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Original Article

Identification of mesenchymal stem cell populations with high osteogenic potential using difference in cell division rate

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ABSTRACT

Introduction: In bone regenerative medicine, mesenchymal stem cells (MSCs) have been widely investigated for their potential in bone regeneration. However, MSCs are a heterogeneous cell population containing a variety of cell types, making it difficult to obtain a homogeneous MSC population sufficient for tissue regeneration. Our group previously reported that by selecting rapidly dividing human auricular chondrocytes, it was possible to enrich for more chondrogenic cells. In this study, we aimed to identify a highly osteogenic MSC population by using a similar approach for mouse bone marrow MSCs.
Methods: Mouse bone marrow MSCs were fluorescently labeled with carboxyfluorescein succinimidyl ester (CFSE) and sorted according to the fluorescence intensity using flow cytometry on day 3 after labeling. To compare the ability to produce bone matrix *in vitro*, osteogenic differentiation cultures were performed and mineral deposition was confirmed by alizarin red staining. Real-time qPCR was also performed to examine the differences in gene expression between the fast- and slow-dividing cell groups immediately after aliquoting and after osteogenic differentiation.
Results: Differences in the growth rate of the fractionated cells were maintained after culture. Results of osteogenic differentiation culture and alizarin red staining showed more extensive mineral deposition in the slow cell group than in the fast cell group. Calcium quantification also showed higher absorbance in the slow cell group compared to the fast cell group, indicating higher osteogenic differentiation potential in the slow cell group. Furthermore, real-time qPCR analysis showed that osteocalcin expression was higher in the slow cell group in cells immediately after preparative differentiation. In addition, the expression of osteocalcin and sclerostin were higher in the slow cells after osteogenic differentiation.
Conclusion: The slow cell population contains many highly differentiated cells that are already more deeply committed to the bone lineage, suggesting that they have higher osteogenic differentiation potential than the fast cell population. This study will contribute to the realization of better bone regenerative medicine by utilizing the high osteogenic differentiation potential of the slow cell population.

1. Introduction

Mesenchymal stem cells (MSCs) are a type of somatic stem cell derived from the mesenchyme and were first identified by Friedenstain in 1976 [1]. MSCs exhibit a fibroblast-like spindle shape when cultured on plastic and can differentiate into three lineages, osteoblasts, adipocytes and chondrocytes, depending on culture conditions [2]. MSCs are found in a wide range of tissues, including bone marrow, adipose tissue, placental chorionic villi, umbilical cord, and dental pulp [3], among which bone marrow-derived MSCs are the most well-studied.

When the existing bone volume to the maxillary sinus mucosa is poor during dental implant placement, a sinus lift is necessary to elevate the maxillary sinus mucosa and build bone in the void

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; ARS, alizarin red s.
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created. Several studies using MSCs for sinus lift have been reported. In an experimental transplantation to the floor of the maxillary sinus in beagle dogs, a composite graft combined with bone marrow MSCs and periodontal ligament MSCs showed a higher bone-forming capacity compared to a single graft of bovine bone grafts [4]. In addition, a composite of bone marrow MSCs and bovine-derived bone fillers was found to promote the formation and maturation of new bone in the rabbit maxillary sinus under compression conditions [5]. Clinical studies have reported that when autologous cells enriched with CD90-positive MSCs and CD14-positive monocytes were administered to the floor of the maxillary sinus of patients, the greater the percentage of MSCs, the greater the bone density [6]. Thus, the efficacy of MSC loading has been demonstrated in several papers.

However, MSCs differ greatly in growth kinetics and gene expression patterns depending on the age and sex of the donor, the tissue supplied, culture conditions, and isolation method, which is thought to affect cellular characteristics and function [7,8]. In addition, repeated passages are necessary to increase the purity of MSCs, which may increase the probability of morphological and genetic changes [9–11]. These changes in cell physiology are said to affect clinical outcomes. For example, there have been reports [12] that MSCs that have increased in size through expanded culture are more difficult to retain in the target tissue, reducing the efficacy of cell therapy.

MSCs are actually a heterogeneous cell population that includes fibroblasts, myofibroblasts, and a small number of stem and progenitor cells, and single-cell RNA sequencing has identified several subpopulations in the MSC population with different functional properties [13]. Given the current difficulty in obtaining a homogeneous MSC population in sufficient quantity for tissue regeneration, we hypothesized that if a population of MSCs with high osteogenic potential could be extracted, regeneration of higher quality of bone could be achieved.

In our previous report, cell labeling of human auricular cartilage with carboxyfluorescein succinimidyl ester (CFSE) revealed that chondrocytes with rapidly decreasing intracellular dye concentration (fast dividing cell group) were smaller in size, less granular, and characterized by higher chondrogenic potential [14,15]. These results suggest that selecting chondrocytes according to their division rate may be a promising approach to enrich chondrocytes with higher chondrogenic potential and improve the quality of regenerated cartilage.

The aim of this study was to apply the CFSE-based cell selection method to mouse bone marrow MSCs in order to identify MSC populations with high osteogenic potential.

2. Materials and methods

2.1. Isolation of mouse mesenchymal stem cells

All procedures and protocols for this experiment were approved by the University of Tokyo Animal Ethics Committee (Ethics approval number P19-114).C57BL/6JJcl mice (6 weeks old, male) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The femur, tibia, and humerus of the mice were harvested, and basic medium: α-MEM (Thermo Fisher Scientific Inc., Waltham, USA) supplemented with 1 % penicillin/streptomycin (Sigma-Aldrich Co., LLC, St. Louis, USA) was injected into the bone marrow cavity to obtain bone marrow cells. Bone marrow cells were filtered through a cell strainer (pore size: 70 μm; Becton, Dickinson and Co., Franklin Lakes, USA), Red blood cells were crushed using RBC Lysis Buffer (0.16 M NH_4Cl: 0.17 M Tris = 9 : 1), and the cells were collected by centrifugation at 1500 rpm for 5 min, seeded on 100 mm cell culture dishes (Becton, Dickinson and Co.) and incubated in growth medium: 20 % fetal bovine serum (FBS) (Thermo Fisher Scientific Inc.), 1 % penicillin/streptomycin supplemented IMDM medium (Thermo Fisher Scientific Inc.). When cells reached confluency, bone marrow mesenchymal stem cells (MSCs) were harvested using 0.5 % trypsin-EDTA (Sigma-Aldrich Co.). Cells were seeded on new cell culture dishes at a density of 1.3×10^6/dish, and cultured for 10 days (passage 1: P1).

2.2. Flow cytometry for cell surface antigens

P1 MSCs were collected using 0.5 % trypsin-EDTA and labeled with antibodies against mouse epitopes according to each manufacturer's protocol. The epitopes and antibody manufacturers are as follows. Positive markers: CD29, CD73 (BioLegend, San Diago, USA), CD90 (Becton, Dickinson and Co.), CD44 (Thermo Fisher Scientific Inc.). Negative markers: CD31 (BioLegend), CD34 (Becton, Dickinson and Co.), CD45 (Thermo Fisher Scientific Inc.). To prevent nonspecific binding, Fc receptor blocking was performed beforehand with CD16/32 (Becton, Dickinson and Co.) and isotype antibodies were used as negative controls. Cells were examined with the BD LSRFortessaTM instrument (Becton, Dickinson and Co.), and collected data were analyzed with BD FACSDiva™ software (Becton, Dickinson and Co.) or FlowJo (Tomy Digital Biology Co.).

2.3. Cell labeling with CFSE

P1 MSCs were collected and stained using CellTrace™ CFSE Cell Proliferation Kit (CFSE; Thermo Fisher Scientific Inc.). Cells were observed with a Leica DM IL (Leica Microsystems K.K., Wetzlar, Germany), and cell number and viability were determined using CellDrop™ Automated Cell Counters (DeNovix, Wilmington, USA) or Bürker-Türk counting chamber (ERMA Inc., Tokyo, Japan). Cell labeling was checked with the BD LSRFortessaTM instrument. After labeling, cells were again seeded and cultured as P2 before analysis.

2.4. Selective separation of target cells

On day 3 of P2 culture, cells labeled with CFSE were collected and sorted using the BD FACSAria™ Fusion instrument (Becton, Dickinson and Co.). The gate was set at the center of the fluorescence histogram so as to 50% of cells were on the both sides. The sorted cells were re-seeded and cultured as P3 cells.

2.5. Cell counting Kit-8 (CCK-8) assay

To examine cell proliferation, fluorescence-activated cell sorted (FACS sorted) P3MSCs were seeded on 96-well microplates (Becton, Dickinson and Co.) at a density of 7.5×10^3 cells/well and cultured in growth medium for 1, 3, 5 and 7 days, respectively. After the medium was replaced with basic medium (100 μl), CCK-8 solution (10 μl) was added to each well and incubated for 2 h in an incubator at 37 °C and 5 % CO_2. Cell activity was evaluated using CCK-8 kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance of the sample solution at 450 nm was measured using a microplate reader (ARVO X3; PerkinElmer Co, Ltd., Waltham, USA).

2.6. Osteogenic differentiation in vitro

FACS-sorted P3MSCs were seeded on 12-well plates (Becton, Dickinson and Co.) at a density of 2×10^5 cells/well and cultured in growth medium up to 80 % confluency, then incubated in osteogenic differentiation medium: IMDM medium supplemented with 10 % FBS, 1 % penicillin/streptomycin, 10 mM β-glycerophosphate (Sigma-Aldrich Co.), 50 μM ascorbic acid (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), and 200 ng/ml BMP2 (Corefront Co., Tokyo, Japan). The medium was changed every 3 days.

2.7. Alizarin red S (ARS) staining and quantification

On day 28 of osteogenesis induction, MSCs were fixed with 4 % paraformaldehyde for 20 min and stained with 1 % ARS (pH 4.3) for 20 min. After washing three times with ion-exchanged water, calcium deposition was observed and imaged with an all-in-one Fluorescence Microscope BZ-X800 (KEYENCE Corp., Osaka, Japan). To quantify calcium deposition, stained cells were destained with 5 % formic acid (Wako, Japan) for 10 min. One hundred microliter aliquots were transferred to 96-well microplates, and a microplate reader (ARVO X3; PerkinElmer Co, Ltd., USA) was used to measure the absorbance at 450 nm.

2.8. RNA isolation and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from the collected samples using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Gene expression was detected using THUNDERBIRD® Next SYBR® qPCR Mix (TOYOBO CO., LTD., Tokyo, Japan). Real-time qPCR was performed using a 7500 Fast Real-Time PCR System (Applied BioSystems, CA, USA). Relative expression levels of each gene were analyzed using the standard curve method. Forward and reverse primers for each gene are listed in Table 1.

2.9. Statistics

All experiments were performed using cells from three or more different lots; comparisons between two groups were made using Welch's *t*-test; comparisons between four groups were made by Tukey's test from one-way analysis of variance (ANOVA) using EZR software, version 1.54 [16]. Data are expressed as mean ± standard deviation. $P < 0.05$ indicated statistical significance.

3. Results

3.1. Confirmation of cell surface markers

To confirm that P1 cells before CFSE labeling were mesenchymal stem cells, we analyzed the expression of cell surface markers [17,18], which have been reported as mouse stem cell markers. The results showed that P1 cells exhibited high expression of positive markers CD29, CD44, CD73 and CD90, and low expression of negative markers CD31, CD34, CD45 compared to isotype controls (Fig. 1). This indicated that the P1 cells used in this study were MSCs.

3.2. Fluorescence labeling of bone marrow mesenchymal stem cells (MSCs) with CFSE

In a previous report, fluorescence intensity of human auricular chondrocytes from day 0 to day 6 after CFSE labeling was measured and showed that the difference in cell division rate increased on day 4 after CFSE labeling, facilitating sorting between rapid and slow cell groups [15]. Since MSCs may differ from human auricular cartilage in terms of division rate and efficiency of staining, we reexamined the CFSE labeling concentration, incubation time, and number of days in culture before aliquoting.

CFSE labeling of P1 MSCs was performed under four combinations of the concentrations of CFSE and incubation times: 5 μM 15 min, 10 μM 20 min, 15 μM 10 min, and 20 μM 5 min. These conditions are based on the recommended concentration of CFSE (0.5–20 μM) by the manufacturer and the optimal staining conditions determined by previous reports: 10 μM 20 min for human auricular cartilage [14,15] and 20 μM 5 min for rat MSCs [19]. Cells were collected after 0, 3, and 7 days of staining, respectively, and fluorescence intensity was measured (Fig. 2A). On 0 day, the fluorescence intensity of CFSE-labeled cells was much stronger than that of unstained cells (Fig. 2B). After 3 days of staining, the histogram became wider and the fluorescence intensity attenuated. After 7 days of staining, the histograms became even wider, and the fluorescence intensity attenuated to almost the same level as that of the unstained cells. The broadening of the histogram over time indicates the presence of cells with different growth rates, indicated by the presence of various fluorescence levels. As for the shape of the histogram, the left and right widths of the histograms after 3 days of staining were more even under the 20 μM 5 min condition than under the other conditions. Therefore, this condition was used for cell sorting hereafter in this study.

3.3. Sorting of fast and slow proliferating cell groups

To compare the characteristics of cell groups with different proliferation rates, cells were sorted into two groups according to fluorescence intensity. Specifically, cells plotted on the left and right sides of the histogram (50 % each) were assigned to the rapid and slow cell groups, respectively. Based on the previous section, P1 cells were stained under the 20 μM 5 min condition and seeded as P2 cells, then collected on 3 days later for sorting by flow cytometry (Fig. 3A–C). In the forward scatter (FSC)/side scatter (SSC) plots, the rapid cell population was generally densely distributed in the lower left corner, whereas the slow cell population was distributed slightly higher and more uniformly (Fig. 3B). Thus, the cells in the slow cell group were larger and have more complexed internal structures than those in the rapid cell group. To evaluate the damage on cells by sorting, we examined cell viability after sorting. Cell viability was nearly 90 % in both the rapid and slow cell groups, with no significant differences (Fig. 3D).

3.4. Differences in growth rate of sorted cells were maintained after culture

The seeded cells were microscopically observed on days 1, 3, 5, and 7 after sorting (Fig. 4A), showing a trend toward increased cell density in the rapid group compared to the slow group. The CCK-8 assay was also used to quantify the cell proliferation rate of the rapid and slow cell groups (Fig. 4B). The results showed that the rapid cell group exhibited higher proliferative capacity than the slow cell group, with significant differences at days 5 and 7. This suggested that both groups were not a population of dead cells or cell debris, and that they maintained their respective mitotic rate characteristics after sorting.

3.5. High osteogenic differentiation potential in vitro was observed in the slow-growing cell group

MSCs of rapid and slow cell groups were cultured as P3 cells in 12-well plates, and their osteogenic differentiation ability was

Table 1
All primer sequences used for qPCR.

Primer	Forward (5′-3′)	Reverse (5′-3′)
Runx2	GAGTCAGATTACAGATCCCA	TGGCTCTTCTTACTGAGAGA
Col1	CCCCAACCCTGGAAACAGAC	GGTCACGTTCAGTTGGTCAAAGG
OC	CCAAGCAGGAGGGCAATA	AGGGCAGCACAGGTCCTAA
SOST	GCCTCATCTGCCTACTTGTG	CTGTGGCATCATTCCTGAAG
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

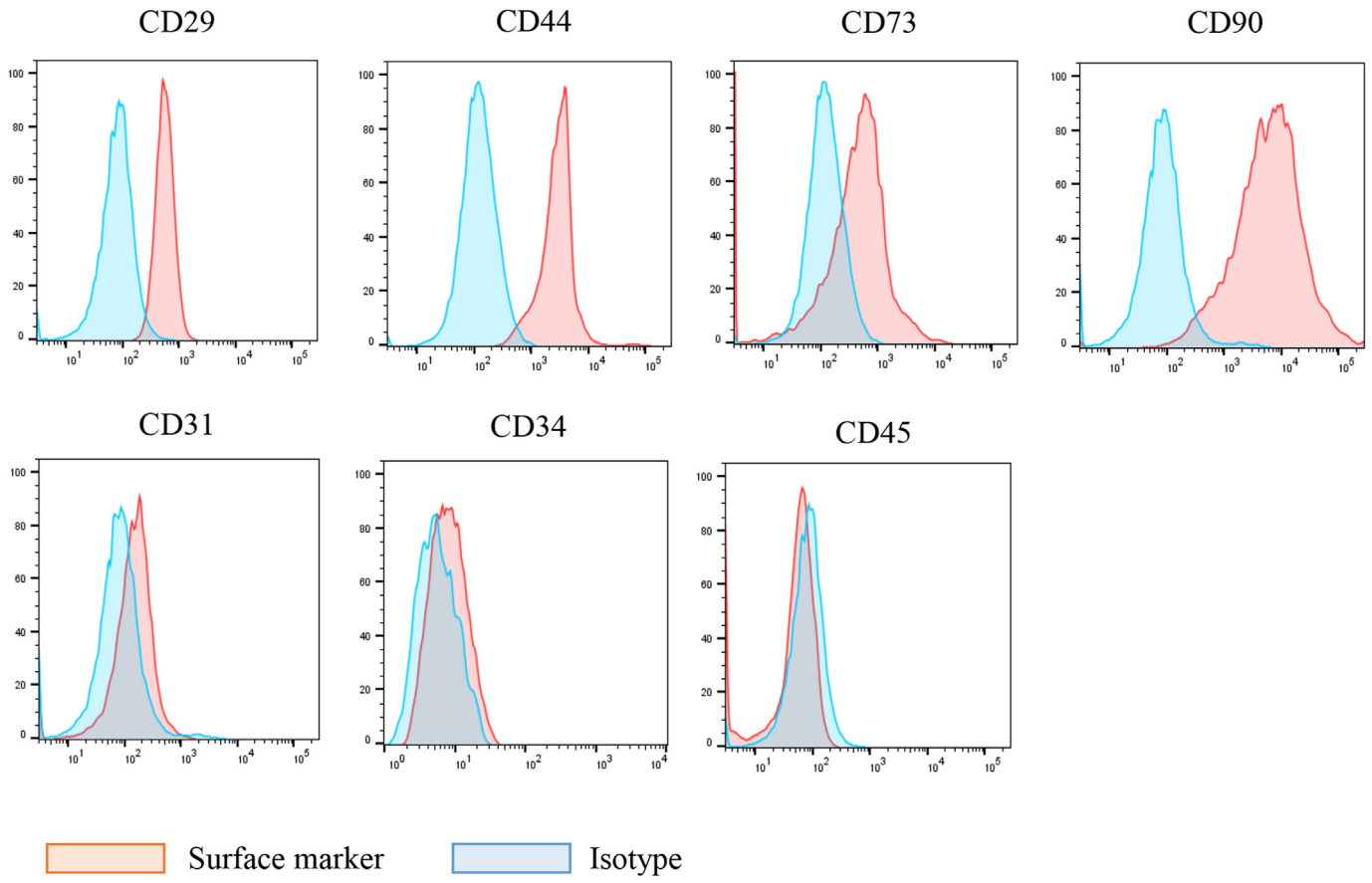


Fig. 1. Flow cytometric analysis of cell surface markers and CFSE fluorescence. Positive markers: CD29, CD44, CD73, and CD90; Negative markers: CD31, CD34, CD45.

examined. Staining of cells at 28 days after the start of osteogenic differentiation with alizarin red showed more extensive mineral deposition in the slow cell group (Fig. 5A). Calcium quantification also showed significantly higher absorbance in the slow cell group compared to the rapid cell group (Fig. 5B). These findings indicate that the osteogenic differentiation potential of cells in the slow cell group is higher than that of cells in the rapid cell group.

3.6. Rapidly and slowly proliferating cell groups showed different trends in gene expression

Real-time qPCR analysis was performed to examine whether there were different trends in the genes contained in the rapid/slow cell population immediately after sorting and at day 28 of osteogenic differentiation culture. In cells immediately after sorting, there was little difference in the expression of Runx2, while the expression of Col1, osteocalcin (OC), and sclerostin (SOST) tended to be higher in the slow cell group. In cells at day 28 of osteogenic differentiation culture, Runx2 expression tended to be higher in the rapid cell group, while OC and SOST expression was significantly higher in the slow cell group (Fig. 6). These results indicated that the slow cell group was the more osteoblast-differentiated cell group immediately after preparative culture, and that osteoblast and osteocyte differentiation was further promoted by differentiation culture.

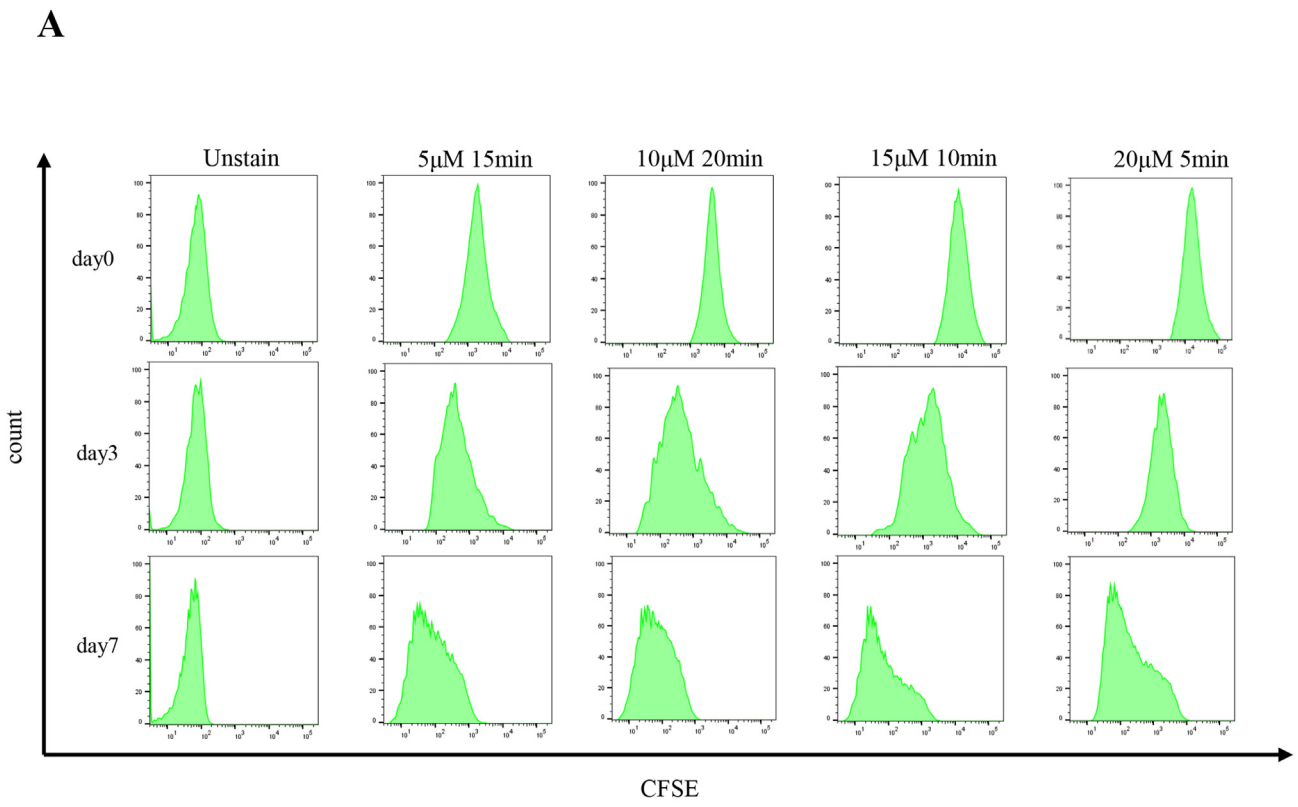
4. Discussion

In previous studies, sorting of human auricular chondrocytes by mitotic rate showed that the fast cell group had higher chondrogenic potential than the slow cell group [14,15]. It has also been shown that the fast cell group is more undifferentiated with less expression levels of CDKN1A, which inhibits cell cycle progression, and CLU and BMP-2, both of which are highly expressed in osteoarthritic cartilage, than the slow cell group [15]. In this study, we applied the same sorting method to mouse bone marrow MSCs and examined the difference in the characteristics of rapid and slow cell groups.

After 28 days of osteogenic differentiation, the slow cell group had higher mineralizing capacity than the fast cell group, and marker genes for mature osteoblasts and osteocytes were also highly expressed. Furthermore, when the two groups were compared immediately after sorting, Col1, OC, and SOST tended to be more highly expressed in the slow cell group. This suggests that the slow cell group contained more highly differentiated cells that were already more deeply committed to the osteoblast lineage.

As mentioned in the introduction, MSCs are a heterogeneous cell population, and studies by Rennerfeldt et al. have reported that even in single cell-derived MSCs with relatively uniform characteristics, cell division and senescence promote heterogeneity within colonies during expanded culture *in vitro*, with functional consequences such as differentiation potential [20]. Simply bisecting these heterogeneous MSCs by mitotic rate alone is likely to result in heterogeneity still remaining within each fractionated cell population. In other words, the uneven distribution of calcified matrix formation may be due to remaining heterogeneity in the cell population.

Stem cells have been reported to be high proliferative *in vitro*. Sekiya et al. stated that human bone marrow MSCs can expand up to several hundredfold when seeded at low densities, while



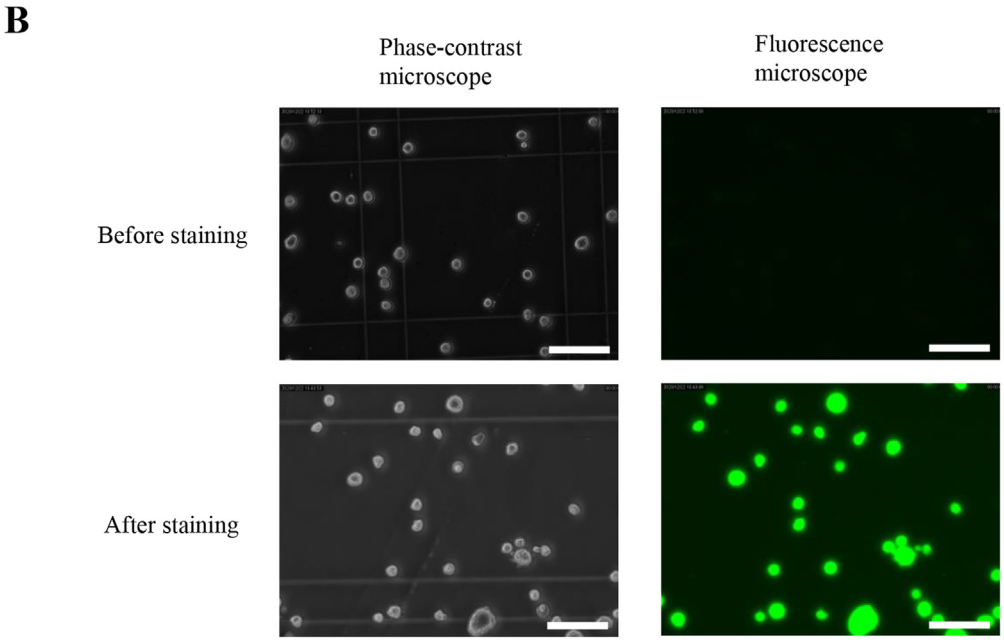
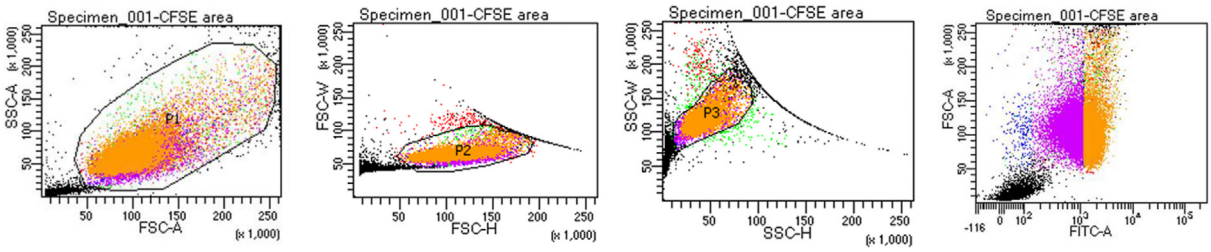


Fig. 2. Cell labeling with CFSE. (A) Flow cytometric analysis of mouse MSCs on the day of CFSE staining and 3 and 7 days after staining. The concentrations of CFSE and incubation times were set to 5 μM 15 min, 10 μM 20 min, 15 μM 10 min, and 20 μM 5 min, respectively. Unstained cells were also analyzed on the same day as negative controls. Horizontal lines indicate the intensity of CFSE fluorescence and vertical lines indicate the percentage of cell count. (B) Phase contrast and fluorescence images of cells stained or unstained with CFSE for 20 μM 5 min. Images were taken immediately before and after staining; Scale bars = 75 μm.

A



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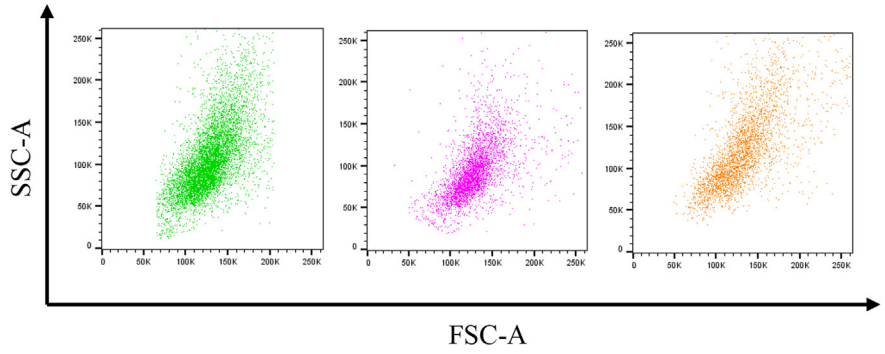

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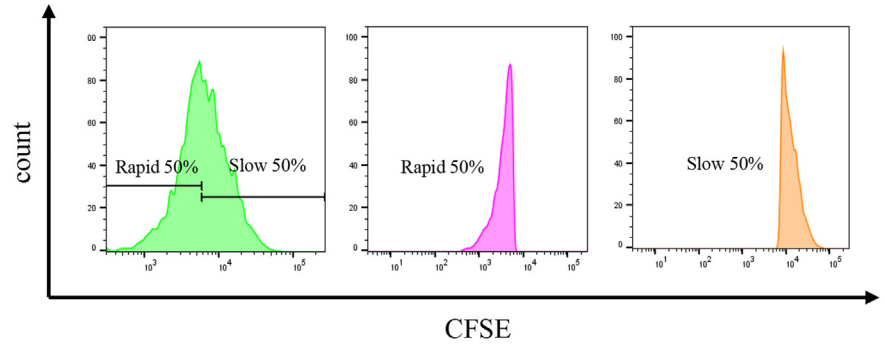
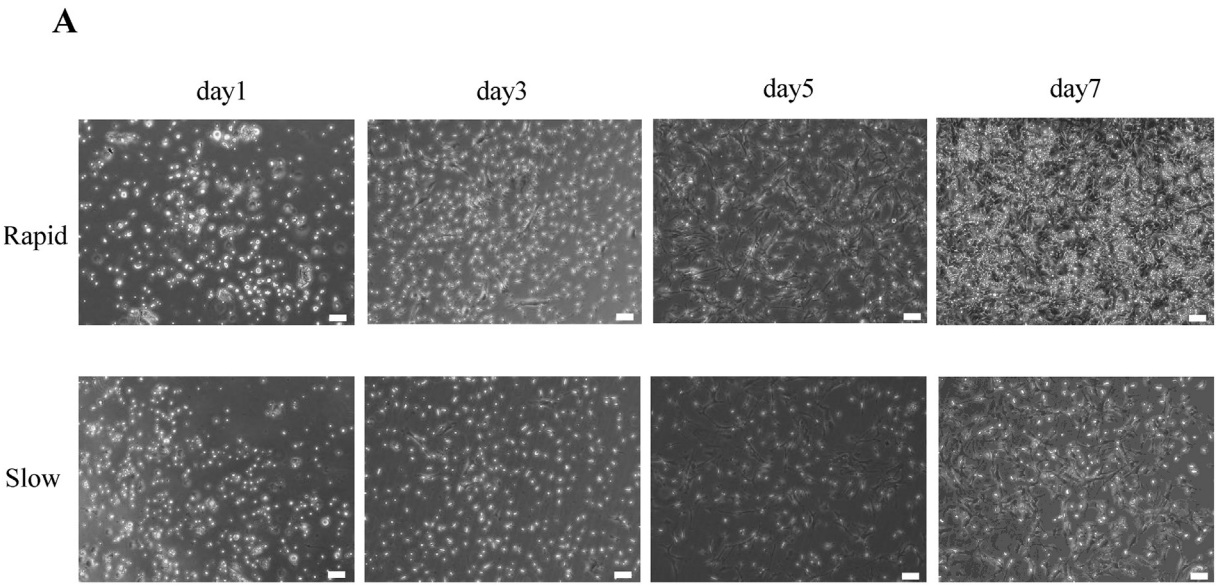


D

	Rapid	Slow
Viability（%）	89.0	89.2
SD	2.5	2.3

Rapid versus Slow p>0.05

Fig. 3. Sorting of rapid cell groups and slow cell groups. (A) Sorting strategy for rapid and slow cell groups by fluorescence intensity. (B) FSC/SSC plots of total cells and rapid and slow cell groups. (C) Histograms of CFSE fluorescence during sorting of all cells and rapid and slow cell groups. Areas of rapid cell group (50 %) and slow cell group (50 %) are shown. (D) Cell viability of rapid and slow cell groups. Values are means ± SD.



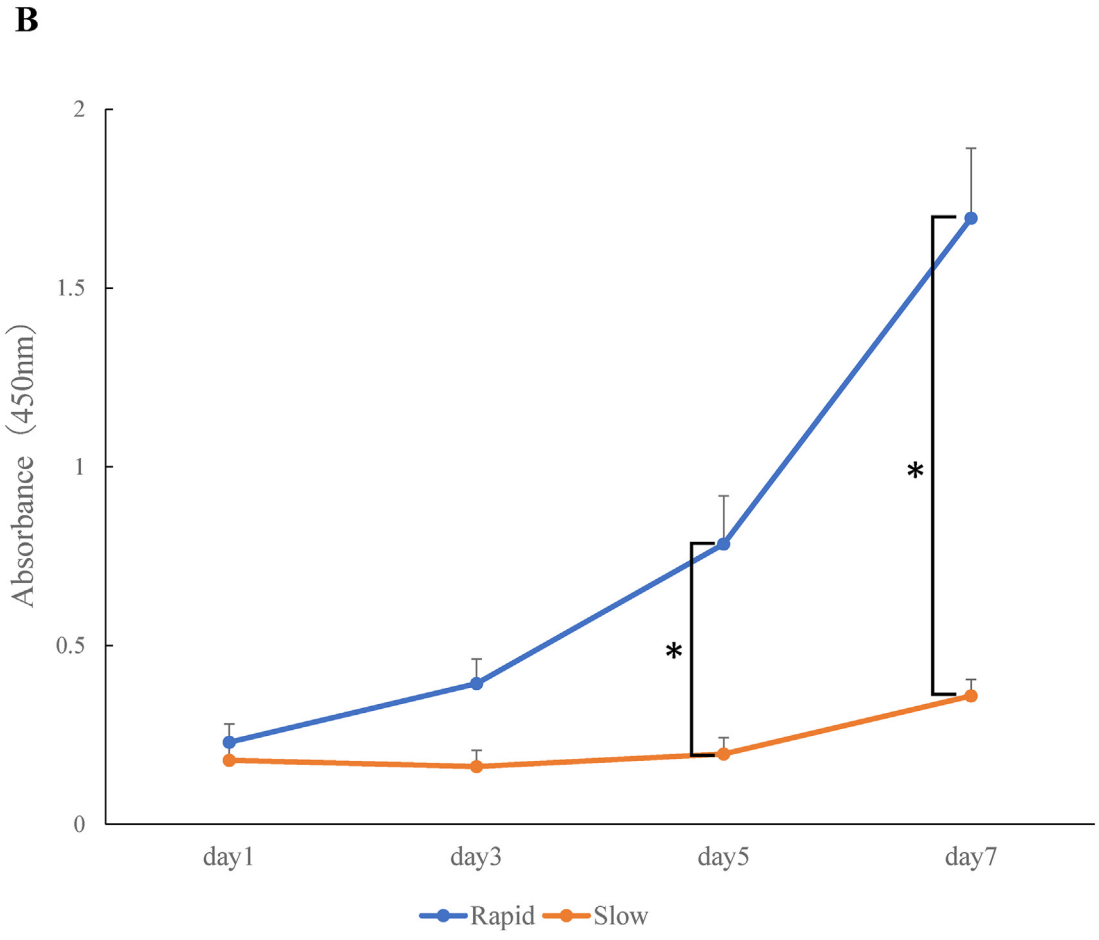


Fig. 4. Proliferation of rapid cell groups and slow cell groups. (A) Phase contrast image of sorted rapid cell group/slow cell group. Photographs were taken after 1, 3, 5, and 7 days of culture. Scale bars = 100 μm. (B) CCK-8 assay of rapid cells/slow cells. Cell proliferation of sorted rapid and slow cell groups was examined by CCK-8 assay on days 1, 3, 5, and 7 ($n = 3$, $^*P < 0.05$).

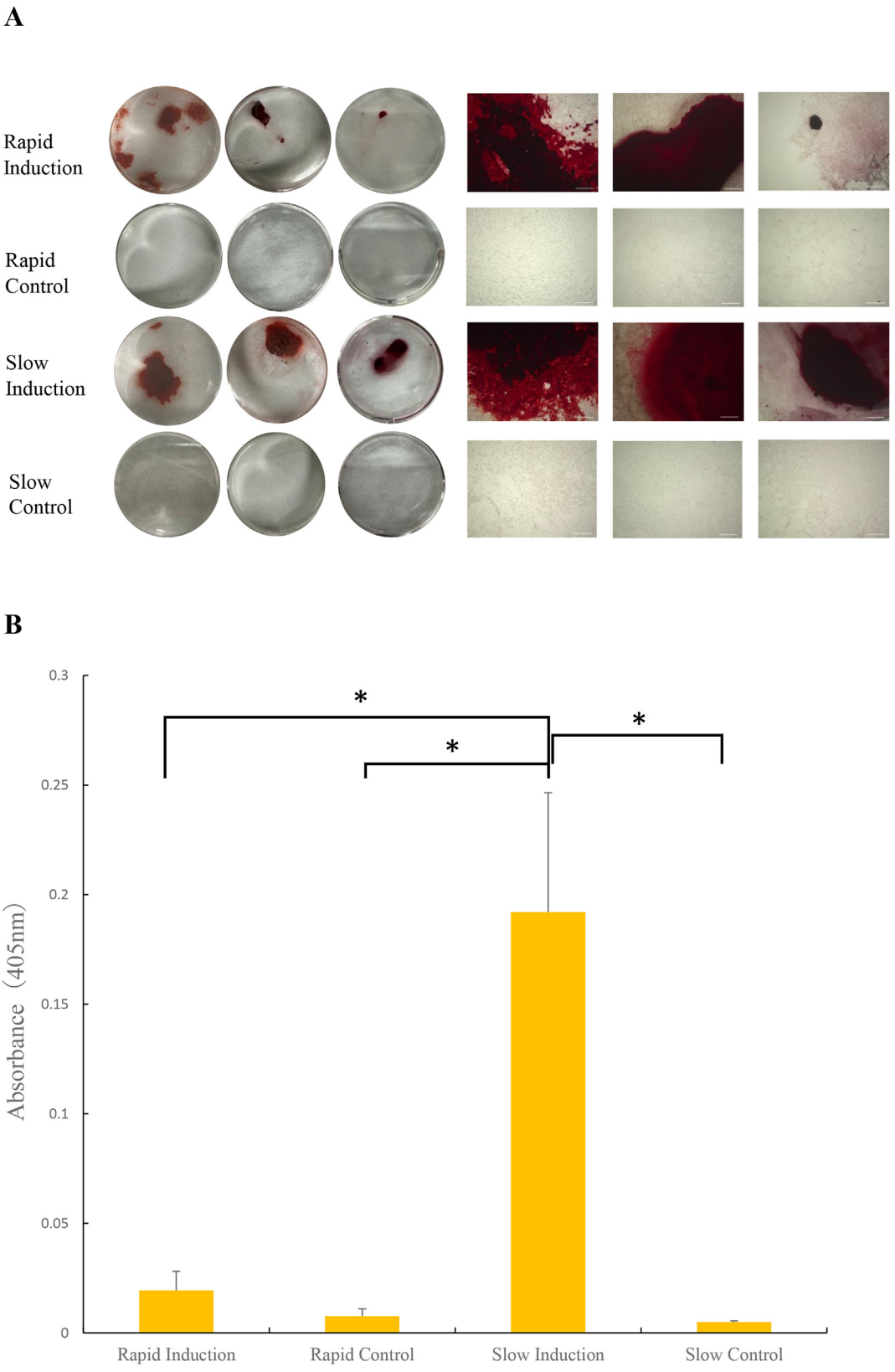


Fig. 5. Osteogenic activity of rapid cell groups and slow cell groups. (A) Alizarin red staining at 28 days after osteogenic differentiation. Left: Photograph of each well (3 wells per group), Right: Micrograph (3 fields of view per group). Scale bars = 500 μm. (B) Quantification of calcium deposition. ($n = 3$, $^*P < 0.01$).

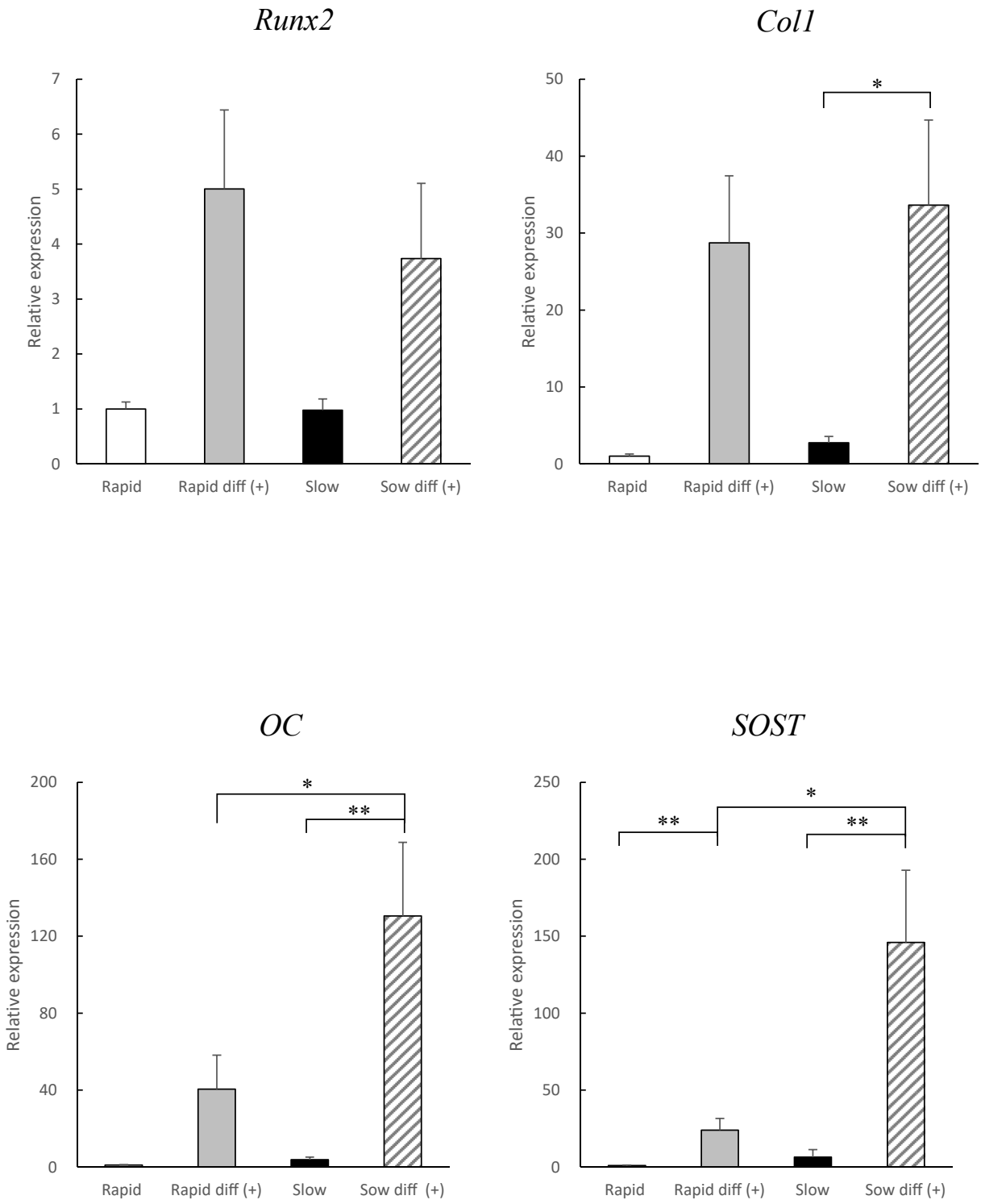


Fig. 6. Gene expression analysis by real-time qPCR. Gene expression of rapid cell group/slow cell group immediately after sorting and on day 28 of bone differentiation culture is shown. ($n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$).

maintaining pluripotency and the ability to form fibroblast-like colonies [21]. Colter et al. reported that when human bone marrow MSCs were seeded at low density, cells amplified 109-fold in 6 weeks, and FACS analysis identified a small population of cells called recycling stem cells [22]. Javazon et al. showed that cells proliferate more than 4000-fold in 12 days when rat bone marrow MSCs were seeded at a lower density than human bone marrow MSCs [23], and Tsai et al. reported that MSCs cultured under low density conditions and at low oxygen concentrations of 1–7% maintained a high growth rate during late passaging [24].

In the present study, osteogenic differentiation markers were lower in the fast cell group immediately after sorting than in the slow cell group, and after differentiation they were also lower except for the early marker Runx2. This finding is consistent with

our findings in chondrocytes. On the other hand, in our previous study regarding human auricular chondrocytes, the capacity of cartilage matrix production of the highly undifferentiated rapid cell group was high both *in vitro* and *in vivo* [14,15]. In contrast, as to the mouse MSCs in this study, the osteogenic capacity was higher in the more highly differentiated slow cell group *in vitro*.

However, we have not been able to confirm whether slow cell populations with advanced osteoblast differentiation show higher bone regenerative potential *in vivo*, which is one of the limitations of this study. This is because the number of viable cells sorted varied among experiments, and no more than 1 million cells per group were obtained. According to a previous report by Kucic et al., they performed subcutaneous injection of mice with 2×10^6 bone marrow MSCs per 400 μl of Matrigel [25]. In addition, Kang et al. transplanted 200 mg of βTCP and 1×10^6 fat MSCs mixed in 300 μl of Matrigel subcutaneously in the back of canine [26], and Nevo et al. also reported subcutaneous injection of mice with 1.5×10^6 cord blood endothelial progenitor cells and 1.5×10^6 bone marrow MSCs in 200 μl of Matrigel [27]. Based on the above reports, it would be difficult to prepare a sufficient number of cells to be transplanted into multiple animals. Possible solutions to this problem include increasing the number of cells before sorting and increasing the cell flow rate during sorting. However, prolonged sorting may damage the cells. In addition, although increase in the cell flow rate can shorten the sorting time, reduction in the time for detection will impair the accuracy of the cell sorting [28]. New technologies for cell sorting with more speed and less cell damages are to be developed.

However, there is a report [29] that pre-differentiation of MSCs prior to transplantation is inhibitory for chondrogenesis and promotive for osteogenesis. One reason that pre-differentiation promotes osteogenesis is that the incorporation of osteogenic cell sources into the scaffold limits the migration and expansion of native bone progenitor cells at the defect site, ultimately increasing the rate of osseointegration. Other transplantation experiments using a rat cranial defect model of bone marrow MSCs and hydrogel mixtures [30] and bone marrow MSCs and apatite-coated chitosan microspheres [31] both reported that the mixture that was pre-differentiated for 14 days *in vitro* showed the highest bone formation In contrast, MSC chondrogenesis depends on their ability to maintain a spherical shape [2], which is diminished by chondrogenic differentiation of MSC. In addition, chondrogenic differentiation may reduce the paracrine action [32] that promotes chondrogenic cell differentiation. Park et al. also reported that transplantation of a composite of human umbilical cord MSCs and hyaluronic acid hydrogel into osteochondral defects in rat femurs resulted in better chondrogenesis in undifferentiated MSCs compared to MSCs that were chondrogenically differentiated for 3 weeks [33]. These reports are consistent with the results of our previous study, which showed that undifferentiated fast cell groups have higher chondrogenic potential, and also suggest that the slow cell groups obtained in this study may have high osteogenic potential after transplantation.

Although this study shows the possibility of fractionation of osteogenic MSCs by mitotic rate, cytotoxicity must be considered for clinical application of CFSE. Yang et al. co-cultured human bone marrow MSCs stained at 2.5 μM for 10 min with human retinal pigment epithelium, and reported that they differentiated into neural-like cells [34]. Although the staining conditions used for mouse MSCs in this study were at a high concentration of 20 μM for 10 min, no obvious effect on cell proliferation and no cytotoxicity was reported 21 days after labeling rat MSCs under similar conditions [19]. However, in actual administration to the human body, safety must be carefully examined. On the other hand, indocyanine green (ICG), which is already widely used in liver function tests and fundus contrast studies, has little cytotoxicity or effect on the human body. However, ICG has a very short half-life of 2–4 min [35], making its application to fractionation by cell division rate difficult. Investigation of dyes that can improve these problems is considered to be a future challenge.

The use of FACS for cell sorting also raises concerns about cell damage. Binek et al. reported that physical effects on cells as they pass through a narrow channel altered the content of metabolites associated with the plasma membrane and upregulated stress genes [36]. Llufrio et al. also reported that FACS treatment altered the ratio of oxidized to reduced glutathione, a cellular oxidative stress indicator, and the concentrations of amino acids, pentose phosphate pathway, and TCA cycle [37]. However, Fister et al. reported no problems with cell viability and proliferation under either condition when human peripheral blood mononuclear cells were sorted at nozzle sizes of 70–100 μm and sheath pressures of 20–70 psi [38]. In the present study, both the fast and slow cell groups after preparative sorting showed nearly 90 % cell viability, and no obvious cell damage by FACS was observed, as both groups retained their osteodifferentiation potential. However, since cell aliquoting in this study took 4–6 h, the effect of prolonged exposure of cells to a suboptimal environment on subsequent transplantation needs to be examined. If these concerns can be solved, better bone regenerative medicine may be realized using the high osteogenic potential of the slow cell population demonstrated in this study.

5. Conclusion

This study shows that the slow cell group in MSCs has higher mineral-forming capacity than the fast cell group. Real-time qPCR analysis showed that mature osteoblast markers were highly expressed in the slow cell group immediately after preparative culture, and mature osteoblast and osteocyte markers were also highly expressed after osteogenic differentiation culture, suggesting high osteogenic potential of the slow cell group. The results obtained in this study will contribute to the realization of better bone regenerative medicine.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Atsuhiko Hikita was affiliated with an endowed chair supported by FUJISOFT INCORPORATED (until October 31, 2020), and is affiliated with an endowed chair supported by CPC corporation, Kyowa Co., Ltd., Kanto Chemical Co. Inc., and Nichirei corporation (July 1, 2021). Yukiyo Asawa is affiliated with an endowed chair supported by SheepMedical Co., Ltd.

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