

Advanced Properties of Urine Derived Stem Cells Compared to Adipose Tissue Derived Stem Cells in Terms of Cell Proliferation, Immune Modulation and Multi Differentiation

Hye Suk Kang,^{1*} Seock Hwan Choi,^{2*}
Bum Soo Kim,² Jae Young Choi,³
Gang-Baek Park,⁴ Tae Gyun Kwon,²
and So Young Chun⁵

¹Department of Physiology, Keimyung University School of Medicine, Daegu; ²Department of Urology, Kyungpook National University School of Medicine, Daegu; ³Department of Urology, Yeungnam University College of Medicine, Daegu; ⁴Department of Biomedical Science, Catholic University of Daegu, Gyeongsan; ⁵BioMedical Research Institute, Kyungpook National University Hospital, Daegu, Korea

*Hye Suk Kang and Seock Hwan Choi contributed equally to this work.

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Address for Correspondence:
So Young Chun, PhD

BioMedical Research Institute, Kyungpook National University Hospital, 135 Dongdeok-ro, Jung-gu, Daegu 41940, Korea
Tel: +82.10-4048-5111, Fax: +82.53-421-9618
E-mail: soyachun99@naver.com

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INTRODUCTION

Stem cells are a population possessing self-renewal capacity, long-term viability, and multilineage potential (1). In recent years, developments of stem cell science and regenerative medicine provided the opportunity for use of embryonic stem cells, induced pluripotent stem cells, and postnatal adult stem cells in repair of tissue injuries and eventually in replacement of organs (2). Among stem cells, multipotent mesenchymal stem cells (MSCs) are non-hematopoietic adult stem cells seen in many postnatal organs and connective tissues, and had no ethical concerns for their use (3).

Human MSCs have been isolated from bone marrow (4), periosteum (5), trabecular bone (6), synovial membrane (7), peri-

Adipose tissue stem cells (ADSCs) would be an attractive autologous cell source. However, ADSCs require invasive procedures, and has potential complications. Recently, urine stem cells (USCs) have been proposed as an alternative stem cell source. In this study, we compared USCs and ADSCs collected from the same patients on stem cell characteristics and capacity to differentiate into various cell lineages to provide a useful guideline for selecting the appropriate type of cell source for use in clinical application. The urine samples were collected via urethral catheterization, and adipose tissue was obtained from subcutaneous fat tissue during elective laparoscopic kidney surgery from the same patient (n = 10). Both cells were plated for primary culture. Cell proliferation, colony formation, cell surface markers, immune modulation, chromosome stability and multi-lineage differentiation were analyzed for each USCs and ADSCs at cell passage 3, 5, and 7. USCs showed high cell proliferation rate, enhanced colony forming ability, strong positive for stem cell markers expression, high efficiency for inhibition of immune cell activation compared to ADSCs at cell passage 3, 5, and 7. In chromosome stability analysis, both cells showed normal karyotype through all passages. In analysis of multi-lineage capability, USCs showed higher myogenic, neurogenic, and endogenic differentiation rate, and lower osteogenic, adipogenic, and chondrogenic differentiation rate compared to ADSCs. Therefore, we expect that USC can be an alternative autologous stem cell source for muscle, neuron and endothelial tissue reconstruction instead of ADSCs.

Keywords: USCs; ADSCs; Stem Cell Characteristics; Multi-lineage Differentiation; Passages

cytes (8), peripheral blood (9), skeletal muscle (10), skin (11), periodontal ligament (12), deciduous teeth (13), umbilical cord (14,15), adipose tissue (16), and urine (17). A transplantable cell source of autologous adult stem cells that is obtainable in large quantities, under local anesthesia, with minimal discomfort would be advantageous (16). Therefore, adipose tissue was considered an attractive source which can be provided in large quantities from adipose tissue fragments (18). Human MSCs isolated from adipose tissue (ADSCs) can differentiate into adipogenic, chondrogenic, myogenic, and osteogenic cells when cultured under appropriate conditions (19), but, the harvest of ADSCs requires invasive procedures and has potential complications, including infection, bleeding, and patient discomfort. To overcome these limitations, cells isolated from urine have

been proposed as an alternative stem cell source for tissue regeneration (17,20).

Urine derived stem cells (USCs) have mesenchymal stem cell characteristics, including SSEA4, OCT4, NANOG, SMAD2, SOX2, and alkaline phosphatase. USCs also showed the capacity to differentiate into various cell lineages, such as myogenic, urothelial, endothelial differentiation. USCs revealed free of malignant cell with cytogenetic study, and cytogenetical stability with an in vivo tumorigenicity assay (17).

USCs have MSC characteristics and can be conveniently obtained through a simple, non-invasive, and low-cost approach that avoids surgical procedures. If USCs have the similar or better characters as ADSCs, it can be the most ideal cell for tissue or organ regeneration. Therefore, we compared USCs and ADSCs from the same patient for stem cell characteristics, immune response and capacity to differentiate into various cell lineages.

MATERIALS AND METHODS

Preparation of USCs and ADSCs

Samples were obtained from 10 healthy humans who underwent live kidney donor nephrectomy, and information for gender and age were listed on Table 1. The urine samples (100 mL) were freshly obtained via urethral catheterization without urine stasis in urinary bladder after washing out initially drained urine. The phosphate-buffered saline (PBS) washed cell pellets were resuspended and plated in 100-mm culture plates with mixed medium composed of keratinocyte serum-free medium (Invitrogen, Carlsbad, CA, USA), DMEM (high glucose) and DMEM/Hamm's F12 (Thermo Scientific, Rockford, IL, USA) in a 2:1:1 ratio with growth factors as previously described (20). The cultured cells at passage 3, 5, and 7 were used for analysis.

Human adipose tissue was obtained from subcutaneous layer of surgical incision line. ADSCs were isolated as previously reported method (21). In brief, adipose tissue was washed in PBS and digested with 2 mg/mL collagenase in 1% bovine serum albumin (BSA) dissolved in PBS for 40 min at 37°C with constant agitation. Mature adipocytes, debris and connective tissue were separated from pellets by centrifugation (1,200 rpm,

15 min). Pellets were resuspended in PBS and passed through a 100- μ m nylon mesh filter. The filtered cells were cultivated in the α -MEM supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) (Invitrogen).

Cell proliferation, colony forming and flow cytometry assay

To generate a growth curve, USCs and ADSCs (cell passage 3, 5, and 7) were seeded at a density of 500 cells/cm². The cell proliferation rates were analyzed using the cell counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions at 1, 3, 5, 7, and 9 days after plating, and doubling time was also measured.

For colony forming analysis, USCs and ADSCs were seeded at a density of 1×10^3 in 24-well plate. After incubation for 14 days, the cells were fixed with 10% paraformaldehyde and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Cells were washed with distilled water and scanned plate after dry. Stained colony was dissolved in 10% acetic acid and absorbance was measured with ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 562 nm.

Flow cytometric evaluation was performed with phycoerythrin (PE)-conjugated mouse monoclonal antibodies specific for embryonic/mesenchymal stem cell marker (SSEA4), mesenchymal stem cell markers (CD44, CD73, CD90, CD105), hematopoietic stem cell markers (CD34, CD45, c-kit), and immunologic marker (HLA-DR) (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Approximately 1×10^4 cells were measured using fluorescence-activated cell sorter (BD Biosciences) system equipped with the CellQuest program. Antibody information was listed at Table 2.

Immune response

USCs or ADSCs were cultured for 24 hr, and proliferation was inhibited by mitomycin C (10 μ g/mL, for 2 hr, Kyowa, Japan). After 3 times PBS washing, peripheral blood mononuclear cells (PBMCs) were co- or separated-(trans-well chamber, Corning,

Table 1. Patient information

No.	ID	Gender	Age (yr)
1	#88	M	49
2	#89	M	72
3	#91	F	31
4	#94	M	69
5	#95	F	52
6	#98	F	58
7	#100	M	23
8	#102	M	44
9	#105	F	39
10	#107	M	61

Table 2. Antibody information for FACS and ICC

Antibody	Company	Dilution
SSEA4	BD Pharmingen	1:20, 1:200
CD44	BD Pharmingen	1:20
CD73	BD Pharmingen	1:20
CD90	BD Pharmingen	1:20
CD105	Abcam	1:20
CD34	BD Pharmingen	1:20
CD45	BD Pharmingen	1:20
C-kit	BD Pharmingen	1:20
HLA-DR	BD Pharmingen	1:20
MyoD	Santa Cruz Biotechnology	1:300
β -Tubulin III	Abcam	1:300
CD34	BD Pharmingen	1:200
UP1 α	Abcam	1:200

Corning, NY, USA) cultured with both stem cells; each well was composed with 1:100, 1:50, 1:25, or 1:12.5 stem cell to PBMC ratio. PBMCs were acquired from AllCells (Emeryville, CA, USA) and activated with 5 mg/mL phytohemagglutinin (PHA) (Sigma-Aldrich). The inhibition efficiency by cell-cell contact or secreted factors was analyzed with Cell Proliferation ELISA, BrdU (colorimetric, Roche, Mannheim, Germany) at 450 nm absorbance at day 3, 5, and 7, and the value was normalized with control (PBMC only). The results were presented in mean \pm SD from three independent experiments.

In vitro differentiation

Cells were seeded at a density of 1×10^5 cells/well in 24-well plates in growth medium. At 80% confluence, the medium was replaced by each differentiation medium. Medium was changed every 3 days and the experiments were terminated at day 7 for neuron, day 14 for adipocyte, osteoblast and myocyte, and day 21 for endothelium and chondrocyte. We purchased the differentiation media for adipocytes, osteoblast and chondrocyte from PromoCell (Heidelberg, Germany), for neuron from NeuroCult NS-A differentiation Kit (StemCell, Vancouver, BC, Canada), and for epithelium from Lonza (Rockland, ME, USA). For myogenic media, we used conditioned medium obtained from human primary skeletal muscle cell culture.

Quantitative real-time PCR, immunocytochemical (ICC) analysis and cytochemical staining

Total RNA was extracted with Trizol (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 2 μ g of RNA was used for cDNA synthesis using DNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). The primers were designed with Primer Express Software (Applied Biosystems, primer sequence list Table 3). The assay was performed using the ABI Prism Sequence Detection System 7500 with SYBR Green Polymerase Chain Reaction Master Mix (Applied Biosystems). To analyze the data, the $2^{-\Delta\Delta Ct}$ method of relative quantification was adapted to estimate the copy numbers.

For ICC, cells were cultured on 8-well chambered slides (Thermo Scientific, Waltham, MA, USA) for staining with stem cell marker (SSEA-4), myogenetic marker (MYOD), neuron-specific marker (β -TUBULIN-III), endothelium-specific marker (CD31), and urothelium-specific marker (UROPLAKIN 1 α , UP1 α). When the cells had become differentiated, they were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 10 min, and washed 3 times with PBS. After blocking with 5% bovine serum albumin, the cells were incubated overnight with primary antibodies. After removal of primary antibodies, cells were washed three times with PBS and incubated with the secondary antibody conjugated to fluorescent for 1 hr. Cells were washed 3 times with PBS and mounted with a medium containing DAPI (4'-6-diamidino-2-phenylindole) to de-

Table 3. Primer sequences for real-time PCR

Genes	Sequences
<i>hPax7</i>	5'-GCAAATTGCTGTCTCTGCTCA 5'-TGAAAATCTGGTCACATCTGCCT
<i>hMyf5</i>	5'-ACCGATTACAGCCTCGAACT 5'-TGTGTATTAGGCCCTCTGGAA
<i>hMyoD</i>	5'-ACAGCGCGGTTTTTCCAC 5'-AACCTAGCCCTCAAGGTTTCAG
<i>hMyogenin</i>	5'-TGGCAGGAACAAGCCTTTTC 5'-ACAGGCAGGTAGTTTTCCCA
<i>hMef2</i>	5'-ATTCACCAGGCAGCAAGAA 5'-GGAGTTGCTACGGAAACCACTG
<i>hMlp</i>	5'-AAGGCTCTTGACAGCAGCAGACG 5'-TGTCATACCCGATCCCTTTG
<i>hβ-Actin</i>	5'-ATCGTCCACCGCAAATGCT 5'-AAGCCATGCCAATCTCATCTTG
<i>mPax7</i>	5'-ACCAAGCTTTCAAGTCCGCA 5'-GCCTTACATCTGGAGGATGGA
<i>mMyf</i>	5'-CTCTGAAGGATGGACATGACGG 5'-ACTGGTCCCAAACTCATCCTC
<i>mMyoD</i>	5'-TTCGGAGTGGCAGAAAGTTAA 5'-TCAAGTCTATGTCGGGAGTGG
<i>mMyogenin</i>	5'-TATCCGGTTCAAAGCCTCTG 5'-GCGGCAGCTTACAAACAACA
<i>mMef2</i>	5'-AACCCCAATCTCTGCCACTG 5'-ATCAGACCGCCTGTGTACCTG
<i>mMlp</i>	5'-GCTGAACAAGTTACTGAGCCGG 5'-ATTTTGACCTCCACCCCA
<i>mGapdh</i>	5'-TGT GTCCGTCGTGGATCTGA 5'-CCTGCTTACCACCTTCTTGA

tect nuclei (VectaShield, Vector Labs, Burlingame, CA, USA).

Alizarin red S staining was carried when cells differentiated into osteoblast at day 14. The cells were fixed with 10% formalin solution for 30 min and were placed in 2% alizarin red S (pH 4.1) solution for 30 min, washed with pure water 3 times and then photographed. Oil red-O staining was achieved when cells were differentiated into adipocytes at day 14. The accumulated cytoplasmic lipid droplets were fixed with 10% formalin for 1 hr, washed with 60% isopropanol, air-dried, stained with filtered Oil red-O staining solution and photographed. Alcian blue staining was carried when cells formed aggregates at day 21. The aggregates were fixed with 4% paraformaldehyde, embedded into 2% agarose to make paraffin blocks, after dehydration steps, blocks were sliced into 6- μ m sections and stained with Alcian blue solution.

Karyotyping

Karyotyping was performed to identify the chromosomal stability of the cultured cells at passage 3, 5, and 7. The cultured cells were treated with 0.02 μ g/mL Colcemid (Life Technologies, Carlsbad, CA, USA) overnight, incubated in hypotonic solution (0.075 M KCl) for 1 hr at 37°C, and fixed in Carnoy's fixative (3:1 methanol/acetic acid, 4°C). Metaphase spreads were placed on glass slides and digested with 0.01% trypsin and then stained with Giemsa to generate G bands. At each passage, 15

cells were chosen for analysis, and the band resolution was 600. Chromosome image capture and karyotyping were performed using CytoVision, version 3.7 (Applied Imaging, San Jose, CA, USA). The same experiment was repeated 3 times, independently.

Statistical analysis

The data were presented as means ± SD. Statistical analysis was conducted by Kruskal-Wallis test and one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered statistically significant. When the value was found to be significant after assessment using the ANOVA, the Tukey’s post-hoc comparison was used.

Ethics statement

The institutional review board of Kyungpook National University School of Medicine approved this study (IRB approved number: KNUH 2012-10-018). All patients submitted informed consents before providing urine and fat samples.

RESULTS

For comparing of cytologic differences between USCs and ADSCs, we used passage number 3, 5, and 7 cells. There were morphological difference in primary cultured USCs and ADSCs; USC showed cobble stone-like shape with frill, and ADSC had fibroblast-like shape (representative images were on Fig. 1A).

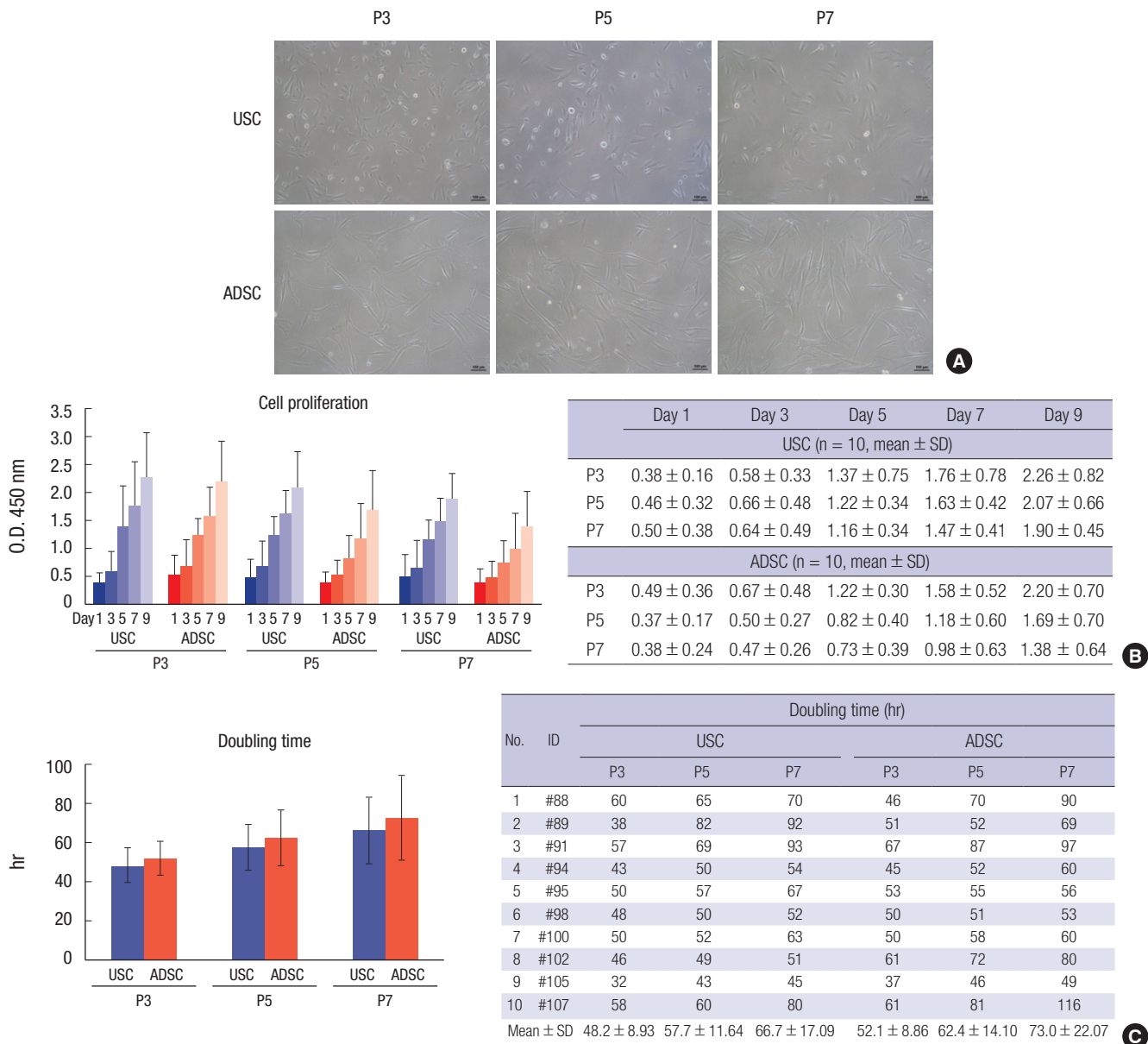


Fig. 1. Comparisons of stem cell characters between USCs and ADSCs at passage 3, 5, and 7 (Representative images came from patient #91). (A) Cell morphology. Scale bars = 100 μm. (B) Cell proliferation analysis at day 1, 3, 5, 7, and 9. (C) Doubling time analysis. (Continued to the next page)

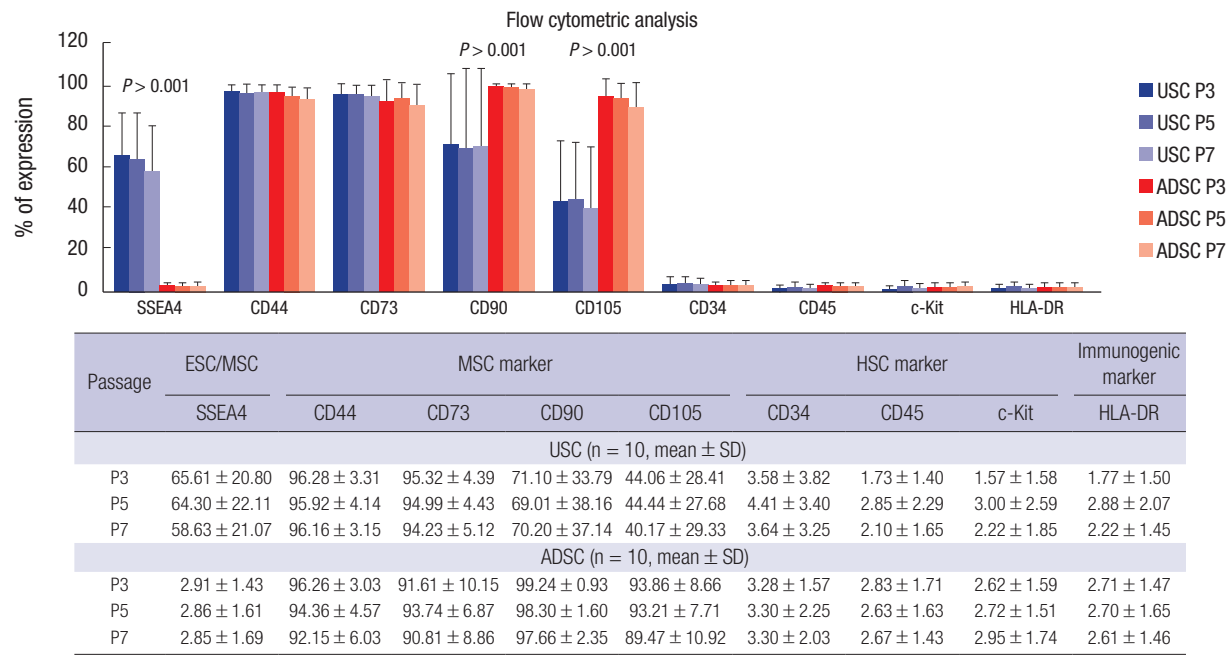
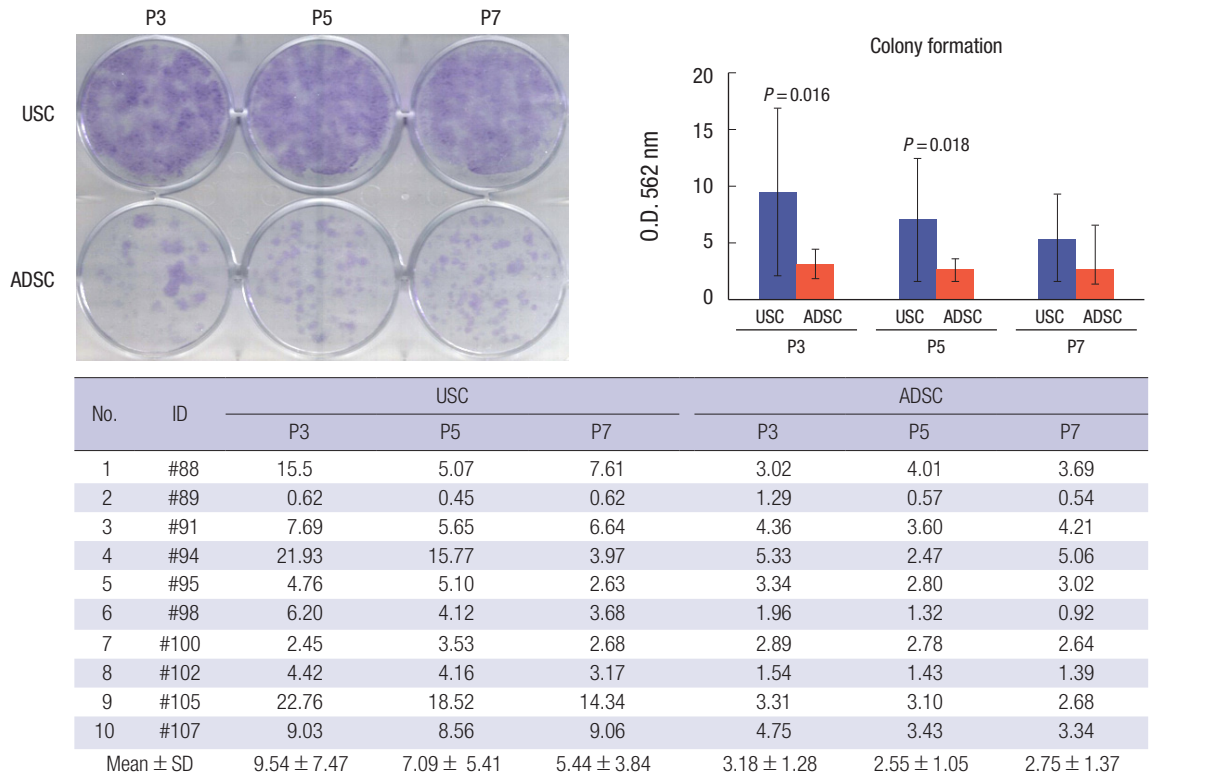


Fig. 1. Continued. (D) Quantity of MSCs colonies. (E) Flow cytometric analysis for analysis of cell surface protein expression. USC, urine stem cell; ADSC, adipose tissue stem cell; P3, passage 3; P5, passage 5; P7, passage 7.

The cell morphology (size and shape) persisted till passage 7. On the cell counting kit-8 assay, both cell types showed more proliferative capacity in early passage number. In comparison of cell proliferation, USCs showed a higher proliferation profile than ADSCs both in 1, 3, 5, 7, and 9 days analysis (Fig. 1B). In doubling time measurement, USCs showed increased prolifer-

ation rate compared to ADSCs at all cell passages (Fig. 1C). In colony formation analysis at passage 3, 5, and 7, USCs showed about 3.00, 2.78, and 1.98 times high value compared to ADSCs (Fig. 1D). Cell surface antigen phenotyping was performed on USCs and ADSCs by flow cytometry (Fig. 1E). Notably, SSEA4 was strongly positive on USCs. USCs and ADSCs revealed very

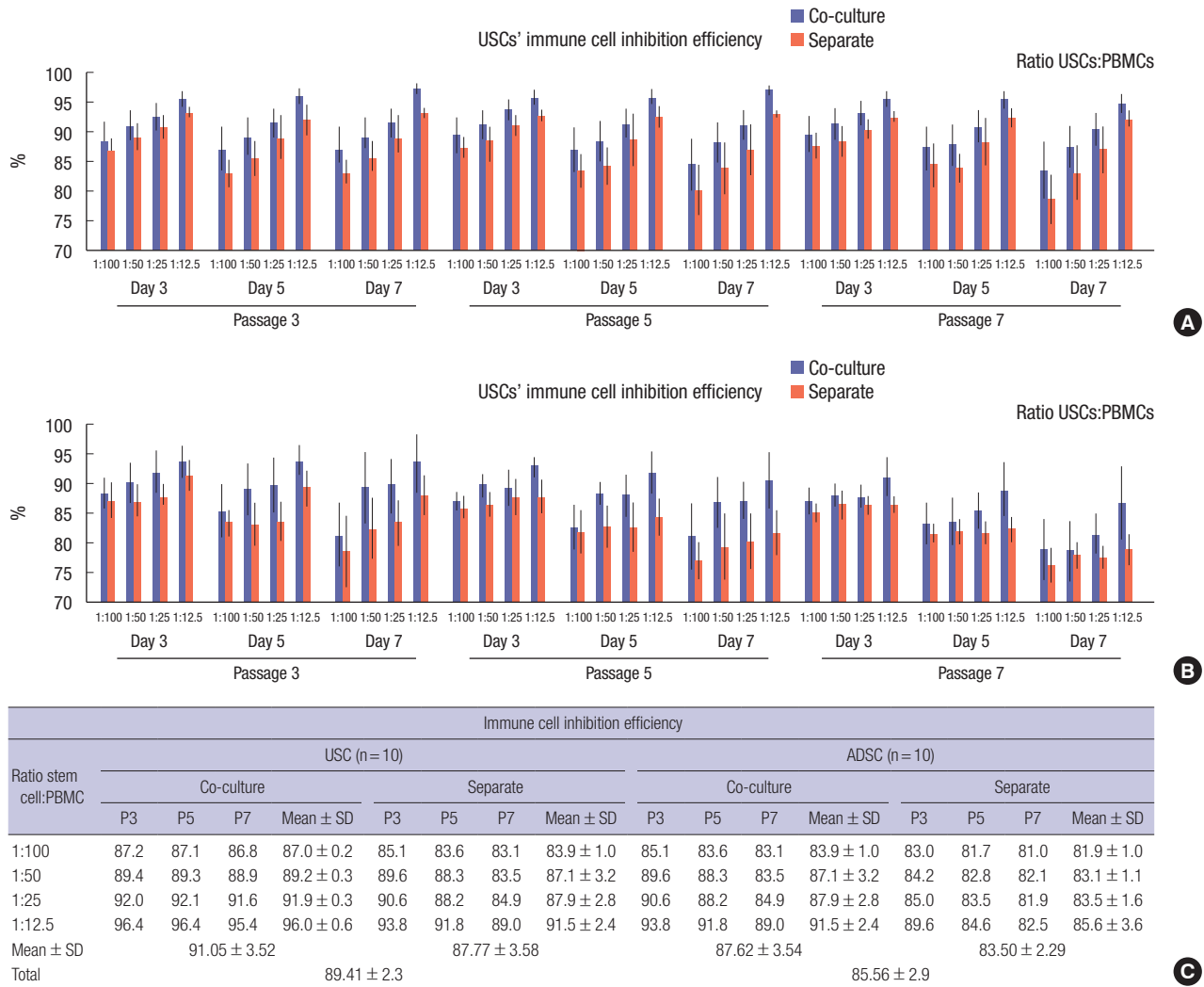


Fig. 2. Immune cell inhibitory effect of MSCs. PHA-activated peripheral blood mononuclear cells (PBMCs) was cultured by co-culture (A) or separate-culture (B), and the percent of inhibition efficiency was compared (C). USCs, urine stem cells; ADSCs, adipose tissue stem cells; PBMCs, peripheral blood mononuclear cells; P3, passage 3; P5, passage 5; P7, passage 7.

similar strongly positive expression for CD44 and CD73 (above 92%), while CD90 and CD105 expression was higher in ADSCs. Hematopoietic and immunogenic markers showed negative expression on both cells.

In passage and cell ratio effect analysis, the passage 3, 5, and 7 USCs (Fig. 2A) and ADSCs (Fig. 2B) induced dose-dependent inhibition of PBMC proliferation at co- and separated- culture system. At low numbers of USCs (1:100 = USC:PBMC), the inhibition percentages on co- and separated-culture were 87.0 ± 0.2 and 83.9 ± 1.0, and ADSCs were 83.9 ± 1.0 and 81.9 ± 1.0. When the stem cell number was increased (1:50, 1:25, 1:12.5), lymphocyte proliferation was further inhibited, and at the highest numbers of USCs, PBMCs proliferation was seriously inhibited (in USCs, 89.2 ± 0.3, 91.9% ± 0.3%, 96.0 ± 0.6 for co-culture, 87.1 ± 3.2, 87.9 ± 2.8, 91.5 ± 2.4 for separated-culture, and in ADSCs, 87.1 ± 3.2, 87.9 ± 2.8, 91.5 ± 2.4 for co-culture, 83.1 ± 1.1,

83.5 ± 1.6, 85.6 ± 3.6 for separated-culture). When compared the two methods, the inhibition degree was higher on the co-culture (USCs 91.05% ± 3.52%, ADSCs 87.62% ± 3.54%) than the separation condition (USCs 87.77% ± 3.58%, ADSCs 83.50% ± 2.29%) (*P* = 0.007). The mean immune cell inhibition efficiency of USCs was 89.41% ± 2.3% and ADSCs was 85.56% ± 2.9% (*P* = 0.004), so USCs showed higher inhibition efficiency than ADSCs (Fig. 2C).

Chromosomal G-band analysis was performed for karyotype investigation. The karyotype from 10 patients consisted with normal diploid complement of autosomes and sex chromosomes. Chromosomal aberrations were not found at any cell and passage. A representative G-banded karyotype of MSCs from patient #91 at passage 3, 5, and 7 was shown in Fig. 3.

We analyzed multi-lineage capability of USCs and ADSCs in vitro. In advance, un-differentiated cells' stem cell markers ex-

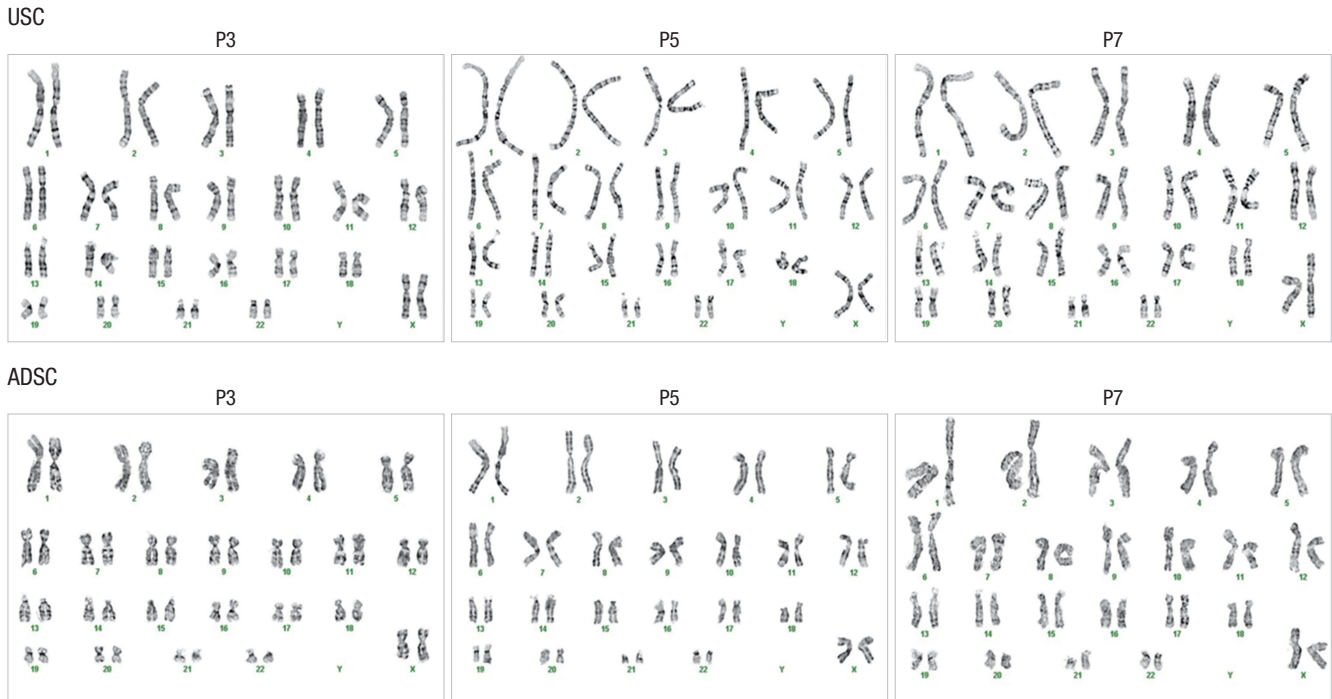


Fig. 3. G-banded karyotypes for USC and ADSC at passage 3, 5, and 7 (Representative images came from patient #91). Both USC and ADSC do not show any chromosomal aberrations. USC, urine stem cell; ADSC, adipose tissue stem cell; P3, passage 3; P5, passage 5; P7, passage 7.

pression level was analyzed through real-time PCR (mean value from 10 patients) and ICC analysis (representative image from patient #91) (Fig. 4A). Although not statistically significant, USCs' stem cell markers expression level was usually higher than that of ADSCs. Both cells' gene expression level was not related to the cell passage, but in ICC analysis with SSEA4, protein expression was dependent to passages. Myogenic differentiation for up to 14 days was resulted higher expression of MyoD in USCs compared with ADSCs (Fig. 4B) (not statistically significant). In vitro neuronal differentiation, USCs revealed enhanced differentiation to neuronal lineage accompanied with neuron-specific morphological changes and β -Tubulin III gene expression (Fig. 4C) (not statistically significant). In endothelial differentiation for 21 days, USCs outgrowths exhibit endothelial-like morphology with an elongated cell population. The CD31 gene was upregulated during endothelial differentiation in USCs compared to ADSCs (Fig. 4D) (not statistically significant). In adipogenic differentiation, oil-deposition capacity was low in USCs compared with ADSCs; lipid vacuoles were more observed in ADSCs than USCs in the quantitative Oil red-O staining. The expression of gene for peroxisome proliferator activated receptor γ (PPAR γ) was upregulated in ADSCs at day 14 (Fig. 4E) (not statistically significant). In osteogenic differentiation, ALP expression was upregulated in ADSCs (at passage 7, statistically significant, $P = 0.04$) and it was confirmed with alizarin red S staining quantitation (Fig. 4F). In the chondrogenic potential assay, the expression of aggrecan and type IV collagen was up-

regulated on ADSCs (Fig. 4G) (at passage 5 and 7, statistically significant, $P = 0.02$ and 0.02), and ADSCs showed bigger aggregate and strong positive alcian blue staining at day 21.

Above results were summarized on Fig. 4H; USCs showed enhanced cell proliferation, stem cell surface markers expression (FACS), stem cell gene expression (real-time PCR), myogenic, neurogenic and endothelial differentiation ability. ADSCs revealed better potential for adipo-, osteo-, and chondrogenesis.

DISCUSSION

In this study, we compared USCs and ADSCs properties from the same patients ($n = 10$), whether USCs could be alternative stem cell source for replacement of ADSCs according to cell morphology, proliferation, colony formation, immune modulation effect, chromosomal stability and in vitro differentiation potency. We used passage 3, 5, and 7 cells, because primary cultured MSCs showed proliferative limitation, and the reported available passage was around 3-5 (22), so we chose passage 7 as a maximum passage for this experiment.

In cell morphology comparison, USC showed cobble stone-like shape with frill indicating an epithelial phenotype (23). This morphology was similar to the previously reported urothelial-like cell shape (20); which indicates that USCs could be epithelial origin (20). ADSC had spindle-shaped morphology, more elongated and dispersed. Such a fibroblast-like morphology

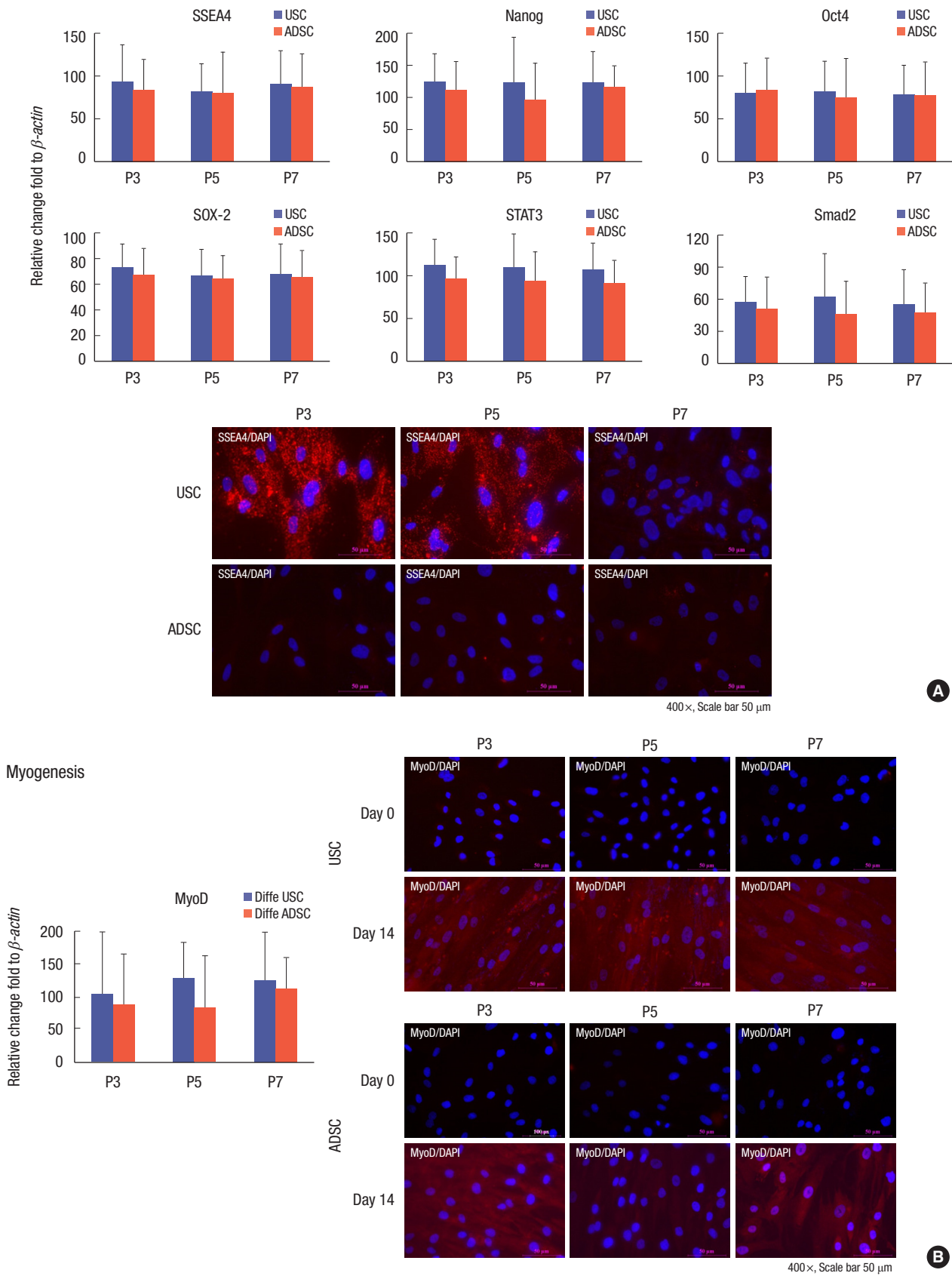
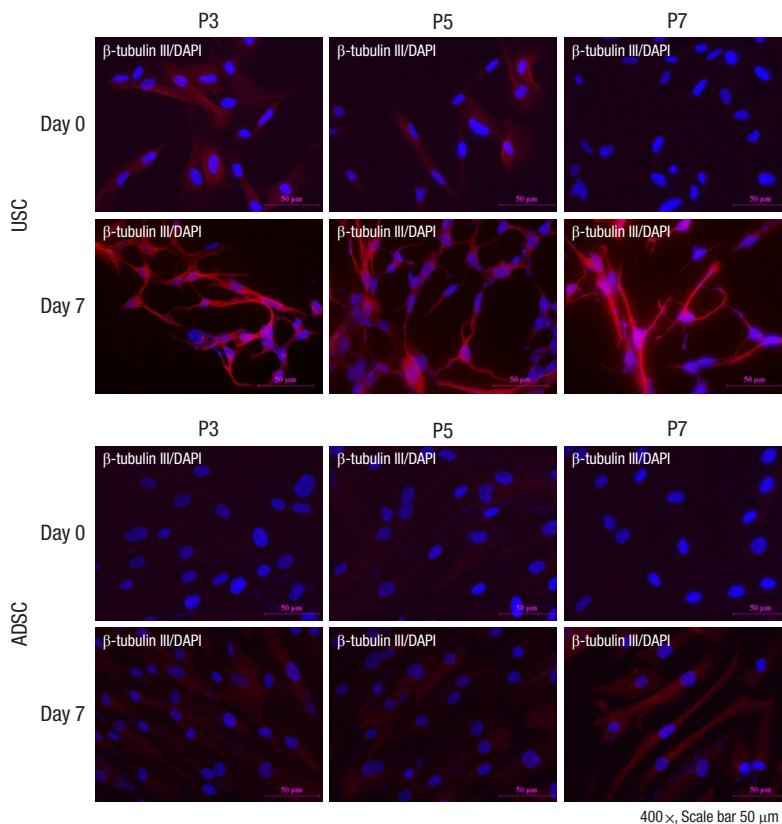
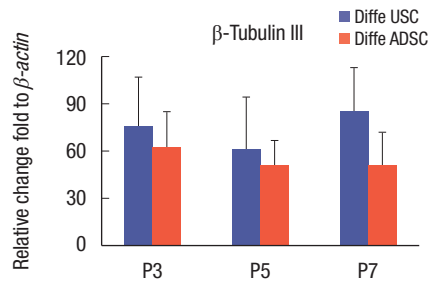


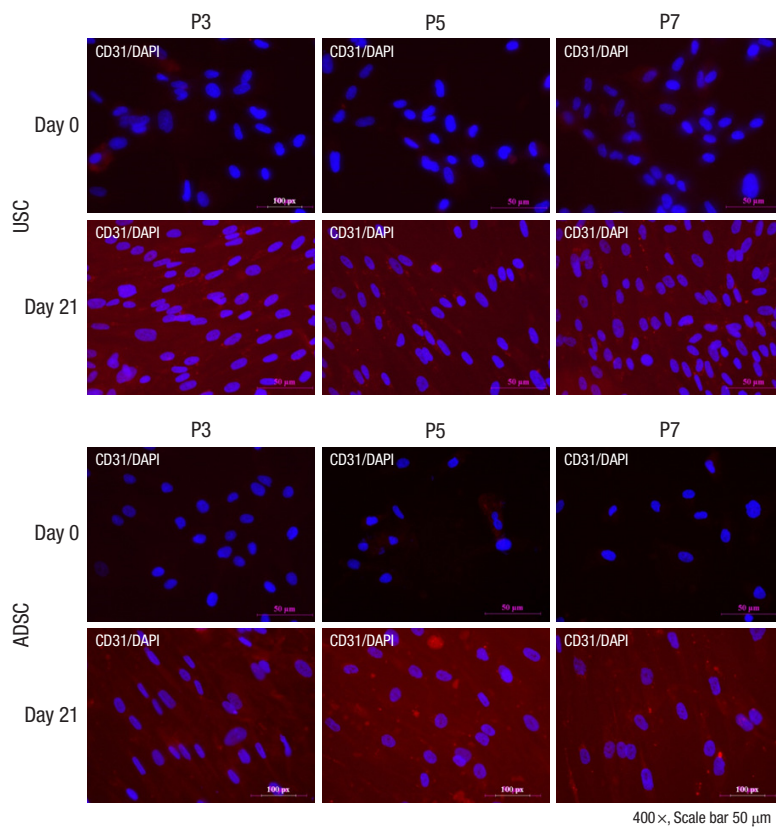
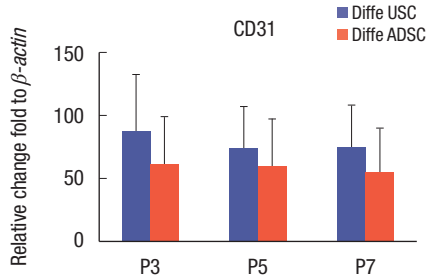
Fig. 4. Comparisons of differentiation ability between USCs and ADSCs at passage 3, 5, and 7 (Representative immunocytochemical [ICC] images came from patient #91). (A) Stem cell markers expression of un-differentiated MSCs. (B) Myogenic differentiation potential analysis with MyoD gene expression and ICC. (Continued to the next page)

Neurogenesis



C

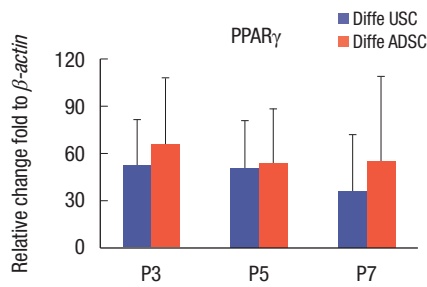
Endothelial genesis



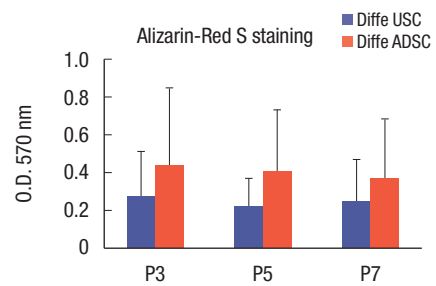
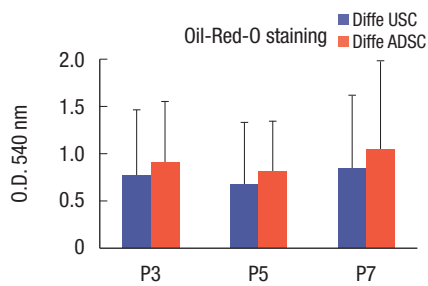
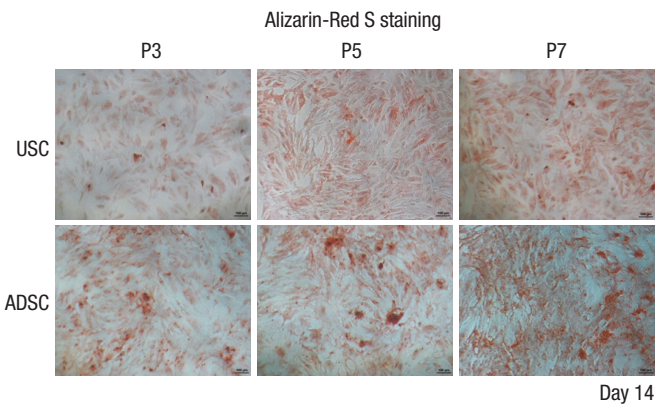
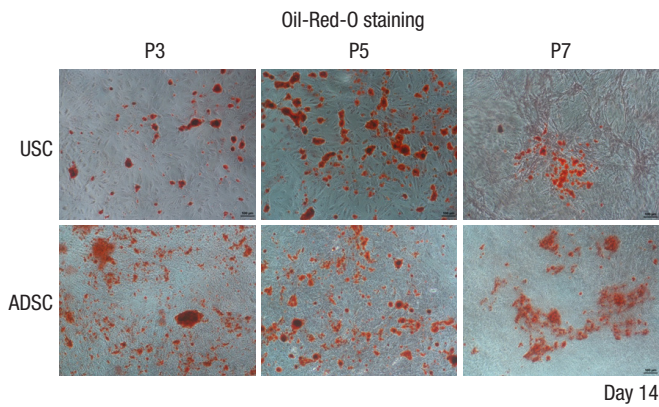
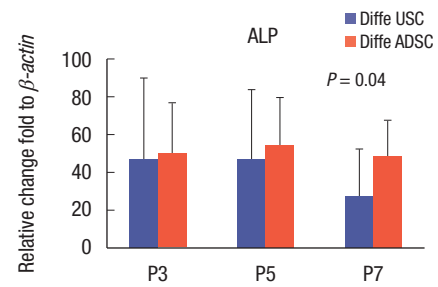
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Fig. 4. Continued. (C) Neurogenic differentiation potential analysis with β -Tubulin III gene expression and ICC. (D) Endothelial differentiation potential analysis with CD31 gene expression and ICC. (Continued to the next page)

Adipogenesis



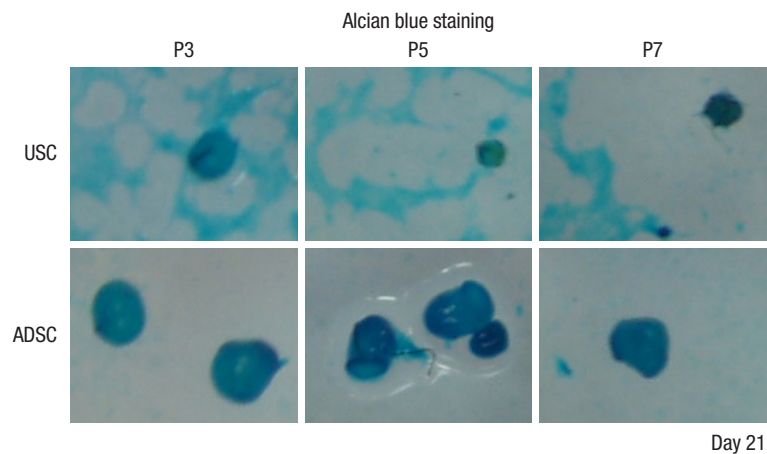
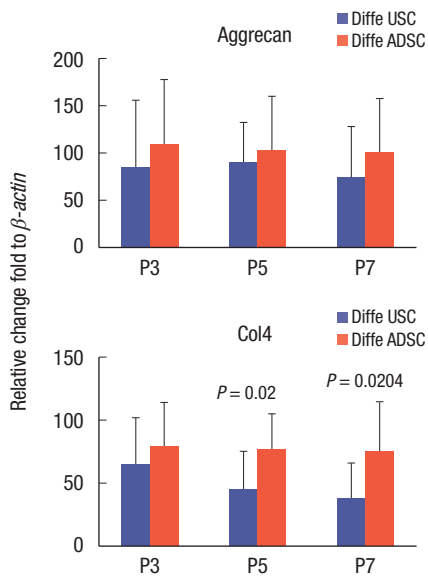
Osteogenesis



E

F

Chondrogenesis



Day 21

G

Fig. 4. Continued. (E) Adipogenic differentiation potential analysis with PPAR γ gene expression and Oil-Red-O staining. (F) Osteogenic differentiation potential analysis with ALP gene expression and Alizarin-Red S staining. (G) Chondrogenic differentiation potential analysis with Aggrecan and Collagen type 4 gene expressions and Alcian blue staining. (Continued to the next page)

No.	ID	Cell proliferation	Stem cell proteins	Stem cell genes	Immune cell inhibition	Differentiation					
						myo	neuron	endothelium	adipo	osteo	chondro
1	#88	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC=ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC=ADSC	USC<<ADSC
2	#89	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC=ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC=ADSC	USC>ADSC
3	#91	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC=ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC<ADSC	USC≤ADSC
4	#94	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC=ADSC	USC<<ADSC	USC<ADSC	USC=ADSC
5	#95	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC=ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC=ADSC	USC≤ADSC
6	#98	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC=ADSC	USC>>ADSC	USC=ADSC	USC<<ADSC	USC=ADSC	USC=ADSC
7	#100	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC<ADSC	USC=ADSC
8	#102	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC=ADSC	USC=ADSC	USC<ADSC	USC<<ADSC	USC<ADSC	USC=ADSC
9	#105	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC=ADSC	USC≤ADSC
10	#107	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC<<ADSC	USC≤ADSC
	Total	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC>>ADSC	USC<<ADSC	USC≤ADSC	USC≤ADSC
		10:0	10:0	10:0	10:0	5:0	9:0	7:0	0:10	0:5	1:6

>> More than 2 times, > about 2 times, ≥ about 1.5 times, = similar.



Fig. 4. Continued. (H) Summarized table. Scale bars = 50 μ m. USC, urine stem cell; ADSC, adipose tissue stem cell; differ USC, differentiated urine stem cell; differ ADSC, differentiated adipose tissue stem cell; P3, passage 3; P5, passage 5; P7, passage 7.

was a frequent character of mesoderm origin mesenchymal cell (24). These different cell morphologies indicated that two stem cells characters would be different in several aspects, because cell shape is decided by the cell properties (25,26).

Cell viability and growth kinetics were assessed by cck-8. The growth ability of the both cells decreased gradually with the increase of passages; which means that the less subculture is required to maintain proliferation ability. We also can see that ADSCs taken for a much longer growth time at tested passages according to the cell doubling time measurement, which indicates that USCs have better growth ability than ADSCs. The analysis of colony formation also showed the similar result to proliferation. Even colony formation was largely dependent between the patients, generally USCs developed higher colony numbers and size than ADSCs.

Cell surface markers were analyzed by flow cytometric analysis. SSEA4 showed distinct expression by about 62.3% positive of the USC population but only by less than 3% of the ADSC; which means that USCs have higher stem cell potency than ADSCs. In CD44 and CD74 comparison, no differences were observed on both cells. In CD90 analysis, USCs showed less abundant, but statistically not significant. The CD105 expression on USCs was significantly lower than ADSCs; according to the references, the expression frequency of CD105 depend on culture medium type (27), and endogenous CD105 expression of USC was low compared to other MSC markers (20). The most of MSC markers showed a tendency to decrease according to cell passage but not significant. Both cells were negative for CD34, CD45, and c-kit; these expression of surface proteins indicated that they were non-hematopoietic origin (28). Less than 3% of both cells expressed the HLA-DR protein, suggesting their low immune rejection and potential for allogeneic transplantation (29).

In analysis of immune cell response with both cells, cell-cell

contact inhibitory efficiency with co-culture of USCs or ADSCs with PBMCs, the PBMCs' proliferation was significantly inhibited at 1:100, 1:50, 1:25, or 1:12.5 stem cell to PBMC ratio with passage 3, 5, and 7, and the inhibitory effect was increased according to the stem cell number. In addition, to investigate the effect of secretory factors by physical separation (with trans-well) also showed significant inhibition at all ratio, but the degree was reduced when compared to the co-culture (30). These results mean that both cells have more inhibitory effect when directly contact to immune cell than secretory molecules.

We compared both cells' multi-lineage ability with three different mesenchymal lineages (adipogenic, osteogenic, and chondrogenic) (27), myogenic, neurogenic, and endothelial differentiation. The un-differentiated cells were expressed stem cell markers (SSEA4, Nanog, Oct4, Sox-2, STAT3, and Smad2) and the gene expression level was higher in USC than ADSC. Interestingly, in gene expression analysis, passage number was not related for these markers expression, but in protein analysis with ICC, early passage cells showed significantly enhanced protein expression; SSEA4 protein expression was decreased depending passages. With this result, we assumed that stem cell property, in case of protein synthesis, could be affected by cell passage, although gene expression was stable (31). For undifferentiated cells' ICC, SSEA4 was selected, because it considers as an embryonic/mesenchymal stem cell specific surface markers, so frequently using for homogeneous stem cell sorting from heterogeneous primary cultured cell (32). In ICC, SSEA4 protein was detected on cytosol as well as cell surface, because this protein expressed both regions (33).

In multi-lineage differentiation analysis, USCs revealed better potential for myogenic-, neurogenic- and endothelial differentiation compared to ADSCs. The reason for these results could be found at the origin of urine stem cells. Urine contained cells came from upper urinary tract (20); this means that USC could

contain ectodermal epithelial stem cell potency as well as mesodermal MSC. The urothelial-like morphology of USC could be an evidence this hypothesis. The most famous epithelial stem cells are found at bulge region of hair follicles, which also has ectodermal potencies (34). The hair follicle stem cells can easily give rise to neurons and smooth muscle cells (35), and this stem cells also can differentiate into endothelial cells expressing von Willebrand factor, vascular endothelial cadherin and CD31 (36). USCs showed the adipo-, osteo-, and chondro-genetic differentiation ability (these three differentiation potency are one of the main character of mesodermal MSC (37), but only less effective differentiation potency than ADSC. ADSC showed fibroblast-like morphology, which can be indicate that ADSCs are close to mesodermal mesenchymal stem cell, instead of ectodermal origin epithelial-like stem cell (21); so, ADSC can be derived more easily into adipo-, osteo-, and chondro-genesis than USC. Therefore, we assumed that USC has both properties (ectodermal epithelial stem cell and mesodermal MSC), and the epithelial stem cell-like character effectively drive USC to differentiate into neuron, muscle and endothelium. Our data provide a useful guideline for selecting the appropriate type of cell source for use in clinical application.

DISCLOSURE

The authors have no potential conflicts of interest to disclose.

AUTHOR CONTRIBUTION

Conceived and designed the experiments: Kwon TG, Chun SY. Performed the experiments: Kang HS, Park GB. Analyzed the data: Choi SH. Drafting of the manuscript: Kang HS, Chun SY. Critical revision of the manuscript for important intellectual content: Kim BS, Kwon TG. Statistical analysis: Choi JY. Receiving grant: Chun SY. Administrative, technical, or material support: Choi JY, Choi SH. Approval of final manuscript: Chun SY.

ORCID

Hye Suk Kang <http://orcid.org/0000-0001-7568-3211>
Seock Hwan Choi <http://orcid.org/0000-0003-3796-2601>
Bum Soo Kim <http://orcid.org/0000-0002-4873-3049>
Jae Young Choi <http://orcid.org/0000-0002-3611-3926>
Gang-Baek Park <http://orcid.org/0000-0001-6708-2256>
Tae Gyun Kwon <http://orcid.org/0000-0002-4390-0952>
So Young Chun <http://orcid.org/0000-0003-4500-4956>

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