Biochemical and Biophysical Research Communications 426 (2012) 468–474

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Establishment of adipose-derived mesenchymal stem cell lines from a *p53*-knockout mouse

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ARTICLE INFO

Article history:
Received 17 August 2012
Available online 6 September 2012

Keywords:
AD-MSC
Cell line
p53-knockout mouse

ABSTRACT

Mesenchymal stem cells (MSCs) can differentiate into a variety of cell types. MSCs exist in several tissues such as the bone marrow, adipose, muscle, cartilage, and tendon. This differentiation potential makes MSCs candidates for cell-based therapeutic strategies for mesenchymal tissue injuries. MSCs can be prepared from bone marrow (BM-MSCs) and adipose (AD-MSCs); however, these MSCs exhibit senescence-associated growth arrest and display inevitable heterogeneity. We established several AD-MSC cell lines from a *p53*-knockout (KO) mouse. These cell lines were immortalized, but no cell lines grew anchorage-independently, suggesting that they are not cancerous. They differentiated into adipocytes, osteoblasts, and chondrocytes by treatment with certain stimuli. Moreover, following injection into the tail vein, the cells migrated into the wounded region of the liver and differentiated into hepatocytes. We succeeded in establishing several AD-MSC clonal cell lines that maintain the tissue-specific markers and characteristics of the developmental phase. These clonal cell lines will serve as important tools to study the mechanism of differentiation of MSCs.

1. Introduction

MSCs exist in several adult mesodermally derived tissues such as the bone marrow, adipose, muscle, cartilage, tendon, and the periosteum [1], and they exist in such tissues of the developing embryo [2,3]. MSCs have multilineage potential and possess the ability to differentiate into nerve cells and β-cells, which are not mesodermally derived cells [4–7], thus making MSCs a potential source of regenerative tissues or organs.

One of the important problems of studying MSCs is that preparation of a sufficient number of MSCs is very laborious and time-consuming. Although BM-MSCs and AD-MSCs are contained in the bone marrow and in the stromal vascular fraction at concentrations of about 0.001–0.002 and 1%, respectively [8], they cause arrest at 38 divisions [9]. Another problem is that MSCs are a mixture of cells at different stages of differentiation, and most stop differentiation at the early phase [10,11]. Thus, to improve this situation, several BM-MSC cell lines have been established [11–13].

We hypothesized that AD-MSC cell lines could be established from the *p53*-KO mouse, because several clones from nerve [14], female reproductive tract [15], and oral [16,17] tissues have been already established. All of these cell lines maintain the tissue-specific markers and characteristics of the developmental phase. In the present study, we successfully established AD-MSC cell lines from *p53*-KO mouse adipose cells. They differentiated into adipocytes, osteoblasts, and chondrocytes *in vitro* by treatment with stimuli. We also succeeded in making the AD-MSC cell lines differentiate into hepatocytes in the liver that had been wounded by their injection into the tail vein of the mouse.

2. Materials and methods

Our gene and animal studies were approved by the Tohoku University recombinant DNA committee and Tohoku University Animal use and care committee (ID76-20-120).

2.1. Animals

p53-KO mice were first generated by Livingstone et al. (1992), and were provided for use in the current study by DuPont Central & Research Development (Wilmington, DE) [18]. They were kept under a 12:12 h light:dark cycle at 22–24 °C. Standard laboratory

Abbreviations: MSC, mesenchymal stem cell; AD-MSC, adipose-derived MSC; BM-MSC, bone marrow-derived MSC; KO, knockout; RT-PCR, reverse transcriptase-polymerase chain reaction; PPARγ, peroxisome proliferator activated receptor gamma; GFP, green fluorescent protein; aP2, fatty acid binding protein.

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http://dx.doi.org/10.1016/j.bbrc.2012.08.094

feed (MR standard, Nousan Ltd., Yokohama, Japan) and tap water were given *ad libitum*. Mice care and handling conformed to the HIH guidelines for animal research.

2.2. Cloning and cell culture

The inguinal fat pads were harvested from a 15-week-old *p53*-KO mouse and extensively washed with phosphate buffered saline [PBS(−)]. They were then minced into pieces and treated with 0.075% type I collagenase (Worthington Biochemical Co., Lakewood, NJ) by vigorous shaking for 30 min at 37 °C. An equal volume of culture medium (Dulbecco's modified Eagle's medium high glucose [D-MEM high glucose]; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum (10% FBS; Life Technologies, Carlsbad, CA), penicillin/streptomycin (Wako), 10 μg/ml insulin (Wako), and 10 μg/ml transferrin (Wako), and 10 μM forskolin (Wako) was added to inactivate the collagenase activity. The reconstituted cell suspension was centrifuged at 1800 rpm (630×*g*) for 3 min, and the pellet was suspended in the culture medium. These cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, maintained in culture medium, and passaged at 10% on culture dishes (FALCON; Becton Dickinson Labware, Franklin Lakes, NJ) (AD-MSC bulk line). Cells were cloned from the bulk line by limited dilution, as previously described [15]. Seventeen clonal cell lines with distinct morphology were established and named #1–#17. The cell lines were maintained using the same method employed for the bulk line.

2.3. GFP infection with a lentiviral vector

HEK293T cells were transiently transfected with pCDH-CMV-MCS-EF1-copGFP, pCMV-VSVG-RSV-REV, and pCAG-HIV-gp by using Lipofectamine™ 2000 (Life Technologies). After 48 h, the transfection solution was added to each AD-MSC line cultured in 24-well plates.

2.4. Soft agar assay

A total of 5×10^3 cells were suspended in 1 ml of 0.35% agarose (FMC Bio Products, Rockland, ME), and seeded onto 60 mm cell culture dishes (FALCON; Becton Dickinson Labware, Franklin Lakes, NJ) that were coated with 0.7% agarose. After 21 days, the colonies were observed.

2.5. Immunochemical staining

Sections were processed for immunohistochemistry as previously described [19]. Cells were fixed in 2% paraformaldehyde (PFA; Sigma, St. Louis, MO)/PBS for 15 min at 4 °C. After washing with PBS(−) for 10 min at 4 °C, cells were treated with 0.05% BSA (Takara, Kyoto, Japan) for 5 min at 25 °C and were blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA)/PBS(−) for 30 min at 25 °C. The primary antibodies used were anti-endoglin (CD105) (1:300; Santa Cruz Biotechnology, Santa Cruz, CA), anti-collagen type II (1:300; Abcam, Boston, MA), and anti-tyrosine aminotransferase (1:300; Santa Cruz Biotechnology) mouse monoclonal antibodies or anti-GFP (1:500; Santa Cruz Biotechnology) rabbit polyclonal antibodies for 15 h at 4 °C. After several washes with PBS(−), cells were incubated with secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG serum (1:500; Life Technologies), Texas Red-conjugated anti-mouse IgG serum (1:500; Life Technologies), and FITC-conjugated anti-rabbit IgG serum (1:500; Life Technologies) for 1.5 h at 25 °C. Nuclei were stained with DAPI.

2.6. RNA isolation and RT-PCR analysis

Total RNA was extracted from each cell line using Trizol (Life Technologies), and reverse transcription was performed by means of RiverTraAce (TOYOBO, Tokyo, Japan) according to the vendor's instructions. The first strand cDNA was used as a template for polymerase chain-reaction (PCR) with the following primer sets: *β-actin* (25 cycles: sense, 5′-CAAGGACAACCGCGAGAAGA-3′; antisense, 5′-GCACTGTGTTGGCGTACAGGT-3′), *CD105* (25 cycles: sense, 5′-CCAGCATTGTCACCTCCTTT-3′; antisense, 5′-GACACGCTCACCTGTACGAA-3′), *CD29* (25 cycles: sense, 5′-GGACAGGAGAAAATGGACGA-3′; antisense, 5′-GCATTCACAAACACGACACC-3′), *CD44* (25 cycles: sense, 5′-GGCGCTAAAGATGCAAGAAG-3′; antisense, 5′-AGCTTTTTCTTCTGCCCACA-3′), PPARγ (30 cycles: sense, 5′-CCCTGGCAAAGCATTTGTAT-3′; antisense, 5′-AATCCTTGGCCCTCTGAGAT-3′), *aP2* (30 cycles: sense, 5′-AAAGTGGCAGGCATGGCCAAGC-3′; antisense, 5′-GCCTTTCATAACACATTCCACC-3′) [20], *Runx2* (25 cycles: sense, 5′-GCAAGATGAGCGACGTGAG-3′; antisense, 5′-AAGGACTTGGTGCAGAGTTCA-3′), osteopontin (22 cycles: sense, 5′-CTCCCGGTGAAAGTGACTGA-3′; antisense, 5′-GACCTCAGAAGATGAACTCT-3′), collagen type II a I (25 cycles: sense, 5′-GGAAAGTCTGGGGAAAGAGG-3′; antisense, 5′-CAGTCCCTGGGTTACCAGAA-3′), aggrecan (25 cycles: sense, 5′-CACTGTTACCGCCACTTCCC-3′; antisense, 5′-ACCAGCGGAAGTCCCCTTCG-3′) [11]. As the positive control, RNA isolated from the whole body of the mouse on embryonic day 14 was used. PCR amplification was performed using Blend Taq polymerase (Takara, Kyoto, Japan) under the following conditions: 22–30 cycles of 94 °C for 30 s, 58–64 °C for 30 s, and 72 °C for 1 min. The amplified products were analyzed by electrophoresis and ethidium bromide staining.

2.7. Cell differentiation assay

The multipotency of each cell line was examined by using the differentiation-induction protocols that have previously described [11–13,21].

2.7.1. Adipogenic differentiation

A total of 2×10^4 cells were seeded onto 35 mm culture dishes and cultured to confluency. The medium was changed to adipogenic induction medium [D-MEM high glucose containing 10% FBS and supplemented with 1 μM dexamethasone (Sigma), 0.2 mM indomethacin (Wako), 10 μg/ml insulin, and 0.5 mM 3-isobutyl-l-methylxanthine (Nakalai Tesque, Kyoto, Japan)] and cultured for 2 days. Subsequently, cells were cultured in culture medium for 8 days, and the medium was replaced every day. Cells differentiated to adipocytes were fixed with 4% PFA for 15 min at 4 °C, washed with PBS(−), treated with 60% isopropanol (Wako) for 5 min at 25 °C, and stained with Oil Red O (Wako) for 15 min at 25 °C. Cells were washed with 40% isopropanol and PBS(−) [21].

2.7.2. Osteogenic differentiation

Cells were seeded at a density of 2×10^4 on 35 mm culture dishes. At 90–100% confluency, the medium was replaced with osteogenic induction medium [D-MEM high glucose containing 10% FBS and supplemented with 10 mM β-glycerophosphate (Wako), 50 mM L-ascorbic acid 2-phosphate (Wako), and 100 nM dexamethasone] and cultured for 9 days; the medium was replaced daily [11]. Cells were induced to form mineralized matrix by osteogenic induction and washed twice with PBS(−). Cells were fixed with 95% ethanol (Wako) for 30 min at −20 °C and stained with 40 mM Alizarin Red S solution (pH 4.2; Sigma) for 1 h at 25 °C. Subsequently, the cells were washed 5 times with deionized water and rinsed with PBS(−) [12].

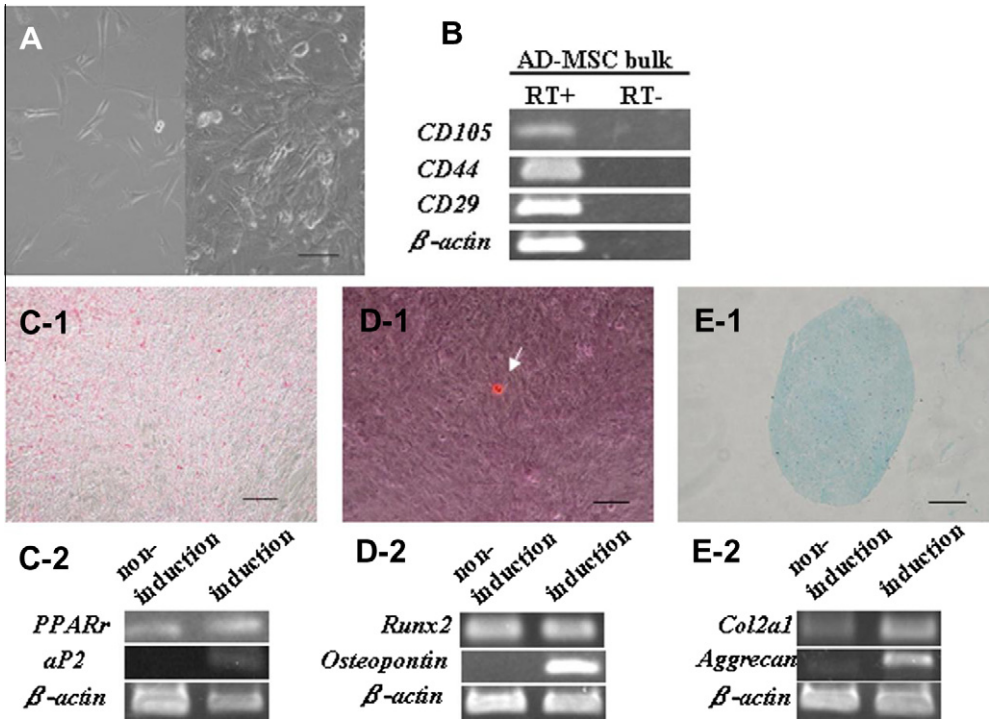

Fig. 1. Characterization of the AD-MSC bulk cell line. Morphology of the cell line established from adult mouse adipose tissue at low (left) and high (right) densities (A). RT-PCR of *CD105*, *CD29*, and *CD44* expression in the cell line (B). Cells proliferated to become confluent and were induced to form adipocytes with adipogenic medium (D-MEM high glucose containing 10% FBS and supplemented with dexamethasone, indomethacin, insulin, and 3-isobutyl-l-methylxanthine) (C). After 8 days of induction, adipocyte induction was analyzed by Oil Red O staining (C-1). Expression of PPARγ and *aP2* by RT-PCR in the adipogenic-induced cell line (C-2). Cells were induced to osteoblasts by culturing with osteogenic medium (D-MEM high glucose containing 10% FBS and supplemented with β-glycerophosphate, L-ascorbic acid 2-phosphate, and dexamethasone) (D). After 9 days of culture, osteogenic induction was analyzed by Alizarin Red S staining of the cell line (D-1; arrow indicates a positive region of Alizarin Red S Staining). Expression of *Runx2* and *osteopontin* by RT-PCR in the osteogenic-induced cell line (D-2). Cells were pelleted and cultured with chondrogenic medium (D-MEM high glucose supplemented with dexamethasone, sodium-pyruvate, L-ascorbic acid 2-phosphate, insulin, transferrin, and TGF-β1) (E). The pellet induced to form chondrocytes was analyzed by Alcian blue staining (E-1). Expression of *Col2a1* (Collagen type II a I) and *aggrecan* by RT-PCR in the chondrogenic-induced pellet (E-2). Scale bars: 100 μm. All images (A, C–E) are at the same magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7.3. Chondrogenic differentiation

A total of 2.5×10^5 cells were placed in V-bottom 96-well plates and centrifuged. These cells were cultured with chondrogenic induction medium [D-MEM high glucose supplemented with 10 μM dexamethasone, 1 nM sodium-pyruvate (Wako), 0.195 mM L-ascorbic acid 2-phosphate (Wako), 10 μg/ml Insulin–Transferrin, and 10 ng/ml TGF-β 1 (Wako)] for 21 days, and the medium was replaced every 3 days. The pellets of chondrocyte differentiated cells were fixed with 4% PFA and embedded in paraffin. Subsequently, 6 μm sections were examined for immunochemical and Alcian blue staining. Sections of chondrocyte differentiated cells were treated with 3% acetic acid and stained with Alcian blue solution (pH 2.5; Sigma) for 1 h. Stained sections were washed with 3% acetic acid and deionized water [13].

2.8. Tail vein injection

The method described previously [22] was performed. GFP-labeled AD-MSC (clonal line #7) (2×10^5 cells) were suspended in PBS(−). The cell suspension was injected into mice whose livers had been wounded (3–4 mm) with scissors through the tail vein. One week later, the liver tissue around the wound was dissected out, fixed with 4% PFA, treated with PBS(−) and sucrose, embedded in O.C.T. Compound (Sakura Finetek, Torrance, CA) and sectioned (6 μm). The section was immunostained with anti-liver tyrosine aminotransferase antibody (Santa Cruz Biotechnology).

3. Results

3.1. Establishment and characterization of an AD-MSC bulk cell line

An AD-MSC cell line was established from the stromal vascular fraction of a *p53*-KO adult mouse (AD-MSC bulk cell line; Fig. 1A). The expression of AD-MSC marker genes, such as *CD105*, *CD29*, and *CD44*, in the cell line was detected by RT-PCR (Fig. 1B).

The multilineage differentiation potential of AD-MSC bulk cells was examined by differentiation induction culture. Following treatment with adipogenic differentiation reagents, the AD-MSC bulk cells changed to express Oil Red O-stained vesicles (Fig. 1C-1), showing that they differentiated into adipose cells. The expression of peroxisome proliferator activated receptor gamma (PPARγ) and fatty acid binding protein (*aP2*) mRNA (biomarkers of adipose cells) was weakly upregulated by treatment with insulin (Fig. 1C-2).

Some of the AD-MSC bulk cells changed to display Alizarin Red S staining (Fig. 1D-1), indicating that they differentiated into osteoblast cells. The biomarkers of osteoblast cells are Runx2 and osteopontin. The expression of Runx2 was detected even in the non-induced cells. Under conditions of osteogenic differentiation, osteopontin was significantly upregulated (Fig. 1D-2).

Alcian blue staining revealed that the AD-MSC bulk cells differentiated into chondrogenic cells. The cells changed to show deposition of extracellular matrix in the pellet culture (Fig. 1E-1). The biomarkers of chondrocytes, collagen type II a I and aggrecan, were detected after induction (Fig. 1E-2).

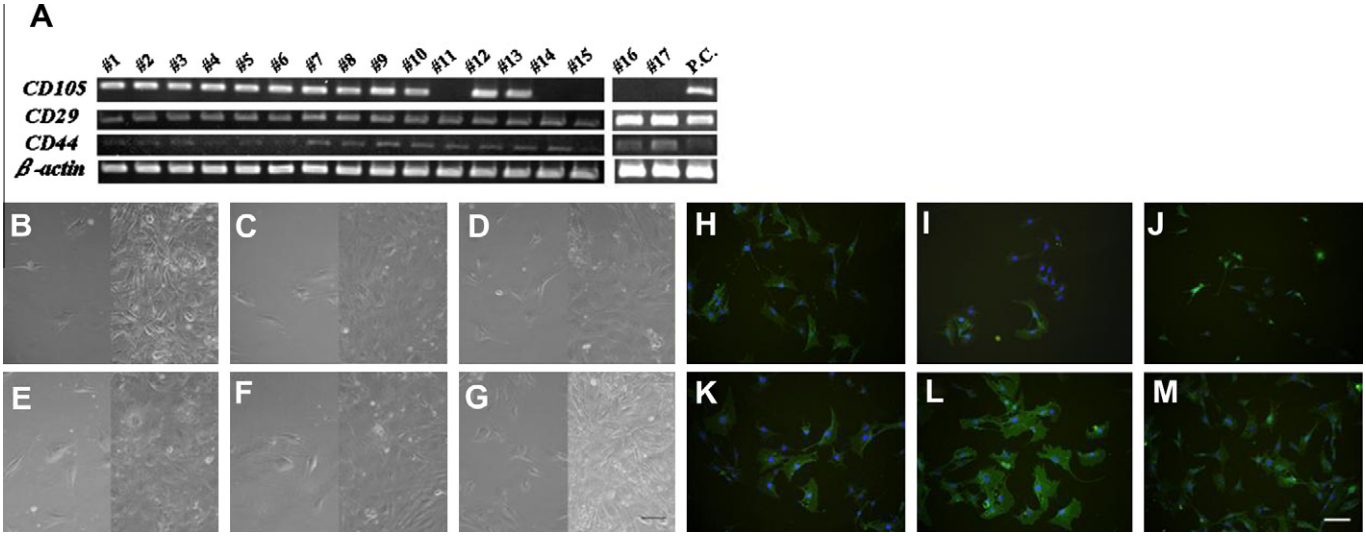

Fig. 2. Characterization of AD-MSC clonal cell lines. Expression of *CD105*, *CD29*, and *CD44* by RT-PCR analysis of the cell lines. The positive control was a whole embryo at embryonic day (E) 14 (A). Morphology of clonal cell lines at low (left) and high (right) densities (B, #3; C, #5; D, #7; E, #8; F, #9; G, #10). Immunocytochemistry with anti-CD105 antibody detected CD105 protein (green) in the AD-MSC cell lines (H, #3; I, #5; J, #7; K, #8; L, #9; M, #10). Nuclei were stained with DAPI (blue). Scale bars (shown in G and M) are 100 μm, and all images are at the same magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Taken together, these results show that the AD-MSC bulk cell line that we established displays multipotency, although the osteogenic potential of the cell line is weak.

3.2. Establishment and characterization of AD-MSC clonal cell lines

To establish a clone cell with high osteogenic induction potency and to facilitate analysis of AD-MSC biology, 17 AD-MSC clonal cell lines were established from the AD-MSC bulk line by limited dilution (AD-MSC #1 to #17). First, these cell lines were grouped by their morphology: Group1 cells showed a broad and bi- or 3-polar shape; Group2 cells showed a flat and 5- to 7-polar shape; Group3 cells were slender and had bi- or 3-polar shape morphology, which was similar to the primary MSCs; Group4 cells were large, with a flat shape, and showed 4- or 5-polar shape morphology; Group5 cells were more spread out and bigger, similar to senescent cells; Group6 cells had a polygonal edge, which resembled epithelioid cells.

The expressions of *CD105*, *CD29*, and *CD44* in the cell lines were analyzed by RT-PCR. The expression of *CD105* mRNA was detected in lines #1 to #10, #12, and #13. The expression of the *CD29* gene was detected in all cell lines. The expression of *CD44* was detected in cell lines except for #1 to #3, #5, #7 to #14, #16, and #17 (Fig. 2A). *CD105*-, *CD29*-, and *CD44*-positive cells were isolated from each group and used in subsequent experiments (Group1, #3; Group2, #5; Group3, #7; Group4, #8; Group5, #9; Group6, #10; Fig. 2B–G). Immunocytochemistry showed that these cell lines were positive for CD105 expression (Fig. 2H–M).

Cells divided at least 150 times and were passaged more than 50 times (data not shown). No cell lines showed anchorage-independent growth on soft agar [23] (data not shown).

3.3. Differentiation potential of AD-MSC clonal cell lines

3.3.1. Adipocyte differentiation

The adipogenic differentiation potential of AD-MSC clonal cell lines was examined by adipogenic induction medium culture. As shown in Fig. 3A, Oil Red O-positive lipid vesicles were detected in all cell lines. The expression of PPARγ and *aP2* was detected in all cell lines. Therefore, these results suggest that the AD-MSC clonal cell lines differentiated into adipocytes.

3.3.2. Osteoblast differentiation

To examine whether these cell lines displayed osteogenic differentiation, the cell lines were cultured with osteogenic induction medium. After induction, the cell lines formed mineralized matrix, which was visualized by Alizarin Red S staining (Fig. 3B). In addition, the expression of osteopontin was detected in all cell lines, and the expression level showed a time-dependent increase.

3.3.3. Chondrocyte differentiation

AD-MSC clonal cell lines were examined for their ability to form chondrocytes in micro-mass culture. The sections of cell pellets were stained with Alcian blue solution and anti-collagen type II antibody, and positive regions were detected (Fig. 3C). Moreover, as shown by RT-PCR, all cell lines were able to upregulate the expression of aggrecan and collagen type II a I.

3.4. Injection of AD-MSC clonal cell lines through the mouse tail vein

GFP-labeled AD-MSC (clonal cell line #7) was injected into mice whose liver had been wounded (Fig. 4) through the tail vein. These GFP- and tyrosine aminotransferase- positive cells were detected by immunohistochemistry respectively (Fig. 4A and B). The merged picture was shown in Fig. 4C. The AD-MSC clonal cell line #7 migrated into the wounded part of the liver and differentiated into hepatocytes. As the control, the non-wounded part of the liver from the same mouse was stained with antibodies against GFP and tyrosine aminotransferase, and merged (Fig. 4D). In the non-wounded area, GFP-labeled AD-MSC was not detected.

4. Discussion

A recent study indicated that the lifespan of MSCs is finite, and the average number of population doublings was reported to be approximately 38 times, at which time the cells finally ceased to divide [9]. In the present study, all of the established AD-MSC lines proliferated continuously without crisis. The cells divided at least 150 times and were passaged more than 50 times (data not

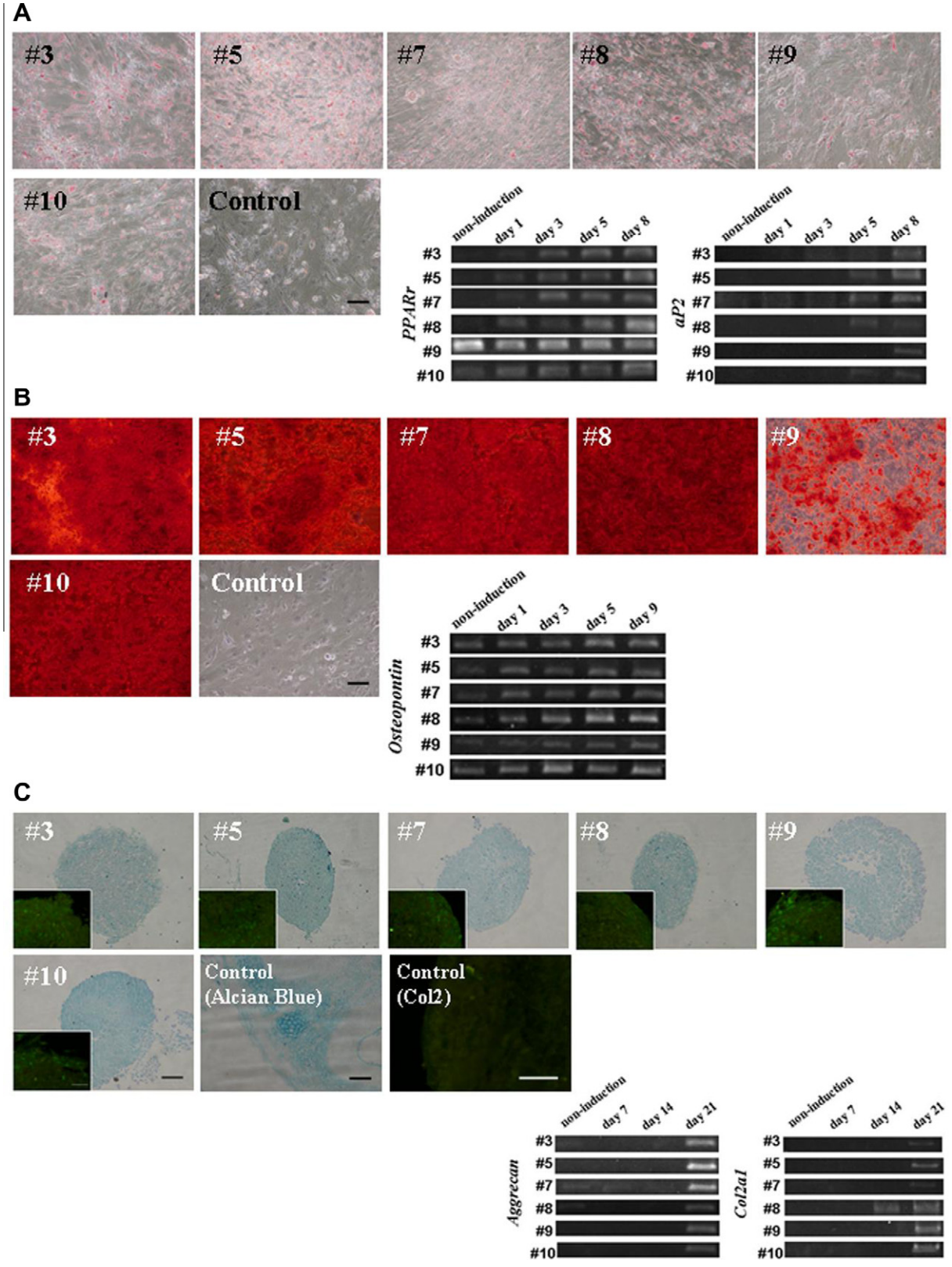

Fig. 3. Differentiation potentials of AD-MSC clonal cell lines. Cells were induced to adipocytes (A), osteoblasts (B) and chondrocytes (C) with differentiation media respectively (adipogenic; D-MEM high glucose containing 10% FBS and supplemented with dexamethasone, indomethacin, insulin, and 3-isobutyl-l-methylxanthine, osteogenic; D-MEM high glucose containing 10% FBS and supplemented with β-glycerophosphate, L-ascorbic acid 2-phosphate, and dexamethasone, chondrogenic; D-MEM high glucose supplemented with dexamethasone, sodium-pyruvate, L-ascorbic acid 2-phosphate, insulin, transferrin, and TGF-β1). (A) After 8 days of induction, adipocyte differentiation was analyzed by Oil Red O staining (Control; [non-induced #3]). Expression of PPARγ and *aP2* by RT-PCR analysis of cell lines. (B) After 9 days of culture, osteogenic induction was analyzed by Alizarin Red S staining of cell lines (Control; [non-induced #3]). RT-PCR analysis of the expression of *osteopontin* in cell lines. (C) Pellets induced to form chondrocytes were analyzed by Alcian blue staining. Collagen type 2 protein expression in each cell line is shown in the square at the lower left section of each figure (green). Cranial chondrocytes from an embryo at E16.5 were stained by Alcian blue staining (Control, Alcian Blue). Negative control of immunostaining for collagen type 2 (Control, Col2). RT-PCR analysis of *aggrecan* and *Col2a1* (Collagen type II a I) expression in the cell lines. Black scale bars are 100 μm. White scale bars are 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

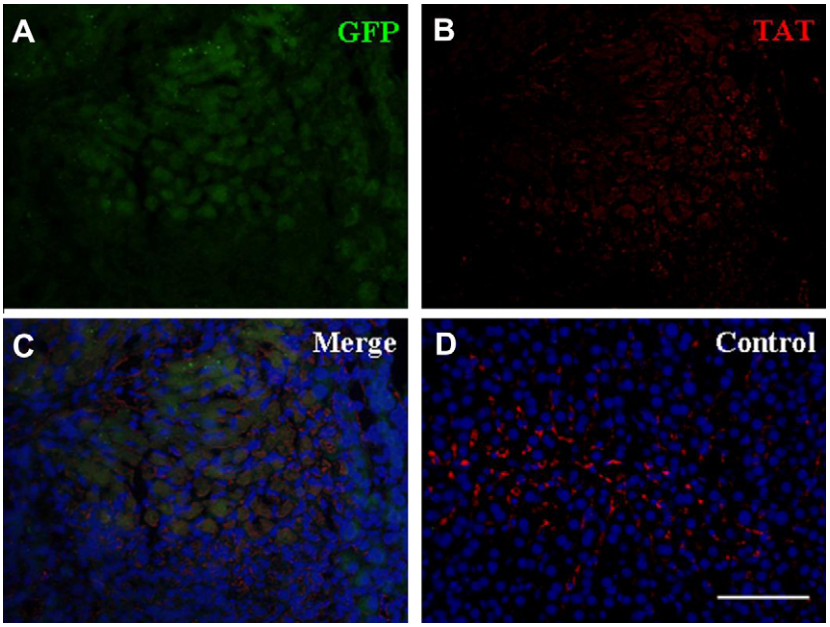

Fig. 4. AD-MSC cell lines differentiated into hepatocytes. GFP-labeled AD-MSC (clonal cell line #7) was injected into mice whose liver had been wounded through the tail vein. The cells-expressing GFP (A) and tyrosine aminotransferase (B) were detected by immunohistochemistry. These photos (A, B) with DAPI stain were merged (C). The photo at the non-wounded part of the liver from the same mice is shown as the control (D). Scale bar: 100 μm, and all images are at the same magnification.

shown), indicating that they are immortalized. In addition, none of cell lines had anchorage-independent growth, suggesting that they are not tumorigenic. *CD105*, *CD29*, and *CD44* are MSC markers; thus, detection of the expression of these genes in the bulk line indicates that MSCs are included in the bulk line. Moreover, clonal cell lines established from the bulk line expressed MSC marker genes, suggesting that clonal cell lines are MSCs.

The adipogenic differentiation assay showed that even the non-induced bulk line expresses PPARγ. In addition, under conditions of osteogenic induction, only a few regions were stained with Alizarin Red S. These results suggest that the stromal vascular fraction that was prepared from adipose contains few AD-MSCs [8], and the prepared AD-MSC bulk cell population consists of mainly adipocyte precursor cells. Therefore, these facts indicate that establishment of clonal cell lines from AD-MSC bulk cells is required for the study of tissue regeneration from AD-MSCs.

The 6 AD-MSC clones we established showed different and characteristic forms. It has previously been reported that there are a variety of BM-MSC clonal cell lines that display differentiation properties, including tri-, bi-, and uni-directional differentiation [11]. In general, MSCs should consist of heterogeneous cells with different differentiation potentials. Therefore, it is quite natural that the AD-MSC clones we established have different characteristics, and this is not caused by their continuous proliferation or lack of *p53*. The AD-MSC clones exhibit long-term growth, while retaining their multilineage potential. Furthermore, since each clonal cell line is homogeneous, these clonal lines represent much better tools than the bulk lines.

When an AD-MSC clonal line #7 was injected into mice whose liver had been wounded through the tail vein, the injected AD-MSCs were observed in the wounded area of the liver. Moreover, injected AD-MSCs differentiated into liver cells, as they changed to express liver tyrosine aminotransferase, a marker of liver cells. These results suggest that the clonal AD-MSC cell line can differentiate into tissues at the wounded region.

Taken together, we conclude that the AD-MSC clonal cell lines we established will be used by many scientists as a model of primary AD-MSCs to study their mechanism of differentiation.

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