Initial Cell Plating Density Affects Properties of Human Primary Synovial Mesenchymal Stem Cells

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ABSTRACT: Synovial mesenchymal stem cells (MSCs) appear to be an attractive cell source in cartilage and meniscus regeneration because of their high proliferative and chondrogenic potentials. Two methods are used to culture synovial nucleated cells in the preparation of primary synovial MSCs. In one method, the cells are plated at low density to make cell colonies. In the other method, the cells are plated at high density. We investigated the effects of initial cell density on proliferation, surface markers, and multipotentiality, including chondrogenesis in primary synovial MSCs. Human synovium was obtained from the knee joints of patients with osteoarthritis after total knee arthroplasty. Immediately after enzyme digestion, the synovial nucleated cells were plated in densities of 10^3, 10^4, or 10^5 cells/60-cm^2 dish and cultured for 14 days. Proliferation, surface markers, chondrogenesis, adipogenesis, and calcification were examined in three populations. The cell colonies were distinct in the 10^3 cells/dish group, faint in the 10^4 cells/dish group, and obscure in the 10^5 cells/dish group. The total number of cells/dish was positively related to plating density, whereas the fold increase was negatively related to plating density ($n = 13$). Among 12 surface markers, a negative relation to plating density was distinct in CD105. The cartilage pellet weight was negatively related to the initial plating density. The oil red-o positive area and alizarin red positive area were positively related to the initial plating density. The initial cell plating density affected the properties of primary synovial MSCs. Synovial nucleated cells proliferated better when plated at low density, and the synovial MSCs obtained by this method contained a high chondrogenic potential. © 2018 The Authors. *Journal of Orthopaedic Research*® Published by Wiley Periodicals, Inc. J Orthop Res 37:1358–1367, 2019.

Keywords: mesenchymal stem cell; synovium; plating density; primary cell; chondrogenesis

Mesenchymal stem cells (MSCs) are attractive as a cell source in regenerative medicine. MSCs were isolated from bone marrow for the first time by Friedenstein 50 years ago.[1] Since then, an increasing number of reports have demonstrated that MSCs can be isolated from various types of adult mesenchymal tissue in addition to bone marrow. Previous in vitro and in vivo chondrogenic assays showed that MSCs derived from synovium (synovial MSCs) had superior chondrogenic ability compared with MSCs from other tissues.[2,3] Moreover, the transplantation of synovial MSCs promoted the repair of cartilage and meniscus.[4,5] Therefore, synovial MSCs appear to be a promising cell source in cartilage and meniscus regeneration.

A fascinating feature of MSCs is their unusual behavior in culture, which was intensively investigated in bone marrow MSCs. When it was plated in extremely low densities, the human bone marrow MSCs expanded, and each cell formed a distinct colony.[6] Therefore, the cells were remarkably different from fibroblasts and other mammalian cells that require plating in a minimum density of 1,000 cells/cm^2 to expand in culture. We previously reported that the proliferation rate of passaged bone marrow MSCs in culture was inversely proportional to the plating density over a broad range.[7] We also obtained similar results in passaged synovial MSCs.[8,9]

The preparation of primary synovial MSCs and the passaging of synovial MSCs may affect the properties of synovial MSCs because immediately after enzyme digestion, synovial nucleated cells consist of diverse kinds of cells, whereas passaged MSCs are much more homogeneous. In the preparation of primary synovial MSCs, two methods are used to culture synovial nucleated cells. In one method, the cells are plated at low density to make cell colonies.[2] In the other method, the cells are plated at high density. The cells obtained by plating at high density are referred to as synovial cells, synovial fibroblasts, and synoviocytes.[10,11] We hypothesized that the initial cell plating density affected the properties of primary synovial MSCs. Hence, the purpose of this study is to investigate the effects of initial cell density on proliferation, surface markers, colony-forming ability, and multipotentiality, including chondrogenesis in synovial MSCs.

METHODS

Human Synovial MSCs

This study was approved by the ethics committee of the Tokyo Medical and Dental University. All patients included in this study provided their informed written consent. The human synovial tissue was harvested from the suprapatellar pouch of the knee joints of 13 patients with osteoarthritis during total knee arthroplasty. The patients included 3 males and 10 females between 68 and 82 years of age. The

synovial membrane was minced and digested in 3 mg/ml of collagenase D (Roche Diagnostics, Mannheim, Germany) in α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA) at 37 °C for 3 h. After digestion, the debris was removed by filtration through a 70-mm nylon filter (BD Bioscience, Franklin Lakes, NJ). The nucleated cells were counted and seeded in densities of 10^3, 10^4, and 10^5 cells in 60-cm^2 culture dishes (Nalgen Nunc International, Rochester, NY) in α-MEM supplemented by 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 units/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B (Invitrogen) and then incubated at 37 °C in a humidified CO_2 chamber. After 24 h, the culture medium was replaced by a fresh culture medium to remove the non-adherent cells.

Surface Antigen Expression Analysis

A flow-cytometric analysis was performed to detect the expression of cell surface antigens (FACS Verse, BD Bioscience). The cells were detached by trypsinization at 37 °C for 5 min and then stained with antibodies against surface antigens according to the manufacturer's protocol. The positive cell populations were analyzed using the FACSuite software (BD Bioscience). The fluorochrome dye conjugated antibodies against the human MSC-related cell surface antibodies used in this experiment: allophycocyanin (APC)-anti-CD44, APC-anti-CD45, FITC (fluorescein isothiocyanate)-anti-CD73, FITC-anti-SSEA3, FITC-anti-CD31, phycoerythrin cyanine 7 (PE-Cy7)-anti-CD90, peridinin chlorophyll protein complex cyanine 5.5 (PerCP-Cy5.5)-anti-CD105, phycoerythrin (PE)-anti-CD140a, PE-anti-CD140b, PE-anti-CD271, PE-anti-CD34, and PE-anti-CD146 (BD Biosciences).

Colony Formation

A total of 100 cells were seeded in 60-cm^2 dishes and maintained for 14 days to form colonies. To visualize the colonies, the cells were fixed by 4% paraformaldehyde and stained with 0.5% crystal violet (Sigma–Aldrich, St. Louis, MO) in methanol for 5 min. The dishes then were rinsed twice with distilled water, and colonies larger than 2 mm in diameter were counted.

Chondrogenesis

Cells that were 2.5×10^5 were placed in a 15-ml polypropylene tube (BD Biosciences), centrifuged at $450 \times g$ for 10 min, and cultured for 14 days in chondrogenic medium containing the insulin-transferrin-selenium (ITS) mixture (BD Biosciences), 1,000 ng/ml rhBMP-2 (Infuse Bone Graft; Medtronic, TN), 10 ng/ml transforming growth factor-β3 (R&D Systems, MN), and 100 nM dexamethasone (Sigma-Aldrich). The medium was replaced by a fresh medium every 3–4 days. At 21 days, the pellets were weighed and then fixed in 4% paraformaldehyde and embedded in paraffin in preparation for further histological assessments.

Adipogenesis

A total of 100 cells were plated in 60-cm^2 dishes and cultured for 14 days in α-MEM supplemented by 10% FBS. The medium was then switched to an adipogenic medium, which consisted of α-MEM supplemented by 10% FBS, 100 nM dexamethasone (Sigma–Aldrich), 0.5 mM isobutyl-methyl-xanthine (IBMX; Sigma–Aldrich), and 50 μM indomethacin (Wako, Japan), which was then and cultured for 21 days. The cells were fixed in 10% paraformaldehyde and stained with fresh oil red-o solution (Sigma–Aldrich) to visualize the lipid droplets in the cytoplasm.[12] The Oil red-o-positive area was calculated using NIH Image J software. Oil red-o dye was eluted by 1 ml of isopropyl alcohol, and the absorbance of 510 nm was measured by spectrometer.[7] The dishes were then counter-stained with crystal violet to visualize all the colonies that were formed. The rate of oil red-o positive colonies was calculated by dividing the number of oil red-o positive colonies by the total number of colonies.[2] Colonies smaller than 2 mm in diameter were excluded from the analysis.

Calcification

One hundred cells were plated in 60-cm^2 dishes and cultured for 14 days. The medium was then switched to a calcification medium consisting of α-MEM, which was supplemented by 10% FBS, 1 nM dexamethasone, 20 mM β-glycerol phosphate, and 50 μg/ml ascorbate-2-phosphate (Sigma–Aldrich) and cultured for 21 days. Calcified nodule formation was visualized by alizarin red staining (Sigma–Aldrich). The alizarin red positive areas were calculated using NIH Image J software. The dishes were then counter-stained with crystal violet to visualize all the colonies that formed. The rate of alizarin red positive colonies was calculated by dividing the number of alizarin red positive colonies by the total number of colonies.[2] Colonies smaller than 2 mm in diameter were excluded from the analysis.

Analysis of the Time-Lapse Images

Immediately after enzyme digestion, the synovial nucleated cells were plated at 16 cells/cm^2 in 6-well plates and cultured for 14 days Time-lapse microscopy was conducted on some colonies were scanned in an environmentally enclosed chamber at 37 °C, 5% CO_2 and humidified (Tokai Hit Co., Shizuoka, Japan) for time-lapse microscopy using a computerized imaging system (IX83ZDC multi-area time-lapse imaging system, Olympus, Tokyo, Japan). Time-lapse photomicrographs were taken every 20 min for 14 days and were reconstructed as time-lapse movie using image analysis software (Dai Nippon Printing Co., Tokyo, Japan).

Statistical Analysis

The Kruskal–Wallis test followed by the Steel-Dwass test were applied in the statistical analyses. p values less than 0.05 were considered significant. All data were presented as mean $\pm$ standard deviations.

RESULTS

Effects of Plating Density on the Proliferation of Synovial MSCs

The cell colonies were distinct in the 10^3 cells/dish group, faint in the 10^4 cells/dish group, and obscure in the 10^5 cells/dish group (Fig. 1A and B). Regarding their morphology, the cells were spindle-shaped independent of the plating density (Fig. 1C). The total number of cells per dish was positively related to the plating density (Fig. 1D), whereas the fold increase was negatively related to the plating density (Fig. 1E).

Effects of Plating Density on the Surface Markers of Synovial MSCs

Among the 12 surface markers, a negative relation to plating density was distinctively observed in CD105 (Fig. 2A). The positive rates of CD73 and SSEA3 were higher in the 10^4 cells/dish group than in the 10^5 cells/dish group. A positive relation to plating density was
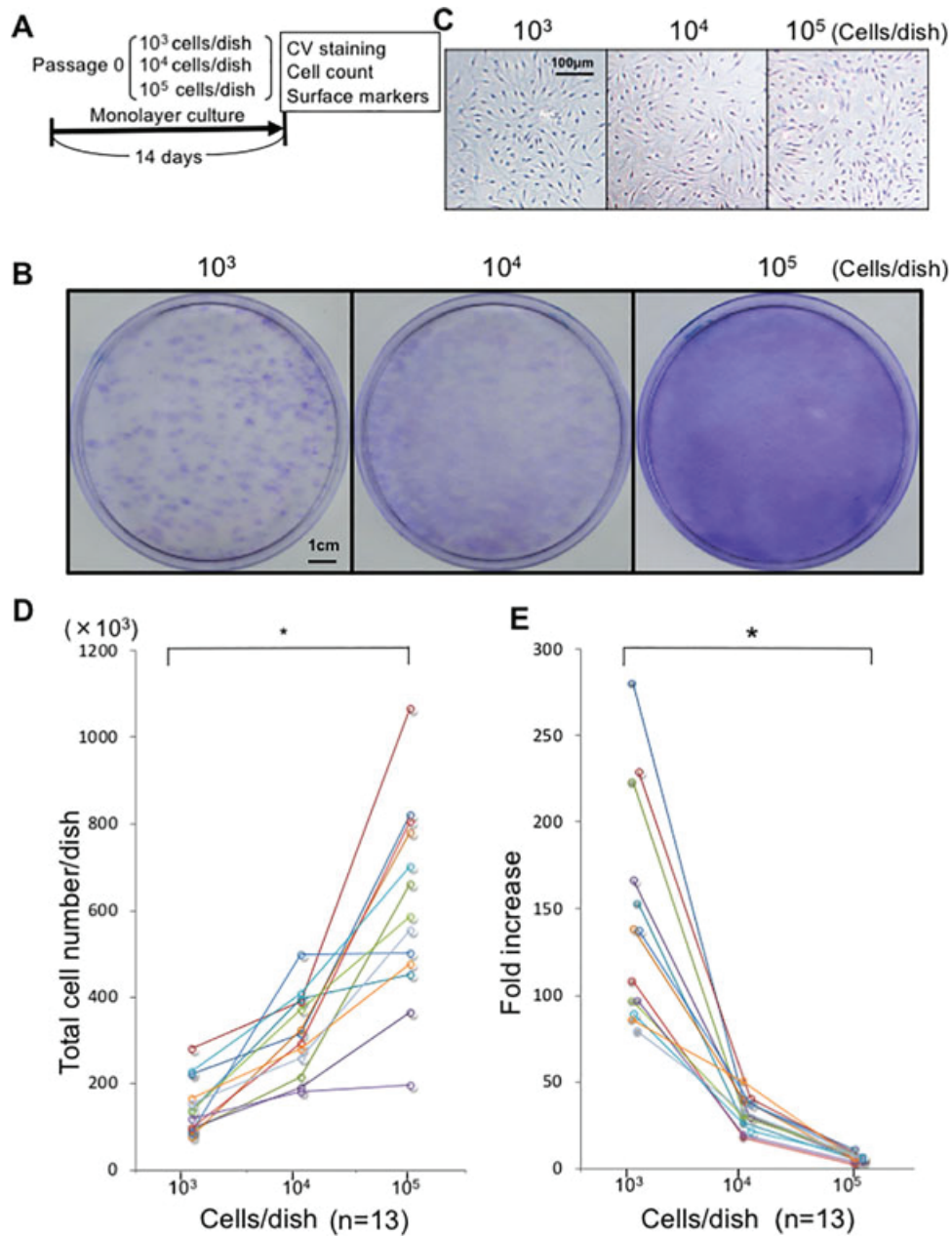
Figure 1. Colony formation and proliferation of synovial MSCs at passage 0. (A) Experimental design. Nucleated cells derived from synovium were plated at 10^3, 10^4, or 10^5 cells/60-cm^2 dish in six dishes and cultured for 14 days. Then three dishes in each condition were stained with crystal violet (CV). The cells from the remaining three dishes were used for further analyses. (B) Representative colonies stained with CV. (C) Morphology of cells composing of cell colonies. (D) Total cell number/60-cm^2. Values derived from 13 donors are shown. * < 0.05 by Kruskal–Wallis test followed by Steel–Dwass test. (E) Fold increase.

observed in CD34. The representative profiles confirmed that the double positive rates of CD105 and CD73 decreased as the cell density decreased although the double positive rates of CD90 and CD44 were unchanged (Fig. 2B). The rates of the CD44+, CD90+, CD73+, CD105+, and CD45- cells were negatively related to cell density (Fig. 2C).

Effects of Initial Plating Density on the Colony Formation of Synovial MSCs at Passage 1
The synovial nucleated cells were cultured at three different densities, then the cells replated at

100 cells/60-cm^2 dish to examine the colony-forming ability (Fig. 3A). Distinct cell colonies were observed in the three groups (Fig. 3B). The number of colonies per dish was not affected by the initial cell density (Fig. 3C).

Effects of Initial Plating Density on the Chondrogenic Potential of Synovial MSCs
The synovial nucleated cells were cultured at three different densities, then differentiated chondrogenically (Fig. 4A). After 21 days, the pellets became spherical and translucent macroscopically (Fig. 3B),
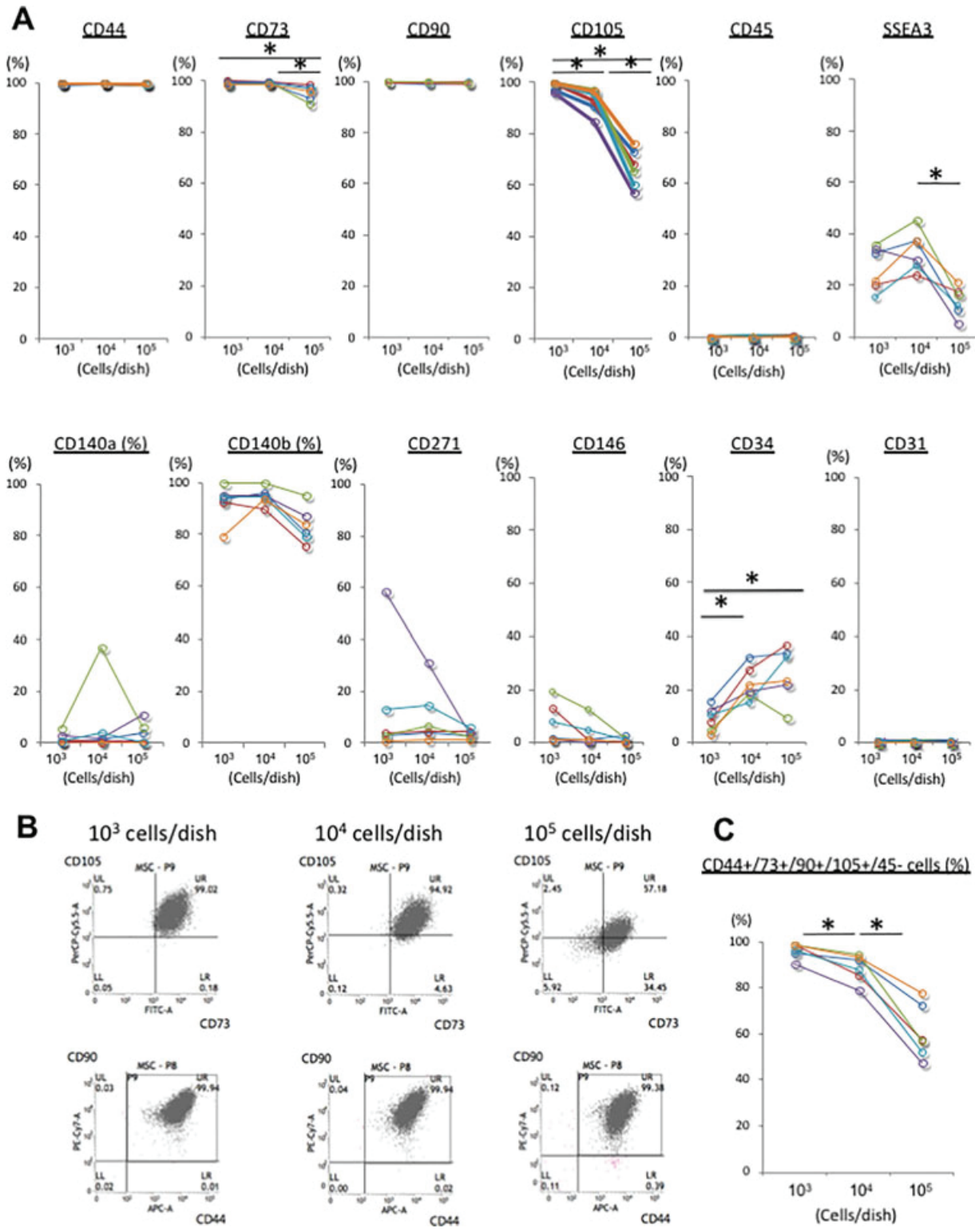
Figure 2. Surface marker analyses. (A) Positive rate for 12 surface markers. Values derived from six donors were shown, respectively. * < 0.05 by Kruskal–Wallis test followed by Steel–Dwass test. (B) Representative profiles for CD105 and CD73 and for CD90 and CD44. (C) The rate for CD44+, CD90+, CD73+, CD105+, and CD45- synovial MSCs. Values derived from five donors are shown, respectively.

and the cartilage matrix was histologically observed in the three groups (Fig. 3C). The pellet size and pellet weight were negatively related to the initial plating density (Fig. 3D and E).

Effects of Plating Density on the Adipogenic Potential of Synovial MSCs

The synovial nucleated cells were cultured at three different densities, then differentiated adipogenically
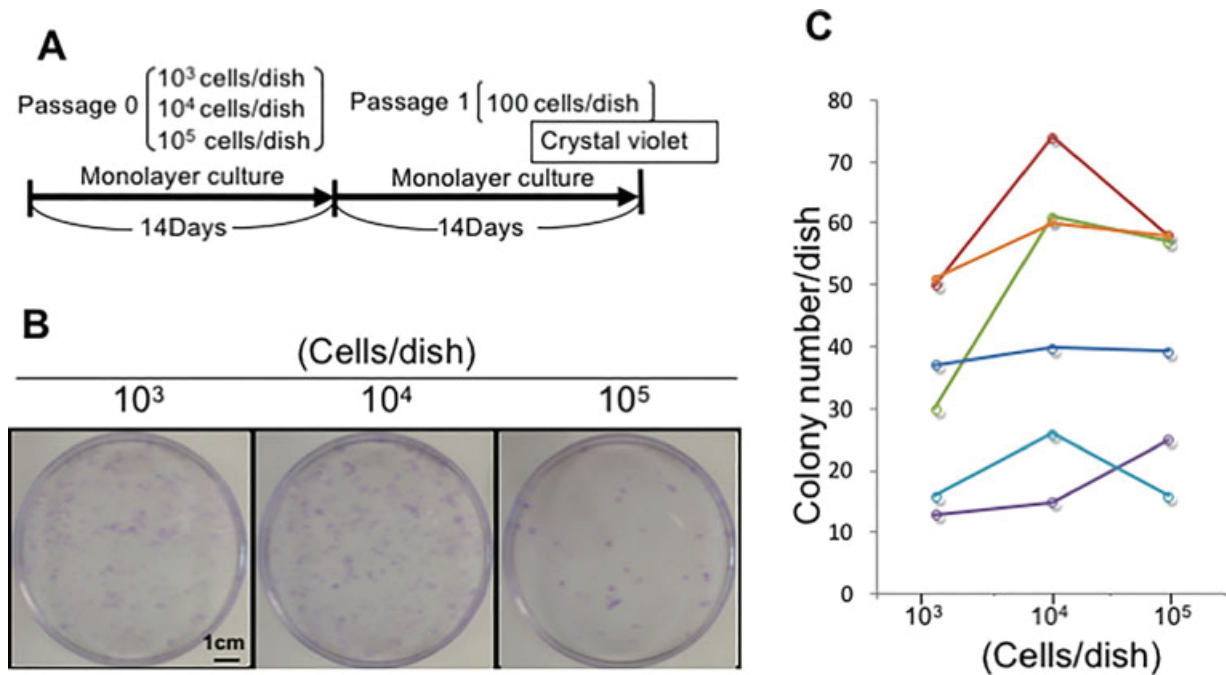
Figure 3. Colony formation of synovial MSCs at passage 1. (A) Experimental design. Nucleated cells derived from synovium were plated at 10^3, 10^4, or 10^5 cells/60-cm^2 dish. Then the cells were replated at 100 cells/60-cm^2 dish in three dishes, cultured for 14 days, and stained with crystal violet. (B) Representative colonies stained with crystal violet. (C) Colony number/dish. Values derived from six donors are shown, respectively.

(Fig. 5A). The cells formed cell colonies, some of which were adipocyte colonies stained with oil red-o (Fig. 5B). The oil red-o positive areas and absorbance at 510 nm (units/dish) were positively related to the initial plating density (Fig. 5C and D). The ratios of the oil red-o positive colony were similar in the three groups (Fig. 5E).

Effects of Plating Density on the Calcification Potential of Synovial MSCs

The synovial nucleated cells were cultured at three different densities, then differentiated for calcification (Fig. 6A). The cells formed large cell colonies, part of which were calcified colonies stained with alizarin red (Fig. 6B). The alizarin red positive areas were positively related to the initial plating density (Fig. 6C). The ratios of alizarin red positive colonies were similar in the three groups (Fig. 6D).

Time-Lapse Images of the Expansion of the Synovial MSCs

To examine whether cell-to-cell contact occurred when the cells were plated at low density, time-lapse images of the expansion of synovial MSCs were analyzed. The first single cells divided, separated, and divided again. The analysis of the time-lapse images showed cell-to-cell contact in the early phase even when they were plated at low density (Fig. 7A, Supplementary movie). Time lapse analysis showed cell to cell contact in the early phase even when plated at low density.

To examine whether the colony was derived from a single cell, the colony was analyzed, retrospectively. A single colony was observed at 8 days, which had

consisted of three colonies at 6 days. Furthermore, at 3 days, the three colonies had consisted of six colonies. Finally, the six colonies were found to be derived from six single cells (Fig. 7B). The analysis of the time-lapse images demonstrated that the colony was derived from multiple cells.

DISCUSSION

We investigated the effects of the initial cell plating density on the properties of primary synovial MSCs. Nucleated cells derived from synovium were plated at 10^3, 10^4, and 10^5 cells/60-cm^2 dish and cultured for 14 days. The initial cell density affected proliferation, surface markers, colony-forming ability, and multi-potentiality, including chondrogenesis in the primary synovial MSCs.

Cell colonies were distinct in the 10^3 cells/dish group, faint in the 10^4 cells/dish group, and obscure in the 10^5 cells/dish group. When the cells were plated at low density, the colony size was not affected by colony-to-colony contact inhibition, and the highest fold increase was obtained. In contrast, when the cells were plated at high density, the cell colonies did not form, but the highest total number of cells /dish was obtained. The initial cell density affected proliferation through the influence of the size of the colonies in primary synovial MSCs.

When precultured at 10^3 cells/dish, the colony-forming ratio was approximately 10% at passage 0 and 10–50% at passage 1. When plated at 10^5 cells/dish, the colony-forming ratio was also 10–50% at passage 1. In the synovial MSCs in passage 1, the
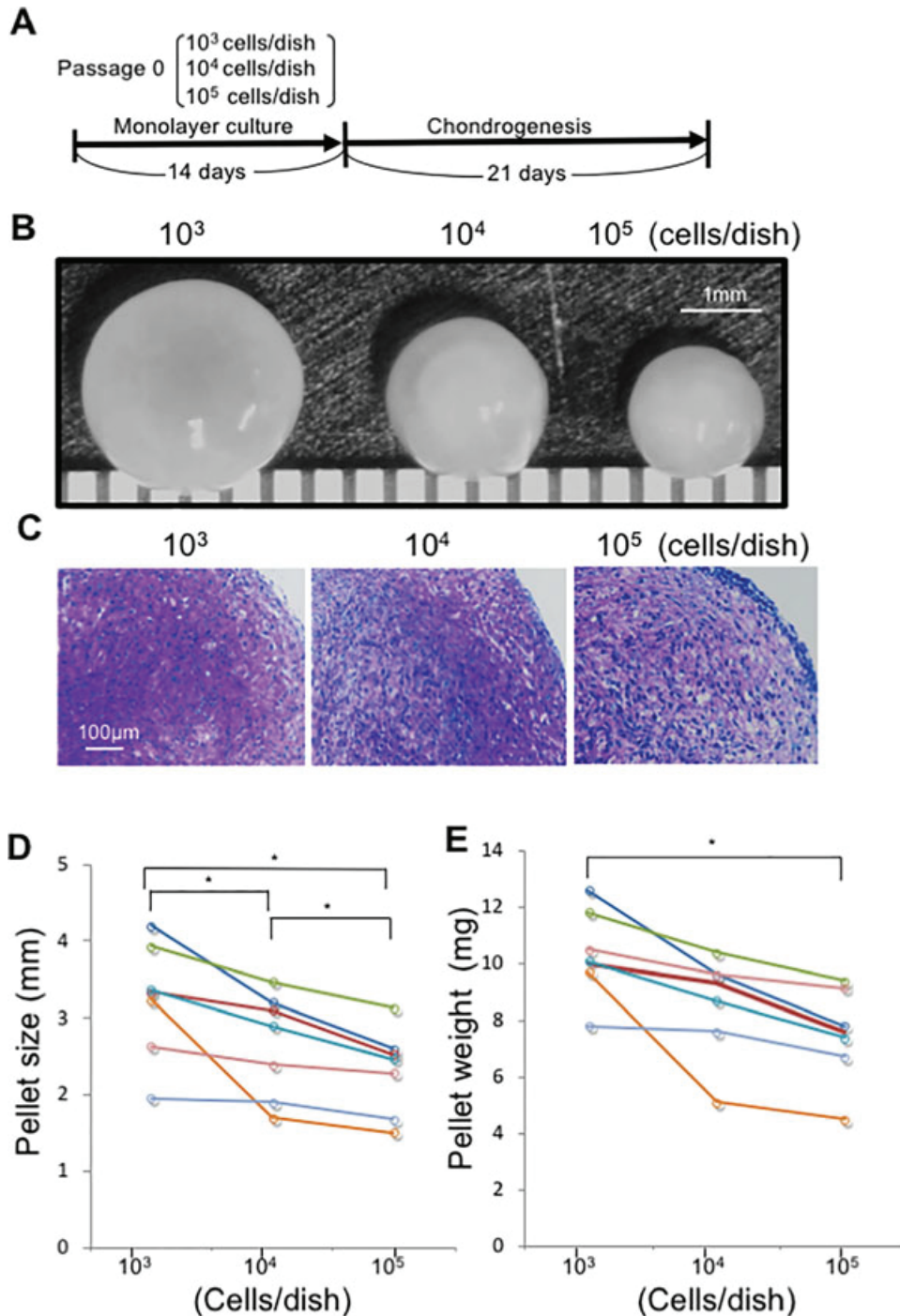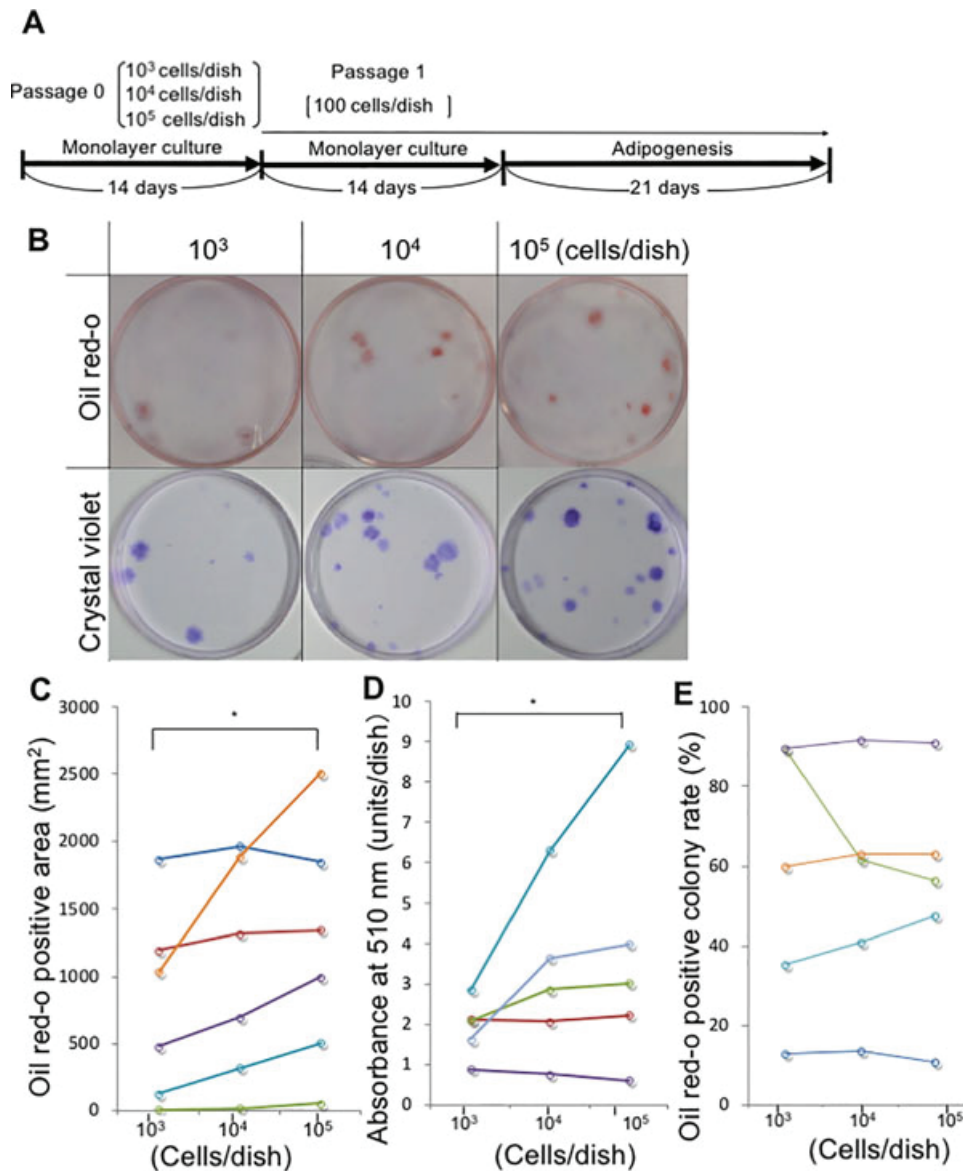
Figure 4. Chondrogenesis. (A) Experimental design. Nucleated cells derived from synovium were plated at 10^3, 10^4, or 10^5 cells/60-cm^2 dish. Then the cells were harvested, pelleted, and cultured in chondrogenic medium for 21 days. (B) Representative pictures of cartilage pellets. (C) Representative histological sections stained with toluidine blue. (D) Pellet size. Values derived from seven donors are shown, respectively. * < 0.05 by Kruskal–Wallis test followed by Steel–Dwass test. (E) Pellet weight.

colony-forming ratio was not affected by the preculture condition.

Among the 12 surface markers, a negative relation to plating density was distinctly observed in CD105. CD105, which is also called endoglin, is a homodimeric membrane glycoprotein that is primarily associated with human vascular endothelium.[13] In addition, CD105 is a component of the transforming growth factor-beta (TGFβ) receptor complex, and it has high binding affinity to TGFβ.[14] In this study, because the chondrogenic medium contained TGFβ, and the high CD105 could have been related to the chondrogenic potential.

The International Society for Cellular Therapy (ISCT) has proposed minimal criteria to define human MSC.[15] First, MSC must be plastic-adherent when it
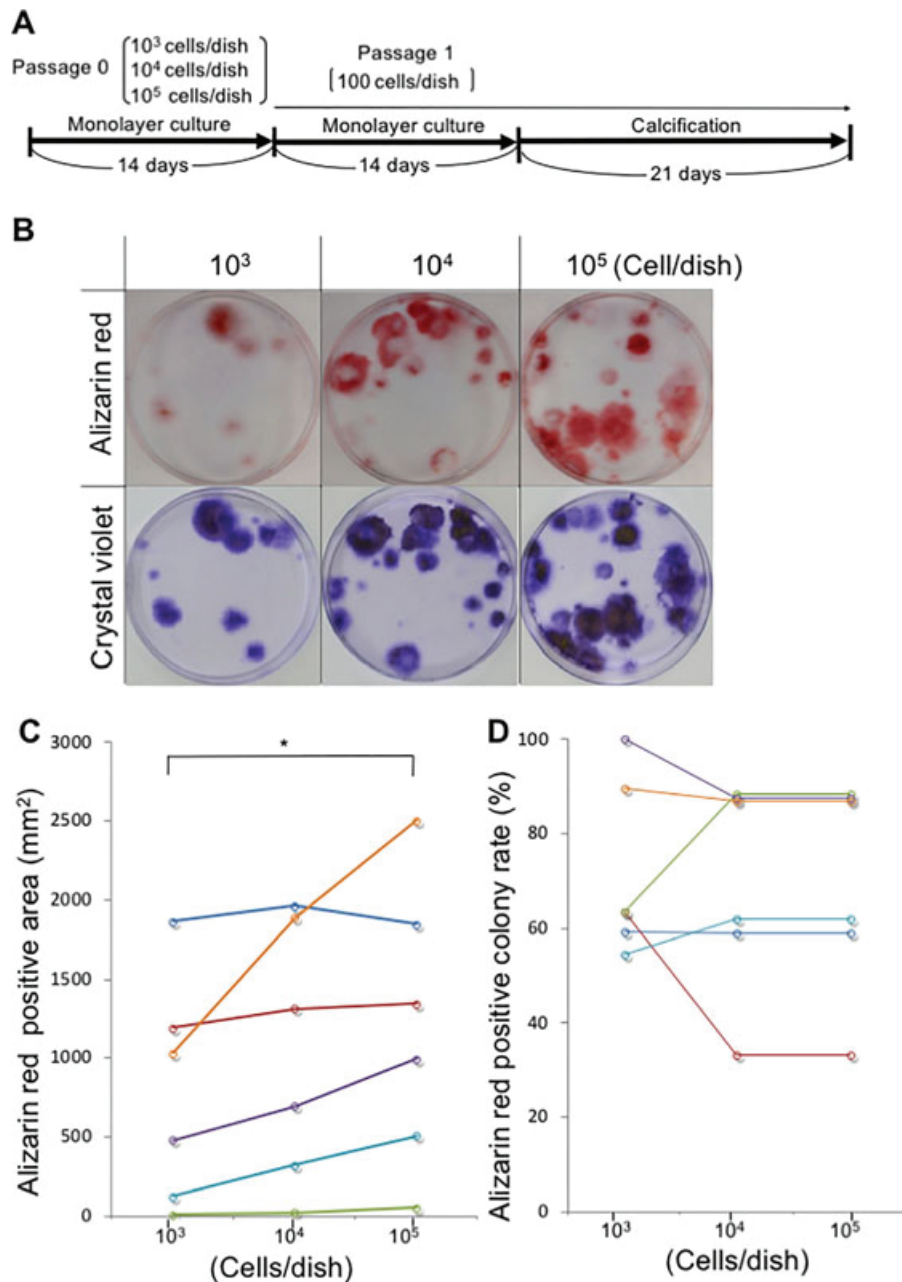
Figure 5. Adipogenesis. (A) Experimental design. Nucleated cells derived from synovium were plated at 10^3, 10^4, or 10^5 cells/60-cm^2 dish for 14 days. The cells were replated at 100 cells/60-cm^2 dish, cultured for 14 days. Then the medium was switched to an adipogenic medium, and the cells were cultured for 21 days. (B) Representative adipocyte colonies stained with oil red-o and total cell colonies stained with crystal violet. (C) Oil red-o positive area. Values derived from six donors are shown, respectively. $^* < 0.05$ by Kruskal–Wallis test followed by Steel–Dwass test. (D) Absorbance at 510 nm. (E) Oil red-o positive colony ratio.

is maintained in standard culture conditions. Second, MSC must be positive ($\geq$95%) for CD105, CD73, and CD90. It must be negative ($\leq$2%) for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR. Third, MSC must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro. In our current study of synovial MSCs, the positive rate of CD105 was affected by the initial plating density. The positive rate of CD105 was less than 95% in all six donors when it was plated at 10^5 cells/60-cm^2 dish. However, these cells, except CD105, met the criteria for defining human MSCs. According to the ISCT, "these criteria will probably require modification as new knowledge unfolds." Therefore, in the future, CD105 might be removed from the ISCT criteria.

In this study, CD105 appeared to be related to colony formation. A specific marker of colony formation would be useful in selecting synovial MSCs. We are currently trying to find a specific marker of colony formation using cell surface marker screening panels.

In another study, we compared the surface makers between synovial cells immediately after enzyme digestion with synovial MSCs 14 days after the synovial cells were cultured. Although the positive rates of CD44, CD73, and CD90 varied before culturing, they all were approximately 100% at 14 days (data not shown). Although the positive rate of CD105 was almost 0% before culturing, it increased to range from 40 to 100% after 14 days. Although the positive rate of CD45 ranged from 0 to 40% before culturing, it

Figure 6. Calcification. (A) Experimental design. Nucleated cells derived from synovium were plated at 10^3, 10^4, or 10^5 cells/60-cm^2 dish for 14 days. The cells were replated at 100 cells/60-cm^2 dish, cultured for 14 days. Then the medium was switched to a calcification medium, and the cells were cultured for 21 days. (B) Representative calcified colonies stained with alizarin red and total cell colonies stained with crystal violet. (C) Alizarin red positive area. Values derived from six donors are shown, respectively. * < 0.05 by Kruskal–Wallis test followed by Steel–Dwass test. (D) Alizarin red positive colony ratio.

decreased to almost 0%. These results indicated that immediately after enzyme digestion, the synovial cells were heterogeneous, whereas after culturing, they became increasingly homogeneous.

The chondrogenic potential was evaluated according to the size and weight of the pellets, which are indicators of chondrogenesis in a population of cells.[16] The size and weight of the pellets decreased in conjunction with initial cell density. This result indicates that in the population of synovial MSCs that were cultured at low density, both the ratio of chondrogenic cells and the chondrogenic potential per cell were high.

The oil red-o positive areas and alizarin red positive areas increased when the initial cell density was high, thus demonstrating the enhancement of the adipogenesis and calcification potentials. However, the initial cell density did not affect the ratio of oil red-o positive colonies or the ration of alizarin red positive colonies. Because these are simple methods, their sensitivity may be too low to detect differences in this study.

To examine whether cell-to-cell contact occurred even when the cells were plated at low density, time-lapse images of the expansion of synovial MSCs were
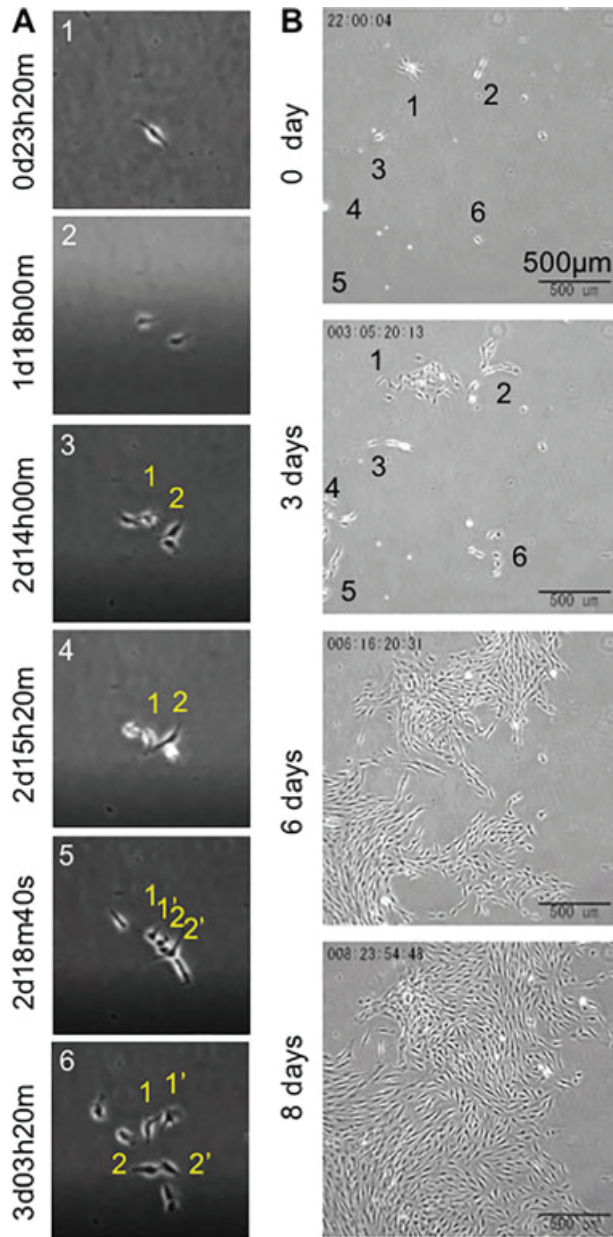
Figure 7. Time-lapse images of the expansion of synovial MSCs. Nucleated cells derived from synovium were plated at 16 cells/cm² dish and cultured (see Supplementary movie). (A) Analysis of cell-to-cell contact. The first single cell (A1) divided and separated (A2), and the two cells divided again (A3). Then No. 1 cells came into contact with No. 2 cells (A4); both cells divided (A5) and separated again (A6). (B) Analysis of colony. The single colony observed at 8 days consisted of 6 single-cell-derived colonies.

analyzed. The analysis showed cell-to-cell contact in the early phase. During the expansion of the synovial MSCs, the cells were in contact and then separated. This behavior was repeated especially in the early phase and in the colonies in the later phase. When the cells came into contact, some organelles (including mitochondria) and/or molecules (including Ca^{2+}, Mg^{2+}, RNAs, proteins/peptides) were transferred for expansion.[17]

Before we determined whether the colony was derived from a single cell, we considered three possibilities. First, the colony was derived from a single cell. Second, some single cell-derived colonies were fused into a single colony. Third, some cells moved away from the single cell-derived colony and formed distinctive colonies. According to our time-lapse movie, when they were plated at 16 cells/cm² dish, some small single cell-derived colonies fused into a single large colony after 14 days (Fig. 7B, Supplementary movie). We did not find a true single cell-derived colony or any distinctive colonies that were derived from a single cell-derived colony.

In our current study, the donors' ages did not affect the in vitro behavior of the synovial MSCs (data not shown). The donors' ages ranged between 68 and 82 years. All donors were patients who had undergone total knee arthroplasty for osteoarthritis. We previously compared the properties of synovial MSCs between young donors (20 years on average) who underwent anterior cruciate ligament injury with elderly donors (70 years on average) who underwent total knee arthroplasty for osteoarthritis. There were no remarkable differences in the proliferative ability and colony-forming efficiency of the cells at passage 1 or in the chondrocyte, adipocyte, and calcification differentiation potential in each MSC population.[9]

Concerning the effects of the donors' sex on the invitro behavior of synovial MSCs, none were found on the colony formation, proliferation, surface marker, chondrogenesis, adipogenesis, and calcification of synovial MSCs (data not shown). The effects of the donors' sex on the properties of synovial MSCs remains to be known. Huard et al. investigated the role of the donors' sex in the in vitro behavior of muscle-derived stem cells (MDSCs). Their results showed the effects of the donors' sex sexual dimorphism on chondrogenic,[18] osteogenic,[19] and myogenic[20] differentiation potential in vitro.

In our opinion, the optimal initial cell density is the condition in which the size of the colony is not affected by contact inhibition, and the greatest number of colonies is obtained. The results of the present study showed that the optimal initial cell density was around 10^3 cells/60-cm² dish. In a future study, we will investigate the optimal initial cell density in increments of 100 cells at approximately 10^3 cells/60-cm² dish.

In clinical situations, the use of passage 0 synovial MSCs could be valuable in reducing the effort required by replating as well as in preventing the possible risks related to chromosomal abnormalities.[21–23] In cartilage and meniscus regenerative therapies, low density plating is recommended because synovial MSCs with high chondrogenic potential can be obtained. In this study, the fold increase was also high although the total number of cells/dish was low.

A limitation of this research is that it did not include an in vivo study to examine the chondrogenic potential. It would be interesting to determine whether the in vitro results of the present study would be reflected in those obtained in vivo. We previously demonstrated the in vitro chondrogenic potential reflected in the vivo

chondrogenic potential with the use of MSCs derived from synovium, bone marrow, muscle, and adipose tissue in a rabbit cartilage defect model.[3]

Based on the results of this study, we conclude that the density of the initial cell plating affected the properties of primary synovial MSCs. The proliferation of the synovial nucleated cells increased when they were plated at low density, and the synovial MSCs cultured at low density showed a high chondrogenic potential.

AUTHORS' CONTRIBUTION
KN contributed to the conception and design, collection of data, analysis and interpretation of data, and manuscript writing. KT contributed to the collection of data, interpretation of data, and analysis of data. MM contributed to time lapse. HK contributed to the interpretation of data. TM contributed to the conception and design, interpretation of data, and administrative support. IS contributed to the conception and design, financial support, manuscript writing, and final approval of manuscript

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of this article.