Inducible Lentivirus-Mediated Expression of the Oct4 Gene Affects Multilineage Differentiation of Adult Human Bone Marrow–Derived Mesenchymal Stem Cells

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Abstract

The octamer-binding transcription factor 4 (Oct4) gene plays an important role in maintaining the undifferentiated state of embryonic stem cells (ESCs) and reprogramming adult somatic cells into induced pluripotent stem cells (iPSCs). In the present study, we transduced human bone marrow–derived mesenchymal stem cells (hMSCs) using tetracycline-on (Tet-On) lentiviruses carrying human Oct4 to examine effects of regulated expression of human Oct4 on the proliferation and differentiation of hMSCs. hMSCs were efficiently transduced by Tet-On lentiviruses to express regulated levels of human Oct4 with doxycycline (Dox), as examined by immunofluorescent staining, flow cytometry, and quantitative real time-PCR (qRT-PCR) assays. Ectopic expression of Oct4 in transduced hMSCs increased the ability of colony formation. Continued expression of Oct4 further enhanced adipogenic differentiation of hMSCs, and transient expression of Oct4 sufficiently enhanced osteogenic differentiation of hMSCs. qRT-PCR analysis showed that ectopic expression of Oct4 in transduced hMSCs temporally increased the expression of Sox2 and c-Myc. Interestingly, ectopic expression of Oct4 reduced neuronal differentiation of hMSCs when incubated under neuronal differentiation conditions. Our results suggest that ectopic expression of human Oct4 leads to temporal changes in multilineage differentiation of hMSCs and may inhibit neuronal differentiation of hMSCs.

Introduction

The octamer-binding transcription factor 4 (Oct4) gene plays a critical role in the undifferentiated state and maintaining pluripotency of embryonic stem cells (ESCs) (Johansson and Simonsson, 2010; Young, 2011). In addition, Oct4 has recently been reported to play a key role in the reprogramming of terminally differentiated somatic cells into an ESC-like state, namely induced pluripotent stem cells (iPSCs) (Kim et al., 2009a; Niwa et al., 2000). Since the pioneering work by Takahashi and Yamanaka, showing that mouse fibroblasts were reprogrammed into iPSCs by introducing Oct4 together with other three pluripotent genes—Sox2, c-Myc, and Klf4—numerous studies have shown that iPSCs can be produced successfully from mouse and human somatic cells by introducing these four genes (Dimos et al., 2008; Lowry et al., 2008; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Because c-Myc and Klf4 are tumorigenic genes, several groups have optimized the Yamanaka protocol without the use of c-Myc or Klf4 (Kim et al., 2009b; Kim et al., 2008; Li et al., 2011; Nakagawa et al., 2008). Different combinations of pluripotent genes have been examined for generating iPSCs, for example, the use of a cocktail of Oct4 and Klf4 successfully induced mouse and human neural stem cells into iPSCs (Kim et al., 2008; Kim et al., 2009b). Li et al. generated iPSCs from mouse fibroblasts using a single transcription factor Oct4 combined with small molecules (Li et al., 2011). Furthermore, it has been demonstrated that overexpression of only Oct4 is enough to induce human neural stem cells into iPSCs (Kim et al., 2009a, b), suggesting that Oct4 plays a central role in reprogramming processes for cells. Oct4 products are essential for controlling early stages of mammalian embryogenesis and pluripotent properties of embryos (Scholer et al., 1989). Different levels of Oct4 proteins lead ESCs into different differentiation fates. For example, transient up-regulation of Oct4 expression in a group of cells in the inner cell mass (ICM) triggers their
determination into primitive endoderm and mesoderm (Jerabek et al., 2014; Pesce and Scholer, 2001). When ESCs were prepared from Oct4 knockout mice, levels of Oct4 expression were low in ESCS, inhibiting expression of naïve pluripotency genes and showing lack of differentiation (Radziszewska et al., 2013). In addition, Niwa et al. showed that increased levels of Oct4 expression led ESCS to differentiate into primitive endoderm and mesoderm, and repression of Oct4 expression resulted in a loss of ESCS pluripotency (Niwa et al., 2000). It has been shown that ESCs are stabilized at an undifferentiated stage by a group of transcription factors, for example, Oct4, Sox2, and Nanog, collaboratively inhibiting differentiation of ESCs to different lineages (Tay et al., 2008; Young, 2011). Overexpression of pluripotent factors in ESCs has been found to induce cell differentiation. For example, the overexpression of Oct4 led to mesodermal differentiation, and the overexpression of Sox2 triggered ESCs to differentiate into neuroectodermal lineages (Kopp et al., 2008; Niwa et al., 2000).

Bone marrow–derived mesenchymal stem cells (MSCs) hold great promise for regenerative medicine (Chang et al., 2014; English et al., 2010; Ohtaki et al., 2008; Zhao et al., 2002). MSCs possess two features—the ability to undergo sustained proliferation in vitro and the potential to differentiate into multilineage cells, including osteocytes, chondrocytes, and adipocytes (Bianco et al., 2008; English et al., 2010; Pittenger, 1999; Prockop, 1997). In addition, it has been reported that MSCs can trans-differentiate into neurons and astrocytes (Dezawa et al., 2004; Shen et al., 2011; Tropel et al., 2006; Zhao et al., 2002). It has been shown that only transient and low levels of Oct4 expression are observed in early passages of MSCs, but undetectable in the late passages of MSCs (Greco et al., 2007; Lengner et al., 2007; Tsai et al., 2012; Wei and Shen, 2011).

How ectopic expression of Oct4 can affect multilineage differentiation of MSCs needs to be examined further. Pochampally et al. reported that the Oct4 gene expressed in human MSCs (hMSCs) in serum-free medium conditions, and these cells had an increased ability for colony formation; they also expressed other genes similar to embryonic cells (Pochampally et al., 2004). Virus-mediated overexpression of Oct4 has been shown to enhance proliferation and multilineage differentiation potential in MSCs (Liu et al., 2009; Pittenger, 1999; Prockop, 1997). In addition, it has been reported that MSCs can trans-differentiate into neurons and astrocytes (Dezawa et al., 2004; Shen et al., 2011; Tropel et al., 2006; Zhao et al., 2002). It has been shown that only transient and low levels of Oct4 expression are observed in early passages of MSCs, but undetectable in the late passages of MSCs (Greco et al., 2007; Lengner et al., 2007; Tsai et al., 2012; Wei and Shen, 2011).

Recently, we developed an inducible lentivirus-mediated gene delivery system that can efficiently transduce hMSCs and tightly regulate transgene expression (Chen et al., 2014; Yang et al., 2013). In the present study, we generated inducible lentiviruses carrying human Oct4 and transduced hMSCs to address the following issues: (1) Efficiency and regulation of Oct4 expression in hMSCs; (2) effects of regulated expression of Oct4 on proliferation and differentiation of hMSCs; (3) effects of ectopic expression of Oct4 on the expression of Sox2 and c-Myc in hMSCs; and (4) effects of ectopic expression of Oct4 on neuronal differentiation of hMSCs.

**Materials and Methods**

In all in vitro experiments, at least three replicates per group were used in each experiment. At least three independent experiments were performed.

**Plasmids and virus vector production**

To produce ectopic expression of Oct4 and regulate Oct4 expression in hMSCs, a binary Tet-On lentivirus vector system was used to transduce hMSCs. We generated a transfer plasmid pNL-TRE/Pitt-hOct4-IRES-hGFP-AU3 on the basis of our previous transfer plasmid pNL-TRE/Pitt-hGDNF-IRES-hGFP-AU3 by removing a BamHI/SwaI fragment containing the human glial cell line–derived neurotrophic factor sequence and replacing it with a BamHI/SwaI fragment containing a human Oct4-coding sequence derived from pLVX-Oct4-ZsGreen-Puro, provided by Biovit Technologies (Shenzhen, China) (Yang et al., 2013). rtTA2S-M2plasmid pNL-CMV-IE-rtTA2S-M2-AU3 was also prepared. Three plasmids, the transfer or rtTA2S-M2 plasmid, pCMVdR8.74, and pMD2G/CS (provided by Dr. Didier Trono, Lausanne, Switzerland), were used for packaging lentivirus vectors. The lentivirus vectors were prepared by the transient transfection of 293T cells using the calcium phosphate precipitation method (Naldini et al., 1996), and then titered and stored in a deep freezer for further use. This binary Tet-On lentivirus vector system enables regulated expression of both the human recombinant OCT4 (hrOCT4) and humanized Renilla reniformis green fluorescent protein (hrGFP) genes.

**Cell culture and inducible lentivirus transduction**

hMSCs were kindly provided by Dr. Darwin J. Prockop (Center for Gene Therapy, Tulane University Health Sciences Center, New Orleans, LA, USA). These hMSCs have been strictly screened, cultured, characterized, and used in numerous studies (Lee et al., 2009; Prockop, 1997; Yang et al., 2013). hMSCs were maintained at 70% confluence with Minimum Essential Medium-α (α-MEM) including 16% fetal bovine serum (FBS), 0.1 mM GlutaMAX, and 0.1 mM Antibiotic-Antimycotic (all the reagents were purchased from Gibco, Grand Island, NY, USA). They were cultured in 75-mL tissue flasks at 37°C under an atmosphere of 5% CO2, iPSCs were purchased from CellAPY Company (CA4002106, Beijing, China) and cultured in PSCeasy hES/iPSc maintaining medium (CA1001500, CellAPY). The culture medium was changed daily.

For lentivirus transduction, hMSCs were seeded at a density of 4 × 10^4 cells per six-well plate and transduced with both pNL-TRE/Pitt-hOct4-IRES-hGFP-AU3 and pNL-CMV-IE-rtTA2S-M2-AU3 lentiviruses at a ratio of 1:2, supplemented with 10 μg/mL Polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 24 h (virally transduced hMSCs were denoted as vhMSCs). Virus particles were washed away with a replacement of new culture medium with or without doxycycline (Dox) (800 ng/mL) (vhMSCs + Dox or vhMSCs – Dox). To examine the efficiency of regulated transgene expression by a binary Tet-On lentivirus vectors, “Off-On” and “On-Off” treatment groups were designed. In the “On-Off” treatment group, vhMSCs were first incubated in the absence (Off) of Dox for 4 days, and then in the presence (On) of Dox for additional 4, 7, and 10 days. In the “On-Off” group, vhMSCs were first incubated in the presence (On) of Dox for 4 days, and then in the absence (Off) of Dox for additional 4, 7, and 10 days, vhMSCs were prepared for flow cytometry (FCM) analysis to determine the mean fluorescence intensity (MFI) units of hrGFP.
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**Colony-forming units**

hMSCs and vhMSCs were seeded at a concentration of 200 cells per 10-cm culture dish and cultured in a 37°C incubator with humidified 5% CO₂ for 14 days. Some cultures of vhMSCs were treated with Dox for 14 days. After rinsing with phosphate-buffered saline (PBS), 3.0% Crystal Violet (Sigma-Aldrich) in 100% methanol was added to culture dishes. After staining at room temperature for 10 min, culture dishes were washed with tap water until the background was clear. The number of colonies with a diameter of more than 1 mm was counted.

**Adipogenic differentiation**

hMSCs or vhMSCs were seeded in six-well plates at a density of 4×10⁴ cells per well. Culture medium was changed for fresh culture medium every 3 days before cells reached 100% confluence. Culture medium was then switched to complete adipogenic differentiation medium: x-MEM containing 16% FBS, 0.5 μM dexamethasone (Sigma), 0.5 μM isobutylmethylxanthine (Sigma-Aldrich), 50 μM indomethacin (Sigma-Aldrich), 0.1 mM GlutaMAX (Gibco), and 0.1 mM Antibiotic-Antimycotic (Gibco). At the same time, Dox was also added to the cultures of vhMSCs. vhMSCs were treated with Dox for either 4 or 10 days. The complete adipogenic differentiation medium was changed every 3 days. Three weeks after differentiation, the cells were rinsed with PBS and were fixed with 4% paraformaldehyde for 1 h at room temperature. After washing with 0.1 M PBS, Oil Red O (Sigma-Aldrich) was added to the wells, and cells were incubated for 20 min at room temperature. After rinsing with PBS, the stained lipid droplets were observed using an inverted microscope (Nikon, Japan). Oil Red O dye was eluted by 100% isopropanol for 10 min by gently shaking. Eluted Oil Red O dye solution was measured at 500-nm wavelengths for quantitative analysis.

**Osteogenic differentiation**

hMSCs or vhMSCs were seeded in six-well plates at a density of 4×10⁴ cells per well. After hMSCs reached 100% confluence, culture medium was switched to complete osteogenic differentiation medium: x-MEM containing 16% FBS, 0.1 mM GlutaMAX (Gibco), and 0.1 mM Antibiotic-Antimycotic (Gibco), 1 mM dexamethasone (Sigma-Aldrich), 20 mM β-glycerolphosphate (Sigma-Aldrich), and 50 μg/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich). At the same time, Dox was also added to the cultures of vhMSCs. vhMSCs were treated with Dox for either 4 or 10 days. Cells were cultured for 3 weeks, and medium was changed every 3 days. After rinsing with PBS and fixing with 4% paraformaldehyde for 1 h at room temperature, Alizarin Red S (Sigma-Aldrich) was added to the wells for 20 min at room temperature. The cells were observed with an inverted microscope. Alierzin Red S–stained mineralization areas of the extracellular matrix (ECM) were analyzed using ImageJ, and the percentage of Alizarin Red S–stained mineralization areas of ECM over the total area in the cultures was calculated.

**Western blot**

Cells were washed three times with cold PBS and collected by scraping with RIPA buffer containing a protein inhibitor on ice. The suspension was then centrifuged at 12,000×g for 10 min. Quantified protein lysates (equal amount of 30 μg) were subjected to 10% sodium dodecylsulfate (SDS)-polyacrylamide gels, and then transferred onto a polyvinylidene difluoride membrane. After blocked in 5% nonfat milk in Tris-HCl–buffered saline (TBS), the membranes were incubated overnight at 4°C with primary antibodies against Oct4 (1:1000, Abcam, Cambridge, MA, USA), Sox2 (1:1000, Cell Signaling Technology, Danvers, MA, USA), c-Myc (1:1000, Cell Signaling Technology), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:3000, Cell Signaling Technology). The membranes were then washed three times and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature with shaking at a speed of 50 rotations per minute (rpm). Membranes were washed three times with TBS + Tween at room temperature. Protein bands were visualized on X-ray film. GAPDH was used as a loading control.

**Immunofluorescent staining**

Cells were cultured on poly-d-lysine– (10 μg/mL; Trevigen, Gaithersburg, MD, USA) coated coverslips in 24-well culture plates, fixed with 4% paraformaldehyde for 20 min at room temperature, and washed with PBS three times. Cells were then permeabilized for 20 min using 0.3% Triton in PBS and later washed with PBS for three times. Non-specific antibody binding was blocked with 10% normal goat serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) containing 0.3% Triton in PBS for 1 h at room temperature. Cells were then rinsed and incubated in primary antibody containing 5% normal goat serum and PBS containing 0.15% Triton overnight at 4°C. After washing in PBS, cells were incubated with secondary antibody containing 5% normal goat serum for 1 h at room temperature. Cells were stained with the following primary antibodies: Monoclonal rabbit anti-Oct4 (1:200, Abcam) and rabbit anti-Tuj1 (1:300, Chemicon, Billerica, MA, USA). Coverslips with immunostained cells were mounted on glass slides in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). For cell counting, stained cells were counted randomly from five fields in a coverslip using a Nikon Eclipse inverted fluorescence microscope (ECLIPSE TE2000-s, Japan) at 200× magnifications. At least three coverslips from each treatment group were used for cell counts.

**Flow cytometry**

Cells in cultures were digested with 0.25% trypsin/EDTA (Sigma-Aldrich) and centrifuged at 1000 rpm for 5 min; the supernatant was then removed. The cells were washed twice with PBS and adjusted to a concentration of 1×10⁶ cells/mL for FCM analysis to determine the MFI units for hrGFP⁺ or hrGFP⁺ cell counts.

**RT-PCR and quantitative real-time-PCR**

Total RNA from cultured cells was isolated using TRIzol (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. Five micrograms of total RNA
was treated with DNase I to remove potential contamination of genomic DNA (DNA Free RNA kit, TIANGEN, Beijing, China). The reverse transcription was carried out with the cDNA synthesis kit (First strand Synthesis kit, TIANGEN).

RT-PCR. For RT-PCR analysis to detect the transgenic gene Oct4, using the cDNA synthesized above, cycling parameters were as follows: Denaturation at 95°C for 2 min, annealing at 60°C for 10 sec, and elongation at 72°C for 1 min. The number of cycles was 35. Primer sequences used for amplification were as follows: Oct4 forward primer 5'-GGAAGGTATTCAGCCAACG-3' and reverse primer 5'-AAACCACACTCGGACCAACATC-3'. To ensure equal loading of cDNA into RT-PCR reactions, we amplified GAPDH mRNA using the following: Forward primer 5'-AGAAGGCTGGGGCTCATTT-3' and reverse primer, 5'-AGGGGGC CATCCACAGTCTC-3'.

Quantitative real-time PCR. The analysis was performed in a Roche 280 with Super Real PreMIX with ROX (TIANGEN). Cycling parameters were as follows: Pre-denaturing at 95°C for 15 min, denaturing at 95°C for 10 sec, annealing at 60°C for 20 sec, and elongation at 72°C for 30 min. The number of cycles was 40. The sequences of primers were as follows: Oct4, forward primer 5'-TGA GAGGCAAACCTGGAGAAAT-3' and reverse primer 5'- ACCACACTCGGACCAACATC-3'; Sox2, forward primer 5'-GCGAACCATCTCTGTGGTCT-3' and reverse primer 5'-TACCACAGGGTGCTAACCCTG-3'; c-Myc, forward primer 5'-CCTCACAATCCGGAAGGACTATC-3' and reverse primer 5'-TTCCCTTCTTGACATTCTCC-3'; hrGFP forward primer 5'-GGCAGAGATGAGCCCAAGAT-3' and reverse primer 5'-GGGGCTVATTTG-3'. To ensure equal loading of cDNA into qRT-PCR reactions, we amplified GAPDH mRNA using the following: Forward primer 5'-CAGGAGGCAATTGCTGTATGAT-3' and reverse primer 5'-GAAGACTGCTGCTTTGCTCTT-3'.

Neuronal differentiation

After vhMSCs were cultured in normal culture medium for 4 days, the culture medium was switched to neural induction medium: Neural basal medium (Gibco) containing 50×B27 (Gibco), basic fibroblast growth factor (bFGF), 50 ng/mL; Gibco), FGF8 100 ng/mL (R&D Systems, Minneapolis, MN, USA), and SHH250 ng/mL (R&D Systems). At the same time, Dox was also added to some cultures of vhMSCs. vhMSCs were treated with Dox for 10 days. After cells were cultured for 10 days, brain-derived neurotrophic factor (BDNF; 50 ng/mL, R&D Systems) was added to the cultures. Cells were cultured for an additional 2 days (total of 12 days from initial induction) (Trzaska and Rameshwar, 2011).

Statistical analysis

All data were presented as mean±standard deviation (SD) and subjected to statistical analysis using GraphPad Prism software (Version 6.0, La Jolla, CA, USA). Student’s t-test was used for group comparisons between two groups. When there were more than two groups, a one-factor analysis of variance (ANOVA) followed by Tukey’s post hoc test was used for comparisons among groups. Statistical significance was defined at $p<0.05$.

**Results**

**Characterization of hMSCs**

Passage 8 hMSCs exhibited spindle-like morphology in the growth medium (Fig. 1a, b). To examine the multilineage differentiation potential of hMSCs, hMSCs were induced into adipocytes and osteoblasts in specific differentiation medium. The cells were cultured in adipogenic or osteogenic differentiation medium and induced to

![FIG. 1. Differentiation and gene expression of hMSCs](image-url)
Effects of OCT4 overexpression on hMSCs

differentiate into adipocytes and osteoblasts, respectively. hMSCs were positive for Oil Red O and Alizarin Red S staining 21 days after either adipogenic or osteogenic differentiation (Fig. 1c, e), suggesting that hMSCs could differentiate into adipocytes and osteoblasts. The cells were negative for both Oil Red O and Alizarin Red S staining after they were cultured in the normal growth medium as a negative control (Fig. 1d, f).

To determine whether OCT4 is expressed in hMSCs, western blotting was conducted in hMSCs in the growth medium. In addition, both Sox2 and c-Myc were also examined in hMSCs. Because these genes were strongly expressed in iPSCs, iPSCs were used as positive controls (Kim et al., 2009b; Shu et al., 2013; Takahashi and Yamanaka, 2006; Yu et al., 2007). There was virtually no expression of OCT4 and Sox2 in hMSCs (Fig. 1g). Only low levels of c-Myc proteins were observed in hMSCs (Fig.1g). In contrast, OCT4, Sox2, and c-Myc were markedly expressed in iPSCs. These results suggest that passage 8 hMSCs retain stem cell properties and do not express OCT4.

Ectopic expression of OCT4 and regulation of OCT4 expression in hMSCs transduced with Tet-On lentiviruses carrying human OCT4

After hMSCs were co-transduced with pNL-TRE-Pitt-OCT4-ires-hrGFP (Fig. 2a) and pNL-CMV-tTA2s-M2 (Fig. 2b) lentiviruses, they were incubated with Dox for 4 days. Immunofluorescent staining showed that numerous hrMSCs were OCT4-positive in nuclei (Fig. 2c–g). Most OCT4-positive cells also showed co-localization with green fluorescent protein (Fig. 2e, f). The percentage of OCT4-positive cells was estimated as 46.43 ± 5.50 in the vhMSCs + Dox cultures (p < 0.05 versus vhMSCs – Dox group, Student’s t-test) (Fig. 2g). Because the expression of both OCT4 and hrGFP was controlled under the same Tet-On–regulated promoter, hrGFP-positive cells were examined to indirectly reflect OCT4 expression using FCM. The results showed the percentage of hrGFP-positive cells was 50.06 ± 6.80 in the vhMSCs + Dox cultures, coinciding with the rate of OCT4-positive cells in the same cultures (p < 0.05 versus vhMSCs – Dox and hMSCs groups, respectively; a one-factor ANOVA followed by Tukey’s post hoc test) (Fig. 2m). Western blot analysis confirmed the expression of OCT4 and hrGFP in the vhMSCs cultures. The vhMSCs were incubated in the presence of Dox at serial doses ranging from 0 to 10^3 ng/mL. FCM analysis of MFI units for hrGFP confirmed these temporal changes in hrGFP transgene expression for the vhMSCs cultures (Fig. 3i–j). For the “Off–On”–treated cultures (Fig. 3i), no MFI units were observed in the vhMSCs cultures 4 days in vitro in the absence of Dox at the beginning of the experiment. After the addition (On) of Dox, MFI units were increased to 36.99 ± 5.02 at 4 days, 37.65 ± 1.11 at 7 days, and 37.08 ± 1.14 at 10 days. For the “On–Off”–treated cultures (Fig. 3j), MFI units for hrGFP were observed in the vhMSCs cultures 4 days in vitro in the presence (On) of Dox were 39.04 ± 2.40. After the removal (Off) of Dox, MFI units were reduced to 25.24 ± 2.13 at 4 days, 7.22 ± 2.58 at 7 days, and 5.34 ± 1.29 at 10 days.

We then examined the induction of transgene expression in the vhMSCs cultures. The vhMSCs were incubated in the presence of Dox at serial doses ranging from 0 to 10^4 ng/mL. FCM analysis showed that MFI units for hrGFP were increased in a Dox dose-dependent manner for vhMSCs (Fig. 3k).

Effects of ectopic expression of OCT4 on colony formation in vhMSCs

In addition to the ability for multilineage differentiation potential of hMSCs, the cells possess the ability of colony formation, which is one of the characteristics of stem cells (Simmons and Torok-Storb, 1991). Colony-forming unit (CFU) analysis showed that the number of colonies in the vhMSCs + Dox cultures was significantly greater (55.17 ± 4.54 colonies, >1 mm diameter in size) than that in the vhMSCs – Dox cultures (44.5 ± 4.37) 14 days in the normal growth medium (p < 0.05, Student’s t-test) (Fig. 4a–c). For the hMSCs cultures, there was no significant difference in colony formation between the cultures in the presence and absence of Dox (data was not shown). These results suggest that ectopic expression of OCT4 facilitates colony formation in vhMSCs + Dox cultures, and Dox per se does not affect colony formation.
FIG. 2. Regulated expression of Oct4 in hMSCs transduced with inducible lentivirus vectors carrying human Oct4. (a) Structure of a Tet-On inducible lentivirus vector carrying a TRE/Pit-hOct4/hrGFP expression cassette. (b) Structure of a transactivator-encoding lentivirus vector, carrying a CMV-IE-rTA2S-M2 expression cassette, hOCT4. (c–i) Oct 4 immunofluorescent staining in virus-transduced hMSCs 4 days in vitro with (c–g) or without (h–i) Dox (800 ng/mL). (c, h) Oct4 staining; (d, i) DAPI; (e, j) hrGFP; (f, k) merge. (g) Percentage of Oct4-positive cells in cultures. (*) p < 0.05 versus vhMSCs-Dox group (Student’s t-test). (i) Phase-contrast photomicrograph prepared from the same fields as h–k. Scale bar, 50 μm. (m–p) FCM analysis of hrGFP-positive cells in different cultures of vhMSCs + Dox (m), vhMSCs − Dox (n), and hMSCs (o) 4 days in vitro. (g) Percentage of hrGFP-positive cells in cultures. (*) p < 0.05 versus vhMSCs-Dox and hMSCs groups, respectively (a one-factor ANOVA followed by Tukey’s post hoc test). (q, r) Reverse transcription PCR showing Oct4 mRNA expression in different cultures of vhMSCs + Dox, vhMSCs − Dox, and hMSCs − Dox 4 days in vitro. (r) Quantitative data of Oct4 mRNA expression. (*) p < 0.05 versus other three groups (a one-factor ANOVA followed by Tukey’s post hoc test). (s, t) Western blot analysis of Oct4 protein in different cultures of vhMSCs + Dox, vhMSCs − Dox, hMSCs + Dox, and hMSCs − Dox 4 days in vitro. (t) Quantitative data of Oct4 protein. (*) p < 0.05 versus other three groups (a one-factor ANOVA followed by Tukey’s post hoc test). (u) Quantitative real-time PCR analysis of mRNA levels of hrGFP and Oct4 in vhMSCs cultures with or without Dox (800 ng/mL) 4 days in vitro.
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vhMSCs + Dox (with Dox for 4 or 10 days), vhMSCs – Dox, and hMSCs were cultured in adipogenic differentiation medium and induced to differentiate into adipocytes (Fig. 5a–d). All of the cells were positive for Oil Red O staining after 21 days of cell culture in the adipogenic differentiation medium, suggesting that the cells could differentiate into adipocytes. hMSCs were negative for Oil Red O staining after they were cultured in the normal growth medium as negative controls (Fig. 5e). The optical density (OD) value of Oil Red O staining was quantified (Fig. 5f). The results showed that the OD value of vhMSCs after 10 days of incubation with Dox was significantly higher than that of vhMSCs without Dox.

FIG. 3. Dynamic expression of Oct4 in vhMSCs cultures with or without Dox (800 ng/mL). (a–d) Confocal microscopic images were prepared from the “Off-On” treatment group in which vhMSCs were first incubated in the absence of Dox (a) and then adding Dox for 4 days (b), 7 days (c), and 10 days (d) and examined using an inverted fluorescence microscope. (e–h) Confocal microscopic images were prepared from the “On-Off” treatment group in which transduced vhMSCs were first incubated in the presence of Dox (800 ng/ml) for 4 days (e) and then in the absence of Dox for another 4 days (f), 7 days (g), and 10 days (h) examined using an inverted fluorescence microscope. Scale bar, 100 μm. Flow cytometry analysis showing dynamic changes in the mean fluorescence intensity (MFI) units for hrGFP in the “Off-On” (i) and “On-Off” (j) groups at different times. (k) Dox dose-dependent mean fluorescence intensities (MFI) of hrGFP were examined by flow cytometry for vhMSCs cultures with doses of Dox ranging from 0, 100, 200, 400, 800, to 1000 ng/mL.

FIG. 4. Effects of overexpression of Oct4 on colony formation in hMSCs. (a) The number of CFU in vhMSCs in the presence of Dox (800 ng/mL) 14 days in vitro. (b) The number of CFU in vhMSCs in the absence of Dox 14 days in vitro. (c) Statistical analysis of the number of CFU in the vhMSCs + Dox and vhMSCs – Dox cultures. (*) p < 0.05 versus the vhMSCs – Dox cultures (Student’s t-test).
treatment of Dox was greater (28.27 ± 4.09) than that in other three types of cultures: vhMSCs + Dox 4 days after treatment of Dox (21.10 ± 2.68), vhMSCs – Dox (9.93 ± 0.99), and hMSCs (9.83 ± 1.49) (*p < 0.05 versus vhMSCs + Dox 4 days, vhMSCs – Dox, and hMSCs cultures, respectively, one-factor ANOVA followed by Tukey’s post hoc test). In addition, the OD value of vhMSCs + Dox 4 days after treatment of Dox was greater than that in other three types of cultures: vhMSCs + Dox 10 days after treatment of Dox, vhMSCs – Dox, and hMSCs cultures, respectively, a one-factor ANOVA followed by Tukey’s post hoc test). No difference was observed in the OD values between the vhMSCs and hMSC cultures, suggesting that virus transduction appeared not to affect the ability of differentiation of hMSCs. These results suggest that ectopic expression of Oct4 can enhance the ability of differentiation of hMSCs into adipocytes, and this effect is further increased following continued expression of Oct4.

FIG. 5. Adipogenic differentiation of hMSCs in adipogenic differentiation medium 21 days after culture as examined by Oil Red O staining. (a–e) Photomicrographs of the vhMSCs in the presence of Dox (800 ng/mL) for 10 days (a) and 4 days (b), in the absence of Dox (c), and hMSCs (d). (e) hMSCs in the growth medium as controls. Scale bars, a–e, 100 μm. (f) Statistical analysis for OD value of Oil Red O. (* ,# ) p < 0.05 versus other three groups (a one-factor ANOVA followed by Tukey’s post hoc test).
into osteoblasts, and this effect is not further increased following continued expression of Oct4.

**Effects of ectopic expression of Oct4 on the expression of Sox2 and c-Myc in hMSCs**

The expression of Oct4, Sox2, and c-Myc was examined by qRT-PCR analysis in the hMSCs, vhMSCs – Dox, vhMSCs + Dox 4-day, and vhMSCs + Dox 10-day cultures (Fig. 7a–c). The levels of Oct4 mRNA were not detected in hMSC and vhMSCs – Dox cultures. The levels of Oct4 mRNA were detected in the vhMSCs + Dox 4-day cultures, and were normalized as 1. The levels of Oct4 mRNA were further increased to 1.99 ± 0.03-fold in vhMSCs + Dox 10-day cultures when compared with vhMSCs + Dox 4-day cultures (Fig. 7a) (*p < 0.05 versus the vhMSCs + Dox 4-day cultures, Student’s *t*-test). Levels of Sox2 mRNA were not detected in the hMSCs and vhMSCs cultures. Levels of Sox2 mRNA were detected in the vhMSCs + Dox 4-day cultures and were normalized as 1. In contrast, levels of Sox2 mRNA were reduced to 0.57 ± 0.14-fold in the vhMSCs + Dox 10-day cultures when compared with the vhMSCs + Dox 4-day cultures (Fig. 7b) (*p < 0.05 versus the vhMSCs + Dox 4-day cultures, Student’s *t*-test). There were very low levels of c-Myc mRNA in the hMSCs cultures (0.06 ± 0.06-fold). Levels of c-Myc mRNA were undetectable in the vhMSCs – Dox cultures. Levels of c-Myc mRNA were increased in the vhMSCs + Dox 4-day cultures and were normalized as 1. However, levels of c-Myc mRNA were reduced to a baseline in the vhMSCs + Dox 10-day cultures (0.11 ± 0.03-fold).
Effects of ectopic expression of Oct4 on neuronal differentiation of hMSCs.

Immunofluorescent staining showed that numerous vhMSCs were induced to differentiate into cells with neural marker Tuj1 in the presence (Fig. 7d–g) or absence (Fig. 7h–k) of Dox after the cells were incubated in neuronal differentiation medium for 12 days. Most of Tuj1-positive cells were not co-localized with hrGFP-expressing cells. The percentage of Tuj1-positive cells was significantly less in the vhMSCs + Dox cultures than that in the vhMSCs − Dox cultures (Fig. 7l) (*p < 0.05, Student’s t-test).

Discussion

Our study examined, for the first time, the effects of temporal expression of human Oct4 on multilineage differentiation of hMSCs. We developed a cellular system in...
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which the expression of human Oct4 was regulated in hMSCs transduced with Tet-On lentiviruses carrying human Oct4. The results demonstrate that inducible lentiviruses carrying human Oct4 can efficiently transduce hMSCs, leading to regulated expression of Oct4 and hrGFP in the presence or absence of Dox. The background expression of the two genes in transduced hMSCs is very low in the absence of Dox. Ectopic expression of Oct4 in transduced hMSCs can enhance proliferation of hMSCs. Adipogenic differentiation of hMSCs is further enhanced following longer ectopic expression of Oct4, but increased osteogenic differentiation of hMSCs is not further enhanced following longer ectopic expression of Oct4. In contrast, ectopic expression of Oct4 in transduced hMSCs reduces neuronal differentiation of hMSCs. In addition, the temporal expression of human Oct4 resulted in transient expression of the other genes, Sox2 and c-Myc, in hMSCs.

It is known that the expression of Oct4 plays a critical role in maintaining pluripotency of ESCs and reprogramming of somatic cells into iPSCs (Johansson and Simonson, 2010; Kim et al., 2009a, b; Niwa et al., 2000; Young, 2011). It is also known that regulation of Oct4 expression is involved in cell proliferation and differentiation (Jerabek et al., 2014; Pesce and Scholer, 2001; Radziszewskaya et al., 2013). Therefore, it is important to examine effects of temporal expression of Oct4 on the properties of stem cells. Several groups have examined the effects of overexpression of Oct4 on proliferation and differentiation of hMSCs using virus-mediated gene transfer approaches (Liu et al., 2009; Palma et al., 2013; Tsai et al., 2012). In addition, Hou et al. reported that small-molecule compounds could stimulate Oct4 expression and therefore induce mouse somatic cells into iPSCs (Hou et al., 2011), avoiding the use of viruses. However, consistent overexpression of Oct4 in stem cells represents a limitation for study of the biological function of Oct4. In agreement with our previous results, the use of a similar binary Tet-On gene delivery system carrying human Oct4 leads to tightly regulated expression of human Oct4 with very low levels of background transgene expression (Chen et al., 2014; Yang et al., 2013). The results of dynamic transgene expression using Dox here suggest that gene transfer with the binary Tet-On gene delivery system is efficient and inducible.

It has been demonstrated that overexpression of Oct4 can increase proliferation and multilineage differentiation potential of hMSCs (Liu et al., 2009; Palma et al., 2013; Pochampally et al., 2004; Tsai et al., 2012). Our study provides supportive evidence for this point using CFU analysis and adipogenic and osteogenic differentiation assays. In the CFU assay, ectopic expression of Oct4 can enhance colony formation and the ability of self-renewal, one of the characteristics of hMSCs. This result was consistent with the previous studies with the same enhancement of CFU (Liu et al., 2009; Pochampally et al., 2004). Pochampally et al. also demonstrated that hMSCs with up-regulation of Oct4 expression in serum-free medium conditions were more clonogenic than hMSCs in normal growth medium (Pochampally et al., 2004). In our study, no difference was observed in CFU for hMSCs with or without Dox treatment, suggesting Dox does not clearly affect clonogenic ability of hMSCs. Interestingly, we observed that the differentiation pattern of adipogenesis differed from that of osteogenesis in the presence of Dox. Induced adipogenesis was further increased following overexpression of Oct4 in hMSCs in the presence of Dox. Although both 4- and 10-day overexpression of Oct4 increased osteogenesis of hMSCs, prolonged overexpression of Oct4 for 10 days did not further increase osteogenesis in hMSCs.

There is a close interaction between Oct4 and Sox2 (Boyer et al., 2006; Lee et al., 2006). It has been shown that Oct4 can form a heterodimer with Sox2, binding to a DNA regulatory site to promote ESCs self-renewal (Rodd et al., 2005). Sox2 is a member of SOXB1 (together with Sox1 and Sox3) required for the maintenance of the early embryos before implantation. Sox2 is expressed in the neuroepithelium and plays an important role in the development of the central nervous system (Avilion et al., 2003; Kim et al., 2009a). In addition, Sox2 plays a critical role in the conversion of somatic cells into iPSCs (Takahashi and Yamanaka, 2006). c-Myc is a regulatory gene associated with cell cycle, apoptosis, and cellular transformation (Qu et al., 2014; Sabo et al., 2014). Overexpression of c-Myc could lead to tumor formation (Chen et al., 2014). c-Myc, together with Oct4, Sox2, and Klf4, has been used recently in induction of somatic cells into iPSCs (Takahashi and Yamanaka, 2006). In the present study, a time-dependent increase in the levels of Oct4 expression in transduced hMSCs with Dox led only to a transient up-regulation of Sox2 expression and then reduced levels of Sox2 expression following extended expression of Oct4. A similar pattern in the expression of c-Myc was observed in hMSCs with Dox. These results suggest that ectopic expression of Oct4 can cause transient expression of endogenous Sox2 and c-Myc in hMSCs. Because overexpression of Oct4 facilitates hMSCs to differentiate into mesoderm, consistent overexpression of Oct4 may inhibit the expression of other genes associated with differentiation of ectoderm. Our results are consistent with previous studies that ectopic expression of Oct4 results in up-regulation of Sox2 and c-Myc expression (Greco et al., 2007).

It has been demonstrated that overexpression of pluripotent factors in ESCs can induce cell differentiation (Niwa et al., 2000). The overexpression of Oct4 led to mesodermal differentiation, and the overexpression of Sox2 triggered ESCs to differentiate into the neuronal lineage (Kopp et al., 2008; Niwa et al., 2000). In the present study, ectopic expression of Oct4 reduced neuronal induction of hMSCs when the cells were incubated in neuronal differentiation medium. These results suggest that ectopic expression of Oct4 favors the maintenance of hMSCs in mesodermal lineage.

In summary, our system enables examination of the effects of temporal expression of Oct4 on the multilineage differentiation of hMSCs and the expression of Sox2 and c-Myc in hMSCs. The ectopic expression of Oct4 in hMSCs enhances proliferation and differentiation of hMSCs into mesoderm lineages (adipogenesis and osteogenesis) and reduces neuronal differentiation of hMSCs.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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AU1: the nomenclature of genes as italic and proteins as roman has been used throughout. Thus, it is not always necessary to indicate “the Oct 4 gene” since using the italic format Oct4 indicates that the gene is meant.

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AU3: —am assuming you mean human mesenchymal stem cells as described in the text and not human mesencephalic stem cells, which has been deleted here. If not, then please reinstate and delete abbreviation.