provide abundant hepatocyte-like cells for drug screening and liver cell transplantation.

Because expandable and multipotent hepatoblasts or hepatic stem cells are of value, suitable culture conditions for the maintenance of hepatoblasts or hepatic stem cells obtained from fetal or adult mouse liver were developed (Kamiya et al., 2009; Tanimizu et al., 2004). Soluble factors, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF), are known to support the proliferation

of mouse hepatic stem cells and hepatoblast (Kamiya et al., 2009; Tanimizu et al., 2004). Extracellular matrix (ECM) also affects the maintenance of hepatoblasts or hepatic stem cells. Laminin can maintain the character of mouse hepatoblasts (Dlk1-positive cells) (Tanimizu et al., 2004). However, the methodology for maintaining HBCs differentiated from hPSCs has not been well investigated. Zhao et al. (2009) have reported that hESC-derived hepatoblast-like cells (sorted N-cadherin-positive cells were used) could be maintained on STO feeder cells. Although a culture system using STO feeder cells for the maintenance of hepatoblast-like cells might be useful, there are two problems. The first problem is that N-cadherin is not a specific marker for human hepatoblasts. N-cadherin is also expressed in hESC-derived mesendoderm cells and definitive endoderm (DE) cells (Sumi et al., 2008). The second problem is that residual undifferentiated cells could be maintained on STO feeder cells. Therefore, their culture condition cannot rule out the possibility of the proliferation of residual undifferentiated cells. Because it is known that hPSC-derived cells have the potential to form teratomas in the host, the production of safer hepatocyte-like cells or hepatoblast-like cells has been required. Therefore, we decided to purify hPSC-derived HBCs, which can differentiate into mature hepatocyte-like cells, and then expand these cells.

In this study, we attempt to determine a suitable culture condition for the extensive expansion of HBCs derived



from hPSCs. We found that the HBCs derived from hPSCs can be maintained and proliferated on human laminin-111 (LN111)-coated dishes. To demonstrate that expandable, multipotent, and safe (i.e., devoid of residual undifferentiated cells) hPSC-derived HBCs could be maintained under our culture condition, the hPSC-derived HBCs were used for hepatic and biliary differentiation, colony assay, and transplantation into immunodeficient mice.

## RESULTS

### Human PSC-Derived Hepatoblast-like Cells Could Adhere onto Human LN111 via Integrin $\alpha 6$ and $\beta 1$

The HBCs were generated from hPSCs (hESCs and hiPSCs) as described in Figure 1A (details of the characterization of hPSC-derived HBCs are described in Figure 3). Definitive endoderm differentiation of hPSCs was promoted by stage-specific transient transduction of FOXA2 in addition to the treatment with appropriate soluble factors (such as Activin A). Overexpression of FOXA2 is not necessary for establishing the hPSC-derived HBCs, but it is helpful for efficient generation of the hPSC-derived HBCs. On day 9, these hESC-derived populations contained two cell populations with distinct morphology (Figure 1B). One population resembled human hepatic stem cells that were isolated from human fetal liver (shown in red) (Schmelzer et al., 2007), whereas the other population resembled definitive endoderm cells (shown in green) (Hay et al., 2008). The population that resembled human hepatic stem cells was alpha-1-fetoprotein (AFP) positive, whereas the other population was AFP negative (Figure 1C, left). On day 9, the percentage of AFP-positive cells was approximately 80% (Figure 1C, right). To characterize these two cell populations (hESC-derived HBC and non-HBC [NHBC] populations), the colonies were manually isolated by using a pipette, and then the gene expression analysis was performed. The gene expression levels of *AFP*, *CD133*, *EpCAM*, *CK8*, and *CK18* in the hESC-derived HBCs were higher than those in the bulk population containing both hESC-derived HBCs and NHBCs (*CD133*, *EpCAM*, *CK8*, and *CK18* were named as pan-hepatoblast markers and are known to be strongly expressed in both human hepatic stem cells and hepatoblasts [Schmelzer et al., 2007; Zhang et al., 2008]) (Figure 1D). On the other hand, the gene expressions of *AFP*, *CD133*, *EpCAM*, *CK8*, and *CK18* in the hESC-derived NHBCs were hardly detected. The gene expression levels of DE, mesendoderm, and pluripotent markers in the hESC-derived NHBCs were higher than those in the hESC-derived HBCs, indicating that the hESC-derived NHBCs could remain in a more undifferentiated state than the hESC-derived HBCs (Figures S1A–S1C available online). These results suggest

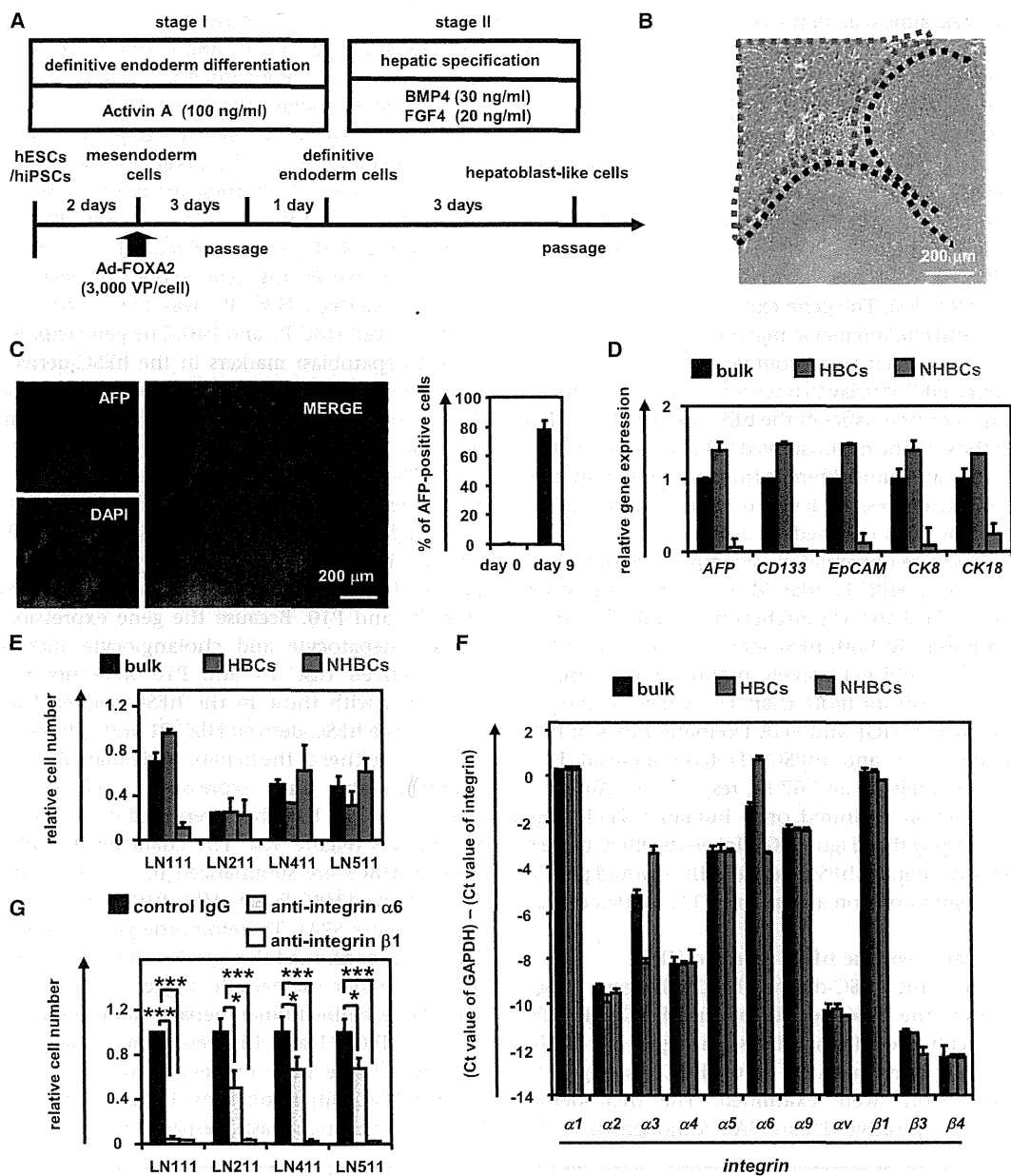
that hepatoblast-like cells could be differentiated from hPSCs.

To purify the hESC-derived HBCs, these cells were plated onto dishes coated with various laminins. There are 15 different laminin isoforms in human tissues. Although laminin is known to be useful to sustain mouse hepatoblasts (Tanimizu et al., 2004), it remains unknown which human laminin isoform has the potential to purify and expand the HBCs. To identify a human laminin isoform that would be useful for purifying hESC-HBCs, the hESC-HBCs and -NHBCs were plated onto dishes coated with various types of commercially available human laminins (Figure 1E). The hESC-derived HBCs could more efficiently adhere onto the human LN111-coated dish compared with hESC-derived NHBCs or unseparated populations (containing both HBCs and NBCs). These data suggest that a hESC-derived HBC population can be purified from the unseparated populations by culturing on human LN111-coated dishes. Because integrins are known to be important molecules for cell adhesion to the ECM including laminins, we expected that certain types of integrins would allow selective adhesion of the hESC-derived HBCs to human LN111-coated dish. The gene expression levels of various integrins were examined (Figure 1F). Among the integrin  $\alpha$  subunits, the gene expression level of *integrin  $\alpha 6$*  in the hESC-derived HBCs was significantly higher than that in the hESC-derived NHBCs. In contrast, among the integrin  $\beta$  subunits, the gene expression level of *integrin  $\beta 1$*  was higher than those of *integrin  $\beta 2$*  and  *$\beta 3$*  in all cell populations. The hESC-derived HBCs, but not NHBCs, expressed both integrin  $\alpha 6$  and  $\beta 1$  (Figure S1D). Almost all adhesion of the hESC-derived HBCs to a human LN111-coated dish was inhibited by both function-blocking antibodies to integrin  $\alpha 6$  and  $\beta 1$  (Figure 1G). These results indicated that the hESC-derived HBCs could attach to a human LN111-coated dish via integrin  $\alpha 6$  and  $\beta 1$ .

### The hPSC-Derived HBCs Could Be Proliferated and Maintained on a Human LN111-Coated Dish

To obtain the purified hESC-derived HBC population, the hESC-derived cells (day 9) were plated onto a human LN111-coated dish, and then unattached cells were removed at 15 min after plating (Figure 2A). Among various laminins, only human LN111 could proliferate (Figure 2B) and purify (Figure 2C) the AFP-positive population in the presence of HGF and EGF. During culture on the human LN111-coated dish, the morphology of the hESC-derived HBCs gradually changed into that of human hepatoblasts (Figure S1E) (Schmelzer et al., 2007). Therefore, the characteristics of hESC-derived HBCs might be changed by culturing on a human LN111-coated dish (details of the characterization of the hESC-derived HBCs are described in Figure 3). After culturing on a human LN111-coated





**Figure 1. The Human ESC-Derived HBCs Selectively Attached to a Human LN111-Coated Dish via Integrin  $\alpha 6$  and  $\beta 1$**   
 (A) The procedure for the differentiation of hESCs (H9) into hepatoblast-like cells (HBCs) is presented schematically. Details are described in the Experimental Procedures.  
 (B) Phase-contrast micrographs of the hESC-derived HBCs (red) and non-HBCs (NHBCs) (green) are shown.  
 (C) The hESC-derived cells (day 9) were subjected to immunostaining with anti-AFP (red) antibodies. The percentage of AFP-positive cells was examined on day 0 or 9 by using FACS analysis. Data represent the mean  $\pm$  SD from ten independent experiments. Cells on "day 0" and "day 9" were compared using Student's t test ( $p < 0.01$ ).  
 (D) On day 9, the hESC-derived HBCs and NHBCs were manually picked, and the gene expression levels of *AFP* and pan-hepatoblast markers (*CD133*, *EpCAM*, *CK8*, and *CK18*) were measured by real-time RT-PCR. The gene expression levels of *AFP* and pan-hepatoblast markers in the hESC-derived cells (day 9; bulk) were taken as 1.0. Data represent the mean  $\pm$  SD from four independent experiments. The gene expression levels in the HBCs were significantly different among the three groups (bulk, HBCs, and NHBCs) based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ( $p < 0.05$ ).  
 (legend continued on next page)



dish for a week, almost all of the cells were still AFP positive (Figures 2C and 2D). To characterize the cells cultured on various types of human laminins for 7 days, the gene expression levels of *AFP* and pan-hepatoblast (*CD133*, *CK8*, *CK18*, and *EpCAM*) markers were examined on day 16 (Figure 2E). The gene expression levels of *AFP* and pan-hepatoblast markers in the hESC-derived HBCs P1 (HBCs passaged once) did not change as compared with those of the hESC-derived HBCs (day 9; HBC P0) (the definitions of HBC P0, P1, P10, and clone in the present study are shown in Figure S3). The gene expression levels of mature hepatocyte and cholangiocyte markers in the hESC-derived HBC P1 did not change as compared with those of the hESC-derived HBC P0 (day 9) (Figure S1F). These results suggest that the characteristics of the hESC-derived HBC P1 are similar to those of the hESC-derived HBC P0, although their morphologies are quite different from each other. Interestingly, the gene expression levels of mature cholangiocyte markers in the cells cultured on human LN411- or 511-coated dishes were upregulated as compared with those of the hESC-derived HBC P0 (day 9) (Figure S1F), suggesting that human LN411 and 511 might promote biliary differentiation. Importantly, both hESC-derived HBCs and hiPSC-derived HBCs could extensively proliferate on a human LN111-coated dish for more than 15 passages (Figure 2F) in the presence of HGF and EGF. Doubling times of hESC (H9)-derived HBCs and hiPSC (Dotcom)-derived HBCs were approximately 78 and 67 hr, respectively. Almost all of the populations cultured on a human LN111-coated dish were AFP positive (Figure 2G). Taken together, these results suggested that the hPSC-derived HBCs would proliferate and be maintained on a human LN111-coated dish.

#### Characterization of the hESC-Derived HBCs

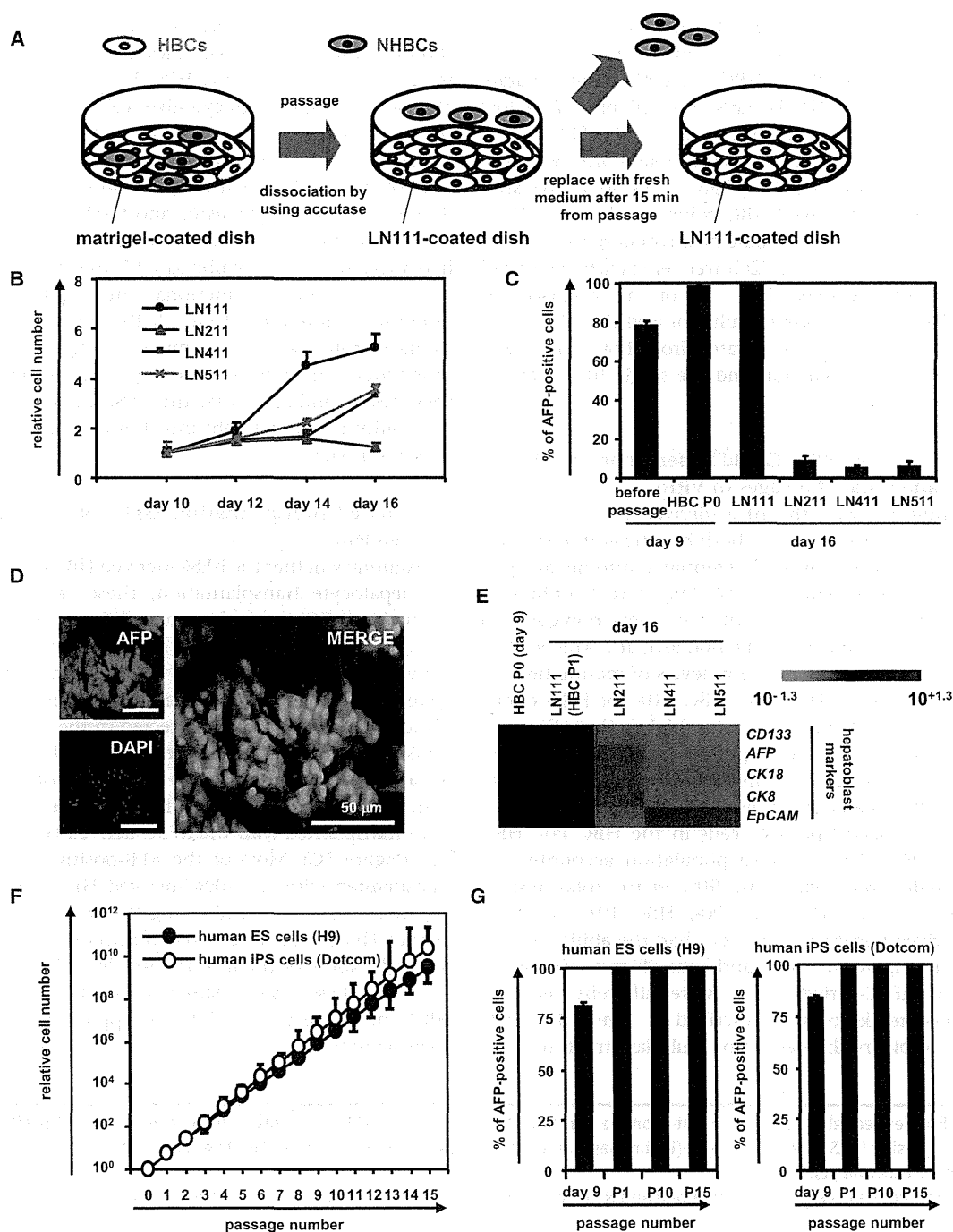
To characterize the hESC-derived HBCs, the gene expression profiles in the hESC-derived purified HBCs (HBC P0), short-term cultured HBCs (HBCs passaged once [HBC P1]), and long-term cultured HBC (HBCs passaged ten times [HBC P10]) were examined. The hESC-derived HBCs were AFP positive (Figure 3A). Although the hESC-

derived HBC P0 were negative for ALB, CK7, and CK19, the hESC-derived HBC P1 and P10 were positive for these genes (Figure 3A). Both integrin  $\alpha 6$  and  $\beta 1$  (receptors of LN111) were strongly expressed in the hESC-derived HBC P0, P1, and P10 (Figure 3B). The gene expression levels of human hepatic stem cell markers (*N-CAM* and *Claudin 3* [Schmelzer et al., 2007]; these are not expressed in human hepatoblasts) in the hESC-derived HBC P0 were higher than those of the hESC-derived HBC P1 and P10 (Figure 3C). However, the gene expression level of *CK19* in the hESC-derived HBC P0 was lower than that of the hESC-derived HBC P1 and P10. The gene expression levels of pan-hepatoblast markers in the hESC-derived HBC P0 were similar to those of the hESC-derived HBC P1 and P10 (Figure 3D). The gene expression levels of human hepatoblast markers (*ALB*, *CYP3A7*, and *I-CAM* [Schmelzer et al., 2007]), none of which were expressed in human hepatic stem cells) in the hESC-derived HBC P1 and P10 were higher than those of the hESC-derived HBC P0 (Figure 3E). However, the AFP expression level in the hESC-derived HBC P0 was similar to that of the hESC-derived HBC P1 and P10. Because the gene expression levels of mature hepatocyte and cholangiocyte markers in the hESC-derived HBC P1 and P10 were not increased as compared with those in the hESC-derived HBC P0 (Figure 3F), the hESC-derived HBC P1 and P10 were not segregated into either of the hepatic and biliary lineages. We also examined the gene expression levels of hepatoblast markers, which have been reported only in mice and not in humans (Figure 3G). The characteristics of the hPSC-derived HBCs are summarized in Figure S3. In addition, hESC-derived HBC P0 and HBC P10 showed normal karyotypes (Figure S2A). Therefore, the genetic stability of the HBCs was confirmed throughout the maintenance period. Taken together, these results suggest that the hESC-derived HBC P0 resemble human hepatic stem cells and the hESC-derived HBC P1 and P10 resemble human hepatoblasts, although some gene expression patterns in the hESC-derived HBCs differ from those in human hepatic stem cells and human hepatoblasts, respectively.

(E) The hESC-derived cells (day 9; bulk), HBCs, and NHBCs were plated onto human LN111-, 211-, 411-, or 511-coated dishes, and the attached cells were counted at 60 min after plating. The cell number that was initially plated was taken as 1.0. Data represent the mean  $\pm$  SD from four independent experiments. The number of attached HBCs on LN111-coated dishes were significantly different among three groups (bulk, HBCs, and NHBCs) based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ( $p < 0.05$ ).

(F) The gene expression levels of the indicated integrins were measured in the hESC-derived cells (day 9; bulk), HBCs, and NHBCs by real-time RT-PCR. Data represent the mean  $\pm$  SD from four independent experiments. The gene expression levels of *integrin  $\alpha 3$*  and  *$\alpha 6$*  in the HBCs were significantly different among three groups (bulk, HBCs, and NHBCs) based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ( $p < 0.05$ ).

(G) The adhesion of the hESC-derived HBCs to human LN111-, 211-, 411-, or 511-coated dishes was examined by using the indicated integrin antibodies. IgG antibodies were used as a control for uninhibited cell adhesion. The number of attached cells was estimated at 60 min after plating. The cell number in the control IgG-treated group was taken as 1.0. Data represent the mean  $\pm$  SD from three independent experiments. "Control IgG" and "anti-integrin  $\alpha 6$  or integrin  $\beta 1$ " were compared using Student's *t* test. \* $p < 0.05$ ; \*\*\* $p < 0.001$ . See also Figure S1 and Tables S2–S5.



**Figure 2. The hESC-Derived HBCs Could Be Proliferated and Maintained on a Human LN111-Coated Dish**

(A) The hESC (H9)-derived cells (day 9) were plated onto a human LN111-coated dish. At 15 min after plating, the unattached cells were removed.

(B) The hESC (H9)-derived cells (day 9) were plated onto a human LN111, 211, 411, or 511-coated dish, and then the cell number were counted on days 10, 12, 14, and 16. The cell number on day 10 was taken as 1.0. Data represent the mean  $\pm$  SD from three independent experiments. "LN111" was significantly different among four groups (LN111, 211, 411, and 511) on day 14 and 16 based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ( $p < 0.05$ ).

(legend continued on next page)



In order to examine whether the hESC-derived HBC P0 have the potential to proliferate clonally on various types of human laminins, single HBCs were plated on separate wells of a human LN111-coated 96-well plate at a low density (one cell per one well) (Table S1). Single cells that attached to the human LN111-coated dish were AFP positive and HNF4 $\alpha$  positive (Figure S2B). At 7 days after plating, the hESC-derived HBC colonies (albumin [ALB]- and cytokeratin 7 [CK7] double positive) (a representative colony is shown in Figure S2C) were efficiently generated from the hESC-derived HBC P0 on a LN111-coated dish. Taken together, these results showed that the hESC-derived HBCs could be generated from both the hESC-derived HBC P0 population and the single hESC-derived HBC P0.

#### The hPSC-Derived HBCs Could Differentiate into Both Hepatic and Biliary Lineages In Vitro

To examine whether the hESC-derived HBCs have the potential to differentiate into both hepatic and biliary lineages, first, these cells were differentiated into hepatocyte-like cells as described in Figure 4A. After 2 weeks of hepatic differentiation, almost all of the cells were polygonal in shape (Figure 4B) and were CYP3A4,  $\alpha$ AT, and ALB positive (Figure 4C). The gene expression levels of mature hepatocyte markers in the HBC P0-, HBC P10-, or HBC clone-derived hepatocyte-like cells were higher than those in the cells that had not undergone hepatic differentiation (Figure 4D), although the gene expression levels of mature cholangiocyte markers in these cells did not change (Figure 4E). The ASGR1-positive cells in the HBC P0-, HBC P10-, and HBC clone-derived population accounted for approximately 60%, 90%, and 90% of the total, respectively (Figure 4F). The HBC P0-, HBC P10-, or HBC clone-derived hepatocyte-like cells had the ability to produce ALB (Figure 4G, left) and urea (Figure 4G, right). Next, the hESC-derived HBCs were differentiated into cholangiocyte-like cells as described in Figure 4H. After 2 weeks of biliary differentiation, tubular structures (Fig-

ure 4I) that were CK7 positive (Figure 4J) were observed. Although the gene expression levels of mature hepatocyte markers (Figure 4K) in the HBC P0-, HBC P10-, or HBC clone-derived cholangiocyte-like cells did not change, the gene expression levels of mature cholangiocyte markers (Figure 4L) in these cells were higher than those in the cells that had not undergone differentiation. Similar results were obtained by using another hESC line (H1) and hiPSC line (Dotcom) (Figure S4). Moreover, HBC-derived hepatocyte-like cells exhibited CYP metabolism capacity (Figure S5A) and a functional urea cycle that could respond to ammonia (Figure S5B) and were considered to have potential to be applied in the prediction of drug-induced hepatotoxicity (Figure S5C). Taken together, these results indicated that the hPSC-derived HBCs have the ability to differentiate into both hepatic and biliary lineages in vitro.

#### In Vivo Cell Transplantation Assays of the hPSC-Derived HBCs

To examine whether the hESC-derived HBCs could be used for hepatocyte transplantation, these cells were transplanted into CCl<sub>4</sub>-treated immunodeficient mice as shown in Figure 5A. The hepatocyte functionality of the hESC-derived HBC P0 or HBC P10 was assessed by measuring secreted human ALB levels in the recipient mice (Figure 5B). Although human ALB was detected in the mice that were transplanted with the hESC-derived HBC P0 or HBC P10, it was not detected in the mice that were not transplanted with these cells. The ALB-positive cells were observed in mice transplanted with the hESC-derived HBC P0 or HBC P10 (Figure 5C). Most of the ALB-positive cells in mice transplanted with the hESC-derived HBC P10 were AFP negative (Figure 5D), indicating that transplanted hESC-derived HBCs were differentiated into mature hepatocyte-like cells (some of them were binuclear [Figure 5E, white arrows]). These results demonstrated that hESC-derived HBCs have the potential to be applied for hepatocyte transplantation.

(C) The hESC-derived cells (day 9) were plated onto a human LN111, 211, 411, or 511-coated dish. The percentage of AFP-positive cells was examined by using FACS analysis on day 9 (before passage and after passage [HBC P0]) or day 16. Data represent the mean  $\pm$  SD from three independent experiments.

(D) The hESC-derived cells cultured on a human LN111-coated dish for 7 days were subjected to immunostaining with anti-AFP (green) antibodies.

(E) The hESC-derived cells (day 9) were plated onto human LN111, 211, 411, or 511-coated dishes. The gene expression levels of *AFP* and pan-hepatoblast markers (*CD133*, *EpCAM*, *CK8*, and *CK18*) were measured by real-time RT-PCR on day 16. The gene expression levels in the hESC-derived HBCs (the LN111-attached cells were collected at 15 min after plating) were taken as 1.0.

(F) The HBCs derived from hESCs (H9) or hiPSCs (Dotcom) were cultured and cell growth was analyzed by obtaining a cell count at each passage. Data represent the mean  $\pm$  SD from three independent experiments.

(G) The percentage of AFP-positive cells was examined by using FACS analysis on day 9 (before passage), P1 (HBCs passaged once), P10 (HBCs passaged ten times), and P15 (HBCs passaged 15 times). Data represent the mean  $\pm$  SD from seven independent experiments.

See also Tables S2 and S3.