TGF-β Signaling Accelerates Senescence of Human Bone-Derived CD271 and SSEA-4 Double-Positive Mesenchymal Stromal Cells

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https://doi.org/10.1016/j.stemcr.2018.01.030

SUMMARY

It is generally thought that the proliferative capacity and differentiation potential of somatic stem cells, including mesenchymal stromal/stem cells (MSCs) and hematopoietic stem cells (HSCs), decline with age. We investigated the effects of aging on human bone-derived MSCs expressing CD271 and SSEA-4 (double-positive MSCs [DPMSCs]). The percentages of DPMSCs in bone tissue decreased significantly with age. The DPMSCs from elderly patients (old DPMSCs) showed cellular senescence, which was evidenced by low growth potential, high senescence-associated β-galactosidase activity, and elevated p16 and p21 CDK inhibitor levels. Moreover, old DPMSCs showed weak osteogenic differentiation potential and less hematopoiesis-supporting activity in comparison with young DPMSCs. Interestingly, the addition of transforming growth factor β2 (TGF-β2) induced cellular senescence in young DPMSCs. With the exception of the adipogenic differentiation potential, all of the aging phenomena observed in old DPMSCs were reversed by the addition of anti-TGF-β antibodies. These results suggest that, in part, old DPMSCs accelerate cellular senescence through TGF-β signaling.

INTRODUCTION

It is well documented that the proliferative capacity and differentiation potential of somatic stem cells, including mesenchymal stromal/stem cells (MSCs) and hematopoietic stem cells (HSCs), decline with age (Oh et al., 2014; Allsopp et al., 2003; Nakamura-Ishizu and Suda, 2014). This process is included in the concept of senescence. However, the process of stem cell senescence remains elusive. Thus, it is important to elucidate the fundamental molecular mechanisms that regulate the cellular functions of stem cells, such as MSCs and HSCs, in order to understand their aging process.

From another point of view, HSCs are thought to be maintained in the specialized bone marrow (BM) microenvironment, also known as the HSC niche (Morrison and Scadden, 2014; Méndez-Ferrer et al., 2010). Collectively, it is considered that the aging of niche cells may affect the cellular senescence of HSCs in terms of their self-renewal activity, and their proliferation and differentiation abilities (Mendelson and Frenette, 2014). In fact, it was reported that aged MSCs exerted weak osteogenic differentiation potential and enhanced adipogenic differentiation potential, both of which are evidence of cellular senescence (Liu et al., 2015). Moreover, aged MSCs produced various tissue-aging stimulating factors. These bioactive mediators are thought to be a component of the senescence-associated secretory phenotype (SASP) (Acosta et al., 2013). The SASP causes an inflammatory response as well as the cell-cycle arrest of surrounding cells through a paracrine mechanism (Acosta et al., 2013). However, the relationship between individual aging and the cellular senescence of MSCs is still unclear. Furthermore, the mechanism of the cellular senescence of MSCs and the HSC-supporting activity of aged human MSCs have remained elusive until now.

Recently, we succeeded in identifying/establishing human BM-derived MSCs that express CD271 and SSEA-4 (double-positive MSCs [DPMSCs]) (Matsuoka et al., 2015). The DPMSCs had an ability to differentiate into mesenchymal cell lineages and supported the activity of human cord blood (CB)-derived CD34-positive and negative (CD34+/−) HSCs in vitro. These DPMSCs expressed higher levels of HSC-supportive genes, including CXCL12 and FOXC1 (Omatsu et al., 2014) and showed the higher supporting ability of human CB-derived CD34+ HSCs in vitro. These DPMSCs expressed higher levels of HSC-supportive genes, including CXCL12 and FOXC1 (Omatsu et al., 2014) and showed the higher supporting ability of human CB-derived CD34+ HSCs in vitro. These DPMSCs expressed higher levels of HSC-supportive genes, including CXCL12 and FOXC1 (Omatsu et al., 2014) and showed the higher supporting ability of human CB-derived CD34+ HSCs in vitro. These DPMSCs expressed higher levels of HSC-supportive genes, including CXCL12 and FOXC1 (Omatsu et al., 2014) and showed the higher supporting ability of human CB-derived CD34+ HSCs in vitro. These DPMSCs expressed higher levels of HSC-supportive genes, including CXCL12 and FOXC1 (Omatsu et al., 2014) and showed the higher supporting ability of human CB-derived CD34+ HSCs in vitro. These DPMSCs expressed higher levels of HSC-supportive genes, including CXCL12 and FOXC1 (Omatsu et al., 2014) and showed the higher supporting ability of human CB-derived CD34+ HSCs in vitro. These DPMSCs expressed higher levels of HSC-supportive genes, including CXCL12 and FOXC1 (Omatzu et al., 2015). It is well documented that steady-state hematopoiesis is sustained by HSCs and niche cells, including DPMSCs and various cytokines (Morrison and Scadden, 2014; Méndez-Ferrer et al., 2010; Lapidot et al., 2005; Wilson and Trumpp, 2006; Smith and Calvi, 2013). Thus, it would be interesting to know how aging affects the niche function of DPMSCs.

In this study, we succeeded in establishing human bone-derived DPMSCs from two different donor groups: young and elderly adults. We then investigated the effects of aging on the functions of human bone-derived DPMSCs in vitro and in vivo to understand the basic mechanisms of cellular senescence. We found that transforming growth factor β2...
(TGF-β2) is one of the candidate factors for the aging of DPMSCs. Interestingly, with the exception of the adipogenic differentiation potential, all of the aging phenomena observed in old DPMSCs were reversed by the addition of anti-TGF-β antibodies (1D11). These results suggest that, in part, old DPMSCs accelerate cellular senescence through TGF-β signaling.

RESULTS

Anatomical Distribution and Proportion of Bone-Derived 11Lin CD45−CD271−SSEA-4− Cells
First, we analyzed/compared the proportion of 11Lin−CD45−CD271−SSEA-4− cells in human femoral neck bone tissues and their BM. The BM cells residing in the bone tissues were collected by vigorous washing. Bone-derived cells were collected from the remaining bone tissue after enzymatic treatment with collagenase and dispase (see Supplemental Experimental Procedures). As shown in Figure S1A, the proportion of 11Lin−CD45−CD271−SSEA-4− cells in bone-derived cells was greater than that of BM cells. Next, we compared the proportions of 11Lin−CD45−CD271−SSEA-4− cells in the human femoral neck, femoral head, and acetabulum. As clearly shown in Figure S1B, the proportion of 11Lin−CD45−CD271−SSEA-4− cells in the femoral neck was higher than the proportions in the femoral head and acetabulum. Based on these findings, we used human femoral neck-derived 11Lin−CD45−CD271−SSEA-4− cells in the following experiments.

Isolation of Bone-Derived 11Lin−CD45−CD271+/−SSEA-4+/− Cells from Young and Elderly Patients and Establishment of Bone-Derived Mesenchymal Stromal/Stem Cells
Human femoral neck bone tissue samples are shown in Figure S2A. As the figure clearly shows, the depth of the cortical bone of the elderly patient was thinner than that of the young patient. In addition, the degree of osteoporotic change in the cancellous bone (Ca) was more severe in the elderly patient than in the young patient. As shown in Figure S2B, the young patient’s bone-derived 11Lin−CD45−cells were further subdivided into CD271−SSEA-4− (SSEA-4 single-positive; SSEA-4 SP, R4), CD271−SSEA-4− (double-positive; DP, R5), CD271−SSEA-4− (double-negative; DN, R6) and CD271−SSEA-4− (CD271 single-positive; CD271 SP, R7) fractions according to their expression levels of CD271 and SSEA-4, as previously reported (Matsuoka et al., 2015). These four cell fractions were cultured in α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS) at 37°C with 5% CO2. We then succeeded in establishing three MSC lines from the R5, R6, and R7 fractions, respectively (Figure S2Cb, c, and d). These three MSC lines showed a similar spindle-shaped morphology. Among them, MSCs from the DN fraction showed poor proliferation. We finally succeeded in establishing DPSC lines from 3 out of 3 young patients and 3 out of 8 elderly patients (Table S1).

We then analyzed the cell-surface marker expression of young and old DPMSCs. Both of the DPMSCs expressed known MSC markers, including CD29, CD44, CD73, CD90, and CD105 (Figure S3Aa). Both DPMSCs also expressed human leukocyte antigen (HLA)-ABC, but not HLA-DR. In contrast, none of these DPMSCs expressed the endothelial/hematopoietic cell markers, including CD31, CD34, CD41, CD45, and CD56 (Figure S3Aa). Interestingly, both DPMSCs expressed perivascular cell markers, including CD146, neuron-glial 2 (NG2), platelet-derived growth factor receptor α (PDGFRα), and PDGFRβ (Figure S3Ab). qRT-PCR confirmed the expression of perivascular cell marker genes, including CSPG4, MCAM, PDGFRα, PDGFRβ, ACTA2, and NES (Figure S4). The expression of CD271 on old DPMSCs was downregulated during in vitro passages (Figure S3B). Conversely, the expression of SSEA-4 on both young and old DPMSCs was unchanged until passage 4.

Percentages of Bone-Derived CD271+/−SSEA-4+/− Cells in Young and Elderly Patients
Next, we compared the percentages of bone-derived CD271+/−SSEA-4+/− cells in young and elderly patients. Representative scattergrams of the expression of CD271 and SSEA-4 on bone-derived 11Lin−CD45− cells from young and elderly patients are shown in Figure 1A. The percentages of CD271−SSEA-4− (R4), CD271−SSEA-4− (R6), and CD271−SSEA-4− (R7) cells were comparable (Figure 1B and Table S1). Interestingly, the percentage of elderly patient-derived CD271−SSEA-4− (R5) cells was significantly decreased in comparison with that in young patient-derived cells (Figure 1Bb). However, we succeeded in establishing bone-derived DPMSCs from both young and elderly patients. As we recently reported (Matsuoka et al., 2015), human BM-derived DPMSCs exerted potent HSC-supportive activity. We therefore focused on the bone-derived DPMSCs from young and elderly patients and analyzed their stem cell characteristics from the viewpoint of cellular senescence.

Stem Cell Characteristics of CD271−SSEA-4− MSCs Established from the Bone Tissue of Young and Elderly Patients
As shown in Figure 2Aa, all three DPMSCs established from young patients (young DPMSCs) showed continuous growth curves until passage 16, at which point they plateaued. In contrast, two of the three DPMSC lines established from elderly patients (old DPMSCs) showed...
continuous growth curves until only passage 11; they then plateaued and stopped proliferating at passage 12. One DPMSC line established from an elderly patient (91 years old) showed poor proliferation ability and stopped proliferating at passage 4. These results clearly demonstrated that the young DPMSCs had a growth advantage in comparison with the old DPMSCs.

Next, we compared the growth curves of three types of MSCs (DPMSCs, CD271SP MSCs, and DN MSCs) established from one young (28F) patient and one old (80F) patient. As shown in Figure 2Ab, the three types of young MSC lines showed continuous growth curves until passage 12. However, the young DPMSCs showed a growth advantage in comparison with the two other types of MSC lines. Two old MSC lines (DPMSCs and CD271SP MSCs) both showed continuous growth curves until passage 11 (Figure 2Ac). Again, old DPMSCs showed a growth advantage in comparison with CD271SP MSCs. In contrast, the old DN MSCs showed poor proliferation ability and their proliferation stopped at passage 9.

From another point of view, the aforementioned growth curve of old DPMSCs suggests their early cellular senescence. Thus, we analyzed the expression of the senescence-associated β-galactosidase (β-gal) activity in young and old DPMSCs (Figure 2B). At passage 4, the mean percentages of β-gal-positive cells in young and old DPMSCs were 1.3% and 20.6%, respectively. At passage 9, the mean percentage of β-gal-positive cells was still 3.1% in young DPMSCs; however, it dramatically increased to 83.2% in old DPMSCs. We then analyzed the expression of cyclin-dependent kinase (CDK) inhibitors, including p16 and p21, which are known to play an important role in arresting the cell cycle in the G1 phase of stem cells upon a variety of stimuli (Collado et al., 2007; van Deursen, 2014; Gil and Peters, 2006). As expected, old DPMSCs expressed significantly higher levels of P16 and P21 in comparison with young DPMSCs (Figure 2C).

Osteogenic, Adipogenic, and Chondrogenic Differentiation Potential of Established Young and Old DPMSCs

We assessed the differentiation potential of established young and old DPMSCs. The DPMSCs (passage 4) were induced to differentiate into osteogenic, adipogenic, and chondrogenic lineages (Figure 3A). We found that the young and old DPMSCs could differentiate into all three lineages. However, the young DPMSCs showed a significantly higher osteogenic differentiation capacity in comparison with old DPMSCs (Figure 3Ba and b). In contrast, old DPMSCs showed significantly higher adipogenic differentiation
capacity in comparison with young DPMSCs (Figure 3Bc and d). Interestingly, the young DPMSCs expressed significantly higher levels of RUNX2 (an osteogenic gene) in comparison with old DPMSCs (Figure 3Ca). On the other hand, the expression levels of PPARG2 (an adipogenic gene) (Figure 3Cb) and SOX9 (an osteo-chondrogenic gene) (Figure 3Cc) were comparable between young and old DPMSCs.

Hematopoiesis-Supporting Abilities of Young and Old DPMSCs: Effects of the Direct Adhesion of DPMSCs to Hematopoietic Cells

The hematopoiesis-supportive abilities of young and old DPMSCs were first assessed using a co-culture system. In these experiments, we used sorted human CB-derived 18Lin^−CD34^CD38^-CD133^+ (abbreviated as 34^−38^-133^+) cells (Figure 4Aa, R5) as a target cell population, which contained primitive long-term repopulating HSCs, as previously reported (Takahashi et al., 2014). The young and old DPMSCs (passage 4) were seeded and cultured for 7 days before the start of co-culturing. The 34^-38^-133^+ cells were cultured with or without feeder DPMSCs for 7 days in serum-free medium with a cocktail of cytokines (Figure 4Ab–e). After co-culturing, the cells were collected, and the hematopoiesis-supportive abilities of the DPMSCs in vitro were assessed by cell proliferation/differentiation assays.

In the co-cultures of 34^-38^-133^+ cells, the numbers of cells were increased with young DPMSCs (130- to 200-fold) and old DPMSCs (80- to 130-fold) (Figure 4Ba). Both young and old DPMSCs supported significantly higher numbers of cells in comparison with the stroma-free
control (mean, 53-fold). Interestingly, young DPMSCs supported significantly higher numbers of cells in comparison with old DPMSCs. The mean percentages of CD34+ cells after 7 days of co-culturing 34°38° 133° cells with young or old DPMSCs (33.9% or 30.9%, respectively) were significantly higher than that of the stroma-free control (17.9%) (Figure 4Bb). As a result, the mean absolute numbers of CD34+ cells after 7 days of co-culturing 34°38° 133° cells with young or old DPMSCs (160 \times 10^3 or 89 \times 10^3, respectively) were significantly higher than that of the stroma-free controls (28 \times 10^3) (Figure 4Bc). Moreover, there was a significant difference between the co-cultures with young and old DPMSCs.

Next, we analyzed the contact-independent hematopoiesis-supportive abilities of young and old DPMSCs using a transwell system. The 34°38° 133° cells were seeded directly or indirectly onto young and old DPMSCs feeders using transwell culture insert. The 34°38° 133° cells that were cultured without feeders were used as a stroma-free control. In the presence of a transwell, the magnitude of cellular proliferation was reduced in both of the co-cultures. Namely, the numbers of cells increased with young DPMSCs (70- to 100-fold) and old DPMSCs (50- to 70-fold). However, both young and old DPMSCs supported higher numbers of cells in comparison with the stroma-free control (mean, 40-fold) (Figure 4Ca).

See also Figure S3.
seemed to be a difference between the co-cultures with young and old DPMSCs even in the presence of a transwell. Interestingly, the mean absolute numbers of CD34+ cells after 7 days of co-culturing 34^+38/C0133+ cells with young and old DPMSCs (30 ± 10^3 and 23 ± 10^3) seemed to be higher than the mean absolute numbers of CD34+ cells in stroma-free controls (12 ± 10^3) (Figure 4Cc). The mean absolute numbers of CD34+ cells after 7 days of co-culturing 34^+38/C0133+ cells with young DPMSCs tended to be higher than that of old DPMSCs, albeit not to a significant degree (Figure 4Cc). These results demonstrated that the contact of the target cells with young and old DPMSCs exerted significant hematopoiesis-supporting abilities. However, this contact was not essential for the hematopoiesis-supporting abilities of either young or old DPMSCs. In addition, it was suggested that some soluble factors secreted/produced by young and old DPMSCs may play an important role in the hematopoiesis-supporting abilities.

Next, we performed co-culturing experiments in which 34^+38/133+ cells were co-cultured with three types of bone-derived MSCs (DPMSCs, CD271SP MSCs, and DN MSCs) for 2 weeks. As shown in Figure 4A, all three types of young and old MSCs supported significantly higher absolute numbers of CD45^+ cells (a to c) and CD34^+ cells (d to f) in comparison with the stroma-free cultures. After 2 weeks of co-culture, the absolute numbers of CD45^+ and CD34^+ cells were greater in comparison with the
numbers after 1 week of co-culturing. Very interestingly, all three types of young MSCs supported significantly higher numbers of CD34+ cells in comparison with the three types of old MSCs. Next, we analyzed the effects of all three types of MSCs on the ex vivo expansion of colony-forming cells (CFCs). Consistent with the effects on the absolute numbers of CD34+ cells, the same trends were observed in the co-cultures with all three types of MSCs (Figure S5B). We then analyzed the proportions of colony types, including CFU-GM, BFU-E, and CFU-Mix, in the co-cultures with the three types of MSCs. After 1 week of co-cultures, the proportions of BFU-E in the co-cultures with all three types of young MSCs were significantly higher than those with the three types of old MSCs. After 2 weeks of co-culturing, however, the proportions of CFU-GM were increased in the co-cultures with all three types of young and old MSCs (Figure S5C). Collectively, these results suggest that the young DPMSCs exerted more potent hematopoietic progenitor cell (HPC)-supporting abilities in comparison with old DPMSCs through direct and/or indirect mechanisms.

**Expression Profiles of Hematopoiesis-Supportive Genes and Perivascular Cell Marker Genes in Young and Old DPMSCs**

We then analyzed the differences in the hematopoiesis-supportive gene expression profiles between young and old DPMSCs. These genes have already been reported to be expressed in mouse and human HSC niche cells (Ren et al., 2011). As shown in Figure S4, the expression of IGF2, TGFβ3, CXCL12, and FOXC1 in young DPMSCs tended to be higher than in old DPMSCs, albeit not to a significant degree. The expression of other hematopoiesis-supportive genes, including WNT3A, JAG1, ANGPT1, TGFβ1, and FGFR2, was comparable in young and old DPMSCs. Among the perivascular cell marker genes, including CSPG4, MCAM, PDGFRA, PDGFRB, ACTA2, and NES, the expression of PDGFRB in young DPMSCs was significantly higher than that in old DPMSCs.

**Hematopoiesis-Supporting Abilities of Young and Old DPMSCs as Evaluated by SCID-Repopulating Cell Activity**

The SCID-repopulating cell (SRC) activities of cultured 34+38-133+ cells were assessed at 20 weeks after transplantation (Table S2). Specified numbers of cells recovered from the co-cultures with or without DPMSCs were transplanted into NOG mice using the intra-bone marrow injection (IBMI) method (Table S2). As shown in Figure 5Aa, all of the five mice that received stroma-free cultured cells showed human CD45+ cell engraftment in the left tibia (median, 3.7%). All of the five mice that received co-cultured cells with young and old DPMSCs showed human CD45+ cell engraftment in the left tibia (median, 43.1% and 10.5%, respectively). Interestingly, the median repopulation level of CD45+ cells in the five mice that received cells co-cultured with young DPMSCs was significantly higher than that of the mice that received cells from stroma-free cultures. In the peripheral blood (PB), the repopulation levels of CD45+ cells in the mice that received cells co-cultured with young and old DPMSCs (median, 4.2% and 11.7%, respectively) were also significantly higher in comparison with that of the mice that received cells from stroma-free cultures (median, 0.0%). We then analyzed the multilineage differentiation capacities of SRCs supported by young DPMSCs through direct and/or indirect mechanisms.

We then performed secondary transplantation. As shown in Figure 5B, all three types of BM cells recovered from all mice that received stroma-free cultured cells and cells co-cultured with young and old DPMSCs were significantly more potent than those with the three types of old MSCs. Among the perivascular cell marker genes, including CB-derived multipotential CD34+ SRCs (HSCs) in co-cultures. As mentioned above, the difference in the HPC/HSC-supportive ability of young and old DPMSCs may be explained by some soluble factors. To investigate the soluble factors secreted/produced from young and old DPMSCs, we performed a global gene expression analysis using a microarray. First, we compared the gene expression profiles of young and old DPMSCs by a gene set enrichment analysis (GSEA) (Figure S6A). The gene sets enriched in young DPMSCs were those related to cell cycle/mitosis, DNA replication, young cell signature, energy metabolism, and osteogenic differentiation, and so on. The gene sets enriched in old DPMSCs included those related to TGF-β signaling, cell adhesion/migration, adipocyte differentiation, vascular signature, and cell-cycle arrest.
We then focused on the gene set of genes associated with cell-cycle arrest. As shown in Figure S6Bb, the gene expression levels of CDK inhibitors (including p21, p16, and p15) in the old DPMSCs tended to be high in comparison with those in young DPMSCs. In concert with this tendency, the expression levels of CDK1 and CDK2, which are regulated by p21, and the target genes of the lower pathway, such as RB1 and E2F1, tended to decrease in the old DPMSCs (Figure S6Bc and d). On the other hand, the expression levels of CDK4 and CDK6, which are regulated by p16, were comparable (Figure S6Bc). Next, we selected 138 differentially expressed genes (Table S3) for which the expression levels (signal value) were significantly different (>2-fold difference) from 60,901 probes (Figure 6). Very interestingly, we identified TGFbeta2 as a gene that was highly expressed in old DPMSCs in comparison with young DPMSCs (Figures 6 and S6Ba). In contrast, the expression level of SMAD6, which suppresses TGF-beta signaling, was significantly low, resulting in the enhancement of TGF-beta signaling in the old DPMSCs. Moreover, the expression level of the ID2 gene, which conveys the downstream signal of bone morphogenetic protein, was also significantly low in the old DPMSCs. These results may provide an important clue for elucidating the molecular background that sustains the differential cellular function of young and old DPMSCs.
Effects of TGF-β2 on the Proliferation, Differentiation, and Hematopoiesis-Supporting Abilities of Young and Old DPMSCs In Vitro

We analyzed the production of TGF-β2 of young and old DPMSCs. As shown in Figure 7A, old DPMSCs produced a significantly higher level (266 pg/mL) of TGF-β2 in comparison with young DPMSCs (118 pg/mL). Next, we analyzed the effects of TGF-β2 on the proliferation of young DPMSCs in cultures. TGF-β2 suppressed the proliferation of young DPMSCs in a dose-dependent fashion (Figure 7B).

We then analyzed the effects of TGF-β2 or anti-TGF-β monoclonal antibody (mAb) (1D11) on the differentiation capacity of young and old DPMSCs. As shown in Figure 7Ca and b, TGF-β2 strongly suppressed the osteogenic differentiation capacity of both young and old DPMSCs. Interestingly, the addition of 1D11 tended to enhance the osteogenic differentiation capacity of both young and old DPMSCs, albeit not to a significant degree (Figure 7Cc and d). In contrast, the addition of TGF-β2 or 1D11 did not affect the adipogenic differentiation capacity of either young or old DPMSCs (Figure 7D).

Finally, we analyzed the effects of TGF-β2 or 1D11 on the hematopoiesis-supporting capacity of both young and old DPMSCs. In co-cultures of 34^+38^+ cells with young and old DPMSCs, the numbers of cells increased with both young DPMSCs (100- to 150-fold) and old DPMSCs (80- to 120-fold) (Figure S7A). Both young and old DPMSCs supported higher numbers of cells in comparison with the stroma-free controls (mean, 53-fold). Interestingly, TGF-β2 seemed to inhibit the proliferation of target cells in all three culture conditions (Figure S7A). In contrast, the addition of 1D11 did not show any significant effects on the fold increase of the target cells in any of the three culture conditions.

Consistent with the data shown in Figure 4Cc, after 7 days of co-culturing 34^+38^+ cells with young and old DPMSCs (80 \times 10^3 and 62 \times 10^3), the mean absolute numbers of CD34^+ cells seemed to be higher in comparison with the stroma-free controls (21 \times 10^3) (Figure S7B). The addition of TGF-β2 showed a tendency to reduce the absolute numbers of CD34^+ cells in the co-cultures with young and old DPMSCs. Conversely, the addition of 1D11 showed a tendency to enhance the absolute number of CD34^+ cells in the co-cultures with young and old DPMSCs, albeit not to a significant degree. Collectively, these results suggest that the hematopoiesis-supporting abilities of young as well as old DPMSCs could be inhibited via TGF-β2 in the BM niche. As shown in Figure 7A, old DPMSCs produced a large amount of TGF-β2 in comparison with young DPMSCs. Thus, it is possible that TGF-β2 may play an important/significant role in hematopoiesis, especially in aged patients.
to elucidate the aforementioned fundamental molecular mechanism (Matsuoka et al., 2015; Tormin et al., 2011).

Peault and colleagues previously reported a prospective method for isolating CD146+ perivascular cells that were able to support the long-term persistence of human myelo-lymphoid HSCs in co-cultures (Corselli et al., 2013). We recently developed a prospective method for isolating BM-derived MSC populations based on the expression of CD271 and SSEA-4 (Matsuoka et al., 2015). Our established DPMSCs expressed perivascular cell markers, including CD146, α-smooth muscle actin, NG2, PDGFRα and PDGFRβ, and NESTIN (Figures S3 and S4). Thus, the DPMSCs may have some relationship with perivascular cells/pericytes. Indeed, we demonstrated that human BM-derived DPMSCs exerted potent HSC-supporting ability (Matsuoka et al., 2015), as did CD146+ perivascular cells. In this mouse study, nestin-positive MSCs expressing a high level of CXCL12 were found in regions adjacent to the bone or within the BM parenchyma (Méndez-Ferrer et al., 2010). These nestin-positive MSCs significantly co-localized with Lin−CD150+CD48− HSCs, suggesting their function as niche cells. Thus, the DPMSCs have some relevance with mouse nestin-positive MSCs.

In this study, we succeeded in establishing bone-derived young DPMSCs from female/male patients with osteonecrosis. In contrast, we only established bone-derived old

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**Figure 7. Production of TGF-β2 by Young and Old DPMSCs and the Effects of TGF-β2 on the Proliferation and Differentiation of Young and/or Old DPMSCs in Cultures**

(A) We used three young and three old DPMSC lines. The concentration of TGF-β2 protein in the cultures of young and old DPMSCs at passage-4 supernatants was measured by ELISA. Error bars represent the mean ± SD of three independent experiments. Data were evaluated by Welch’s t test. *p < 0.05.

(B) (a) We performed the same experiments three times using three young DPMSC lines. The growth curve of one representative young DPMSC line (31F) in the presence of various concentrations (1, 10, and 100 ng/mL) of recombinant human TGF-β2 is shown. Each dot represents the mean ± SD of three culture dishes. Data were evaluated by one-way ANOVA with Tukey’s multiple comparison procedure. *p < 0.05. (b) Representative phase-contrast images of young DPMSCs in the presence or absence of TGF-β2 (1, 10, and 100 ng/mL) at passage 7. Scale bars, 200 μm.

(C and D) We used two young and two old DPMSC lines. The effects of TGF-β2 and anti-TGF-β mAb (1D11) on the osteogenic (C) and adipogenic (D) differentiation potentials of young and old DPMSCs are shown. Representative fluorescence images of cellular calcium deposition staining by calcein (C, a and c) and cellular lipid droplet staining (BODIPY) (D, a and c) in the presence or absence of TGF-β2 (100 ng/mL) or 1D11 (10 μM/mL) are shown. Scale bars, 500 μm. The mean intensity of calcein (C, b and d) and BODIPY (D, b and d) fluorescence of the differentiated DPMSCs of the three pictured fields of each DPMSC line were analyzed by fluorescence microscopy. The data show the MFI of two young or old DPMSC lines. Error bars represent the mean ± SD of six measurements (3 per one young or old DPMSC line) in two independent experiments. Data were evaluated by two-way ANOVA with Tukey’s multiple comparison procedure. See also Figure S7.
DPMSCs from female patients with osteoarthritis. Interestingly, young DPMSGs exerted more potent effects on the expansion of CD34+ cells and CFCs in comparison with old DPMSGs (Figures S5A and S5B). However, both young and old DPMSGs showed significant HSC-supporting abilities upon primary and secondary transplantation for the SRC assay (Figure 5). These results suggest that hematopoiesis-supporting abilities of young and old DPMSGs may differ between HPCs and HSCs. It was suggested that bone-derived DPMSGs play an important role in bone tissue maintenance, regeneration, and steady-state hematopoiesis. Unexpectedly, the percentages of bone-derived DPMSGs significantly decreased with age (Figure 1 and Table S1), suggesting that this reduction of DPMSGs may cause or be related to the abnormal hematopoiesis observed in elderly patients (Guralnik et al., 2004).

Moreover, old DPMSGs exerted higher adipogenic potential and lower osteogenic potential in comparison with young DPMSGs (Figure 3). In young adults, MSC differentiation favors osteoblastogenesis; however, in elderly adults the differentiation is balanced toward adipogenesis (Bethel et al., 2013). As previously reported, osteoblasts play an important role as a BM niche to support primitive HSCs (Morrison and Scadden, 2014; Méndez-Ferrer et al., 2010). In contrast, adipocytes negatively regulate hematopoiesis (Naveiras et al., 2009). Thus, a balance between osteoblastogenesis and adipogenesis that changes with aging is important for maintaining steady-state hematopoiesis.

As shown in Figure 4Ca and c, after 7 days of co-culturing 34°38’133° cells with young and old DPMSGs, the fold increase of cells and the mean absolute numbers of CD34+ cells seemed to be higher in comparison with stroma-free controls. It is of interest that there was still a tendency toward a difference between the co-cultures with young and old DPMSGs, even in the presence of a transwell. We paid attention to this difference and tried to elucidate the basic mechanisms underlying this observation. It is well documented that senescent cells secrete bioactive mediators, which negatively affect stem cell function with aging (Coppé et al., 2010). Our microarray analysis accurately extracted the TGFβ2 gene from 60,901 probes (Figure 6), which was highly expressed by old DPMSGs in comparison with young DPMSGs. We then focused on the cellular senescence induced by TGF-β2 signaling. Very interestingly, TGF-β2 significantly suppressed the growth of young DPMSGs (Figure 7B). Moreover, TGF-β2 strongly suppressed the osteogenic differentiation potential of both young and old DPMSGs (Figure 7Ca and b). Old DPMSGs produced a significantly greater amount of TGF-β2 in their cultures in comparison with young DPMSGs (Figure 7A). In addition, old DPMSGs expressed significantly higher levels of P16 and P21 (Figure 2C). It was also reported that TGF-β activated the p16 and p21 pathways, which induced cellular senescence (Acosta et al., 2013; van Deursen, 2014). Collectively, it is suggested that TGF-β2 can induce autocrine senescence mediated by p16 and p21. Based on these results, TGF-β2 is considered to be one of the SASP components, as recently reported (Acosta et al., 2013).

From another point of view, stem cell aging has long been considered to be irreversible. However, in this study we demonstrated that the addition of 1D11 (anti-TGF-β mAb) reversed the osteogenic differentiation potential in cultures of young and old bone-derived DPMSGs (Figure 7Cd). Thus, the suppression of TGF-β2 signaling may rejuvenate bone-derived old DPMSGs and enhance the function of young DPMSGs. It is well documented that TGF-β is an abundant bone matrix protein that affects the interaction of osteoblasts and osteoclasts (Centrella et al., 1991). Thus, TGF-β can change the balance of adipogenesis and osteogenesis in vivo. As recently reported, the in vivo administration of 1D11 increased the bone mineral density, trabecular thickness, and bone volume in mice (Edwards et al., 2010). These results were accompanied by an increase in the number of osteoblasts and a decrease in the number of osteoclasts. These findings suggest that in the near future, the administration of 1D11 that can specifically target TGF-β in vivo may be a therapeutic approach to the treatment of osteoporosis and other bone diseases. As shown in this study, young DPMSGs showed higher proliferative and osteogenic differentiation abilities in comparison with old DPMSGs. Thus, the ex vivo expansion of young DPMSGs may have a potential therapeutic advantage in comparison with the clinical application of regular batches of MSCs.

In conclusion, the present study demonstrated that old DPMSGs showed a higher adipogenic potential and lower osteogenic potential in comparison with young DPMSGs. In other words, old DPMSGs accelerated cellular senescence earlier than young DPMSGs and TGF-β2 may be partly involved in this process. Importantly, the inhibition of TGF-β signaling by 1D11 reversed this cellular senescence. These results suggest that rejuvenation of old DPMSGs may be possible through the suppression of TGF-β2 signaling.

EXPERIMENTAL PROCEDURES

Animals

The animal experiments were approved by the Animal Care Committees of Kansai Medical University. The details of the breeding and SRC assays of NOG mice are described in Supplemental Experimental Procedures.

Preparation of Human Bone Tissue-Derived Cells

The human femoral neck bone tissue samples were obtained from young and elderly patients who had undergone total hip
replacement arthroplasty. The patients’ characteristics are shown in Table S1. This study was approved by the Research Ethics Committee of Kansai Medical University, and written informed consent was obtained from patients at the time of enrollment in all cases. The details of the treatment of the bone tissue specimens are described in Supplemental Experimental Procedures.

Isolation of Human Bone-Derived 11Lin<sup>CD45<sup>C</sup>CD271<sup>C</sup>SSEA-4<sup>C</sup> Cells by Fluorescence-Activated Cell Sorting

Human bone-derived 11Lin<sup>CD45<sup>C</sup>CD271<sup>C</sup>SSEA-4<sup>C</sup> cells were sorted as described previously (Matsuoka et al., 2015). The fluorescence-activated cell sorting data were processed using the FlowJo software program (FlowJo, OR, USA). The various mAbs used in this study are listed in Table S4.

Cell Culturing and Cell Proliferation Analysis

The sorted cells were seeded onto 6-well cell culture plates (BD Biosciences, CA, USA) and cultured in modified α-MEM (Nakalai Tesque, Kyoto, Japan) with 10% FBS (BioWest, Kansas City, MO, USA) and 1× Antibiotic-Antimycotic solution (Nacalai Tesque) at 37°C with 5% CO₂. For the cell proliferation assay, the cells were dissociated using 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA) and seeded at a density of 2 × 10⁴ cells per well at passage 2. During culturing, every 5 days cells were collected, counted, and reseeded at a density of 2 × 10⁴ cells per well. At each passage, we calculated the number of population doublings based on the total number of cells.

Senescence-Associated β-Gal Assay

The details of the staining methods are described in Supplemental Experimental Procedures.

Real-Time RT-PCR

The details of the real-time RT-PCR are described in Supplemental Experimental Procedures and the primer sets used in the PCR reactions are listed in Table S5.

Analysis of the Differentiation Potentials of the Established Human Bone-Derived DPMSCs

The osteogenic, adipogenic, and chondrogenic differentiation capacities of young and old DPMSCs were assessed as described in Supplemental Experimental Procedures.

Co-culturing of 18Lin<sup>CD34(CD38<sup>C</sup>CD133<sup>C</sup>) Cells with DPMSCs

The detailed methods that were used for the co-culturing and purification of 18Lin<sup>CD34<sup>C</sup>CD38<sup>C</sup>CD133<sup>C</sup> cells are described in Supplemental Experimental Procedures.

Microarray Analysis

Details of the gene expression data analysis are described in Supplemental Experimental Procedures. The microarray data were uploaded at the GEO, accession number GEO: GSE101694.

Effects of TGF-β2 and 1D11 on Proliferation and/or Differentiation of Young and Old DPMSCs

The details of the effects of TGF-β2 and 1D11 on the proliferation and/or differentiation of young and old DPMSCs are described in Supplemental Experimental Procedures.

Statistical Analysis

The statistical methods used to analyze the data are described in each of the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.01.030.

AUTHOR CONTRIBUTIONS

H.K. designed and performed a majority of the experiments and analyzed and interpreted the data; R.N. designed and performed the experiments, analyzed and interpreted the data, and contributed to the writing of the manuscript; K.S., Y.M., and T.F. provided study material and contributed to some parts of the experiments; H.A. provided advice on the statistical analyses; H.I. contributed to the analysis and interpretation of the data; Y.S. conceived of and designed the study, analyzed and interpreted the data, provided financial and administrative support, and wrote the paper.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research C (grant nos. 21591251, 24591432, and 16K09881) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; a grant from the Strategic Research Base Development Program for Private Universities from MEXT; and MEXT-supported Program for the Strategic Research Foundation at Private Universities (S1101034 and S1201038). The authors are grateful to the Japanese Red Cross Kinki Cord Blood Bank for providing the CB samples that were used in this study.

Received: August 8, 2017
Revised: January 23, 2018
Accepted: January 24, 2018
Published: February 22, 2018

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