The "window-shift type" model evaluation revealed a critical period for obtaining the most informative morphological features (Fig. 3). When attempting prediction with only 108 features (i.e., 16% of the information compared to that used in our original study), we found that the morphological changes in culture days 8–10 (D14_ALP model) and days 9–11 (D21_ALP model) are most informative and enhance prediction performance. This result largely matched the performance of "skipping type" models (Fig. 4). Most "skipping type" models exceeded the performance of models from the later stages in the "shortening type" analyses, which lost predictive strength in this portion of the time-course. The lack of later-stage morphological features correlated with decreased prediction performance in both D14_ALP and D21_Ca models, but more so in D21_Ca models, when derived from "shortening type" evaluations. This suggests morphological changes are informative in the first 2 days, but incorporation of morphological features from later stages of differentiation can further improve prediction accuracy. From this observation we conclude that acquiring additional images from the later stages of differentiation culture are not essential, but can add value to prediction models. Considering both observations from "shortening type" and "skipping type" evaluations, we conclude the image-acquisition from the first 2 days of a "shortening type" model, can be reduced by loosening the image acquisition schedule to a frequency of 48 h intervals without significant impact to correctly classifying candidate differentiation potential. An advantage to widening the sampling frequency to 48 h intervals is the ability to implement a manual version of this method in a clinical setting that frees up weekend time and mitigates resource burden on operators and facilities.

We also identified limitations of the morphological features reflecting cellular potential. As shown in Figure 4, the "shortening type" analysis revealed that including changes in morphological features from the first day (16–32 h) undermines the prediction performance. These observations suggest morphological changes in the first day of differentiation culture are likely more random compared to subsequent changes in morphology that more directly contribute to prediction of osteogenic potential. Since cells had attached sufficiently during the expansion culture period in the same plate wells prior to day 0 in our experiment, these extraordinary morphological changes are likely a response to changing the culture environment to differentiation medium. We therefore further examined the morphological responses to medium changes (Fig. 5). When the differentiation period started, medium was changed at 64 h (day 3) and at 216 h (day 10; Fig. 5a). Surprisingly, many morphological features showed irregular changes after the medium was refreshed, which we interpret as clear morphological responses to the medium change. When viewed collectively these morphological responses to medium change were limited to particular morphological features. The morphological parameter, "fiber lengths," was one parameter sensitive to medium changes (Fig. 5b). Even though these changes were small from a statistical point of view, this parameter, which indicates cell shrinkage, was most sensitive to medium change across all cell lots. Although most of these features were insensitive to the second medium change at time-point 26, fiber length still reflected this change in the environment. We found a drift in model performance in the "shortening type" and "window-shift type" analyses, which was due to parameter disturbances reflecting this response to medium change. Based on our observations we suggest correcting for these types of morphological parameters, which may be a source of noise when used in predictions of cellular ostogenic potential. This finding also strongly indicates the possibility that cellular morphology changes can also be used to detect changes in cellular microenvironment as a way to quality check animal cells during high-throughput assays.

The utility of morphology-based cell quality evaluation has also been studied by Seiler et al. where they reported a systematic evaluation method for hBMSCs' differentiation potential based on morphological features measured from non-labeled time-course images (Seiler et al., 2012). Building on the work of Seiler et al. and their algorithm approach (node-harvest method), our original report greatly expands the utility of the image-based modeling concept by advancing the field in three areas: (i) improved model performance by data accuracy, (ii) various modeling scenarios for practical implementation in clinics, and (iii) quantitative prediction of osteogenic potential (versus classifications). The further iterations presented here add three more advancements: (i) identification of a critical period for measuring morphological features, (ii) the sensitivity of particular morphological features, and (iii) the most resource-efficient data collection method.

In conclusion, in this study, we were able to define the key characteristics of hBMSC morphological features to advance our image-based computational prediction model. The detailed characterization of morphological features in this study has demonstrated a way that allows practical, flexible and efficient introduction of our method, allowing users to most appropriately match facilities and protocols. Our next investigation to universalize this application should be the confirmation of our method under different imaging platforms. We believe that this is a benchmark study highlighting the importance of real-time morphological features for use in prediction modeling, which may trigger wider implementation of this approach to address the need for cell quality evaluation methods in regenerative medicine.

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

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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

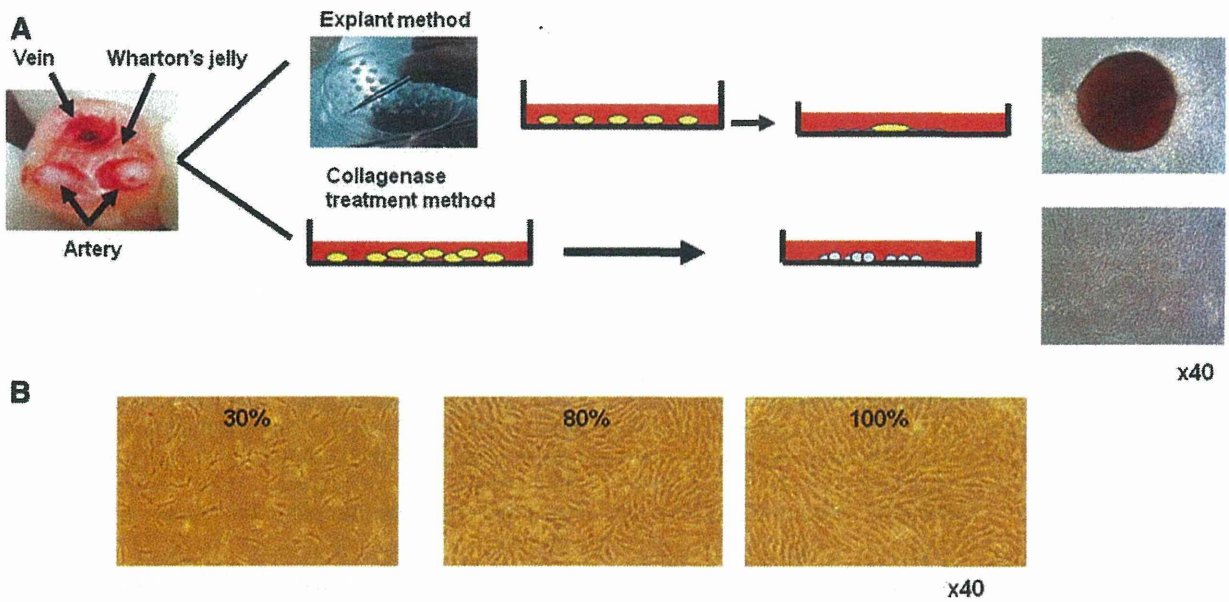

FIG. 1. Umbilical cord Wharton's jelly–derived mesenchymal stem cells (WJ-MSCs). (**A**) MSCs were collected from umbilical cord WJ tissue by the explant method (WJe-MSCs) and by the collagenase-treatment method (WJc-MSCs). Photographs of migrating cells from the minced tissue in the explant method and 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

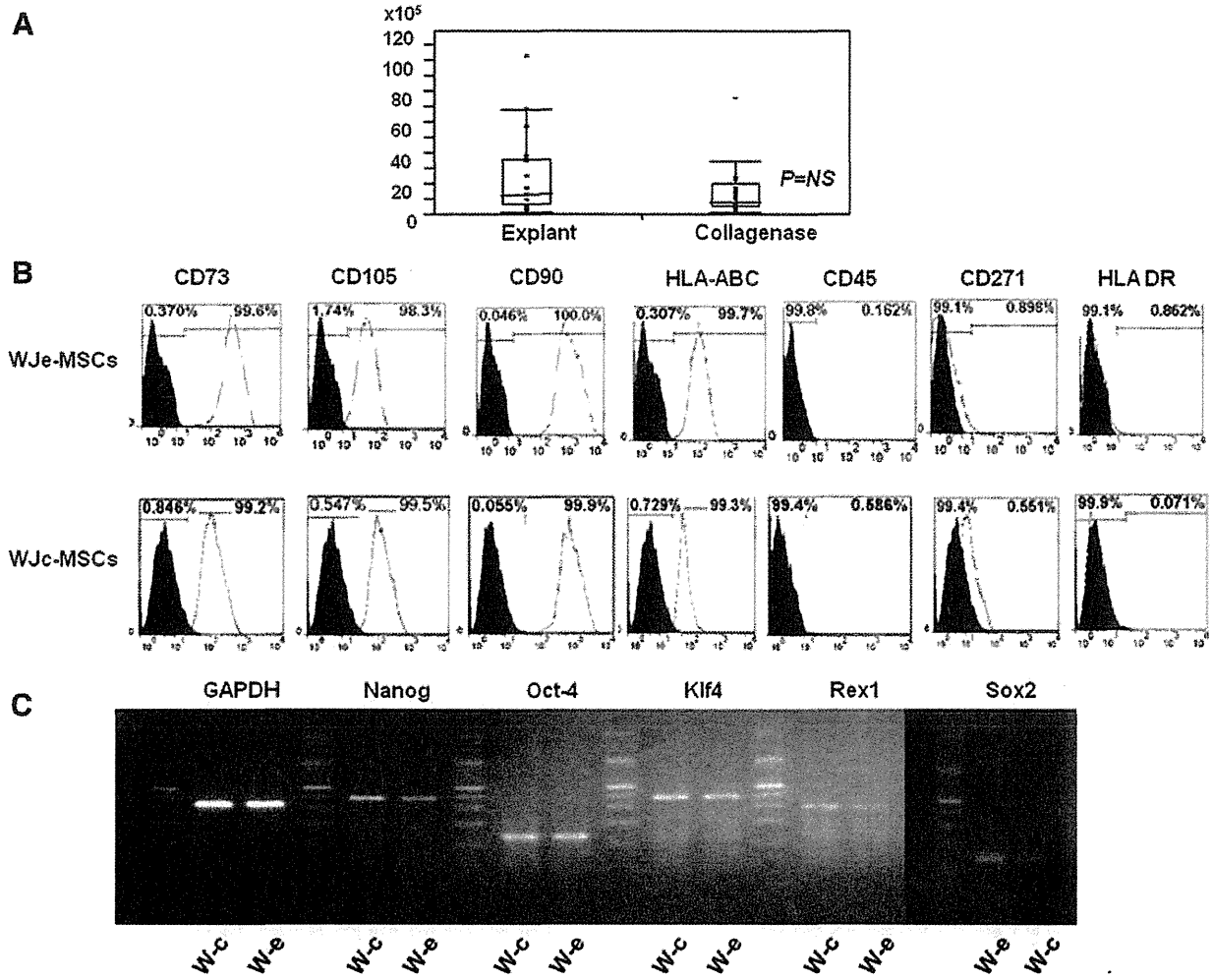

FIG. 2. Characterization of WJ-MSCs collected by the explant and the collagenase-treatment methods. **(A)** Median cell numbers of WJ-MSCs collected by the explant method ($n=23$) and the collagenase-treatment method are shown ($n=20$). **(B)** Both WJe-MSCs and WJc-MSCs were positive for CD73, CD90, 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

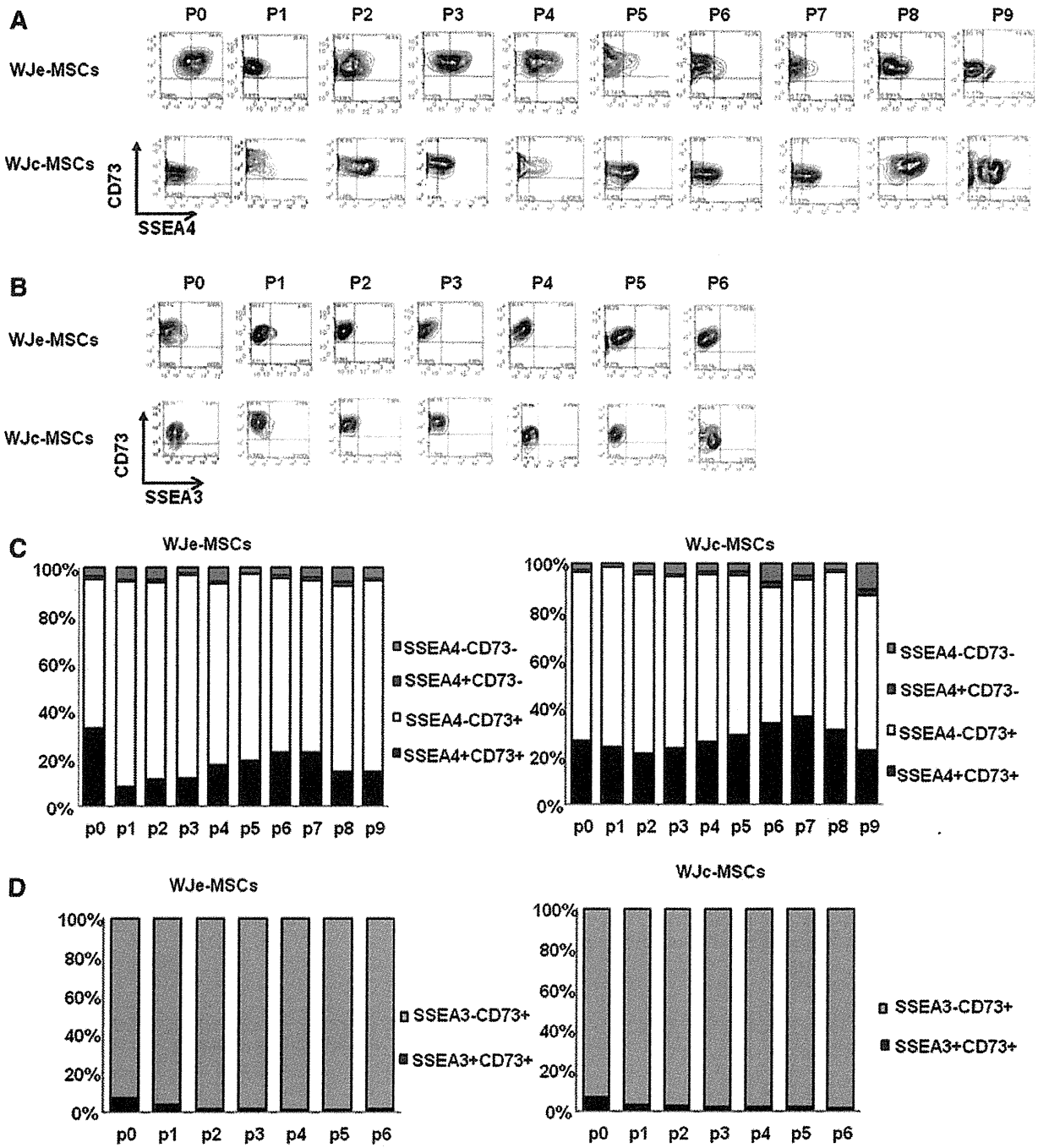

FIG. 3. Stage-specific embryonic antigen-4 expression during passages. **(A)** Stage-specific embryonic antigen 4 (SSEA4) and CD73 expression was monitored in WJ-MSCs collected by the explant (WJe-MSCs) and the collagenase-treatment methods (WJc-MSCs) during P0–P9. **(B)** SSEA3 and CD73 expression was 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

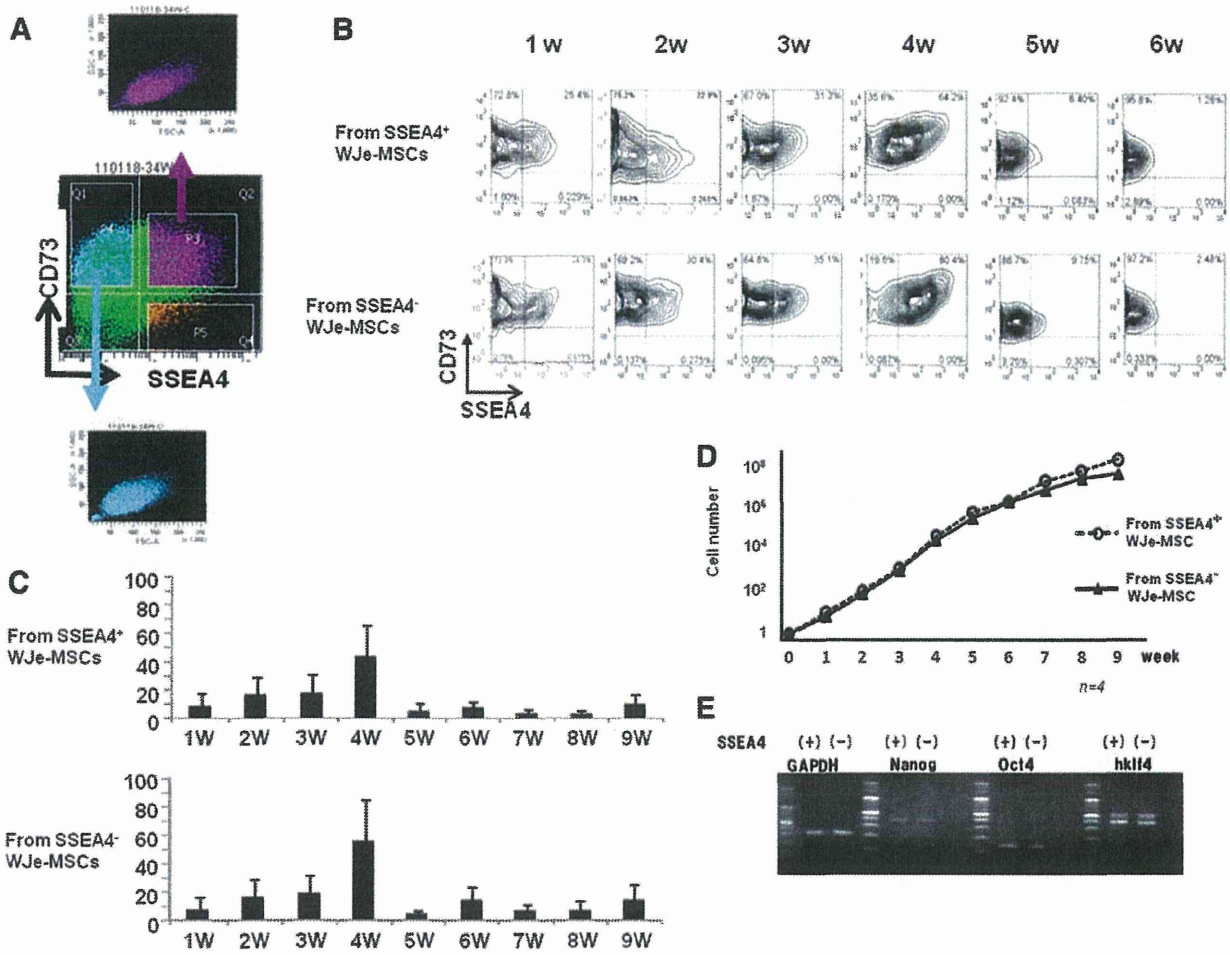

FIG. 4. Sorted SSEA4$^+$ and SSEA4$^-$ WJ-MSCs collected by the explant method. (**A**) SSEA4$^+$CD73$^+$ WJ-MSCs and SSEA4$^-$CD73$^+$ WJe-MSCs were sorted by FACSAria. (**B**) SSEA4 and CD73 expression in sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs during passages 1–6 weeks (w) is shown. (**C**) The percentages 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

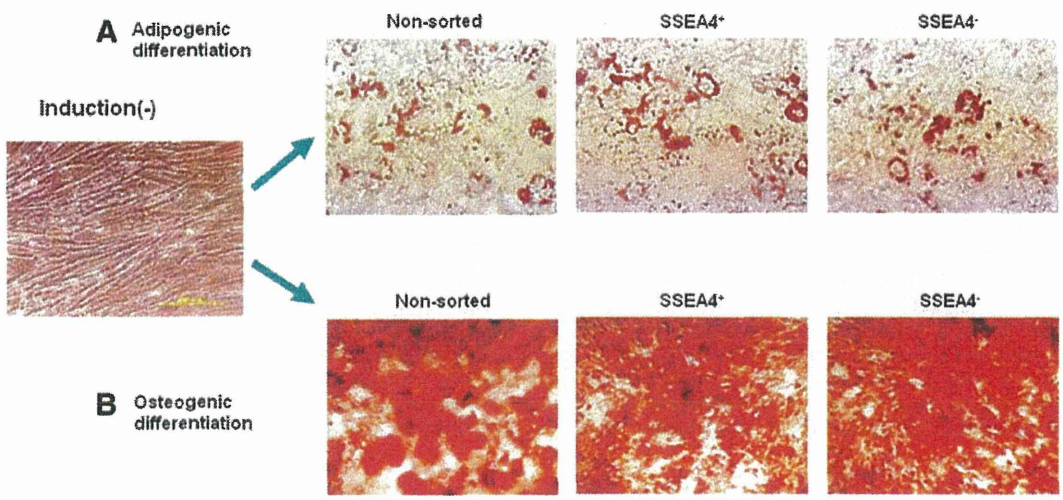

FIG. 5. Adipogenic and osteogenic differentiation of sorted SSEA4$^+$ and SSEA4$^-$ WJ-MSCs. (**A**) Adipogenic differentiation of nonsorted and SSEA4$^+$- and SSEA4$^-$-sorted WJe-MSCs is shown by Red O-stained lipid drops at 3 weeks. (**B**) Osteogenic differentiation of unsorted and SSEA4$^+$- and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

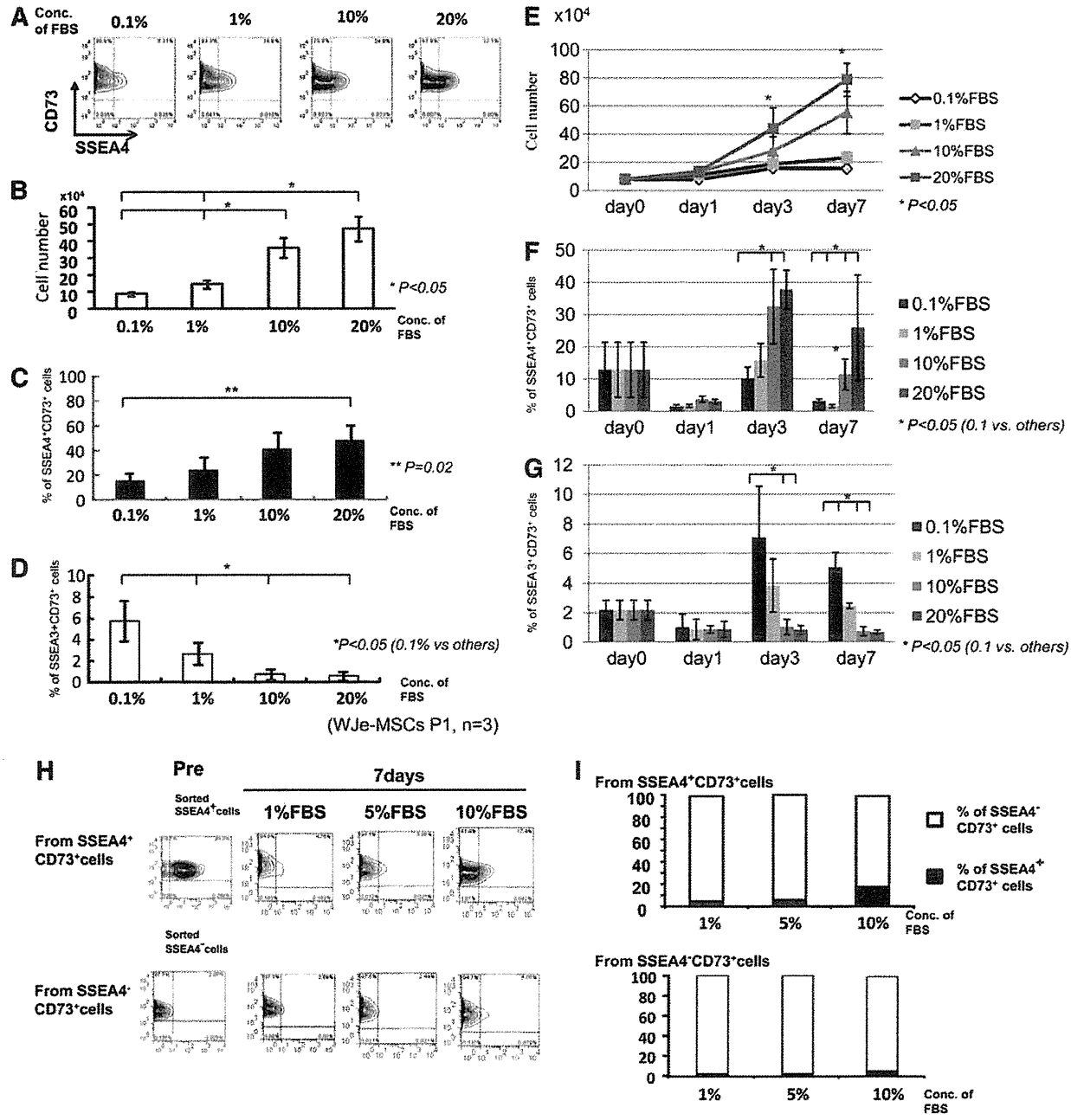

FIG. 6. Influence of fetal bovine serum (FBS) on SSEA4 and SSEA3 expression. **(A)** WJ-MSCs collected by the explant method (WJe-MSCs) were cultured for 1 week in media containing 0.1%, 1%, 10%, and 20% FBS, and SSEA4 expression patterns were analyzed by flow cytometry. The data are representative of three individual experiments. **(B)** WJe-MSC numbers in media with different concentrations of FBS were determined. **(C, D)** Percentages of $SSEA4^+$ and $SSEA3^+$ cells, respectively, among the WJe-MSCs in media with different concentrations of FBS were determined. **(E)** Growth curves of WJe-MSCs with different concentrations of FBS. **(F, G)** Percentage of $SSEA4^+$ and $SSEA3^+$ cells during the proliferation of WJe-MSCs, respectively. **(H, I)** Flow cytometry patterns and percentages of SSEA4 expression in the sorted $SSEA4^+$ and $SSEA4^-$ WJe-MSCs in media with 1%, 5%, and 10% FBS were determined. $*P<0.05$, $**P=0.02$.

cells with low incidence of MSCs and also low incidence of SSEA4+cells. As expected, the proportion of $CD45^+$ hematopoietic cell expression declined during the passages; instead, $CD45^-$ cells increased (Fig. 7A). The latter cells expressed $CD45^-CD73^+$ MSCs (data not shown). However, in α-MEM with 10% FBS, we could not observe the predominant growth of $SSEA4^+$ cells regardless of CD45 expression (Fig. 7A). Further, in consistent with the results in WJ-MSCs, the SSEA4 expression in BM-MSCs was correlated with the FBS concentration, whereas SSEA3

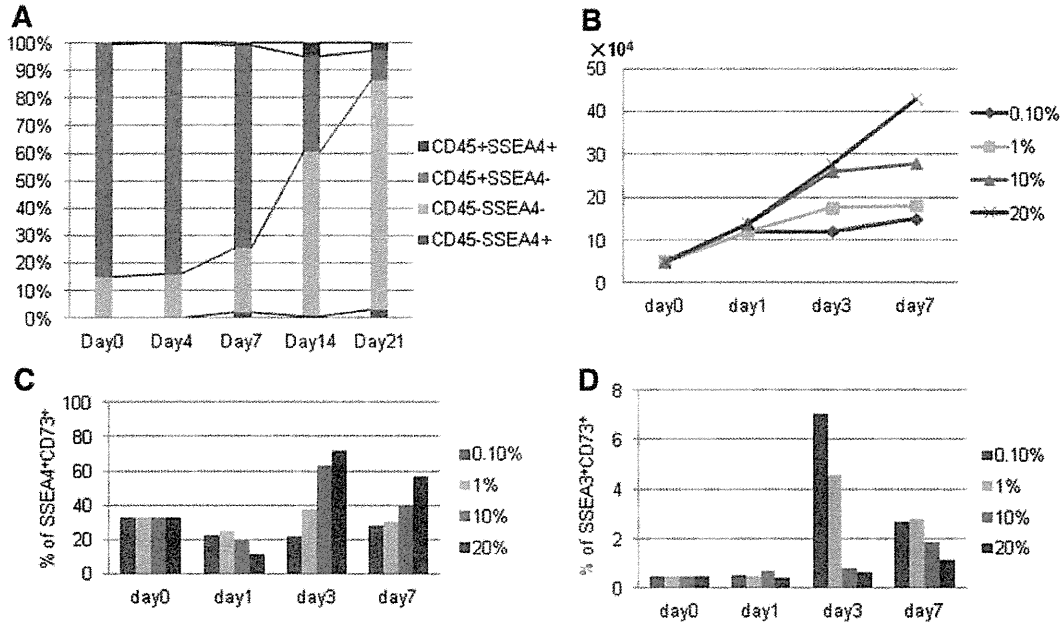

FIG. 7. Influence of FBS on SSEA4 and SSEA3 expression in bone-marrow-derived mesenchymal stem cells. **(A)** Shift of the proportion of $CD45^+$ hematopoietic cells and $CD45^-$ cells with or without SSEA4 expression during the passages. **(B)** Growth curves of BM-MSCs with different concentrations of FBS. **(C, D)** Percentage of $SSEA4^+$ and $SSEA3^+$ cells during the proliferation of BM-MSCs, respectively. The data are representative of two individual experiments.

expression was inversely correlated (Fig. 7B–D). The relationship between the growth curve and SSEA4/3 expression was also consistent with the results in WJ-MSCs (Figs. 6E–G and 7B–D).

Discussion

To gain insight into the role of SSEA3 and SSEA4 in UC WJ-MSCs, we examined the SSEA3 and SSEA4 expression on WJ-MSCs obtained by different methods and compared the differentiation abilities of $SSEA4^+$ and $SSEA4^-$ cells.

First, we compared SSEA3 and SSEA4 expression in WJe-MSCs and WJc-MSCs. The explant method has several advantages. It does not require nonhuman collagenase derived from *Clostridium histolyticum* and saves the time for lysing the tissue with enzyme in the procedure. The disadvantages of the explant method are that collecting adequate cell numbers is dependent on the amount of WJ tissue fragments that attach firmly to the bottom of the dish, which in turn, is dependent on the individual ability. Although the collagenase process from cutting tissue to plating can be unified, we found that some samples are more sensitive to collagenase, resulting in reduced viability. We did not find any significant differences between WJe-MSCs and WJc-MSCs in cell numbers, MSC surface markers as defined by the ISCT, or ES-cell-related gene expression at P0. In addition, the incidence of $SSEA4^+$ and $SSEA3^+$ at P0 was similar between the two types. However, SSEA3 disappeared rapidly in the early culture passages, as described previously.[14,20] It is known that SSEA3 disappears more rapidly from the cell surface compared with SSEA4, if GSL synthesis is blocked by inhibitors, and a similar phenomenon has also been detected during ES cell differentiation.[14,21] Interestingly, the incidence of WJe-MSC $SSEA4^+$ cells was reduced after the first passage, and, by P7, the original levels were recovered. In contrast, the incidence of WJc-MSC $SSEA4^+$ cells was relatively stable until P9. The reason for the depression of SSEA4 expression in WJe-MSCs at P1 is unknown. However, $SSEA4^+$ MSCs in both WJe-MSCs and WJc-MSCs did not proliferate predominantly in our culture medium. As previously reported, osteogenic differentiation was difficult with WJ-MSCs,[8] requiring a longer induction period, additional cytokines such as BMP2, and specific FBS concentrations. However, both sorted $SSEA4^+$ and $SSEA4^-$ WJ-MSCs eventually differentiated into osteocytes and adipocytes in a similar manner, and there were no differences in ES-marker gene expression between the $SSEA4^+$ and $SSEA4^-$ MSCs. Interestingly, $SSEA4^+$ cells appeared even from the $SSEA4^-$ MSCs, and the incidence of $SSEA4^+$ cells derived from the $SSEA4^-$ MSCs demonstrated a similar transition pattern as those derived from the $SSEA4^+$ MSCs. This result suggested that the culture medium may have been the source of SSEA4 antigens.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Rostovskaya *et al.* also suggested SSEA4-marked adipogenic progenitor lacking osteogenic capacity.[22] In ES cell study, Ramirez *et al.* demonstrated that both SSEA3 and SSEA4 are markers of immature ES cells, but particularly SSEA3 together with OCT4 and TRA-1–60 were good tracers for validating pluripotent stem cells, whereas SSEA4 was expressed for long during the differentiation of ES cells.[20] Gang *et al.* reported that $SSEA4^+$ cells proliferated predominantly when the culture was initiated from primary BM cells, which were mostly hematopoietic cells.[11] But in our culture condition, the incidence of $SSEA4^+$ cells, which were characterized as MSCs, was not increased dramatically

as reported by Gang's group. Their results might be induced by the special cocktail of the medium, consisted of MCDB-201, 10% FBS, ITS, linoleic acid–bovine serum albumin, dexamethasone, ascorbic acid, hPDGF-BB, and hEGF, followed by the medium with relatively high FBS concentration. In other articles, Schrobback *et al.* assessed the SSEA4 in human articular chondrocytes, osteoblasts, and BM-derived MSCs and characterized their differentiation potential. But their results showed that SSEA4 levels in these cells were not unrelated to the cells' chondrogenic and osteogenic and proliferation potentials *in vitro*.[23] Suila *et al.* reported that SSEA4, and not SSEA3, was expressed on the surface of cord-blood-derived MSCs, whereas SSEA3 was expressed at very low levels in cord blood hematopoietic stem cells.[24] They also suggested that FBS contains detectable amounts of globoseries GSLs and showed that the SSEA3 was influenced and upregulated by culturing with FBS overnight, even though they did not demonstrate an influence on SSEA4 expression. In our study, we demonstrated that SSEA4 expression significantly correlated with FBS concentration, whereas SSEA3 appeared to be negatively correlated with FBS concentration. The possibility that FBS stimulated the proliferation of WJe-MSCs, resulting in the increase of SSEA4 expression, cannot be denied completely. But the fact that the SSEA4 expression was declined during the proliferation of WJe-MSCs and BM-MSCs in each medium did not support this hypothesis. Regardless of cell growth or cell concentration, consistently, the SSEA4 expression was clearly associated with FBS concentration not only in WJ-MSCs but also in BM-MSCs. Reversely to the SSEA4 expression, SSEA3 expression appeared to be negatively correlated with FBS concentration. The reason why the data of SSEA3 elevation upon the higher FBS concentration are not coincident with Suila's data remained unresolved. We add a partial speculation that SSEA4 is derived from SSEA3; thus, the increase of SSEA4 means the waste of SSEA3, or MSCs with high concentration of FBS are differentiated accompanied with decrease of SSEA3. But we need the scrupulous attention to analyze SSEA3 by FCM in various FBS concentrations. We also found that SSEA4 could be induced from pure $SSEA4^-$ WJ-MSCs. This suggests the presence of substrate for SSEA4 in $SSEA4^-$ cells and also the influence of FBS on the SSEA4 expression in $SSEA4^-$ cells. Brimble *et al.* reported that the depletion of these two molecules by the addition of GSL synthesis inhibitors apparently did not affect the ES cell pluripotency.[14] In conclusion, these results indicate that SSEA4 may display altered expression profiles in response to culture medium including FBS and may not be an essential marker of WJ-MSC pluripotency.

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Disclosure Statement

The authors have no conflicts of interest.

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LETTER TO THE EDITOR

Pretransplant hyperferritinemia has no effect on the outcome of myeloablative cord blood transplantation for acute leukemia and myelodysplastic syndrome

Takaaki Konuma · Seiko Kato · Maki Oiwa-Monna · Arinobu Tojo · Satoshi Takahashi

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Dear Editor,

Pretransplant hyperferritinemia has been associated with an increased incidence of morbidity and mortality following allogeneic hematopoietic stem cell transplantation (allo-HSCT) after myeloablative or reduced-intensity conditioning [1–3]. Its effect on the outcome of myeloablative cord blood transplantation (CBT) has yet to be clarified. In this study, we retrospectively analyzed whether hyperferritinemia affected the outcome of myeloablative CBT.

Pretransplant serum ferritin level measured within 100 days preceding single-unit CBT for adult patients with acute leukemia or myelodysplastic syndrome (MDS) in our institute between the year 2001 and 2013 was available for 133 patients in this study. All patients received 12 Gy total body irradiation-based myeloablative conditioning regimens and cyclosporine and short-term methotrexate as a graft versus host disease (GVHD) prophylaxis. Cord blood units were selected as reported previously [4, 5]. In multivariate analysis, the following variables were considered: age (<45 vs. ≥45 years), cytomegalovirus serostatus (negative vs. positive), disease status at CBT (standard risk vs. high risk), cord blood nucleated cell count (<2.5 vs. ≥2.5×10^7/kg), cord blood CD34+ cells (<1 vs. ≥1×10^5/kg), HLA disparities based on antigen level HLA-A and -B and allele level HLA-DRB1 (≤2 vs. ≥3), and pretransplant ferritin level (<1,000 vs. ≥1,000 ng/ml). All statistical analyses were performed with EZR, a graphical user interface for R 2.13.0 [6]. $P<0.05$ was considered significant. Analysis of data was performed on August 2013.

The median pretransplant serum ferritin level was 751(range, 58–6,285) ng/ml, the median age was 40(range, 16–55) years, the median number of nucleated cells was 2.52(range, 1.32–5.69)×10^7/kg, and the median number of CD34+ cells was 0.91(range, 0.28–7.75)×10^5/kg. Disease types were acute myelogenous leukemia in 74 patients, acute lymphoblastic leukemia in 45, and MDS in 25. The median follow-up of surviving patients was 42(range, 3–103) months after CBT. In univariate and multivariate analysis, we found no impact of hyperferritinemia on overall survival (OS), relapse, transplant-related mortality (TRM), grades II–IV acute GVHD, extensive chronic GVHD, and neutrophil engraftment (Table 1). We also analyzed the effect of hyperferritinemia using different ferritin threshold (500, 1,500, 2,000 ng/ml). But, we were unable to find any impact of hyperferritinemia on outcomes (data not shown).

The effect of hyperferritinemia on outcome might differ depending on the kinds of stem cell sources in allo-HSCT. It has been reported that hyperferritinemia was associated with inferior OS in CBT recipients [7]. However, the cumulative incidence of TRM was very low in our entire cohort, which might have contributed to hyperferritinemia not being shown to influence outcome in our study.

Ferritin is not only a marker for iron overload but also an inflammatory indicator. Moreover, iron overload correlates better with magnetic resonance imaging-measured liver iron content (LIC) than hyperferritinemia. Recently, prospective studies demonstrated that LIC did not affect outcomes of allo-HSCT [8, 9], suggesting that it is unclear whether iron overload

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Table 1 Univariate and multivariate analysis of pretransplant ferritin levels on outcomes of myeloablative CBT

		Univariate analysis[b]		Multivariate analysis[c]	
	Ferritin[a]	% (95 % CI)	*P*	HR (95 % CI)	*P*
Overall survival					
	<1,000 ng/ml	77 (66–85) at 3 years	0.71	1	Reference
	≥1,000 ng/ml	78 (62–87) at 3 years		0.77 (0.37–1.60)	0.49
Relapse					
	<1,000 ng/ml	20 (12–30) at 3 years	0.67	1	Reference
	≥1,000 ng/ml	17 (8–29) at 3 years		0.76 (0.33–1.75)	0.52
Transplant-related mortality					
	<1,000 ng/ml	8 (3–15) at 3 years	0.70	1	Reference
	≥1,000 ng/ml	8 (2–19) at 3 years		0.68 (0.23–2.04)	0.50
Grades II–IV acute GVHD					
	<1,000 ng/ml	70 (59–78) at 100 days	0.34	1	Reference
	≥1,000 ng/ml	63 (47–75) at 100 days		0.79 (0.48–1.29)	0.36
Extensive chronic GVHD					
	<1,000 ng/ml	20 (12–29) at 3 years	0.42	1	Reference
	≥1,000 ng/ml	28 (16–42) at 3 years		1.07 (0.54–2.14)	0.83
Neutrophil engraftment					
	<1,000 ng/ml	92 (82–96) at 60 days	0.28	1	Reference
	≥1,000 ng/ml	95 (81–99) at 60 days		1.13 (0.79–1.62)	0.47

GVHD graft-versus-host disease, *CI* confidence interval, *HR* hazard ratio

[a] Among the 133 patients, pretransplant serum ferritin level was categorized as ≥1,000 ng/ml (n=49) or <1,000 ng/ml (n=84)

[b] The probability of overall survival was estimated according to the Kaplan–Meier method, and the groups were compared using the log-rank test. The probabilities of the others were estimated based on a cumulative incidence method to accommodate competing risks

[c] Multivariate analysis was performed with a Cox proportional hazard model adjusted for overall survival, and Fine and Gray proportional hazards model for the others

affects outcomes of allo-HSCT. Although hyperferritinemia was not associated with adverse outcomes after CBT in this study, further studies are warranted to evaluate the effect of iron overload on the outcome of CBT for acute leukemia and MDS.

Conflict of interest The authors have no conflicts of interest.

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TOPIC HIGHLIGHT

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Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility

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Abstract

Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). Apart from their prominent advantages, such as a painless collection procedure and faster self-renewal, UC-MSCs have shown the ability to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining and subamnion and perivascular regions. In this review, we give a brief introduction to various compartments of UC as a source of MSCs and emphasize the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

Key words: Umbilical cord; Mesenchymal stem cells; Wharton's Jelly; Multipotency; Immunotherapy

Core tip: Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). UC-MSCs have shown the ability of faster self-renewal and to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining membrane and subamnion and perivascular regions. In this review, we introduce various compartments of UC and discuss the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

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INTRODUCTION

Mesenchymal stem cells (MSCs) originate in the human embryo and are considered multipotent stem cells. MSCs are a heterogeneous subset of stromal stem cells, which can be isolated from the bone marrow[1], mobilized peripheral blood[2], cord blood[3], umbilical cord (UC)[4,5], placenta[6], adipose tissue[7], dental pulp[8], and even the fetal liver[9] and lungs[10]. UC contains two umbilical arteries (UCAs) and one umbilical vein (UCV), both embedded within a specific mucous connective tissue, known as Wharton's jelly (WJ), which is covered by amniotic epithelium (Figure 1). UC is considered medical waste and the collection of UC-MSCs is noninvasive; furthermore, the access to UC-MSCs has not been encumbered with ethical problems. UC-MSCs, similarly to MSCs derived

from other sources, have distinct capacity for self-renewal while maintaining their multipotency, *i.e.*, the ability to differentiate into adipocytes, osteocytes, chondrocytes, neurons and hepatocytes, although some differentiation abilities are known to be partial[11-13]. Moreover, UC-MSCs have also attracted great interest because of their immunomodulatory properties. Nowadays, UC-MSCs are proposed as a possible versatile tool for regenerative medicine and immunotherapy.

HISTORY OF UC-MSCs

During pregnancy, the fetus and mother are connected by UC. UC prevents umbilical vessels from compression, torsion and bending, while providing good blood circulation. McElreavey *et al*[4] for the first time reported isolation of fibroblast-like cells from WJ of human UC in 1991. The UC-derived cells have the similar surface phenotype, plastic adherence and multipotency as those of MSCs derived from other sources. It was 3 years earlier that the first cord blood (CB) transplantation was performed in France in 1988[14]. After that, together with the development of CB banking, CB transplantation has become the alternative source of hematopoietic stem cells. Although CB-derived MSCs cannot be consistently isolated[15-18], MSCs were considered to circulate in the blood of preterm fetuses and able to be successfully isolated and expanded[3]. Where these cells home at the end of gestation is not clear[13]. Thus, UC has inevitably become a focus of attention as a source of MSCs because it contains CB[18]. One key study appeared concerning CB-derived MSCs appeared around 2003[19]. Mitchell *et al*[20] successfully isolated matrix cells from UC WJ using explant culture and Romanov *et al*[19] isolated MSCs-like cells from the subendothelial layer of UCV.

ADVANTAGES OF UC-MSCs

Stem cell populations can be isolated from embryonic, fetal and adult tissues. Embryonic stem cells (ESCs) are a leading candidate for tissue engineering because of their high self-renewal capacity and pluripotency (ability to differentiate into all germ layers) *in vitro* and *in vivo*. Nonetheless, in addition to ethical restrictions, the clinical applications of ESCs are severely limited by technical difficulties with the depletion of immature cells that may result in the formation of a teratoma.

In contrast, adult stem cells, such as those in the skin, bone marrow (BM) and adipose tissue, may have wider clinical applications. BM-MSCs have been used for autologous and allogeneic purposes. Recently, successful clinical application of autologous BM-MSCs was reported for conditions such as cardiac infarction[21], graft-versus-host disease (GVHD)[22,23], Crohn's disease[24] and bone tissue engineering[25]. On the other hand, the autologous use was sometimes limited by cell numbers and age-related changes such as decreased growth and differentiation capacity[26,27].

Compared with BM-MSCs and ES cells, UC-MSCs show a gene expression profile more similar to that of ESCs and faster self-renewal rather than BM-MSCs[11,12].

It is easy to obtain a substantial number of UC-MSCs after several passages and extensive *ex vivo* expansion[28]. The most appreciable advantage is that the collection procedure is noninvasive and ethically acceptable.

Similar to BM-MSCs, UC-MSCs can be considered for autologous and allogeneic use. Autologous UC-MSCs might be used as gene therapy for genetic diseases and as regenerative or anti-inflammatory therapy for neonatal injury, such as cerebral palsy or hypoxic brain damage. On the other hand, allogeneic UC-MSCs can be expanded and cryopreserved in a cell bank for patients in need. The only disadvantage is that physicians need to confirm the baby's health as a donor because it cannot be ascertained in advance whether the donor will grow normally without health problems; thus, genomic or chromosomal tests need to be performed. In contrast, in the case of a BM donor, the physician can directly see and examine the donor and then decide to collect BM. In the case of CB banking, many CB banks monitor the baby's health after birth. Thus, it is important to know the advantages and disadvantages of UC-MSCs for each clinical application.

DIFFERENT METHODS FOR ISOLATION OF MSCs FROM DIFFERENT COMPARTMENTS OF UC

There are two methods to obtain MSCs from various UC compartments or from the whole UC: the explant method and the enzymatic digestion method.

The explant method

UC, or its compartments, is manually minced into small fragments 1-2 mm^3. These fragments are aligned and seeded regularly on the tissue culture-treated dishes. After the tissue fragments are attached to the bottom of the dish, the culture medium is poured slowly and gently, so as not to detach the fragments, and the culture is started[29-31]. The culture medium is replaced every 3-7 d for 2-4 wk until fibroblast-like adherent cells reach 80%-90% confluence. The adherent cells and tissue fragments are rinsed once with PBS and detached using a trypsin solution, followed by washing with the medium. The cells and tissue fragments are filtered to remove the tissue fragments.

The disadvantage of this method is that the fragments often float in the medium, resulting in poor cell recovery. No MSCs can be obtained from the floating fragments. To collect a consistent number of MSCs each time, it is important to prevent the exfoliation of the tissue fragments from the bottom of plastic dishes.

The enzymatic digestion method

WJ is either directly exposed to enzymatic solutions to

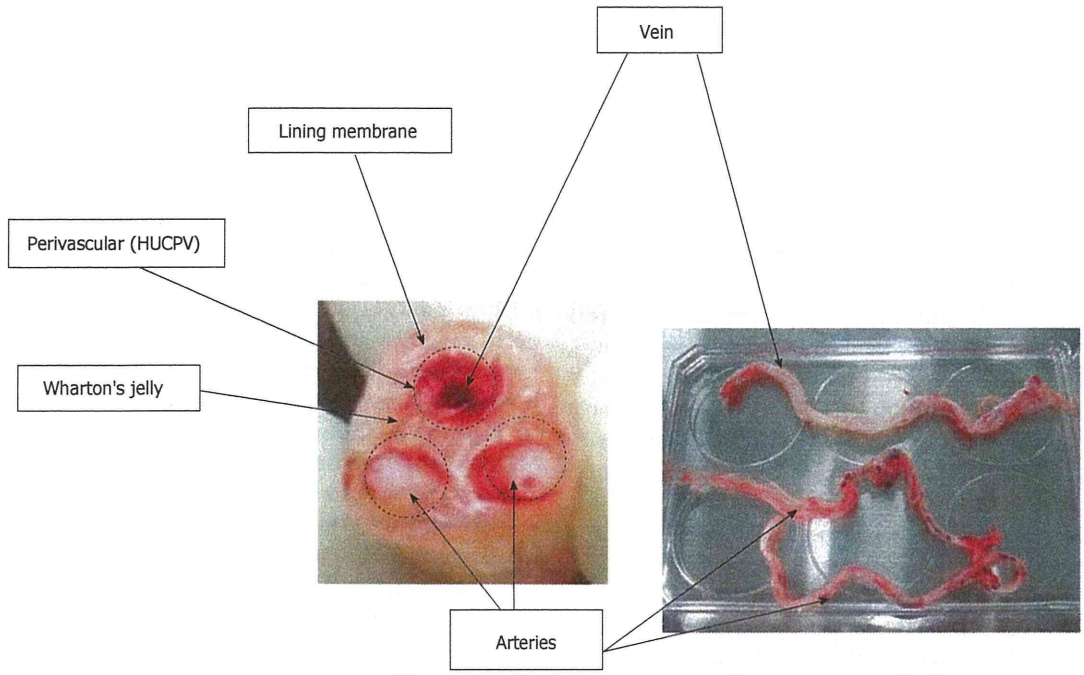

Figure 1 Various compartments of umbilical cord from which mesenchymal stem cells can be isolated. HUCPV: Human umbilical cord perivascular.

release the cells or it is cut into small pieces followed by enzymatic digestion. The enzymes used for digestion vary from simple collagenase[31,32] to a combination of either collagenase and hyaluronidase with or without trypsin[33,34] or collagenase, dispase II and hyaluronidase[33]. The digestion time and concentrations varied by researchers.

There are four compartments of UC as a source of MSCs: (1) Whole UC-MSCs: the whole UC is cut into smaller pieces followed by either an explant procedure or enzyme digestion[30,35,36]; (2) UCWJ-, UCA- and UCV-MSCs: UCWJ-MSCs are obtained after removing umbilical vessels. Umbilical vessels [two arteries (UCAs) and one vein (UCV)] can also be minced into 1-2 mm^3 fragments. The fragments are aligned regularly on the plate and cultured until MSCs start growing; (3) UC lining and subamnion-derived MSCs: the subamnion region of UC lining membrane is removed with a razor blade and then cut into small pieces. These fragments are plated in plastic culture dishes until MSCs start growing (explant method). With this method, however, it might be difficult to remove the adjacent region underneath the amniotic epithelium completely[37,38]; and (4) Human UC perivascular stem cells (HUCPVC): the vessels are extracted from UC and tied at both ends into loops. The loops are then placed into an enzymatic solution for a defined period of time to allow the cells to separate from the perivascular region. The detached cells are cultured and collected as HUCPVCs[26,28,39].

It is still controversial whether the isolation of the cells from the whole or some compartment is superior to others with respect to their proliferation ability, differentiation ability and immunosuppressive capacity.

Proliferation assays

The frequency of colony-forming unit fibroblasts (CFU-F) is significantly higher in whole UC-derived MSCs than in BM-MSCs with limiting dilution[26,30,40]. The authors first compared UCWJ-MSCs, UCA-MSCs and UCV-MSCs. UCV-MSCs exhibited a significantly higher frequency of CFU-F than UCWJ-MSCs and UCA-MSCs, but the doubling time was not different among these cell types[5]. The Mennan group also reported that there are no significant differences among the various compartments of UC, although the cells derived from any UC compartment proliferate significantly faster than BM-MSCs, with mean doubling times of 2-3 d at P0 through P3[41]. Depending on the purpose, researchers need to select either a compartment or the whole UC.

Biomarkers of UC-MSCs

The immunoprofile of UC-MSCs is analyzed using flow cytometry, according to the standard definitions for MSCs described by the position paper of the International Society for Cellular Therapy[42]. There are no single specific markers that can be used to identify multipotent MSCs. MSCs are positive for adhesion markers such as CD29 and CD44; mesenchymal markers such as CD90, CD73 and CD105; and human leukocyte antigen class I (HLA-ABC). MSCs are negative for endothelial cell marker CD31; hematopoietic cell markers such as CD34, CD45 and CD117; and human leukocyte differentiation antigen class II (HLA-DR)[43]. Among the different UC compartments, UCWJ-, UCV- and UCA-derived MSCs show a similar fibroblast-like spindle shape and the MSCs from these three types of tissues demonstrate no significant differences in the immunoprofile. These cells are positive for CD13, CD29 (integrin β1), CD73 (SH3), CD90 (Thy-1), CD105 (SH2; endogrin) and HLA-ABC at the cellular frequency greater than 90% and are negative for CD34,

CD45, CD133 and HLA-DR, with the cellular frequency less than 1%[5]. Mennan *et al*[41] also confirmed that MSC immunophenotypes showed no significant differences among different sources: BM, umbilical cord arteries, vein, WJ and UC lining membrane. Even although the authors could not find any major differences in their immunophenotypes, the cell populations derived from the different compartments may consist of different proportions of multipotent stem cells. Karahuseyinoglu *et al*[44] demonstrated that CD73 is expressed throughout the vessels and endothelium and is absent in the perivascular region, but the strongest expression is observed in the epithelial and subepithelial regions of WJ. CD90 is positive in most compartments but negative in the endothelial lumen lining. A high expression of vimentin, CKs (1, 4, 5, 6, 8, 10, 13, 18 and 19), desmin and SMA has been detected in the subamniotic layer and the perivascular region. Schugar *et al*[45] reported that CD146 (endothelial progenitor marker) is expressed in the vessel walls (100%) and the perivascular region of UC (62%) but is no longer expressed in UCWJ-MSCs[26,46]. These markers might aid in determining the multipotency of the isolated cell population. Phenotypic characterization of UC-MSCs might be influenced by the culture passage number, culture medium and culture method.

Furthermore, ESC markers such as Oct4, Nanog, Sox-2 and KLF4 are expressed only at low levels in UC-MSCs[47]. This suggests that MSCs are primitive stem cells, somewhere between ESCs and mature adult stem cells. Nonetheless, a precise isolation of pluripotent MSCs using specific markers remains a challenge.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Gang *et al*[48] reported that $SSEA4^+$ cells proliferate predominantly when the culture is initiated from primary BM cells in the medium supplemented with special cocktails of cytokines. In contrast, the authors failed to reproduce the same phenomena in UCWJ-MSCs in the medium consisting of α-MEM and 10% FBS. Furthermore, SSEA4 expression in UCWJ-MSCs significantly correlates with the FBS concentration in the culture medium, whereas SSEA3 expression was inversely correlated. We concluded that SSEA4 in UCWJ-MSCs is not a marker of either proliferation capacity or multipotency[31]. Schrobback *et al*[49] assessed SSEA4 expression in human articular chondrocytes, osteoblasts and BM-derived MSCs and characterized their differentiation potential. Their results showed that SSEA4 levels in these cells are not related to the capacity for chondrogenic and osteogenic differentiation and the proliferation potential *in vitro*[49].

THE ABILITY OF UC-MSCs TO DIFFERENTIATE INTO ADIPOGENIC, CHONDROGENIC AND OSTEOGENIC LINEAGES

UC-MSCs originating from the extraembryonic mesoderm and their capacity for differentiation into adipogenic, chondrogenic and osteogenic lineages have been extensively studied[50]. Regarding the osteogenic differentiation ability, Hsieh *et al*[11] demonstrated that the gene profiles of UC-MSCs are close to ESCs; UC-MSCs show delayed and insufficient differentiation into osteocytes. On the other hand, BM-MSCs have already expressed an osteogenic gene profile and can easily differentiate into osteocytes. Among the three compartments, UCWJ, UCV and UCAs, UCWJ-MSCs demonstrate an obviously defective ability to differentiate into osteocytes, even although the expression of osteocyte-related genes is detected by reverse-transcriptase PCR, at levels similar to those in the other two tissues/compartments[5]. Mennan *et al*[41] compared the osteogenic differentiation among cord regions in six samples and found that the best differentiation is seen with UCWJ-MSCs and whole UC-derived MSCs, rather than with UCA-, UCV- and UC lining MSCs.

As for adipocytic differentiation, Mennan *et al*[41] reported that UC-MSCs produce small lipid vacuoles, whereas BM-MSCs produce more mature adipocytes (unilocular lipid vacuoles). UC-MSCs might maintain their multipotency for longer periods than BM-MSCs can[51], although there were no obvious differences among MSCs derived from UC compartments in our research[5].

With respect to chondrogenic differentiation, UC-MSCs show no apparent differences among the different cord regions (sources)[41]. Moreover, the comparison of the chondrogenic potential between BM-MSCs and UC-MSCs revealed that UC-MSCs produce thrice as much collagen as BM-MSCs; this finding indicates that the former may be a better option for fibrocartilage tissue engineering[52].

In relation to other differentiation abilities, UCWJ-MSCs are the most studied cell type among various UC compartments and many papers have been published[53,54]. In addition to differentiating into osteocytes, chondrocytes and adipocytes, UCWJ-MSCs can differentiate into cardiomyocytes (with the gene expression of N-cadherin and cardiac troponin I [55]), neurons and glia[20], oligodendrocytes[56] and hepatocytes[57]. Recently, clinical trials have been conducted using UC-MSCs for neurogenic disorders (spinocerebellar ataxia and multiple system atrophy of the cerebellar type)[58] and liver disorders[59,60].

IMMUNOSUPPRESSIVE PROPERTIES OF UC-MSCs

Immunosuppressive effects have now become the most popular property of MSCs for potential clinical use. First, MSCs themselves are weakly immunogenic owing to the lack of HLA-DR and low expression of MHC class I molecules. MSCs have been shown to have immunomodulatory properties *in vitro*[61]. Furthermore, MSCs lack both CD80 and CD86 proteins[36,62], which

are costimulatory molecules inducing T cell activation and survival. The lack of HLA-DR, CD80 and CD86 suggests that MSCs do not elicit acute rejection and are suitable for allogeneic cell-based therapy.

Second, UC-MSCs have immunosuppressive properties *in vitro* and *in vivo*. Many studies have been published about the immunosuppressive effect of UCWJ-MSCs[63], UC lining-MSCs[37,64], HUCPV[65] and whole UC-derived MSCs[66]. The immunosuppressive effect of UC-MSCs is mediated by soluble factors and cell-to-cell contacts. PGE2, galectin-1 and HLA-G5 are released from MSCs and serve as effective factors of immunosuppression[67]. Among these factors, indoleamine 2,3-dioxygenase (IDO) is one of the most relevant because it is inducible by IFN-γ and catalyzes conversion from tryptophan to kynurenine[62,68]. This depletion of tryptophan from the environment can suppress T cell proliferation. UCWJ-MSC-mediated immunosuppression may require preliminary activation by proinflammatory cytokines, such as IFN-γ, with or without TNF-α, IL-1α or IL-1β.

It was recently suggested that the inflammatory environment produced by the upregulation of cytokines such as IFN-γ and TNF-α might alter the biological activity of MSCs from immunosuppression to immunostimulation[68]. In this case, UC-MSCs maynot prevent GVHD *in vivo*. It is known that upon stimulation by activated immune cells or cytokines (priming), MSCs are primed and become functional immunosuppressors. The extent of immunosuppression is greater with UCWJ-MSCs than with BM-MSCs[62]. Polchert *et al*[68] demonstrated that MSCs primed with IFN-γ are effective in a mouse GVHD model despite upregulated MHC class II molecules. In order to ensure the effective and safe therapeutic use of UC-MSCs, more *in vivo* experiments need to be conducted because of the many discrepancies with *in vitro* data.

CONCLUSIONS

Compared with the counterparts of other origins, UC-MSCs have attractive advantages as MSCs and as UC-derived cells: (1) a noninvasive collection procedure for autologous or allogeneic use; (2) a lower risk of infection; (3) a low risk of teratoma; (4) multipotency; and (5) low immunogenicity with a good immunosuppressive ability. It is still unclear which compartment in UC is the best for clinical use; nonetheless, the era of the clinical use of UC-MSCs is approaching quickly.

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