Importance of Sox2 in maintenance of cell proliferation and multipotency of mesenchymal stem cells in low-density culture

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Received 28 December 2010; revision accepted 26 April 2011

Abstract

Objectives: This study has aimed to repopulate 'primitive' cells from late-passage mesenchymal stem cells (MSCs) of poor multipotentiality and low cell proliferation rate, by simply altering plating density.

Materials and methods: Effects of low density culture compared to high density culture on late-passage bone marrow (BM)-derived MSCs and pluripotency markers of multipotentiality were investigated. Cell proliferation, gene expression, RNA interference and differentiation potential were assayed.

Results and conclusions: We repopulated 'primitive' cells by replating late-passage MSCs at low density (17 cells/cm²) regardless of donor age. Repopulated MSCs from low-density culture were smaller cells with spindle shaped morphology compared to MSCs from high-density culture. The latter had enhanced colony-forming ability, proliferation rate, and adipogenic and chondrogenic potential. Strong expression of osteogenic-related genes (*Cbfa1*, *Dlx5*, *alkaline phosphatase* and *type I collagen*) in late-passage MSCs was reduced by replating at low density, whereas expression of three pluripotency markers (*Sox2*, *Nanog* and *Oct-4*), *Osterix* and *Msx2* reverted to levels of early-passage MSCs. Knockdown of *Sox2* and *Msx2* but not *Nanog*, using RNA interference, showed significant decrease in colony-forming ability. Specifically, knockdown of *Sox2* significantly inhibited multipotentiality and cell proliferation. Our data suggest that plating density should be considered to be a critical factor for enrichment of 'primitive' cells from heterogeneous BM and that replicative senescence and multipotentiality of MSCs during *in vitro* expansion may be predominantly regulated through *Sox2*.

Introduction

Mesenchymal stem cells (MSCs) isolated from 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 during *in vitro* expansion, with increasing passages (14–19). After several passages, MSCs enter senescence, characterized by enlarged and irregular cell shape, and cessation of cell division (20).

To isolate BM-derived primitive cells during *in vitro* culture, several approaches have been reported, including assessment of surface markers such as *stro-1* (21), cell shape and size (22), donor age (23,24), and plating density (25–27). Some studies have demonstrated the importance of plating density for *in vitro* cultures for cell proliferation and differentiation potential. MSCs seeded at cell density less than 5000 cells/cm² undergo apoptotic cell death (25); however, low-density culture appeared to be sufficient for maintaining colony-forming ability of early-passage MSCs and their stem cell properties ('stemness') (26). Nevertheless, most studies have used passage-limited MSCs (less than five passages) (28,29) and/or have expanded MSCs at various cell densities ranging from 10³ to 10⁵ cells/cm² (27,30–33). Loss of stem cell properties of MSCs poses significant limitations in their use for cell-based regenerative medicine and in studies aimed at understanding their differentiation mechanisms.

Recently, reprogramming of somatic cells into induced pluripotent stem cells (iPS) has been achieved by forced expression of embryonic stem (ES) cell factors

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(*Oct4*, *Sox2*, *cMyc* and *Klf4*), and combinations of these genes has been shown to be a critical factor for high reprogramming efficiency. Pluripotency markers, such as *Sox2*, *Nanog* and *Oct-4* are expressed in adult stem cells and ES cells; however, their expression in MSCs is dependent on passage number and tissue source (34). Go *et al.* (35) observed that when passage 5 (P5) MSCs, which displayed flattened, aged morphology and reduced proliferation rates, overexpressed a retrovirus encoding *Sox2* or *Nanog*, cells had restoration of their normal morphology and proliferation level. However, these changes were only apparent in presence of bFGF, and retroviral silencing is still a problem in achieving pluripotent states and superior iPS cells. Despite recent successes to overcome the senescence of MSCs, in various trials, and to maintain or enhance their stemness, little is known about correlation between cell fate based on culture conditions and expression of pluripotency markers.

Thus, our current study was aimed at repopulating a subpopulation that was similar to early-passage MSCs, from late-passage MSCs, and rescuing some transcription factors, including pluripotency markers, in the repopulation process.

Materials and methods

Isolation and culture of MSCs from human bone marrow aspirates

Bone marrow 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% foetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% antibiotic-antimycotic solution (Invitrogen) at 37 °C in a 5% CO₂ atmosphere. Cells were grown to 80–90% confluence and then harvested by incubation with 0.25% trypsin/EDTA (Invitrogen) centrifuged at 188 g for 3 min. Harvested cells (P1) were replated at density of 5000 cells/cm² and subcultured when they were 80–90% confluent up to P7. Late passage MSCs (P7) were replated at high density (HD; 5000 cells/cm²) or low density (LD; 17 cells/cm²) and maintained for 10–12 days.

Colony-forming unit fibroblast (CFU-F) assay

MSCs from each passage (P1, P3, P5 and P7) were plated in 10 cm culture dishes at the appropriate density

(1×10^3 cells/dish) in DMEM-LG containing 20% FBS. Medium was changed every 3 days and cultures were maintained for approximately 10–12 days. 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.

Cell proliferation assay

Cell proliferation was determined using a hexosaminidase assay. Proliferative ability of density-controlled 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- β -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.

Alkaline phosphatase 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.

Calcium content assay

To evaluate calcium content, cells were washed twice in PBS and incubated in 800 μ l 0.5 N acetic acid for 24 h at room temperature. After incubation, 300 μ l fresh reagent (O-Cresolphthalein Complexon, ethanolamine/boric acid, hydroxyquinol; Sigma) was added to 50 μ l 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 μ g total protein. Experiments were performed in triplicate.

Multi-lineage differentiation

To identify multi-lineage differentiation potential of MSCs, cells were seeded at 8×10^4 cells/well in 12-well culture plates. For osteogenic differentiation, cells were maintained for 14 days in osteogenic medium [DMEM-LG containing 10% FBS, 1% antibiotic-antimycotic

solution, 100 nM dexamethasone (Sigma), 10 mM β -glycerophosphate (Sigma), and 50 μ g/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. For adipogenic differentiation, cells were maintained for 14 days in adipogenic medium [DMEM-LG containing 10% FBS, 1% antibiotic-antimycotic solution, 1 μ M dexamethasone, 0.5 mM isobutyltethylxanthin (Sigma), 5 μ g/ml insulin (Gibco), and 200 μ M 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 de-stained 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. For chondrogenic differentiation, cells were maintained for 14 days in chondrogenic medium [DMEM-high glucose containing 1 $\times$ insulin-transferrin-selenium-A (Gibco), 1% antibiotic-antimycotic solution, 50 μ g/ml ascorbic acid and 10 ng/ml TGF- β 3 (R&D Systems, Minneapolis, MN, USA)]. For pellet culture, 8×10^4 cells in 15-ml tubes were harvested after centrifugation at 160 g for 3 min, then chondrogenic medium with TGF- β 3 was then 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.

Cell cycle analysis and cell sorting

Cells were harvested by incubation with 0.25% trypsin/EDTA and washed twice in PBS. Cells from each group (1×10^6) were fixed in ice-cold 70% ethanol for 1 h at -20°C , stained with 50 μ g/ml propidium iodide (PI; Sigma) containing 100 μ g/ml RNase A (Sigma) for 40 min at 4°C , and then analysed using FACS Calibur instrumentation (Becton Dickinson Instrument, San Jose, CA, USA) to detect cell cycle distribution. All samples were tested in triplicate ($n = 3$). P7-HD and P7-LD MSCs were harvested by incubation with 0.25% trypsin/EDTA and washed twice in PBS. Cells were resuspended in pre-warmed PBS, then small, middle and large cells were sorted and analysed using FACS (Beckman Coulter, Fullerton, CA, USA).

Real-time quantitative polymerase chain reaction

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 and pluripotency-related transcription factors. Primer sets used were validated by and purchased from Bioneer (Bioneer, Daejeon, 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); *Osterix* (P150104, NM_152860.1); *Msx2* (P300127, NM_002449.4); *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*, and thus the primer was separately designed as follows: 5'-GCAA GCCCTCATTTACCA-3' (*Oct4*, sense, NM_002701) and 5'-GCCCATCACCTCCACCAC-3' (*Oct4*, antisense). PCR reaction mixtures consisted of 1 $\times$ SYBR Green PCR premix (Bioneer), 10 pM specific primers and 2 μ l of cDNA in the Bioneer Exicycler Real-Time PCR system (Bioneer). Real-time PCR analysis underwent 40 cycles of amplification. Mean cycle threshold (CT) values from triplicate ($n = 3$) measurements were used to calculate gene expression, with normalization to GAPDH as internal control.

RNA interference

On-TargetPlus SmartPool siRNAs for *Sox2* (Cat. L-011778) and *Nanog* (Cat. L-014489) were purchased from Dharmacon (Boulder, CO, USA). siRNA for *Msx2* was purchased from Genepharma (Shanghai, China) and targeted the following sequences: *Msx2* siRNA sense: 5'-CGCUCAUGUCCGACAAGAATT-3' and *Msx2*-siRNA antisense: 5'-UUCUUGUCGGACAUGAGCGCC-3'. Scramble-siRNA was obtained by Bioneer Inc. and targeted the following sequences: scramble-siRNA sense: 5'-CCUACGCCACCAAUUUCGU-3' and scramble siRNA antisense: 5'-ACGAAAUUGGUGGCGUAGG-3'. P7-LD MSCs were used for siRNA experiments. Briefly, P7-LD MSCs were plated to obtain 70–80% confluency in six-well plates, and transfected with 100 nM of *Sox2*, *Nanog*, *Msx2*, or scramble (Neg) siRNA using Lipofectamine^{TM} 2000. After 6 h transfection, fresh media were

added to plates, and transfection efficiency was confirmed by western blot analysis.

Lentivirus-mediated overexpression

Lentivirus encoding human Sox2 was purchased from SLB (Seoulin Bioscience, Seoul, South Korea; Service Code: SRDL-IV). Sox2 was amplified from cDNA of MSCs and then cloned into pCDH-EF1-MCS-T2A-cop-GFP. Mock vector with no insert was used as control (Mock). Infection of MSC was performed by 12 h exposure to dilutions of viral supernatant in presence of Polybrene (5 µg/ml). Lentivirus-infected cells were confirmed by GFP expression (Data not shown).

Western blot analysis

Cells were lysed in Passive lysis buffer (Promega, Madison, WI, USA). Protein concentrations were determined by BioRad protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA), and total 30 µg protein was applied and analysed by 10% SDS-PAGE (Sigma). Transferred membranes were blocked with 5% skimmed milk (BD, Sparks, MD, USA) and incubated for 4 h with antibodies to Sox2 (R&D System), Nanog (BD), and Msx2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were further probed with antibody to β-actin (Santa Cruz Biotechnology), which was provided as loading control. Sox2, Nanog and Msx2 protein expression were confirmed in 3 donors, and data shown are representative.

Statistical analysis

Statistical analysis for all results was performed using Student's *t*-test, and data were expressed as means ± SD. Values of *P < 0.05 or **P < 0.01 were considered statistically significant.

Results

Reduced stemness of human BM-derived MSCs via repeated serial subculture

We investigated colony-forming abilities and multi-differentiation potential of early- and late-passage MSCs. Colony-forming ability of P7 MSCs was markedly lower compared to that of P1 MSCs (Fig. 1a,b). Both P1 and P7 MSCs readily differentiated into osteoblastic cells when cultured in osteogenic medium for 14 days, but osteogenic potential of P7 MSCs was higher than that of P1 MSCs, suggesting that P7 MSCs might have a subpopulation of more committed osteoblastic cells that increases during subsequent passage. However, adipogenic poten-

tial of P7 MSCs was lower than that of P1 MSCs, and chondrogenic differentiation capacity was not present in P7 MSCs (Fig. 1c).

Changes in cell morphology and enhanced colony-forming ability of late-passage MSCs in LD culture

We confirmed changes in cell morphology and proliferation of late-passage MSCs by altering cell culture methods. We observed that when cells were cultured at densities less than widely used density of 5000 cells/cm², specifically, at 17 cells/cm², cells displayed markedly enhanced cell population doubling (data not shown). Based on these results, cells were subcultured at high density (HD) (5000 cells/cm²) up to P7, at which point they had dramatically lower colony-forming and multi-differentiation abilities; P7 MSCs were then replated at either HD (5000 cells/cm², P7-HD MSC) or LD (17 cells/cm², P7-LD MSC) (Fig. 2a). Colony-forming ability of P7 MSCs was remarkably lower compared to that of P1 MSCs, but this ability recovered by switching to LD culture conditions, but not to HD culture conditions (Fig. 2b). We also observed that P7-HD MSCs appeared flattened and enlarged, whereas many small spindle shaped MSCs reappeared in P7-LD MSC populations (Fig. 2b, right). BM-derived MSCs have heterogeneously sized cell populations, and the property of having small cells has been thought to indicate more efficient stem cell properties (26,36). Using FACS analysis of P7-HD MSCs, small cell-sized populations (A-region, 5–10 µm) represented approximately 10.9% of entire populations and large cell-sized populations (B-regions, >30 µm) represented approximately 21.2% of entire populations. In contrast, in P7-LD MSCs, small cell-sized population represented approximately 30.9% and large cell-sized populations approximately 8.7% of entire populations (Fig. 2c). These results suggest that plating density is critical to enrichment of more primitive cells in heterogeneous MSC populations and that we can repopulate much higher levels of primitive cells, even from late-passage cells, using low-density culture conditions.

Changes in proliferative capacity and cell cycle distribution of late-passage MSCs under LD culture conditions

Proliferative ability of human BM-derived MSCs gradually diminishes over continuous expansion. In addition to increasing passage number, donor age is also a critical factor for proliferative ability of MSCs (23) as colony-forming ability of MSCs decreases noticeably in donors older than 20 (24). To verify whether the LD method was effective in recovering proliferative ability of late-passage

Figure 1. Decreased colony-forming ability and multi-lineage differentiation potential of P7 human BM-derived MSCs. (a, b) P1, P3, P5 and P7 MSCs from the same donor were seeded at 1×10^3 cells in 10 cm culture dishes. Cells were then cultured in DMEM-LG containing 20% FBS for 12 days to examine cell colony formation (violet). * $P < 0.05$, ** $P < 0.01$ compared to P1 MSCs. (c) P1 and P7 MSCs from the same donor were seeded at 8×10^4 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 potentials respectively. The above data have been confirmed on all 10 of the donors tested, whereas representative data shown here are those of a 19-year-old male donor.

MSCs, from donors of different ages, a proliferation assay was performed in samples organized by their respective age group [10–20 years old ($n = 3$), 20–50 years old ($n = 3$) and 50–70 years old ($n = 3$)]. P1 MSCs were cultured up to P7 as shown in Fig. 2a. At this point, cells were harvested and replated at either 5000 (HD method) or 17 (LD method) cells/cm² in 10 cm dishes. Evaluation of proliferative capacities of P7-HD and P7-LD MSCs was performed using a hexosaminidase assay. Compared to continuous HD culture, P7-LD MSCs showed significantly higher proliferation in every age group (Fig. 3a). These results suggest that the LD culture method both favours cell proliferation and increases numbers of small cells in a heterogeneous population of late-passage MSCs (Fig. 2c) regardless of donor age.

Next, we analysed the effect of HD or LD culture on cell cycle distribution in late-passage MSCs, from each age group. Cell cycle analysis showed that on average, 87.78%, 9.53% and 2.69% P7-HD MSCs were in G₀/G₁, S and G₂/M phases respectively. In comparison, for P7-LD MSCs, G₀/G₁, S and G₂/M phases represented 73.53%, 20.35% and 6.12% cell population respectively (Fig. 3b). Proportions of S-phase cells was higher in P7-LD MSCs compared to P7-HD MSCs. To further

assess effects of the LD method on expression of genes involved in cell cycle distribution, we utilized real-time PCR to monitor their expression patterns. Progression of proliferation during the cell cycle is closely regulated by cyclins, proteins that activate cyclin-dependent kinases (CDKs). Results from the real-time PCR analysis showed marked decrease in expression of genes known to be involved in S-phase and mitosis, such as *cyclin A2* (*CCNA2*) and *cyclin B1* (*CCNB1*), when cells were cultured from P1 until P7 (Fig. 4a,b). *Cyclin D1* (*CCND1*), a gene associated with most proliferating cells, did not show any statistically significant changes with serial subculture, while expression was higher in LD cultures (Fig. 4c). In addition, we analysed expression of two genes (*CDK2* and *CDK4*) known to be involved in G₁/S phase transition. *CDK4* expression decreased with serial subculture up to P7, but expression of both *CDK2* and *CDK4* recovered in LD culture (Fig. 4d,e). Thus, we found that in P7 cells cultured using the LD method, there were much higher recovery levels of genes related to the cell cycle compared to their expression in HD-cultured cells (Fig. 4a–c). Collectively, these data suggest that restoration of cell proliferation by plating density may be mediated by cell cycle-related genes, such as *cyclin D1*, *CDK2* and *CDK4*.

Figure 2. Changes in cell morphology, cell size, colony-forming ability and ALP expression in late-passage MSCs using density-controlled culture method. (a) Overall culture scheme of cells from early to late passage followed by replating cells under either HD or LD conditions. This culture scheme was maintained throughout the experiment. (b) P1 MSCs, P7 MSCs, P7-HD and P7-LD cells were seeded at 1000 cells in 10 cm culture dishes. Cells were then cultured in DMEM-LG containing 20% FBS for 12 days to examine cell colony formation ($\times 100$). (c) P7-HD and P7-LD cells were sorted into small ($<10 \mu\text{m}$), middle ($10\text{--}30 \mu\text{m}$) and large (over $30 \mu\text{m}$) cells using FACS, and size distribution of the sorted cells was analysed. Cell size was observed using an optical microscope ($\times 100$) after trypan blue staining. The above data have been confirmed on all 10 donors tested, whereas the representative data shown here are those of a 35-year-old male donor.

Differentiation potential of late-passage MSCs into three lineages recovers using LD culture methods

We next examined differentiation potential of P7-HD and P7-LD MSCs. Cells were cultured following the general scheme shown in Fig. 2a, up to P7 using the HD method. Cells were then grown using either HD or LD method. Each repopulated cell culture was then replated in 12-well plates at identical cell densities, grown to confluence, and induced to differentiate into osteoblasts and adipocytes. Both P7-HD and P7-LD MSCs expressed ALP and showed accumulation of calcium-containing mineralized nodules, after von Kossa staining, 14 days of osteogenic induction (Fig. 5a). In the calcium content assay, P7-HD MSCs had higher calcium accumulation compared to P7-LD MSCs (Fig. 5b). Although multi-differentiation ability of BM-derived MSCs decreases

after expansion in culture, potential for osteogenic differentiation is retained or increased (15,16,37). These reports indicate that MSCs differentiate into osteogenic cells with continued cell passaging. Adipogenic differentiation was demonstrated here using morphology and oil red O staining. P7-LD MSCs had increased lipid droplet formation and oil red O-stained cells compared to P7-HD MSCs (Fig. 5c). In addition, absorbance of oil red O staining detected after de-staining was significantly different between the two cell populations (Fig. 5d). Chondrogenic differentiation was analysed using safranin O staining. Similar to the results of adipogenic differentiation, P7-LD MSCs had higher chondrogenic differentiation capacity than P7-HD MSCs (Fig. 5e), and absorbance of proteoglycans detected after de-staining was also significantly different between the two groups (Fig. 5f).

Figure 3. Effects of LD culture on cell proliferation and cell cycle distribution in late-passage MSCs from donors of different ages. (a) P7 MSCs from donors aged 10–20, 20–50 and 50–70 were replated at appropriate density (approximately 5000 or 17 cells/cm²) on 10 cm dishes. Replated cells were then seeded into 24-well plates (1×10^4 cells/well). A hexosaminidase assay was performed to determine proliferative potential of density-controlled MSCs (P7-HD and P7-LD cells). * $P < 0.05$ compared to P7-HD MSCs. (b) P7-HD and P7-LD cells from each age group were harvested, and 1×10^6 cells were used for cell cycle analysis. Each experiment was performed in triplicate. Tabulated data provided here shows mean value from three donors.

Figure 4. Effects of LD culture on mRNA expression in late-passage MSCs. mRNA expression patterns of cell cycle-related genes were examined using real-time PCR in undifferentiated P1, P3, P7, P7-HD and P7-LD MSCs. After subculturing from each previous passage, P1, P3, P7, P7-HD and P7-LD cells were grown in DMEM-LG containing 10% FBS for 3 days. Cells were harvested when 80–90% confluent to extract RNA. Each experiment was performed in triplicate ($n = 3$). * $P < 0.05$ compared to P1 or P7-HD MSCs.

Figure 5. Three-lineage differentiation potentials of P7-HD and P7-LD cells subcultured from late-passage MSCs (P7). (a) P7-HD and P7-LD cells (8×10^4 cells/well in 12-well plates) were incubated in osteogenic medium for 14 days. After incubation, ALP and von Kossa staining were performed to detect mineral deposition. (b) A calcium content assay was performed to confirm results of von Kossa staining. (c) P7-HD and P7-LD cells (8×10^4 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. (d) For quantitative analysis of oil red O staining, absorbance was detected at 500 nm following de-staining with isopropanol for 30 min. (e) P7-HD and P7-LD cells (8×10^4 cells/well in 12-well plates) were incubated in chondrogenic medium containing 10 ng/ml TGF β -3 for 14 days. After incubation, safranin O staining was performed to detect proteoglycans ($\times 100$, Con, undifferentiated control MSCs; CH, chondrogenic-differentiated MSCs). (f) 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.

Changes in mRNA expression patterns of osteogenic and pluripotent markers in P1, P3, P7, P7-HD and P7-LD MSCs

We examined RNA expression of genes related to osteogenic differentiation and stemness in P1, P3, P7, P7-HD and P7-LD MSCs. Results indicated that levels of genes involved in osteogenesis, such as *Runx2*, *Dlx5*, *ALP* and *type I collagen*, increased gradually from P1 to P7 (Fig. 6a,b,e,f). *Osterix*, a crucial transcription factor in osteogenic differentiation, was expressed constantly from P1 to P3, whereas its expression was somewhat lower in P7 MSCs (Fig. 6c). Expression of *Msx2* was significantly lower in P7 MSCs (Fig. 6d). Genes whose expression was higher during HD culture, such as *Runx2*, *Dlx5*, *ALP* and *type I collagen* were lower during LD culture to similar expression levels, as those observed in P1 MSCs (Fig. 6a,b,e,f). However, expression levels of both *Osterix* and *Msx2* during LD culture were upregulated to levels observed in early-passage MSCs (Fig. 6c,d). These results indicate that LD culture represses increased expression of

osteogenic marker genes, diminishing or reversing MSC commitment to osteogenic precursor cells.

Figure 7 shows changes in expression of pluripotential markers in P1, P3, P7, P7-HD and P7-LD MSCs. Expression of *Sox2* and *Nanog* gradually decreased as cells progressed from P1 to P7. Lower expression of *Sox2* and *Nanog* in P7 MSCs was recovered during LD culture to levels similar to those observed in P1 MSCs. Although expression of *Oct4* did not change significantly with increased cell passage, its expression was slightly upregulated after LD culture. Upregulation of pluripotent marker genes during LD culture of late-passage MSCs might be related to enhanced cell proliferation and recovered differentiation potential, including ability to undergo adipogenesis and chondrogenesis. Thus, we evaluated whether these genes played key roles during osteogenic differentiation, and found that expression of *Msx2*, *Sox2* and *Nanog* gradually decreased over the course of osteogenic differentiation (data not shown). However, expression level of *Oct4* remained constant throughout the differentiation process. In summary, these data indicate that *Msx2*, *Sox2*

Figure 6. Expression changes in osteogenesis-related genes in P7-HD and P7-LD cells subcultured from late-passage MSCs (passage 7). mRNA expression patterns of osteogenesis-related genes were evaluated using real-time PCR in undifferentiated P1, P3, P7, P7-HD and P7-LD MSCs. After subculturing cells from each previous passage, P1, P3, P7, 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 P1 or P7-HD MSCs.

and Nanog proteins may play roles in maintenance of stemness.

Effect of RNA interference of Sox2, Nanog and Msx2 on P7-LD MSC proliferation and colony-forming ability

We found that *Msx2* expression decreased with increasing passage and could then be re-expressed at high levels in MSCs repopulated in LD culture, as shown in Fig. 6. Similarly, *Sox2* and *Nanog* but not *Oct4* were significantly down-regulated up to P7, and expression could be restored to high levels in LD culture. To investigate effects of *Sox2*, *Nanog*, and *Msx2* on MSC proliferation and differentiation, repopulated cells were treated with siRNAs targeting each gene (Fig. 7b, left). Protein expression of Sox2, Nanog and Msx2 were decreased in *Sox2* (45% reduction)-, *Nanog* (43% reduction)-, and *Msx2* (67% reduction)-siRNA-transfected cells compared to scramble-siRNA-treated cells (data not shown). Proliferation rate of *Sox2*-siRNA-transfected cells was lower than that of scramble-siRNA-transfected cells (Fig. 7b, right, $P < 0.05$). However, there was no inhibitory effect on proliferation in response to knockdown of *Nanog* or *Msx2* (Fig. 2b). In a CFU assay, number of *Sox2*- and

Msx2-siRNA-transfected cell colonies was significantly lower compared to that of scramble-siRNA-transfected cells, but this was not the case for *Nanog*-siRNA-transfected cells (Fig. 7c, $P < 0.05$). Next, we tested potential of siRNA-transfected MSCs to differentiate into osteogenic, adipogenic and chondrogenic lineages. *Sox2*-siRNA-transfected cells had lower osteogenic, adipogenic and chondrogenic potentials in all donors tested, compared to scramble-siRNA-transfected cells, whereas differentiation potential of *Nanog*-siRNA-transfected cells towards the three lineages was not noticeably different (Fig. 7d). However, results from multiple donors showed donor variations in the three differentiation potentials (data not shown). *Msx2*-siRNA-transfected cells had increase in osteogenic potential (Fig. 7d), but there was donor variation in differentiation potentials to all three lineages (data not shown). In addition, *Sox2* overexpression using lentiviral vector showed increased colony-forming ability in P7-HD MSCs compared to mock vector-infected P7-HD MSCs (Fig. 7e, $P < 0.05$). These results suggest that Sox2 may have an important role in proliferation and multi-differentiation potential of BM-derived MSCs indicated by reproducibility of results from multiple donors.

Figure 7. Expression changes in stemness (pluripotency markers in ES cell)-related genes in P7-HD and P7-LD cells subcultured from late-passage MSCs (P7). (a) mRNA expression patterns of stemness-related genes were observed using real-time PCR in undifferentiated P1, P3, P7, P7-HD and P7-LD MSCs. After subculturing cells from each previous passage, P1, P3, P7, 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 P1 or P7-HD MSCs. (b) Protein expression patterns of scramble-, Sox2-, Nanog-, and Mx2-siRNA-transfected cells were observed using western blotting in P7-LD MSCs. A hexosaminidase assay was performed to determine proliferative potential of siRNA-transfected MSCs. Each experiment was performed in triplicate ($n = 3$). * $P < 0.05$ compared to scramble-siRNA-transfected MSCs. (c) Scramble-, Sox2-, Nanog- and Mx2-siRNA-transfected cells were seeded at 1×10^3 cells in 10 cm culture dishes. Cells were then cultured in DMEM-LG containing 20% FBS for 12 days to examine cell colony formation (violet). Each experiment was performed in triplicate ($n = 3$). * $P < 0.05$, ** $P < 0.01$ compared to scramble-siRNA-transfected MSCs. (d) Scramble-, Sox2-, Nanog- and Mx2-siRNA-transfected cells were seeded at 8×10^4 cells/well in 12-well culture plates. Cells were then cultured in osteogenic, adipogenic and chondrogenic medium to determine their osteogenic, adipogenic and chondrogenic differentiation potentials respectively. Each experiment was performed in triplicate ($n = 3$). (e) Effect of Sox2 overexpression on colony-forming ability of P7-HD MSC. Western blot analysis shows increased levels of Sox2 in P7-HD MSC. Lentivirus-mediated overexpression of Sox2 caused improvement of self-renewal ability in P7-HD MSC. CV staining showed increased colony-forming ability (2-fold) in Sox2-overexpressed cells compared to mock vector-infected cells. Each experiment was performed in triplicate ($n = 3$).

Discussion

In vitro expansion capacity of MSCs fulfills prerequisite requirements for *in vivo* transplantation. However, despite these advantages, BM-derived MSCs gradually lose their stemness and progress to senescence after extended passaging (14–19,23,24,38). A previous study has found that when MSCs were repeatedly subcultured at low density (1.5 or 3.0 cells/cm²) for 6 weeks, cells amplified 10^9 fold (36). In contrast, a further group reported 25) that optimal density for MSC culture was approximately

5000 cells/cm², and MSCs cultured at lower densities underwent apoptotic cell death, however, based on our results, late-passage MSCs that were re-plated at low density (17 cells/cm²) had restored colony-forming activity, cell proliferation and multi-lineage differentiation potential, with small, spindle-shaped cells. To determine which subpopulation of late-passage MSCs could be restored, we isolated three populations by cell size [small (<10 μm), middle and large (>30 μm)] and found that the small population (<10 μm) had higher colony-forming ability than the others, suggesting that small and rapidly

dividing subpopulation could be enriched by controlling plating density, even from late-passage MSCs (data not shown). A previous study has reported that rapid expansion of low-density MSCs is largely dependent on a minor population of small cells (36). Collectively, these results suggest that only small cells within a heterogeneous MSC population can exploit the increased spatial gap between cells and are thus able to proliferate with higher efficiency compared to their larger counterparts, resulting in enhanced proliferation of small cells, under LD conditions. However, it is reasonable to assume that there may be key factors involved in this pathway in addition to spatial distribution, such as cell-to-cell interactions within the heterogeneous cell populations or release of cytokines during the culture period. Further study will be needed to fully identify all of the key factors involved in this process.

The cell cycle is a critical process for determining cell proliferation and cell senescence. In our study, culture under LD condition enhanced S-phase ratio of late-passage MSCs compared to cells grown under HD conditions. mRNA expression levels of *Cyclin A*, *Cyclin B*, *Cyclin D*, *CDK2* and *CDK4* were also enhanced by culturing late-passage MSCs under LD conditions. CDK2 protein is known to be activated by binding to Cyclin A to induce DNA replication in a variety of cell systems (39). Additionally, Cyclin D protein is a critical regulator at transition from G₁ to S phase, when CDK4 is activated by binding to Cyclin D to transfer the DNA replication signal (40). Endogenous promoter activity of Cyclin B is high during the mitotic stage in mammalian cells (41). Our results suggest that proliferative capacity of late-passage MSCs can also be restored using LD culture methods, suggesting that this recovery might be correlated with expression of cyclin genes of the repopulated cells.

Recently, iPS cells have been described comprising a unique class of stem cells in which fully differentiated somatic cells are de-differentiated to recover pluripotency (42,43). Numerous studies have identified a number of transcription factors, such as Oct-4, Sox2 and Nanog, which play key roles in the process of dedifferentiation (44,45). In our study, we also observed that mRNA expression levels of *Sox2* and *Nanog* gradually decreased as passage number increased, while these genes were higher when late-passage MSCs were replated at low cell density. Interestingly, we did not observe any significant change in expression of *Oct4* as passage number increased, but *Oct4* expression was higher under LD conditions. However, a previous study by Lengner *et al.* (46) reported that Oct4 was likely to be dispensable for maintenance of self-renewal capacity in somatic cells. Although it is difficult to directly compare data of their study with ours (as they used mouse cells instead of human BM-

derived MSCs), it is possible that expression of Oct4 may be independent of the specific role it plays in maintaining stemness. However, because *Sox2* and *Nanog* showed marked changes in expression during prolonged cell passage and under LD conditions, it is likely that these two genes play key roles in self-renewal and multi-differentiation capacity of human MSCs. Knockdown of *Sox2* gene using siRNA in P7-LD MSCs showed that level of proliferation and efficacy of differentiation to the three lineages were lower compared to groups treated with siRNAs against *Nanog* and *Msx2*, and scramble siRNA. Recently, a novel function for Sox2 has been identified in maintenance of self-renewal of cells of a mouse osteoblastic lineage (47). 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 MSCs were small and showed high proliferative and osteogenic capabilities from human BM-derived MSCs (35). Liu *et al.* reported that Nanog and Oct4 overexpression using a lentiviral system enhanced proliferation and colony-forming ability in BM-derived MSCs (48). In addition, they confirmed that Oct4 overexpression enhanced adipogenesis, whereas Nanog overexpression impaired efficiency of adipogenesis. In contrast, chondrogenesis was enhanced in both Oct4- and Nanog-overexpressing cells. However, our data showed that *Oct4* expression patterns during long-term culture did not change significantly, and *Nanog*-siRNA-transfected cells did not show meaningful results from multiple donors. Our results suggest that *Sox2* gene is essential for MSC proliferation and osteogenic differentiation and that without it MSCs may lose their stem cell function.

Transcription factors known to be critical in the MSC differentiation process, such as Cbfa1 and Dlx5, which act during osteogenesis, also displayed significant expression changes with increasing passage. In early-passage MSCs, cells are highly multipotent and are naturally uncommitted to any differentiation lineage. However, even in absence of specific differentiation medium, late-passage MSCs gradually committed themselves to osteogenic lineage with increased expression of osteogenic markers, such as ALP and *type I collagen*, and further culture of these cells induced complete commitment to osteogenesis. These data correlate with previous reports indicating gradual commitment of late-passage MSCs to osteogenic lineage (15,16,49). A previous study (37) also found increased expression of Cbfa1 in late-passage MSCs. However, unlike other key transcription factors involved in osteogenesis, such as *Cbfa1*, *Msx2* had the opposite expression profile. We observed gradual decrease in *Msx2* expression with prolonged passaging, and recovery of its expression under LD culture conditions. Although a further previous study (50) reported that *Msx2* knockout mice had

delayed cranial bone formation, the exact role of this gene during osteogenic differentiation is not well-known. Liu *et al.* (51) reported that *Msx2* protein repressed differentiation of immature pre-osteoblasts while increasing their proliferation, and that *Msx2* enhanced both proliferation and differentiation of osteoblasts (52,53). Moreover, it has been reported that *Msx* genes enhance cell proliferation, and their expression is inversely correlated with differentiation (54,55). In our study, we observed significantly reduced MSC proliferation and *Msx2* expression in late-passage cells. When these cells were re-cultured using the LD method, we observed recovered cell proliferation and *Msx2* expression, similar to our findings with *Sox2* and *Nanog*. These results indicate that *Msx2* gene might also be correlated with maintenance of cell proliferation and differentiation potentials of undifferentiated MSCs, along with *Sox2* and *Nanog*.

In conclusion, repopulated cells in LD conditions showed extensive recovery of both proliferation and differentiation capacities, and reactivation of genes involved in cell cycle distribution, pluripotency and differentiation. By controlling plating density, increase in pluripotency-related genes, such as *Sox2*, that were reduced in late-passage MSCs is expected to maintain or increase proliferation and differentiation potential of early- or late-passage MSCs over expanded culture periods.

Acknowledgements

This work was supported by a grant (code: SC3210) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology, Republic of Korea.

References

- Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D *et al.* (1997) Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J. Bone Miner. Res.* **12**, 1335–1347.
- Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J. Cell. Biochem.* **64**, 295–312.
- Haynesworth SE, Goshima J, Goldberg VM, Caplan AI (1992) Characterization of cells with osteogenic potential from human marrow. *Bone* **13**, 81–88.
- Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* **238**, 265–272.
- Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM *et al.* (1998) The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J. Bone Joint Surg. Am.* **80**, 1745–1757.
- Wang WG, Lou SQ, Ju XD, Xia K, Xia JH (2003) In vitro chondrogenesis of human bone marrow-derived mesenchymal progenitor cells in monolayer culture: activation by transfection with TGF-beta2. *Tissue Cell* **35**, 69–77.
- Neubauer M, Hacker M, Bauer-Kreisel P, Weiser B, Fischbach C, Schulz MB *et al.* (2005) Adipose tissue engineering based on mesenchymal stem cells and basic fibroblast growth factor in vitro. *Tissue Eng.* **11**, 1840–1851.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR *et al.* (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41–49.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD *et al.* (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.
- Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71–74.
- Conget PA, Minguell JJ (1999) Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J. Cell. Physiol.* **181**, 67–73.
- Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI (1995) Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Bone Marrow Transplant.* **16**, 557–564.
- Minguell JJ, Erices A, Conget P (2001) Mesenchymal stem cells. *Exp. Biol. Med. (Maywood)* **226**, 507–520.
- Mets T, Verdonk G (1981) In vitro aging of human bone marrow derived stromal cells. *Mech. Ageing Dev.* **16**, 81–89.
- Bruder SP, Jaiswal N, Haynesworth SE (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J. Cell. Biochem.* **64**, 278–294.
- Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br. J. Haematol.* **107**, 275–281.
- Rombouts WJ, Ploemacher RE (2003) Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* **17**, 160–170.
- Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ, Bellantuno I (2004) Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. *Stem Cells* **22**, 675–682.
- Ksiazek K (2009) A comprehensive review on mesenchymal stem cell growth and senescence. *Rejuvenation Res.* **12**, 105–116.
- Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **37**, 614–636.
- Dennis JE, Carbillet JP, Caplan AI, Chabord P (2002) The STRO-1+ marrow cell population is multipotential. *Cells Tissues Organs* **170**, 73–82.
- Prockop DJ, Sekiya I, Colter DC (2001) Isolation and characterization of rapidly self-renewing stem cells from cultures of human marrow stromal cells. *Cytotherapy* **3**, 393–396.
- Stolzing A, Jones E, McGonagle D, Scutt A (2008) Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech. Ageing Dev.* **129**, 163–173.
- Nishida S, Endo N, Yamagiwa H, Tanizawa T, Takahashi HE (1999) Number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation. *J. Bone Miner. Metab.* **17**, 171–177.
- van den Bos C, Silverstetter S, Murphy M, Connolly T (1998) p21(cip1) rescues human mesenchymal stem cells from apoptosis induced by low-density culture. *Cell Tissue Res.* **293**, 463–470.

- 26 Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* **20**, 530–541.
- 27 Neuhuber B, Swanger SA, Howard L, Mackay A, Fischer I (2008) Effects of plating density and culture time on bone marrow stromal cell characteristics. *Exp. Hematol.* **36**, 1176–1185.
- 28 Chen L, Tredget EE, Wu PY, Wu Y (2008) Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* **3**, e1886.
- 29 Mueller MB, Tuan RS (2008) Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis Rheum.* **58**, 1377–1388.
- 30 Dickhut A, Pelttari K, Janicki P, Wagner W, Eckstein V, Egermann M *et al.* (2009) Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage. *J. Cell. Physiol.* **219**, 219–226.
- 31 Goessler UR, Bieback K, Bugert P, Heller T, Sadick H, Hormann K *et al.* (2006) In vitro analysis of integrin expression during chondrogenic differentiation of mesenchymal stem cells and chondrocytes upon dedifferentiation in cell culture. *Int. J. Mol. Med.* **17**, 301–307.
- 32 Wagner W, Bork S, Horn P, Kronic D, Walenda T, Diehlmann A *et al.* (2009) Aging and replicative senescence have related effects on human stem and progenitor cells. *PLoS One* **4**, e5846.
- 33 Pierantozzi E, Gava B, Manini I, Roviello F, Marotta G, Chiavarelli M *et al.* (2010) Pluripotency regulators in human mesenchymal stem cells: expression of NANOG but not of OCT-4 and SOX-2. *Stem Cells Dev.* **20**, 915–923.
- 34 Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM *et al.* (2006) Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J. Cell. Biochem.* **99**, 1285–1297.
- 35 Go MJ, Takenaka C, Ohgushi H (2008) Forced expression of Sox2 or Nanog in human bone marrow derived mesenchymal stem cells maintains their expansion and differentiation capabilities. *Exp. Cell Res.* **314**, 1147–1154.
- 36 Colter DC, Class R, DiGirolamo CM, Prockop DJ (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA* **97**, 3213–3218.
- 37 Kozhevnikova MN, Mikaelian AS, Paiushina OV, Starostin VI (2008) Comparative characterization of mesenchymal bone marrow stromal cells at early and late stages of culturing. *Izv. Akad. Nauk Ser. Biol.* **2**, 156–162.
- 38 Banfi A, Muraglia A, Dozin B, Mastrogiacomio M, Cancedda R, Quarto R (2000) Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. *Exp. Hematol.* **28**, 707–715.
- 39 Wang Q, Hirohashi Y, Furuuchi K, Zhao H, Liu Q, Zhang H *et al.* (2004) The centrosome in normal and transformed cells. *DNA Cell Biol.* **23**, 475–489.
- 40 Marzec M, Kasprzycka M, Lai R, Gladden AB, Wlodarski P, Tomczak E *et al.* (2006) Mantle cell lymphoma cells express predominantly cyclin D1a isoform and are highly sensitive to selective inhibition of CDK4 kinase activity. *Blood* **108**, 1744–1750.
- 41 Sciortino S, Gurtner A, Manni I, Fontemaggi G, Dey A, Sacchi A *et al.* (2001) The cyclin B1 gene is actively transcribed during mitosis in HeLa cells. *EMBO Rep.* **2**, 1018–1023.
- 42 Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676.
- 43 Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K *et al.* (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872.
- 44 Shi W, Wang H, Pan G, Geng Y, Guo Y, Pei D (2006) Regulation of the pluripotency marker Rex-1 by Nanog and Sox2. *J. Biol. Chem.* **281**, 23319–23325.
- 45 Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S *et al.* (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920.
- 46 Lengner CJ, Camargo FD, Hochedlinger K, Welstead GG, Zaidi S, Gokhale S *et al.* (2007) Oct4 expression is not required for mouse somatic stem cell self-renewal. *Cell Stem Cell* **1**, 403–415.
- 47 Basu-Roy U, Ambrosetti D, Favaro R, Nicolis SK, Mansukhani A, Basilico C (2010) The transcription factor Sox2 is required for osteoblast self-renewal. *Cell Death Differ.* **17**, 1345–1353.
- 48 Liu TM, Wu YN, Guo XM, Hui JH, Lee EH, Lim B (2009) Effects of ectopic Nanog and Oct4 overexpression on mesenchymal stem cells. *Stem Cells Dev.* **18**, 1013–1022.
- 49 Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R *et al.* (2008) Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PLoS One* **3**, e2213.
- 50 Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K *et al.* (2000) Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat. Genet.* **24**, 391–395.
- 51 Liu YH, Tang Z, Kundu RK, Wu L, Luo W, Zhu D *et al.* (1999) Msx2 gene dosage influences the number of proliferative osteogenic cells in growth centers of the developing murine skull: a possible mechanism for MSX2-mediated craniosynostosis in humans. *Dev. Biol.* **205**, 260–274.
- 52 Ishii M, Merrill AE, Chan YS, Gitelman I, Rice DP, Sucov HM *et al.* (2003) Msx2 and Twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. *Development* **130**, 6131–6142.
- 53 Ichida F, Nishimura R, Hata K, Matsubara T, Ikeda F, Hisada K *et al.* (2004) Reciprocal roles of MSX2 in regulation of osteoblast and adipocyte differentiation. *J. Biol. Chem.* **279**, 34015–34022.
- 54 Dodig M, Tadic T, Kronenberg MS, Dacic S, Liu YH, Maxson R *et al.* (1999) Ectopic Msx2 overexpression inhibits and Msx2 antisense stimulates calvarial osteoblast differentiation. *Dev. Biol.* **209**, 298–307.
- 55 Mina M, Gluhak J, Rodgers B (1996) Downregulation of Msx-2 expression results in chondrogenesis in the medial region of the avian mandible. *Connect. Tissue Res.* **35**, 79–84.