Research article

Effect of CD44 signal axis in the gain of mesenchymal stem cell surface antigens from synovial fibroblasts *in vitro*

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HIGHLIGHTS

- Fibroblasts isolated from synovial membrane give rise to cells with MSC antigen-positive cells during *in vitro* culture.
- MSC antigen-positive cells are formed from CD44-positive cell fraction.
- Hyaluronic acid, a CD44 ligand, controls MSC antigen expression.

ARTICLE INFO

ABSTRACT

Tissue-residing mesenchymal stromal/stem cells (MSCs) have multipotent characteristics that are important for adult tissue homeostasis and tissue regeneration after injury. We previously reported that fibroblastic cells isolated from the synovial membrane in the knee joint give rise to cells with MSC characteristics in a two-dimensional culture. To explore the molecular mechanisms underlying these hyperplastic properties, we performed time-course surface antigen expression analyses during *in vitro* culture. Cells freshly isolated from the synovial membrane rarely contained cells that met the criteria (CD45$^-$CD73$^+$CD90$^+$CD105$^+$). However, the number of cells expressing MSC antigens increased on day 7. Flow cytometric analysis indicated that cells positive for either CD73 or CD90 were specifically derived from cells positive for CD44. CD44 expression was upregulated during culture, and CD105$^+$ cells were specifically derived from the CD44 highly expressing cells. In addition, depletion of hyaluronic acid (HA), a major ligand of CD44, decreased the number of CD105$^+$ cells, whereas supplementation with HA increased their number. These data suggest that intracellular signals activated by CD44 play an important role in the formation and/or maintenance of MSCs.

1. Introduction

Osteoarthritis (OA) cause a decline in activities of daily living and quality of life due to persistent pain and restricted range of motion [1]. Most current conservative treatment strategies for knee OA are based on symptom management by weight control, education, anti-inflammatory analgesics, and improvement of joint mobility and flexibility by programmed exercise rather than disease modification (cartilage regeneration) [1]. Recent progress in the field of mesenchymal stem cell (MSC) biology has resulted in the development of

new clinical applications for regenerating damaged tissues in the knee joint [2].

Synovial tissues in the knee joint consist of two different cell types, synovial macrophages and synovial fibroblasts [3]. Synovial fibroblasts play a central role in joint homeostasis as they produce synovial fluid and provide nutrition to intra-articular avascular tissues, such as articular cartilage [3]. Recent studies have shown that synovial tissues contain dormant cells that can proliferate upon activation and contribute to tissue regeneration after articular cartilage injury [4]. These cells are tissue-residing MSCs [5]. However, the molecular mechanisms and

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cellular components involved in the maintenance of dormant cells in synovial tissues are not yet fully understood.

We previously reported that fibroblastic cells isolated from synovial tissues have strong growth potential and give rise to cells with MSC characteristics *in vitro* [6]. These cells have superior chondrogenic capacity compared to those from the bone marrow, and can thus be used in cartilage regeneration therapy in humans [7, 8]. In our previous studies, transplantation of autologous synovial MSCs effectively accelerated cartilaginous tissue regeneration in the knee joint of both humans and experimental animals [8, 9, 10, 11, 12, 13, 14, 15]. However, we found that there are individual differences in the proliferation potential of MSCs *in vitro* and the efficacy of cartilage regeneration by intra-articular transplantation [8, 16]. To successfully conduct cartilage regeneration therapy using synovial MSCs, precise control of the number and quality of these cells in two-dimensional (2-D) cultures is important. However, little is known about how the MSC population develops from synovial fibroblasts during *in vitro* culture.

To answer this question, we analyzed the time-course of MSC-related cell surface antigen expression profiles, which were positive for CD73, CD90, and CD105 and negative for CD45, during 2-D cultures of synovial fibroblasts [5]. In the present study, we demonstrated that synovial fibroblasts have high plasticity and give rise to cells with MSC characteristics. In addition, we showed that these cells (CD45$^-$CD73$^+$CD90$^+$CD105$^+$) derive from CD44 expressing cells, and hyaluronic acid (HA), a major ligand for CD44, enhanced the CD105$^+$ cell population. These data suggest an important role for the HA-CD44 signaling axis in the maintenance of MSCs *in vitro*.

2. Methods

2.1. Ethics

This study was approved by the Ethics Committee of Tokyo Medical and Dental University (No. M2017-142). All the patients provided written informed consent to participate in the study.

2.2. Isolation and expansion of synovial MSCs

Primary human synovial cells were isolated from the suprapatellar synovium of patients who underwent total knee arthroplasty as described previously with minor modifications [17]. Briefly, nucleated cells were isolated from the synovial membrane (0.5–1 g) dissected from the suprapatellar bursae using collagenase digestion (C9263; Sigma-Aldrich, St. Louis, MO, USA) for 3 h at 37 °C. The cells were seeded onto 15 cm-diameter dishes at 10^4-10^5 cells/dish and cultured in complete growth medium (MEM-alpha 12561-056; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS 26140079; Thermo Fisher Scientific) and antibiotics (15240062; Thermo Fisher Scientific). The growth medium was replaced with fresh medium every 3–4 days during culture. We have reported that these synovial cells form a cell population that does not express the surface antigens of hematopoietic cells (CD45) and vascular endothelial cells (CD31), but expresses surface antigens that meet the definition of MSCs (positive for CD73, CD90, and CD105) [5, 16].

2.3. The time-course of cell surface antigen expression during cultures of primary synovial fibroblasts

Primary synovial cells obtained by collagenase digestion were seeded onto 15 cm-diameter dishes at 10^4–10^5 cells/dish. The cells were dispersed using trypsin (25300062; Thermo Fisher Scientific) for 5 min at 37 °C on day 1, 4, 7, and 14. Dispersed cells were stained with fluorochrome-conjugated antibodies against cell surface markers, including CD44 (559942; BD Biosciences, Franklin Lakes, NJ, USA), CD73 (561254; BD Biosciences), CD90 (561558; BD Biosciences), CD105 (560819; BD Biosciences), CD45 (555483; BD Biosciences), CD140a

(556002; BD Biosciences), CD140b (558821; BD Biosciences), CD146 (550315; BD Biosciences), CD271 (557196; BD Biosciences), and SSEA3 (560236; BD Biosciences) for 30 min [17]. Cells were used as day 0 samples immediately after isolation from the synovial membrane and prior to cell culture. The surface antigen-positive cell fraction was measured using a FACS Verse flow cytometer (BD Biosciences). Information on the patients who participated in this study is provided in Supplemental Table 1 (n = 6).

2.4. The effect of HA-signal for maintain the surface marker of synovial MSCs

To examine the effect of HA on the regulation of MSC-related surface antigen expression, half a million synovial MSCs (CD45$^-$CD44$^+$ CD73$^+$CD90$^+$CD105$^+$, passage 2–4) were seeded onto 10 cm-diameter dishes and incubated for two days in complete growth medium. To remove HA from the medium, cells were rinsed twice with phosphate-buffered saline (PBS; Thermo Fisher Scientific), and incubated in serum-free medium (MEM-alpha supplemented with antibiotics) in the presence or absence of hyaluronidase (Sigma-Aldrich) for one day. Cells were dispersed using trypsin, and MSC-related surface antigen expression was measured using a flow cytometer (FACS Verse).

To examine the positive effects of HA on MSC-related surface antigen expression, HA (1 mg/mL, Supartz FX, Seikagaku Corporation, Tokyo, Japan) was added to depleted cells and incubated for one day. Cells were dispersed using trypsin, and MSC-related surface antigen expression was measured using a flow cytometer (FACS Verse). Information on the patients who participated in this study is provided in Supplemental Table 1 (n = 9).

2.5. Quantitation of HA in the culture medium

The concentration of HA in the culture medium was quantified by enzyme-linked immunosorbent assay (ELISA) (DHYAL0; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Briefly, 50 μL of assay diluent and 50 μL of sample were incubated for 2 h at room temperature in aggrecan-pre-coated 96-well plates. The wells were rinsed five times with washing buffer, incubated for 2 h with hyaluronan-conjugated solution, and then rinsed again. To determine the HA concentration, the wells were incubated with 100 μL of substrate solution for 30 min. The calorimetric reaction was terminated by adding 100 μL of the stopping solution. Absorbance was measured at 450 nm and 570 nm using a microplate reader (InfiniteF50-R; Tecan, Mannedorf, Switzerland).

2.6. Cell viability assay

Cell viability was calculated using trypan blue staining. Ten microliters of 0.4% trypan blue solution (15250061; Thermo Fisher Scientific) was added to 10 μL of the cell suspension and incubated for 2 min. Viable cells were counted using a hemocytometer under a light microscope.

2.7. Statistical analysis

Statistical analyses were performed using the EZR software [18]. Dunnett's test and Paired t-tests with Bonferroni correction were used for the analyses. Numerical data are shown as the mean $+/-$ standard deviation (SD) and are presented in Supplemental Table 2.
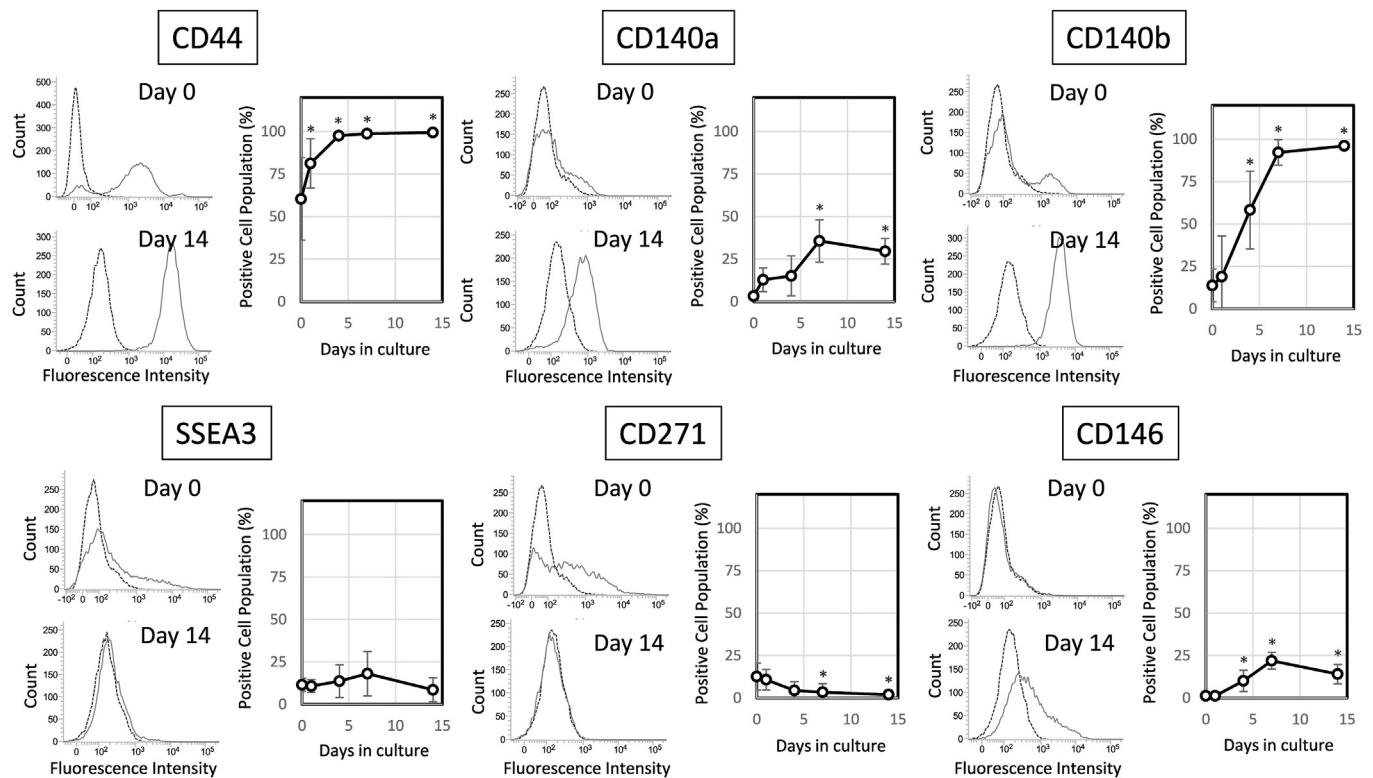
3. Results

3.1. Changes in morphology and surface antigen expression in synovial fibroblasts during 2-D culture

To assess how synovial fibroblasts acquire MSC characteristics during 2-D culture, time-course flow cytometric analysis was performed
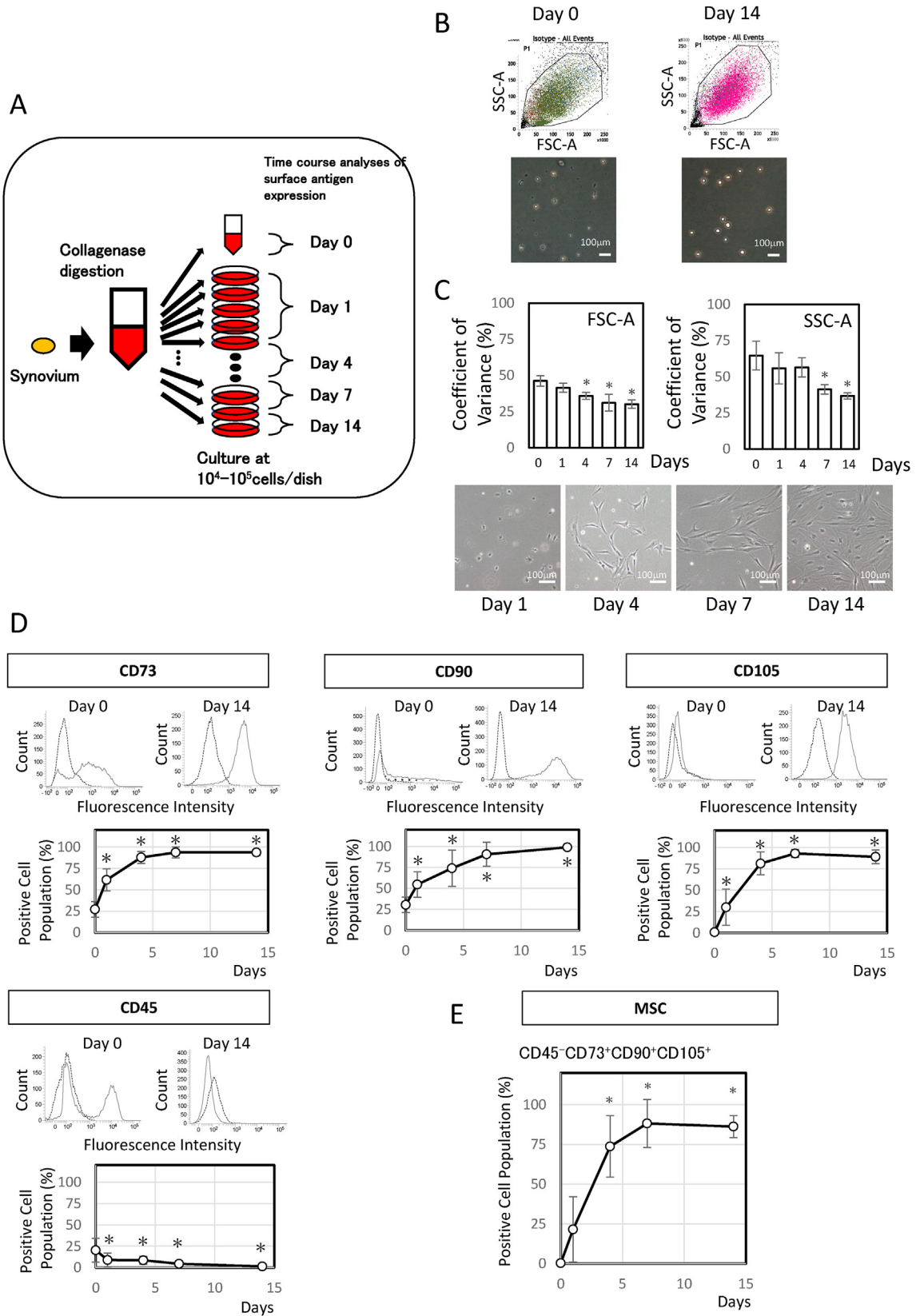
(caption on next page)

Figure 2. Time-course changes of the cell population positive for other MSC-related antigens, CD44, CD140a, CD140b, SSEA3, CD271, and CD146. Positive cell population at each time point is indicated as mean $+/-$ SD (n = 6). Numerical data are summarized in Supplemental Table 2. Asterisks indicate the p value is less than 0.05 by Dunnett's test.

(Figure 1A). As indicated in Figs. 1B and C, the coefficient of variance of both forward scatter (FSC) and side scatter (SSC) decreased with the culture period, indicating that the size and complexity of synovial cells became much more homogeneous during 2-D culture.

Time-course flow-cytometric analysis indicated that the cell population that was positive for either CD73 or CD90 was 25%, whereas CD105$^+$ cells were rarely observed on day 0 (Figure 1D). However, CD105$^+$ cells developed as high as CD73$^+$ and CD90$^+$ cells by day 7 (Figure 1D). Cells positive for CD45, a negative marker for MSCs, disappeared during the culture. Multicolor flow-cytometric analysis was in line with these data, since synovial fibroblasts with MSC characteristics developed during 2-D culture, whereas they rarely existed in the synovial tissues (Figure 1E).

Figure 2 shows the time-course changes in CD44, CD140a, CD140b, SSEA3, CD271, and CD146, which have been reported to be MSC-related surface antigens, during 2-D culture [17, 19]. Among the antigens tested, most cells were positive for CD44 and CD140b by day 7. In addition, we found that not only the positive population but also the fluorescence intensity of CD44 in each cell increased during culture (Figures 2 and 3A). This seemed to be specific to the CD44 antigen in synovial cell culture. qPCR experiments also indicated a transient increase in CD44

mRNA expression on day 1 (Supplementary Figure 1). As indicated in Figure 3A, the fluorescence spectrum of CD44$^+$ cells was bimodal in the early stages of culture (arrows in blue for lower fluorescence and arrows in green for higher fluorescence in Figure 3A). The lower fluorescence peak disappeared by day 7 and a unimodal higher peak appeared at a later stage. To examine the biological significance of CD44 upregulation during culture, two-color flow cytometric analysis was performed. As indicated in Figure 3B, cells positive for CD73, CD90, and CD105 were specifically derived from the CD44$^+$ cell population. CD73$^+$and CD90$^+$ cells were detected in both CD44^{Low} and CD44^{High} cells; however, CD105$^+$ cells were derived only from CD44^{High} cells (arrows in black [CD73], gray [CD90], and red [CD105] in Figure 3B).

To further analyze the hierarchical expression of MSC-related markers, CD44$^-$, CD44^{Low}, and CD44^{High} cell populations were independently analyzed for CD73, CD90, and CD105 expression. Cells positive for any of the MSC markers rarely existed in the CD44$^-$ cell population (Figure 3C, left column). In the early stage of culture, we observed certain numbers of CD44^{Low}CD73$^+$ and CD44^{Low}CD90$^+$ cells (Figure 3C, middle column); however, these cells appeared to be in a transition state since they disappeared by day 7. No or very few CD105$^+$ cells arose from CD44$^-$ or CD44^{Low} cells (Figure 3B, left and middle columns). CD105$^+$ cells

Figure 1. Time-course analyses of mesenchymal stromal/stem cells (MSC)-related surface antigen expression during two-dimensional (2-D) culture. (A). Experimental design. Primary human synovial cells were isolated from the suprapatellar synovium from patients who underwent total knee arthroplasty and seeded onto 15 cm-diameter dishes. On days 1, 4, 7, and 14, cells were dispersed by trypsin and surface antigen analyses were performed by a flow cytometer. Cells before culture were subjected to analysis on day 0. (B). Representative patterns of forward scatter (FSC) and side scatter (SSC) at days 0 and 14. Microscopic images of cell suspension were also shown. (C). Coefficient of variance of both FSC and SSC were decreased along with the culture period. Coefficient of variance at each time point are indicated as mean $+/-$ SD (n = 6). Numerical data are summarized in Supplemental Table 2. Asterisks indicate the p value is less than 0.05 by Dunnett's test. Microscopic images of cell morphology were also shown. (D) Time-course flow-cytometric analysis for MSC antigens. Positive cell population at each time point is indicated as mean $+/-$ SD (n = 6). Numerical data are summarized in Supplemental Table 2. Asterisks indicate the p value is less than 0.05 by Dunnett's test. (E) Time course of the cells positive for CD45$^-$CD73$^+$CD90$^+$CD105$^+$ during 2-D culture. Cells negative for CD45 were analyzed for CD73 and CD90 expression and then the CD105 positive population in CD45$^-$CD73$^+$CD90$^+$ cells was calculated and plotted. Values at each time point are indicated as mean $+/-$ SD (n = 6). The numerical data are summarized in Supplemental Table 2. Asterisks indicate the p value is less than 0.05, by Dunnett's test.
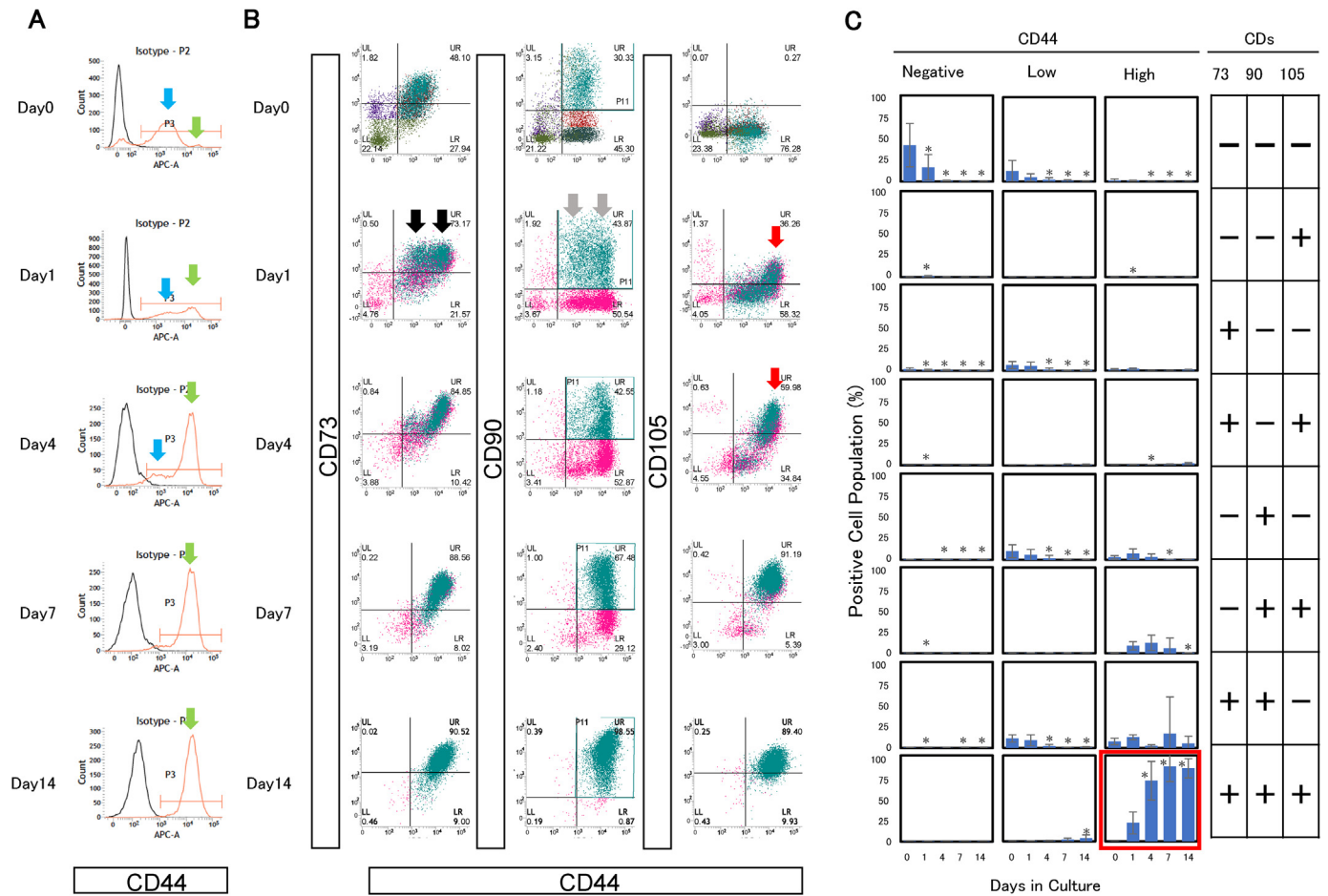
Figure 3. CD44 plays pivotal roles in the development of MSC-related surface antigen-positive cells during 2-D culture. (A) Time-course of CD44 expression during 2-D culture. Not only the population of CD44$^+$ cells but also the fluorescence intensity in each cell were increased with culture time. Blue and green arrows indicate the lower and higher fluorescence peaks, respectively. Representative charts are indicated. (B) Cells positive for CD73, CD90, and CD105 were derived from the CD44$^+$ cell population. CD73$^+$, CD90$^+$, and CD105$^+$ cells were rarely observed in the CD44 negative fraction (upper left of each scatter plot). Of note CD73$^+$ and CD90$^+$ cells were derived from both CD44^{Low} and CD44^{High} cells (arrows in black and gray), while CD105$^+$ cells were specifically observed in the CD44^{High} population (red arrows). Representative charts are indicated. (C) Time-course of each surface antigen-expressing cell population. The value at each time point is indicated as mean $+/-$ SD (n = 6). Numerical data are summarized in Supplemental Table 2. Asterisks indicate the *p* value is less than 0.05 by Dunnett's test.

appeared to arise specifically from CD44^{High}CD73$^+$CD90$^+$ cells (Figure 3C, boxed in red in the right column).

### 3.2. HA-CD44 signal axis regulates CD105 expression in synovial MSCs

To examine whether intracellular signals mediated by CD44 play a role in MSC maintenance, we examined the effect of HA on MSC-related surface antigen expression. Since FBS contains HA and synovial cells themselves produce HA [20, 21], we incubated MSCs in the absence of FBS and in the presence of hyaluronidase for one day and analyzed the surface antigen expression (Figure 4A). Under these culture conditions, no obvious decrease in cell viability was observed (Figure 4B). ELISA indicated the successful depletion of HA in the culture medium of experimental groups III and IV (Figure 4C). Flow cytometric analysis indicated that depletion of HA from the culture medium reduced the number of CD105$^+$ cells, and thus reduced the MSC population (Figures 4D and 4E). To further investigate the physiological roles of HA-CD44 signals in the expression of MSC-related surface antigens, HA was supplemented after hyaluronidase treatment (Figure 5A). As shown in Figure 5B, cell viability was not altered after this treatment. ELISA indicated an increase in HA concentration after supplementation (Figure 5C). Flow cytometric analysis indicated that CD105$^+$ cell populations were slightly but significantly increased after HA supplementation, thereby increasing the MSC population (Figures 5D and 5E).

4. Discussion

In the present study, we analyzed the time-course changes in MSC-related surface antigen expression during 2-D culture. We showed that cells satisfying the MSC criteria were rarely present in freshly isolated synovial cells; however, synovial fibroblasts gave rise to cells with MSC characteristics by day 7 in culture. Since we have shown that expanded synovial cells are multipotent and can differentiate into osteoblast-like, chondrocyte-like, and adipocyte-like cells *in vitro* [22], these data suggest that fibroblastic cells isolated from synovial tissues have hyperplastic phenotypes and acquire an undifferentiated status *in vitro*. These characteristics have also been observed in other types of cells of mesenchymal origin. Fibroblastic cells isolated from adipose tissues have also been reported to have MSC characteristics after 2-D culture, and have been coined as adipose-derived MSCs (Ad-MSCs) [23]. Watanabe et al. further described that even fully differentiated adipocytes, which contain lipid droplets in the cytoplasm, can be de-differentiated and gain MSC characteristics *in vitro* (DFAT cells: de-differentiated fat cells) [24]. Cultured bone marrow stromal cells have also been reported to exhibit stem cell characteristics [25]. These hyperplastic characteristics have also been reported in hematopoietic stem cells (HSCs) [26] and may play important roles in adult tissue homeostasis. Therefore, elucidating the molecular basis of MSC hyperplasticity is critical in future studies.
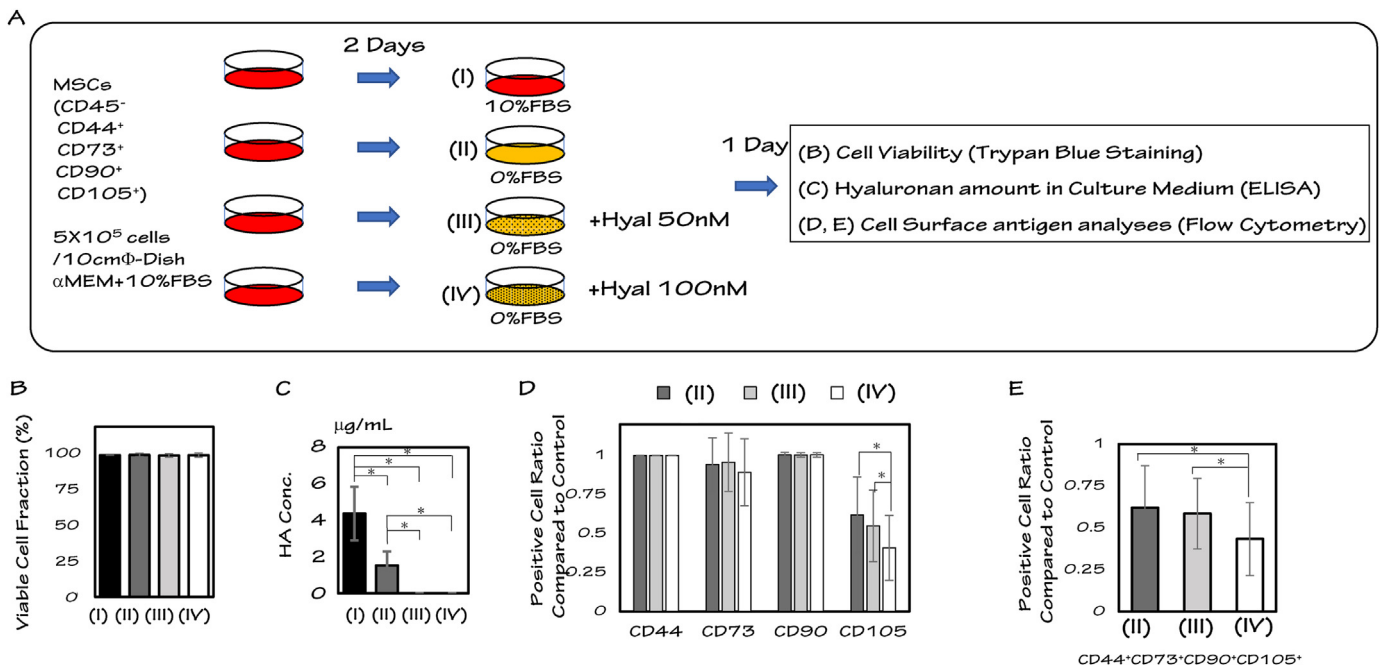
Figure 4. Removal of hyaluronic acid (HA) from culture medium reduced the CD105$^+$ cell population. (A) Experimental plan. Half a million cells were incubated for two days in normal growth medium. On the third day, culture media were replaced to (I) 10% FBS (control), (II) 0% FBS, (III) 0% FBS and 50 nM of hyaluronidase, and (IV) 0% FBS and 100 nM of hyaluronidase. On the next day, cell viability (B), hyaluronan amount in culture medium (C), and surface marker-positive cell ratio compared to that of control (Group I) were analyzed (D, E). The value at each time point is indicated as mean +/− SD (n = 9). Numerical data are summarized in Supplemental Table 2. Asterisks indicate the *p* value is less than 0.05 by paired t-test. The multiplicity was adjusted by the Bonferroni correction.
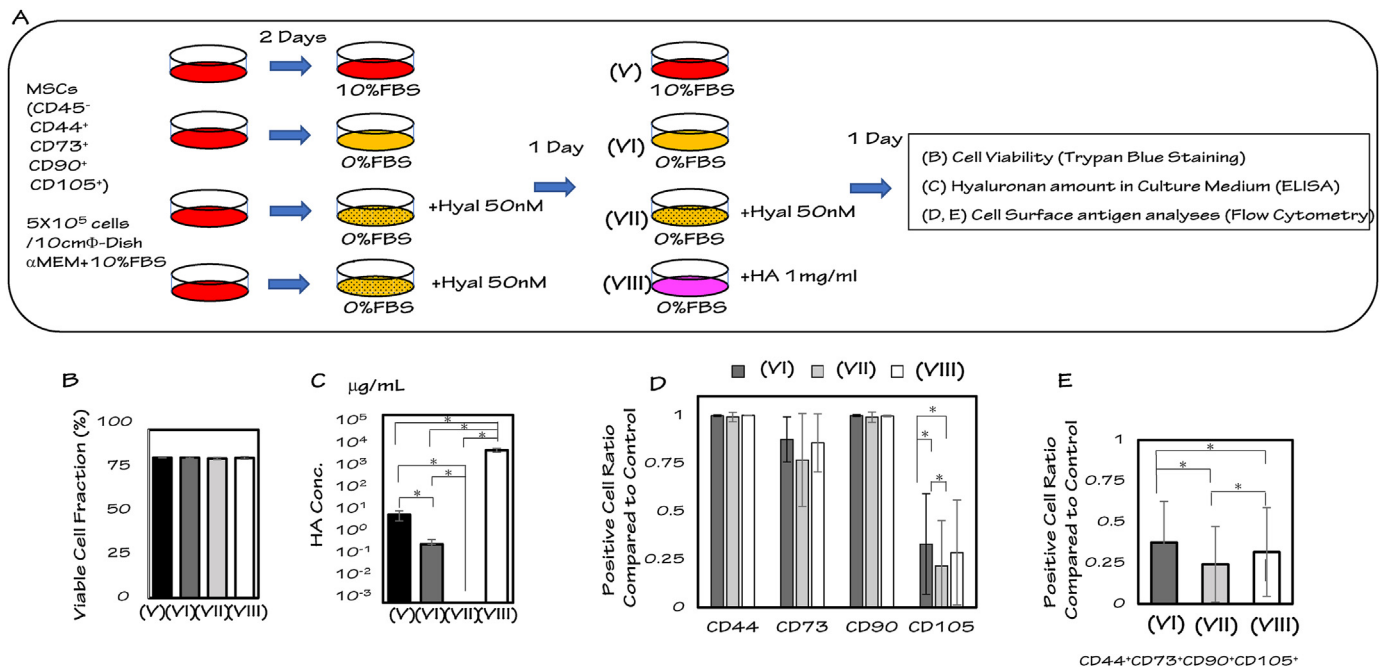
Figure 5. Supplementation of HA in culture medium enhanced the CD105$^+$ cell population. (A) Experimental plan. A half million cells were incubated for two days in normal growth medium. On the third day, culture media were replaced to (V) 10% FBS (control), (VI) 0% FBS, (VII, and VIII) 0% FBS and 50 nM of hyaluronidase. To analyze the effect of HA on the expression of CD105, 1 mg/ml of HA was supplemented to the culture medium and incubation continued for an additional 1 day (VIII). On the fourth day, cell viability (B), hyaluronan amount in culture medium (C), and surface marker-positive cell ratio compared to that of control (Group V) were analyzed (D, E). The value at each time point is indicated as mean +/− SD (n = 9). Numerical data are summarized in Supplemental Table 2. Asterisks indicate the *p* value is less than 0.05 by paired t-test. The multiplicity was adjusted by the Bonferroni correction.

We have started clinical trials to induce regeneration of articular cartilage and/or meniscus by transplanting autologous mesenchymal cells. In these clinical studies, transplanted cells were obtained by enzymatic digestion of the suprapatellar synovial membrane and expanded in 2-D culture for two weeks [8, 15]. Since the cell culture procedures are the same as those employed in the current study, we consider that the results observed here strongly support that the protocol we employ in clinical trials is rational.

The present study showed that cells expressing MSC antigens arise specifically from the CD44$^+$ population. In particular, CD105$^+$ cells were specifically derived from the CD44^{High} cell population. These data suggested that the signals transduced by CD44 may regulate the hyperplastic characteristics of synovial fibroblasts. CD44 is an N- and O-glycosylated cell adhesion molecule that functions as a receptor for various extracellular matrix proteins [27]. The signals activated by CD44 remain largely unknown; however, physiological responses triggered by CD44 have been reported to be mediated by the MAPK and NF-κB pathways. CD44 expression has also been observed in various types of tumor cells, and CD44 expression in each cell has been reported to be positively correlated with the proliferation potential of the cells [28]. In the present study, upregulation of the CD44 antigen in each cell was observed along with the proliferation of synovial cells during culture. These results suggest that signals transduced by CD44 may play an important role in regulating the differentiation and proliferation of synovial fibroblasts.

HA, a major ligand of CD44 in the synovial joint [29], is a non-sulfated glycosaminoglycan that consists of disaccharide repeats (N-acetyl-D-glucosamine and N-glucuronic acid) and is ubiquitously expressed throughout the tissues of the body. Synovial fluid contains abundant HA, which is produced by synovial fibroblasts and articular chondrocytes. HA in synovial fluid is considered to play an essential role in the regulation of its physicochemical properties, such as shock absorption and lubrication of articular cartilage; however, recent studies have highlighted its physiological roles in joint homeostasis. HA binding to CD44 has been reported to suppress synovial inflammation, infra-patellar fat pad fibrosis, and articular cartilage degeneration [30, 31, 32, 33]. In addition to these physiological functions, our results further indicate new aspects of the HA-CD44 signaling axis in the development of MSCs from synovial fibroblasts. First, the levels of CD44 in each cell increased between days 1 and 4 in culture (from CD44^{Low} to CD44^{High} cells, Figure 3). Second, CD73$^+$ and/or CD90$^+$ cells were selectively derived from CD44$^+$ cells (both CD44^{Low} and CD44^{High} cells, Figure 3). Third, cells positive for CD105 were derived mainly from CD44^{High}CD73$^+$CD90$^+$ cells during culture (Figure 3). Fourth, depletion of HA downregulated CD105$^+$ cells, whereas supplementation with HA enhanced CD105$^+$ cells (Figures 4 and 5).

Among the MSC-related surface antigens, CD105 expression was closely associated with HA-CD44 signals. CD105, also known as endoglin, is a coreceptor of the transforming growth factor beta (TGFb) superfamily [34]. It is predominantly expressed in activated endothelial cells and plays a crucial role in angiogenesis during development. Previous studies have indicated that CD105 signals enhance the proliferation and migration of vascular endothelial cells [35] and play a significant role in tumor angiogenesis in adults [35]. In addition, recent studies have suggested the critical role of CD105 in the proliferation and differentiation of cells of mesenchymal origin. Notably, CD105 expression has been reported to be upregulated in sarcomas [36], whereas downregulation of CD105 expression leads to differentiation (osteogenic phenotype) of MSCs in humans and mice. CD105$^+$ MSCs demonstrate stronger chondrogenic potential, which implies their greater intrinsic capability to repair cartilage defects [37, 38]. These data further support the biological importance of HA-CD44-CD105 signaling in the hyperplastic phenotypes of synovial cells.

In conclusion, the HA-CD44 signaling axis plays an important role in the formation and maintenance of MSC antigen-positive cells *in vitro*.

The present study had several limitations. We did not perform single-cell lineage analyses during 2-D culture. Thus, it is still difficult to eliminate the possibility that CD45$^-$CD44^{High}CD73$^+$CD90$^+$CD105$^+$ cells expanded from a very small number of MSCs, but not from the CD44$^+$ cell fraction during culture. We still do not know the underlying molecular mechanisms of CD105$^+$ cell formation during 2-D culture. Analyses of *in vivo* cell dynamics were not performed.

Declarations

Author contribution statement

Masaaki Isono: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jun Takeuchi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ami Maehara: Performed the experiments; Analyzed and interpreted the data.

Yusuke Nakagawa; Hiroki Katagiri; Kazumasa Miyatake; Ichiro Sekiya; Hideyuki Koga and Yoshinori Asou: Analyzed and interpreted the data.

Kunikazu Tsuji: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Kunikazu Tsuji reports financial support was provided by Seikagaku Corporation.

Jun Takeuchi reports a relationship with Seikagaku Corporation that includes:employment.

Additional information

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