# Importance of the culture method for differential expression of osteogenic and stemness genes in the lineage commitment of bone marrow-derived mesenchymal stromal cells

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# Importance of the culture method for differential expression of osteogenic and stemness genes in the lineage commitment of bone marrow-derived mesenchymal stromal cells

Directed by Professor Jin Woo Lee

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Dong Suk Yoon

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This certifies that the Doctoral Dissertation
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2006년 10월 이진우 교수님 실험실로 입실해서 6년 반이라는 시간이 흐른 지금에서야 이렇게 감사의 글을 적을 수 있게 되었습니다. 지난 시간을 돌아보면 저에겐 이 논문에 담긴 내용들 보다 더 많은 것을 얻고 보다 많은 것을 배울 수 있는 소중한 시간들이었습니다. 생물학과에 편입해서 2년이라는 짧은 시간 동안 생물학을 배우고 이곳으로 오게 되었습니다. 그만큼 부족한 저를 많은 것을 배우고 경험할 수 있게 이끌어 주시고, 긴 시간 동안 지켜봐 주시고 지도해주신 이진우 교수님께 깊은 감사를 드립니다. 이곳이 아니었다면 배울 수도 경험할 수도 없는 많은 소중한 것들을 경험하였기에 긴 시간이 전혀 아깝지 않고 뜻 깊었습니다. 그리고, 논문이 나오기까지 따뜻한 관심을 가져주시고 세심한 지도를 해주신 박상욱 교수님, 이유미 교수님, 이재면 교수님, 그리고 김재우 교수님께 깊은 감사를 드립니다. 교수님들께 부끄럽지 않은 훌륭한 연구자가 될 수 있도록 노력하고 매진할 수 있도록 하겠습니다.

어느 날 뉴스에서 줄기세포 관련 기사를 보고 관심이 생겨 생물학과로의 편입을 결심하게 되었습니다. 그 과정에서 제가 올바른 선택을 할 수 있도록 조언해주신 김천대학교 임병철 교수님과, 편입생인 저에게 처음으로 실험실이라는 곳을 경험할 수 있게 해주신 세종대학교 소문수 교수님께도 깊은 감사를 드립니다.

끝날 것 같지 않았던 학위과정을 이렇게 마무리하면서 함께 했던 많은 인연들이 생각납니다. 처음 실험실에 들어와서 적응하고 많은 실험을 가르쳐 주고, 그리고 무엇보다 혼자서 해나갈 수 있는 방법과 참된 연구자로써의 길을 걷게 해주신 김윤희 박사님께 정말 감사하다고 전하고 싶습니다. 지금은 외국에 있지만 항상 다정하게 대해주고 제가 많은 실험들을 시도해 볼 수 있게 배려해준 정호선 박사님과 언제나 한결같던 박민성 박사님께도 정말 감사하다고 전하고 싶습니다. 첫번째 제 후배이자 첫 외국인 친구였던 백승일군과 언제나 밝고 열심히 하는 모습이 보기 좋은 황지숙양, 그리고 오랜 기간 동안 실험실 막내 생활을 불평 없이 해온 이슬기양에게도 정말 졸업 축하하고 앞으로도 함께 하고 싶은 인연이라고 전하고 싶습니다. 고된 실험실 생활에 언제나 버팀목이 되어준 경미 누나와 앞으로 오랫동안 많은 일들을 해 줄 현애, 곧 학위를 들어올 실험실 막내 최유림 학생, 앞으로 저희 랩에서 많은 일들을 해주시고 이끌어 가실 장연수 박사님께도 감사의

인사를 전하고 싶습니다. 그리고 항상 저희 실험실에 관심 가져주시고 많은 도움 주셨던 한승환 교수님, 처음 이곳에 와서 지금까지도 자주 뵙고 항상 친절하게 대해주신 최우진 교수님께도 감사하고 졸업 축하한다고 전하고 싶습니다. 저희 실험실 중대형 동물 수술은 다 맡아서 최선을 다해주시는 김성환 교수님께도 정말 감사를 드립니다.

공부만 한다고 자식 노릇 제대로 못했지만, 항상 믿고 격려해주고 지금까지 기다려주신 제 아버지와 어머니, 그리고 장모님께도 정말 감사하다고 전하고 싶고, 항상 힘이 되어준 든든한 내 동생 경석이와 앞으로 우리 가족이 될 미선이에게 감사의 인사를 전하고 싶습니다. 마지막으로 학생남편을 만나서 결혼한지 4년이 지났지만 단 한번도 저한테 부담주지 않고 항상 격려해 주었던 제 아내에게 정말 사랑하고 고맙다고 전하고 싶습니다. 이렇게 많은 고마운 분들께 자랑스런 과학자가 될 수 있도록 노력하겠습니다.

기회는 찾아야 만들어지고, 행운은 노력하는 자에게 찾아온다고 합니다. 지금도 그림 하나를 얻기 위해서 그 이상의 노력과 시간을 바쳐야 하는 많은 후배들에게 큰 격려를 보내고 싶습니다.

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## ABSTRACT

# Importance of the culture method for differential expression of osteogenic and stemness genes in the lineage commitment of bone marrow-derived mesenchymal stromal cells

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Bone marrow-derived mesenchymal stem or stromal cells (BM-MSCs) are considered good sources for cell therapy in clinical applications. However, BM-MSCs lose their self-renewal capacity and multi-lineage differentiation potential during prolonged cell passage *in vitro*. Furthermore, BM-MSCs that are cultured for a long time become committed to the osteogenic lineage as they approach senescence and lose their potential to differentiate along the other lineages. The decreased stem cell properties of MSCs pose significant limitations to their application in cell-based regenerative medicine. BM-MSCs are composed of heterogeneous cell populations. However, little is known about the effects of the interaction between the different BM-MSC populations on cellular senescence. Thus, the purpose of this study was to identify mechanisms that induce cellular senescence in BM-MSCs via cell-cell interactions between the heterogeneous BM-MSC populations. In addition,

this study proposes an alternative BM-MSCs culture method, which involves repopulating a “primitive” cell population from late-passage MSCs with poor multipotentiality and low cell proliferation rate by simply altering the plating density.

First, we confirmed senescence in BM-MSCs following repeated serial subculture. Late-passage MSCs include a subpopulation of more committed osteogenic cells with increased expression of osteogenic transcription factors (*Runx2* and *Dlx5*) that is further elevated during subsequent passage and diminishing stemness with decreased expression of stemness genes (*Sox2* and *Nanog*). Specifically, knockdown of Sox2 significantly inhibited multipotentiality and cell proliferation of BM-MSCs. This result indicated that Sox2 is important for maintaining the stemness of BM-MSCs. Next, we hypothesized that cytokines secreted by the large-cell BM-MSC population would affect the cellular senescence and osteogenic lineage commitment of primitive cells (small-cell population) in heterogeneous BM-MSCs because the large-cell population has been considered the senescent population. Indeed, senescence and osteogenic lineage commitment of BM-MSCs were strongly induced in the presence of cytokines secreted by the large-cell population. Among the cytokines, the level of interleukin-6 (IL-6) secreted by large-cell population was significantly higher than that secreted by the small-cell population. Therefore, we focused our attention on the ability of IL-6 to induce cellular senescence and osteogenic lineage commitment in the small-cell population. IL-6 induced osteogenic lineage commitment and cellular senescence in the BM-MSC population by increasing Runx2 and Dlx5 protein levels, and reduced stemness by decreasing Sox2 protein expression. Furthermore, the IL-6-induced Runx2 and Dlx5 proteins decreased the transcriptional activity of Sox2. These results indicate that IL-6, one of the cytokines secreted from the large-cell population of BM-MSCs, can induce osteogenic lineage commitment by up-regulating the expression of osteogenic transcription factors (Runx2 and Dlx5) and decrease the self-renewal capacity and multi-lineage differentiation potential of primitive cells by

down-regulating the transcriptional activity of *Sox2*, stemness-related gene.

Further, we determined the effects of low-density culture (compared to high density culture) on late-passage BM-MSCs. We repopulated "primitive" cells (small-cell population) by replating late-passage BM-MSCs at low density (10-20 cells/$cm^2$) regardless of donor age. The repopulated BM-MSCs derived from low-density cultures were smaller than the cells from high-density cultures had spindle-shaped morphology, and exhibited enhanced colony-forming ability, proliferation rate, and adipogenic and chondrogenic potentials. The strong expression of osteogenic genes (*Runx2*, *Dlx5*, *alkaline phosphatase* and *type I collagen*) in late-passage BM-MSCs was reduced by replating at low density, whereas expression of 3 stemness markers (*Sox2*, *Nanog* and *Oct-4*) reverted to levels observed in early-passage BM-MSCs.

In conclusion, among the cytokines secreted by the large-cell population, IL-6 may play important roles in cellular senescence and osteogenic lineage commitment of BM-MSCs during prolonged cell passage *in vitro*. Therefore, molecules targeting the IL-6 signaling pathway would be useful in maintaining the primitive BM-MSC population in long-term culture. In addition, plating density should be considered a critical factor in the enrichment of "primitive" cells in heterogeneous BM-MSC populations.

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Key words: BM-MSCs, Heterogeneous population, Lineage commitment, Cellular senescence, Self-renewal, Multi-potency, Stemness, IL-6, Runx2, Dlx5, Sox2, Low density cylture

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## I. INTRODUCTION

Mesenchymal stem cells (MSCs), also referred to as mesenchymal stromal cells, isolated from the bone marrow (BM) are believed to possess self-renewal properties and have the ability to generate multipotential progeny for musculoskeletal tissues such as bone[1-3], cartilage[4-6], and adipose tissues[7-13]. However, BM-derived MSCs lose their self-renewal capacity and multi-lineage differentiation potential with increasing passage during *in vitro* expansion[14-19]. After several passages, MSCs enter the process of senescence, characterized by enlarged and irregular cell shapes, and cessation of cell division[20].

MSCs comprise a heterogeneous population of cells that that differ in size, morphology, and differentiation potential. As MSCs approach senescence in *in vitro* culture, they lose their multi-lineage differentiation potential. Despite the decreased multi-lineage differentiation potential, BM-MSCs have been reported to retain their ability to differentiate along the osteogenic lineage during prolonged cell passage *in vitro*[17,18]. These results indicate that MSCs that are

cultured for a long time become committed to the osteogenic lineage as they approach senescence, and they lose the potential to differentiate along other lineages. The loss of stem cell properties significantly limits the utility in cell-based regenerative medicine and in studies aimed at understanding their differentiation mechanisms.

It has been reported that cytokines secreted by senescent cells can alter the fates of neighboring cells[21-23]. Cytokines secreted by human BM cultures have been shown to exhibit certain patterns according to age or estrogen status[24], and cytokines secreted by senescent cells can stimulate tissue aging and tumor formation[25]. Furthermore, it has been shown that cytokines secreted by senescent cells, such as interleukin-6 (IL-6)[26,27] and -11 (IL-11)[28], are capable of inducing cells to commit to the osteogenic lineage[24]. However, less is known about the roles of the cytokines secreted by senescent cells in osteogenic lineage commitment induced prior to cellular senescence.

Recently, somatic cells have been reprogrammed into induced pluripotent stem cells (iPS) via forced expression of embryonic stem (ES) cell factors (*Oct4*, *Sox2*, *cMyc*, and *Klf4*), and the combination of these genes has been shown to be critical for high reprogramming efficiency. Pluripotency markers, such as *Sox2*, *Nanog*, and *Oct-4*, are expressed in both adult stem cells and ES cells; however, their expression in MSCs is dependent on passage number and tissue source[29]. Go et al.[30] observed that overexpression of a retrovirus encoding Sox2 or Nanog in passage 5 (P5) MSCs, which displayed flattened aged morphology and reduced proliferation rates, resulted in restoration of normal morphology and proliferation levels. However, these changes were only apparent in the presence of basic fibroblast growth factor, and retroviral silencing remains an obstacle to achieving the pluripotent state and superior iPS cells.

To isolate BM-derived primitive cells during *in vitro* culture, several approaches have been reported, including assessment of surface markers such as stro-1[31], cell shape and size[32], donor age[33,34], and plating density[35-37]. Some studies have demonstrated the importance of *in vitro* plating density in cell proliferation and differentiation potential. MSCs seeded at a cell density less

than 5000 cells/cm$^2$ undergo apoptotic cell death[35]; however, low-density (LD) cultures were shown to be sufficient for maintaining the colony-forming ability and stem cell properties ("stemness") of early passage MSCs[36]. Nevertheless, most studies have used passage-limited MSCs (less than 5 passages)[38,39] and/or have expanded MSCs at various cell densities (ranging from $10^3$ to $10^5$ cells/cm$^2$)[37,40-43]. However, little is known about the relationship between cell culture condition and cellular senescence in BM-MSCs.

Despite various trials to overcome MSC senescence and maintain their stemness, little is known about environmental factors that can negatively affect MSC quality, such as MSC senescence, commitment to the osteogenic lineage, or loss of multi-potency, in heterogeneous MSC populations. In addition, some researchers do not use late-passage MSCs cultured for a long time despite the presence of a small primitive cell population because multi-potency and renewal capacity are reduced in late-passage cell populations. Thus, the purpose of our current study was to (1) investigate whether cytokines secreted from a senescent BM-MSC population can regulate cellular senescence and stemness (or osteogenic lineage commitment) of neighboring primitive cells, and (2) repopulate a primitive cell population similar to early-passage BM-MSCs from late-passage BM-MSCs using a density-controlled culture method.

## II. MATERIALS AND METHODS

### 1. Isolation and culture of MSCs from human bone marrow aspirates

BM aspirates were obtained from posterior iliac crests of 14 adult donors (nine males, five females) 19-69 years of age, with approval of the Institutional Review Board (IRB) of our institution. MSCs from human BM were selected based on their ability to adhere to plastic cell culture flasks. Cells were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM-LG, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% antibiotic-antimycotic solution (Invitrogen) at 37℃ in a 5% $CO_2$ atmosphere. Cells were grown to 80-90% confluence and then harvested by incubation with 0.25% trypsin/EDTA (Invitrogen) centrifuged at 1300 rpm for 3 min. Harvested cells (P1) were replated at density of 5000 cells/$cm^2$ and subcultured when they were 80-90% confluent up to P7. Late passage BM-MSCs (P7) were replated at high density (HD, 5000 cells/$cm^2$) or low density (LD, 17 cells/$cm^2$) and maintained for 10-12 days.

### 2. Colony-forming unit fibroblast (CFU-F) assay

After being fixed in 1:1 acetone:methanol fixative, cultures were stained with 20% crystal violet solution (Merck, Darmstadt, Germany) for 30 min in the dark. After being washed in distilled water (DW), colony-forming ability of the stained cells was evaluated.

### 3. Cell proliferation assay

Cell proliferation was determined using a hexosaminidase assay. Proliferative ability of density-controlled BM-MSCs was examined after 1, 4 and 7 days. Briefly, after being washed in PBS, a mixture of 0.1 M citrate buffer (Sigma, St.

Louis, MO, USA) containing 7.5 mM p-nitrophenyl-N-acetyl-b-D-glucosaminide (PNAD; pH 5.0, Sigma) and 0.5% Triton X-100 (Sigma) was added to each well and incubated at 37 _C for 3 h. After incubation, 50 mM glycine buffer (pH 10.4; Amresco, Solon, OH, USA) containing 5 mM ethylenediaminetetra acetic acid (EDTA; Sigma) was added to each well. Absorbance of released hexosaminidase was measured at 405 nm. All samples were tested in triplicate.

4. Senescence-associated-β-galactosidase assay (SA-β-gal assay)

SA-β-gal assay was performed using a Cellular Senescence Assay kit (Millipore, Temecula, CA, USA) following the manufacturer's protocol. Briefly, Cells were washed with PBS, and then were fixed for 15 min at room temperature with 1X fixing solution. After washing with DW, cells were stained with freshly prepared 1X SA-β-gal detection solution [X-gal (1) : Solution A (4) : Solution B (4)] for 4h in the dark at 37℃ incubator without $CO_2$. SA-β-gal–positive cells exhibited a blue color. The number of positive cells was counted under a phase-contrast microscope. Experiments were performed in triplicate.

5. Alkaline phosphatase (ALP) staining

After being fixed in 2:3 citrate buffer:acetone fixative, cultures were stained for alkaline phosphatase (ALP) using alkaline staining solution (Sigma) for 30 min in the dark. After washing in DW, cells were stained in Mayer's haematoxylin solution (Sigma) for 5 min, then rinsed in tap water.

6. Calcium contents assay

To evaluate calcium content, cells were washed twice in PBS and incubated in 800 μL 0.5 N acetic acid for 24h at room temperature. After incubation, 300

μL fresh reagent (O-Cresolphthalein Complexon, ethanolamine ⁄ boric acid, hydroxyquinol; Sigma) was added to 50 μL of sample supernatant, and absorbance was measured at 560 nm. Standards were prepared from a CaCl2 solution, and results were expressed as mg⁄ml calcium equivalent per μg total protein. Experiments were performed in triplicate.

7. Multi-lineage differentiation

To identify multi-lineage differentiation potential of BM-MSCs, cells were seeded at $8 \times 10^4$ cells/well in 12-well culture plates.

A. *In vitro* osteogenic differentiation

For osteogenic differentiation, cells were maintained for 14 days in osteogenic medium [DMEM-LG containing 10% FBS, 1% antibiotic-antimycotic solution, 100nM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), and 50 μg/mL ascorbic acid (Gibco)]. For von Kossa staining, after being fixed in 1:1 acetone:methanol, 1 ml freshly prepared 3% silver nitrate (wt/vol) (Sigma) was added, and for alizarin red S staining, 1 mL freshly prepared 3% alizarin red S solution (wt/vol) (Sigma) was added, then incubated in the dark for 30 min.

B. *In vitro* adipogenic differentiation

For adipogenic differentiation, cells were maintained for 14 days in adipogenic medium [DMEM-LG containing 10% FBS, 1% antibiotic-antimycotic solution, 1μM dexamethasone, 0.5mM isobutyltethylxanthin (Sigma), 5 μg/mL insulin (Gibco), and 200 μM indomethasin (Sigma)]. To detect lipid droplets by oil red O staining, after being fixed in 10% neutral buffered formalin, 1 ml 0.18% oil red O solution (Sigma) was added and incubated for 30 min. For quantitative analysis, absorbance was detected at 500 nm after de-staining with isopropanol for 30 min. To normalize for cell number, cells were stained with crystal violet

(CV) for 10 min and destained with 95% ethanol; absorbance was measured at 595 nm. Each oil red O optical density (OD) value was then divided by its respective CV measurement for normalization.

C. *In vitro* chondrogenic differentiation

For chondrogenic differentiation, cells were maintained for 14 days in chondrogenic medium [DMEM-high glucose containing 1X insulin-transferrin-selenium-A (Gibco), 1% antibiotic-antimycotic solution, 50 μg/mL ascorbic acid, and 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, MN, USA)]. For pellet culture, $8 \times 10^4$ cells in 15-mL tubes were harvested after centrifugation at 1200 rpm for 3 min, then chondrogenic medium with TGF-β3 was added. To detect proteoglycan synthesis 0.1% safranin O solution (Sigma) was added and incubated for 1 h. For quantitative analysis, absorbance was detected at 490 nm following de-staining with 100% ethanol for 20 min. Each safranin O value was normalized to absorbance from CV staining.

8. Cell cycle analysis

Cells were harvested by incubation with 0.25% trypsin/EDTA and washed twice in PBS. Cells from each group ($1 \times 10^6$) were fixed in ice-cold 70% ethanol for 1 h at -20°C, stained with 50 μg/ml propidium iodide (PI, Sigma) containing 100 μg/ml RNase A (Sigma) for 40 min at 4°C, and then analyzed using a FACS Calibur instrumentation (Becton Dickinson Instrument, San Jose, CA) to detect the cell cycle distribution. All samples were tested in triplicate (n=3).

9. Size-fractionized cell sorting

BM-MSCs were harvested by incubation with 0.25% trypsin/EDTA and washed twice in PBS. Cells were resuspended in pre-warmed PBS, then small

or large cell population was sorted and analysed using FACS (Beckman Coulter, Fullerton, CA, USA).

10. Flow cytometry

Cultured cells were harvested with 0.02% EDTA and washed twice in PBS containing 1% FBS and 0.05% sodium azide (FACS buffer). Single cells were labeled for 20 min 4℃ in FACS buffer with following conjugated antibodies: Sox2-PerCP-Cy (BD Bioscience, San Jose, CA, USA) or PerCP-Cy-mouse-IgG isotype control (BD Bioscience). After being washed in FACS buffer, cells were analysed using FACS (Beckman Coulter, Fullerton, CA, USA).

11. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using an Omniscript kit (Qiagen). Real-time polymerase chain reaction (PCR) was performed to determine changes in mRNA expression of cell cycle-related genes, early osteogenic markers, differentiation-related genes, pluripotency-related transcription factors, and interleukin family (IL-6 and IL-11). Primer sets used were validated and purchased from Bioneer (Bioneer, Daejon, South Korea, http://sirna.bioneer.co.kr/). Primers used and product information are as follows: (*GAPDH* (P267613, NM_002046.3); *CCNA2* (P212796, NM_001237.2); *CCNB1* (P275460, NM_031966.2); *CCND1* (P298560, NM_053056.2); *CDK2* (P136765, NM_001798.2); *CDK4* (P268249, NM_000075.2); *Runx2* (P229954, NM_001015051.1); *Dlx5* (P199945, NM_005221.5); *PPAR-γ* (P102359, NM_005037.4); *Adiponectin* (P160254, NM_004797.2); *Sox9* (P232240, NM_000346.2); *IL-6* (P211161, NM_000600.1); *IL-11* (P229401, NM_000641.2); *ALP* (P324388, NM_000478.2); *Collagen I* (P157768, NM_000088.2); *Sox2* (P200205, NM_003106.2); and *Nanog* (P255522, NM_024865.1). There are no validated

primers for *Oct4*, *type* // *collagen*, and *Osteocalcin*, and thus the primer was separately designed as follows: 5′-GCAAGCCCTCATTTCACCA-3′ (*Oct4*, sense), 5′-GCCCATCACCTCCACCAC-3′ (*Oct4*, antisense), 5′-GAGTGGAA GAGCGGAGACTA-3′ (*type* // *collagen*, sense), 5′-CTCCATGTTGCAGAAG ACTT-3′ (*Type* // *collagen*, antisense), and 5′-AGAGCCCCAGTCCCCTACC C-3′ (*Osteocalcin*, sense), 5′-AGGCCTCCTGAAAGCCGATG-3′ (*Osteocalcin*, antisense). PCR reaction mixtures consisted of 2× SYBR Green PCR premix (Bioneer), 10 pM specific primers and 2 μl of cDNA in the ABI7500 real-time machine by AppliedBiosystem (ABI, Carlsbad, CA, USA). Real-time PCR analysis underwent 40 cycles of amplification. Mean cycles threshold (CT) values from triplicate (n=3) measurements were used to calculate gene expression, with normalization to GAPDH as internal control.

12. RNA interference

On-TargetPlus SmartPool siRNAs for *Sox2* (Cat. L-011778) and *Nanog* (Cat. L-014489) were purchased from Dharmacon (Boulder, CO, USA). Scramble, Runx2 (siRNA No: 1132367), and Dlx5 (siRNA No: 1042423) siRNA were purchased from Bioneer Inc., and targeted the following sequences: scramble-siRNA sense: 5`-CCUACGCCACCAAUUUCGU-3` and scramble siRNA antisense: 5`-ACGAAAUUGGUGGCGUAGG-3`. Briefly, cells were plated to obtain 70-80% confluency in six-well plates and transfected with 100 nM of *Sox2*, *Nanog*, *Runx2*, *Dlx5*, or scramble (Neg) siRNA using Lipofectamine™ 2000 (Invitrogen). After 6 h transfection, fresh media were added to plates, and transfection efficiency was confirmed by western blot analysis.

13. Western blot

Cells were lysed in the Passive lysis buffer (Promega, Madison, WI, USA). Protein concentrations were determined by the BioRad protein assay (Bio-Rad

Laboratories Inc., Hercules, CA, USA), and total 30 μg protein was applied and analysed by 10% SDS-PAGE (Sigma, St. Louis, MO, USA). Transferred membranes were blocked with a 5% skim milk (BD, Sparks, MD, USA) and incubated for 4 h with antibodies of Sox2 (abCAM, Cambridge, UK), Nanog (abCAM), Runx2 (abCAM), Dlx5 (abCAM), STAT3 (BD Bioscience), Tyr-p-STAT3 (Cell Signaling Technology, Inc. Boston, MA, USA) and Ser-p-STAT3 (Cell Signaling Technology, Inc.). Membranes were further probed with antibody of β-actin (Santa Cruz Biotechnology) or GAPDH (Research Diagnostics, Flanders, NJ, USA), which was provided as loading control. Sox2, Nanog, Runx2, Dlx5, STAT3, Ser-p-STAT3, and Tyr-p-STAT3 protein expression were confirmed in 3 donors, and data shown are representative.

14. Proteome Profiler Human Cytokine Array

The Proteome Profiler Human Cytokine Array Panel A Array Kit (R&D Systems Inc., Cat. No; ARY005) were used to analyze cytokines secreted in small, large, and mixed cell population of BM-MSCs. 36 different anti-cytokine antibodies have been spotted in duplicate on nitrocellulose membrane provided. Membranes were incubated in blocking solution for 1h on a rocking plate at room temperature. After washing membranes with 1X wash buffer three times, each media samples were added to membrane and incubated at 4℃ for overnight. After being washed with 1X wash buffer, the streptavidin-HRP was added to each membrane for 30min at room temperature. Cytokine spots bound to membranes were visualized on X-ray film using *Chemi Reagent Mix* provided. Signal quantification of each cytokine was measured by subtracting the background signal using the TINA 2.0 program from Fuji image scanners.

15. Interleukin-6 enzyme-linked immunosorbent assay (ELISA)

In order to measure amount of IL-6 secreted into culture medium from small, large, or mixed-cell population, we used cell culture supernatant for experiment using IL-6 ELISA kit (KOMA Biotech Inc., Seoul, Korea) according to the manufacture's instruction. In brief, cell culture supernatants were loaded into wells coated with antibody, and wells were incubated at room temperature for 4h on a microplate shaker. After being washed with provided wash buffer, all wells were incubated with detection antibody at room temperature for 2h. Finally, plates were read by a 96-well spectrophotometric microplate reader at 450 nm wavelength. All the assays were performed in duplicate ($n$ = 3). IL-6 concentrations were calculated from the standard curve.

16. Determination of IL-6 concentration

It is known that IL-6 induces the phosphorylation of STAT3 protein. To optimize the concentration of IL-6 (KOMA Biotech Inc.) for induction of STAT3 phosphorylation in BM-MSCs *in vitro*, the experimental groups were organized into control group (no treatment) and 3 experimental groups (IL-6 treatment; 1, 10, 50 ng/mL). The cells were cultured in cell culture plates for 24 h and the extent of STAT3 and pSTAT3 expressions was measured at 1, 4, 12, and 24 h after culture by western blotting.

17. Determination of IL-6 receptor antibody concentration

Tocilizumab (Actemra), humanized anti-human IL-6 receptor monoclonal antibody, was provided by Chugai Pharma Manufacturing CO., LTD via JW Pharmaceutical (Seoul, Korea). To optimize the concentration of Tocilizumab for inhibition of IL-6 signaling, the experimental groups were organized into control group (no treatment) and 2 experimental groups (Tocilizumab treatment; 25 or 50 ug/mL) under the presence of 50ng/mL of IL-6. The cells were cultured in cell culture plates for 24 h and the extent of STAT3 and pSTAT3 expressions

was measured at 24 h after culture by western blotting.

18. Cignal reporter assay for Sox2-dependent GFP reporter activity

The Cignal Reporter Assay for Sox2-dependent GFP reporter activity was performed following the manufacture's manuals (SABiosciences, Frederick, MD, USA). This reporter contains transcriptional regulatory elements for Sox2 (TRE: *AACAAAGAGT*). rhIL-6 or plasmids of Runx2 and Dlx5 (100ng) plus Sox2-dependent cignal reporter (50ng) were transfected into BM-MSCs or Hela cells using Lipofectamine$^{TM}$ 2000 (Invitrogen). After 36h, transfected cells were analyzed using a simple dual-luciferase assay (Promega) to determine activity of Sox2 signal for IL-6 treatment or siRNA effect of *Runx2* and *Dlx5*.

19. Statistical analysis

The statistical analysis for all results was performed using Student's t-test, and the data were expressed as means ± SD. Values of *, $p<0.05$ were considered statistically significant.

## III. RESULTS

### 1. Confirmation of cellular senescence in BM-MSCs during prolonged passage

First, we investigated senescence of BM-MSCs during prolonged passages. It is well-known that β-galactosidase (β-gal) activity is associated with *in vitro* senescence of cells[44]. In MSCs, β-gal activity was shown to increase during prolonged passage, but no difference was observed between BM-MSCs isolated from young and old donors[45]. Furthermore, senescent BM-MSCs exhibited increased cell size[46-48] and a flattened morphology with more actin stress fibers[45]. Given these results, we examined BM-MSC senescence by measuring senescence-associated (SA)-β-gal activity and observing the change in cell size during prolonged passage. SA-β-gal activity was significantly increased in late-passage BM-MSCs (P7) (Figure 1A). Cell size changes were also observed between early- and late-passage BM-MSCs. The increase in cell size was much higher in the late-passage BM-MSCs (16.2%) than in the early-passage BM-MSCs (2.75%) (Figure 1B). These results indicate that BM-MSCs senesce during prolonged serial subculture.

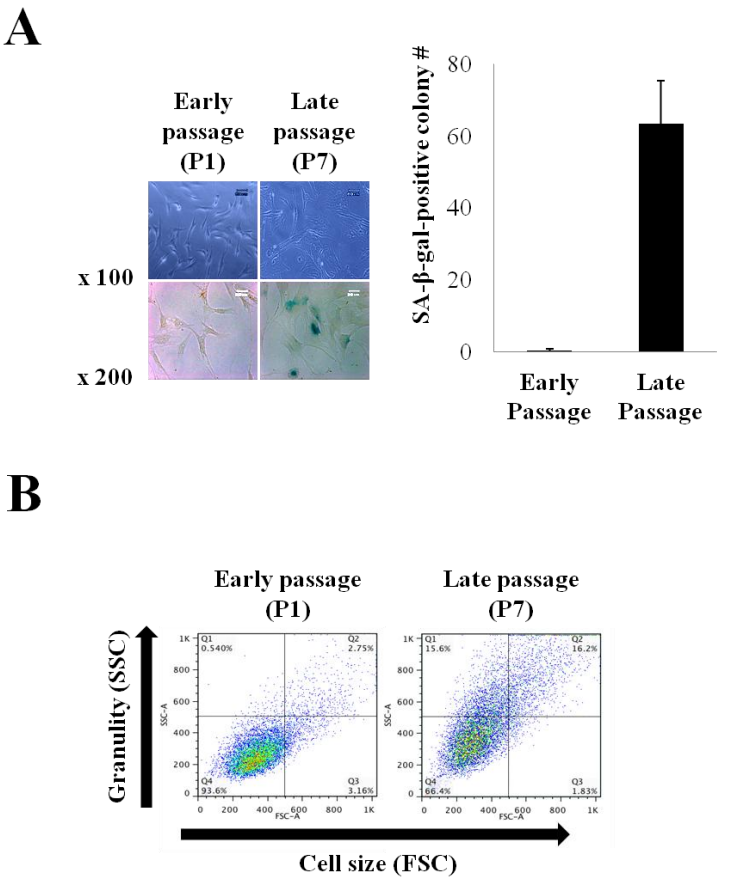



Figure 1. *In vitro* senescence of BM-MSCs during prolonged passage. (A) SA-β-gal staining was used to examine the effects of increasing passage number on BM-MSC senescence. SA-β-gal activity is increased in late-passage (P7) BM-MSCs compared to early passage (P1) BM-MSCs (left panel). SA-β-gal–positive cells were counted in triplicate by 3 independent observers (right panel). (B) FACS analysis was used to compare the distribution of cell sizes between early- and late-passage BM-MSCs. FSC indicates the size of cells analyzed by FACS, and SSC indicates the granularity of cells analyzed. These data have been confirmed in all the 3 donors tested. Representative data shown here are those of a 64-year-old female donor (A) and a 34-year-old female donor (B).

2. Decreased renewal capacity during prolonged cultivation of BM-MSCs

Next, we investigated the colony-forming ability and mRNA expression of cell cycle-related genes during prolonged passage of BM-MSCs. The colony-forming abilities of later passage BM-MSCs (P3 and P7) were markedly lower than that of early-passage (P1) BM-MSCs (Figure 2A). To further assess the effects of prolonged cultivation on the expression of genes involved in cell cycle progression, real-time PCR was performed. Cell cycle progression is closely regulated by cyclins, proteins that activate cyclin-dependent kinases (CDKs). Real-time PCR analysis revealed a marked decrease in the expression of genes known to be involved in S-phase and mitosis, such as cyclin A2 (CCNA2), during the passage of cells from P1 to P7. Cyclin D1 (CCND1), a gene associated with actively proliferating cells, did not show any statistically significant changes during serial subculture (Figure 2B). In addition, we analyzed the expression of 2 genes (*CDK2* and *CDK4*) known to be involved in G1/S phase transition. *CDK2* and *CDK4* expression decreased during serial subculture up to P7 (Figure 2B). Collectively, these data suggest that decrease in cell proliferation during prolonged passage is accompanied by a decrease in the expression of cell cycle-related genes, such as *CCNA2*, *CDK2*, and *CDK4*.

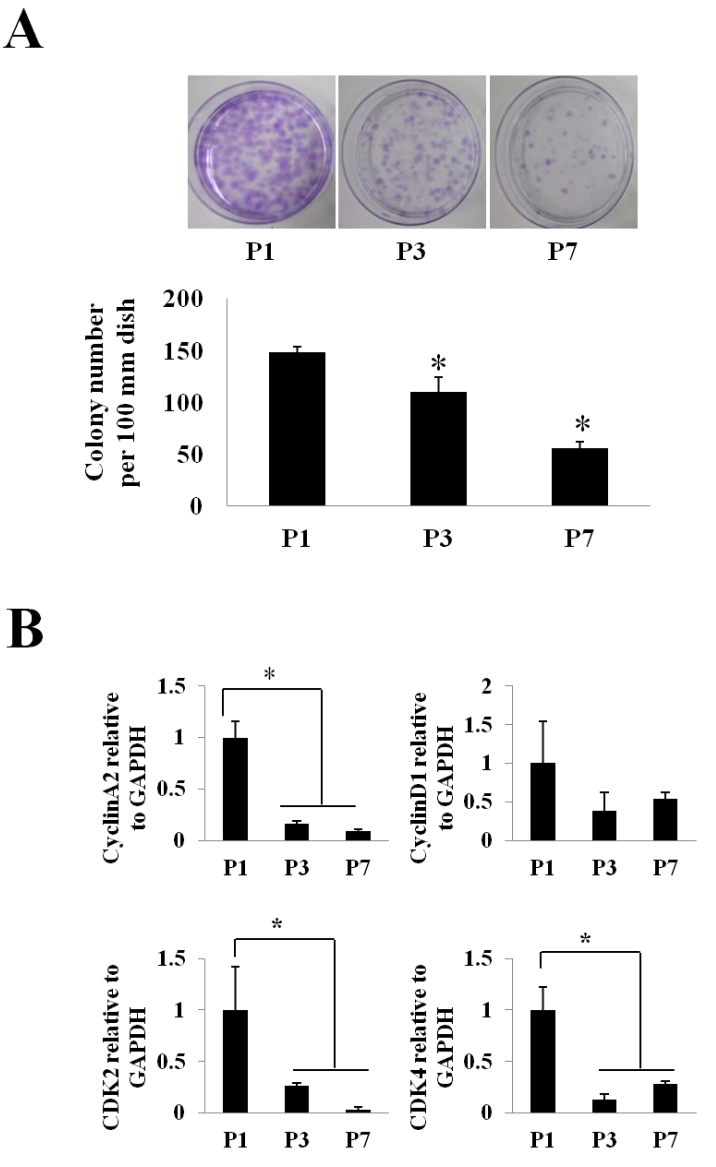
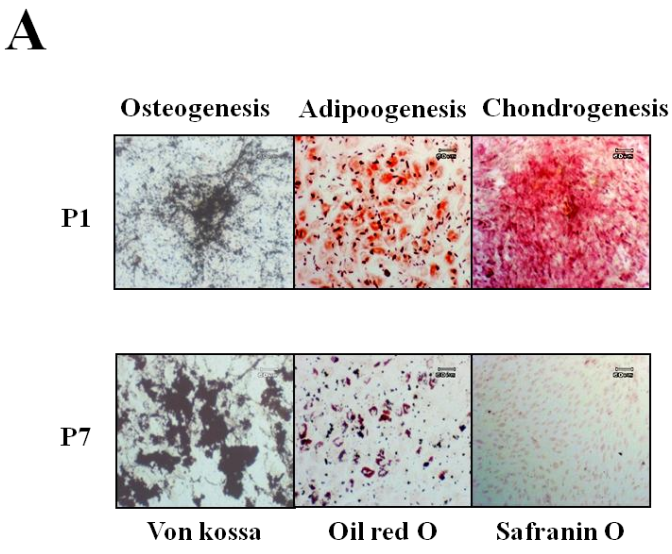



Figure 2. Decrease in the number of colony-forming cells and expression of cell cycle-related genes during prolonged passage. (A) P1, P3, and P7-MSCs from the same donor were seeded at 1 x $10^3$ cells in 100-mm culture dishes. Cells were then cultured in DMEM-LG containing 20% FBS for 12 days to examine cell colony formation (violet). *, $P < 0.05$ compared to P1 MSCs. (B) The

mRNA expression patterns of cell cycle-related genes in undifferentiated P1, P3, and P7-MSCs were examined using real-time PCR. After subculturing from each previous passage, P1, P3, and P7-MSCs were grown in DMEM-LG containing 10% FBS for 3 days. Cells were harvested at 80%–90% confluency for RNA extraction. Each experiment was performed in triplicate ($n = 3$). * $P < 0.05$ compared to P1-MSCs.

3. Decrease in multi-potency and commitment to the osteogenic lineage in late-passage BM-MSCs

Both early-passage (P1) and late-passage (P7) BM-MSCs readily differentiated into osteoblastic cells when cultured in osteogenic medium for 14 days, but the osteogenic potential of late-passage BM-MSCs was higher than that of early-passage BM-MSCs. The adipogenic potential of late-passage BM-MSCs was lower than that of early-passage BM-MSCs, and chondrogenic differentiation capacity was absent in late-passage BM-MSCs (Figure 3A). Furthermore, late-passage BM-MSCs expressed ALP, an early osteogenic marker, in the undifferentiated state, with decrease in colony-forming ability and increase in cell size (Figure 3B). These results indicate that BM-MSCs become committed to the osteogenic lineage as they approach senescence, and they lose their potentials to differentiate along the other lineages (adipogenic and chondrogenic lineages).



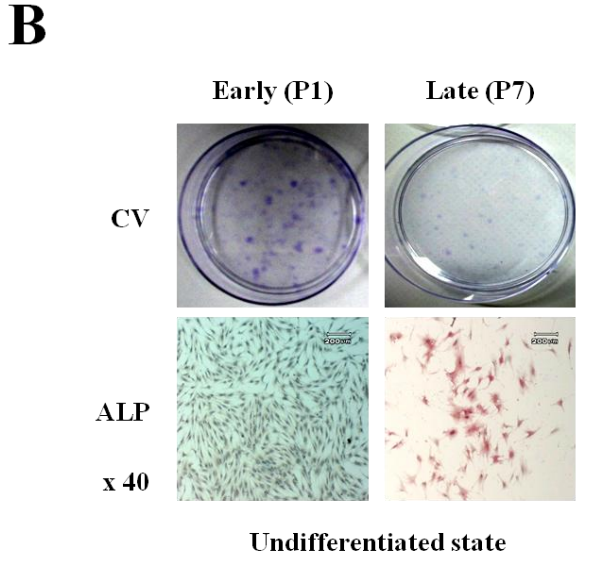




Figure 3. Decrease in multi-potency and expression of alkaline phosphatase in late-passage BM-MSCs. (A) P1 and P7 MSCs from the same donor were seeded at 8 x $10^4$ cells/well in 12-well culture plates. Cells were then cultured in osteogenic, adipogenic, and chondrogenic media to determine their osteogenic,

adipogenic and chondrogenic differentiation potential, respectively. The above data have been confirmed in all the 3 donors tested. Representative data shown here are those of a 19-year-old male donor. (B) Crystal violet (CV) staining was used to compare the colony-forming abilities of early- and late-passage BM-MSCs (upper). Alkaline phosphatase (ALP) staining was used to compare the degree of osteogenic lineage commitment in early- and late-passage BM-MSCs (lower) (n = 3, in triplicate).

4. Expression of osteogenic and stemness genes during prolonged passage

Commitment of BM-MSCs to osteogenic lineage occurs during prolonged expansion *in vitro* prior to cellular senescence[17] (Figure 3). Commitment to the osteogenic lineage is accompanied by increased expression of osteogenic genes, such as *Runx2* and *ALP*, whereas expression of stemness genes involved in renewal capacity and multi-potency, such as *Sox2*, *Oct4*, and *Nanog*, decreased in late passage MSCs[49]. Despite these reports, the correlation between osteogenic and stemness genes has not been investigated. Therefore, we examined the mRNA levels of genes related to osteogenic differentiation and stemness in P1, P3, and P7 MSCs. The mRNA levels of genes involved in osteogenesis, such as *Runx2*, *Dlx5*, *ALP*, and *type I collagen*, increased gradually from P1 to P7. Expression of *Sox2* and *Nanog* gradually decreased as cells progressed from P1 to P7. The down-regulation of stemness genes with increase in the passage number of BM-MSCs may be related to the decrease in cell proliferation and differentiation potential, including the ability to undergo differentiation along the adipogenic or chondrogenic lineage. However, *Oct4* expression levels remained constant throughout the senescence process. These data suggest that *Sox2* and *Nanog* are involved in maintaining the renewal capacity and multi-potency of BM-MSCs.

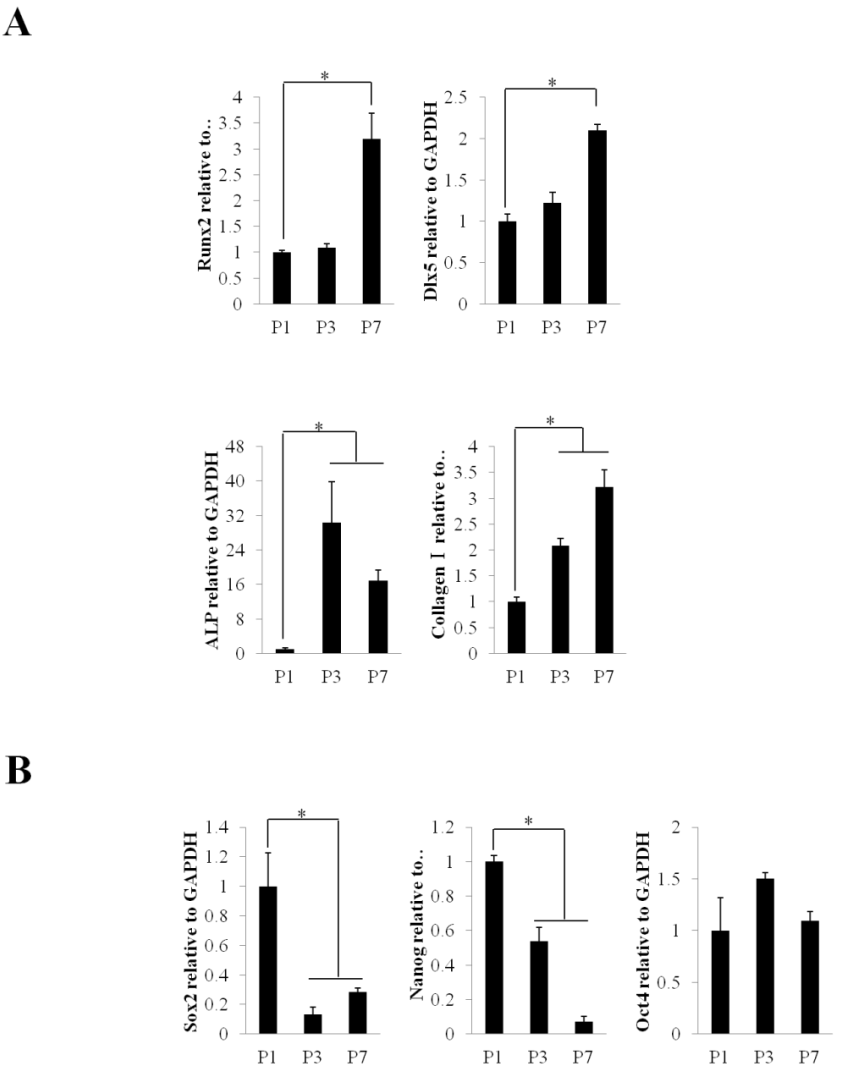



Figure 4. Expression of osteogenic and stemness genes during prolonged passage of BM-MSCs. The mRNA expression patterns of osteogenic (A) and stemness (B) genes in undifferentiated P1, P3, and P7 MSCs were examined using real-time PCR. After subculturing cells from each previous passage, P1, P3, and P7 MSCs were grown in DMEM-LG containing 10% FBS for 3 days. Each experiment was performed in triplicate (n = 3). *, $P < 0.05$ compared to P1.

5. Development of an alternative culture method to repopulate primitive cells in late-passage BM-MSCs.

We examined the changes in cell morphology and proliferation of late-passage BM-MSCs after altering the cell culture method. We observed that when BM-MSCs were cultivated at 17 cells/$cm^2$ (LD), which is less than the widely used density of 5000 cells/$cm^2$ (High-density; HD), cells displayed markedly enhanced cell population doubling. Based on these results, BM-MSCs were subcultured under HD conditions (5000 cells/$cm^2$) up to P7, at which point they had dramatically lower colony-forming and multi-differentiation abilities. The P7 BM-MSCs were then replated at either HD (5000 cells/$cm^2$; P7-HD MSC) or LD (17 cells/$cm^2$; P7-LD MSC) (Figure 5). The colony-forming ability of P7 BM-MSCs was remarkably lower than that of P1 BM-MSCs; this ability was restored by switching to LD culture conditions, but not HD culture conditions (Figure 6A). We also observed that P7-HD MSCs appeared flattened and enlarged, whereas many small spindle-shaped cells reappeared in the P7-LD MSCs (Figure 6A). BM-MSCs comprise heterogeneous-sized cell populations, and the existence of small cells has been thought to indicate more efficient stem cell properties[36,50]. FACS analysis of P7-HD MSCs revealed that the small-cell population (SCP) (region A, 5-10 μm) and the large-cell population (LCP) (region B, >30 μm) represented approximately 10.9% and 21.2% of the entire population, respectively. By contrast, in P7-LD MSCs, the SCP and the LCP represented approximately 30.9% and 8.7% of the entire population, respectively (Figure 6B). These results suggest that plating density is critical to the enrichment of primitive cells in heterogeneous BM-MSCs and that a much higher number of primitive cells can be repopulated, even from late-passage cells, using LD culture conditions.

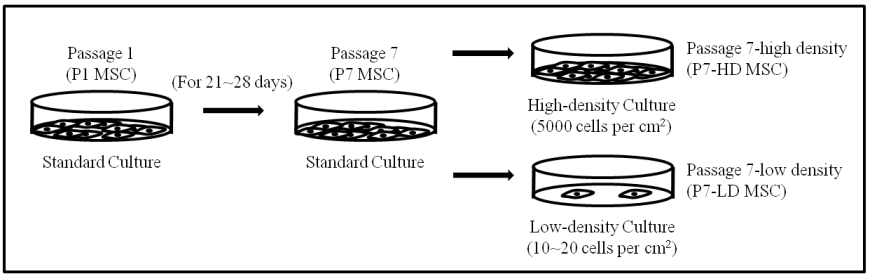
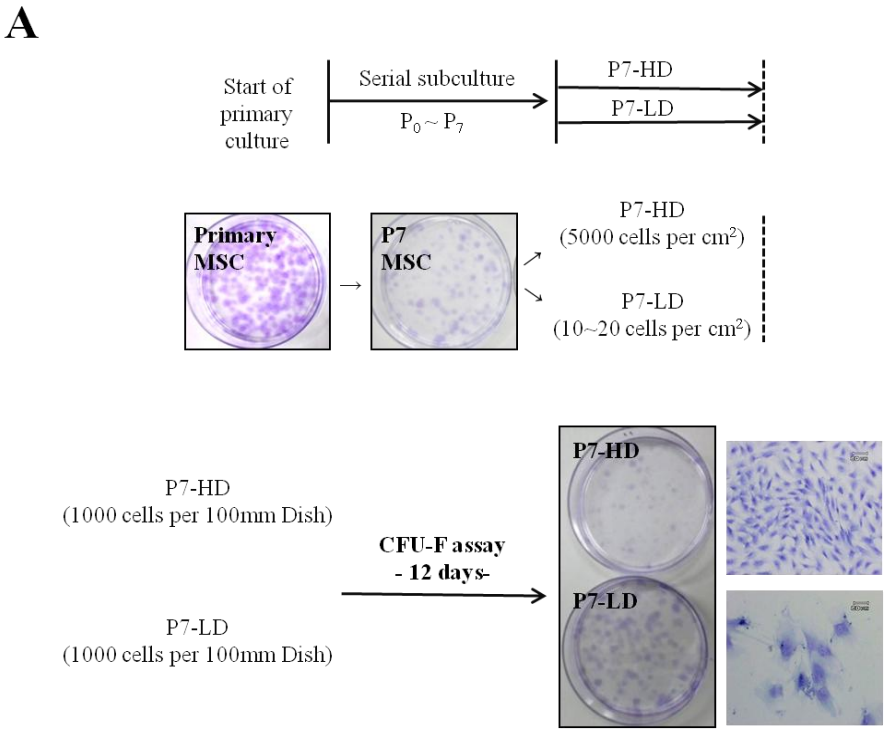
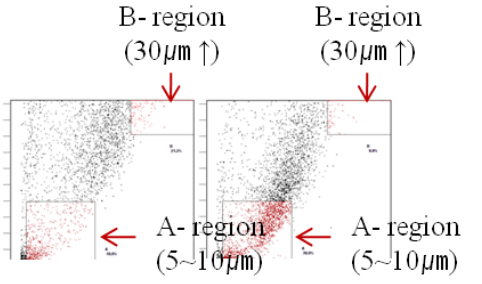


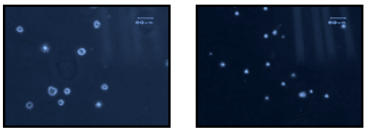

Figure 5. Development of an alternative culture method to repopulate the SCP in late-passage BM-MSCs. The overall cell culture scheme from early to late passage followed by replating under HD or LD conditions is shown. This culture scheme was maintained throughout the study.



**B**




| Cell distribution (%) | A region | B region |
|---|---|---|
| P7-HD cell | 10.9% | 21.2% |
| P7-LD cell | 30.9% | 8.7% |



Cell size observed through trypan blue staining

Figure 6. Application of LD culture conditions in late-passage BM-MSCs. (A) P1 MSCs, P7 MSCs, P7-HD, and P7-LD cells were seeded at 1 x $10^3$ cells in 100-mm culture dishes. Cells were then cultured in DMEM-LG containing 20% FBS for 12 days to examine colony formation (100x). (B) P7-HD and P7-LD cells were sorted into small (<10 μm) and large (>30 um) cells using FACS, and the size distribution of the sorted cells was analyzed. Cell size was determined using an optical microscope at (100x) magnification after trypan blue staining. The above data have been confirmed in all the 10 donors tested. Representative data shown here are those of a 35-year-old male donor.

6. Change in proliferative capacity and cell cycle distribution of late-passage BM-MSCs under LD culture conditions

The proliferative ability of human BM-MSCs gradually diminishes with continuous expansion. Besides the passage number, donor age is also a critical determinant of the proliferative ability of MSCs[33], given that the colony-forming ability of MSCs noticeably decreases in donors older than 20 years[34]. To determine whether the LD method was effective in restoring the proliferative ability of late-passage BM-MSCs from donors of different ages, a proliferation assay was performed in samples organized into age groups [10-20 years old (n = 3), 20-50 years old (n = 3), and 50-70 years old (n = 3)]. P1 BM-MSCs were cultured up to P7 as described in Figure 6A. Then, cells were harvested and replated at either 5000 (HD method) or 17 (LD method) cells/$cm^2$ in 100-mm dishes. The proliferative capacities of P7-HD and P7-LD MSCs were evaluated using a hexosaminidase assay. Compared to continuous HD culture, P7-LD MSCs showed significantly higher proliferation in every age group (Figure 7A). These results suggest that the LD culture method both favors cell proliferation and increases the number of small cells in a heterogeneous population of late-passage BM-MSCs, regardless of donor age.

Next, we analyzed the effect of HD and LD culture conditions on the cell cycle distribution of late-passage BM-MSCs from each age group. Cell cycle analysis revealed that on average, 87.78%, 9.53%, and 2.69% P7-HD MSCs were in $G_0/G_1$, S, and $G_2$/M phases, respectively, whereas, in the P7-LD MSCs, the $G_0/G_1$, S, and $G_2$/M phases constituted 73.53%, 20.35%, and 6.12% of the cell population respectively. The proportion of S-phase cells was higher in P7-LD MSCs than in P7-HD MSCs (Figure 7B). To further assess the effects of the LD method on the expression of genes involved in cell cycle distribution, we performed real-time PCR. The analysis revealed a marked increase in the expression of *CCNA2* (cyclin A2), *CCND1* (cyclin D1), *CDK2*, and *CDK4* (Figure 7C). Thus, in P7 cells cultured under LD conditions, the expression levels of cell cycle-related genes were much higher than that in HD-cultured

cells. Collectively, these data suggest that restoration of cell proliferation in response to altered plating density is mediated by cell cycle-related genes.

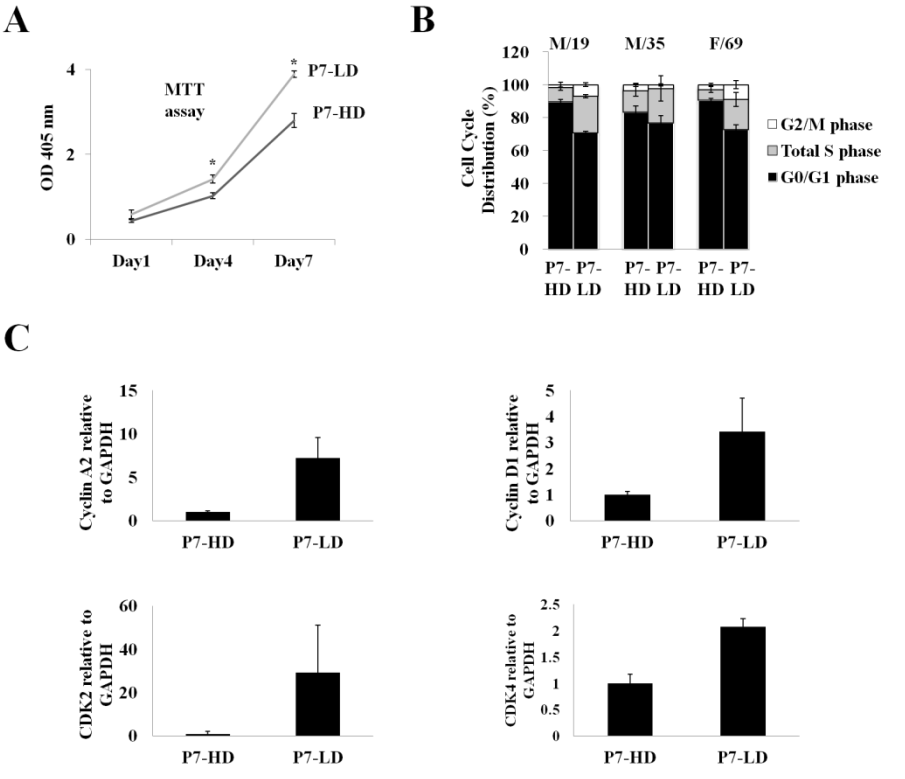



Figure 7. Effect of LD culture condition on the proliferation of late-passage BM-MSCs. (A) P7 MSCs from donors aged 10–20, 20–50 and 50–70 years were replated at an appropriate density (approximately 5000 or 17 cells/$cm^2$) on 100-mm dishes. The replated cells were then seeded into 24-well plates (1 x $10^4$ cells/well). Hexosaminidase assay was performed to determine the proliferative potential of density-controlled BM-MSCs (P7-HD and P7-LD cells). *, $P < 0.05$ compared to P7-HD MSCs. The representative data shown here are those of a 19-year-old male donor. (B) P7-HD and P7-LD cells from each age group were

harvested, and 1 x $10^6$ cells were used for cell cycle analysis. Each experiment was performed in triplicate. The tabulated data presented here show the mean values from 3 donors. (C) The mRNA expression patterns of cell cycle-related genes in undifferentiated P7-HD and P7-LD MSCs were examined using real-time PCR. After subculturing from each previous passage, P7-HD and P7-LD cells were grown in DMEM-LG containing 10% FBS for 3 days. Cells were harvested at 80%–90% confluency for RNA extraction. Each experiment was performed in triplicate ($n = 3$). *, $P < 0.05$ compared to P7-HD MSCs.

7. Enhanced differentiation potential in late-passage BM-MSCs cultured under LD conditions

We next examined the differentiation potential of P7-HD and P7-LD MSCs. Cells were cultivated up to P7 by using the HD method, according to the general scheme shown in previous data (Figure 6A). Cells were then grown using either the HD or LD method. Each repopulated cell culture was then replated in 12-well plates at identical cell densities, was grown to confluence, and was induced to differentiate into osteoblasts and adipocytes. Both P7-HD and P7-LD MSCs expressed ALP and showed accumulation of calcium-containing mineralized nodules on performing von Kossa staining after 14 days of osteogenic induction (Figure 8A, left). In the calcium content assay, P7-HD MSCs exhibited higher calcium accumulation compared to P7-LD MSCs (Figure 8B left). Although the multi-differentiation potential of BM-MSCs decreases after expansion in culture, the potential for osteogenic differentiation is retained or increased[17,18,51]. These reports indicate that MSCs differentiate into osteogenic cells with continued cell passaging. Adipogenic differentiation was examined using morphology and oil red O staining. P7-LD MSCs exhibited increased lipid droplet formation and more oil red O-stained cells compared with P7-HD MSCs (Figure 8A, middle). In addition, the absorbance of oil red O staining detected after de-staining was significantly different between the 2 cell populations (Figure 8B, middle). Chondrogenic differentiation was analyzed using safranin O staining. Similar to the results of adipogenic differentiation, P7-LD MSCs exhibited a higher chondrogenic differentiation capacity than P7-HD MSCs (Figure 8A right), and the absorbance of proteoglycans detected after de-staining was also significantly different between the 2 groups (Figure 8B, right).

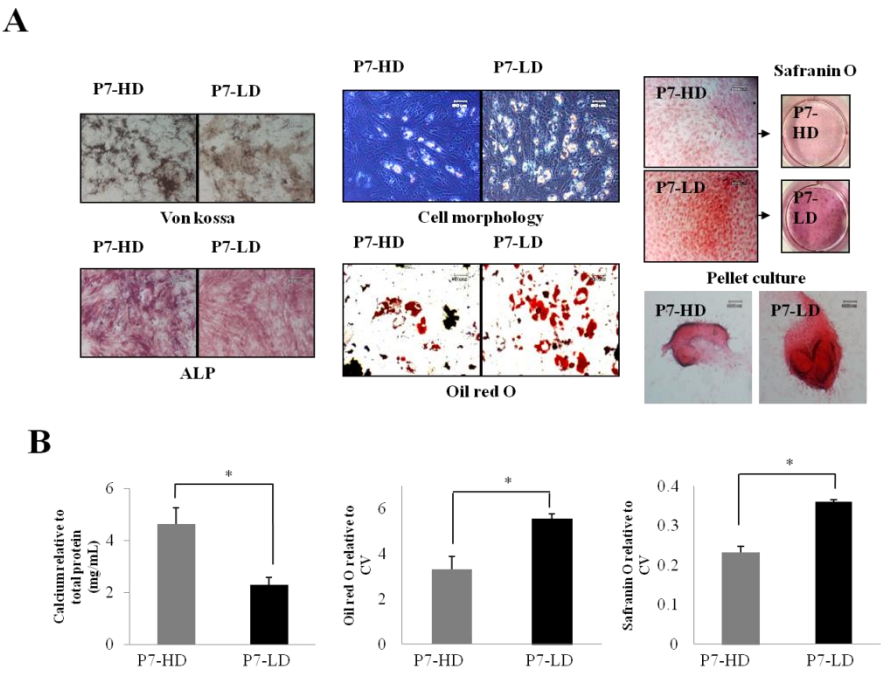



Figure 8. Effects of LD culture conditions on the multi-lineage differentiation potential of late-passage BM-MSCs. (A; left) P7-HD and P7-LD cells (8 x $10^4$ cells/well in 12-well plates) were incubated in osteogenic medium for 14 days. Then, ALP and von Kossa staining were performed to detect mineral deposition. (B; left) A calcium content assay was performed to confirm the results of von Kossa staining. (A; middle) P7-HD and P7-LD cells (8 x $10^4$ cells/well in 12-well plates) were incubated in adipogenic medium for 14 days. After incubation, oil red O staining was performed to detect lipid droplets. (B; middle) For quantitative analysis of oil red O staining, absorbance was detected at 500 nm following de-staining with isopropanol for 30 min. (A; right) P7-HD and P7-LD cells (8 x $10^4$ cells/well in 12-well plates) were incubated for 14 days in chondrogenic medium containing 10 ng/mL TGFβ-3. After incubation, safranin O staining was performed to detect proteoglycans (100x, Con, undifferentiated control BM-MSCs; CH, chondrogenic-

differentiated BM-MSCs). (B; right) For quantitative analysis of safranin O staining, absorbance was detected at 490 nm following de-staining with 100% ethanol for 20 min. Each experiment was performed in triplicate (n = 3). *, $P < 0.05$ compared to P7-HD MSCs.

8. Decrease in osteogenic gene expression and increase in stemness gene expression in late-passage BM-MSCs cultured under LD conditions.

We examined the mRNA levels of genes related to osteogenic differentiation and stemness in P7-HD and P7-LD MSCs. Genes that were expressed at higher levels during HD culture, such as *Runx2*, *Dlx5*, *ALP*, and *type I collagen*, exhibited lower expression following LD culture (Figure 9A). These results indicate that LD culture can repress the expression of osteogenic marker genes, thereby diminishing or reversing the commitment of the osteogenic precursor cells. The lower expression of *Sox2* and *Nanog* observed in P7 BM-MSCs was restored to normal levels following LD culture. Although the expression of *Oct4* did not significantly change with increase in passage number, its expression was slightly up-regulated after LD culture (Figure 9B). The up-regulation of stemness genes during LD culture of late-passage BM-MSCs may be related to the enhanced cell proliferation and restored differentiation potential, including the ability to undergo adipogenesis and chondrogenesis.

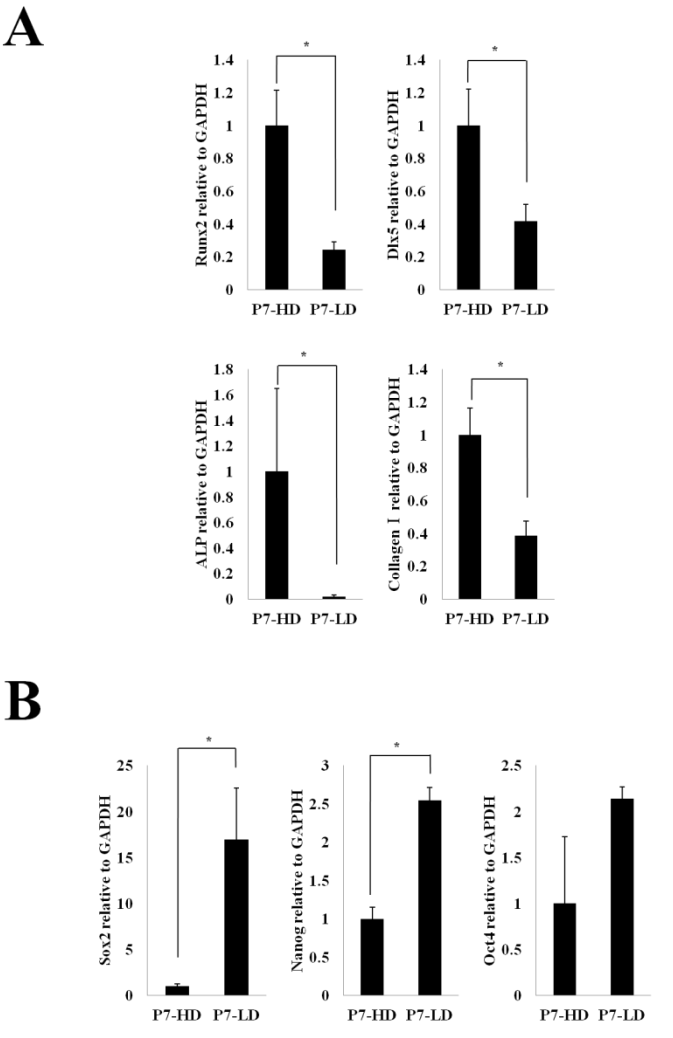
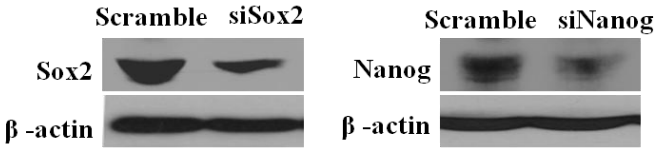



Figure 9. Effects of LD culture conditions on the commitment to the osteogenic lineage and stemness loss of late-passage BM-MSCs. The mRNA expression patterns of (A) osteogenic and (B) stemness genes in undifferentiated P7-HD and P7-LD MSCs were examined using real-time PCR. After subculturing, P7-HD and P7-LD cells were grown in DMEM-LG containing 10% FBS for 3 days. Each experiment was performed in triplicate (n = 3). *, $P < 0.05$ compared to P7-HD MSCs.

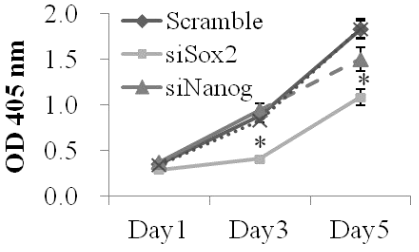
9. Importance of stemness genes for maintenance of renewal capacity and multi-potency in BM-MSCs

As described above, *Sox2* and *Nanog* but not *Oct4* were significantly down-regulated from P1 to P7 (Figure 4). To investigate the roles of *Sox*2 and *Nanog* in BM-MSC proliferation and differentiation, early-passage BM-MSCs were treated with small interfering RNAs (siRNAs) targeting each gene. Expression of *Sox2* and *Nanog* was lower in *Sox2* and *Nanog*-siRNA-transfected cells (45% and 43% reductions, respectively) than in scrambled-siRNA-treated cells (Figure 10A). The proliferation rate of *Sox2*-siRNA- transfected cells was lower than that of scrambled-siRNA-transfected cells (Figure 10B). However, Nanog knockdown had no inhibitory effect on proliferation. In a CFU assay, the number of *Sox2*-siRNA-transfected cell colonies was significantly lower compared to that of scrambled-siRNA-transfected cells, but this was not the case for *Nanog*-siRNA-transfected cells (Figure 10C). Next, we examined the potential of siRNA-transfected BM-MSCs to differentiate along the osteogenic, adipogenic and chondrogenic lineages. *Sox2*-siRNA-transfected cells had lower osteogenic, adipogenic and chondrogenic potential in all the donors tested, compared to scrambled-siRNA-transfected cells, whereas the differentiation potential of *Nanog*-siRNA-transfected cells along the 3 lineages was not noticeably different (Figure 10D). However, results from multiple donors showed variations in the differentiation abilities (data not shown). In addition, Sox2 overexpression using a lentiviral vector enhanced colony-forming ability in late-passage (P7) BM-MSCs compared to that for mock vector-infected BM-MSCs (data not shown). These results suggest that Sox2 plays an important role in the proliferation and multi-potency of BM-MSCs, as seen by the reproducibility of results from multiple donors.

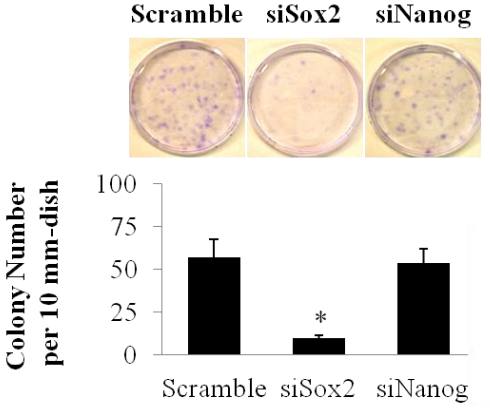
**A**


Scramble
siSox2
Sox2
β -actin
Scramble
siNanog
Nanog
β -actin

**B**


OD 405 nm
2.0
1.5
1.0
0.5
0.0
Scramble
siSox2
siNanog
*
*
Day1
Day3
Day5

**C**


Scramble
siSox2
siNanog
Colony Number per 10 mm-dish
100
75
50
25
0
*
Scramble
siSox2
siNanog

**D**

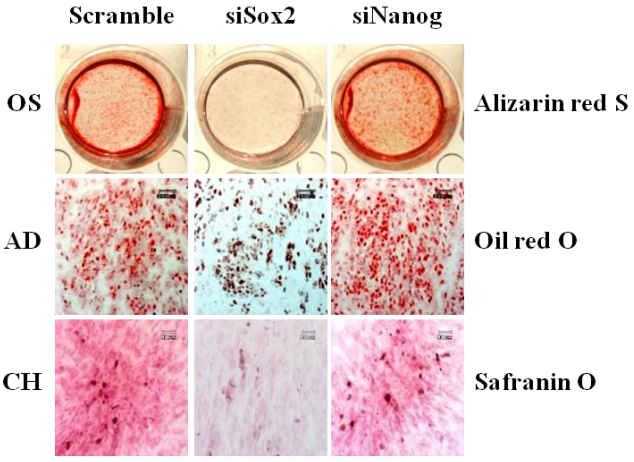



Figure 10. Effect of Sox2 and Nanog knock-down on BM-MSC proliferation and colony-forming ability. (A) The protein expression patterns of scrambled-, Sox2-, and Nanog-siRNA-transfected BM-MSCs were examined by western blot analysis. (B) Hexosaminidase assay was performed to determine the proliferative potential of siRNA-transfected BM-MSCs. Each experiment was performed in triplicate (n = 3). *, $P < 0.05$ compared to scrambled-siRNA-transfected BM-MSCs. (C) Scrambled-, Sox2-, and Nanog-siRNA -transfected cells were seeded at 1 x $10^3$ cells in 100-mm culture dishes. Cells were then cultured in DMEM-LG containing 20% FBS for 12 days to examine colony formation (violet). Each experiment was performed in triplicate (n = 3). *, $P < 0.05$ compared to scrambled-siRNA-transfected BM-MSCs. (D) Scrambled-, Sox2-, and Nanog-siRNA transfected cells were seeded at 8 x $10^4$ cells/well in 12-well culture plates. Cells were then cultured in osteogenic, adipogenic and chondrogenic media to determine their osteogenic, adipogenic, and chondrogenic differentiation potential, respectively. Each experiment was performed in triplicate (n = 3).

10. Change in the Sox2-positive cell population of heterogeneous BM-MSCs during prolonged passage

Late-passage BM-MSCs exhibited increased expression of ALP, an early osteogenic marker, in the undifferentiated state. To confirm this phenomenon, we next examined the protein expression of genes related to osteogenic differentiation and stemness in early- and late-passage BM-MSCs. Runx2 and Dlx5, which are important osteogenic transcription factors, were expressed at higher levels in late-passage BM-MSCs compared to early-passage BM-MSCs. On the other hand, expression of the Sox2 protein, one of the important factors for stemness maintenance in MSCs[30,52-55], was dramatically decreased in late-passage BM-MSCs (Figure 11A). In addition, FACS analysis of Sox2 expression revealed differences between the 2 groups: 52.84% in early-passage BM-MSCs vs. 10.72% in late-passage BM-MSCs (Figure 11B). These results suggest that late-passage BM-MSCs contain a subpopulation of more committed osteogenic cells that increases during subsequent passage, and they lose stemness with a decrease in the numbers of Sox2-positive BM-MSCs.

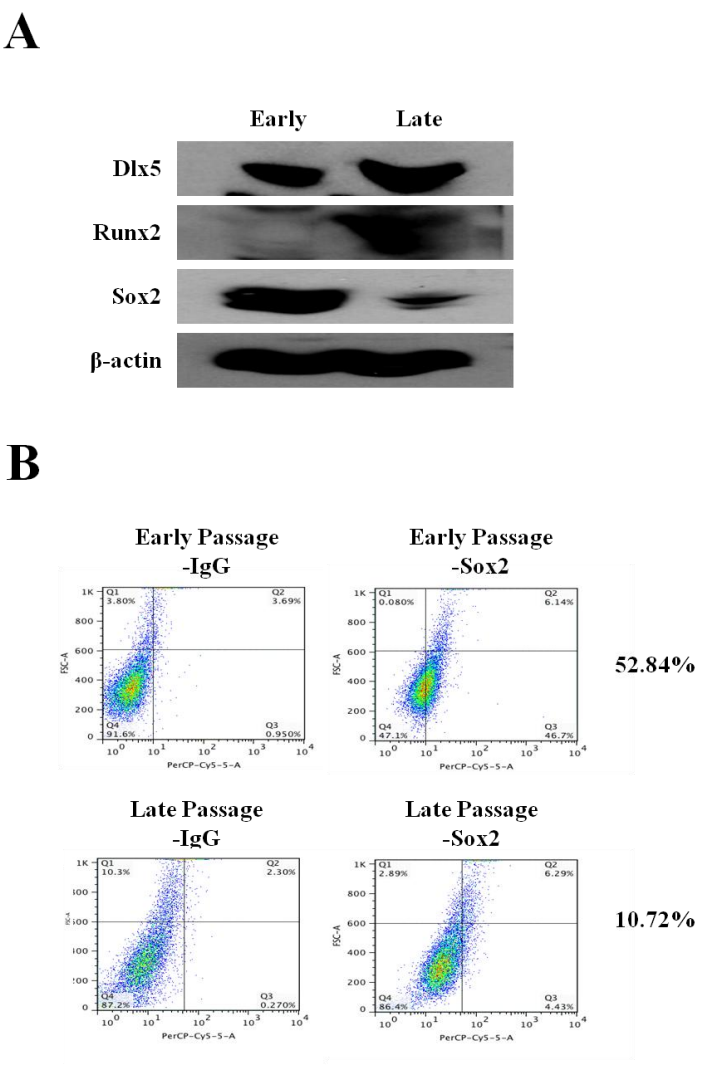



Figure 11. Comparison of Sox2 expression between early and late passage BM-MSCs. (A) Protein expression levels of a stemness gene (*Sox2*) and osteogenic genes (*Runx2* and *Dlx5*) were examined by western blot analysis. β-actin was used as the loading control. (B) Early- and late-passage BM-MSCs were harvested from *in vitro* culture, stained with an antibody against Sox2-PerCP-Cy or control IgG-PerCP-Cy, and subjected to FACS analysis.

11. Characterization of the different subpopulations of heterogeneous BM-MSCs by cell size

BM-MSCs are a heterogeneous cell population comprising cell type with different surface markers, cell size, and differentiation potential. Using these features, BM-MSCs can be divided into 2 cell types according to size and morphology[19]. The SCP exhibits spindle-shaped morphology and rapid proliferation, whereas the LCP shows flattened morphology and slow proliferation. Colter et al. designated these cell populations as RS cells (rapidly self-renewing cells/small in size) and mMSCs (mature MSC/large in size), respectively[56]. RS cells can differentiate along the osteogenic, adipogenic, and chondorgenic lineages, whereas mMSCs fail to differentiate along the adipogenic and chondrogenic lineages. These results indicate that the LCP is committed to the osteogenic lineage compared to the SCP. In a heterogeneous cell population, senescent cells can affect the fate (proliferation, senescence, and differentiation status) of neighboring cells through secretion of cytokines[21,57,58]. Based on these reports, we hypothesized that the decrease in renewal capacity and multi-potency of BM-MSCs during prolonged expansion *in vitro* is due to interaction between the different cell populations of BM-MSCs. To determine if this was the case, we sorted BM-MSCs by cell size using the FACS cell sorter (Beckman Coulter) (Figure 12). The sorted cells exhibited small, spindle-shaped (SCP) or large, round morphology (LCP) (Figure 13A). SA-β-gal assay revealed that the LCP was a more senescent cell population compared to the SCP (Figure 13B). Furthermore, the SCP had a higher colony-forming ability than the LCP (Figure 13C). FACS analysis revealed differences in ALP levels between the 2 populations: 3.87% in the SCP and 31.4% in the LCP (Figure 13D). These results suggest that only the SCP in heterogeneous BM-MSCs can proliferate with higher efficiency compared to its larger counterparts. In addition, the LCP is thought to be senescent and committed to the osteogenic lineage.

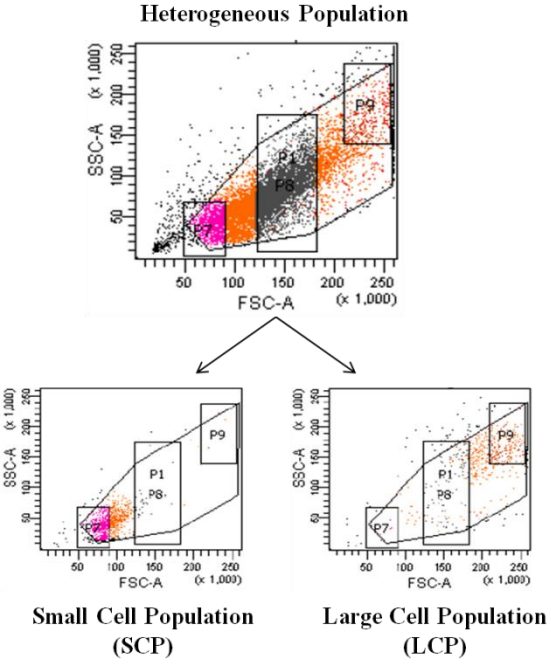
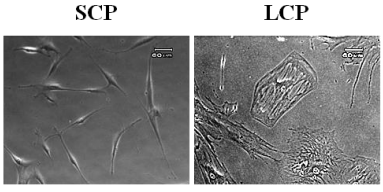
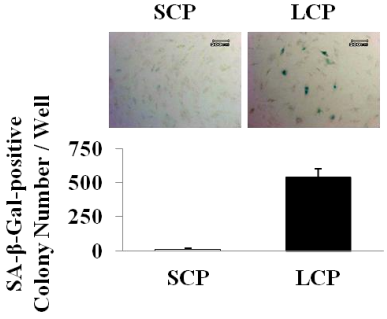



Figure 12. Scheme for the isolation of SCP and LCP from heterogeneous BM-MSCs. BM-MSCs were harvested by incubation with 0.25% trypsin/EDTA and washed twice in PBS. Cells were resuspended in pre-warmed PBS, and the SCP and LCP were sorted and analyzed using FACS.

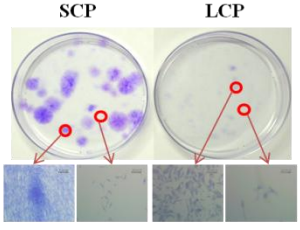
A


SCP
LCP

B


SCP
LCP
SA-β-Gal-positive
Colony Number / Well
750
500
250
0
SCP
LCP

C


SCP
LCP

D

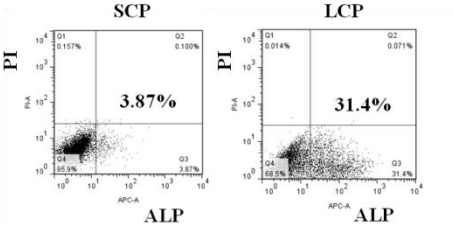

SCP
LCP
PI
3.87%
31.4%
ALP
ALP

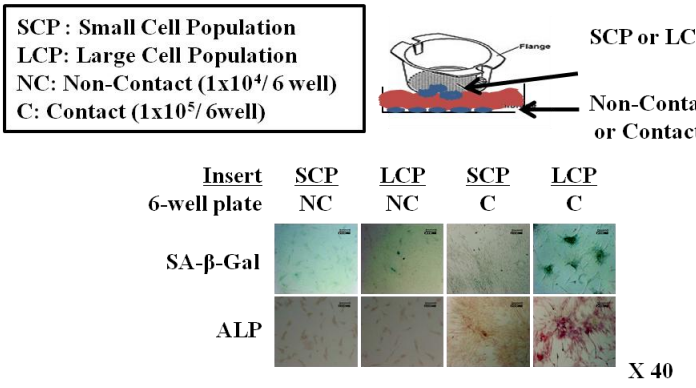
Figure 13. Characteristics of SCP and LCP isolated from heterogeneous BM-MSCs. (A) The morphology of cells fractionated by size using FACS was examined under a phase-contrast microscope. Cells in the SCP are small and spindle-shaped, whereas cells in the LCP are large and exhibit a flattened morphology. (B) SA-β-gal staining was used to determine the extent of cellular senescence in the SCP and LCP. SA-β-gal activity is higher in the LCP than in the SCP. SA-β-gal-positive cells were counted in triplicate by 3 independent observers. (C) CV staining was used to evaluate the colony-forming ability of cells fractionated by size using FACS. The SCP shows better colony-forming ability than the LCP. (D) The SCP and LCP were stained with an antibody against MSCA-1-APC (PI, propidium iodide). Each experiment was performed in triplicate (n = 2).

12. Effects of LCP-secreted cytokines on cellular senescence and osteogenic lineage commitment of the SCP (primitive cell population)

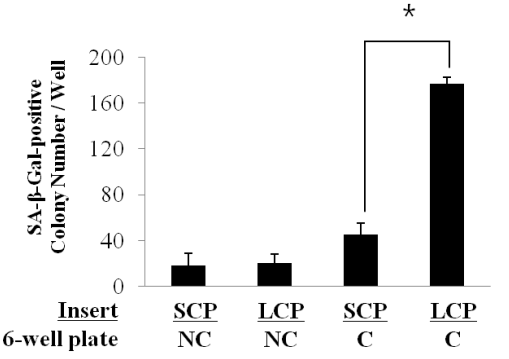
We sought to determine why LD-cultured MSCs had higher stemness potential than HD-cultured MSCs. Based on previous results, we hypothesized that LCP-secreted cytokines would affect the osteogenic lineage commitment and stemness loss of the SCP in heterogeneous BM-MSCs, given that the LCP, considered the senescent cell population, was more abundant than the SCP at later passages. Therefore, we designed an appropriate experiment to investigate the effect of LCP-secreted cytokines on the cellular senescence and osteogenic lineage commitment of the SCP without direct cell-to-cell contact. Briefly, the SCP was seeded in 6-well culture plates, and cell culture inserts with 0.4-μm pore size (Falcon, Franklin, NJ, USA) were inserted into the SCP-seeded culture plates. The LCP was then seeded into the cell culture insert with 0.4-μm pore size to avoid direct contact with the SCP; the culture insert is permeable to medium, thereby exposing cells to the secreted cytokines. In this experiment, the β-gal assay showed that LCP-secreted cytokines induced cellular senescence in the SCP under conditions of confluent cell density ($1 \times 10^5$ cells/well of a 6-well plate), but not under non-contacting conditions ($1 \times 10^4$ cells/well) (Figure 14A and B). The LCP-secreted cytokines also induced osteogenic lineage commitment in the SCP by increasing ALP activity and upregulating *Runx2* and *Dlx5* mRNAs, whereas *Sox2* mRNA expression was decreased under confluent cell density conditions (Figure 14A and C). Ho et al. reported that the cell-to-cell contact-induced senescence model is a useful system for investigating the molecular mechanisms of MSC senescence[59]. They reported that cell-to-cell contact accelerates the senescence of human MSCs independently of telomere shortening and p53 activation. In the present study, we found that cell-to-cell contact alone can induce senescence and osteogenic gene expression in MSCs. However, senescence and osteogenic lineage commitment of BM-MSCs were more strongly induced by a combination of confluent cell density and LCP-secreted cytokines than by confluent density

alone.

**A**


SCP : Small Cell Population
LCP: Large Cell Population
NC: Non-Contact (1x10⁴/ 6 well)
C: Contact (1x10⁵/ 6well)
Flange
SCP or LCP
Non-Contact
or Contact
Insert
6-well plate
SCP NC
LCP NC
SCP C
LCP C
SA-β-Gal
ALP
X 40

**B**


SA-β-Gal-positive Colony Number / Well
200
160
120
80
40
0
*
Insert
6-well plate
SCP NC
LCP NC
SCP C
LCP C

**C**

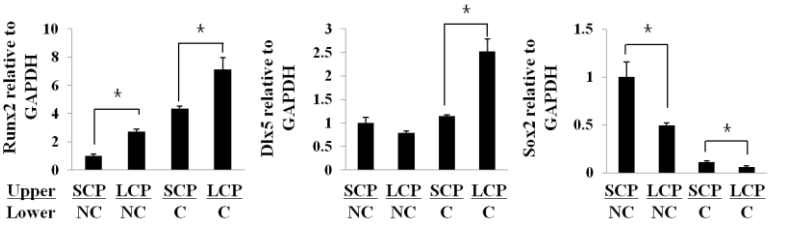
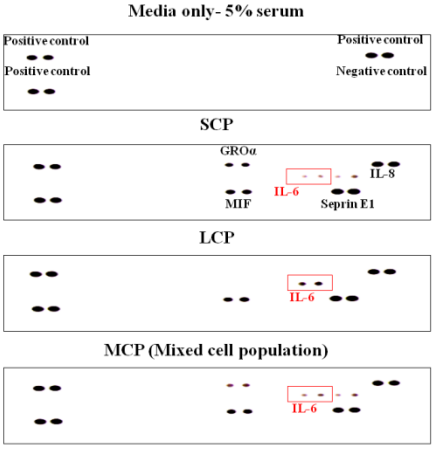

Runx2 relative to GAPDH
Dlx5 relative to GAPDH
Sox2 relative to GAPDH
*
Upper
Lower
SCP NC
LCP NC
SCP C
LCP C

Figure 14. Effects of LCP conditioned medium on cellular senescence and osteogenic lineage commitment of the SCP. (A) Cell culture inserts were used to investigate whether LCP-secreted cytokines affected cellular senescence and osteogenic lineage commitment of the SCP isolated by FACS. SCPs were seeded in 6-well culture plates at 1 x $10^4$ (non-contact; NC) or 1 x $10^5$ (contact; C) cells per well. The SCPs were allowed to attach to the culture plates, and cell culture inserts (0.4-μm pores) were inserted into the wells. Then, the LCPs or SCPs were seeded in the cell culture inserts at 1 x $10^5$ cells per well. The cells were stained for SA-β-gal and ALP at day 7. (B) SA-β-gal-positive cells were counted in triplicate by 3 independent observers. (C) Quantitative real-time PCR was performed to investigate the expression of *Runx2*, *Dlx5*, and *Sox2* mRNAs. The mRNA expression levels for each gene were normalized to *GAPDH* expression.

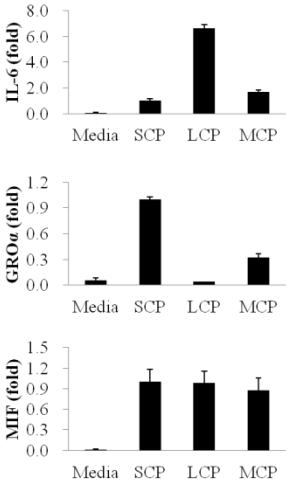
13. Lineage commitment of late-passage BM-MSCs during cellular senescence

We analyzed cytokines secreted from the SCP, LCP, and a mixed-cell population (MCP) using a human cytokine array. This analysis revealed the production of 5 cytokines (Groα, IL-6, IL-8, MIF, and Seprin E1) from the SCP and MCP compared to medium containing 5% serum (Figure 15A). Only 3 cytokines (IL-6, MIF, and Seprin E1) were secreted from the LCP (Figure 15A). Of these, IL-6 levels were significantly increased compared to that secreted from the SCP (6.7-fold) and MCP (3.9-fold). We focused our attention on the ability of IL-6 to induce cellular senescence and osteogenic lineage commitment in the SCP because IL-6 can induce growth arrest in lung carcinoma cells[60] and stimulate mesenchymal progenitors differentiation along the osteogenic lineage[61]. We next examined the expression of *IL-6* mRNA in P4 MSCS, P5 SCP, P5 MCP, and P5 LCP. *IL-6* mRNA was highly expressed in the LCP compared to the SCP and MCP (Figure 16A). To measure the amounts of IL-6 secreted from the SCP, MCP, and LCP into the medium, the IL-6 enzyme-linked immunosorbent assay was performed using cell culture supernatants at 24 h and 48 h. IL-6 was secreted at much higher levels in the LCP (24h: 5.4-fold; 48h: 3.9-fold) than in the SCP and MCP (Figure 16B). These results suggest that the synthesis of IL-6 in the BM-MSC populations is LCP-dependent.

**A**




**B**




| Order | Cytokines | SCP | LCP | MCP | Array No. |
|---|---|---|---|---|---|
| 1 | IL-6 | weak | O | weak | [B15, B16] |
| 2 | GROα [CXCL1] | O | — | O | [A11, A12] |
| 3 | IL-8 [CXCL8] | O | — | O | [B17, B18] |
| 4 | MIF [GIF / DER6] | O | O | O | [D11, D12] |
| 5 | Seprin E1 [PAI-1] | O | O | O | [D17, D18] |

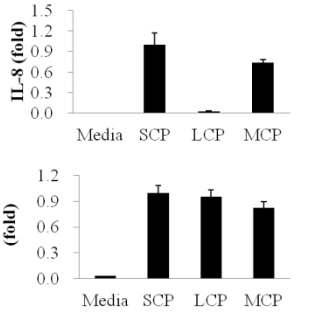



Figure 15. Identification of cytokines secreted from the LCP using a Human Cytokine Array. (A) Human cytokine array panels at 48 h using conditioned media from the SCP, LCP, or MCP cultured with DMEM-LG containing 5% FBS. (B) Cytokine signals were quantified by subtracting the background signal using the TINA 2.0 program of a Fuji image scanner.

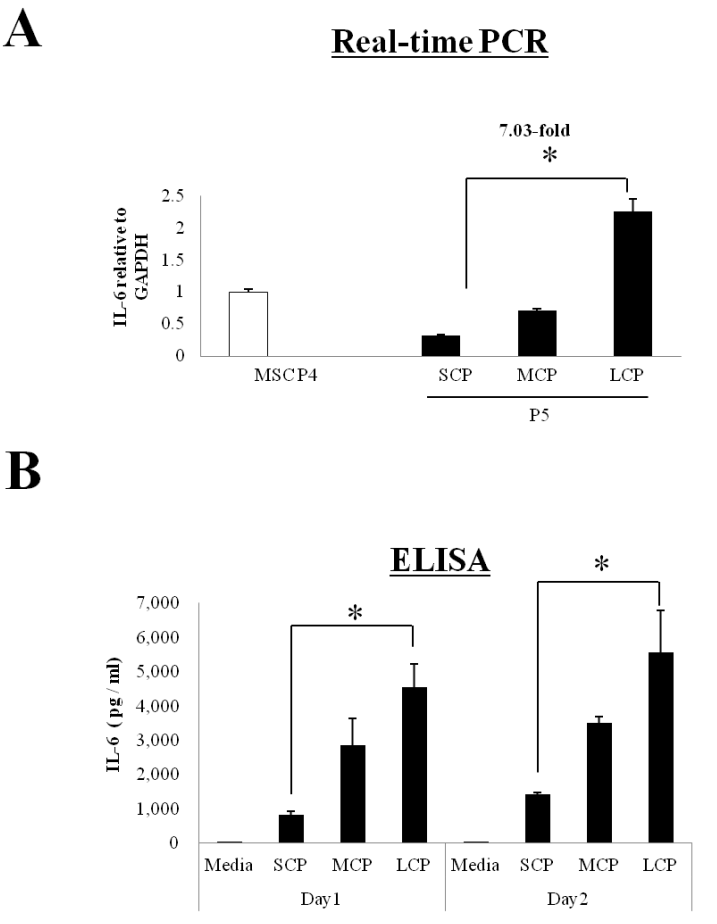



Figure 16. Changes in IL-6 expression in the LCP assayed by real-time PCR and ELISA. (A) Real-time PCR and (B) ELISA were performed to confirm IL-6 secretion from the LCP.

14. Effects of IL-6 on the renewal capacity and multi-potency of BM-MSCs

To establish conditions for IL-6 activity in BM-MSCs, human recombinant IL-6 was used. Treatment with 50 ng/mL IL-6 significantly increased $STAT3^{tyr705}$ phosphorylation at 12 to 24 h in BM-MSCs, as determined by western blot analysis, whereas total STAT3 and $STAT3^{ser727}$ phosphorylation remained constant. By contrast, IL-6 at 1 and 10 ng/mL had no significant effect on $STAT3^{tyr705}$ phosphorylation (Figure 17). Therefore, 50 ng/mL IL-6 was determined as an appropriate dose for enhancing IL-6 activity in BM-MSCs.

We next performed SA-β-gal staining after culturing BM-MSCs in the presence or absence of IL-6 for 7 days to examine the effects of IL-6 on BM-MSC senescence. The number of SA-β-gal–positive cells was increased in IL-6-treated BM-MSCs (Figure 18A). Furthermore, the CFU-F assay showed that 50 ng/mL IL-6 decreased the number of colony-forming cells in BM-MSCs (Figure 18B, upper). Proliferation of BM-MSCs was measured using MTX assay. The results revealed that the proliferative capacity of BM-MSCs was somewhat inhibited by 50 ng/mL IL-6 (Figure 18B, lower). These observations indicate that IL-6, one of the candidate senescence-inducing factors in the LCP-secreted cytokines, can induce cellular senescence and decrease the self-renewal capacity of the SCP.

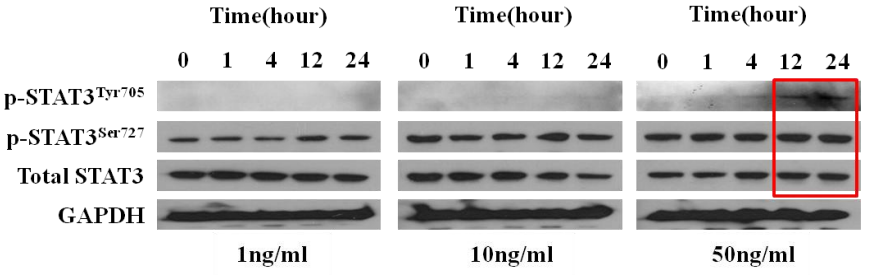



Figure 17. Dose- and time-dependent effects of IL-6 on STAT3 modulation in BM-MSCs. BM-MSCs were treated with different doses of IL-6 for 12 and 24 h. Expression of p-STAT3$^{Tyr705}$ is not affected by 1 and 10 ng/mL IL-6, but is enhanced at 4 to 24 hours by 50 ng/mL IL-6. However, total STAT3 and p-STAT3$^{Ser727}$ are constitutively expressed, regardless of the IL-6 concentration. The data shown here are representative of that observed in all the 3 donors tested.

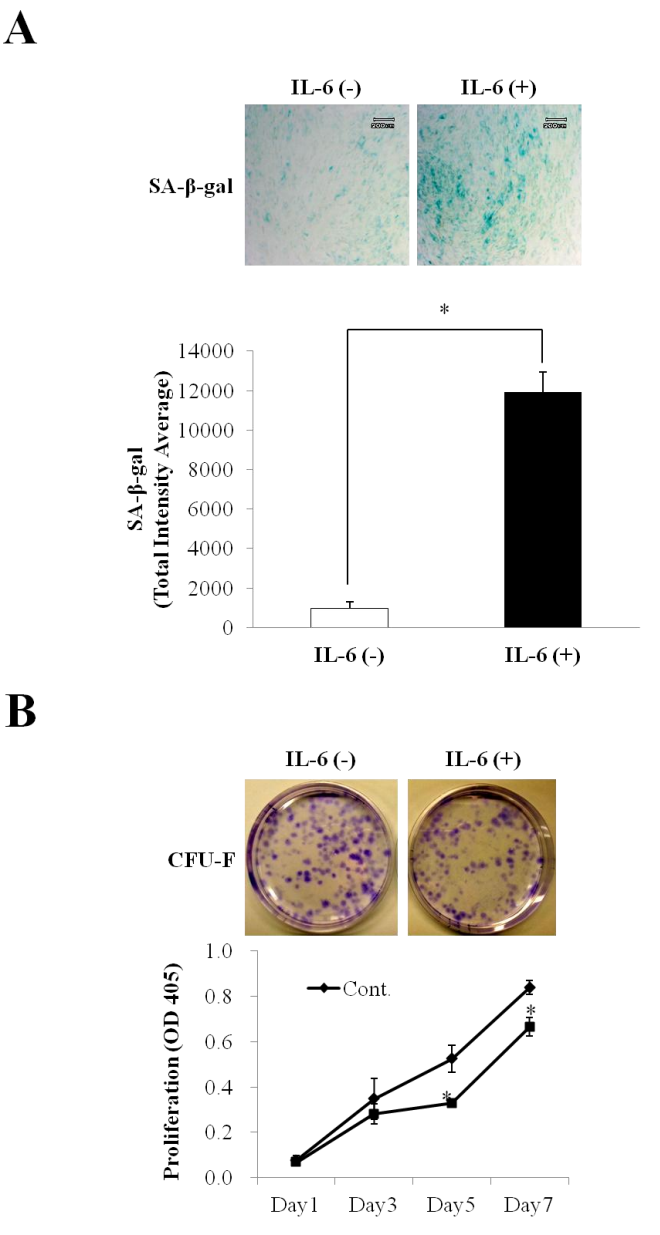



Figure 18. Effects of IL-6 on cellular senescence and renewal capacity of the SCP. (A) SA-β-gal staining was performed to examine the effects of IL-6 on BM-MSC senescence. SA-β-gal activity was increased in IL-6-treated SCPs (upper). The total area of SA-β-gal-positive cells was measured using

Metamorph Image Analyzer (Lower). (B) CV staining was performed to examine the colony-forming ability of IL-6-treated SCPs (upper). MTX assay was performed to determine the proliferative potential of IL-6-treated SCPs. Each experiment was performed in triplicate.

15. Effects of IL-6 on the multi-lineage differentiation potential of BM-MSCs

We next examined the multi-lineage differentiation potential of BM-MSCs in the presence or absence IL-6 (50 ng/mL). BM-MSCs were cultured in osteogenic medium with or without 50 ng/mL IL-6. At day 14, IL-6 enhanced the osteogenic differentiation potential by enhancing calcium deposition as seen in alizarin red S staining and by enhancing the expression of *Runx2* and o*steocalcin* mRNAs (Figure 19A). Adipogenic differentiation potential was decreased in IL-6 treated BM-MSCs compared to the cells cultured in adipogenic medium without IL-6. IL-6 inhibited the formation of lipid droplets in adipogenesis-induced BM-MSCs and suppressed the expression of *PPAR-γ* and a*diponectin* mRNAs (Figure 19B). During chondrogenic differentiation of BM-MSCs in the presence of IL-6, a significant decrease in proteoglycan synthesis was observed by safranin O staining. Real-time PCR analysis of *Sox9* and *type II collagen* expression confirmed the chondrogenic differentiation -inhibitory effect of IL-6 (Figure 19C). These results indicate that IL-6 induces osteogenic lineage commitment of BM-MSCs by increasing the expression of *Runx2* and o*steocalcin* mRNAs, and it inhibits adipogenic and chondrogenic differentiation of BM-MSCs by suppressing the expression of *PPAR-γ /adiponectin* and *Sox9/type II collagen* mRNAs, respectively.

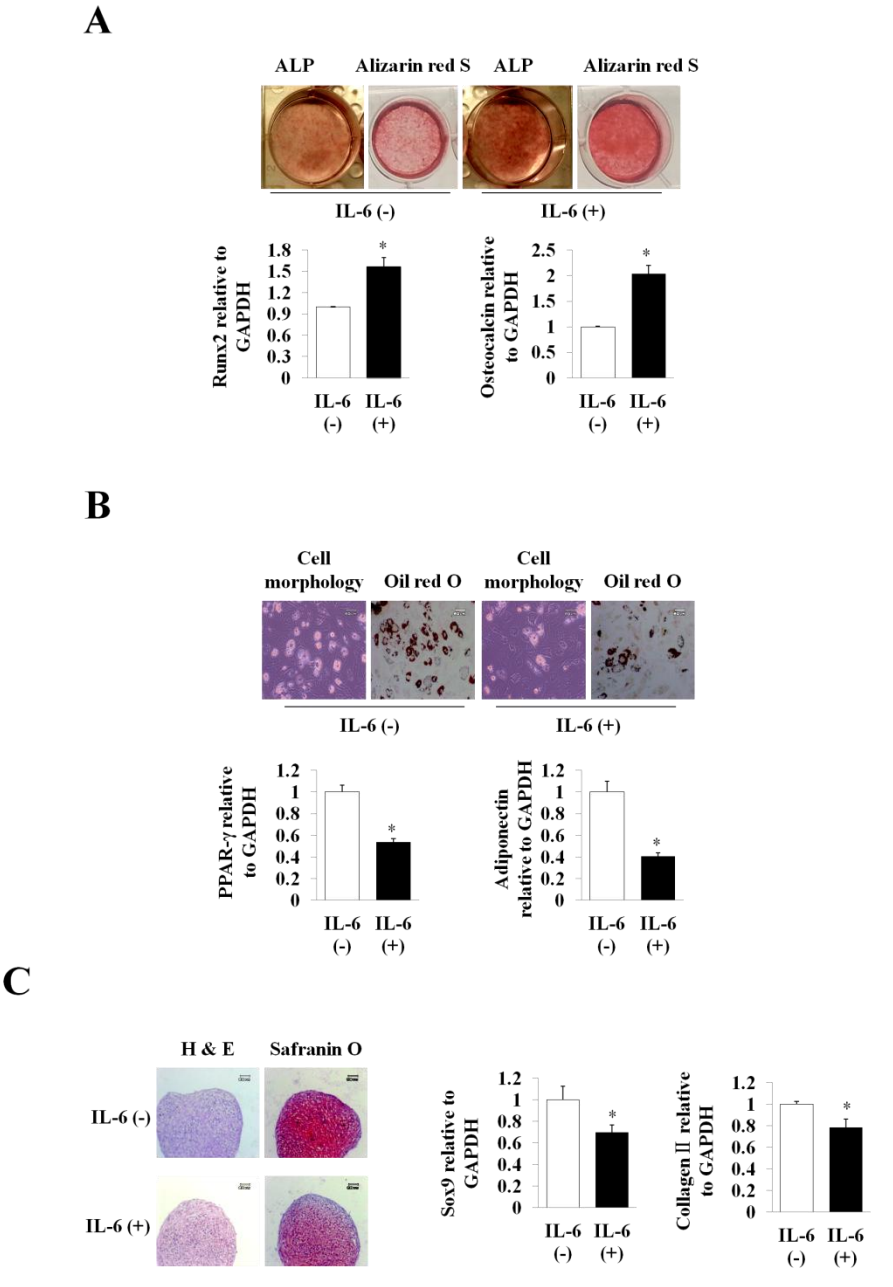



Figure 19. Effects of IL-6 on multi-potency of the SCP. (A) IL-6-treated or untreated SCPs (8 x $10^4$ cells per well in 12-well plates) were incubated in osteogenic medium for 14 days. After 14 days, alizarin red S staining was

performed to detect mineral deposition (upper). For quantitative analysis of alizarin red S staining, absorbance was measured at 595nm following de-staining with 10% cetylpyridinium chloride monohydrate for 30 min (lower). Each experiment was performed in triplicate (n = 3), and representative data are shown. (B) IL-6-treated or untreated SCPs (mass culture using 8 x $10^4$ cells per well in 24-well plates) were incubated in chondrogenic medium containing 10 ng/mL TGFβ-3 for 14 days. After 14 days, safranin O staining was performed to detect proteoglycans (upper). For quantitative analysis of safranin O staining, total area of safranin O-positive cells was measured using Metamorph Image Analyzer (lower). Each experiment was performed in triplicate (n = 3), and representative data are shown. (C) IL-6-treated or untreated SCPs (8 x $10^4$ cells per well in 12-well plates) were incubated in adipogenic medium for 14 days. After 14 days, oil red O staining was performed to detect lipid droplets (upper). For quantitative analysis of oil red O staining, the total area of safranin O-positive cells was measured using Metamorph Image Analyzer (lower). Each experiment was performed in triplicate (n = 3), and representative data are shown.

16. Effect of IL-6 on protein expression of osteogenic and stemness genes

Next, we investigated the expression of Runx2, Dlx5, and Sox2 proteins in IL-6-treated BM-MSCs. IL-6 increased the expression of Runx2 and Dlx5, and decreased the expression of Sox2 in a dose-dependent manner (Figure 20), independent of $STAT3^{Tyr705}$ phosphorylation (data not shown). These results suggest that IL-6 induces BM-MSC commitment to the osteogenic lineage by increasing the levels of Runx2 and Dlx5, and reduces stemness by decreasing the level of Sox2 protein expression. However, these phenomena do not appear to be STAT3 pathway-dependent.

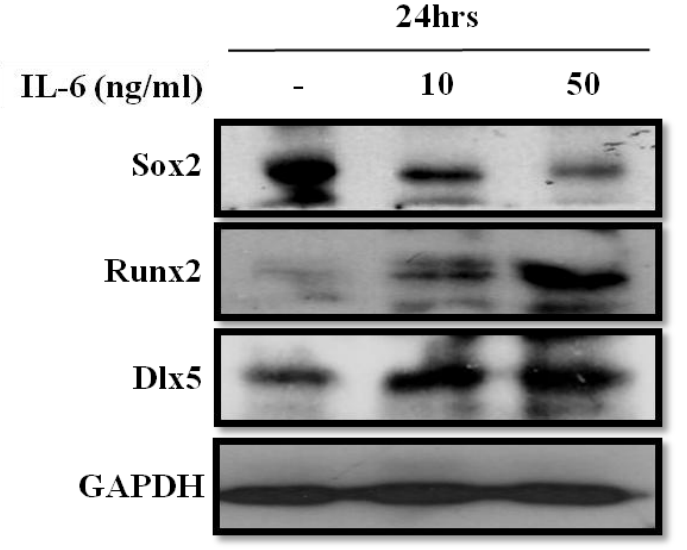
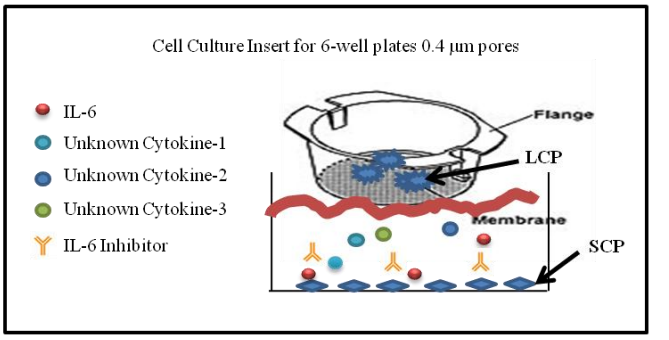



Figure 20. Changes in the protein levels of Sox2, Runx2, and Dlx5 in the presence of IL-6. Expression of Sox2, Runx2, and Dlx5 in IL-6-treated SCPs were examined by western blot analysis. GAPDH was used as the loading control.

17. Importance of LCP-secreted IL-6 in regulating osteogenic lineage commitment and stemness loss of the SCP

To block the activity of LCP-secreted IL-6, a humanized IL-6 receptor antibody (tocilizumab) was used. Tocilizumab binds to the IL-6 receptor and competitively inhibits IL-6 signal transduction[62]. Treatment with 25 or 50 μg/mL tocilizumab significantly decreased $STAT3^{tyr705}$ and $STAT3^{ser727}$ phosphorylation in the presence of IL-6 (50ng/mL) (Figure 21B). In particular, 50 μg/mL tocilizumab decreased total STAT3 protein levels. Therefore, we used both concentrations of tocilizumab (25 and 50 ng/mL) in this experiment. To study the role of IL-6 in the commitment to the osteogenic lineage induced by LCP-secreted cytokines, we performed the ALP assay under blockade of IL-6 signaling by tocilizumab using the cell culture insert model. As shown in Figure 22A, LCP-secreted cytokines significantly increased ALP activity in the SCP. However, tocilizumab inhibited this increase in ALP activity (Figure 22A). We also examined the protein expression levels of osteogenic (*Runx2* and *Dlx5*) and stemness genes (*Sox2*) in the SCP treated with LCP-secreted cytokines in the presence of tocilizumab. LCP-secreted cytokines increased the expression of Runx2 and Dlx5, and decreased the expression of Sox2 in the SCP. The inhibitory effect of the LCP-secreted cytokines on Sox2 protein expression was overcome by the addition of 50 μg/mL tocilizumab (Figure 22B). These results suggest that IL-6 plays an important role in the induction of commitment to the osteogenic lineage and loss of stemness in the SCP of heterogeneous BM-MSCs.

**A**




**B**

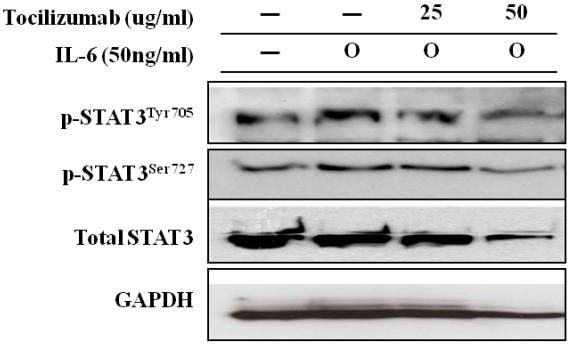



Figure 21. Effects of tocilizumab, an IL-6 receptor antibody, on STAT3 modulation in BM-MSCs. (A) Scheme for inhibition of IL-6 signaling in BM-MSCs. (B) Expression patterns of total STAT3, STAT3$^{tyr705}$, and STAT3$^{ser727}$ in the SCP treated with tocilizumab in the presence of IL-6 were examined by western blot analysis. GAPDH was used as the loading control.

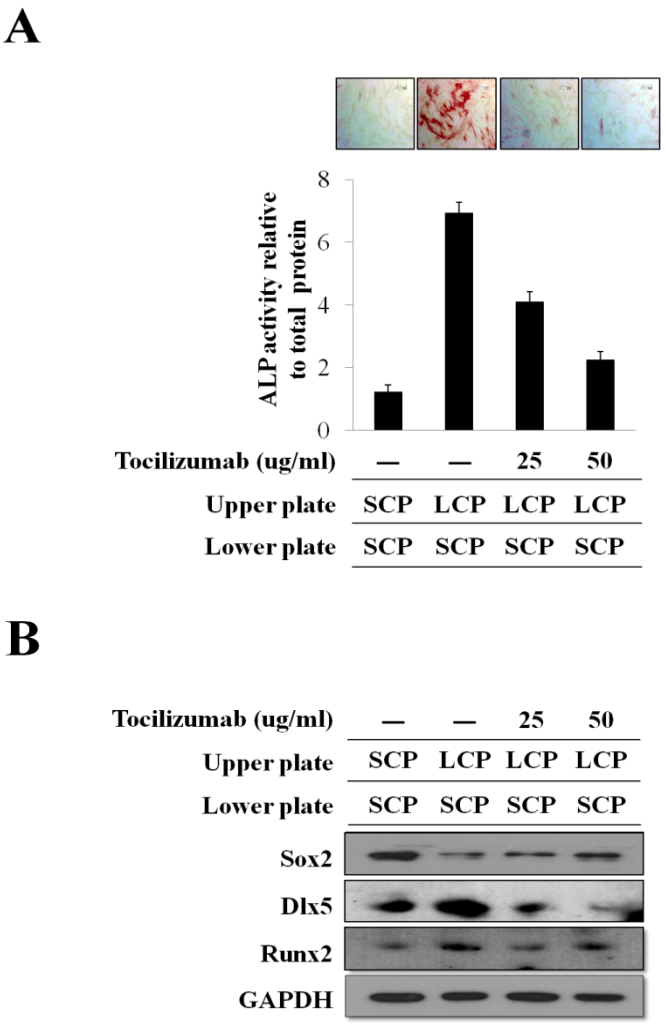



Figure 22. Importance of LCP-secreted IL-6 in regulating osteogenic lineage commitment and stemness loss in BM-MSCs. (A) ALP activity in SCPs treated with LCP-secreted cytokines in the absence or presence of tocilizumab. Each experiment was performed in triplicate, and representative data are shown. (B) Protein expression patterns of Sox2, Runx2, and Dlx5 in SCPs treated with LCP-secreted cytokines in the absence or presence of tocilizumab were examined by western blot analysis. GAPDH was used as the loading control.

18. Correlation between the stemness gene *Sox2* and the osteogenic transcription factors genes *Runx2* and *Dlx5*

In the *in vitro* senescence model of BM-MSCs, we observed significant increase in the expression of osteogenic genes, *Runx2* and *Dlx5*, and a significant decrease in the expression of the stemness gene, *Sox2* with increasing passage number. To determine the correlation between osteogenic and stemness genes, we knocked down the *Runx2*, *Dlx5*, and *Sox2* genes using siRNAs. Knockdown of the *Runx2* or *Dlx5* gene in BM-MSCs increased *Sox2* mRNA expression (Figure 23A), whereas knockdown of the *Sox2* gene had no significant effect on *Runx2* or *Dlx5* mRNA expression (Figure 23B). These data suggest the possibility that the IL-6-induced osteogenic genes, *Runx2* and *Dlx5* regulate the expression of *Sox2* gene via downstream signaling.

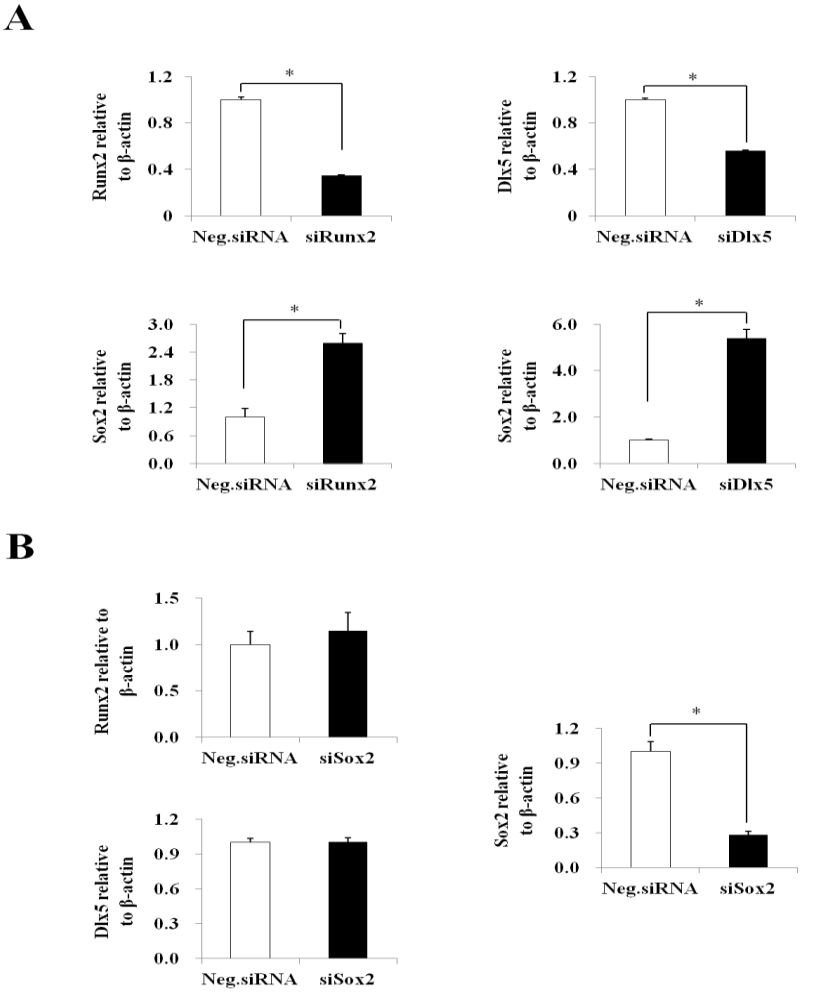


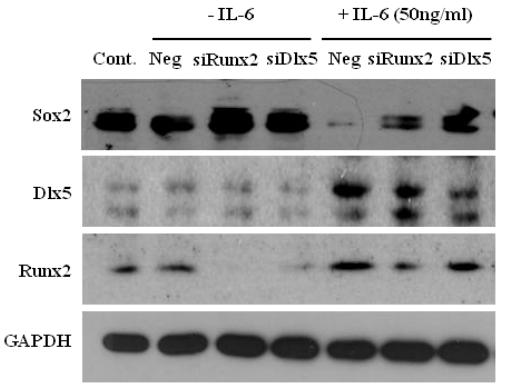

Figure 23. Correlation between stemness and osteogenic genes. (A) Quantitative real-time PCR was performed to investigate changes in *Runx2*, *Dlx5*, and *Sox2* mRNA expression following *Runx2* or *Dlx5* siRNA transfection. (B) Quantitative real-time PCR was performed to investigate changes in *Runx2*, *Dlx5*, and *Sox2* mRNA expression following *Sox2* siRNA transfection. The mRNA expression levels of each gene were normalized to β-actin expression and the experiments were performed in triplicate.

19. Effect of IL-6-induced osteogenic transcription factors, Runx2 and Dlx5 on Sox2 protein expression and transcriptional activity

IL-6 induced cellular senescence in BM-MSCs by increasing the number of SA-β-gal-positive cells, and commitment to the osteogenic lineage by increasing the expression of major osteogenic transcription factors (Runx2 and Dlx5). Furthermore, IL-6 caused loss of BM-MSC self-renewal capacity and multi-lineage differentiation potential by repressing Sox2 expression. Therefore, we hypothesized that IL-6-induced Runx2 and Dlx5 regulate expression of *Sox2* gene. To determine whether the repressive effects of IL-6 on *Sox2* gene expression occurred via IL-6-induced Runx2 and Dlx5, we simultaneously treated BM-MSCs with IL-6 and *Runx2* and *Dlx5* siRNAs. Introduction of *Runx2* and *Dlx5* siRNAs into BM-MSCs without IL-6 slightly increased Sox2 protein levels. However, IL-6 successfully induced the expression of Runx2 and Dlx5 proteins, and significantly decreased Sox2 protein expression. The IL-6-induced repression of Sox2 protein was overcome by introduction of *Runx2* and *Dlx5* siRNAs (Figure 24A). Next, we hypothesized that the transcriptional activity of Sox2 is controlled by Runx2 and Dlx5 expressed via IL-6 downstream signaling. To monitor Sox2 transcriptional activity in BM-MSCs, we performed a Cignal Sox2 Reporter Assay to determine the effects of IL-6 treatment and knockdown/ over-expression of *Runx2* and *Dlx5*. When BM-MSCs were transfected with the Sox2 reporter vector along with *negative* siRNA, *Runx2* siRNA or *Dlx5* siRNA, transcriptional activity of Sox2 was increased, but not significantly, in the *Runx2* and *Dlx5* siRNA-treated groups compared to the *negative* siRNA-treated group. IL-6 suppressed Sox2 reporter activity in the *negative* siRNA-transfected group compared to the *negative* siRNA-transfected group (i.e., without IL-6). Introduction of *Runx2* or *Dlx5* siRNA restored Sox2 reporter activity in the IL-6-treated BM-MSC groups compared to the groups without IL-6 treatment (Figure 24B and C). In HeLa cells, we confirmed that introduction of Runx2 (pCMV6) or the Dlx5 (pcDNA3.1) over-expression vector could significantly

decrease the activity of the Sox2 reporter to a similar extent as in IL-6-treated HeLa cells, compared to the respective vector controls (Figure 24D). These results indicate that IL-6 can induce osteogenic lineage commitment by up-regulating the expression of osteogenic transcription factors (*Runx2* and *Dlx5*) and decrease the self-renewal capacity and multi-lineage differentiation potential of primitive cells by down-regulating the transcriptional activity of the stemness-related gene product, Sox2. In summary, LCP-secreted cytokines (IL-6) can induce the expression of osteogenic transcription factors (Runx2 and Dlx5) in the primitive cell population of heterogeneous BM-MSCs. IL-6-induced Runx2 and Dlx5 increase the expression of an early osteogenic marker (ALP), thereby committing the primitive cell population (SCP) to the osteogenic lineage. At the same time, IL-6-induced Runx2 and Dlx5 suppress the transcriptional activity of Sox2, an important factor in maintaining the renewal capacity and multi-potency of the SCP, resulting in stemness loss in the SCP (Figure 25).

A


- IL-6
+ IL-6 (50ng/ml)
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siRunx2
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Sox2
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Runx2
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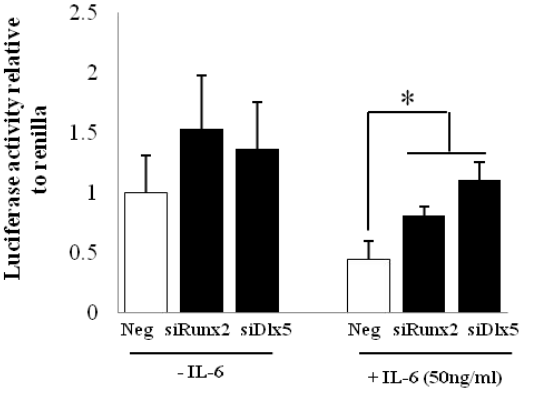
B


Luciferase activity relative to renilla
2.5
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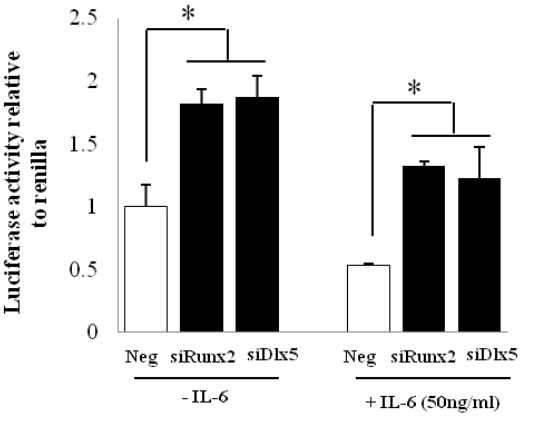
C


Luciferase activity relative to renilla
2.5
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- IL-6
+ IL-6 (50ng/ml)

**D**

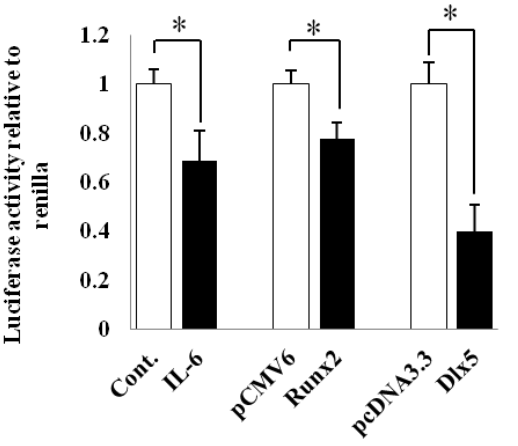


Figure 24. Regulation of Sox2 expression by IL-6-induced Runx2 and Dlx5. (A) Western blot analysis was performed to investigate the expression patterns of Sox2, Runx2, and Dlx5 proteins in response to IL-6 treatment, and *siRunx2* and *siDlx5* transfection in BM-MSCs. The above data have been confirmed in all the 3 donors tested, and representative data are shown. (B and C) BM-MSCs or HeLa cells were co-transfected with the Sox2 reporter and *siRunx2*, *siDlx5*, or negative control siRNA in the absence or presence of IL-6. This reporter contains transcriptional regulatory elements for Sox2 (TRE: *AACAAAGAGT*) After 36 h, dual-luciferase assays were performed, and relative firefly activities were normalized to *Renilla* luminescence. (D) HeLa cells were co-transfected with the Cignal Sox2 reporter and PCMV6-control, PCMV6-Runx2, pcDNA3.3, or pcDNA3.3-Dlx5. After 36 h, dual-luciferase assays were performed, and relative firefly activities were normalized to *Renilla* luminescence.

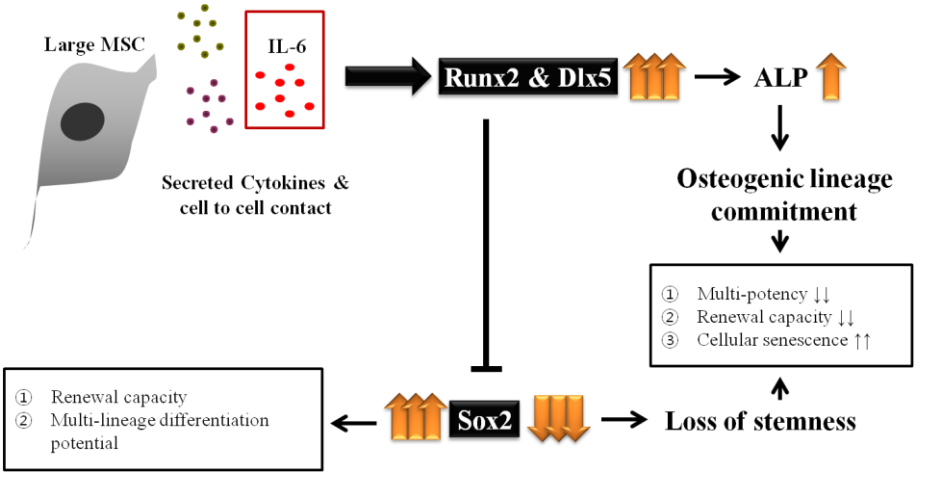


Figure 25. The proposed model of lineage commitment and stemness loss during senescence of heterogeneous BM-MSCs

## IV. DISCUSSION

The *in vitro* expansion capacity of BM-MSCs fulfills the prerequisites for *in vivo* transplantation. However, despite this advantage, BM-MSCs gradually lose their stemness and progress to senescence after extended passaging[14-19,33,34,63]. Senescent cell-secreted cytokines alter the fates of neighboring cells[21-23]. Furthermore, cytokines secreted from senescent cells stimulate tissue aging and tumor formation[25]. In the present study, the LCP isolated from a BM-MSC culture exhibited senescent morphology, such as increased SA-β-gal activity and reduced colony-forming ability. The LCP with senescent morphology induced cellular senescence and expression of ALP, an early osteogenic marker, in the SCP, which did not exhibit senescence characteristics. Among the cytokines secreted from the BM-derived LCP, IL-6 induced both cellular senescence and osteogenic lineage commitment in BM-MSCs. The IL-6/soluble IL-6 receptor (sIL-6R) complex has been shown to promote osteogenic differentiation in BM-derived MSCs[26] and periodontal ligament cells[27] by increasing Runx2 and ALP. Erices et al. reported that IL-6 alone cannot induce the differentiation of BM-MSCs along the osteogenic lineage because the cells do not express membrane-bound or sIL-6R[26]. Although BM-MSCs do not express receptors related to IL-6, we confirmed that IL-6 can activate the STAT pathway[64,65], one of the main pathways induced by IL-6, in BM-MSCs by increasing STAT3 phosphorylation. STAT3 has also been shown to promote osteogenic differentiation of human MSCs via a rapid increase in both Runx2 and ALP levels[66]. In contrast to its effect on osteogenic differentiation, IL-6 decreased the differentiation potential of BM-MSCs along both the adipogenic and chondrogenic lineages by decreasing the expression of *PPAR-γ*/*adiponectin* and *Sox*9/*type II collagen*, respectively. Overall, our results suggest that IL-6 induces osteogenic lineage commitment of BM-MSCs *in vitro*.

Transcription factors known to be critical in the MSC differentiation process, such as Runx2 and Dlx5, which act during osteogenesis, also displayed significant expression changes with increasing passage. At the early-passage

stage, MSCs were highly multi-potent and were naturally uncommitted to any differentiation lineage. However, even in the absence of specific differentiation medium, late-passage BM-MSCs gradually committed themselves to the osteogenic lineage with increase in osteogenic markers, such as ALP and type I collagen, and further culture of these cells induced complete commitment to the osteogenic lineage. These data are consistent with previous reports showing the gradual commitment of late-passage MSCs to the osteogenic lineage[17,18,67]. A previous study also reported the increased expression of Runx2 in late-passage MSCs[51]. In addition to an increase in Runx2, the expression of stemness markers, such as Sox2 and Oct4, has been shown to be decreased in senescent MSCs[49]. In addition, down-regulation of stemness-related genes contributes to the senescence process via a decline in stem cell properties[68]. Based on these results, we hypothesized that the transcriptional activity of stemness genes may be affected by osteogenic genes during senescence in BM-MSCs. Our results show that RNA interference of the *Runx2* or *Dlx5* gene restores the IL-6-repressed protein levels and transcriptional activity of Sox2. These results are consistent with those of Sayyed, who found that Runx2-deficient cells failed to undergo senescence and became immortalized, as confirmed by absence of the β-gal activity and p16 tumor suppressor expression[69]; however, our Dlx5 results are inconsistent with that of a previous study, in which over-expression of Dlx5 in human BM stromal cells induced the persistent expression of Sox2[70]. Nevertheless, Dlx5 is an important regulator that induces osteogenic differentiation by regulating BMP-induced Runx2[71], ALP[72], and osteocalcin[73]. Thus, our findings suggest that Runx2 and Dlx5 function as both osteogenic inducers and stemness suppressors during cellular senescence of BM-MSCs *in vitro*.

A previous study reported that MSCs were amplified $10^9$-fold when repeatedly subcultured under LD conditions (1.5 or 3.0 cells/cm$^2$) for 6 weeks[50]. Contrary to this, another group reported that the optimal density for MSC culture was approximately 5000 cells/cm$^2$ and that MSCs cultured at lower densities underwent apoptotic cell death[35]; however, based on our results,

late-passage BM-MSCs that were re-plated at low cell density (17 cells/cm$^2$) recovered their colony-forming activity, cell proliferation, and multi-lineage differentiation potential, and were small, with spindle-shaped morphology. To determine which subpopulation of late-passage BM-MSCs could be restored, we isolated 2 populations by cell size [small (about <10 μm) and large (about >30 μm)] and found that the SCP had higher colony-forming ability than LCP, suggesting that the small and rapidly dividing subpopulation could be enriched by controlling the plating density, even in late-passage BM-MSCs. A previous study reported that rapid expansion of LD MSCs is largely dependent on a minor population of small cells[50]. Collectively, these results suggest that only the small cells in heterogeneous MSC populations exploit the increased spatial gap between cells and are thus able to proliferate with higher efficiency compared to their larger counterparts, resulting in the enhanced proliferation of small cells under LD conditions. However, it is reasonable to assume that there may be other key factors involved in this pathway in addition to spatial distribution, such as cell-to-cell interactions between the heterogeneous cell populations or release of cytokines from heterogeneous populations during the culture period. Further studies will be needed to fully identify all the key factors involved in this process.

The cell cycle is a critical process that determines cell proliferation and cell senescence. In our study, culture under LD conditions enhanced the S-phase ratio of late-passage BM-MSCs compared to cells grown under HD conditions. The levels of *CCNA2*, *CCNB1*, *CCND1*, *CDK2*, and *CDK4* mRNAs were also elevated by LD culture of late-passage BM-MSCs. Cyclin A binding activates the CDK2 protein to induce DNA replication in a variety of cell systems[74]. Additionally, cyclin D is a critical regulator of the G1/S transition, during which cyclin D binding activates CDK4 to convey the DNA replication signal[75]. The endogenous promoter activity of cyclin B is high during the mitotic stage in mammalian cells[76]. Our results suggest that the proliferative capacity of late-passage BM-MSCs can also be restored using the LD culture method,

suggesting that this recovery may correlate with the expression of cyclin genes in the repopulated cells.

Recently, iPS cells have been described comprising a unique class of stem cells[77,78]. Numerous studies have identified a number of transcription factors, such as Oct-4, Sox2, and Nanog, that play key roles in de-differenitation processes[79,80]. Indeed, in the present study, we observed that *Sox2* and *Nanog* mRNA levels gradually decreased with increasing passage number, whereas the expression levels of these genes were higher in late-passage BM-MSCs that were replated at low cell density. Interestingly, we did not observe any change in the expression of *Oct4* with increasing passage number, but *Oct4* expression was higher under LD conditions. However, a previous study by Lengner et al.[81] reported that Oct4 was likely to be dispensable for the maintenance of self-renewal capacity in somatic cells. Although it is difficult to directly compare their data with ours (because they used mouse cells instead of human BM-MSCs), it is possible that Oct4 expression is independent of the specific role it plays in stemness maintenance. Nevertheless, given the marked changes in *Sox2* and *Nanog* expression during prolonged cell passage and under LD conditions, it is likely that these 2 genes play key roles in the self-renewal and multi-differentiation capacities of human BM-MSCs. Knockdown of the *Sox2* gene using siRNA in P7-LD MSCs resulted in lower levels of proliferation and decreased the ability to differentiate along the 3 lineages compared to that for the groups treated with *Nanog* or scrambled siRNAs. Recently, a novel function for Sox2 was identified in the maintenance of self-renewal in a mouse osteoblastic lineage[82]. In addition, Sox2-knockout cells could not form colonies, and their population growth was arrested with a senescent phenotype. In another study, most Sox2-overexpressing BM-derived MSCs were small and showed high proliferative and osteogenic capabilities[30]. Liu et al. reported that Nanog and Oct4 overexpression using a lentiviral system enhanced the proliferation and colony-forming ability of BM-derived MSCs[83]. In addition, they confirmed that Oct4 overexpression enhances adipogenesis, whereas Nanog overexpression impaired the efficiency of adipogenesis. By contrast,

chondrogenesis was enhanced in both Oct4- and Nanog-overexpressing cells. However, in the present study, *Oct4* expression did not significantly change during long-term culture, and *Nanog*-siRNA-transfected cells did not show meaningful results from multiple donors. Our results suggest that the *Sox2* gene is essential for BM-MSC proliferation and osteogenic differentiation and that without it the cells may lose their stem cell function.

## V. CONCLUSION

Under an *in vitro* culture system, BM-MSCs were committed to the osteogenic lineage during prolonged culture, resulting in loss of multi-potency. In addition, late-passage BM-MSCs exhibited increased cell size and flattened morphology compared to early-passage BM-MSCs. However, previous studies on MSC senescence did not focus on the change in cell size during long-term culture *in vitro*. Among the heterogeneous BM-MSCs, the LCP was senescent. Furthermore, LCP-secreted cytokines altered the fate of the primitive cell population extant among the BM-MSCs. Especially, IL-6 induced the commitment to the osteogenic lineage and decreased the multi-potency of BM-MSCs. In addition, IL-6 up-regulated the expression of Runx2 and Dlx5 and down-regulated the expression and transcriptional activity of Sox2. Our results indicate that Sox2 is essential for maintaining the stem cell properties of BM-MSCs. Thus, this study suggests that autocrine secretion of IL-6 by the LCP may be a step in the osteogenic lineage commitment of BM-MSCs toward cellular senescence *in vitro*. Furthermore, blockade of IL-6 signaling between the different BM-MSC populations may be a way to maintain the primitive cell population in long-term culture.

In addition, under LD conditions, the repopulated cells showed extensive recovery of both proliferation and differentiation capacities as well as reactivation of genes involved in cell cycle distribution, stemness, and differentiation. By controlling the plating density, increased expression of stemness-related genes, such as *Sox2*, that were downregulated in late-passage BM-MSCs is expected to maintain or increase the proliferation and differentiation potential of early- or late-passage MSCs over extended culture periods.

In conclusion, an understanding of the mechanisms underlying cellular senescence and the development of conventional cell culture methods would be useful in maintaining the stemness of primitive BM-MSC.

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ABSTRACT (IN KOREAN)

# 골수유래 중간엽 줄기세포의 노화 과정에서 골분화와 줄기성 관련 유전자의 서로 다른 발현과 세포 배양법의 중요성

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윤동석

인간 골수로부터 유래된 중간엽 줄기세포는 자가 증식능력을 가지고 있으며 골, 지방, 연골 세포 등 다양한 계열로의 분화가 가능한 다능성세포이다. 이러한 이유로, 인간 골수유래 중간엽 줄기 세포는 임상적용을 위한 가장 이상적인 세포치료제로서 여겨진다. 하지만 오랜 기간 동안 시험관배양을 하게 되면 자가 증식능력과 분화능력을 잃고 급격하게 노화가 진행되는 것으로 알려져 있다. 또한, 노화가 진행되는 과정에서 골세포 계열로 분화 능력이 제한되는 특징을 보이며, 지방과 연골 세포로의 분화능력은 감소되게 된다. 이러한 현상들은 대량의 다능성세포를 필요로 하는 임상 적용에 있어 큰 제약이 될 수 있다. 또한 골수유래 중간엽 줄기세포는 이질적인 세포들의 집단 (heterogeneous cell population)으로 이루어져 있다. 이질적인 세포집단을 크게 두 가지로 분류하면 다능성을 가진 원시세포집단 (primitive cell population)과 다능성을 잃은 노화된 세포집단으로 나누어 볼 수 있다. 원시세포들은 세포의 크기가 작고 방추형의 모양을 가지고 있으며, 노화된 세포들은 상대적으로 세포의 크기가 크고 퍼진 모양을 지니고 있다. 노화된 세포들은 특정 시토킨 (cytokine)을 분비함으로써 주변 이웃 세포들의 세포 운명 (cell fate)에 영향을 줄 수 있다고 알려져 있다. 하지만 아직까지 골수유래

중간엽 줄기세포집단에서 노화된 세포들이 다능성세포인 주변 원시세포들과 어떠한 상호작용을 하는지에 대하여 알려진 것이 없다. 그러므로 이 연구는 골수유래 중간엽 줄기세포의 노화과정에서 노화된 세포들이 주변에 이웃한 다능성 원시세포들의 세포운명에 영향을 줄 수 있는지 보고자 하였다. 추가적으로, 기존 골수유래 중간엽 줄기세포의 배양방법과 비교하여 후기계대 골수유래 중간엽 줄기세포의 배양 밀도를 조절해 주는 것이 다능성 원시세포집단을 재군집 (repopulation) 시키는데 있어 효율적인지 확인해 보고자 하였다.

골수유래 중간엽 줄기세포는 평균적으로 7번의 계대배양 후, 노화된 현상들을 보여주었다. 특히, 크기가 큰 세포집단들이 증가되었으며 골분화를 제외한 다른 계열로의 분화 효율은 급격히 감소되었다. 골분화와 연관된 전사인자인 Runx2와 Dlx5의 발현은 후기계대 세포집단에서 증가되어 있었으며, 다능성 유지에 중요한 역할을 할 것으로 생각되는 줄기성인자인 Sox2와 Nanog의 발현은 감소되어 있었다. 골수 유래 중간엽 줄기세포의 자가증식능력과 다능성에 Sox2와 Nanog가 중요한 역할을 하는지 확인해 보고자 RNA 저해 실험을 수행하였으며, 그 결과 Sox2가 골수유래 중간엽 줄기세포의 줄기성을 유지하는데 있어 필수적이라는 것을 확인하였다. 배양기간이 길어짐에 따라서 노화가 되는 원인을 찾고자 하였으며, 그 원인으로 이미 노화된 세포들이 주변 다능성원시세포들의 세포운명을 변화시켜 노화된 세포집단들이 증가될 것이라고 가정하였다. 이러한 가정을 증명하고자 우리는 세포를 크기에 따라 분리하였으며 각각 SCP (small cell population)와 LCP (large cell population)로 명명하였다. 결과적으로 우리는 LCP에서 분비되는 시토킨들에 의하여 다능성 원시세포인 SCP의 세포 운명이 변할 수 있다는 것을 확인하였다. 그 중 interleukin-6 (IL-6)가 LCP에서 과발현되고 있다는 것과, IL-6는 SCP의 다능성을 잃게 하고 골아세포 계열로 분화능력을 제한 (osteogenic lineage commitment) 시켜주는 것을 확인하였다. IL-6에 의한 영향들은 골수유래 중간엽 줄기세포의 노화가 진행되면서 나타나는 현상들과 동일하였다. 뿐만 아니라, IL-6는 골분화 전사인자인 Runx2와 Dlx5의 발현을 증가시켰으며 반대로 줄기성인자인 Sox2의 발현은 감소시켰다. 우리는 골수유래

중간엽 줄기세포가 노화과정에서 골아 세포 계열로 분화능이 제한되고, 다능성이 감소되는데 있어 Runx2와 Dlx5의 발현 증가, 그리고 Sox2의 발현 감소가 원인일 것이라고 생각했다. 기존 중간엽 줄기세포의 노화 연구에서 Runx2와 Sox2의 발현 패턴이 반대로 움직인다는 보고들이 있었다. 하지만 아직까지 두 유전자의 상호작용에 대하여 연구된 것은 없었다. Runx2, Dlx5, 그리고 Sox2에 대한 RNA 억제 실험을 통하여 우리는 Sox2의 발현이 Runx2와 Dlx5에 의존적으로 변할 수 있다는 것을 확인하였다. IL-6가 처리된 골수유래 중간엽 줄기세포에서 선별적으로 Runx2와 Dlx5를 억제시켜주면 감소되었던 Sox2의 발현이 다시 회복되는 것을 확인할 수 있었다. 또한, IL-6에 의하여 유도된 Runx2와 Dlx5는 Sox2의 전사활성을 조절할 수 있었으며 Sox2 프로모터 부위에 결합할 수 있는 것으로 확인되었다. 이러한 결과들은, 골수유래 중간엽 줄기세포집단의 노화과정동안 LCP에 의하여 분비된 시토킨인 IL-6가 다능성 원시세포인 SCP의 Runx2와 Dlx5의 발현을 증가시킨다. 증가된 Runx2와 Dlx5는 SCP의 초기골분화를 유도하고, 동시에 줄기성인자인 Sox2의 발현을 억제함으로써 자가증식능력과 다능성을 잃게 할 것으로 생각된다.

또한 우리는 후기 계대의 노화된 골수유래 중간엽 줄기세포집단을 적은 밀도로 계대배양 하였을 때, 다능성 원시세포인 SCP의 밀도를 높일 수 있다는 것을 발견하였다. 기존 배양방법 (high density; HD-5000 cells/$cm^2$)과 비교할 때, 저밀도 배양법 (low density; LD-10~20 cells /$cm^2$)은 전체 세포집단의 증식능력과 분화능력을 회복시켰다. 뿐만 아니라, Runx2와 Dlx5같은 골분화 관련 인자들의 발현을 감소시켜 주었으며 줄기성 인자인 Sox2와 Nanog의 발현을 증가시켜 주었다. 이러한 현상들은 앞선 결과들에서 관찰했듯이 LD 배양법이 전체 세포집단에서 SCP의 비율을 증가시킴으로써 증식능력과 다능성 유지에 도움을 주는 것으로 생각된다.

결론적으로, 노화된 세포집단인 LCP에서 분비되는 시토킨인 IL-6는 골수유래 중간엽 줄기세포의 노화와 다능성 유지에 있어 중요한 역할을 하는 것으로 보여진다. 그러므로, IL-6 신호전달 단계를 표적으로 하는 분자들을 적용하면 오랜 배양기간 동안 다능성 원시세포인 SCP를 유지하는데 있어 효율적일 것으로 생각된다. 뿐만

아니라, 세포의 배양 밀도를 조절하는 것은 이종적인 골수유래 중간엽 줄기세포 집단에서 다능성 원시세포들의 비율을 증가시키고 유지 하는데 있어 중요한 요인이 될 것으로 사료된다.

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핵심되는 말: 골수 유래 중간엽 줄기세포, 이질적 세포집단, 다능성, 세포 노화, 자가증식, 줄기성, IL-6, Runx2, Dlx5, Sox2, 저밀도배양