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Characterization of adipose tissue-derived stromal vascular fraction for clinical application to cartilage regeneration

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Abstract Bone marrow concentration (BMC) is the most recognized procedure to prepare mesenchymal stem cells for cartilage regeneration. However, bone marrow aspiration is highly invasive and results in low stem cell numbers. Recently, adipose tissue-derived stromal vascular fraction (AT-SVF) was studied as an alternate source of stem cells for cartilage regeneration. However, AT-SVF is not fully characterized in terms of functional equivalence to BMC. Therefore, in this study, we characterized AT-SVF and assessed its suitability as a one-step surgical procedure for cartilage regeneration, as an alternative to BMC. AT-SVF contained approximately sixfold less nucleated cells than BMC. However, adherent cells in AT-SVF were fourfold greater than BMC. Additionally, the colony-forming unit frequency of AT-SVF was higher than that of BMC, at 0.5 and 0.01%, respectively. The mesenchymal stem cell (MSC) population (CD45−CD31−CD90+CD105+) was 4.28% in AT-SVF and 0.42% in BMC, and the adipose-derived stromal cell (ASC) population (CD34+CD31−CD146−) was 32% in AT-SVF and 0.16% in BMC. In vitro chondrogenesis demonstrated that micromass was not formed in BMC, whereas it was clearly formed in AT-SVF. Taken together, uncultured AT-SVF could be used in one-step surgery for cartilage regeneration as a substitute for BMC.

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Introduction

Articular cartilage has limited healing potential (Hunziker 2002), and damaged articular cartilage cannot heal naturally. For cartilage regeneration, surgical approaches such as microfracture or autologous chondrocyte transplantation (ACT) have been developed (Brittberg *et al.* 1994; Steadman *et al.* 2001; Bartlett *et al.* 2005; Bartha *et al.* 2006). However, these therapies have shown insufficient efficacy or drawbacks in clinical use. Microfracture, a marrow stimulation technique which induces migration of subchondral bone-derived mesenchymal stem cells (MSCs) via an inflammatory reaction, often results in fibrocartilage and not hyaline cartilage (Murawski *et al.* 2010). ACT has been used recently due to its effectiveness, but requires expensive and time-consuming ex vivo cell expansion (Brittberg 1999). Due to the above reasons, stem cells that can differentiate into specialized cells and form various tissues including cartilage have been considered as a potential cell source for cell-based cartilage regeneration. Currently, stem cell therapy is considered the most effective approach for cartilage regeneration.

Mesenchymal stem cells (MSCs) from bone marrow have been widely used as a source of stem cells. However, bone marrow aspiration must be concentrated for clinical use as the quantity of MSCs is small (Pittenger *et al.* 1999). Bone marrow concentration (BMC) has been developed in response and proven effective for cartilage regeneration; however, BMC has intrinsic drawbacks in clinical applications (Fortier *et al.* 2010) such as painful aspiration and the cost of concentration (Hermann *et al.* 2008). Various other tissues have been

examined to determine an alternate source of MSCs, including adipose tissue, which is known to be abundant in adult stem cells. Furthermore, preparation of adipose tissue-derived stromal cells (ASCs) is easier and less expensive than that of bone marrow-derived MSCs (bMSCs) (Zuk *et al.* 2001). Cultured ASCs showed a similar function to bMSCs in multi-lineage differentiation (Zuk *et al.* 2002; Kokai *et al.* 2013), and it was recently suggested that uncultured adipose tissue-derived stromal fraction (AT-SVF) can be applied as a one-step surgical procedure for cartilage regeneration. In patients with knee osteoarthritis (OA), uncultured AT-SVF treatment improved function and reduced pain (Koh and Choi 2012; Koh *et al.* 2013). In addition, it has been suggested that AT-SVF can be a useful therapeutic option for chronic ischemic heart disease and skin regeneration (Premaratne *et al.* 2011; Leblanc *et al.* 2012; Sheng *et al.* 2013; Atalay *et al.* 2014).

Uncultured AT-SVF has been utilized clinically for cartilage regeneration, though it has not been fully characterized and compared with BMC in detail. The effect of non-MSC populations in uncultured AT-SVF on cartilage regeneration also needs to be investigated before clinical use. In this study, we characterized uncultured AT-SVF and compared it to BMC. Finally, we evaluated the suitability of uncultured AT-SVF for clinical applications of cartilage regeneration.

Materials and Methods

Tissue sample preparation. Human buttock fat (n=22, age; 47–60 yr, median age; 56.5 yr) was aspirated by gentle suction as previously described (Klein 1990; Khan 2012), and bone marrow (n=6, age; 47–55 yr, median age; 53.6 yr) was obtained under general anesthesia. Informed consent from all patients and approval of the Institutional Review Board (IRB) were obtained. Routinely collected liposuctioned adipose tissue (120 mL) and bone marrow (60 mL) were subjected to laboratory analyses. BMC was concentrated using the Marrowstim™ Concentrate Kit (Biomet Biologics, Warsaw, IN) according to the manufacturer's instructions.

Cell isolation and cell counting. Adipose tissues were prepared as previously described (English *et al.* 2007; Koh and Choi 2012). Adipose tissue was excised from donor buttocks, digested using 0.1% collagenase (collagenase type I; Worthington Biochemical, Lakewood, NJ) at 37°C with continuous agitation for 3 h. Floating adipocytes were removed after centrifugation (450×g) for 3 min. The pellet was washed three times to remove any remaining collagenase. Before the last round of centrifugation, the pellet was filtered through 70-μm nylon cell strainers (BD Biosciences, Bedford, MA). The pellet was resuspended in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS; Gibco), 100 IU penicillin (P), and 100 μg/mL streptomycin (S). The resulting SVF cell pellet was treated with red blood cell lysis buffer (ACK lysis buffer; Gibco), washed with PBS, and then resuspended in DMEM. The BMC were also treated with ACK lysis buffer, washed with PBS, and resuspended in DMEM. The total number of AT-SVF and BMC-nucleated cells was determined using a Cellometer Auto T4 system (Nexcelom Bioscience LLC, Lawrence, MA). Freshly isolated cells from each sample were resuspended in 4 mL of media (volume for a single BMC treatment) and then counted. In order to confirm the number of stem cells, some of isolated cells were plated in 100-mm culture dishes (passage 0; P0) with growth media (DMEM supplemented with 10% FBS, 1% P/S) and cultured in a humidified 5% CO_2 incubator at 37°C. After 3 d, adherent cells were counted.

Flow cytometry. Human anti-CD34 (FITC), CD31 (PE), CD146 (APC), CD90 (APC-vio770), CD45 (PerCP), and isotype control antibodies were purchased from MACS (MiltenyiBiotec. Auburn, CA). Human anti-CD105 (PE/Cy7) antibody was purchased from Biolegend (San Diego, CA). Freshly isolated cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% BSA and 0.05% sodium azide) and cells were resuspended in 50 μL of FACS buffer followed by antibody incubation in the dark for 30 min at 4°C. Cells were washed with FACS buffer, centrifuged at 300×g for 10 min at 4°C, and resuspended in 300 μL of FACS buffer. Stained samples were analyzed using FACS BD FACSVerse (BD Bioscience, San Jose, CA). Isotype and positive controls stained with a single antibody were used to set the lower limit of positive fluorescence and compensation for spectral overlap of fluorochromes. Data based on 20,000–100,000 events were acquired from each sample and analyzed using BD FACSuite software (BD Bioscience).

Colony forming unit assay. Colony-forming unit assay for MSCs was performed with AT-SVF and BMC as described previously (Yoon *et al.* 2011). In brief, freshly prepared or cultured cells from AT-SVF and BMC were resuspended in growth medium (DMEM supplemented with 20% FBS and1% P/S), and plated in 100-mm dishes at densities of 1×10^4 for P0 or 1×10^3 for P1 in triplicate. The medium was changed every 3–4 d. Twelve days after plating, cells were stained with 3% crystal violet (Sigma, St. Louis, MO) in methanol. The frequency of ASC or MSC within AT-SVF or BMC was expressed as a percentage of seeded cells.

Multi-lineage differentiation capacity. For osteogenesis, cells were plated at a seeding density of 3.2×10^4 cells per well in 12-well plates with DMEM media (10% FBS, 1% P/S) for 24 h. As uncultured AT-SVF and BMC are heterogeneous

mixtures of cells, the contribution of stem cells is relatively low. To add similar numbers of stem cells to culture wells, AT-SVF and BMC cells were plated at fourfold higher densities than cultured bMSCs for in vitro differentiation. Cells were incubated in basic medium containing 100 nM of dexamethasone (Sigma), 50 μM of ascorbic acid-2-phosphate (Gibco), and 10 mM of β-glycerophosphate (Sigma) for 14 d. Ascorbic acid was added daily and osteogenic differentiation medium was changed every 2–3 d. Osteoblast differentiation was confirmed by Alizarin Red S and von Kossa staining. For Alizarin Red S staining, cells were fixed in 1:1 acetone: methanol, stained with 3% Alizarin Red S solution for 2 min, and then washed with PBS. For von Kossa staining, cells were fixed in 1:1 acetone:methanol, incubated in fresh 3% silver nitrate (Sigma), and then incubated in the dark for 30 min. Each staining was performed in triplicate. For adipogenesis, cells were plated at a seeding density of 4.8×10^5 cells per well in 12-well plates with DMEM media (10% FBS, 1% P/S) for 24 h and treated with the standard protocol of 3-isobutyl-1-methylxanthine (IBMX, 500 μM, Sigma), dexamethasone (1 μM), indomethacin (100 μM; Sigma), and insulin (5 μM; Gibco) for 14 d. Medium was changed every 2–3 d. Oil red O (Sigma) staining was performed to confirm lipid droplet formation in cells. For chondrogenesis, 5×10^5 cells were pelleted by centrifugation at $300\times g$ for 3 min in a 15-mL tube, and chondrogenic differentiation induced by incubation in DMEM-high glucose containing 1% antibiotic-antimycotic solution, 1% insulin transferrin selenium-A (ITS; Gibco), 50 μM of ascorbic acid-2-phosphate, 10 ng/mL of transforming growth factor-beta 3 (TGF-β3; R&D Systems, Minneapolis, MN) for 14 d with daily addition of ascorbic acid and TGF-β3. Chondrogenic differentiation was confirmed by Safranin O (Sigma) and Alcian blue (Sigma) staining.

Quantitative real-time polymerase chain reaction. Total RNA from cultured AT-SVF and BMC cells were isolated using RNA iso Plus (Takara, Shiga, Japan) reagent according to the manufacturer's protocol. Complementary DNA was synthesized from the total RNA using Omniscript Reverse-Transcription Kit (Qiagen, Venlo, Netherlands), and then used for real-time polymerase chain reaction (PCR) with SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed using an ABI7500 real-time system (Applied Biosystems). Primers were purchased from Bioneer (Daejeon, South Korea). The primers were as follows: *GAPDH* (P267613, NM_002046.3), *SOX2* (P200205, NM_003106.2), *NANOG* (P255522, NM_024865.1), and *OCT4* [forward; 5′-GCAAGCCCTCATTTCACCA-3′, reverse; 5′-GCCCATCACCTCCACCAC-3′ (NM_002701)]. There were no validated primers for *COL2A1* and *AGCN*. Therefore, we designed the primers as follows: *COL2A1*, 5′-GTCCTCTCCCAAGTCCACACAG-3′ (sense) and 5′-GGGCACGAAGGCTCATCATTC-3′ (antisense); and *AGCN*, 5′-CCACTGTTACCGCCACTT-3′ (sense) and 5′-GTAGTCTTGGGCATTGTTGT-3′ (antisense). The PCR was initiated for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 20 s at 60°C. Mean cycle threshold (CT) values from triplicate measurements were used to calculate gene expression normalized to *GAPDH* as an internal control.

Micromass staining. Micromass pellets were washed twice with PBS, and fixed for 24 h in 10% formalin. After fixation, pellets were paraffin-embedded and then sectioned. Micromass pellet sections were deparaffinized, rehydrated, washed with PBS, and then stained with 1% Safranin O solution and 3% Alcian blue for 30 min. Hematoxylin and eosin solution (Sigma) was used as a counterstain.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized, rehydrated, and washed twice with PBS. To reduce nonspecific background, sections were incubated in blocking reagent (20% FBS) for 1 h and then washed twice with PBS. Sections were incubated with rabbit anti-type II collagen A1(COL2A1) (Santa Cruz, Santa Cruz Biotechnology Inc, CA) or mouse anti-aggrecan (Santa Cruz) overnight at 4°C, washed with PBS three times, treated with goat anti-rabbit secondary antibody (Santa Cruz) or goat anti-mouse secondary antibody for 30 min, and then stained using DAB Substrate Kit (Vector Laboratories, Burlingame, CA). Nuclei were stained with hematoxylin. Images were captured using a digital camera (TL-4, Olympus).

Statistical analysis. Statistical analysis was performed using an independent Student's *t* test. The data were presented as the mean±standard deviation (SD). For all tests, $p<0.05$ was considered to be statistically significant.

Results

Cell yield of AT-SVF and BMC. To compare the nucleated cell yield of AT-SVF and BMC, the amount of adipose tissue and bone marrow obtained from a single surgical procedure were analyzed (Buda *et al.* 2010; Gobbi *et al.* 2011; Koh *et al.* 2013). The number of total nucleated cells in AT-SVF was 42.9×10^6 ($\pm31.6\times10^6$), and BMC was 251×10^6 ($\pm175\times10^6$). Three days after incubation, adherent cells were counted as potential stem cells. Adherent cells from AT-SVF and BMC were 1.28×10^6 ($\pm0.99\times10^6$) and 0.36×10^6 ($\pm0.46\times10^6$) per mL, respectively (Table 1). The total nucleated cell number of AT-SVF was approximately sixfold lower than that of BMC, whereas adherent cell number of AT-SVF was approximately fourfold higher than that of BMC.

Table 1 Cell yield of AT-SVF and BMC

	Adipose tissue -SVF	Bone marrow concentrate	Mean days in culture
Sample (*n*)	22	6	
Average age	56.5	53.6	
Tissue/bone marrow	68.5 g	60 ml	
Harvested volume (ml)	4	4	
Total nucleated cells	42.9 (±31.6)×10^6	251 (±175)×10^6	3 d
Adherent cells (P0)	5.11 (±3.98)×10^6	1.44 (±1.85)×10^6	
Yield adherent cells/ml	1.28 (±0.99)×10^6	0.36 (±0.46)×10^6	

Immunophenotype of freshly isolated AT-SVF and BMC. To characterize the phenotype of uncultured AT-SVF and BMC, flow cytometric analysis was performed on cells freshly isolated from fat tissue and BMC (Fig. 1, Table 2). First, we analyzed the MSC fraction of AT-SVF and BMC using CD45 and CD31 as negative markers and CD90 and CD105 as positive markers. In AT-SVF, the CD45−CD31− subpopulation constituted 50±17% (mean±SD) of total cells, while 4.28±1.6% cells were positive for CD90 and CD105 from the CD45−CD31− subpopulation. In BMC, the CD45−CD31− subpopulation constituted 17±2.47% (mean±SD) of total cells, and 0.42±0.32% were positive for CD90 and CD105 of the CD45−CD31− subpopulation

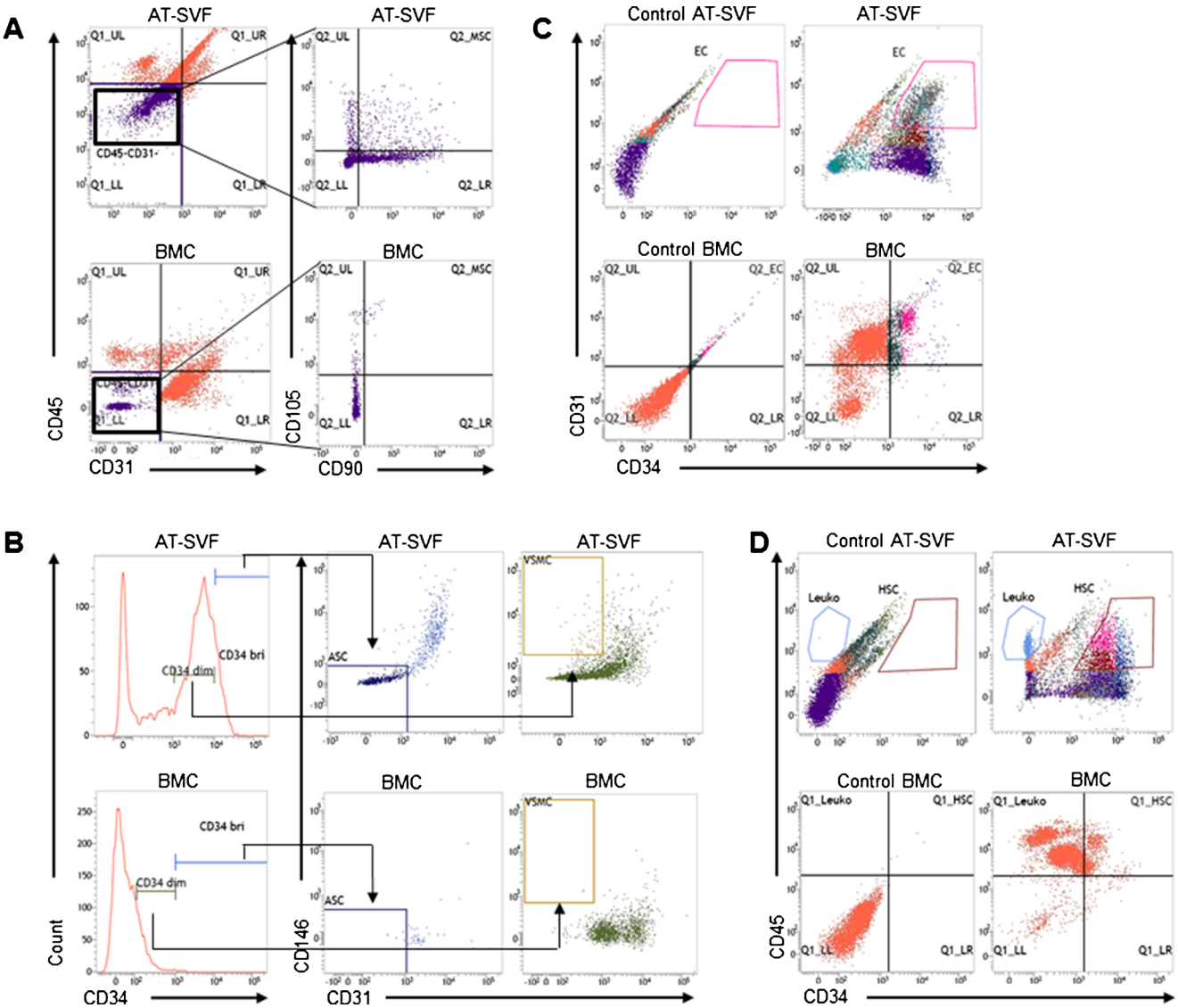

Figure 1. Immunophenotypic analysis of AT-SVF and BMC. The cell population was defined by gates using indicated CD markers. (*A*) CD45−CD31− gated cells were further analyzed using CD90 and CD105 for MSC. (*B*) For ASCs, the CD34^{bright} profile was further characterized using CD31 and CD146. For VSMCs, the CD34^{dim} profile was further characterized using CD31 and CD146. (*C*) ECs were distinguished using CD34 and CD31. *D*) CD45+CD34− and CD45+CD34+ represented leukocytes and HSCs, respectively.

Table 2 Cells population in AT-SVF and BMC

	AT SVF (%)	BMC (%)	
CD45− CD31− CD90+ CD105+	4.28(±1.6)	0.42(±0.32)	Mesenchymal stem cell (MSC)
$CD34^{bright}$ CD31− CD146−	32(±13.35)	0.16(±0.08)	Adipose tissue-derived stromal cell (ASC)
CD34+ CD31+	6.73(±5.04)	9.43(±10.84)	Endothelial cell (EC)
$CD34^{dim}$ CD31− CD146+	3.32(±4.61)	0.22(±0.42)	Vascular smooth muscle cell (VSMC)/pericyte
CD45+ CD34+	5.92(±5.83)	5.17(±5.64)	Hematopoietic stem cell-like (HSC)
CD45+ $CD34^{+}$	9.07(±457)	49.18(±32.08)	Leukocyte

Event: 20,000~100,000 cells

(Fig. 1*A*). The ASC fraction of AT-SVF and BMC was analyzed using CD34, CD31, and CD146 (Varma *et al.* 2007). Cell populations were sorted on the basis of CD34 expression and then divided into $CD34^{bright}$ and $CD34^{dim}$. For ASCs, the $CD34^{bright}$ profile was further characterized into CD31− and CD146− subpopulations, with 32±13.35% in AT-SVF and 0.16±0.08% in BMC characterized as CD31−CD146−. Additionally, the non-stem cell population in AT-SVF and BMC was analyzed. For vascular smooth muscle cells (VSMCs), the $CD34^{dim}$ profile was further characterized into CD31− and CD146+ subpopulations, with 3.32±4.61% in AT-SVF and 0.22±0.42% in BMC characterized as CD31−CD146+ (Fig. 1*B*). Endothelial cells (EC) were identified using CD34+ and CD31+. ECs constituted 6.73±5.04% of AT-SVF and 9.43±10.84% of BMC (Fig. 1*C*). Hematopoietic stem cells (HSCs) and leukocytes were analyzed using CD45 and CD34. CD45+CD34+ cells (HSCs) were 5.92±5.83% in AT-SVF and 5.17±5.64% in BMC, and leukocytes (CD45+CD34−) were 9.07±4.57% in AT-SVF and 49.18±32.08% in BMC (Fig. 1*D*).

Colony-forming unit evaluation. We compared the colony-forming capacity of AT-SVF and BMC using a colony formation assay. Twelve days after seeding, colonies were stained and counted. As shown in Fig. 2, AT-SVF showed a higher frequency of colony formation than BMC in both P0 and P1 cells. The colony-formation frequency of P0 cells was 0.5%±0.05% in AT-SVF and 0.01%±0.005% in BMC. The colony-formation frequency of P1 cells was 6.55%±0.8% in AT-SVF and 4.63%±0.68% in BMC.

Comparison of multi-lineage differentiation. To assess the osteoblastic phenotype, mineralized matrix and calcium

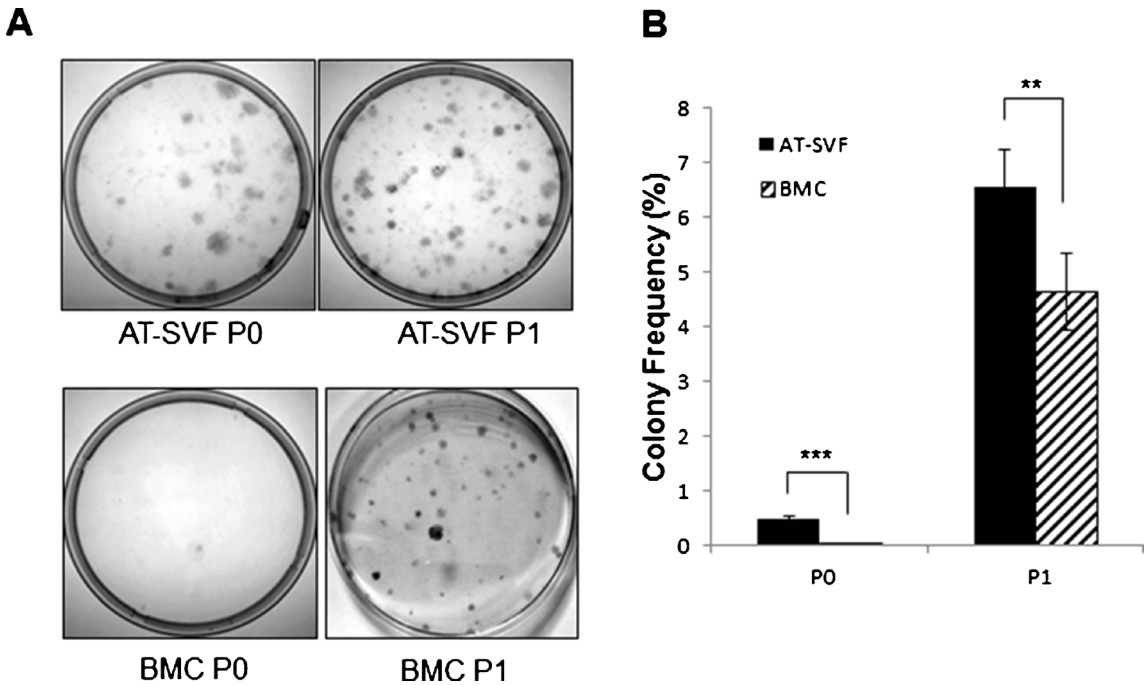

Figure 2. Colony-forming unit assay in AT-SVF and BMC. *P0* (1×10^4 cells) and *P1* (1×10^3 cells) of AT-SVF and BMC cells were seeded in 100-mm plates and cultured in DMEM-LG containing 20% FBS for 12 d to examine cell colony formation. Formed colonies were stained and counted. (*A*) Images are representative of crystal violet-stained colonies. (*B*) The number of violet-stained colonies was expressed as a percentage of seeded cells; $^{**}p<0.01$ and $^{***}p<0.001$.

deposition were estimated. As shown in Fig. 3A, both uncultured AT-SVF and BMC were able to differentiate into osteoblasts, though AT-SVF was more osteogenic than BMC. To evaluate the adipogenic phenotype, lipid droplet frequency in cells was evaluated. AT-SVF showed a higher frequency of lipid droplet formation than BMC (Fig. 3B). To assess the chondrogenic phenotype, three-dimensional pellet culture was performed. AT-SVF pellets began to form micromass within 1–2 d and proteoglycan, a major component of the cartilage extracellular matrix (ECM), was detected at 14 d (Fig. 3C). The expressions of chondrogenic differentiation markers, *aggrecan* (*AGCN*) and *collagen type2 alpha1* (*COL2A1*) mRNA, were upregulated in 14-day culture pellets compare to the control, the freshly isolated cell fraction of AT-SVF (Fig. 3D). AT-SVF was differentiated into the chondrogenic lineage without any scaffold, whereas BMC did not form micromass until 14 d (data not shown). Therefore, we further examined chondrogenic differentiation capacity using cultured cells at P1 (Fig. 3E–G). Proteoglycan was detected in both AT-SVF and BMC, but proteoglycan synthesis was not different between AT-SVF and BMC at P1 (Fig. 3E). AGCN and COL2A1 were expressed at the similar levels in AT-SVF and BMC at P1 (Fig. 3F). The corresponding mRNA, *AGCN* was upregulated in AT-SVF and BMC at P1 compare to the controls, non-differentiated AT-SVF and BMC, respectively. The expression level of *AGCN* was not different between AT-SVF and BMC, whereas that of *COL2A1* was upregulated in AT-SVF and higher in AT-SVF than BMC (Fig 3G).

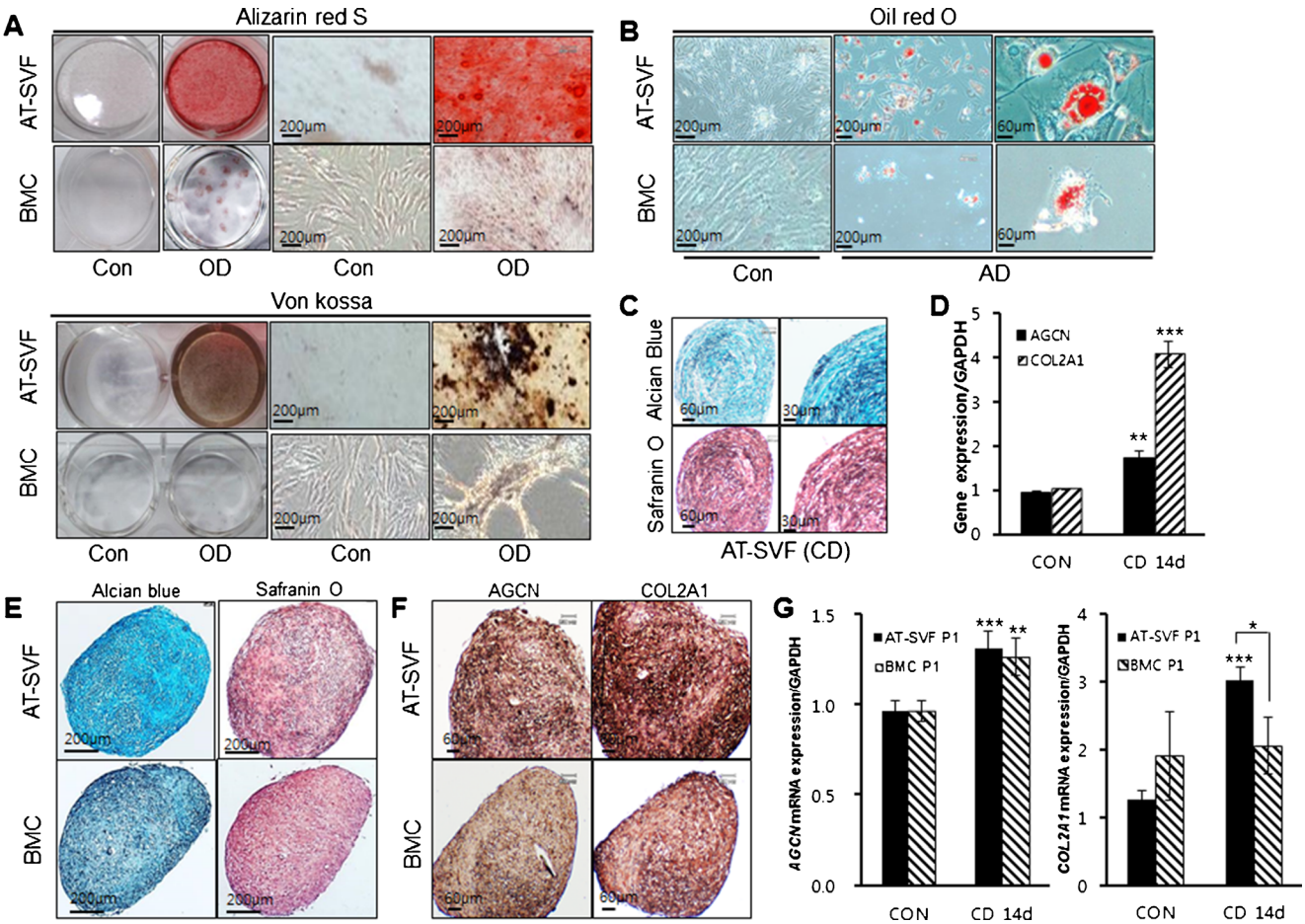

Figure 3. Multi-lineage differentiation capacity of uncultured AT-SVF and BMC. Multi-lineage differentiation capacity of AT-SVF and BMC. *A*) Osteogenic differentiation (*OD*), *B*) adipogenic differentiation (*AD*), and *C*) chondrogenic differentiation (*CD*) of AT-SVF and BMC. *D*) Expression of *AGCN* and *COL2A1* in chongenic differentiated AT-SVF at day 14. Freshly isolated cells of AT-SVF and BMC were cultured in 12-well tissue culture plates with DMEM media (10% FBS, 1% P/S) for 24 h. For osteogenic differentiation, the cells were induced by changing DMEM media to osteogenic media and then demonstrated by Alizarin Red S and von Kossa staining. For adipogenic differentiation, the cells were induced by changing DMEM media to adipogenic media, and then confirmed by the Oil Red O staining of lipid droplet. For chondrogenic differentiation, AT-SVF cells were cultured in chondrogenic medium using pellet culture and then indicated by Alcian blue and Safranin O staining. *E*) Proteoglycan staining of AT-SVF and BMC by Alcian blue and Safranin O. *F*) Immunohistochemistry of aggrecan and type II collagen A1. *G*) Real-time PCR analysis of *AGCN* (*left panel*) and *COL2A1* (*right panel*). Chondrogenic differentiation was induced in AT-SVF and BMC at P1 for 14 d. Control (*Con*) from AT-SVF and BMC were cultured in growth medium (DMEM supplemented with 10% FBS and 1% P/S); *$p<0.05$ and **$p<0.01$, and ***$p<0.001$.

Stemness gene expression. To compare the pluripotency of AT-SVF and BMC, we examined stemness-related gene expression in uncultured cells using real-time PCR. The stem cell pluripotency markers SOX2, NANOG, and OCT4 were analyzed. Messenger RNA expression levels of *SOX2*, *NANOG*, and *OCT4* in uncultured AT-SVF were much higher than in BMC (Fig. 4)

Discussion

Sufficient introduction of stem cells into damaged sites is the most critical aspect of one-step surgical cartilage regeneration. Therefore, it is necessary to obtain an adequate number of stem cells in a limited time during surgery. BMC is a procedure used to concentrate nucleated cells from bone marrow aspiration and can provide at least 2.5-fold higher number of nucleated cells than the traditionally used Ficoll isolation (Hermann *et al.* 2008). However, bone marrow aspiration contains only a small portion of MSC (0.001–0.01%) of the total nucleated cells (Pittenger *et al.* 1999; Martin *et al.* 2002). For this reason, various tissues have been investigated to replace bone marrow (Jankowski *et al.* 2002; Jiang *et al.* 2002; Lechner and Habener 2003), such as adipose tissue, which is not only easier to access but also more abundant. AT-SVF has recently become popular in one-step surgery for cartilage regeneration. In spite of increased clinical use, AT-SVF has not been fully characterized or compared with BMC. In this study, we characterized AT-SVF and compared it to BMC using measures such as isolated cell yield, immunophenotyping using multiple CD markers, multi-lineage differentiation capacity, and stemness-related gene expression.

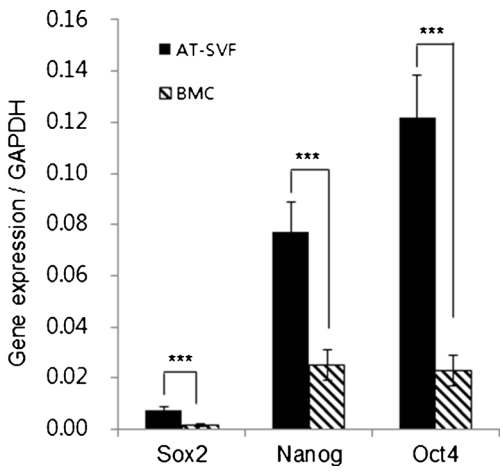

Figure 4. Expression of stemness-related genes in uncultured AT-SVF and BMC. Messenger RNA expression of stemness-related genes was evaluated using real-time PCR in uncultured AT-SVF and BMC. Each experiment was performed in triplicate; ***$p<0.001$.

In the present study, the total nucleated cells in AT-SVF were approximately sixfold lower than in BMC. However, adherent cells were fourfold higher in AT-SVF compared to BMC in proliferation-free culture conditions (3 d). It was previously reported that total nucleated cells in bone marrow aspiration (not BMC) was 60-fold higher than that of AT-SVF, but adherent cell number was not significantly different 2 wk after cell culture (De Ugarte *et al.* 2003). In most previous studies, only a single set of CD markers for bMSC or AT-SVF was used to identify stem cells (Mitchell *et al.* 2006; Bourin *et al.* 2013), and MSCs of the AT-SVF have not been analyzed and compared with BMC. In our study, for accurate comparison of total stromal/stem cell portion between AT-SVF and BMC, MSCs in AT-SVF were identified on the basis of high expression of CD90 and CD105 and lack of CD45 and CD31 expression (Bourin *et al.* 2013). AT-SVF contains a higher proportion of MSCs than BMC (Table 2) and it has been shown that CD90+ and/or CD105+ cells are important indicators of chondrogenic differentiation potential (Diaz-Romero *et al.* 2005; Nagase *et al.* 2008; Jiang *et al.* 2010). It has also been shown that the functional characteristics of ASCs are similar to MSCs (De Ugarte *et al.* 2003). Unlike bMSCs, $CD34^{bright}$, CD31-, and CD146- were suggested as phenotype markers for ASCs to distinguish from non-stem cell subpopulations in AT-SVF such as pericytes, ECs, and others (Bourin *et al.* 2013). ASCs were identified at a proportion of 32±13.35% of AT-SVF.

Jurgens and colleagues demonstrated the chondrogenic differentiation potential of uncultured AT-SVF using scaffolds, as with BMC (Jurgens *et al.* 2009). In our study, we induced differentiation of uncultured AT-SVF into the chondrogenic lineage using a pellet culture system without a scaffold. Uncultured AT-SVF begins to form micromasses at 1–2 d after chondrogenic differentiation, and chondro-specific markers such as proteoglycan can be detected at day 14. Considering that BMC begins to express chondrogenic markers in chondrogenic differentiation conditions with hyaff-11 scaffold at day 40 (Cavallo *et al.* 2013), chondrogenic differentiation of AT-SVF may be more rapid than with BMC. Transcription factors such as SOX2, NANOG, and OCT4 play key roles in the process of differentiation in MSC (Shi *et al.* 2006; Yu *et al.* 2007). In our study, mRNA expression levels *of SOX2*, *NANOG*, and *OCT4* were higher in uncultured AT-SVF than in BMC (Fig 4).

Taken together, uncultured AT-SVF has abundant stem cells, which can be rapidly differentiated into multiple lineages, as compared to BMC. Therefore, we suggest that uncultured AT-SVF may be a good option for cartilage regeneration in articular cartilage defect.

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Conflict of interest The authors declare no conflict of interests.

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