AsiaCORD 2013

"New Perspectives in Mesenchymal Stem Cell Research"

Date April 19(Fri)-20(Sat), 2013
Venue Kobe Portopia Hotel, Kobe, Japan
President Shigetaka Asano

Day 1

Workshop: Progress in CBT

WS1. Current Status of CBT
Chairs: Hideki Kodo, Kaiyan Liu, Satoshi Takahashi
Satoshi Takahashi, Jun Ooi & Makoto Ootsu *(The Institute of Medical Science, The University of Tokyo)*
Shuichi Taniguchi *(Toranomon Hospital)*
Tomohiro Morio *(Tokyo Medical and Dental University)*
Kaiyan Liu *(Peking University People's Hospital)*
Satoshi Yanagida, Hirokazu Kurata & Shigetaka Asano *(Central Research Laboratories Sysmex Corporation)*

WS2. Quality Control of Public CBB
Chairs: Kai-Hsin Lin, Tokiko Nagamura, Il-Hoan Oh
Young-Ho Lee *(Hanyang University Medical Center)*
Kai-Hsin Lin *(National Taiwan University Hospital)*
Il-Hoan Oh *(Catholic High Performance Cell Therapy Center)*
Tokiko Nagamura *(The Institute of Medical Science, The University of Tokyo)*
Hong Hoe Koo *(Sungkyunkwan University School of Medicine & KoreaCORD)*
Soisaang Phikulsod *(National Blood Centre, The Thai Red Cross Society)*

– Open Discussion on Future of CBT –
Discussants: Po-Min Chen, Hong Hoe Koo, Kaiyan Liu, Keiya Ozawa

Symposium I: New Insights into Stem Cell Biology

1. Stem Cell Development & Differentiation
Chairs: Tzeon-Jye Chiou, Mari Dezawa, Young-Joon Kim, Il-Hoan Oh
Yi-Chin Toh *(Institute of Bioengineering and Nanotechnology)*
Mari Dezawa *(Tohoku University Graduate School of Medicine)*
Masataka Kasai *(The University of Tokyo)*
Naoyuki Yao *(Kyoto University Hospital)*
Satoshi Yoshioka *(Kyoto University Hospital)*

2. Interactions between TSC/LSC & Stromal Cells
Chairs: Po-Min Chen, Hideo Harigae, Surapol Issaragrisil, Arinobu Tojo
Hideo Harigae *(Tohoku University Graduate School of Medicine)*
Yasuo Miura *(Kyoto University Hospital)*
Luc Gailhouste & Takahiro Ochiya *(National Cancer Center Research Institute)*

Day 2

3. Epigenetic Regulation
Chairs: Masaaki Muramatsu, Akihiro Umezawa
Young-Joon Kim *(Yonsei University)*
Akihiro Umezawa *(National Center for Child Health and Development)*
Noriko Sato *(Tokyo Medical and Dental University)*
Jun Ohgane *(Meiji University)*

– Discussion on Future of Stem Cell Biology –
Discussants: Yong-Mook Choi, Surapol Issaragrisil, Yuzuru Kanakura

Symposium II: Basic & Clinical Research of MSC

1. Current Status of MSCT
Chairs: Kaiyan Liu, Keiya Ozawa, Akihiro Umezawa, Chandra Viswanathan
Zhong-Chao Han *(Chinese Academy of Medical Sciences & Peking Union of Medical College)*
Kazuo Muroi *(Jichi Medical University Hospital)*
Kang-Hsi Wu *(China Medical University Hospital)*
Liang Lu *(National Engineering Research Center of Cell Products/AmCellGene Co. Ltd)*

2. Significance of Semi-Public MSC Banking
Chairs: Yong-Mook Choi, Zhong-Chao Han, Shinichiro Okamoto, Akihiro Umezawa
Chandra Viswanathan *(Reliance Life Sciences Pvt. Ltd)*
Yukio Kato & Koichiro Tsuji *(Hiroshima University and Two Cells Co., Ltd.)*
You-Wei Wang *(Chinese Academy of Medical Sciences & Peking Union of Medical College)*
Wei-Chun Chen *(Steminent Biotherapeutics Inc.)*
Tokiko Nagamura & Haiping He *(The Institute of Medical Science, The University of Tokyo)*
Yutaka Hishiyama *(Ministry of Education, Culture, Sports, Science and Technology)*

– Discussion on Future of MSCT –
Discussants: Shigetaka Asano, Soon Keng Cheong, Surapol Issaragrisil, Hong Hoe Koo

General Comment: Keiya Ozawa & Yuzuru Kanakura

Sponsored by Strategic Network for Consolidated Research of Medical Sciences in Asia (CRMSA)
Organization for University Research Initiatives, WASEDA University
Advanced Institute for Environmental Science and Medical Engineering
Public Health Research Foundation

Secretariat: AsiaCORD2013 Managing Office
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April 19, 2013

9:00-10:40 **≪Workshop: Progress in CBT≫**

1. Current Status of CBT

Chairpersons; Hideki Kodo (JP), Kaiyan Liu (CN), Satoshi Takahashi (JP)

Satoshi Takahashi, Jun Ohi & Makoto Ostu (15')
Shuichi Taniguchi (15')
Tomohiro Morio (15')
Kai-Yan Liu(15')
Satoshi Yanagida, Hirokazu Kurata & Shigetaka Asano(15')

9:00-10:40

2. Quality Control of Public CBB

Chairpersons; Kai-Hsin Lin (TW), Tokiko Nagamura (JP), Il-Hoan Oh (KR)

Young-Ho Lee(15')
Kai-Hsin Lin(15')
Il-Hoan Oh(15')
Tokiko Nagamura(15')
Soisaang Phikulsod(15')

10:50-11:00 **≪Opening Remarks≫**

By Shigeteka Asano

11:00-11:50 ***-Open Discussion on Future of CBT-***

Discussants; Po-Min Chen (TW), Hong Hoe Koo (KR), Kaiyan Liu (CN), Keiya Ozawa (JP)

- Lunch -

13:00-15:00 **≪Symposium1: New Insights into Stem Cell Biology (1~3)≫**

1. Stem Cell Development& Differentiation

Chairpersons; Tzeon-Jye Chiou (TW), Mari Dezawa (JP), Young-Joon Kim (KR), Il-Hoan Oh (KR)

Masataka Kasai(30')
Yi-Chin Toh(30')
Mari Dezawa(30')
Hisayuki Yao (15')
Satoshi Yoshioka (15')

15:10-16:40

2. Interactions between TSC/LSC & Stromal Cells

Chairpersons; Po-Min Chen (TW), Hideo Harigae (JP), Surapol Issaragrisil (TH), Arinobu Tojo (JP)

Hideo Harigae(30')
Yasuo Miura(30')
Luc Gailhouste(30')

19:00-21:00 **- Welcome Reception: Dinner Cruise –**

April 20, 2013

9:00-9:30 **≪General Assembly≫**

9:45-11:25

3. Epigenetic Regulation

Chairpersons; Masaaki Muramatsu (JP), Akihiro Umezawa (JP)

Young-Joon Kim30')
Akihiro Umezawa(30')
Noriko Sato(20')
Jun Ohgane(20')

11:25-12:15 ***-Discussion on Future of Stem Cell Biology-***

Discussants; Yong-Mook Choi (KR), Surapol Issaragrisil (TH), Yuzuru Kanakura (JP)

- Lunch -

13:10-14:50 **≪Symposium 2: Basic & Clinical Research of MSC (1~2)≫**

1. Current Status of MSCT

Chairpersons; Kaiyan Liu (CN), Keiya Ozawa (JP), Akihiro Umezawa(JP), Chandra Viswanathan (IN)

Zhong-Chao Han (20')
Kazuo Muroi (20')
Kang-Hsi Wu (20')
Liang Lu (20')
Hong Hoe Koo (15')

- Coffee Break -

15:00-17:00

2. Significance of Semi-Public MSC Banking

Chairpersons; Yong-Mook Choi (KR), Zhong Chao Han (CN), Akihiro Umezawa (JP), Shinichiro Okamoto (JP)

Chandra Viswanathan (30')
Yukio Kato & Koichiro Tsuji (15')
You-Wei Wang (15')
Wei-Chun Chen (15')
Tokiko Nagamura & Haiping He (15')
Yutaka Hishiyama (30')

17:00-17:40 ***-Discussion on Future of MSCT-***

Discussants; Shigetaka Asano (JP), Soon Keng Cheong (MY), Surapol Issaragrisil (TH), Hong Hoe Koo (KR)

17:40-17:55 **≪General Comment ≫**

By Keiya Ozawa & Yuzuru Kanakura

17:55-18:00 **≪Closing Remarks≫**

By Shigetaka Asano

TISSUE ENGINEERING: Part A
Volume 20, Numbers 7 and 8, 2014

DOI: 10.1089/ten.tea.2013.0333

Original Article

Stage-Specific Embryonic Antigen 4 in Wharton's Jelly–Derived Mesenchymal Stem Cells Is Not a Marker for Proliferation and Multipotency

Haiping He, MD,[1–3] Tokiko Nagamura-Inoue, MD, PhD,[2] Hajime Tsunoda, MD, PhD,[4] Miki Yuzawa, MT,[2] Yuki Yamamoto, MT,[2] Pariko Yorozu, BNS,[2] Hideki Agata, PhD,[5] and Arinobu Tojo, MD, PhD[1,2]

Background: Umbilical cord Wharton's jelly (WJ) is a rich source of mesenchymal stem cells (MSCs) similar to bone marrow (BM) and adipose tissues. Stage-specific embryonic antigen (SSEA)4 has been reported as a stem cell marker in BM-derived MSCs, but whether $SSEA4^+$ cells have growth and differentiation advantages over $SSEA4^-$ cells remains controversial. To gain insight into the role of SSEA4, we studied $SSEA4^+$ cells in WJ-derived MSCs (WJ-MSCs).

Methods: WJ-MSCs were collected by the explant (WJe-MSCs) or collagenase methods (WJc-MSCs) and analyzed by flow cytometry and reverse-transcription polymerase chain reaction (RT-PCR). To evaluate whether culture conditions influenced the SSEA4 expression, WJe-MSCs were cultured in the medium supplemented with different fetal bovine serum (FBS) concentrations.

Results: SSEA4 was expressed for a long-term culture. In contrast, $SSEA3^+$ disappeared rapidly in early passages of the culture. The incidence of $SSEA4^+$ and $SSEA3^+$ cells was similar between WJe-MSCs and WJc-MSCs at passages P0–P9, except for transient depletion of SSEA4 expression in early passages of WJe-MSCs. These were $CD73^+CD105^+$ cells that express embryonic stem cell markers detected by RT-PCR. No differences in growth and differentiation ability of osteocytes and adipocytes were observed between the sorted $SSEA4^+$ cells and $SSEA4^-$ cells. Further, SSEA4 expression in WJe-MSCs was significantly correlated with FBS concentration in the culture medium.

Discussion: SSEA4, which may display altered expression profiles in response to culture conditions, may not be an essential marker of WJ-MSC multipotency.

Introduction

Umbilical cord (UC) Wharton's jelly (WJ) is a rich source of mesenchymal stem cells (MSCs) along with bone marrow (BM) and adipose tissue.[1–3] WJ-derived MSCs (WJ-MSCs) exhibit the characteristics of MSCs as defined by the International Society for Cellular Therapy (ISCT) criteria. First, MSCs are plastic adherent when maintained in standard culture conditions; second, they are positive for CD105, CD73, HLA-class I, and CD90 and negative for CD45 and HLA-DR surface molecules; and third, MSCs have the pluripotent ability of various mesoderm lineages to generate adipocytes, osteoblasts, and chondrocytes.[4,5] Similar to BM-derived MSCs (BM-MSCs), WJ-MSCs have the potential to differentiate into mesoderm-derived tissues, endoderm, and ectoderm lineages, such as endothelial cells, cardiac myoblasts, pancreatic cells, hepatocytes, and neurogenic cells.[6,7] Hsieh *et al.* compared the gene expression profiles of BM-MSCs and WJ-MSCs and reported that WJ-MSCs were more primitive and more similar to embryonic stem (ES) cells than BM-MSCs.[8,9] On the basis of this data, we isolated the primitive MSCs in WJ-MSCs that are similar to ES cells. Markers of pluripotent, undifferentiated ES cells express several nuclear transcription factors, such as *Oct4, Nanog*, and *SOX2*, and cell surface antigens that have been used to define ES cells, including stage-specific embryonic antigen (SSEA)3 and

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4. The latter two cell surface antigens are present on human ES cells and human embryonic carcinoma cells and are downregulated as these cells differentiate. SSEA3 and SSEA4 are epitopes on the related glycosphingolipids (GSLs), GL-5 and GL-7, respectively. GSLs consist of a carbohydrate moiety or a chain linked to ceramide[10] and appear to be attractive surface markers for sorting live ES-like primitive cells from WJ-MSCs. However, the role of SSEA3 and SSEA4 as pluripotent markers remains controversial, with different laboratories reporting variable results. Gang *et al.* reported that SSEA4 is a marker for BM-MSCs,[11] and Wakao *et al.* showed that SSEA3 is a pluripotent stem cell marker on MSCs defined as multilineage-differentiating stress-enduring (Muse) cells.[12,13] In contrast, Brimble *et al.* demonstrated that both SSEA3 and SSEA4 are not essential for human ES cell pluripotency, as proven by GSL inhibitors.[14]

To obtain $SSEA3^+/4^+$-rich MSCs, we compared the following two major collection methods from the UC: the explant method (WJe-MSCs) and the collagenase-treatment method (WJc-MSCs). We previously reported that WJe-MSCs by the explant method were preferred over that by the collagenase method because WJ-MSCs treated with collagenase sometimes showed decreased cell viability due to the lytic activity of collagenases. However, we could not determine whether the cells migrating from the tissue in the explant method could be selected and induced to differentiate to some degree.

In this study, we compared SSEA3/4 expression in WJ-MSCs collected by different methods. To examine the potential role of SSEA4 in WJ-MSCs, we studied the growth and differentiation ability of cells sorted by SSEA4 expression and factors that influence its expression.

Materials and Methods

Isolation and culture of adherent cells

The present study was approved by the Ethics Committee of the Institute of Medical Science, University of Tokyo, Japan, and the NTT Medical Center Tokyo hospital. Informed consent was obtained from mothers planning to have cesarean sections. UCs were collected aseptically from full-term cesarean section patients after informed consent. The UCs were transferred after collection and the process was initiated within 24 h of delivery. The UC surface was rinsed with phosphate-buffered saline (PBS; Gibco–BRL) containing antibiotics and antifungal reagents Anti–Anti (Antibiotic–Antimycotic, 100×; Gibco–BRL). After removing two arteries and one vein, the remaining WJ tissues were minced into 1–2 mm^3 fragments and divided into two groups for the explant and collagenase-treatment methods (Fig. 1A). In the explant method, the minced fragments were aligned and attached at regular intervals in 10-cm culture dishes. After the fragments were semi-dried and firmly attached to the bottom, the culture medium was gently poured into the dishes.[1,15] In the collagenase-treatment group, the minced WJ tissues were incubated in 1 mg/mL collagenase type I solution (Sigma–Aldrich) in α-MEM (Gibco–BRL) with shaking at 37°C for 2–3 h.[15,16] The cells were then washed with α-MEM supplemented with 10% fetal bovine serum (FBS) and seeded in 10-cm tissue culture dishes with the culture medium as described earlier.[1] The culture medium was refreshed once a week for 3–4 weeks until fibroblast-like adherent cells reached 80–90% confluence. The first-harvested master cells were defined as passage 0 (P0; Fig. 1B). The adherent cells and tissue fragments (Fig. 1A) were rinsed once with PBS and detached using 10% trypsin solution (TrypLE Express; Invitrogen) followed

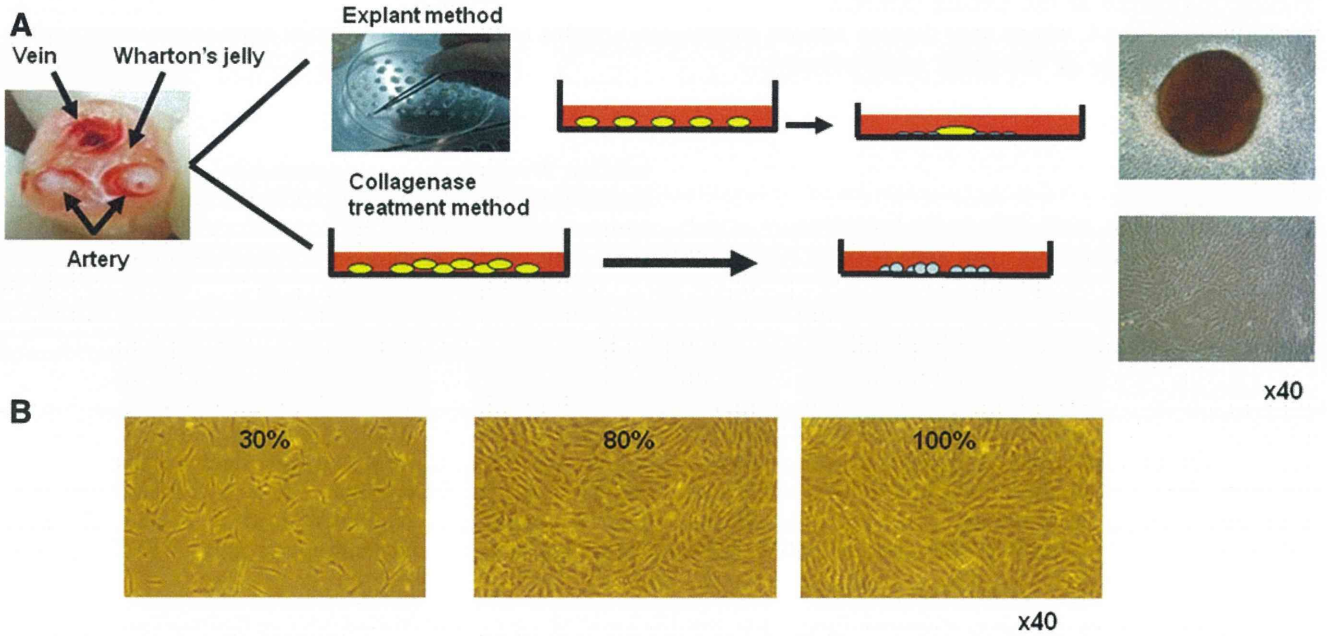

FIG. 1. Umbilical cord Wharton's jelly–derived mesenchymal stem cells (WJ-MSCs). **(A)** MSCs were collected from umbilical cord WJ tissue by the explant method (WJe-MSCs) and by the collagenase-treatment method (WJc-MSCs). Photographs of migrating cells from the minced tissue in the explant method and adherent cells from the scattered cells in the collagenase-treatment method are shown. **(B)** Both WJe-MSCs and WJc-MSCs were spindle-shaped fibroblast-like cells. Color images available online at www.liebertpub.com/tea

by washing with α-MEM supplemented with 10% FBS. In the explant method, the cells and tissue fragments were filtered to remove the tissue fragments. The harvested cells, other than those undergoing further analysis, were immediately cryopreserved in 10% DMSO/5% dextran 40 solution. For serial cultures, the cells were inoculated at 2×10^5 cells per 10-cm-diameter dish and counted at each passage.

Flow cytometry analysis and sorting

Standard flow cytometry (FCM) techniques were used to determine the typical cell surface markers of WJ-MSCs. WJ-MSCs were stained with the following mouse monoclonal antibodies (mAbs): phycoerythrin (PE)–conjugated anti-human CD73 (BD), CD271 (Miltenyi), and HLA-ABC (BD); fluorescein isothiocyanate (FITC)–conjugated anti-human CD90 (BD), CD105 (eBioscience), HLA-DR (BD), and CD45 (BD), FITC-, PE-, and Alexa Fluor–conjugated anti-mouse IgGs (BD) were used as isotypic controls. Dead cells were identified by staining with propidium iodide. To detect the ES cell markers in WJ-MSCs, Alexa Fluor–conjugated mouse anti-human SSEA4 (Clone MC813-70; BD) and FITC-conjugated rat anti-human SSEA3 (Clone MC631; BD) together with the MSC markers CD73 or CD105 mAbs were used. The stained cells were acquired with an FACSCaliber (BD) and analyzed by FlowJo (Tomy Digital Biology, Co. Ltd.). For cell sorting, WJe-MSCs were stained with Alexa-conjugated anti-human SSEA4 and PE-conjugated anti-human CD73 antibodies. The cells were acquired with an FACSAria cell sorting system (BD) and sorted by SSEA4, SSEA3, and CD73 expression.

Proliferation assays of sorted SSEA4$^+$ and SSEA4$^-$ MSCs

To evaluate the proliferative abilities of sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs, the sorted cells were plated at 1×10^4/well in six-well plates (Greiner Bio-one) and cultured in α-MEM supplemented with 10% FBS. The cells were harvested every week and the cell numbers were counted with trypan blue (Gibco–BRL) for 9 weeks.

SSEA4 expression in WJ-MSCs with different FBS concentrations

To evaluate whether culture conditions influenced the SSEA4 expression, WJe-MSCs (P0) were cultured at 1×10^5 cells/well in six-well plates ($n=3$) in α-MEM with the indicated FBS concentrations. After 1 week, the SSEA4, SSEA3, and CD73 expression was analyzed by FCM. To study the influence of the proliferation of WJe-MSCs on SSEA4 expression, we explored the time-course experiment to see the relationship between SSEA4 expression and WJe-MSC growth curve. WJe-MSCs were plated in six-well plates with indicated concentrations of FBS, and the cell number was counted to figure the growth curve on indicated days. The cells were analyzed by FCM to analyze the expression of SSEA3, SSEA4, and CD73.

Further, to analyze the influence of FBS on SSEA4$^-$ WJe-MSCs, SSEA4$^+$ and SSEA4$^-$ WJe-MSCs were cultured in 12-well plates with different concentrations of FBS followed by FCM.

SSEA4 expression in BM-MSCs with different FBS concentrations

To clarify that the phenomena of SSEA4 expression are limited to the WJe-MSCs, we studied the SSEA4 expression in BM-MSCs obtained from BM mononuclear cells (MNCs). Frozen BM-MNCs were purchased from Lonza Walkersville, Inc. BM-MNCs (8×10^5/well) were seeded in six-well plates and grown at 37°C with 5% CO_2. On days 0–21, the proportion of CD45-, SSEA4-, and CD73-positive or -negative cells were analyzed by FCM. To see the influence of FBS on BM-MSCs, we continued to culture BM-MNCs in α-MEM supplemented with 10% FBS and obtained the MSCs. BM-MSCs at p2 were plated in six-well plates to figure the growth curve and analyzed the incidence of CD45, SSEA3, SSEA4, and CD73 by FCM, as described in WJe-MSCs.

RNA isolation and reverse-transcription polymerase chain reaction analysis

Total RNAs were extracted from WJ-MSCs at P3 and from sorted SSEA4$^+$ and SSEA4$^-$ MSCs using TRIzol® Reagent (Invitrogen Corp.). Reverse-transcription polymerase chain reaction (RT-PCR) was performed using the PrimeScript RT-PCR Kit (Takara Shuzou) according to the manufacturer's instructions. The ES markers *Nanog*, *Oct4*, *Klf4*, and *Sox2*[17] and *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) as the control were amplified from the synthesized cDNAs by PCR with the primer pairs shown in Table 1. The amplification conditions were 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and

TABLE 1. HUMAN PRIMER SEQUENCES USED FOR REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Gene	*Accession*		*Primer sequence*	*Product size (bp)*
hOCT3/4	NM-002701	Sense	5′ GACAGGGGGAGGGGAGGAGCTAGG 3′	144
		Anti-sense	5′ CTTCCCTCCAACCAGTTGCCCCAAAC 3′	
REX1	NM-174900	Sense	5′ CAGATCCTAAACAGCTCGCAGAAT 3′	306
		Anti-sense	5′ GCGTACGCAAATTAAAGTCCAGA 3′	
NANOG	NM-024865	Sense	5′ CAGCCCCGATTCTTCCACCAGTCCC 3′	391
		Anti-sense	5′ CGGAAGATTCCCAGTCGGGTTCACC 3′	
hSOX2	NM-003106	Sense	5′ GGGAAATGGGAGGGGTGCAAAAGAGG 3′	151
		Anti-sense	5′ TTGCGTGAGTGTGGATGGGATTGGTG 3′	
hKLF4	NM-004235	Sense	5′ ACGATCGTGGCCCCGGAAAAGGACC 3′	397
		Anti-sense	5′ TGATTGTAGTGCTTTCTGGCTGGGCTCC 3′	
hGAPDH	NM-002046	Sense	5′ AACAGCCTCAAGATCATCAGC 3′	338
		Anti-sense	5′ TTGGCAGGTTTTTCTAGACGG 3′	

extension at 72°C for 1 min. The PCR products were separated by electrophoresis on 2.0% agarose gels and visualized by staining with ethidium bromide.[1]

Adipogenic differentiation

WJe-MSCs were cultured at 2×10^4 cells/well in six-well plates in α-MEM supplemented with 10% FBS. When the cells achieved 80% confluence, the medium was replaced with adipogenesis induction medium,[8] consisting of 100 μM indomethacin (Sigma–Aldrich), 1 μM dexamethasone (Sigma–Aldrich), 0.5 mM IBMX (Sigma–Aldrich), and 10 μg/mL insulin (Sigma–Aldrich). The medium was refreshed every 3 days. After 3 weeks, the cells were fixed with 10% formaldehyde, washed with PBS and 60% isopropanol, and stained with Oil Red O (Sigma–Aldrich).

Osteogenic differentiation

WJe-MSCs at 2×10^4/well (P2) and SSEA4$^+$ and SSEA4$^-$ WJe-MSCs were cultured in 24-well plates in α-MEM supplemented with 10% FBS. On the following day, the medium was replaced with osteogenic induction medium including 10 nM dexamethasone (Sigma–Aldrich), 10 mM β-glycerol phosphate (Sigma–Aldrich), 100 μM ascorbic acid (Sigma–Aldrich), and 50 ng/mL human BMP2 (rhBMP2; Peprotech).[18,19] Human-BM-derived MSCs were used as the positive control. The induction medium was refreshed every 3 days. After 5 weeks, the cells were fixed with 2.5% glutaraldehyde for 15 min at room temperature followed by rinsing with PBS. The bone matrix was stained with 2% Alizarin Red S solution (Sigma–Aldrich) with pH adjusted to ~4.1–4.3 with 1% ammonium hydroxide (Sigma–Aldrich).[19]

Statistical analysis

Differences between groups were analyzed with JMP 6.0.2 software (SAS Institute). Statistical analyses were performed with Turkey–Kramer tests, and a p-value of 0.05 was regarded as statistically significant.

Results

Collection efficiency and WJe-MSC and WJc-MSC biomarkers

Both WJe-MSCs and WJc-MSCs were spindle-shaped fibroblast-like cells (Fig. 1B). There was no significant difference between these two methods in the collected cell numbers at P0, even though the collected cell numbers of WJe-MSCs varied (Fig. 2A). The median number of collected WJe-MSCs from 1 g of WJ was 2×10^6 (range, from 9.1×10^4 to 10.3×10^6; $n=23$) and of collected WJc-MSCs was 1.7×10^6 (range, from 9.2×10^4 to 7.5×10^6; $n=20$). Further, we compared their surface markers as defined by the ISCT. Both WJe-MSCs and WJc-MSCs were positive for CD73, CD90, CD105, and HLA-ABC with a small percentage of cells also positive for CD271 and negative for CD34, CD45, and HLA-DR (Fig. 2B). In addition, both WJe-MSCs and WJc-MSCs expressed the ES-related genes *Nanog*, *Oct4*, *Klf4*, *Rex1*, and *Sox2* (Fig. 2C).

Expression of SSEA4 and SSEA3 in WJ-MSCs

Because SSEA4$^+$ and SSEA3$^+$ cells have been considered as representative of immature cells, we periodically monitored SSEA4, SSEA3, and CD73 expression in WJe-MSCs and WJc-MSCs during P0–P9. The percentages of SSEA4$^+$ cells at P0 were similar in WJe-MSCs and WJc-MSCs. At P0, the WJe-MSCs included 32.4%±17.5% SSEA4$^+$CD73$^+$, 62.8%±18.9% SSEA4$^-$CD73$^+$, and 1.3%±1.8% SSEA4$^+$CD73$^-$, whereas the WJc-MSCs included 26.1%±16.1%, 70.4%±16.2%, and 0.7%±0.8% cells, respectively ($n=8$, $p=0.21$). However, the percentage of WJe-MSC SSEA4$^+$ cells decreased after the first passage and recovered to the original level by P7, whereas the incidence of WJc-MSC SSEA4$^+$ cells was relatively stable until P9 (Fig. 3A, C). In contrast, the percentage of SSEA3$^+$ cells among both WJe-MSCs and WJc-MSCs was highest at P0 that declined and disappeared by P5 (Fig. 3B, D). At P0, the percentage of SSEA3$^+$ cells among WJe-MSCs was 6.7%±6.3% and among WJc-MSCs was 6.1%±6.1% ($n=6$).

Comparison of SSEA4$^+$ and SSEA4$^-$ cells sorted from WJe-MSCs

Further, we sorted SSEA4$^+$ and SSEA4$^-$ cells from WJe-MSCs at P4 using FACSAria and analyzed the SSEA4 expression in the sorted cells every week. The mean purity of SSEA4$^+$CD73$^+$ was 89% and that of SSEA4$^-$CD73$^+$ was 97.7%. The SSEA4$^+$ MSCs and SSEA4$^-$ MSCs were of similar sizes (Fig. 4A).

The percentage of SSEA4$^+$ cells derived from the sorted SSEA4$^+$ WJe-MSCs decreased rapidly in the first week and then increased gradually until week 4 (Fig. 4B, C). Interestingly, SSEA4$^+$ MSCs were present in the SSEA4$^-$ WJe-MSCs, and the incidence of SSEA4$^+$ cells in the subsequent cultures was similar to that in the SSEA4$^+$-sorted cells. After week 4, the incidence of SSEA4$^+$ cells from both the SSEA4$^+$ and SSEA4$^-$ MSCs decreased to <10%. The incidences of SSEA4$^+$ cells derived from SSEA4$^+$ WJe-MSCs and SSEA4$^-$ WJe-MSCs were 8.5%±8.8% and 8.5%±8.8% at week 1 and 43.5%±21.6% and 55.1%±29.4% at week 4, respectively ($n=3$; Fig. 4C). In addition, there was no difference in proliferation ability between SSEA4$^+$ and SSEA4$^-$ WJe-MSCs (Fig. 4D).

RT-PCR analysis showed that the sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs expressed *Nanog*, *Oct4*, and *Klf4* (Fig. 4E).

Adipocyte differentiation

To determine the differentiation ability of SSEA4$^+$ WJe-MSCs, we induced sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs into adipocytes. With induction medium, we observed the accumulation of Oil Red O-stained lipid drops in SSEA4$^+$, SSEA4$^-$, and nonsorted WJe-MSCs, whereas there were no changes in WJe-MSCs cultured without induction medium (Fig. 5A).

Osteogenic differentiation

We also compared the osteogenic differentiation abilities of SSEA4$^+$ and SSEA4$^-$ WJe-MSCs. There was no difference between SSEA4$^+$ and SSEA4$^-$ WJe-MSCs after histochemical staining with Alizarin red, even though WJe-MSCs were difficult to differentiate into osteoblasts, as previously reported. Induction of osteogenic differentiation in WJe-MSCs required a relatively high concentration

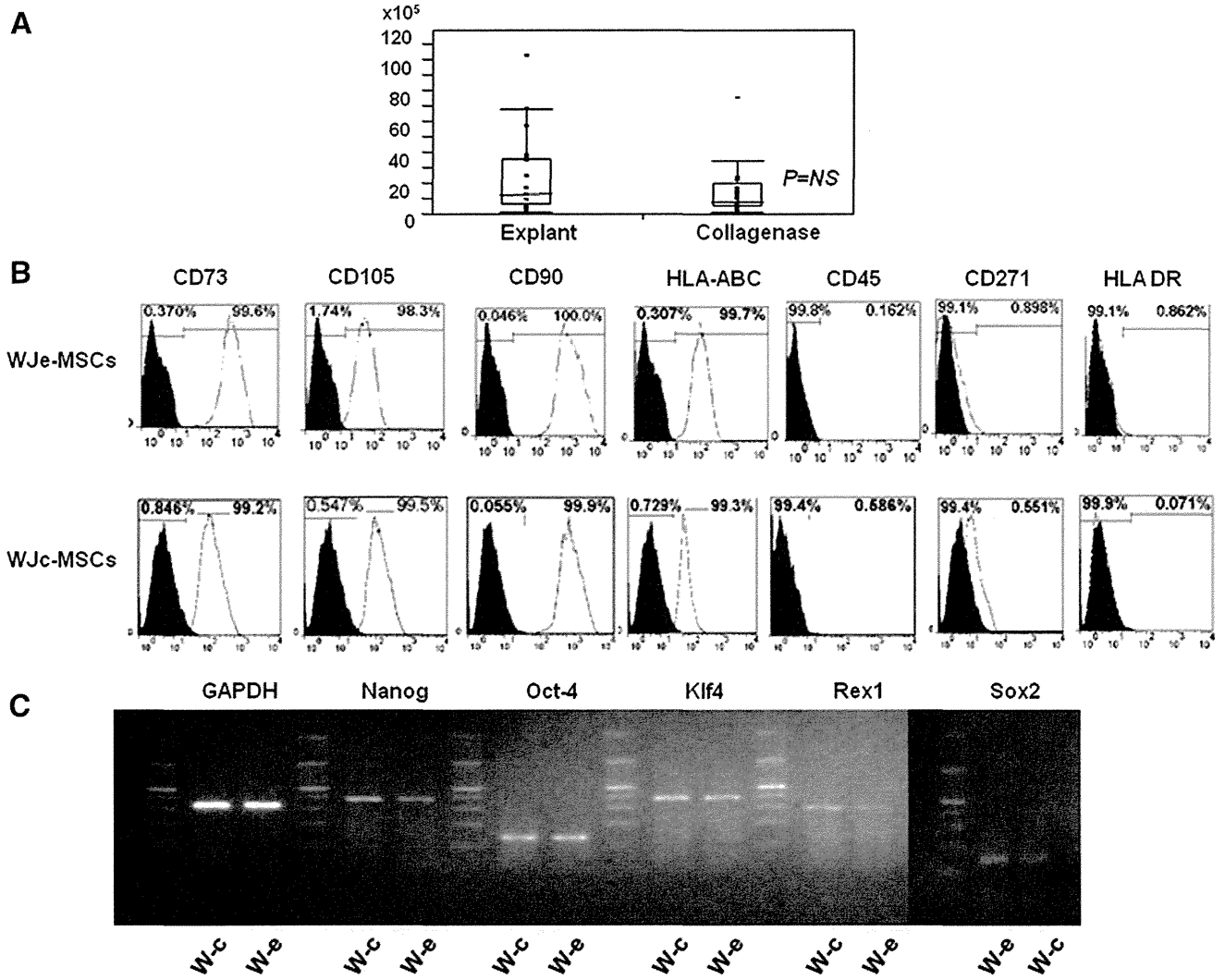

FIG. 2. Characterization of WJ-MSCs collected by the explant and the collagenase-treatment methods. **(A)** Median cell numbers of WJ-MSCs collected by the explant method ($n=23$) and the collagenase-treatment method are shown ($n=20$). **(B)** Both WJe-MSCs and WJc-MSCs were positive for CD73, CD90, CD105, CD271, and HLA class I and negative for CD45 and HLA class II. **(C)** Expression of embryonic stem cell–related markers *Nanog*, *Oct4*, *Klf4*, *Rex1*, and *Sox2* is shown. The data are representative of three individual experiments.

(100 nM/mL) of BMP2 and a longer culture period of 5 weeks ($n=3$; Fig. 5B).

Expression of SSEA4/3 in media with different FBS concentrations

We examined SSEA4/3 expression after 1 week in cultures containing 0.1%, 1%, 10%, and 20% FBS, which contains GSL. The cultured WJe-MSCs were all positive for CD73, and cell proliferation of WJe-MSCs was associated with FBS concentration (Fig. 6A, B). The SSEA4 expression was positively correlated with the FBS concentration (Fig. 6C), whereas SSEA3 expression was negatively correlated (Fig. 6D). The WJe-MSCs were 15.8%±6.2% $SSEA4^+CD73^+$ and 5.8%±1.9% $SSEA3^+CD73^+$ in 0.1% FBS medium, 24.5%±9.8% and 2.7%±1.0% in 1% FBS, 41.5%±13.1% and 0.7%±0.5% in 10% FBS, and 48.3%±12.0% and 0.6%±0.4% in 20% FBS, respectively ($n=3$).

To see that the increase in SSEA4 expression upon increasing FBS concentration is caused by the change in expression alone but not because of increased WJe-MSC proliferation, we analyzed SSEA4 expression associated with growth curve with different FBS concentrations. In consistent with the prior data, SSEA4 expression was correlated with FBS concentration, while SSEA3 was inversely correlated (Fig. 6E–G). The higher FBS concentration accelerated the proliferation of WJe-MSCs with higher expression of SSEA4 (Fig. 6E, F). The incidence of $SSEA4^+$ and $SSEA3^+$ cells was highest on day 3, followed by the decline from days 3 to 7, still during proliferation. The possibility of the substrate shortage for SSEA4 and SSEA3 during culture could be denied, because we replaced the fresh medium on day 4. The data are representative of three individual experiments.

Next, when the sorted $SSEA4^+$ and $SSEA4^-$ WJe-MSCs were cultured in 1%, 5%, and 10% FBS for 1 week, SSEA4

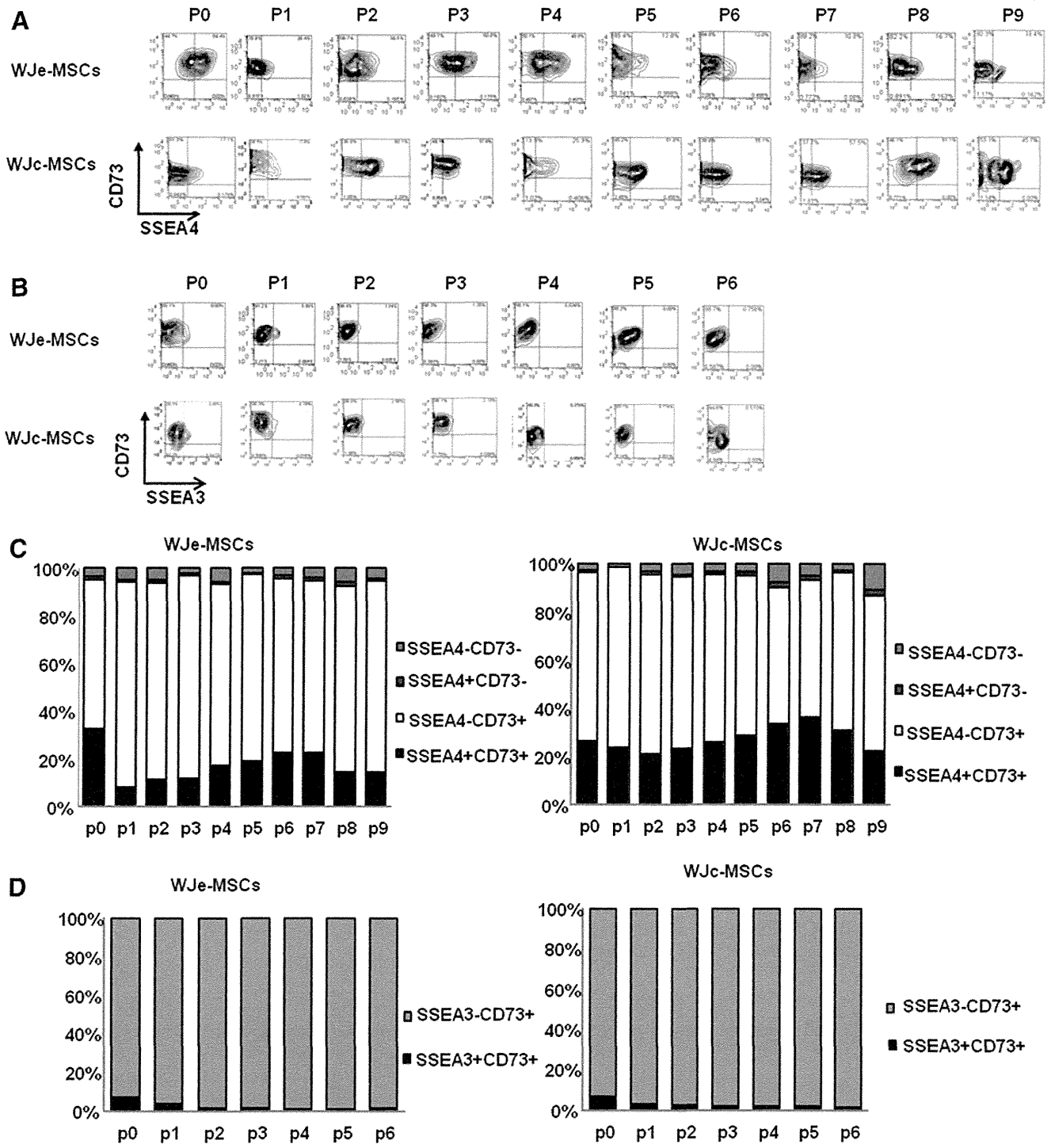

FIG. 3. Stage-specific embryonic antigen-4 expression during passages. **(A)** Stage-specific embryonic antigen 4 (SSEA4) and CD73 expression was monitored in WJ-MSCs collected by the explant (WJe-MSCs) and the collagenase-treatment methods (WJc-MSCs) during P0–P9. **(B)** SSEA3 and CD73 expression was periodically monitored in WJe-MSCs and WJc-MSCs during P0–P6. **(C)** The mean incidence of SSEA4 and CD73 in WJe-MSCs and WJc-MSCs is shown. **(D)** The mean incidence of SSEA3 in $CD73^+$ WJe-MSCs and WJc-MSCs is shown. The data are representative of eight individual experiments.

expression in the sorted $SSEA4^+$ WJe-MSCs was 4.74% and 17.4% in 1% and 10% FBS medium, respectively, whereas SSEA4 expression in the sorted $SSEA4^-$ WJe-MSCs was 2.69% and 5.08% in 1% and 10% FBS, respectively ($n=3$; Fig. 6H, I).

Further, to clarify whether the phenomena of SSEA4 expression influenced by FBS concentration were limited to WJe-MSCs, we performed the same experiments using BM-MSCs derived from frozen-thawed MNCs. As shown in Figure 7A, BM-MNCs before culture included hematopoietic

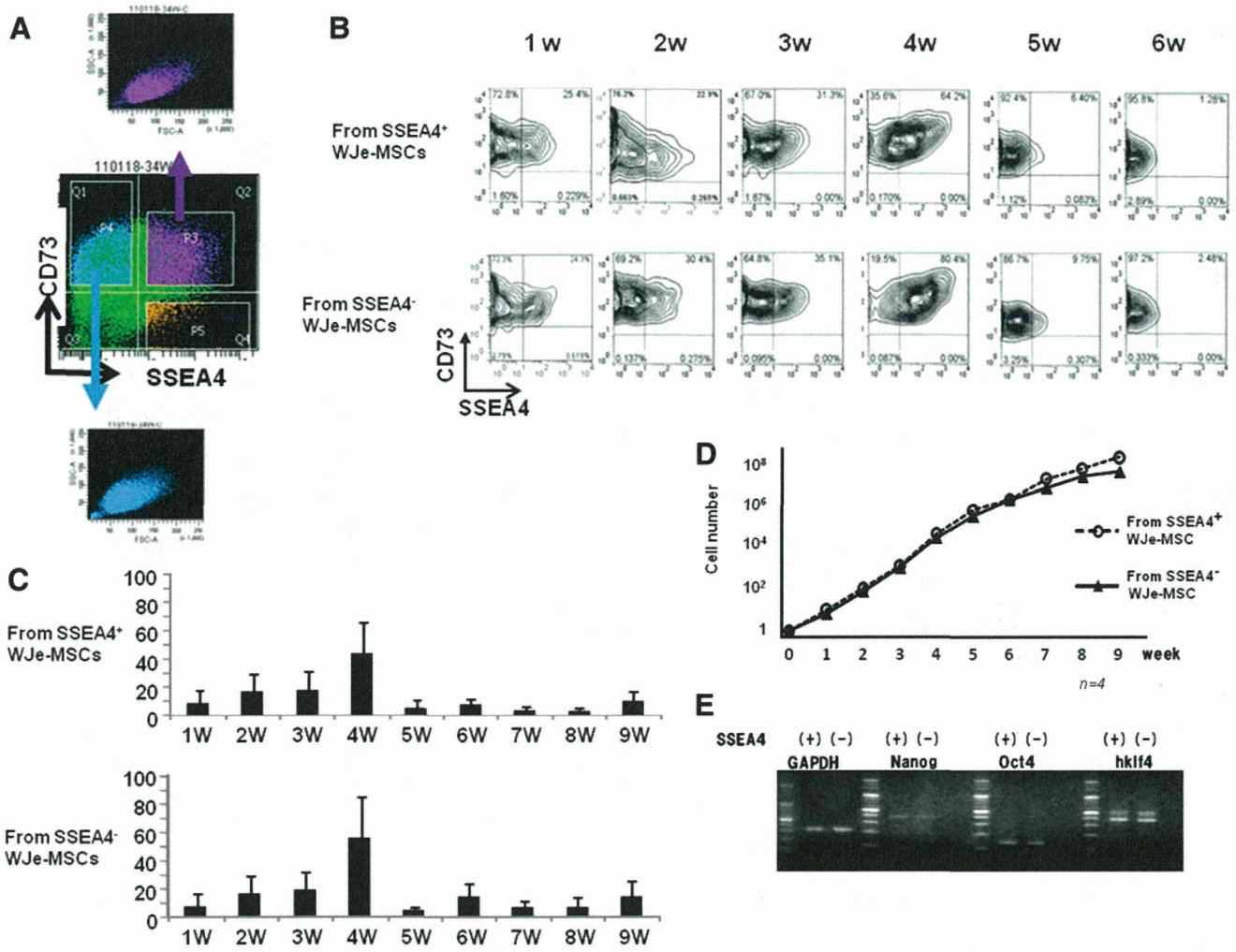

FIG. 4. Sorted SSEA4$^+$ and SSEA4$^-$ WJ-MSCs collected by the explant method. **(A)** SSEA4$^+$CD73$^+$ WJ-MSCs and SSEA4$^-$CD73$^+$ WJe-MSCs were sorted by FACSAria. **(B)** SSEA4 and CD73 expression in sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs during passages 1–6 weeks (w) is shown. **(C)** The percentages of SSEA4$^+$ cells in the sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs during weeks 1–9 are shown ($n=3$). **(D)** Growth curves of the sorted SSEA4$^+$ (open circles) and SSEA4$^-$ (closed triangles) WJe-MSCs over 9 weeks are shown. **(E)** Gene expression analysis of the sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs showed that both were positive for *Oct4*, *Nanog*, and *Klf4*. Color images available online at www.liebertpub.com/tea

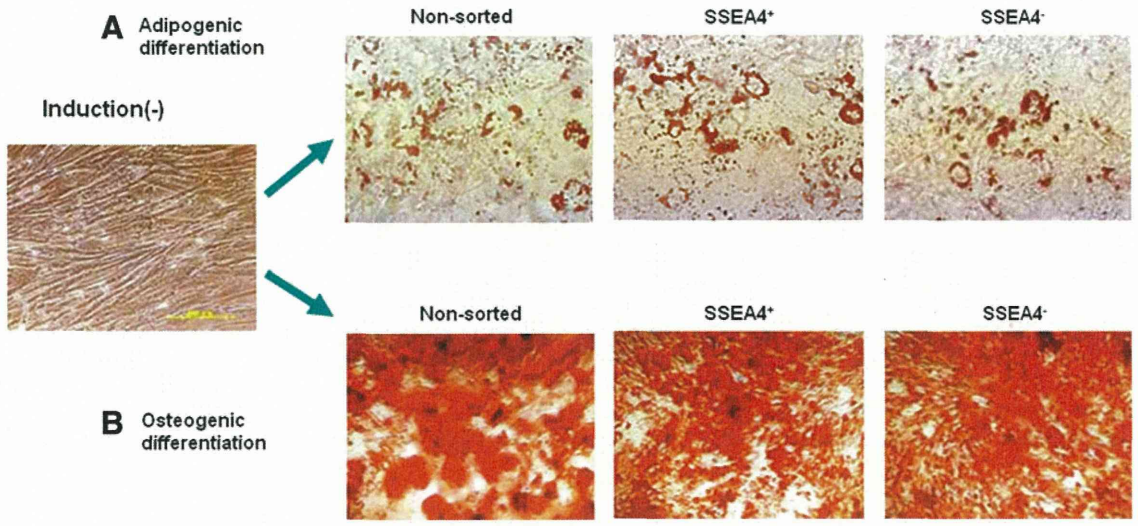

FIG. 5. Adipogenic and osteogenic differentiation of sorted SSEA4$^+$ and SSEA4$^-$ WJ-MSCs. **(A)** Adipogenic differentiation of nonsorted and SSEA4$^+$- and SSEA4$^-$-sorted WJe-MSCs is shown by Red O-stained lipid drops at 3 weeks. **(B)** Osteogenic differentiation of unsorted and SSEA4$^+$- and SSEA4$^-$-sorted WJe-MSCs is shown. Color images available online at www.liebertpub.com/tea