Original Article

High capacity of purified mesenchymal stem cells for cartilage regeneration

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Mesenchymal stem cells (MSCs) are a heterogeneous population of cells that proliferate and differentiate into bone, cartilage, and fat *in vitro*. Because of this multi-potency, the therapeutic applications of MSCs are under intensive exploration. The most common and redundant method for MSC cultivation requires prolonged culture on plastic dishes. The current study compared the differentiation/proliferative potency of purified mouse MSCs (CD45$^-$/ TER119$^-$/PDGFRα$^+$/ Sca-1$^+$ cells, or PαS cells) with whole bone marrow (WBM)-derived, plastic-adherent MSCs. After three passages, the surface expression levels of CD45, TER119, PDGFRα, and Sca-1 were evaluated in WBM and PαS cells. While PαS cells maintained high expression levels of both PDGFRα and Sca-1, WBM cells exhibited less expressed levels of these stem cell makers. Additionally, WBM cell cultures were frequently contaminated by CD45$^+$ hematopoietic cells. Both cell migration and proliferation were significantly higher in PαS vs. WBM cells, indicating the enhanced differentiation potential of PαS cells for the mesenchymal lineage, and suggesting that WBM cell heterogeneity may regulate and limit the stemness of their MSC progeny. Consistent with this hypothesis, PαS cells transplanted locally at sites of cartilage defects displayed higher cartilage regeneration capacity than WBM cells in a rat osteochondral defect model. This is the first report to demonstrate its improved contribution to cartilage repair *in vivo*. Thus, the protocol employed for MSC isolation is crucial for the effective translation of MSC multi-potency into clinical therapeutics.

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Introduction

The clinical treatment of cartilage injury is still challenging for orthopedic surgeons, because cartilage shows a weak competency for self-repair. Bone marrow (BM) contains both hematopoietic stem cells and non-hematopoietic mesenchymal stem cells (MSCs). These BM-derived MSCs can be isolated from the adult BM, expanded *in vitro*, and differentiated into several types of specialized cells, including osteocytes, chondrocytes, and adipocytes[1]. The most commonly used isolation technique for MSCs requires a prolonged period of culture on plastic dishes. However, MSCs expanded in this manner gradually undergo differentiation, exhibiting altered morphological features and functions from the parent stem cells. The differentiation process alters the nature of immature MSCs, making it difficult to identify and study their *in vivo* functional characteristics. Currently, several clinical studies have been performed with MSCs without even understanding their role in tissue regeneration.

Articular cartilage covers the ends of the bone and provides a flexible connective tissue, and is composed of extracellular matrix molecules, ground substance, and chondrocytes[2]. Osteoarthritis occurs following the loss of articular cartilage, leading to boney injury with pain, stiffness, and loss of movement[3]. Osteoarthritis is the most common cause of arthritis worldwide and engenders a significant burden on health care budgets[4]. Due to the avascular nature of cartilage, there is little hope for spontaneous self-repair, and joint replacement is therefore a major option for osteoarthritis patients.

In vivo studies using rabbits and mice demonstrated that MSC aggregates can promptly adhere to osteochondral defects and thereafter promote the regeneration of cartilage[5, 6]. This methodology may theoretically be incorporated into a medical treatment for osteoarthritis patients. MSCs hold significant promise for cartilage repair, and various ongoing trials are investigating their ability to improve osteoarthritis outcomes. Intuitively, it is important to isolate the most potent subpopulation of MSCs for use in regenerative strategies, including those intended for cartilage repair. Although cells isolated by the plastic-adherence protocol lose much of their stemness, prospectively-isolated, purified mouse MSCs (i.e., cluster of differentiation 45 $(CD45)^-$/erythroid cell marker, Ly76 $(TER119)^-$/platelet-derived growth factor receptor α $(PDGFR\alpha)^+$/stem cell antigen-1 $(Sca\text{-}1)^+$ cells, or PαS cells) have recently garnered attention due to their significantly enhanced colony-forming unit-fibroblast (CFU-F) ability[7, 8]. PαS cells also differentiate more efficiently into adipocytes, osteocytes, and chondrocytes relative to $PDGFR\alpha^+$ or $Sca\text{-}1^+$ single-positive cells *in vitro*. Given the large number of researchers performing mouse MSC investigations by using PαS cells, PDGFRα and Sca-1 are becoming the standard markers for the identification of MSCs, especially when they are co-expressed[9-11]. However, it still remains unexplained the regenerative effects of the PaS cells on tissue defect model.

The application of MSCs to cartilage regeneration is one of the more advanced fields in MSCs clinical study. In the present study for the first time, we compared the differentiation and proliferative potency of mouse PαS cells with whole BM (WBM)-derived MSCs *in vitro*. We then separately transplanted the mouse PαS and WBM cells into an osteochondral defect in rats and monitored the chondrogenic differentiation of the cells *in vivo*. Our results indicate that purified MSCs have enhanced therapeutic potential for cartilage repair. This protocol employed for MSC isolation is crucial for the effective translation of MSC multi-potency into clinical therapeutics.

Materials and methods

1)Animals

Adult C57BL/6-Tg (cytomegalovirus enhancer (CAG)-enhanced green fluorescent protein (EGFP)) mice at 6-8 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan). LEW/CrlCrlj rats at 10-12 weeks of age were purchased from Japan Charles River (Yokohama, Japan). Animal care was in strict accordance with the guidelines of the Animal Care and Use Committee of Tokyo Medical and Dental University (Tokyo, Japan).

2)Isolation and culture of mouse MSCs

Femurs, tibias, and ilia were dissected from mice and crushed with a muddler. The fragments were gently washed once in HBSS+ solution containing 2% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 10 mM HEPES (Wako Pure Chemicals, Ltd., Osaka, Japan), and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY, USA). The washed fragments were filtered through a Falcon™ 70-μm cell strainer (BD, Franklin Lakes, NJ, USA), and the filtrate was discarded. The bone fragments were collected into Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) containing 0.2% collagenase (Wako), 10 mM HEPES, 1% penicillin/streptomycin, and 25 U/mL DNase1 (Sigma

Chemical Co., St. Louis, MO, USA). The mixture was incubated for 1 h at 37°C with shaking. The suspension was filtered through a 70-μm cell strainer to remove the bone fragments and centrifuged at 280 × g for 7 min. The pellet was mechanically disrupted, and sterile water (1 mL) was added for 6 sec. Next, 2× phosphate buffered saline (PBS) containing 4% FBS was added, and the mixture was filtered through a 70-μm cell strainer and centrifuged at 280 × g for 7 min. The pellet was then used for antibody staining, as described below.

For antibody-mediated isolation of PαS cells, the cell pellet was diluted in HBSS+ and stained for 30 min on ice with the following mouse primary antibodies: anti-phycoerythrin (PE)-conjugated Sca-1 and anti-PE-conjugated CD31, anti-PE-Cy7-conjugated CD45, anti-PE-Cy7-conjugated TER119, and anti-allophycocyanin (APC)-conjugated PDGFRα (eBioscience, San Diego, CA, USA). For cell surface marker analysis, we used anti-PE-conjugated CD31. Flow cytometry and cell sorting were performed by using a MoFlo instrument (Beckman Coulter, Miami, FL, USA). Propidium iodide (PI) fluorescence was used for gating dead cells. Sorted cells were plated at a density of 3000 cells per dish in non-coated 10-cm culture dishes and incubated at 37°C in MSC media containing DMEM-GulutaMAX (Life Technologies), 20% FBS, 20 ng/mL basic fibroblast growth factor (ReproCELL, Kanagawa, Japan), and 1% penicillin/streptomycin.

3)Multi-lineage differentiation of WBM and PαS cells

For adipogenic induction, 1.0×10^4 cells at passage 3 were plated into 24-well plates with adipogenic induction media (Lonza, Walkersville, MD, USA) and incubated at 37°C in an atmosphere containing 5% humidified CO_2. For osteogenic induction, 0.7×10^4 cells at passage 3 were plated into 24-well plates with osteogenic induction media (Lonza) and incubated at 37°C with 5% humidified CO_2. Both adipogenically- and osteogenically-differentiated cells were cultured for 2 weeks. For the chondrogenesis assay, 5.0×10^5 cells at passage 3 were placed into a 15-mL polypropylene tube (Nalge Nunc International, Rochester, NY, USA) and centrifuged at 200 × g for 4 min. The pellet was cultured in chondrogenic induction media (Lonza) containing 500 ng/mL bone morphogenic protein (BMP) 6 plus 10 ng/mL transforming growth factor beta 3 (TGFβ3; R&D Systems, Minneapolis, MN, USA) and incubated at 37°C with 5% humidified CO_2 for 4 weeks.

4)Quantitative analysis of *in vitro* cell differentiation

Adipogenically-differentiated cells were stained with Oil red O (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) and extracted by using 2-propanol (Wako). The absorbance at 510 nm was then measured. Osteogenically-differentiated cells were stained with alizarin red (Millipore, Billerica, MA, USA) and extracted by using 10% acetic acid. The absorbance at 405 nm was then determined. Chondrogenically-differentiated cell pellets were embedded in paraffin and cut into 4-μm-thick sections. The paraffin-embedded sections were then double-stained with primary mouse anti-chick collagen 2 (Col2) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and 4',6-diamidino-2-phenylindole (DAPI).

5)Immunofluorescence staining

The anti-Col2 antibody was employed for immuno-fluorescence staining of MSC aggregates. After fixation in 4% paraformaldehyde (Wako), cells on slides were treated with hyaluronidase for 1 h at 37°C and blocked with Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 30 min at room temperature. Next, the slides were incubated with primary antibody at a 1:100 dilution in 2 mg/ml hyaluronidase solution overnight at 4°C, followed by an Alexa Fluor-conjugated anti-mouse immunoglobulin G (IgG) (Invitrogen, Carlsbad, CA, USA) at a 1:500 dilution for 30 min at room temperature. Slides were mounted with Vector shield (Vector Laboratories, Burlingame, CA, USA) containing DAPI and examined on a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Biberach, Germany).

6)Migration assay

To analyze the proliferative potential of MSC donor cells, MSC aggregates were plated into 24-well plates, and colony size was measured each day. Cell division was analyzed by staining with an antibody against Ki67 (BD), a marker of cell proliferation.

7)MSC transplantation into rats

Mouse MSCs at passage 3 were cultured in hanging drops (25×10^4 cells/drop) with MSC media and cultured for 3 days at 37°C with 5% humidified CO_2. Under isoflurane anesthesia, the rat knee joint was incised, and the patella was dislocated. An osteochondral defect (1.8 mm in diameter) was created in the medial condyle of the femur. Four MSC aggregates (100×10^4 cells) were inserted into

Table 1 Primer sequences for quantitative RT-PCR

Target		Sequence
HPRT	Forward	5' TCAGTCAACGGGGGACATAAA 3'
	Reverse	5' GGGGCTGTACTGCTTAACCAG 3'
TGFb3	Forward	5' CCCTGGACACCAATTACTGC 3'
	Reverse	5' TCAATATAAAGGGGGCGTACA 3'
BMP6	Forward	5' AACCGGTCCACCCAGTCGCA 3'
	Reverse	5' TCCATcACAGTAGTTGGCAGC 3'
MMP13	Forward	5' CAGTCTCCGAGGAGAAACTATGA 3'
	Reverse	5' GGACTTTGTCAAAAAGAGCTCAG 3'

the defect. The defect was left empty in control animals. The patella was then relocated, and the wound was closed. Rats were sacrificed at 4 or 8 weeks after the operation.

8)Histological examination

The dissected femurs were immediately embedded in Super Cryo Embedding Medium (Section-lab, Co., Ltd., Hiroshima, Japan) and frozen with dry ice and hexane. The tissue block was cut into 12-μm-thick sections, fixed in a 4% paraformaldehyde solution, and washed in PBS. The frozen sections were stained 1 with anti-Col2 antibody.

9)RT-PCR

Total RNA was prepared with TRI reagent (Sigma-Aldrich). The quantitative RT-PCR was performed using the StepOne real-time PCR system (Life Technologies). cDNA was amplified with 50 PCR cycles at 95°C for 3 seconds, 60°C for 30 seconds using FAST SYBR Green Master Mix (Life Technologies) and gene-specific primers (Table 1).

10)Statistical analysis

Quantitative data are presented as the means ± the standard deviation (SD) from at least 3 representative experiments. For statistical analysis, the data were evaluated with Student's *t*-test. In all cases, *p*-values of < 0.05 were considered significant.

Results

1)Isolation and characterization of WBM and PαS cells

Mouse WBM was stained with antibodies against the following antigens: CD45, Ter119, PDGFRα, and Sca-1. Flow cytometry analysis demonstrated the presence of PDGFRα and Sca-1 in a subpopulation of cells and allowed the isolation of PDGFRα$^+$Sca-1$^+$ PαS cells and all living

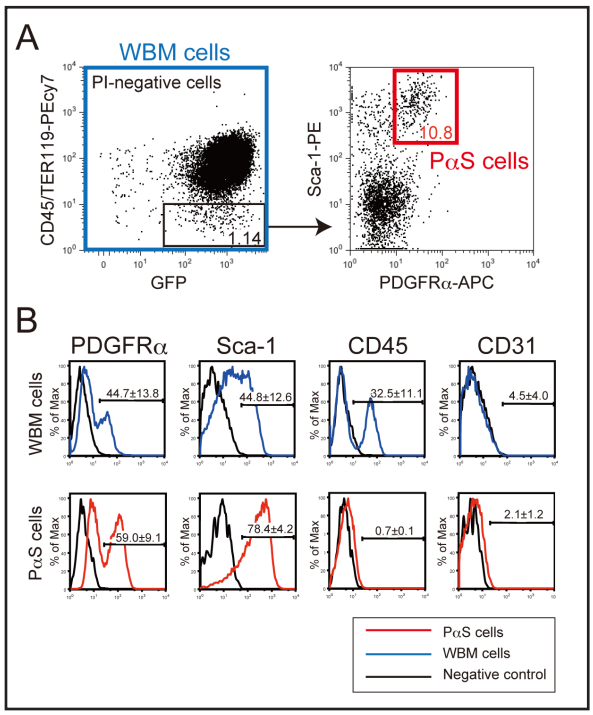

Fig.1 Characterization of mouse MSCs derived from WBM cells and the prospectively-isolated PDGFRα$^+$Sca-1$^+$ cell fraction

(A) Representative flow cytometric profiles of the PI-negative WBM cell population (WBM: blue gated) and the CD45$^-$Terr119$^-$ PDGFRα$^+$Sca-1$^+$ cell population (PαS: red gated) derived from CAG-EGFP mice. Numbers adjacent to the outlined areas indicate the percentage of gated cells in total WBM mononuclear cells.

(B) Surface marker expression in WBM and PαS cells after three passages. Unlabeled cells (negative control) are included for comparison. Numbers indicate the means ± the SEM.

PI$^-$ (WBM) cells (Fig. 1A). Analysis of surface marker in both populations in culture (passage 3) demonstrated that PαS maintain expression of PDGFRα and Sca-1 makers in contrast to WBM cells (Fig. 1B). Cultured WBM-MSCs contained a substantial percentage of contaminating CD45$^+$ hematopoietic cells (32.5 ± 11.1%).

2)Multi-lineage potential differences between WBM and PαS cells

After three passages, PαS and WBM cells were transferred into an adipogenic induction medium. Both cell types adopted a rounded shape and showed evidence of lipid vesicle formation after 14 days in differentiation medium and staining with Oil red O (Fig. 2A). Adipocyte generation was quantitatively analyzed via absorption spectrometry analysis

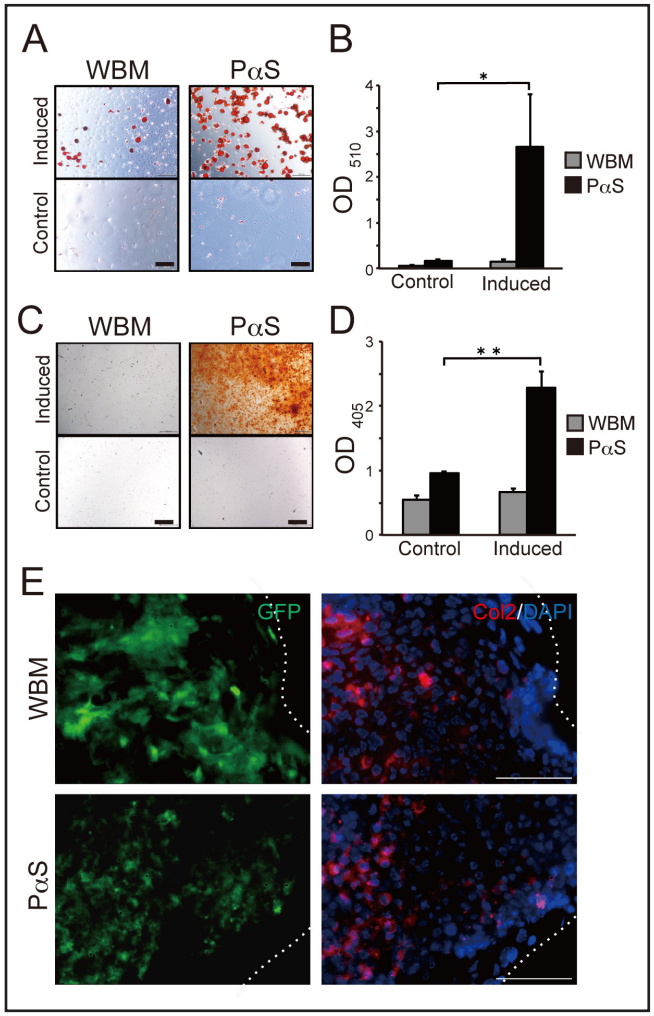

Fig.2 PαS-MSCs show high differentiation potency for the mesenchymal lineage

(A, B) Adipogenically-differentiated WBM and PαS cells were stained with Oil red O, and adipocyte formation was analyzed by using absorption spectrometry at 510 nm.

(C, D) Osteogenically-differentiated WBM and PαS cells were stained with alizarin red, and osteoblast formation was analyzed by absorption 30 spectrometry at 405 nm.

(E) Chondrogenically differentiated WBM and PαS cells were immunofluorescently stained with an antibody against Col2.

The scale bar is 500 μm in (A) and (C), 15 μm in (E). Data in (B) and (D) indicate the means ± the SEM. *p<0.05, **p<0.01.

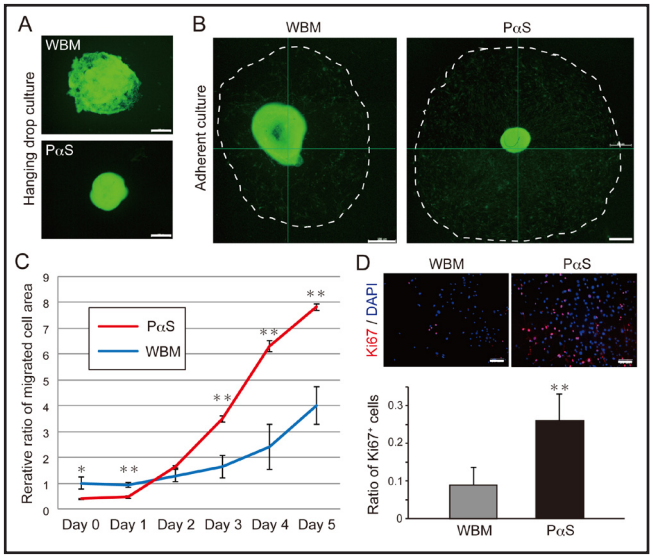

Fig.3 PαS cells have high growth capacity *in vitro*

(A) WBM and PαS sphere were cultured by the hanging drop method for 3 days and visualized under a fluorescence microscope.

(B) GFP+ 8 MSCs derived from hanging drop culture were grown on glass slides for 5 days. Representative neurosphere cultures are shown in the four panels. The scale bar is 500 μm in A and B, and 50 μm in D.

(C) The radii of the aggregates were measured every day for 5 days (blue line, WBM cells; red line, PαS cells).

(D) Double fluorescence staining with anti-Ki67 antibody (red) and DAPI (blue). The ratio of the Ki67+ area to the DAPI+ area is shown.

Data in (C) and (D) are given as the means ± the SEM (n=3, p<0.01).

at 510 nm of Oil red O-stained cells, revealing a significantly higher adipogenic differentiation potential for PαS vs. WBM cells (Fig. 2B). Osteogenic differentiation potential, as assessed by alizarin red staining, was also enhanced in PαS vs. WBM cells (Fig. 2C). Spectrometric quantification of the alizarin red-stained cells at 405 nm revealed 2.3-fold higher staining intensity for the PαS cells (Fig. 2D). Lastly, chondrogenic differentiation was analyzed by sectioning and staining of cell pellets with anti-Col2 antibody. PαS and WBM cells had differentiation ability to chondrocyte at the same level (Fig. 2E). Taken together, these results suggest that PαS cells have an augmented differentiation potential for the mesenchymal lineage relative to WBM-derived MSCs.

3)Cell migration and proliferation *in vitro*

Cell migration and proliferation ability are important for the proper functioning of MSCs. A cell migration assay revealed significantly greater motility for PαS-derived spheres compared with WBM-MSC-derived spheres (Fig. 3A), with greater spreading on plastic dish (Fig. 3B). No difference was found for cell motility between the groups within 3 days of culture. After 5 days in culture, cell migration was significantly increased in PαS cells (2-fold) (Fig. 3C). Ki67 (proliferation marker) immunohistological staining indicated a higher proliferation in PαS cells compared with WBM cells (Fig. 3D).

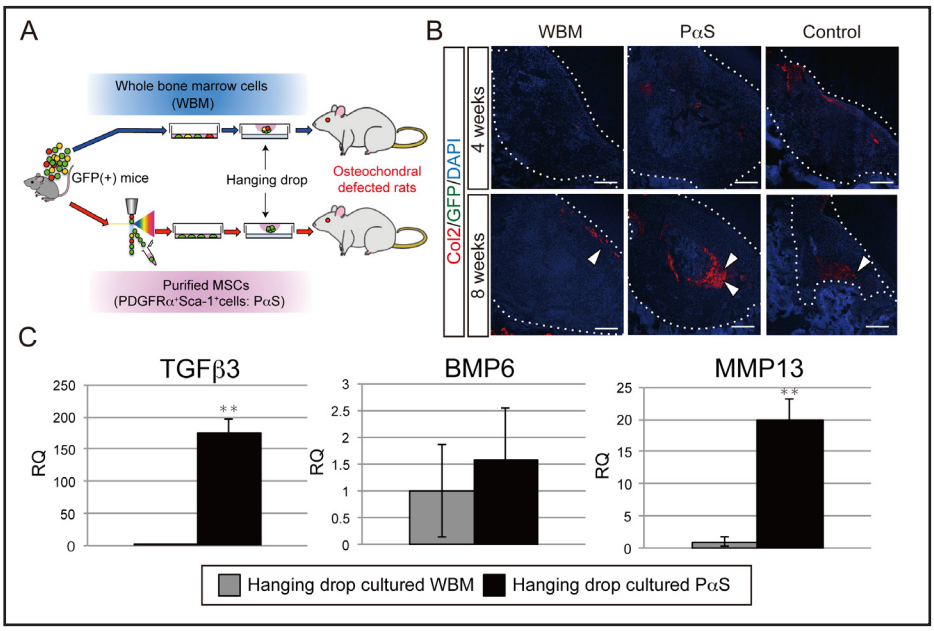

Fig.4 PαS cells promote cartilage reconstitution *in vivo*

(A)Scheme of experiment MSCs were transplanted into a rat cartilage defect model.
(B) Double fluorescence staining is shown for the chondrogenic marker, Col2 (red), and GFP (green). Chondrocyte-like cells are indicated by the white arrowheads. The scale bar is 250 μm.
(C) Quantitative analysis of mRNA expression of TGFβ3, BMP6, and MMP13 of hanging drop cultured WBM-MSCs and hanging drop cultured PαS-MSCs. The mRNA expression of each gene was normalized using hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression. The gene expression of hanging drop cultured WBM-MSCs was set as 1.0. $^{**}p<0.01$.

4)Cartilage regenerating effects of MSCs *in vivo*

To analyze the regenerative potential of PαS and WBM cells *in vivo*, we separately transplanted both cell types into a rat cartilage defect model (Fig. 4A). Cultured MSCs (100 × 10^4 cells in four aggregates) were transplanted into each defect, and the wounds were surgically closed. After 4 or 8 weeks, the cartilage was surgically removed and analyzed by immunohistochemistry. Immunohistochemical staining with Col2 antibody demonstrated that PαS MSCs generated markedly improved cartilage relative to WBM-MSCs *in vivo*, especially at 8 weeks after transplantation (Fig. 4B). These findings are strongly indicative of PαS cell-promoted cartilage regeneration *in vivo*. Nonetheless, we failed to identify GFP^+ cells in the defect site, even though the stem cells were derived from CAG-EGFP mice. It is likely that PaS-MSCs exert a paracrine effect on surrounding tissues to stimulate chondrogenic regeneration. We quantitatively analyzed mRNA expression of the endogenous cytokines in hanging drop cultured WBM-MSCs and hanging drop cultured PaS-MSCs (Fig.4C). PaS-MSCs express significantly higher TGFβ3 and matrix metalloproteinase 13 (MMP13), and somewhat higher BMP6 than WBM-MSCs.

Discussion

This study compared the basic properties and therapeutic aptitude of prospectively-isolated vs. plastic-adherent MSCs. Our data showed that prospectively-isolated MSCs exhibit enhanced growth, differentiation, and regenerative potential. These data are not altogether unexpected. Although flushed WBM undoubtedly contains immature MSCs, we previously demonstrated that other cell populations or “contaminating cells” can dramatically affect the fundamental characteristics of human MSCs in culture[12]. These contaminating actions appear to be independent of cell density. In this report, we also showed that an unpurified population of mouse WBM-MSCs expresses lower levels of the PDGFRα and Sca-1 antigens than PαS cells, and also contains $CD45^+$ hematopoietic cells. By contrast, prospectively-isolated MSCs are free from contaminating cells, revealing the true therapeutic potential of these multi-potent stem cells.

Traditional MSC isolation by plastic adherence requires prolonged time in culture to exclude contaminating cells.

Unfortunately, this culture step diminishes the "stemness" of MSCs[13]. The optimal culture conditions to expand MSCs while maintaining their undifferentiated state has not yet been established. Previous work indicated that aggregation of MSCs into three-dimensional spheroids enhances their stem cell potency, especially their CFU-F capacity and differentiation ability[14]. The current data showed that PαS cell aggregates can spread to a greater extent *in vitro* than WBM-MSC aggregates, and also have greater proliferative capacity *in vitro* and chondrogenic regenerative potency *in vivo*. Possible explanations for these findings include the negative effect of contaminating cell populations on MSC properties and the presence of additional plastic-adherent cells (e.g., fibroblasts and macrophages) that lack stem cell-like properties.

Although the transplanted PαS cells significantly improved chondrogenic regeneration in the present animal model, we could not identify GFP^+ transplanted cells in the defect area. Therefore, the difference in cartilage regeneration between PαS cells and WBM-MSCs cannot merely be explained by long-term engraftment. Indeed, previous data demonstrated that the number of transplanted MSCs diminishes with time after transplantation[15]. It is likely that PαS-MSCs exert a paracrine effect on surrounding tissues to stimulate the chondrogenic regeneration when we transplanted the cell. TGFβ and BMP6 are necessary for chondrogenic differentiation[16-20], and MMP13 is important for wound healing[21]. If Elevation of TGFβ3, BMP6, and MMP13 may therefore be a possible mechanism by which PαS cells mediate their pro-chondrogenic effects.

In conclusion, our findings imply that transplanting purified MSCs might improve chondrogenic regeneration via the production growth factors, although this hypothesis requires further investigation. In clinical applications, evaluation of the production of autocrine and paracrine factors by transplanted stem cells is essential to long-term success, because the therapeutic efficacy of MSCs depends on both autocrine and paracrine molecular and cellular events. Overall, however, our work shows that prospectively-isolated MSCs, which are free of contaminating cell populations, have enhanced stemness and augmented capacity for bone repair relative to plastic-adherent, WBM-derived MSCs.

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Competing interests

The authors declare that they have no competing financial interests in association with this manuscript.

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