Downregulation of Melanoma Cell Adhesion Molecule (MCAM/CD146) Accelerates Cellular Senescence in Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells

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ABSTRACT

Therapeutic applications of mesenchymal stem cells (MSCs) for treating various diseases have increased in recent years. To ensure that treatment is effective, an adequate MSC dosage should be determined before these cells are used for therapeutic purposes. To obtain a sufficient number of cells for therapeutic applications, MSCs must be expanded in long-term cell culture, which inevitably triggers cellular senescence. In this study, we investigated the surface markers of human umbilical cord blood-derived MSCs (hUCB-MSCs) associated with cellular senescence using fluorescence-activated cell sorting analysis and 242 cell surface-marker antibodies. Among these surface proteins, we selected the melanoma cell adhesion molecule (MCAM/CD146) for further study with the aim of validating observed expression differences and investigating the associated implications in hUCB-MSCs during cellular senescence. We observed that CD146 expression markedly decreased in hUCB-MSCs following prolonged in vitro expansion. Using preparative sorting, we found that hUCB-MSCs with high CD146 expression displayed high growth rates, multilineage differentiation, expression of stemness markers, and telomerase activity, as well as significantly lower expression of the senescence markers p16, p21, p53, and senescence-associated β-galactosidase, compared with that observed in hUCB-MSCs with low-level CD146 expression. In contrast, CD146 downregulation with small interfering RNAs enhanced the senescence phenotype. In addition, CD146 suppression in hUCB-MSCs caused downregulation of other cellular senescence regulators, including Bmi-1, Id1, and Twist1. Collectively, our results suggest that CD146 regulates cellular senescence; thus, it could be used as a therapeutic marker to identify senescent hUCB-MSCs.

SIGNIFICANCE

One of the fundamental requirements for mesenchymal stem cell (MSC)-based therapies is the expansion of MSCs during long-term culture because a sufficient number of functional cells is required. However, long-term growth inevitably induces cellular senescence, which potentially causes poor clinical outcomes by inducing growth arrest and the loss of stem cell properties. Thus, the identification of markers for evaluating the status of MSC senescence during long-term culture may enhance the success of MSC-based therapy. This study provides strong evidence that CD146 is a novel and useful marker for predicting senescence in human umbilical cord blood-derived MSCs (hUCB-MSCs), and CD146 can potentially be applied in quality-control assessments of hUCB-MSC-based therapy.

INTRODUCTION

Mesenchymal stem cells derived from human umbilical cord blood (hUCB-MSCs) are characterized by self-renewal [1], a potential for differentiation into multiple cell lineages [2], low immunogenicity [3], and paracrine functions [4–6], suggesting that they may be beneficial in allogeneic MSC-based therapy. However, enabling MSC-based therapy requires expanding MSCs in large-scale production via long-term in vitro cultivation. During this process, MSCs inevitably enter cellular senescence, which adversely affects the biological properties of stem
cells associated with therapeutic outcomes, including their stemness, proliferation, differentiation potency, migration, and cytokine-production profiles [7, 8]. Thus, the senescence process of MSCs is a major issue that needs to be addressed to achieve improved outcomes in MSC-based therapy.

Typically, cellular senescence induces morphological changes that result in enlarged, multinucleated cells with a fried-egg morphology [9, 10]. Along with these morphological changes, growth arrest occurs during senescence, which is mediated by the inhibition of cell cycle progression. The senescence pathway underlying cell cycle progression proceeds via two major pathways that involve the p53/p21 and p16/retinoblastoma (Rb) proteins [11, 12]. When senescence is well advanced, the level of activated (phosphorylated) p53 increases, leading to phosphorylation of its downstream target, p21, which in turn mediates cell cycle arrest during senescence [13]. Indeed, several previous studies have demonstrated that activation of the p53/p21 pathway inhibits the growth of bone marrow-derived MSCs (BM-MSCs) [14, 15]. Phosphorylated Rb drives cell cycle progression by releasing E2F transcription factors that mediate the transcription of a variety of genes important for progression from G1 to S phase [16, 17], and p16 inhibits Rb phosphorylation, which consequently induces senescence [18]. A recent report showed that the level of phosphorylated Rb was high in early-passage MSCs, but that phosphorylated Rb levels decreased during later passages [19]. Such characteristic molecular phenotypes are often used as markers for cellular senescence. In addition, another commonly employed senescence-associated (SA) biomarker is β-galactosidase (SA-β-gal) [20]. Many research groups have endeavored to establish a standard set of criteria for confirming senescence in MSCs and in doing so have focused on investigating the mechanisms underlying the senescence process in MSCs. However, despite some advances made in recent studies, knowledge regarding MSC senescence and associated biomarkers is currently limited.

Here, we sought to identify senescence-related factors in hUCB-MSCs. Because the analysis of cell surface-expression phenotypes is widely used to rapidly identify or characterize cell statuses and identify senescence factors, we analyzed the expression of surface proteins in hUCB-MSCs during long-term culture by using fluorescence-activated cell sorting (FACS) with 242 different cell surface antibodies. Among the surface proteins studied, we found that the expression of melanoma cell adhesion molecule (MCAM/CD146) gradually decreased after multiple passages of hUCB-MSCs. Furthermore, we demonstrated that CD146 suppression accelerated cellular senescence in hUCB-MSCs. To our knowledge, this study is the first to demonstrate a possible role for CD146 in determining the senescence fate of hUCB-MSCs.

Materials and Methods

Cell Culture

The Institutional Review Board of MEDIPOST Co., Ltd., approved this study (MP-2014-07-1). We collected hUCB from umbilical veins after neonatal delivery after first obtaining informed maternal consent. The hUCB harvests were processed within 24 hours of collection. The hUCB was isolated by separating mononuclear cells (MNCs) with Ficoll-Hypaque solution (density = 1.077 g/cm³; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). The separated MNCs were washed and suspended in minimal essential medium α (Gibco/Invitrogen/ThermoFisher Scientific, Grand Island, NY, https://www.thermofisher.com), supplemented with 10% fetal bovine serum (Gibco/ThermoFisher Scientific). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂, wherein the culture medium was changed twice a week [21]. The expansion of live cells was analyzed by using the trypan blue exclusion method. For each passage (P), MSCs were cultured for 5 days, harvested with trypsin-EDTA (Gibco), counted, and then reseeded at a cell density of 2,000 cells/cm². Cumulative population doubling (PD) was calculated for each passage on the basis of the total number of cells at each passage [22]. This procedure was repeated until the cells stopped proliferating. Cell areas were analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD, http://imagej.net) by drawing cell margins on T75 flasks from images of cultured cells and measuring total cell areas from three fields [10]. The pictures shown are representative images of cultured cells. A total of 27 lots of hUCB-MSCs was used in our experiments. Basic information related to the hUCB-MSCs is summarized in supplemental online Table 1.

Cell Surface Antibody Screening With Lysoplates

Antibodies against 242 human cell surface markers, which were lyophilized in 96-well plates at 0.5 µg/well, were acquired from BD Biosciences (San Diego, CA, http://www.bdbiosciences.com). For screening, we selected cells from two different donors, which showed typical MSC features, including the potential for multilineage differentiation, representative MSC marker expression, and proliferation (supplemental online Fig. 1). For screening, MSCs were dispensed into 96-well round-bottom plates (BD Lysoplates, BD Biosciences) at 500,000 cells/well. Subsequently, the antibodies were reconstituted and the cells were stained on ice for 20 minutes. Next, cells were washed and stained for 20 minutes with an Alexa Fluor 647-conjugated goat-anti-mouse IgG secondary antibody (Molecular Probes/ThermoFisher Scientific, Eugene, OR, https://www.thermofisher.com). Surface markers were measured by flow cytometry on a FACSCalibur instrument (BD Biosciences), and the percentage of cells expressing each cell surface antigen was calculated for 10,000 cell events. Results were analyzed in Excel 2013 (Microsoft, Redmond, WA, http://www.microsoft.com) to generate heat maps.

Flow Cytometry and Sorting

For flow cytometric analysis of cultured cell phenotypes, cells were stained for 15 minutes at room temperature with fluorescein isothiocyanate-conjugated antibodies against human CD14, CD45, CD47, CD71, and CD146 (BD Biosciences); phycoerythrin-conjugated antibodies against human CD29, CD44, CD73, CD90, CD106, CD165, CD274, the epidermal growth factor receptor (EGFR; BD Biosciences), and CD105 (Serotec, Kidlington, U.K., https://www.abdsan.com); and an Alexa 647-conjugated antibody against human CD49f (BD Biosciences). Corresponding isotype-match mouse antibodies were used as controls. The cells were washed with phosphate-buffered saline (Gibco) and fixed with 1% (vol/vol) paraformaldehyde (Sigma-Aldrich). MSC immunotypes were determined by flow cytometry on a FACScalibur instrument, and then the percentage of expressed cell surface antigens was calculated for 10,000 gated-cell events. For
Nitrocellulose membranes. Each membrane was incubated with antibodies against phospho-p53 (pho-p53), p21, p16, and Rb (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com); p53 and phospho-Rb (pho-Rb, Abcam, Cambridge, U.K., http://www.abcam.com); and β-actin (Sigma-Aldrich).

RESULTS

Expansion of hUCB-MSC Induced Cellular Senescence

To assess the growth rate of hUCB-MSCs (n = 3), we continuously monitored cumulative PD until the cells stopped proliferating for individual lots of hUCB-MSCs. All cells eventually ceased proliferating in culture, with the number of passages being dependent on the donor (Fig. 1A). During the process of expansion, we analyzed fold-increases in cell counts at P5, P9, and P13. The fold-increases in cell growth gradually diminished from P5 to P13 (Fig. 1B). The expression of stemness markers in hUCB-MSCs, including Oct4, Nanog, and Sox2, was demonstrated at the mRNA level, which significantly decreased from P5 to P13 (Fig. 1C). The telomerase activities of hUCB-MSCs also significantly decreased during passing (Fig. 1D). The levels of pho-p53, p16, and p21 significantly increased during passages P5 to P13, whereas the level of pho-Rb decreased between P5 and P13 (Fig. 1E). In parallel, the expression of senescence-related markers was further confirmed by quantitative real-time PCR (qPCR) analysis of p16 and Bmi-1 mRNA (Fig. 1F). Moreover, to assess the general cellular-senescence phenotypes, we monitored SA β-gal activities and cell areas during passing from P5 or P9 to P13. SA β-gal staining and cell areas gradually increased from P5 to P13 (Fig. 1G). We also tested for multilineage differentiation with passaged hUCB-MSCs. ALP activity, a marker of

In Vitro Multilineage Differentiation

To assess multilineage potential, cells were incubated under specific conditions to induce differentiation into osteocytes or adipocytes. After differentiation, multilineage potential was evaluated as previously described [23]. Briefly, osteoblast or osteocyte formation was assessed by measuring the level of alkaline phosphatase (ALP; Sigma-Aldrich) activity or by von Kossa staining (Sigma-Aldrich). Quantitation of ALP or von Kossa staining was analyzed by calculating the overall percentage of cells that were positively stained. Assessment of adipocyte formation was based on the staining of accumulated lipid vacuoles with Oil Red O (Sigma-Aldrich). Lipid vacuole accumulation was quantified by calculating the percentage of stained cells in the total population. All quantitation of stained cells was performed by using i-Solution software (IMT I-Solution Inc., Daejeon, Republic of Korea, http://imt-isolution-lite.software.informer.com).

SA β-gal Staining

SA β-gal staining was used as a biomarker for senescence. A histochemical staining kit (Sigma-Aldrich) was used according to the manufacturer’s instructions to qualitatively assess SA β-gal activity, and cells were examined on an inverted microscope. To assess senescent-cell formation, the overall percentage of stained cells in the cell populations was averaged from four fields [24].

Telomerase Activity

Telomerase activity was analyzed by a telomeric repeat amplification protocol (TRAP) assay using a telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay kit (Roche, Mannheim, Germany, http://www.roche.com), according to manufacturer’s protocol [24]. Briefly, cells (2 × 10^6) were harvested for each reaction and centrifuged at 3,000 g for 10 minutes at 4°C, washed twice with PBS, incubated for 20 minutes at 4°C with 200 μl lysis buffer, and centrifuged at 16,000 g for 20 minutes. Telomeric repeats were added to a biotin-labeled primer during the first reaction. The PCR product was denatured, hybridized to a digoxigenin-labeled telomeric repeat-specific probe, and immobilized on a microplate. Finally, the immobilized PCR product was incubated with an anti-digoxigenin peroxidase antibody and visualized by colored-reaction product formation after substrate addition. Absorbsances for the final products were measured at 450 nm by using a microplate reader. Cellular extract from 293 cells was used as a positive control (included in the kit), and the lysis reagent served as a negative control.

Western Blotting

Cell extracts were prepared in buffer containing 9.8 M urea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, 130 mM dithiothreitol, 40 mM Tris-HCl, and 0.1% sodium dodecyl sulfate (SDS). Protein concentrations were measured by using the bicinchoninic acid kit (Sigma-Aldrich). Protein extracts (10 μg) were separated by SDS-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to nitrocellulose membranes. Each membrane was incubated with antibodies against phospho-p53 (pho-p53), p21, p16, and Rb (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com); p53 and phospho-Rb (pho-Rb, Abcam, Cambridge, U.K., http://www.abcam.com); and β-actin (Sigma-Aldrich).

Quantitative Real-Time PCR and Small Interfering RNA Experiments

Quantitative real-time PCR (qPCR) was performed by using a LightCycler 480 (Roche). TaqMan probes were designed with the Universal Probe Library Assay Design Center (Roche) (supplemental online Table 2) and used to quantitatively detect mRNA for the following genes: Bmi-1, p16, Oct4, Nanog, Sox2, inhibitor of DNA binding 1 (Id1), Id2, Id3, Id4, Twist1, Twist2, and CD146. Relative expression levels of mRNAs of interest were calculated by using the comparative threshold cycle method (2^(-ΔΔCt)), with normalization to β-actin mRNA expression. GE Dharmacon (Chicago, IL, http://dharmacon.gelifesciences.com/) designed a CD146 siRNA and scrambled siRNA for use in the siRNA experiments. siRNAs were transfected using the Dharmafect Reagent (GE Dharmacon) according to the manufacturer’s instructions. The siRNA pools consisted of four different siRNA duplexes (supplemental online Table 2).

Statistical Analysis

All data are reported as mean ± SD and were analyzed in SPSS software, version 18 (IBM, Chicago, IL, http://www-01.ibm.com). Significant differences were verified by one-way analysis of variance followed by the least-significant-difference post hoc test. p values less than .05 were considered to represent statistically significant differences.
osteoblast-associated differentiation, decreased in culture between P5 and P13. Adipogenesis, which is characterized by a decrease in lipid vacuole formation, was demonstrated by Oil Red O staining (Fig. 1H). Collectively, these data showed that the expansion of hUCB-MSCs induced the senescent phenotype.
Passaging of hUCB-MSCs Downregulated Cell-Surface CD146 Expression

A variety of surface proteins have been proposed to govern MSC features, such as stemness, differentiation potency, and primitive selection [25]. Thus, we hypothesized that senescent hUCB-MSCs may use cell-surface proteins to actively control the aging process. To test this hypothesis, we first analyzed the cell-surface expression of CD29, CD73, CD90, CD105, and CD166 in early-stage (P4) and late-stage (P10) hUCB-MSCs, which are the minimal criteria established by the International Society for Cellular Therapy (ISCT) [26]. No change in the expression of these surface markers was observed in hUCB-MSCs between P4 and P10 (Fig. 2A). We also used a surface marker array containing antibodies against 242 CD markers to screen for expression differences during the passaging of hUCB-MSCs (n=2) (supplemental online Table 3). As a result, we identified 7 cell-surface proteins that were markedly downregulated after passaging in culture: CD47, CD71, CD106, CD146, CD165, CD274, and EFGR (Fig. 2B). To verify these screening results, we measured the expression levels of the 7 surface proteins in early- and late-stage hUCB-MSCs from an additional 25 different donors by flow cytometry (Fig. 2C; supplemental online Table 4). The expression level of 3 markers (CD47, CD106, and CD165) did not show significant differences between the early and late stages (Fig. 2C). Although CD274 was not decreased with statistical significance, 5 lots of hUCB-MSCs showed decreased CD274 expression at P10. In particular, CD274 expression of hUCB-MSCs showed relative variation among 25 lots of hUCB-MSCs depending on the donors. CD71, CD146, and EFGR expression significantly decreased at a late stage (Fig. 2C). Notably, CD146 was the most significantly downregulated surface marker as the number of passages increased. The expression of CD146 was reduced at P10 in all hUCB-MSCs lots tested (Fig. 2C; supplemental online Table 4). In fact, the percentage of CD146-positive hUCB-MSCs gradually decreased during long-term culture, with the following percentages observed: P5 (92.3% ± 7.1%), P9 (66.0% ± 7.1%), and P13 (26.1% ± 11.4%), as shown in Figure 2D. Thus, we selected CD146 as a marker to further investigate the involvement of CD146 in the senescence of hUCB-MSCs.

Senescence Increased in CD146—hUCB-MSCs Compared With That Observed in CD146+ hUCB-MSCs

To determine whether CD146 expression is related to senescence in hUCB-MSCs, we sorted CD146+ and CD146—hUCB-MSCs by flow cytometry with an anti-CD146 antibody at P6. The sorted cells (i.e., CD146+ or CD146—) were purified to ≥95% (Fig. 3A). To analyze whether MSC surface markers were altered by CD146 expression, we tested the expression of MSC-specific antigens (CD29, CD73, CD90, CD105, CD166, CD14, and CD45) in sorted cells. Surface antigen staining showed that no difference occurred between the two populations (Fig. 3B). We also analyzed several cell-senescence phenotypes, including proliferation, stemness gene-expression levels, telomerase activity, expression levels of senescence-related proteins, SA β-gal activities, cell areas, and differentiation potentials. A comparative analysis between both populations showed that the growth rate of CD146+ cells was faster than that of CD146—cells. Specifically, the growth of CD146—cells ceased at P12, whereas CD146+ cells continued to grow until P15 (Fig. 4A). In qPCR analysis, CD146+ cells showed high expression levels of stemness genes compared with those observed in CD146—cells (Fig. 4B). Similarly, telomerase activities of CD146+ cells were significantly higher than those of CD146—cells (Fig. 4C). Western blot analysis indicated that CD146—cells showed enhanced expression of pho-p53 and p16 relative to that observed in CD146+ cells (Fig. 4D), as well as significantly augmented SA β-gal activity (Fig. 4F) and cell areas (Fig. 4G). In addition, CD146+ cells showed relatively low p16 expression but high Bmi-1 expression compared with that found in CD146—cells (Fig. 4E).

The functional effects of CD146 expression on the multilineage differentiation of hUCB-MSCs were evaluated by assessing CD146+ and CD146—cells under osteogenic- and adipogenic-specific conditions. As expected, under identical osteogenic conditions, ALP activity was significantly higher in CD146+ cells than in CD146—cells. Moreover, the level of calcium observed after von Kossa staining was also significantly higher in CD146+ cells than CD146—cells under the levels observed in CD146—cells. Under adipogenic conditions, significantly larger lipid drops were formed within CD146+ cells than in CD146—cells, as determined by Oil Red O staining (Fig. 4H). Collectively, our results implied that CD146—cells have a higher potential for developing the senescence phenotype than do CD146+ cells.

Knockdown of CD146 Expression Accelerates Cellular Senescence in hUCB-MSCs

To confirm that CD146 functionally contributed to the senescence phenotype of hUCB-MSCs, we blocked CD146 expression using a siRNA (n = 3). Control experiments showed that treatment with the target siRNA also effectively inhibited CD146 expression at the protein level, as shown by flow cytometry (supplemental online Fig. 2A), with suppression being maintained for up to 28 days (supplemental online Fig. 2B). Cells with silenced CD146 expression exhibited a reduced duration of proliferation during the culture period and lower growth rates, compared with the respective measures made in naïve cells or scrambled siRNA-transfected cells (Fig. 5A). For example, the growth of CD146-silenced cells ceased at P12, whereas the other two cell populations continued to grow until P15. Thus, under the same conditions, the cumulative PD of CD146 siRNA transfectants (9.8 ± 0.3) was significantly shorter than that of naïve (15.0 ± 0.7) or scrambled siRNA (14.8 ± 0.5) transfectants (Fig. 5A). CD146 knockdown in hUCB-MSCs also resulted in decreased expression levels of stemness genes, such as Oct-4, Nanog, and Sox2 (Fig. 5B). In particular, CD146 suppression significantly reduced the telomerase activity of hUCB-MSCs to 40% of the level found in the control groups (Fig. 5C). At the protein level, CD146 silencing significantly enhanced pho-p53 production and p16 expression compared with their relative expression levels in naïve or scrambled siRNA transfectants at P9 (Fig. 5D). As expected, CD146 siRNA treated cells showed increased p16 mRNA expression levels and inhibited Bmi-1 mRNA expression (Fig. 5E). In addition, SA β-gal activities (Fig. 5F) and cell areas (Fig. 5G) were significantly increased in CD146 siRNA transfectants. CD146 silencing significantly reduced both osteogenesis and adipogenesis, as confirmed by ALP staining, von Kossa staining, and Oil Red O staining (Fig. 5H). Taken together, these data demonstrate that inhibiting CD146 expression accelerated the senescence phenotype in hUCB-MSCs.

CD146 Suppression in hUCB-MSCs Downregulated Senescence Regulators

To further examine the mechanistic relationship between CD146 expression and cellular senescence, we investigated whether...
CD146 expression regulates the expression of Id1- or Twist-family genes, which can regulate the cellular senescence process [27]. During the onset of cellular senescence in hUCB-MSCs, the expression of Id1 and Twist family genes significantly decreased (0.2–0.6-fold lower expression at P12 than at P5), which was confirmed by qPCR (Fig. 6A). After sorting of hUCB-MSCs for CD146 expression, Id1 and Twist1 expression was lower in the CD146− subset than in CD146+ cells (Fig. 6B). To confirm these
observations, we also analyzed the expression of Id- and Twist-family genes, with or without CD146 siRNA treatment. We found that Id1 and Twist1 expression levels were significantly decreased only in CD146 siRNA transfectants and not in the control groups (negative and scramble siRNA; Fig. 6C). Collectively, these data imply that CD146 expression might be involved in maintaining expression of the Id1 and Twist1 transcription factors that are related to cellular senescence regulation.

DISCUSSION

Currently, the cell-surface markers suggested by the ISCT are the most widely used markers, not only as the minimal criteria for defining or characterizing MSCs but also as quality-control markers for producing functional MSCs [26, 28]. Indeed, growing evidence has suggested that these surface proteins are useful biomarkers for predicting various biological characteristics of MSCs. For instance, our previous results indicated that endoglin (CD105) is a useful marker for characterizing the differentiation status of hUCB-MSCs [29]. Another report demonstrated that neural gan-glioside (GD2) is associated with neural differentiation of hUCB-MSCs [23]. Moreover, several surface markers, including CD140b [30, 31] and CD271 [25, 32, 33], have been suggested as new positive markers for identifying and isolating MSCs. However, there have been few reports of markers that are functionally related to the senescence status of MSCs, and no direct evidence has previously been provided that CD146 expression is related to the cellular senescence regulation.

In the present study, we sought to identify cell-surface senescence-marker proteins in hUCB-MSCs during long-term culture through a large-scale, FACS-screening approach. Because we were interested in negative regulators of cellular senescence, we mainly focused on surface proteins with decreased expression during long-term culturing of hUCB-MSCs. Consequently, during screening we identified 7 surface markers that were downregulated at a late passage (P10). Because individual variations can result from unidentified factors in each hUCB-MSC lot used for screening, we validated the results with these candidate markers with 25 lots of hUCB-MSCs. After validation, we found that CD71, CD146, and EGFR significantly decreased during passaging. In particular, among these 3 validated markers, only CD146 was markedly and reproducibly decreased after passaging all lots of hUCB-MSCs to P10; hence, CD146 was selected for further investigation, specifically for its role in the senescence process. Although the passage-dependent reduction of CD274 expression in hUCB-MSCs did not reach statistical significance, 5 of 25 lots of hUCB-MSCs showed dramatically decreased CD274 expression at a late passage (supplemental online Table 4). Because CD274 has been reported to be involved in the hypomunogenic features of MSCs [34, 35], these late-passage cells may not be suitable for transplantation as a result of the lack of these properties. However, the overall expression levels of CD274 were in fact relatively low both in early- and late-passage naive hUCB-MSCs. Similarly, the results from several studies demonstrated that naive MSCs express low levels of CD274 but that proinflammatory factors such as interferon-γ stimulated CD274 expression in MSCs [36–38]. Thus, the induction potential of CD274 expression may decrease in late passages of hUCB-MSCs after stimulation with proinflammatory conditions, a possibility that future studies should address.

Here, we provide evidence that CD146 expression is markedly decreased in hUCB-MSCs passaged through senescence. Similarly, results from a recent study showed that the expression of vascular cell adhesion molecule-1 (CD106) is markedly reduced in senescent BM-MSCs, which results in low homing activity [39]. Although CD106 is also expressed in hUCB-MSCs, its expression did not change significantly during long-term culture. We found for the first time that CD146 siRNA-transfected
hUCB-MSCs and the natural CD146− subset of hUCB-MSCs exhibit accelerated cellular senescence phenotypes. In addition, we further demonstrated the direct role of CD146 in regulating the cellular senescence of hUCB-MSCs. These results indicate that CD146 acts as a negative regulator of cellular senescence. Collectively, our results suggest that CD146 can be a useful alternative marker for predicting the senescence status of hUCB-MSCs.

To use CD146 as a marker for evaluating the senescence status of hUCB-MSCs, it is important to define the level of CD146 expression that occurs at senescence. Thus, we determined the cutoff CD146 expression level for defining nonsenescent hUCB-MSCs. When the proliferation of each of the 25 lots of hUCB-MSCs ceased, we measured CD146 expression at that passage by FACS analysis (supplemental online Fig. 3). Although the hUCB-MSC lots showed different passage numbers before

Figure 4. Senescence phenotypes of cells, based on CD146 expression. (A): Cell growth was measured by determining the cumulative PD. (B): Stemness genes were assessed by quantitative real-time polymerase chain reaction (qPCR) at passage (P) 9 (mean ± SD; n = 3; **, p < .01). (C): Telomerase activities was measured at P9 by using the TRAP assay (mean ± SD; n = 4; **, p < .01). (D, E): Expression of cell cycle inhibitors was measured by immunoblotting (D) and qPCR at P9 (E) (mean ± SD; n = 3; **, p < .01). (B, D, E): Expression levels were normalized to β-actin, with the expression levels in CD146+ cells defined as 1. (F): CD146+ cells showed strong SA β-gal staining at P9 and P12 (right panel; mean ± SD; n = 4; *, p < .05; **, p < .01). (G): Cell areas were normalized to the mean area in CD146+ cells, which was defined as 1 at P9 (right panel; mean ± SD; n = 30; **, p < .01). The black lines indicate the cell margins. (H): In each population, multilineage differentiation was examined by ALP staining, von Kossa staining, and Oil Red O staining. Quantitative results was significantly reduced in CD146− cells at P9 (lower panel; mean ± SD; n = 3; *, p < .05; **, p < .01). (F–H): Scale bar = 50 μm. Abbreviations: ALP, alkaline phosphatase; OD, optical density; PD, population doubling; pho-p53, phospho-p53; SA β-gal, senescence-associated β-galactosidase; TRAP, telomeric repeat amplification protocol.
Figure 5. CD146 knockdown in human umbilical cord blood-derived mesenchymal stem cells accelerates the senescence process. (A): Cell growth was monitored by measuring cumulative PD. (B): Cells were assessed for their expression of stemness genes by quantitative real-time polymerase chain reaction (qPCR) at passage (P) 9 (mean ± SD; n = 3; *, p < .01). (C): Telomerase activities was measured at P8 using the telomerase PCR enzyme-linked immunosorbent assay kit (mean ± SD; n = 4; **, p < .01). (D): Expression of the cell cycle inhibitors was measured by immunoblotting at P9, with β-actin serving as a loading control fold (right panel; mean ± SD; n = 4; *, p < .05; **, p < .01). (E): qPCR data showing the mRNA expression levels (p16 and Bmi-1) at P9 (mean ± SD; n = 4; **, p < .01). (B, D, E): Expression levels were normalized to β-actin, with the expression levels in naïve defined as 1. (F): SA-β-gal-positive cells were measured at P9 and P12. Results are shown as mean ± SD (n = 4; **, p < .01). (G): Cell areas compared at P9, which was normalized to the mean area in naïve cells, defined as 1 (mean ± SD; n = 25; **, p < .01). The black lines indicate the cell margins. (H): Multilineage differentiation was assessed by quantifying the percentage of positively stained cells at P9 (mean ± SD; n = 3; *, p < .05). (F–H): Scale bar = 50 μm. Abbreviations: OD, optical density; PD, population doubling; pho-p53, phospho-p53; SA-β-gal, senescence-associated β-galactosidase; siCD146, CD146 small interfering RNA; siCon, small interfering scrambled RNA; siRNA, small interfering RNA; TRAP, telomeric repeat amplification protocol.
proliferation stopped or senescence was induced, we suggest that predictions for senescence of hUCB-MSCs are possible according to this cutoff value. In addition, because freezing and thawing processes are necessary for MSC-based therapy, understanding the stability of markers during these processes is also required. Thus, we also examined the effects of freezing and thawing on CD146 expression in hUCB-MSCs, and we found that CD146 expression in hUCB-MSCs was not altered (supplemental online Fig. 4) by either process. Collectively, these results suggest the potential for using CD146 in evaluating quality-control assessments of MSC-based therapy.

Originally, CD146 was characterized as a cell-adhesion molecule that was useful as a biomarker for endothelial lineages, and it was found to play key roles in various biological processes, including cell growth, migration, and angiogenesis [40, 41]. Results from several studies have shown that CD146 augments motility, metastasis, and tumorigenesis of cancer cells, including melanoma and breast cancer cells [42, 43]. Recently, growing evidence has indicated that CD146 expression is related to the multilineage differentiation potential, proliferation, and stemness of MSCs [44–49]. Indeed, we also observed that CD146 downregulation led to decreases in the osteogenic and adipogenic differentiation potential of hUCB-MSCs, implying that such multilineage differentiation potential depends on CD146 expression levels in hUCB-MSCs (supplemental online Figs. 5 and 6). Furthermore, we showed that CD146 regulates hUCB-MSC proliferation, and stemness markers in hUCB-MSCs were decreased by CD146 suppression. Our data demonstrated that cellular senescence following long-term culture reduced the biological potentials of hUCB-MSCs, which were further accelerated by CD146 suppression. Thus, we conclude that CD146 regulates the senescence of hUCB-MSCs, which affects the osteogenic and adipogenic differentiation, proliferation, and stemness of hUCB-MSCs.

Because unsorted hUCB-MSCs have a subpopulation of CD146- cells and CD146 plays a role in stem cell phenotypes, sorted CD146+ hUCB-MSCs showed high levels of stemness markers and differentiation potentials compared with unsorted naive hUCB-MSCs (supplemental online Fig. 7). These results

Figure 6. Involvement of CD146 in Id and Twist-family gene expression. (A): The expression of Id- and Twist-family genes was analyzed after human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) expansion (both at passage [P] 5 and P12, mean ± SD; n = 3; **, p < .01). The expression levels of all genes were normalized to that of β-actin in hUCB-MSCs at P5, which was defined as 1-fold expression. (B): CD146+ and CD146- hUCB-MSCs were sorted by flow cytometry and examined for Id- or Twist-family gene expression at P9 (mean ± SD; n = 3; **, p < .01). The expression levels of all genes were normalized to that of β-actin in CD146+ cells, which was defined as 1-fold expression. (C): The gene-expression levels of Id1 and Twist1 significantly decreased siCD146 compared with the expression levels in naive and siCon-treated cells (mean ± SD; n = 3; **, p < .01). The expression levels of all genes were normalized to that of β-actin in naive cells, which was defined as 1-fold expression. Abbreviations: siCD146, CD146-specific siRNA; siCon, control siRNA; siRNA, small-interfering RNA.
suggest that hUCB-MSCs contain a subpopulation of cells expressing stemness-related factors such as CD146 that show increased stem cell characteristics compared with other MSCs within the population, which further indicates that hUCB-MSCs are heterogeneous. In particular, according to the results of several studies, CD146 expression distinguishes MSCs from non-MSCs in BM cells, such as fibroblasts, as well as bone cells that express several MSC markers, but not CD146 [25, 50]. Furthermore, several research groups have suggested that CD146 not only is an enrichment marker for MSCs but also serves as an additional positive marker for defining MSCs [25]. In the context of MSC heterogeneity, the overall population of MSCs includes the more comitted cells with basic features of MSCs; ES-like cells with high expression of Oct4 and Sox2; and non-MSCs, such as fibroblast cells [51]. In fact, this MSC heterogeneity has led to limited and variable therapeutic outcomes in clinical trials [52–54]. Several research groups have endeavored to enrich MSCs using factors associated with stemness or multipotent differentiation to increase their therapeutic effects [55, 56]. Thus, CD146 can probably be used to enrich MSCs and improve their therapeutic potentials by reducing MSC senescence and heterogeneity.

In this study, we clearly demonstrated that CD146 expression is closely related to the cellular senescence of hUCB-MSCs. To investigate the downstream CD146-dependent mechanisms underlying cellular senescence, we also examined the expression of helix-loop-helix transcription factors, such Id1 and Twist1, during long-term culturing of hUCB-MSCs. Results from a previous study demonstrated that Id1 and Twist1 expression are downregulated during the expansion of BM-MSCs [27]. Similarly, we observed that the expression of Id- and Twist-family proteins decreased during passageing through senescence. These data indicate that Id- and Twist-family proteins may govern the senescence process of hUCB-MSCs. In agreement, data from previous reports showed that Id1 and Twist1 serve inhibitory functions during senescence in melanoma cells or MSCs by regulating key cell-cycle regulators, such as p53 and p16 [57–60].

Importantly, we also found that CD146 downregulation resulted in decreased Id1 and Twist1 mRNA levels. Although a previous report showed that CD146 regulates Id1 expression during melanoma progression [61], a link between CD146 and Twist1 expression had not been previously demonstrated. Thus, our results provide new, indirect evidence that Twist1 is a downstream target protein of CD146 during the development of cellular senescence in hUCB-MSCs. Interestingly, the results of a recent study suggested a post-translational mechanism for Id1 regulation that involved Smurf-2-mediated ubiquitination and Id1 degradation, which promoted cellular senescence by increasing p16 activity [62]. Furthermore, recent data showed that high Bmi-1 expression in adult stem cells protects them against senescence by suppressing senescence-related genes, including p16 [63, 64]. We observed a novel correlation between Bmi-1 and CD146, in that the expression level of Bmi-1 decreased following CD146 suppression. Collectively, our findings support an alternative mechanism whereby CD146 may delay cellular senescence via the transcriptional regulation of Bmi-1, Id1, or Twist1. However, further studies, which are in progress, are necessary to clarify the downstream mechanism(s) underlying the negative regulation of cellular senescence by CD146.

CONCLUSION

Our data showed that CD146 expression is gradually decreased during the long-term expansion of hUCB-MSCs in culture and that enforced CD146 downregulation accelerated senescence in hUCB-MSCs, which affected their multilineage differentiation potential, growth, and stemness. Collectively, these data indicate a possible role for CD146 in inhibiting senescence in hUCB-MSCs, which may occur via the regulation of Bmi-1, Id1, and Twist1 expression. Thus, we suggest that CD146 is a novel marker for predicting senescence in hUCB-MSCs, and it could be valuable in quality-control assessments and for improving the therapeutic potential of hUCB-MSC-based therapy.

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AUTHOR CONTRIBUTIONS

H.J.J.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; J.H.K., M.K., and Y.K.B.: collection and/or assembly of data; S.J.C. and W.O.: data analysis and interpretation; Y.S.Y.: data analysis and interpretation, financial support; H.B.J.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

S.J.C. has compensated employment. W.O. has compensated employment, is an intellectual property and patent holder, and has stock options with MEDIPOST, Co., Ltd. The other authors indicated no potential conflicts of interest.

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