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Original Article

Human auricular chondrocytes with high proliferation rate show high production of cartilage matrix

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ABSTRACT

Cartilage has a poor capacity for healing due to its avascular nature. Therefore, cartilage regenerative medicine including autologous chondrocyte implantation (ACI) could be a promising approach. Previous research has proposed various methods to enrich the cultured chondrocytes for ACI, yet it has been difficult to regenerate homogeneous native-like cartilage in vivo. The cell populations with an increased ability to produce cartilage matrix can show somatic stem cells-like characteristics. Stem cells, especially somatic stem cells are able to grow rapidly in vitro yet the growth rate is drastically reduced when placed in in vivo conditions [14]. Thus, in this study we investigated whether proliferation rate has an impact on in vivo regeneration of cartilage constructs by sorting human chondrocytes. The human chondrocytes were fluorescently labeled with CFSE and then cultured in vitro; once analyzed, the histogram showed a widening of fluorescence level, indicating that the cells with various division rates were included in the cell population. To compare the characteristics of the cell groups with different division rates, the chondrocytes were sorted into groups according to the fluorescence intensity (30 or 45 percent of cells plotted in the left and right sides of histogram). Then the cells of the rapid cell group and slow cell group were seeded into PLLA scaffolds respectively, and were transplanted into nude mice. Metachromatic regions stained with toluidine blue were larger in the rapid cell group compared to the slow cell group, indicating that the former had higher chondrogenic ability. We proposed a new method to enrich cell population with high matrix production, using proliferation rate alone.

1. Introduction

Due to its avascular nature, cartilage has a poor capacity for healing once it has been damaged. Therefore, autologous chondrocyte implantation (ACI) could be a promising approach in the field of cartilage regenerative medicine. Human ACI was first reported in 1994 by Brittberg et al., in which autologous chondrocytes from a healthy non-bearing site of cartilage in a knee joint were cultured in vitro, and then transplanted into the defective sites [1,2]. While transplantation of autologous cells is advantageous in terms of controlling immune response, it requires the process of isolating and expanding the cells to the amounts that would suffice for transplantation. Meanwhile, changes in cell morphology or dedifferentiation could occur during culture, leading to reduced matrix production [3,4].

It is difficult to make native cartilage-like tissue with three-dimensional structure and uniform cartilaginous properties, however cartilaginous tissue has been regenerated by transplanting the dedifferentiated chondrocytes into the body [5]. A probable explanation for the nonuniform cartilaginous properties seen in previous research could be the inconstancy of cells used for transplantation. If the primary chondrocytes taken from cartilage contain multiple cell populations, the regenerated tissue may also

Abbreviation: CFSE, Carboxyfluorescein diacetate succinimidyl ester; PLLA, poly-L-lactic acid scaffolds.

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become heterogeneous. As a breakthrough for this issue, methods should be established to enrich the cells that have more potential to produce cartilage matrix. There have been studies on enriching the cells, in which culture conditions or cell surface markers have been examined [6–12]. Culture conditions have been regulated in which the cells should be seeded at low density, and cultured with low glucose medium or under hypoxia [6–9]. These in vitro studies have proved to be effective, however the efficacy has not been sufficiently replicated for in vivo transplantation thus far. There have been reports in which cell populations with high cartilage matrix capacity were identified, focusing on the enrichment of MSC-like and progenitor cells [10–13]. Yet the expression of cell surface markers was not consistent due to changes in culture conditions and passage numbers. Therefore, thus far, it has not been feasible to regenerate homogenous tissue-engineered cartilage in vivo.

The population with high ability to produce cartilage matrix shows somatic stem cell-like characteristics. Stem cells, especially somatic stem cells are able to grow rapidly in vitro yet the growth rate is drastically reduced when placed in in vivo conditions [14]. Thus, in this study it was imperative to investigate whether proliferation rate has an impact on in vivo regeneration of cartilage constructs. To concentrate the chondrocytes according to the proliferation rate, we sorted human chondrocytes with the high proliferation rate and then evaluated the regeneration of cartilage constructs in mice.

2. Materials and methods

2.1. Isolation of human auricular chondrocytes

This study was approved by the Research Ethics Committee of the University of Tokyo Hospital. Auricular cartilages were provided as excised remnant auricular cartilage tissue from the surgery of microtia patients in NAGATA Microtia and Reconstructive Plastic Surgery Clinic. We obtained informed consent from all patients. After the excision of soft tissues and perichondria by scalpel and scissors, auricular cartilage was minced, and digested by shaking with 0.3% collagenase solution for 18 h at 37 °C. The solution was filtered with a cell strainer (100 μm pore size, BD Falcon), centrifuged at 1500 rpm for 5 min and the supernatant was removed to obtain human auricular chondrocytes. Cells were seeded at 2.0×10^5 cells/dish to φ100 mm collagen Type I Coated dich (AGC Techno Glass Co., Ltd.), and cultured in the cartilage growth medium (HFI; Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Sigma–Aldrich Co.) supplemented with 5% Human Serum (Sigma–Aldrich Co.), 100 ng/mL FGF-2 (Kaken Pharmaceutical Co, Ltd.), 5 μg/mL Insulin (Novo Nordisk Pharma Ltd.), 1% Penicillin/Streptomycin (Sigma–Aldrich Co.)) at 37°C in 5% CO_2. After 10 days, cells reached the confluence, and they were detached by Trypsin-EDTA (Sigma–Aldrich Co.), recovered by centrifugation, and stored frozen at −80°C in CELLBANKER (Nippon Zenyaku Kogyo Co., Ltd.).

2.2. Cell labeling

The stored cells were thawed and cultured under the conditions described above (passage 1: P1). The cells were collected using Trypsin-EDTA at the confluence on day 7, and stained by CellTrace™ CFSE Cell Proliferation Kit (CFSE; Thermo Fisher Scientific Inc.) or the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific Inc.), according to the protocols provided by the supplier. The cells were observed by Leica DM IL (Leica Microsystems K.K.). Viability of cells was examined by NucleoCounter (Chemometec A/S). The labeling of cells was confirmed with BD™ LSR II (Becton, Dickinson and Co.) or BD FACSAria™ Fusion (Becton, Dickinson and Co.). After labeling, the cells were seeded again as P2 and cultured after which they were analyzed or transplanted.

2.3. Flow cytometry for cell surface antigens

On day 4 of the P2 culture, cells stained with CFSE were collected and labeled with allophycocyanin-conjugated antibodies against human epitopes in accordance with the protocol provided by each manufacturer. Epitopes and the manufacturers of antibodies were as follows: CD14, CD90, CD166, STRO-1 (BioLegend Japan Inc.), CD29, TRA-1-60, TRA-1-81 (Affymetrix Inc.), CD44, CD73, CD105, SSEA-4 (Miltenyi Biotec K.K.), CD31, CD45 (Cymbus Biotechnology Ltd.), CD34, SSEA-1 (Sony Biotechnology Inc.). The cells were examined by BD™ LSR II, and collected data were analyzed by BD FACSDiva™ software (Becton, Dickinson and Co.) or FlowJo (Tomy Digital Biology Co., Ltd.).

2.4. Cell sorting

On day 4 of the P2 culture, cells stained with CFSE were collected and sorted using BD FACSAria™ (Becton, Dickinson and Co.). The gates were set to 45 percent (left spaces 10 percent between both sides, rapid and slow group) or 30 percent of both ends in fluorescence histogram. For longer observation, the cells collected on day 7 of the P1 culture were labeled with Violet, and cultured until day 4 of the P2 culture. Then, the cells were sorted using BD FACSAria™ Fusion, with the gates set to 45 percent of both ends in fluorescence histogram. The sorted cells were seeded again, and the cells in P3 were analyzed by flow cytometry.

2.5. Preparation of cartilage regenerative constructs and transplantation

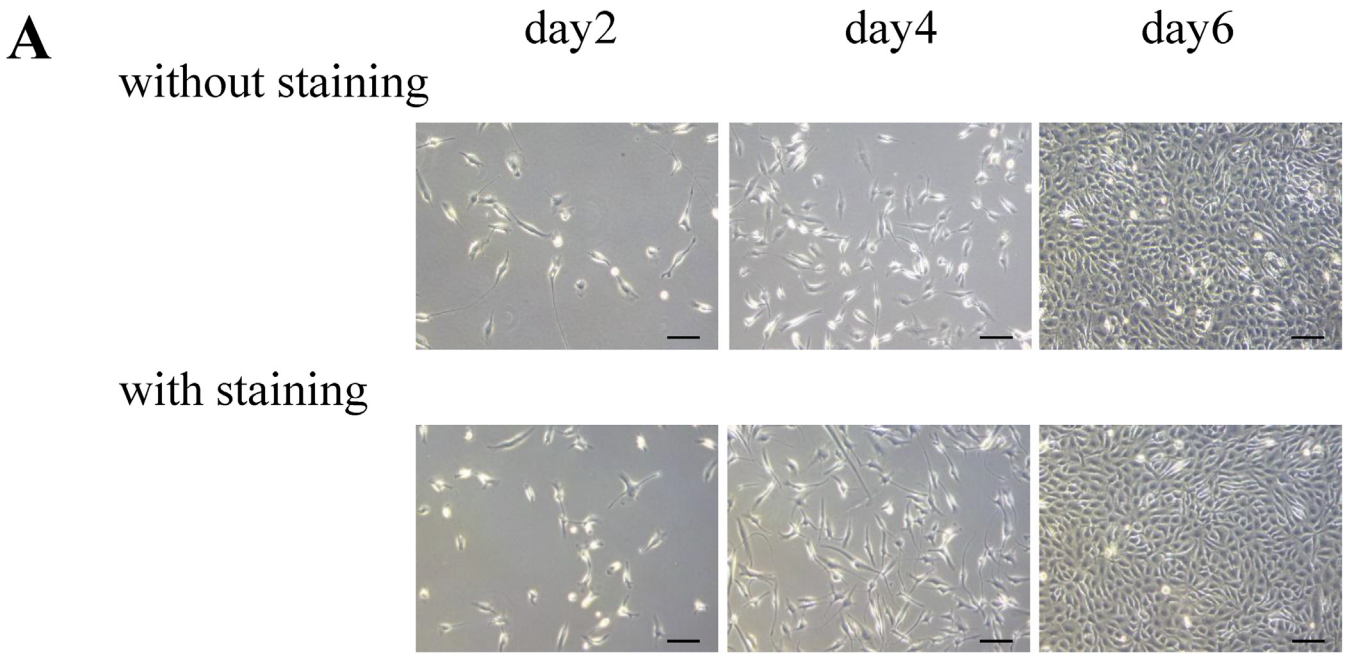
The cells collected on day 7 of the P1 culture were labeled with CFSE, and cultured until day 4 of the P2 culture. Then, the cells were sorted into 2 groups (rapid and slow cells) according to their fluorescence intensities with the gates set to 30 or 45%. The sorted cells were mixed with 1% atelocollagen solution respectively at the concentration of 1×10^7 cells/mL, and 3×10^6 cells/300 μL and were embedded in poly-L-lactic acid scaffolds (PLLA; KRI) ($5 \times 5 \times 3$ mm) to make the cartilage regenerative constructs. After 2 h of incubation, constructs were implanted subcutaneously into 6-week-old male BALB/cA-nu nude mice (Nippon Bio-Supp. Center). The mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Then a small incision was made on the back at the midline, and cartilage regenerative constructs, were implanted subcutaneously. The grafts were harvested 4 and 8 weeks post-implantation.

2.6. Histology

The recovered grafts were split in half by a scalpel, and fixed for 2 h with 4% paraformaldehyde solution. The samples were dehydrated and embedded in paraffin. Sections were sliced to a thickness of 5 μm, and stained with Toluidine blue. Positive area of staining was quantified by the Kompaktes Fluoreszenz-Mikroskop HS-Modellreihe BZ-9000 and BZ-II Analyzer (KEYENCE Corp.).

2.7. Statistics

Data were expressed as mean ± SD, and statistically analyzed using Student's t test. A value of $p < 0.05$ was used to indicate statistical significance.



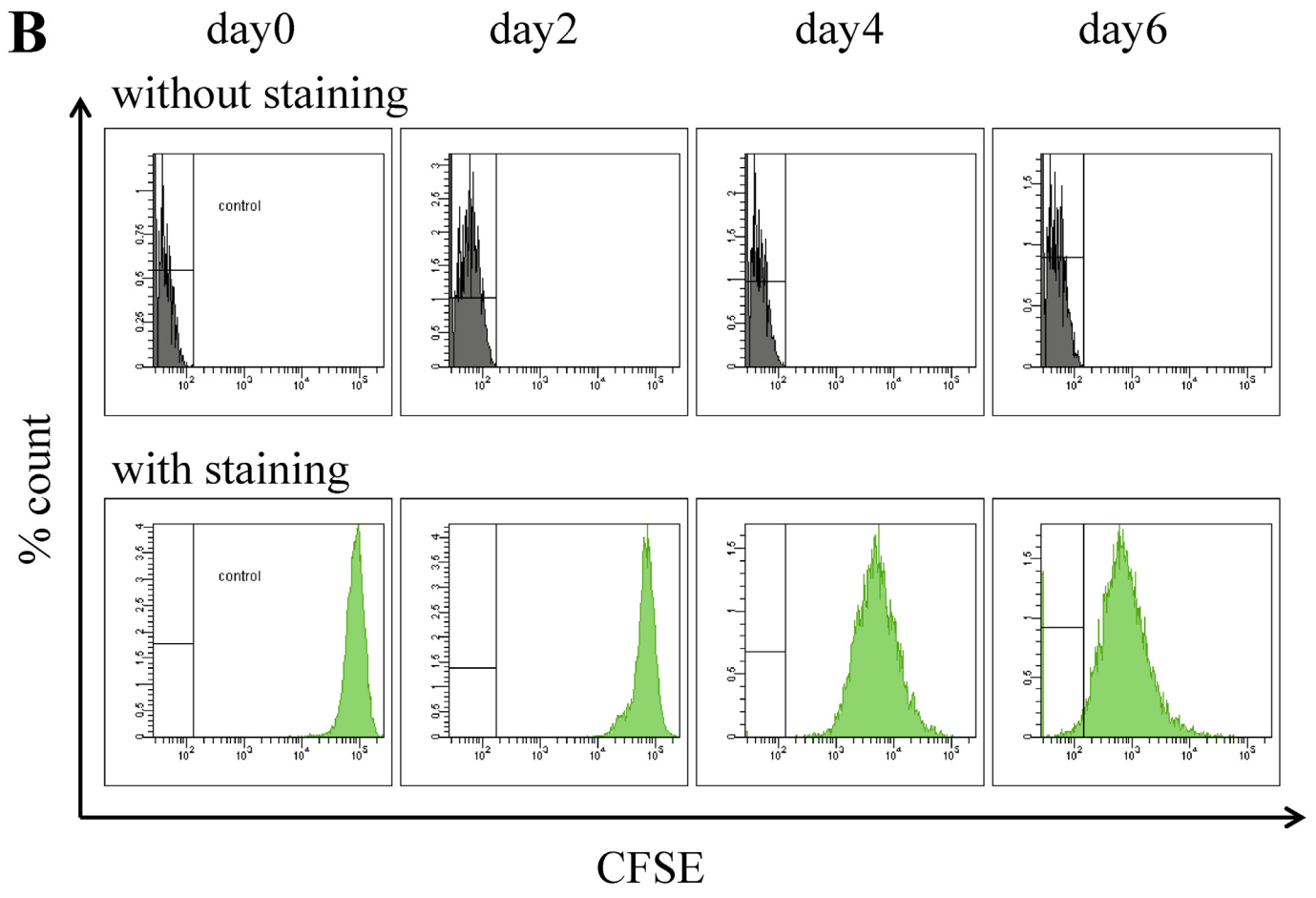
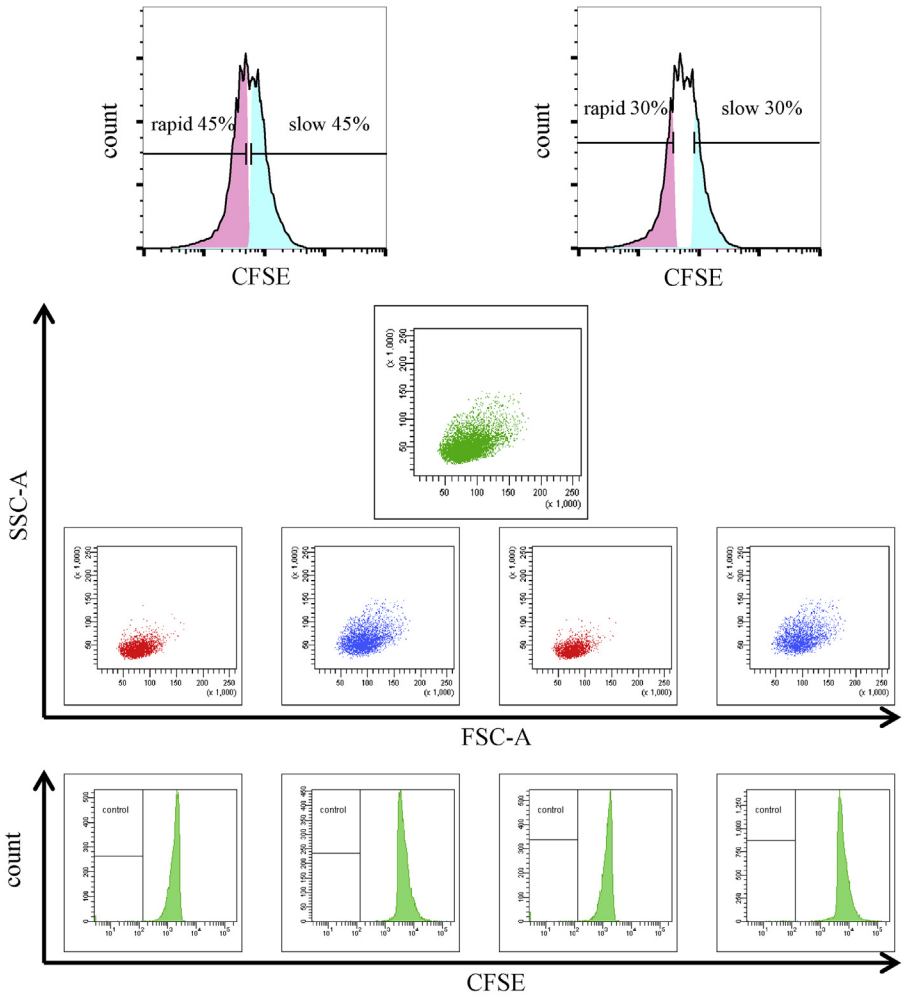


Fig. 1. Cell labeling with CFSE. (A) Phase contrast images of auricular chondrocytes with (lower panels) or without (upper panels) CFSE staining on day 7 of P1 culture. Pictures were taken 2, 4 and 6 days after staining. Sale bar = 100 μm. (B) Flow cytometry analysis of auricular chondrocytes on the day of CFSE staining, and on 2, 4 and 6 days after staining (lower panels). Cells without staining were also analyzed on the same days as negative controls (upper panels). The horizontal line showed intensity of CFSE fluorescence, and the vertical line represented percent of cell number.

A



B

sorting percentage	rapid 45 %	slow 45 %	rapid 30 %	slow 30 %
viability (%)	93.6	94.1	94.4	95.5
SD	3.1	1.6	2.9	1.3

rapid versus slow p>0.05

C

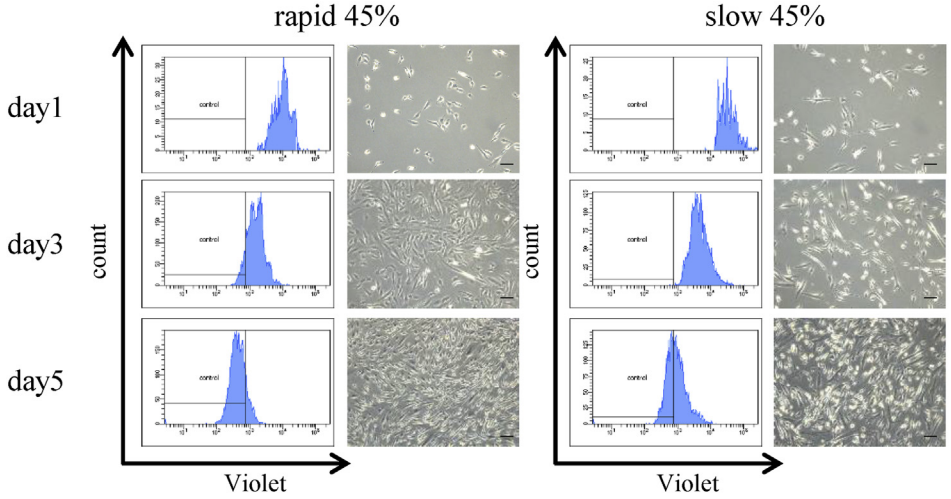


Fig. 2. Sorting of rapid and slow cell groups. (A) Sorting of rapid and slow cell groups according to the intensity of fluorescence. Upper panels: Areas for rapid cell groups and slow cell groups were indicated by pink and blue, respectively. Middle panels: FSC/SSC plot for whole cells (middle upper panel), rapid (45%), slow (45%), rapid (30%) and slow (30%) cell

3. Results

3.1. Fluorescence labeling of auricular chondrocytes with CFSE

To see the diversity of proliferation rate in chondrocytes cultured in monolayer, we first conducted flow cytometry using CFSE. Cell viability was more than 95 percent. Then the cells were cultured and examined by microscopy and flow cytometry until they reached confluence on day 6. Observations using a microscope showed that cells were polygonal-shaped, arranged like cobblestones, and attached to the bottom of the culture dishes in a single layer (Fig. 1A). The cell densities were not seemingly different between cells with and without staining. The fluorescence intensity of CFSE was much stronger than cells without staining, and the shape of the histogram was narrow and sharp (Fig. 1B). This result indicated that cells were dyed uniformly by CFSE fluorescent labeling. Each day during the observation period, the width of the histogram became wider, and the intensity of fluorescence attenuated. These trends were similarly observed in independent 6 lots. The attenuation of fluorescence intensity was most likely due to the dilution of fluorescent dyes by cell proliferation, supported by the fact that the fluorescent labeling seemingly does not affect cell morphology and proliferation by CFSE staining according to the microscopic observation. The widening of the histogram over time indicates the existence of cells with different proliferation rates indicated by the presence of various fluorescence levels.

3.2. Sorting of rapid and slow cell groups

To compare the characteristics of cell groups with different proliferation rates, cells were sorted into 2 groups according to the fluorescence intensity. Specifically, cells plotted in the left and right sides of histogram (30 or 45 percent) were assigned to rapid and slow cell groups, respectively (Fig. 2A). In the forward scatter (FSC)/side scatter (SSC) plotting, the rapid cell group tended to distributed to the left lower part in whole cell population, and this tendency was more remarkable when 30 percent of cells were sorted from both sides of the histogram. Fifteen independent lots were examined and similar results were obtained. It was therefore suggested that the cells in the rapid cell group could be smaller and have more uniform internal structures than those of slow cell group. Precision of sorting was confirmed by secondary flow cytometry, in which the forms of histogram were found to be quite similar to the both sides of original histograms (Fig. 2A). To evaluate the effect of cell sorting to cell death, cell viability after sorting was examined (Fig. 2B). In all groups, cell viabilities were more than 90 percent, and there was no significant difference among the groups. We then examined the change of fluorescence in the sorted cells for a longer period. Because CFSE could not retain its fluorescence long enough for this purpose (data not shown), CellTrace™ Violet, which can be trackable for a longer-term than CFSE, was used to observe the histogram. Both groups gradually lost fluorescence, and the histogram of cells in the rapid cell group approached the intensity displayed by the control group by day 3. The slow cell group took 5 days to reach the same intensity, suggesting that the rapid cell group continued to divide faster, and could maintain their characteristics during cell proliferation. These results showed that both groups were not populations of dead cell or cell debris. Most of the rapid cell group and about the half of the slow cell group were negative for the fluorescence by day 5, indicating that both groups seemed to have done cell division (Fig. 2C).

3.3. Analysis for the correlation of cell surface markers and cell division rate

Stem cells grow rapidly for in vitro [14]. Thus, we hypothesized that stem cells would be included in the rapid cell group. We analyzed the expression of cell surface markers previously reported as stem cells/progenitor cell markers and examined the correlation between the cell surface markers and the cell division rate indicated by fluorescence of CFSE. The following were the candidates of cell surface markers: CD14, CD29, CD34, CD45, CD73, CD90, CD105, CD166, STRO-1 as MSC markers, CD31, CD34, CD45, CD90, CD105 as ASC marker, CD44, CD90 as cartilage stem cells/progenitor cell markers, SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 as ES/iPS markers. As indicated in Fig. 3, none of the markers seemed to match the cell division rate in the chondrocytes on day 4 in the P2 culture. Similar results were obtained from 3 independent batches, indicating consistency.

3.4. The difference of chondrogenic abilities in rapid and slow cell groups

In order to examine whether the chondrogenic abilities were different between rapid and slow cell groups, chondrocytes were fluorescently labeled with CFSE on day 7 of P1 culture, and sorted on day 4 of P2 culture. Cells of rapid cell group (30 or 45 percent) and slow cell group (30 or 45 percent) were seeded to PLLA scaffolds and cartilage regenerative constructs were transplanted to nude mice. The grafts were harvested on 4 and 8 weeks after the transplantation, and histologically examined with toluidine blue staining (Fig. 4A and B). In all grafts, metachromatic region and cartilage lacunae containing the chondrocyte were observed. The grafts harvested 8 weeks after implantation showed greater accumulation of proteoglycan than those of after 4 weeks. In addition, metachromatic regions were larger in the rapid cell group compared to the slow cell group in both weeks, and greater differences were observed in the comparison between both 30 percent-sorting groups. These results indicated higher chondrogenic ability in the rapid cell group.

4. Discussion

This study demonstrated that the rapid cell group, that was separated from the cultured human auricular chondrocyte population, was a good candidate as an alternative cell source for cells that have chondrogenic ability. By using the CFSE, some groups of cultured chondrocytes were classified, based on the proliferation rate (Fig. 1B). The cells in the high proliferation rate group presented structures that were small in size and had uniform internal structure (Fig. 2A). Under the conventional observation by microscopy, cultured chondrocytes looked homogeneous. However, our results suggested that there was a mixture of multiple cell groups with different properties. The signal intensity of CFSE attenuated, as the periods of culture increased, which can be seen in Fig. 1B with the widening of the histogram. Because cell viability was more than 90 percent after the CFSE staining or sorting, the CFSE fluorescent labeling did not seem to deteriorate cell functions, such as proliferation and survival, implying that the decrease in CFSE signal intensity was due to cell division and dilution of CFSE solution. This result indicated that the chondrocytes isolated from native

groups from the left (middle lower panels). Lower panels: Histograms for CSFE fluorescence after sorting for rapid (45%), slow (45%), rapid (30%) and slow (30%) cell groups from the left. (B) Cell viability of each group. Values are mean ± SD. (C) Histograms for Violet fluorescence of rapid (45%) and slow (45%) cell groups analyzed on 1, 3 and 5 days after sorting. Sale bar = 100 μm. The horizontal line of the histogram showed the intensity of CFSE or Violet, while the vertical line showed percent of cell number.

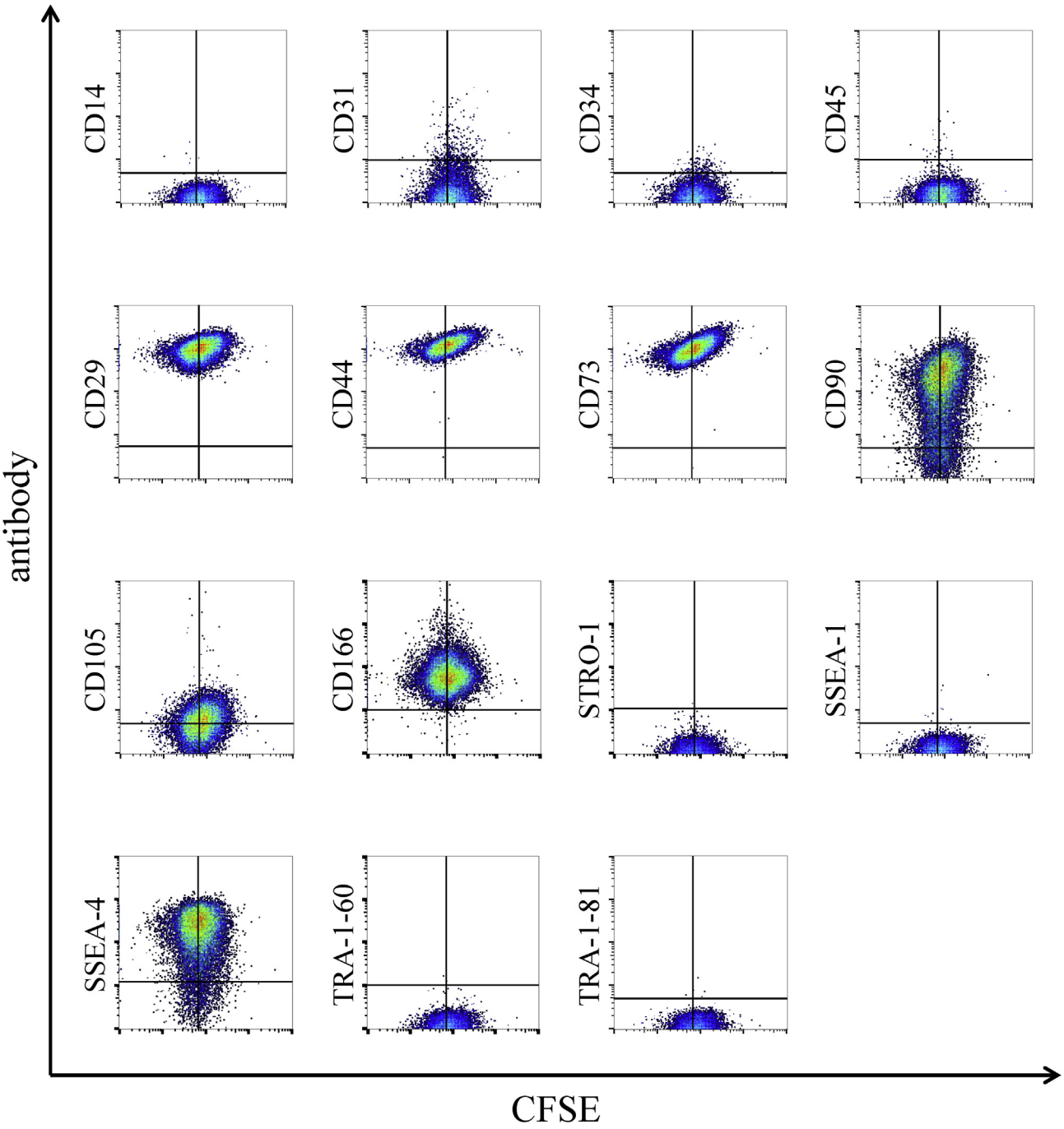


Fig. 3. Flow cytometry analysis of cell surface markers and CFSE fluorescence. Cells were stained on day 7 of P1 culture, and labeled with antibodies against cell surface markers indicated in each panel on day 4 on P2.

cartilage tissue were heterogeneous, containing cells with different proliferation rates. Among them, the rapid cell group showed high production of cartilage matrix (Fig. 4). Our study showed that cartilage made by cells from the rapid group were more than two times larger than those from slow group. One of the speculations why the rapid cell group showed larger areas of regenerative cartilage was that the cells of the rapid cell group may proliferate more rapidly, compared with those of the slow group, even after the transplantation into the body. As another possibility, matrix production per cell was higher in the cells of rapid group, than in those of slow group, implying that the former cells possessed the characteristics resembling the somatic stem/progenitor cells. We could not obtain a clear conclusion in this study, but a pellet culture may be conducted in the future to compare the matrix production between the groups. We will continue to examine whether transplanted cells are somatic stem/progenitor cells or merely highly active chondrocytes. Colony forming ability or pluripotency should be also examined to demonstrate the characteristic of the somatic stem cell in the rapid cell group.

Somatic stem cells possess the ability of self-renewal, and usually stay in a quiescent state. However, once the tissue is injured, they begin to proliferate and supply progenitor cells or differentiated

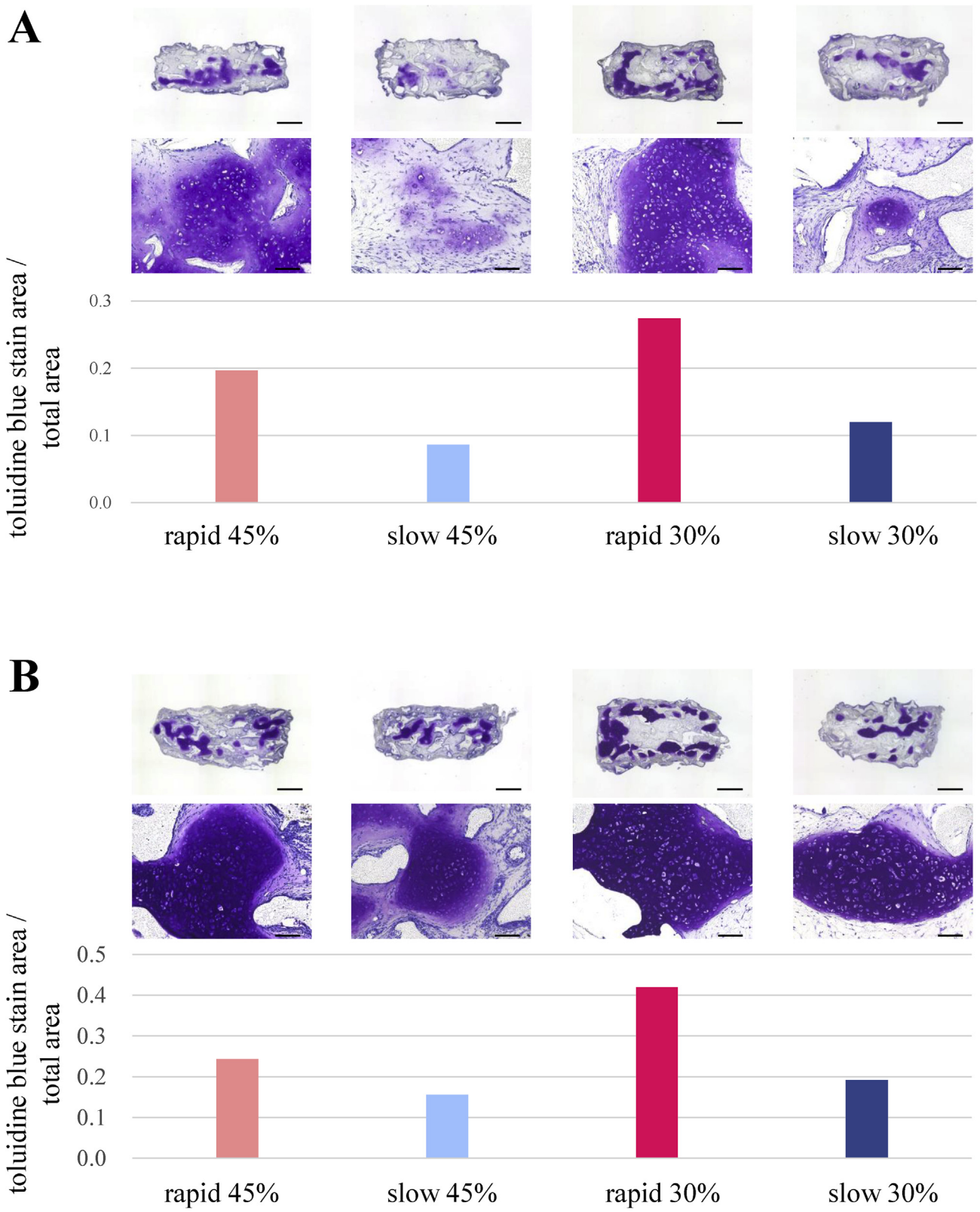


Fig. 4. Histological analysis of cartilage regenerative constructs implanted in nude mice for 4 weeks (A) and 8 weeks (B). Histological slices were prepared and stained with toluidine blue. Upper and middle panels: low and high magnification images of toluidine blue staining of histological sections, respectively. Scale bar = 1 mm (upper panels) and 100 μm (middle panels). Lower panels: quantification of areas positive for metachromasia with toluidine blue staining. Values were divided by those for total areas.

cells, contributing to repair. In the 1960s [15], Becker et al. found the hematopoietic stem cells from the bone marrow, spreading the concept of somatic stem cells. The somatic stem/progenitor cells were isolated from various tissues, such as nerves and fat [16–19], all of which are expected to be taken advantage of as a potential cell source in regenerative medicine. In order to enrich the somatic stem/progenitor cells form heterogeneous cell populations, many studies have been reported thus far. Although most of them have used cell surface markers, specific markers for the cells of rapid cell group could not be detected in our study (Fig. 3). Whether cell surface markers can reflect and be screened for characteristics of somatic stem/progenitor cells is a very important question. Romero [20] and Hongzhe [21] described that positivity of markers depended on the culture environment and passage numbers. The positivity was unstable, and it essentially represented “the cell phenotype at that moment”. In our experiment, we could not obtain a specific surface marker that is expressed in the rapid cell group, which could reflect the chondrocytes with high matrix production.

Although many issues has yet to be discussed, we proposed a new method to enrich the cell population with high matrix production using proliferation rate alone. As was also described above, research about purification or concentration of somatic stem/progenitor cells has been mainly based on cell surface markers. However, the selection by the combination of many markers needs complicated processes and takes ample time. Moreover, a decisively useful marker has not been discovered yet. In our study, we also used a cell sorting process, where we were easily able to obtain a sufficient amount of cells necessary for transplantation that displayed a single strain. Sorting cells using cell proliferation rate as the key variable can be the major step towards clinical application. After sorting, the cells of each group maintained the characteristic of the cell proliferation rate (Fig. 2C). In the future, we will make efforts to establish this expansive method to effectively maintain the characteristics of rapid cell proliferation.

Concentrating numerous chondrocytes with high matrix production has been efficiently produced using our methods. Thus this stable and easy approach may contribute to future development of functional cartilaginous tissue with physiological cartilage characteristics comparable to the mechanical strength and three-dimensional shape of native cartilage. In addition, it would contribute to the development of cartilage regenerative medicine with site-specific and custom-made properties, by 3D manufacturing techniques.

Acknowledgments

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