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Original Article

Characterization of stage-specific embryonic antigen-4 (SSEA-4)-positive very small embryonic-like stem cells isolated from human Wharton's jelly

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Abstract: Objective: Recent studies have demonstrated that several subpopulations of adult stem cells display pluripotent properties. Methods: In this study, we isolated and characterized a small population of human umbilical cord mesenchymal stem cells (hUC-MSCs) expressing stage-specific embryonic antigen-4 (SSEA-4). Results: These cells expressed multiple stem cell markers including CD105, CD90, CD73, Oct4, Nanog, Sox2, and SSEA-4. These cells had a high ability to self-renew and form embryoid bodies in suspension culture. Most interestingly, a minor proportion of SSEA-4$^+$ cells could spontaneously differentiate into cell types of all three germ layers in vitro. However, SSEA-4$^-$ cells did not show these properties. The comparison of osteogenic and adipogenic differentiation ability showed that SSEA-4$^+$ cells were easier to differentiate into osteogenic and adipogenic cells than SSEA-4$^-$ cells. Conclusions: In conclusion, SSEA-4$^+$ cells derived from hUC-MSCs have the possibility acting as an excellent source for multipotent stem cells. SSEA-4$^+$ cells may serve as a promising source for cell-based therapies.

Keywords: Characterization, differentiation, mesenchymal stem cells, umbilical cord

Introduction

Mesenchymal stem cells (MSCs) have the capacity to self-renew and differentiate into various lineages including bone, adipocytes, osteoblasts, cardiomyocytes, as well as sperm-like cells [1, 2]. Human umbilical cord provides an important source of MSCs [3]. It has been documented that a subpopulation of human marrow stromal cells can be differentiated in vitro into cell lineages derived from all three germ layers [4]. Several other studies further reported that a rare population of multipotent cells with pluripotent properties can be isolated from human skin fibroblasts and bone marrow [5-7]. These cells express pluripotency markers and CD105. Very small embryonic stem-like cells (VSELs) have been detected in human bone marrow and cord blood [8, 9]. Many proteins have been suggested to serve as markers of VSELs, including OCT4, CXCR4, SSEA-1, and SSEA-4 [10, 11]. SSEA-4 was shown to be an efficient marker for enriching VSELs from bone marrow of acute myocardial infarction patients [12]. However, it is still unclear whether VSELs exist in human umbilical cord-derived MSCs (hUC-MSCs) and whether SSEA-4 can be served as a marker to isolate them from hUC-MSCs.

In the present study, SSEA-4$^+$ hUC-MSCs were separated by magnetic-activated cell sorting (MACS) and examined for the multipotency. It was revealed that SSEA-4$^+$ cells expressed typical mesenchymal markers CD105, CD90, CD73 and CD29, as well as pluripotent stem cell markers Nanog, Oct4, and Sox2. They spontaneously differentiated into cells representative of all three germ layers but did not form teratomas.

Materials and methods

Ethical statement

The study was approved by the Ethical Committee of Jiangsu University (Zhenjiang, China; approval no.: 2012258).

Table 1. The sequences of primers used in PCR

Gene	Primers (5'-3')
β-Actin	Forward: GCGGCATCCACGAAACTAC
	Reverse: TGATCTCCTTCTGCATCCTGTC
Oct4	Forward: CCACACTCGGACCACGTCTT
Sox2	Forward: CTATGACCAGCTCGCAGA
	Reverse: GGAAGAAGAGGTAACCACG
Nanog	Forward: GATTCTTCTACCAGTCCCAAAC
	Reverse: CTGTCTCTCCTCTTCCCTCCTC
C-myc	Forward: CACGCTGACCAAGGTAT
	Reverse: CTGAGGTGGTTCATACTGA
AFP	Forward: AGAACCTGTCACAAGCTGTG
	Reverse: GACAGCAAGCTGAGGATGTC
β-III-tubulin	Forward: CTTTTGGCCAGATCTTTAGACC
	Reverse: CTCGTTGTCAATGCAATAGGTC
Desmin	CCTACTCTGCCCTCAACTTC
	AGTATCCCAACACCCTGCTC

Cell isolation and culture

Umbilical cords were obtained after full-term birth with the consent of parents (n = 18). Umbilical cords were stored aseptically in cold Hanks' balanced salt solution, and cellular isolation was performed within 12 h of collection. hUC-MSC were isolated as previously described [13]. Briefly, after the removal of blood vessels, the cord pieces were centrifuged at 250 g for 5 min and washed twice with phosphate-buffered saline (PBS). The cord pieces were cultured in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 20 ng/ml EGF (Sigma), 10 ng/ml bFGF (Sigma), 2 mM L-glutamine (Gibco), and 100 IU/ml penicillin/streptomycin at 37°C in a 5% CO_2 incubator. When the cells grew to 80% confluency, hUC-MSC were digested with 0.25% trypsin, centrifuged and subcultured at a dilution of 1:3. Thereafter, the medium was changed every two days.

Enrichment of SSEA-4$^+$ hUC-MSCs using MACS

SSEA-4$^+$ cells were isolated as previously described [14]. For cell sorting, hUC-MSC at the first passage (P1) were dissociated into a single-cell suspension. Cells were centrifuged at 300 g for 5 min and resuspended in ice-cold PBS with 0.5% bovine serum albumin (BSA), and 2 mM EDTA. Anti-SSEA-4 Microbeads (MiltenyiBiotec) were added per 10^7 total cells and incubated for 15 min at 4°C. AMACS column was placed in the magnetic field of a suitable MACS Separator and prepared by rinsing with 3 ml of buffer. The column was washed three times with 3 ml of buffer. The flow-through of unlabeled cells (SSEA-4$^-$ cells) was collected. The magnetically labeled cells (SSEA-4$^+$ cells) were flushed out by firmly pushing the plunger into the column. SSEA-4$^+$ and SSEA-4$^-$ cells were cultured in medium containing DMEM/F12 (1:1) supplemented with 15% knockout serum replacement (KSR; Gibco, USA), B-27 (Gibco), 20 ng/ml EGF (Sigma), 10 ng/ml bFGF (Sigma), 2 mM L-glutamine (Gibco), and 100 IU/ml penicillin/streptomycin.

Flow cytometric analysis

The DNA content and surface antigen expression of cells was examined by flow cytometry. To detect the DNA content, cells were digested, collected, washed twice with PBS, and fixed with 1 ml of 70% of ice-cold ethanol at 4°C for at least 12 h. Cells were then washed twice with PBS, resuspended in 0.5 ml of PBS containing propidium iodide (PI) and RNase A at a final concentration of 50 mg/ml, and incubated for 30 min at 37°C. For determination of phenotypes of cell surface antigens, cells were detached and stained with fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated antibodies (Becton Dickinson) against CD73, CD105, CD44, CD71, CD90 and CD29, according to the manufacturer's instructions. The binding of primary antibodies was detected using anti-mouse immunoglobulin G (IgG) conjugates (Becton Dickinson). To detect the expression of Oct4, cells were detached and stained with Anti-Oct4 (Alexa Flour 488 conjugate). Cells were analyzed using a flow cytometer.

RNA extraction and reverse-transcription PCR (RT-PCR) analysis

Total RNA was extracted from cells using the RNAprep Pure Micro Kit (Qiagen). First-strand cDNA was generated using the PrimeScript RT Perfect Real Time reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. PCR reactions were performed using Ex Taq DNA polymerase (TaKaRa). The PCR primers and length of the amplified products are shown in **Table 1**.

Quantitative real-time PCR (qRT-PCR) analysis

Real-time PCR was performed using the ABI Step One Plus PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex TaqII kits (Takara). The qRT-PCR reactions were set

up in 25-μl reaction mixtures containing 12.5 μl of 1 × SYBR Premix Ex Taq (TaKaRa), 0.5 μl of sense primer, 0.5 μl of antisense primer, 11 μl of distilled water, and 0.5 μl of template. The reaction conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 58°C for 30 s. All of the data were normalized to β-actin. The primers used for qRT-PCR analysis are listed in **Table 1**.

Cell size analysis

Cell size analyses were performed using the CASY TT cell counter (Innovatis-Roche). An aliquot of the cell suspension was mixed with the buffer, and the cell number and size were determined according to manufacturer's instructions. The CASY TT cell counter can determine the complete cell number, the ratio of cells with damaged cell membranes and the mean volume of cells.

Alkaline phosphatase staining

Cells were fixed in 4% PFA for 15 min and washed at least three times with PBS. Staining was performed at room temperature in the dark using the 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) Alkaline Phosphatase Color Development Kit (Beyotime, Jiangsu, P. R. China) according to the manufacturer's instructions.

Immunofluorescence staining

Cells were fixed in 4% PFA for 15 min, permeabilized with 0.1% Triton X-100 for 30 min, and then incubated with blocking solution (PBS+1% BSA) at 4°C for 30 min followed by incubation with primary antibodies at 4°C overnight. Primary antibodies were diluted in Immunol Fluorescence Staining Primary Antibody Dilution Solution (Beyotime, P0108) at the followings ratios: Oct4 (1:500, Abcam), Sox2 (1:500, Abcam), Nanog (1:500, Abcam), SSEA-4 (1:500, Abcam), β-III-tubulin (1:500, Chemicon), AFP (1:500, Chemicon), and α-actin (1:200, Sigma). After washing three times with PBS, cells were incubated with the appropriate secondary antibodies for 1 h at room temperature in the dark. Finally, DNA was stained with Hoechst33342 (Beyotime, C1005) for 3 min. Negative controls were only stained with secondary antibodies.

Lineage differentiation in vitro and teratoma formation

Expression of the following germ layers markers was analyzed by RT-PCR: β-III-tubulin (ectoderm), desmin (mesoderm), and alpha-fetoprotein (AFP; endoderm). To assess differentiation of the three germ layers, SSEA-4$^+$ cells were seeded in gelatin-coated 24-well culture tissue plates, and the medium was changed every 2 days. After 14 days, the three germ layer markers (β-III-tubulin (ectoderm), AFP (endoderm), α-actin (mesoderm)) were examined by immunofluorescence. SSEA-4$^+$ cells have been cultured under conditions for adipocyte, osteoblast, and cartilage differentiation in previous studies [15, 16].

Karyotype analysis

Briefly, cells grown to 70-80% confluence were treated for 2.5 h with 0.1 μg/ml colchicine (Sigma), digested with 0.25% trypsin solution, resuspended in 0.075 mol/L KCl and incubated at 37°C for 20 min before fixation with methanol and acetic acid (3:1) for 5 min. After centrifugation, the cells were resuspended, spread on slides, stained with Giemsa dye, and air-dried. Next, they were observed and analyzed using a Leica microscope.

Osteogenic and adipogenic differentiation in vitro

hUC-MSCs and SSEA-4$^+$ hUC-MSC were seeded at 5000 cells for Osteogenic differentiation and 2 × 10^5 for adipogenic differentiation. They were cultured for 2 weeks in DMEM with 10% FBS, 1% antibiotics and osteogenic supplement (0.1 mM dexamethasone, 10 mM b-glycerophosphate, 50 mg/L ascorbic acid) or adipogenic differentiation medium (GUXMX-90031, Cyagen Biosciences, CA, USA) according to the manufacturer's instructions. At the end of the experiment, cells were tested for osteogenic or adipogenic differentiation by measuring Osterix, Osteopontin, ALP and Adiponectin expression.

Animal experiments

Adult female Sprague-Dawley rats (weighing 220 ± 20 g) were purchased from the Animal Centre of Chinese Academy of Sciences (Shanghai, China). Rat model of skin deep second-degree burn wound was established as described previously with added modifications [17]. Rats were anesthetized with sodium thiopental at a dose of 40 mg/kg body weight. After the hair on their upper back was shaved, the back skin of rats were injured with 80°C water for 8 s to create a 16 mm diameter wound, then

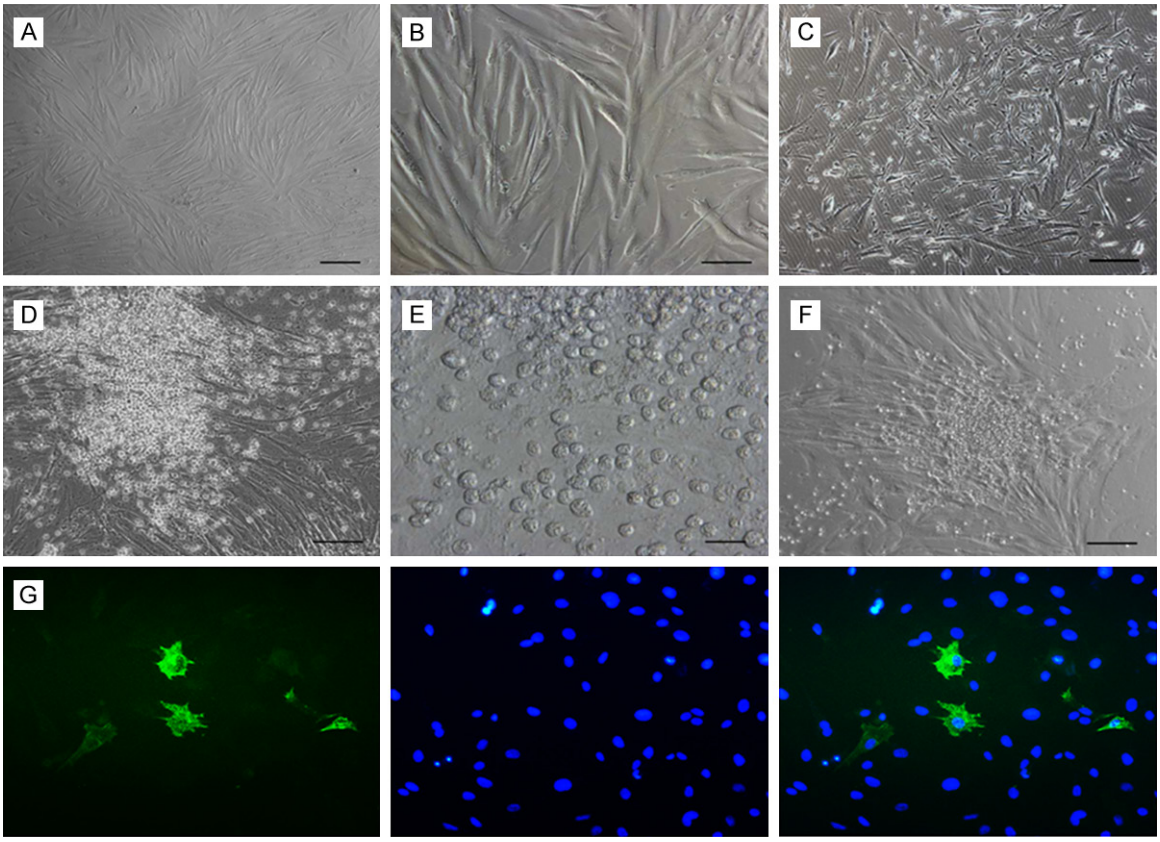

Figure 1. Small cells existed in Wharton's Jelly of human umblilical cord-derived cells. A, B. The morphology of hUC-MSCs cultured in vitro at P1. C. Small cell proliferation among thawing cryopreserved hUC-MSCs at P1. D, E. Small round cells grew on the top of hUC-MSCs. F. Clones formed spontaneously by small cells in adherent culture of naive hUC-MSCs. G. Immunocytochemical detection of SSEA-4 expression (green, left) among hUC-MSCs. Nuclei were counterstained with DAPI (middle). Right, merged image. A, C, D. Scale bar, 100 μm; B, F. Scale bar, 50 μm; E. Scale bar, 20 μm.

covered with gauze soaking saline for 6 min on the wound. Meanwhile, 1×10^6 cells (hUC-MSC, SSEA-4$^-$ cells and SSEA-4$^+$ cells) suspended in 200 μl PBS, or 200 μl PBS were injected subcutaneously at three sites. The normal group had no treatment. The animals were housed individually. At 1 week after treatment, the rats were sacrificed and the wound area was cut for further analysis. The wound skin and surrounding skin (4 mm^2) were fixed in 4% paraformaldehyde (pH 7.4), and gradually dehydrated, embedded in paraffin, cut into 4-μm sections and stained with hematoxylin and eosin (H&E).

Statistical analysis

Using SPSS 18.0 statistical software (IBM Corporation, Somers, NY, USA), the data were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) tests and were reported as means ± SEM. For all of the analyses, a *P* value less than 0.05 was considered statistically significant.

Results

Isolation and culture of hUC-MSCs

Primary hUC-MSCs grew to 70-80% confluence at 12 days using the culture conditions described in our report. The results reported were representative of the results obtained using 18 umbilical cords, and no significant differences were observed between samples in terms of cell numbers or viability. The hUC-MSCs appeared as typical fibroblasts with unique phenotypes, and most of the cells exhibited mononuclear fibroblast morphology (**Figure 1A**, **1B**). hUC-MSCs proliferated up to passage 20 and maintained normal phenotypes.

Small cells existed in hUC-MSCs

In the process of culturing hUC-MSCs, we found that some small cells existed in hUC-MSCs. These cells were much more proliferative than

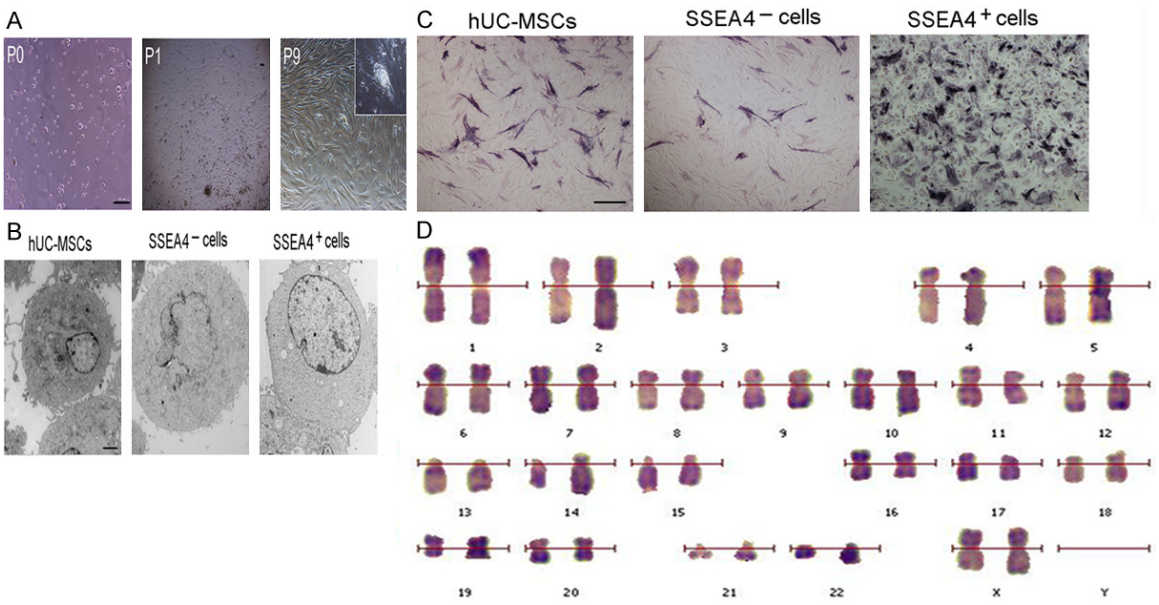

Figure 2. Characterization SSEA-4$^+$ cells from hUC-MSCs. A. The morphology of SSEA-4$^+$ cells derived from hUC-MSCsby MACS. B. TEM of hUC-MSCs, SSEA-4$^-$ and SSEA-4$^+$ cells at P3. C. AP staining of hUC-MSCs at P1, SSEA-4$^-$ cells and SSEA-4$^+$ cells at P3. D. The karyotype of SSEA-4$^+$ cells at P18. A. Scale bar, 100 μm; B. Scale bar, 20 μm; C. Scale bar, 2 μm.

hUC-MSCs and could form cell clones. The primary hUC-MSCs is consisted of a heterogeneous cell population. When the cryopreserved hUC-MSCs were thawed, small cells were easily discovered (**Figure 1C**). Although the majority of cells exhibited typical spindle-shaped fibroblast phenotypes, a few of the cells were small and exhibited different phenotypes (**Figure 1D**). When these cells were further cultured on plastic plates, some floating cells grew on top of the adherent cells (**Figure 1D**, **1E**). When naive hUC-MSCs grew in adherent culture, colonies (**Figure 1F**) spontaneously formed by the small cells at a low frequency, which appeared similarly to the colony formed by ES cells at an early stage.

Interestingly, we found these small cells highly express SSEA-4. SSEA-4 is a cell surface glycosphingolipid considered as an embryonic/pluripotency marker. Overlaying phase contrast and SSEA-4 immunofluorescence images revealed that the SSEA-4 expression was detected across the entire cell surface (**Figure 1G**). These results indicated that the heterogeneous population of hUC-MSC may contain a multipotent subpopulation of cells.

Characterization of SSEA-4$^+$ hUC-MSC

SSEA-4$^+$ cells were isolated from hUC-MSCs at P1-P3 by MACS. We observed that SSEA-4$^+$ cells contained a large amount of small cells and spontaneously formed typical cell clones when grown in adherent culture (**Figure 2A**). The nuclei of SSEA-4$^+$ cells were relatively large, though the SSEA-4$^+$ cells were smaller than hUC-MSCs and SSEA-4$^-$ cells, with diameters of 8-12 μm (**Figure 2B**). Additionally, SSEA-4$^+$ cells showed a polygonal-shaped morphology and were positive for AP staining, however, AP were rarely expressed in hUC-MSC (**Figure 2C**). SSEA-4$^+$ cells at the 18th passage showed a normal karyotype containing 46 chromosomes (46, XX) with no acquired numerical or structural aberrations or chromosome/chromatid breaks (**Figure 2D**).

Stem cell markers expressed in SSEA-4$^+$ hUC-MSC

Under suspension culture conditions, SSEA-4$^+$ cells formed embryoid bodies (EBs) at 7 days with the size of approximately 50-100 μm. Immunofluorescence analysis demonstrated that cell clusters from SSEA-4$^+$ cells were consistently positive for the pluripotent like markers OCT4, NANOG, SOX2, and SSEA-4 (**Figure 3A**). RT-PCR analysis also showed strong expression of Nanog, Oct4, Sox2 and C-myc in SSEA-4$^+$ cells, which was higher than total hUC-MSCs (**Figure 3B**). qRT-PCR analysis further confirmed that SSEA-4$^+$ cells expressed higher levels of Oct4, Nanog and Sox2 than SSEA-4$^-$

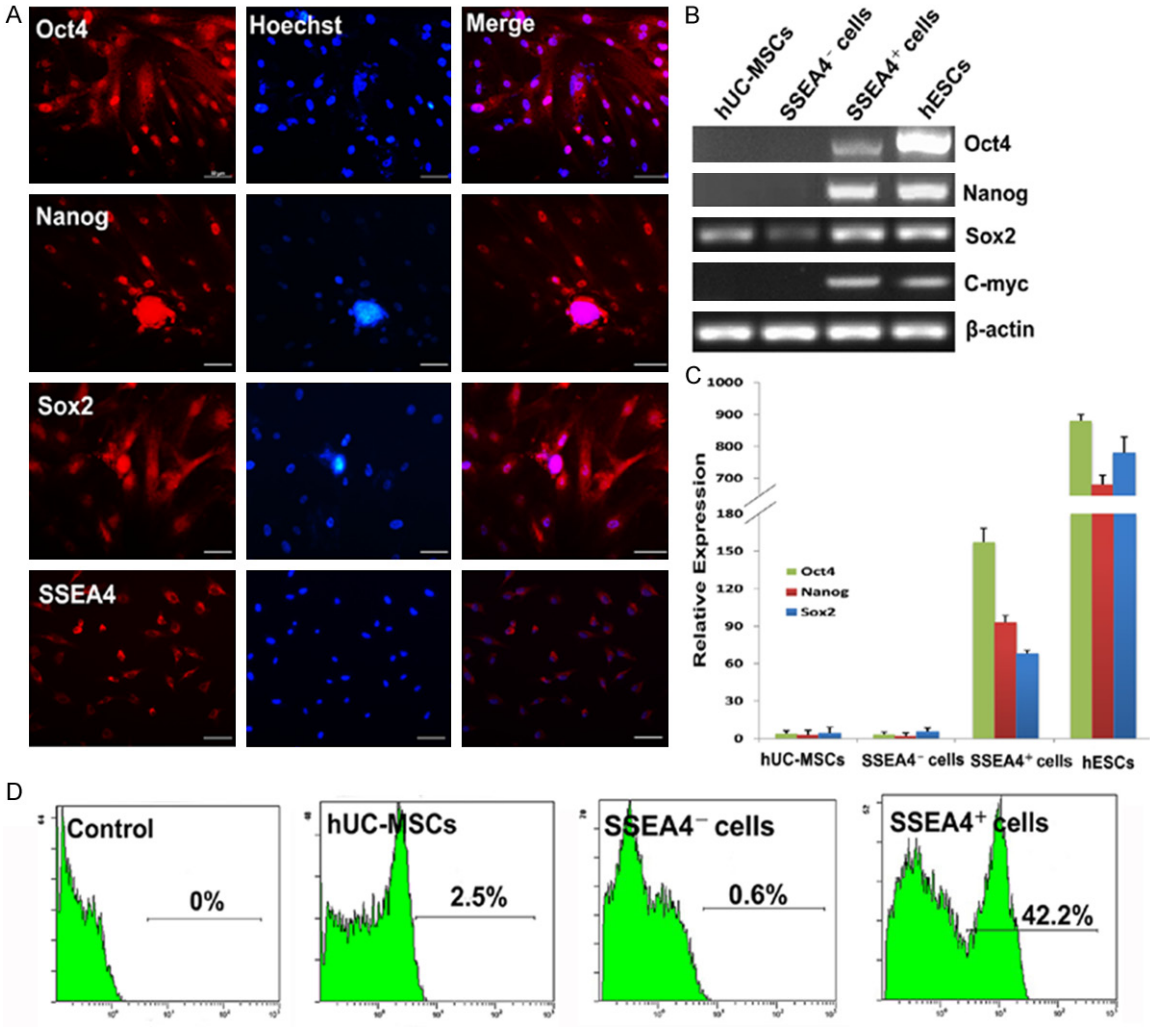

Figure 3. Multipotenty of SSEA-4$^+$ hUC-MSCs. A. Immunofluorescence assay of SSEA-4$^+$ cells at P8. Oct4, Nanog, Sox2 and SSEA-4 were labeled with Alexa Fluor 555 (red). DNA was stained with Hoechst33342 (blue). Scale bar, 20 μm. B. RT-PCR analysis of Oct4, Nanog, Sox2 and C-myc expression in hUC-MSCs at P1, SSEA-4$^-$ cells and SSEA-4$^+$ cells at P3. C. The relative expression of Oct4, Nanog, and Sox2 in hUC-MSCs at P1, SSEA-4$^-$ cells, SSEA-4$^+$ cells at P3, and hESCs (H19). D. Oct4-positive cells among hUC-MSCs at P1, SSEA-4$^-$ cells and SSEA-4$^+$ cells at P3 were analyzed by flow cytometry.

cells (**Figure 3C**). Flow cytometry analysis showed that the Oct4 positive rates of hUC-MSC, SSEA-4$^-$ cells, and SSEA-4$^+$ cells at P1 were 2.5%, 0.6%, and 42.2%, respectively (**Figure 3D**). Under adherent culture conditions, SSEA-4$^+$ cells at P12 showed MSC phenotype with strong expression of CD90, CD105, CD73, CD29, and matrix receptor CD44 (**Figure 4**).

SSEA-4$^+$ hUC-MSC show greater proliferation capacity than total hUC-MSCs

The percentages of S-phase SSEA-4$^+$ cells and hUC-MSC were 50.58% and 28.01%, respectively (**Figure 5A**). Furthermore, the cell growth curve assay showed that SSEA-4$^+$ cells underwent strong proliferation in vitro (**Figure 5B**).

SSEA-4$^+$ cells have the ability to differentiate into various cell types

To assess the ability of SSEA-4$^+$ cells to differentiate in vitro, cells were cultured under various conditions to induce adipocyte, osteocyte, or chondrocyte differentiation. In the adipogenic induction medium, lipid droplets appeared in the cytoplasm after one week and stained positive with Oil red O. When cultured under osteo-

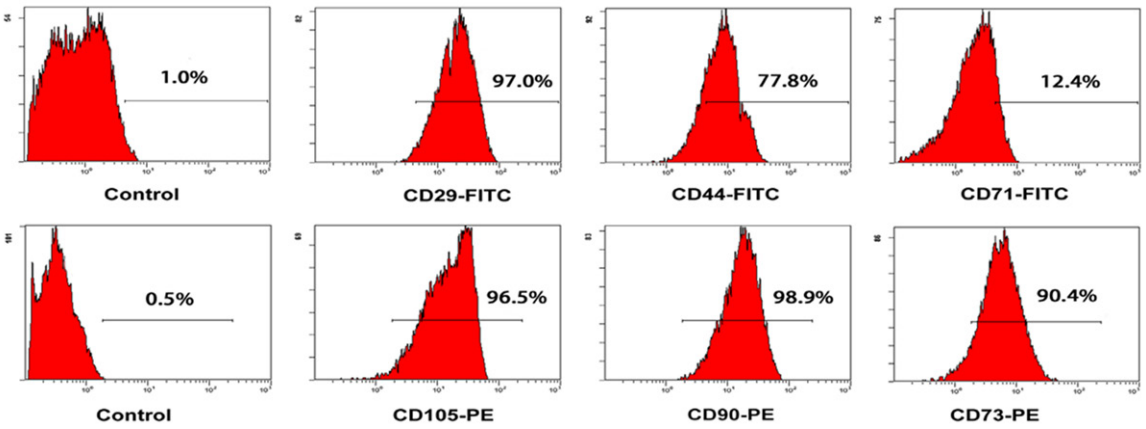

Figure 4. Flow cytometry analysis of SSEA-4$^+$ hUC-MSCs. The expression of cell surface antigens was examined by flow cytometry. Antibodies against CD29 (97.0%), CD44 (77.8) and CD71 (12.4%) were conjugated with FITC, and antibodies against CD105 (96.5%), CD90 (98.9%), and CD73 (90.4%) were conjugated with PE.

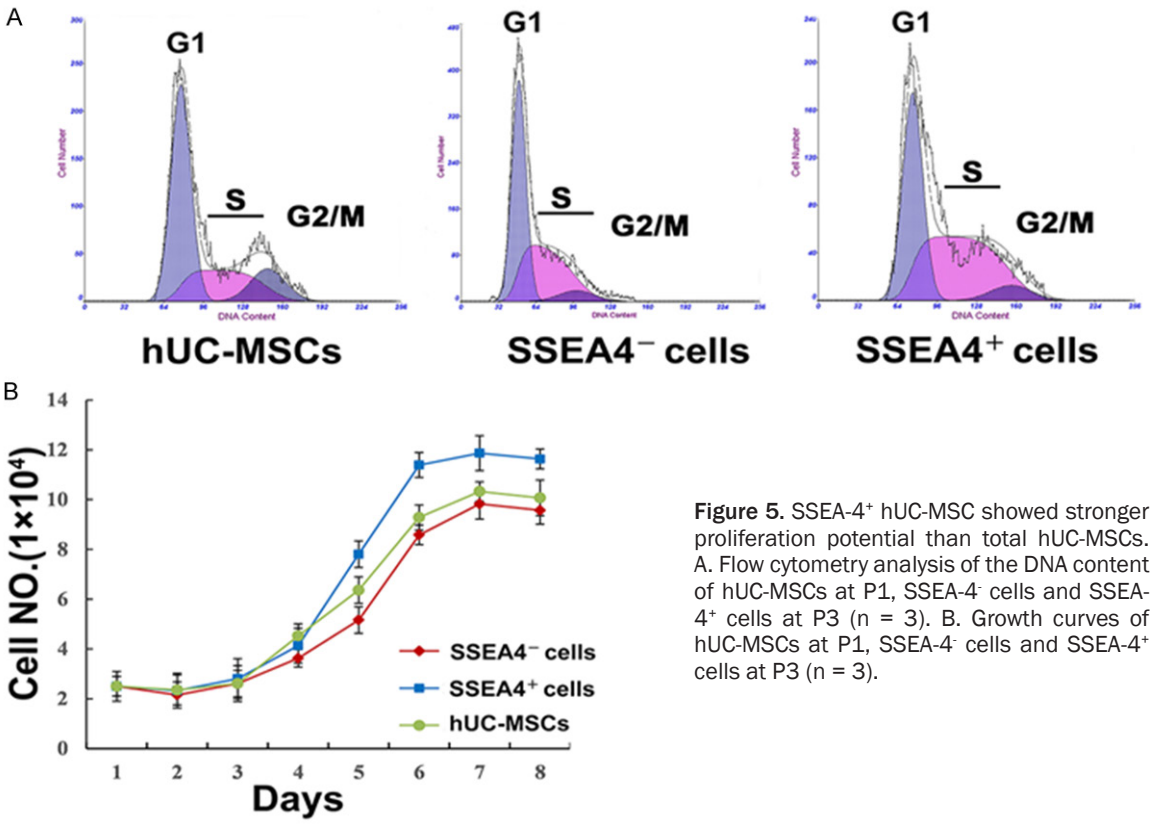

Figure 5. SSEA-4$^+$ hUC-MSC showed stronger proliferation potential than total hUC-MSCs. A. Flow cytometry analysis of the DNA content of hUC-MSCs at P1, SSEA-4$^-$ cells and SSEA-4$^+$ cells at P3 (n = 3). B. Growth curves of hUC-MSCs at P1, SSEA-4$^-$ cells and SSEA-4$^+$ cells at P3 (n = 3).

genic conditions, SSEA-4$^+$ cells acquired an osteoblastic phenotype with the deposition of a calcium-rich, mineralized extracellular matrix, as revealed by Alizarin Red staining. SSEA-4$^+$ cells cultured as pellets under chondrogenic conditions stained positive for Alcian blue (**Figure 6A**). To further address the differentiation potential of SSEA-4$^+$ cells, cell clusters at P1 and P3 were transferred onto gelatin-coated dishes. The results revealed that EBs had spontaneously differentiated into the three types of germ layers and expressed the corresponding specific markers, including β-III-tubulin (ectoderm), AFP (endoderm), α-actin and desmin (mesoderm) (**Figure 6B**). RT-PCR analysis also confirmed that induced cells expressed mark-

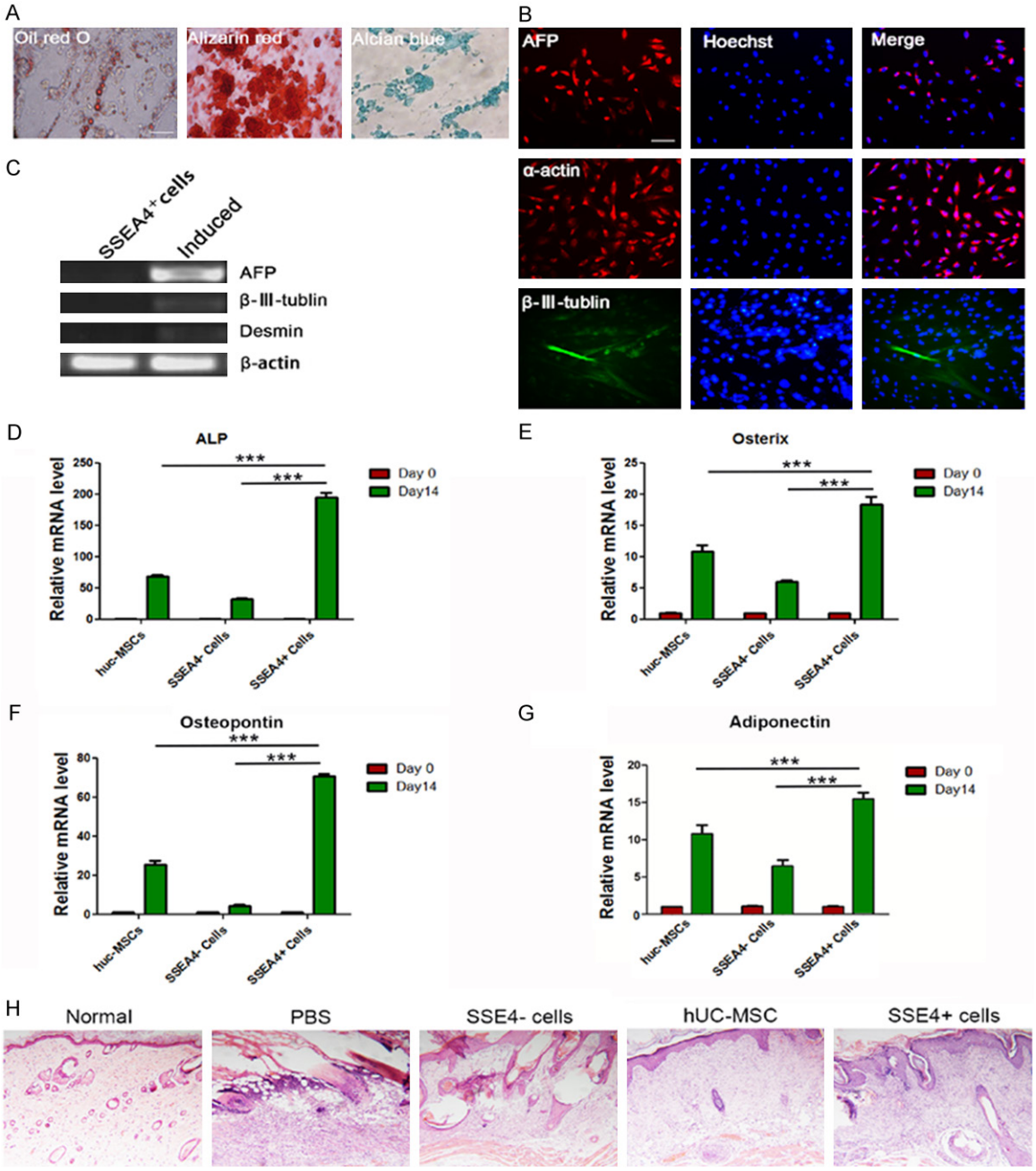

Figure 6. The differentiation potential of SSEA-4$^+$ hUC-MSCs. A. *In vitro* differentiation of SSEA-4$^+$ hUC-MSC at P12 into adipocytes, osteocytes, and chondrocytes assayed using Oil red O staining for adipogenesis, Alizarin Red staining for osteogenesis, and Alcian blue staining for chondrogenesis. B. SSEA-4$^+$ hUC-MSC spontaneously differentiated into the three germ layers. Immunofluorescence was performed to analyze the three types of germ layers using special markers, including β-III-tubulin (ectoderm), AFP (endoderm) andα-actin (mesoderm). C. RT-PCR assay for the three germ layer-specific markers AFP, β-III-tubulin and desmin. Scale bar, 50 μm. D-G. qRT-PCR assay for the expression of osteogenic and adipogenic differentiation makers (Osterix, Osteopontin, ALP and Adiponectin). H. Representative micrographs of wounds (H&E staining) at 1 week following treatment with hUC-MSC, SSE4$^-$ cells, SSEA-4$^+$ cells or the same volume of PBS. Scale bar, 100 μm.

ers specific for the three germ layers including the ectodermal marker, β-III-tubulin, the mesodermal markers, Desmin, and the endodermal markers, AFP (**Figure 6C**).

All above results confirmed that SSEA-4$^+$ cells hold the pluripotent characteristics. However, the molecular characterization of the lineage-specific markers is superficial and the advan-

tage of SSEA-4$^+$ cells was not reflected. Thus, we compared the capacity of osteogenic and adipogenic differentiation. The results showed that the expression of osteogenic index, Osterix, Osteopontin and ALP, was higher than total hUC-MSC cells and SSEA-4$^-$ cells (**Figure 6D-F**). Adipogenic marker (Adiponectin) also showed similar results (**Figure 6G**). Our previous work has shown that hUC-MSC and its exosome could enhance repair of skin burn [18, 19]. So we hypothesized that SSEA-4$^+$ cells may also induce cutaneous wound healing. We established a rat deep second-degree burn injury model and infused hUC-MSC cells, SSEA-4$^-$ cells and SSEA-4$^+$ cells into the injured rats, separately. The results of histological evaluation of wounds at 1 week post-infusion showed that the number of epidermal and dermal cells significantly increased in hUC-MSC cells and SSEA-4$^+$ cells treated wounds (**Figure 6H**), while wounds that were treated with PBS were still in second-degree burn injury state (**Figure 6H**). This preliminary result suggested that SSEA-4$^+$ cells may be an ideal stem cells source for regenerative medicine for skin wound healing.

Discussion

In this study, hUC-MSCs at P0-P2 represented a heterogeneous cell population. They showed a fibroblast-like morphology when cultured over three passages in vitro. Previous report has showed that culture conditions influence multipotent differentiation capacity [20]. To investigate the early mesenchymal population present among hUC-MSCs, we cultured hUC-MSCs in medium containing 20 ng/ml EGF, 10 ng/ml bFGF, and 2 mM L-glutamine, which was suitable for pluripotent stem cell survival. The culture conditions likely contributed to small cells survival and affected the phenotype of MSCs in the present study. We observed that small cells existed in early hUC-MSCs. They could form colonies at a low frequency. Two types of small stem cells have been identified in previous studies, spore-like cells and very small embryonic-like stem cells (VSELs). Spore-like cells with greater than 50% nucleic acids by volume are present in all tissues. They can proliferate and differentiate into any cell type under extreme conditions such as low oxygen and high or low temperatures [21]. VSELs exist in many types of adult tissues and may play an important role in tissue homeostasis [22]. Using flow cytometric analysis, we also found that mesenchymal cells isolated from the umbilical cord consisted of two main populations when expanded in vitro. Both of these populations express a high level of matrix markers (CD73, CD105) and a low level of hematopoietic markers (CD34, CD45). In contrast, VSELs in human umbilical cord blood have been shown to be CD34 positive [23]. Therefore, the small cells in hUC-MSCs resistant to freeze-thawing that were similar to the spore-like cells in previous report that remained viable after being frozen and then thawed. These spore-like cells were also regarded as VSELs [24].

SSEA-4 was previously shown to be expressed in a small subset of cells from the human fetal forebrain and forebrain-derived neurosphere cells [25]. Virant-Klun et al. confirmed that SSEA-4-positive cells isolated from human adult ovaries showed the potential embryonic-like character of small putative stem cells [26]. Amiri F *et al.* isolated MSCs from umbilical cord tissue at the single-cell level, by treatment with trypsin, followed by cultivation under suspension conditions to form a colony. These cells which formed the colony expressed SSEA-3 and other stem markers. But they did not detect the expression of SSEA-4 [27]. Many other studies reported that SSEA-4-positive cells can be isolated from umbilical cord. Different from our results, He H *et al.* considered that SSEA-4-positive MSCs is not a marker for proliferation and multipotency [28]. South Korean scholars isolated multipotent mesenchymal stem cells from canine umbilical cord matrix by collagenase digestion, which expressed pluripotency markers such as Oct3/4, Nanog, Sox-2 and SSEA-4. However, the morphology of these cells were plastic adherent, spindle-shaped and fibroblast-like cells rather than small round cell in our study [29]. Gonzalez R *et al.* reported an efficient approach to isolation and characterization of pre- and postnatal umbilical cord lining stem cells for clinical applications, which expressed Oct4, nanog and SSEA-4 [30]. All above studies showed that SSEA-4$^+$ cells can be isolated from umbilical cord, but few researches explored whether SSEA-4 can be a marker for isolating pluripotent-like cells and further prove its role in wound healing. In the current study, we demonstrated that hUC-MSCs contained a small percentage of stem cells expressing SSEA-4 with the capacity for triplo-

blastic differentiation and self-renewal. In particular, SSEA-4$^+$ cells were small, with a high nuclear/cytoplasmic ratio, and were positive for AP. These SSEA-4$^+$ cells could also form EBs in suspension culture, and they were consistently positive for the pluripotent stem cell markers Nanog, Oct4, Sox2, and SSEA-4. MSCs have been reported to express certain pluripotency markers characteristic of ES cells, including the transcription factor Oct-4 [31]. However, most hUC-MSCs express Oct4 in the cytoplasm and only rarely in the nucleus. Upon continued culture, SSEA-4$^+$ cells expressed higher levels of these markers, and they could differentiate into adipocytes, osteocytes, and chondrocytes, as well as cells comprising the three germ layers (endoderm, mesoderm, and ectoderm). In addition, the morphology of undifferentiated SSEA-4$^+$ cells differed from that of MSCs, whereas differentiated SSEA-4$^+$ cells were morphologically similar to MSCs. Previous reports have shown that stem cells derived from the skin could be isolated via the selective growth of floating spheres [5, 32]. In this study, it was difficult to ensure that SSEA-4$^+$ cells would remain undifferentiated in adherent culture. Overall, our results showed that SSEA-4$^+$ cells represented a rare population of stem cells among hUC-MSCs and that these cells exhibited a very primitive morphology, a relatively small size and characteristics of pluripotency. Thus, SSEA-4$^+$ cells represent an early developmental cell population from hUC-MSCs. These multi-potent cells are thought to be deposited early during development in the bone marrow as a mobile pool of circulating pluripotent like stem cells that play a crucial role in postnatal tissue turnover and regeneration, in both non-hematopoietic and hematopoietic tissues. In our study, we evaluated the role of SSEA-4$^+$ cells in skin injury repair by a rat burn model. The results showed that SSEA-4$^+$ cells promoted cutaneous wound healing. However, the related mechanism needs to be further clarified.

In conclusion, our results show that SSEA-4$^+$ cells with the capacity for differentiation into cells of the three germ layers can be obtained from accessible hUC-MSCs without the requirement for exogenous genes. Thus, SSEA-4$^+$ cells may serve as an excellent source for cell-based therapies and may promote the effectiveness of current hUC-MSC transplantation to replenish lost cells for tissue repair.

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Disclosure of conflict of interest

None.

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