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Supplemental Information

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

Hiroshi Kawamura, Ryusuke Nakatsuka, Yoshikazu Matsuoka, Keisuke Sumide, Tatsuya Fujioka, Hiroaki Asano, Hirokazu Iida, and Yoshiaki Sonoda

Figure S1

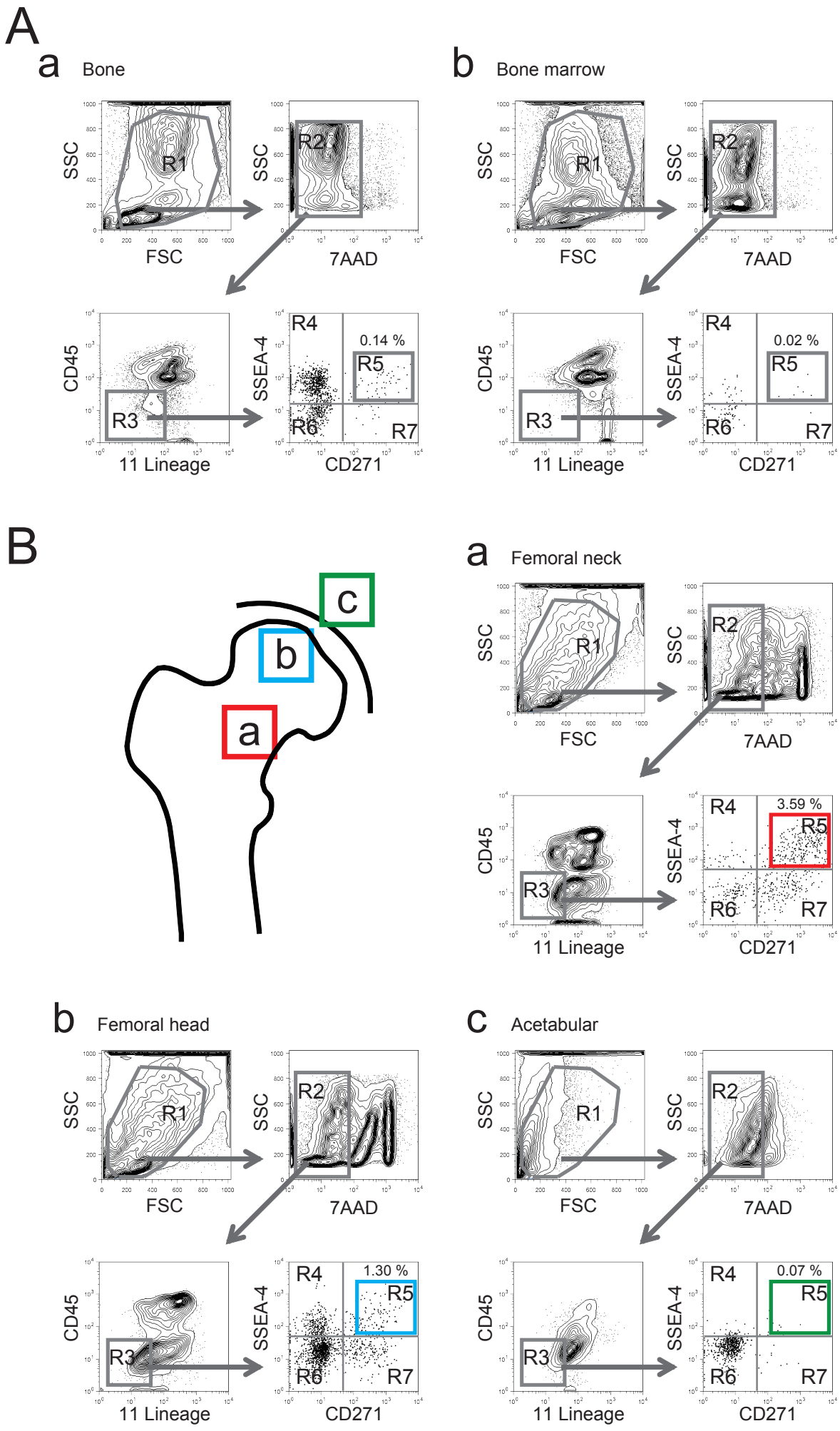
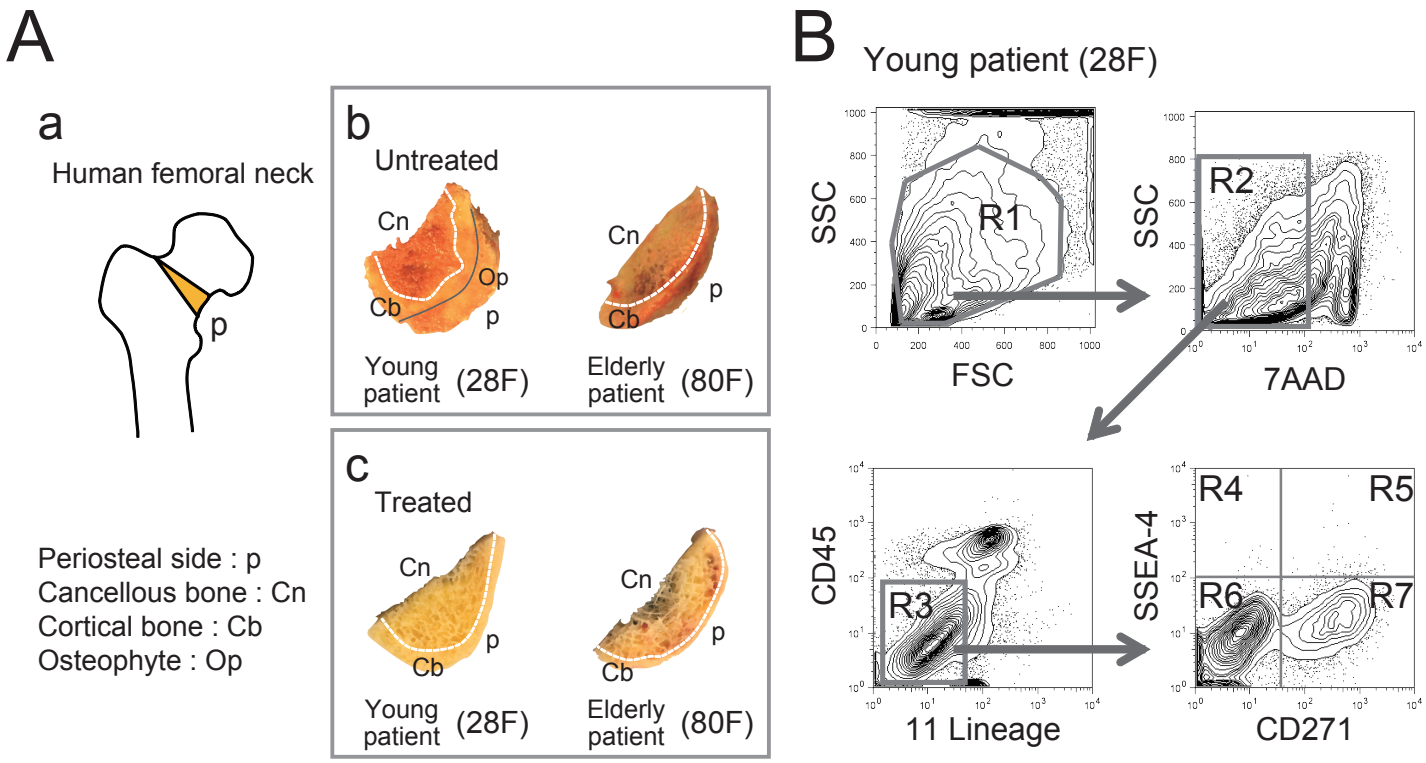

A
a Bone
b Bone marrow
SSC
FSC
R1
R2
7AAD
CD45
R3
11 Lineage
SSEA-4
R4
R5
R6
R7
CD271
0.14 %
0.02 %
B
a
b
c
Femoral neck
Femoral head
Acetabular
3.59 %
1.30 %
0.07 %

Figure S2


A
a
Human femoral neck
p
Periosteal side : p
Cancellous bone : Cn
Cortical bone : Cb
Osteophyte : Op
b
Untreated
Cn
Op
Cb
p
Young patient (28F)
Cn
Cb
p
Elderly patient (80F)
c
Treated
Cn
p
Cb
Young patient (28F)
Cn
p
Cb
Elderly patient (80F)
B
Young patient (28F)
SSC
FSC
R1
SSC
7AAD
R2
CD45
11 Lineage
R3
SSEA-4
CD271
R4
R5
R6
R7

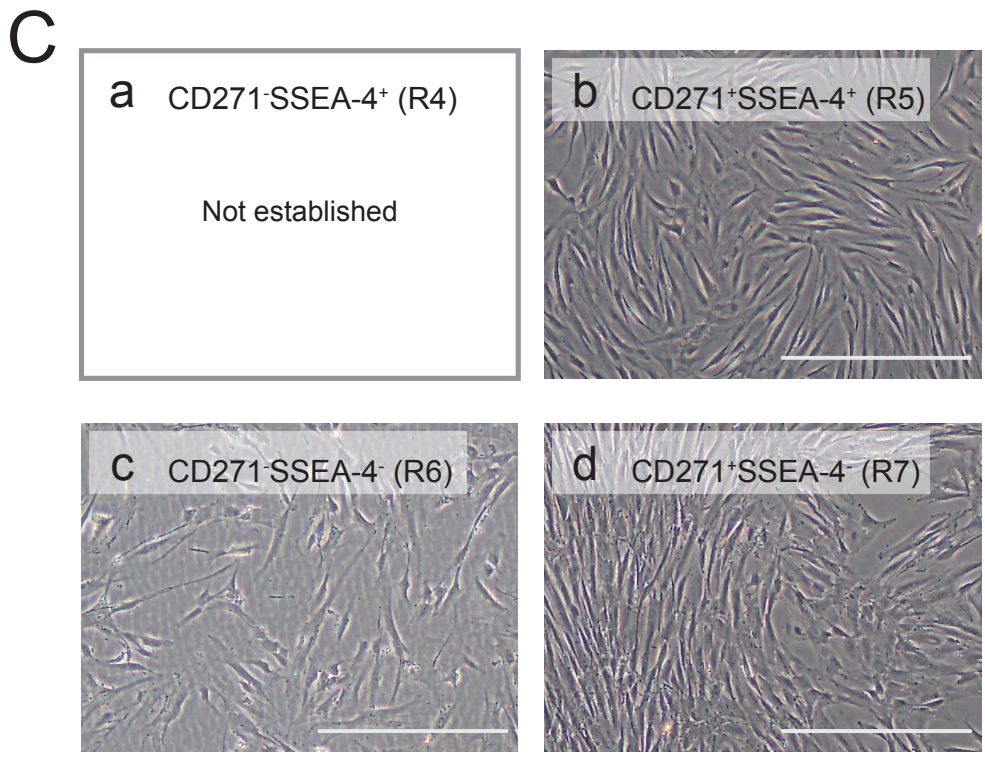
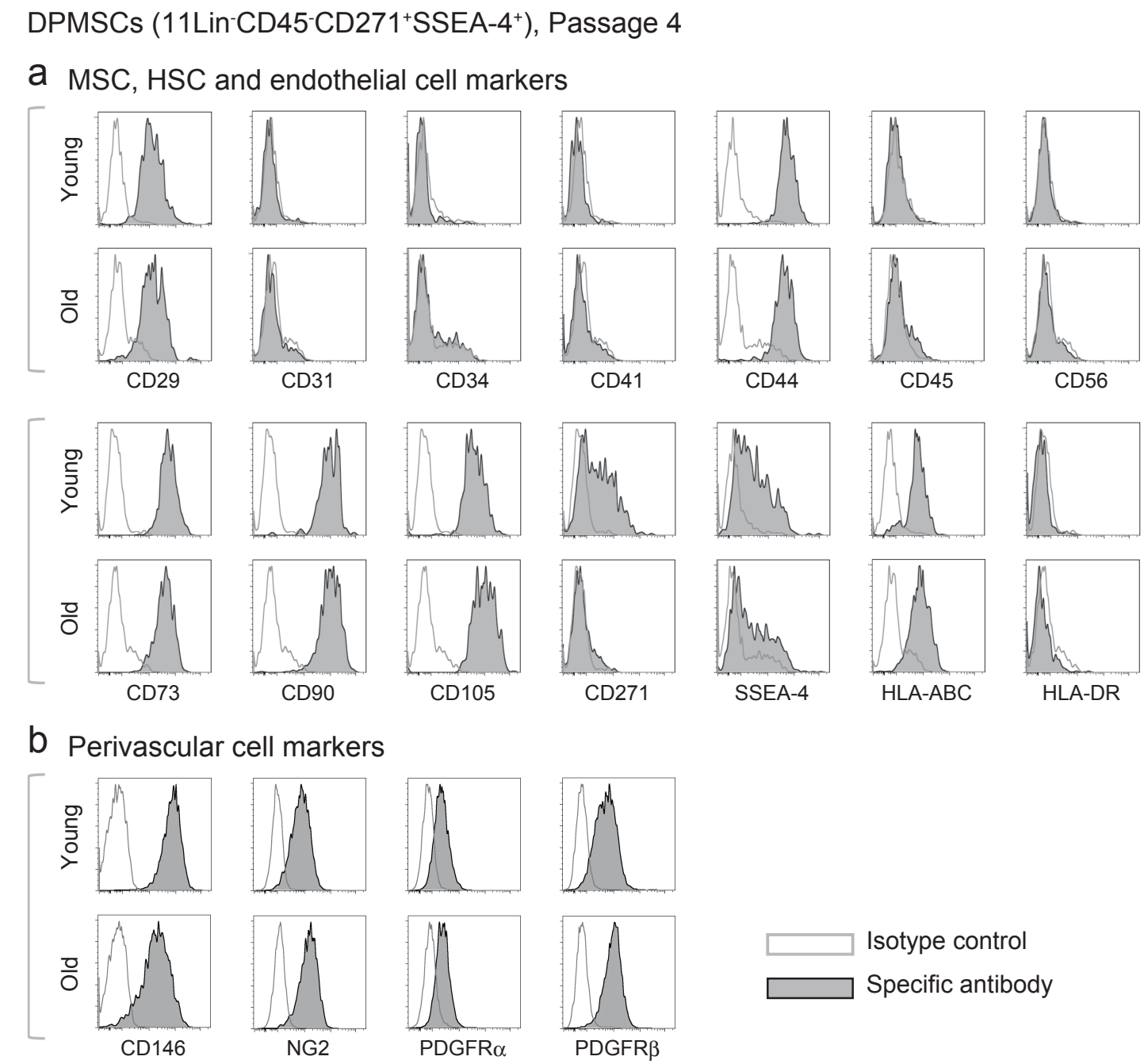

C
a CD271-SSEA-4+ (R4)
Not established
b CD271+SSEA-4+ (R5)
c CD271-SSEA-4- (R6)
d CD271+SSEA-4- (R7)

Figure S3


A
DPMSCs (11Lin-CD45-CD271+SSEA-4+), Passage 4
a MSC, HSC and endothelial cell markers
Young
Old
CD29
CD31
CD34
CD41
CD44
CD45
CD56
CD73
CD90
CD105
CD271
SSEA-4
HLA-ABC
HLA-DR
b Perivascular cell markers
CD146
NG2
PDGFRα
PDGFRβ
Isotype control
Specific antibody

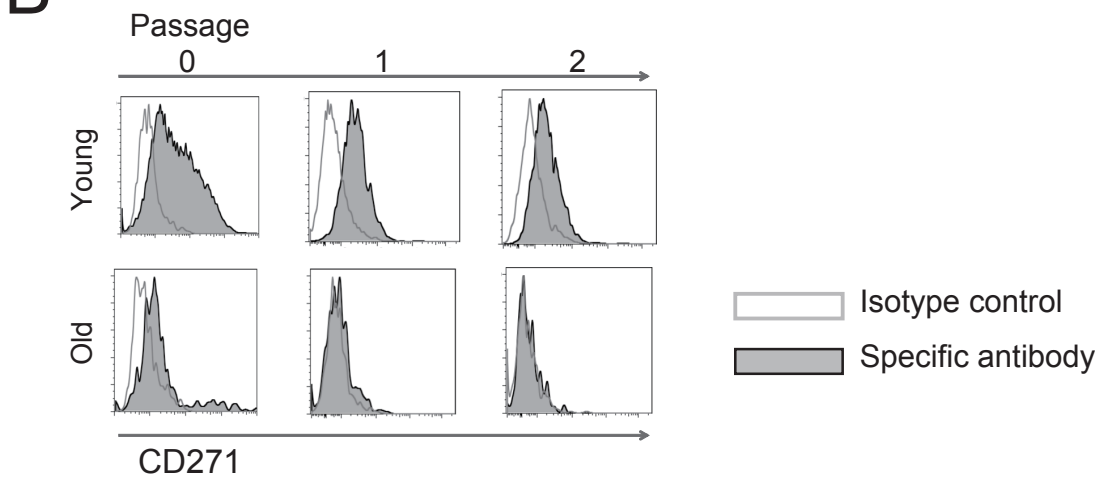

B
Passage
0
1
2
Young
Old
CD271
Isotype control
Specific antibody

Figure S4

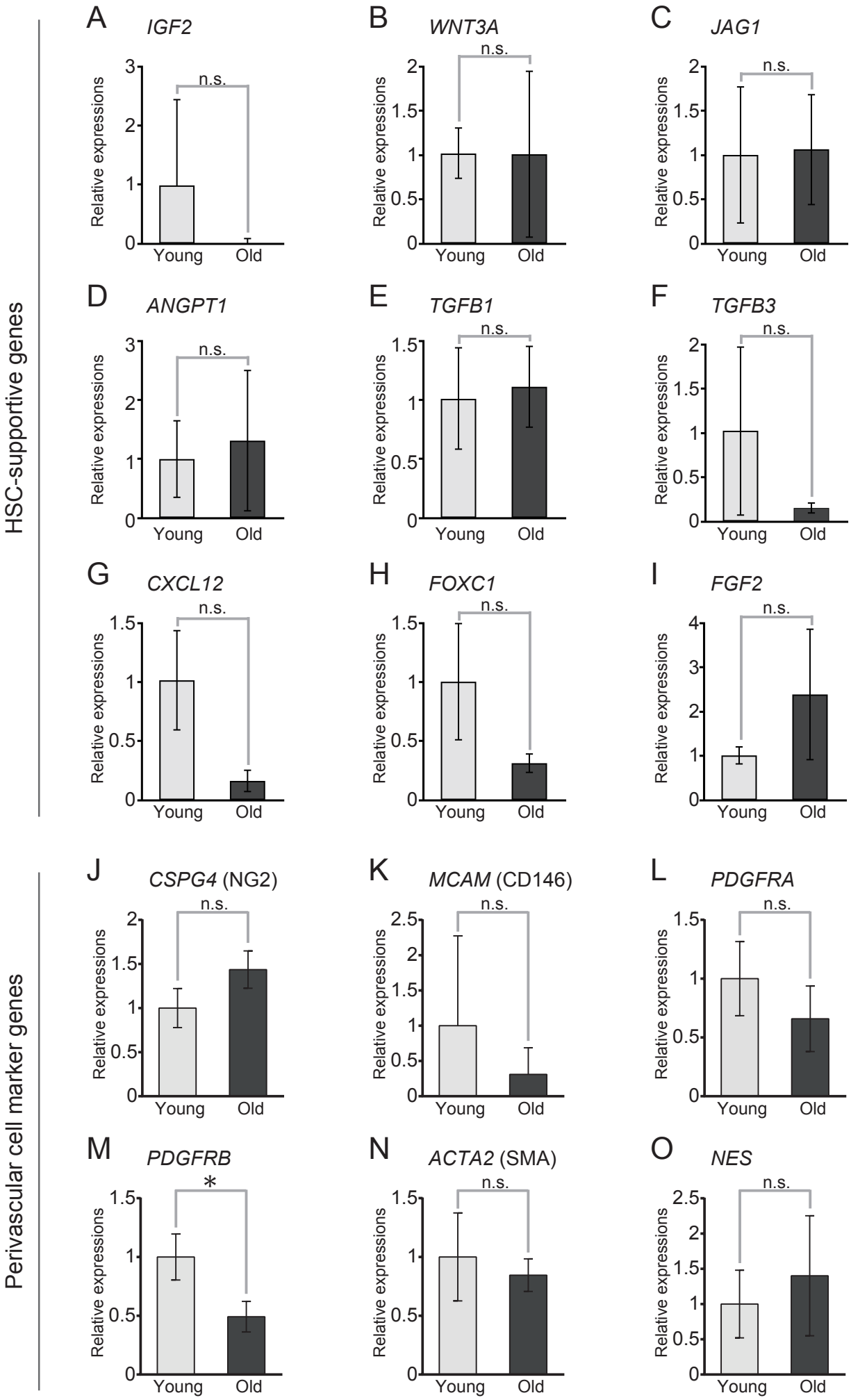

HSC-supportive genes
Perivascular cell marker genes
A
IGF2
B
WNT3A
C
JAG1
D
ANGPT1
E
TGFB1
F
TGFB3
G
CXCL12
H
FOXC1
I
FGF2
J
CSPG4 (NG2)
K
MCAM (CD146)
L
PDGFRA
M
PDGFRB
N
ACTA2 (SMA)
O
NES
Relative expressions
n.s.
*
Young
Old

Figure S5

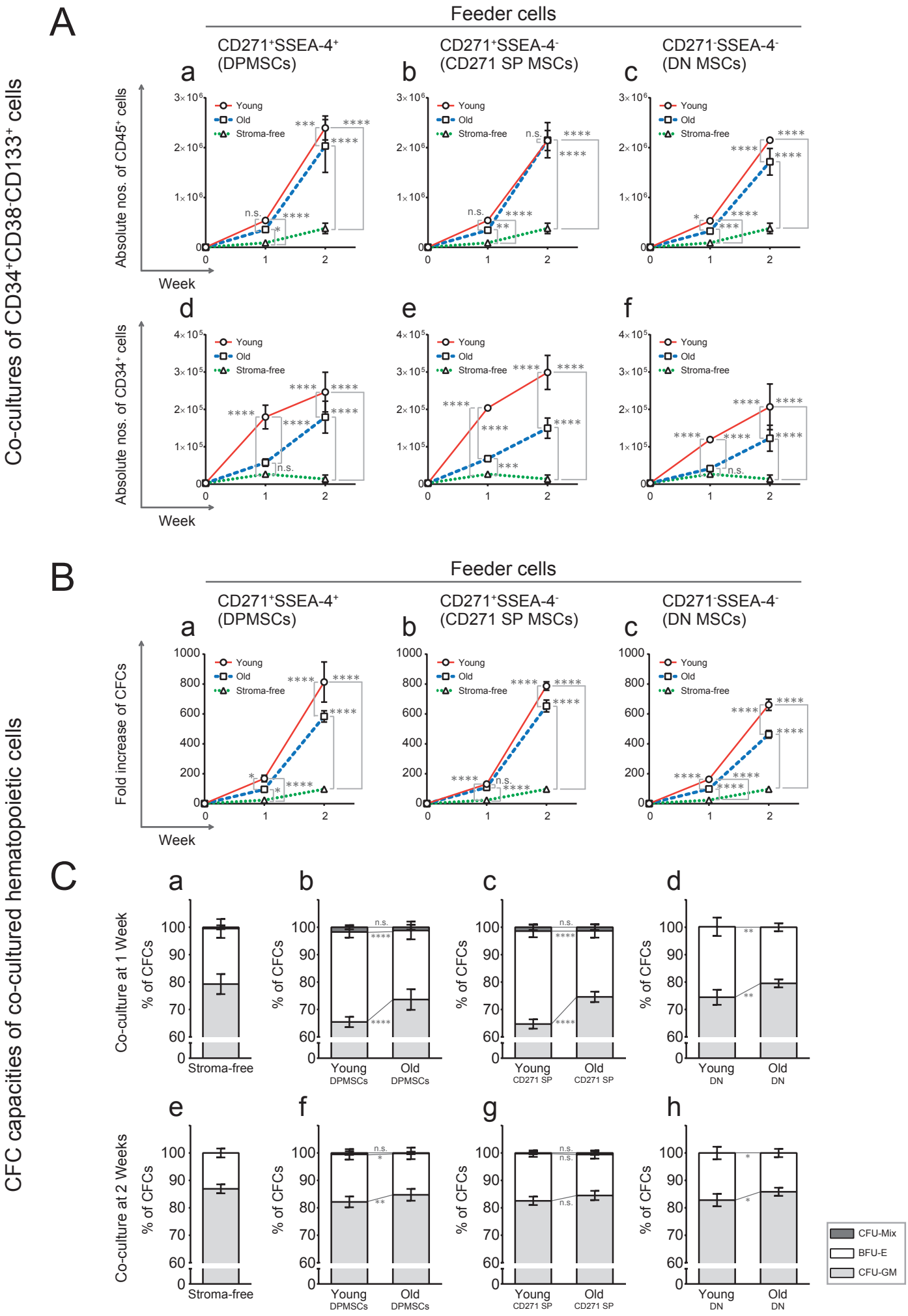
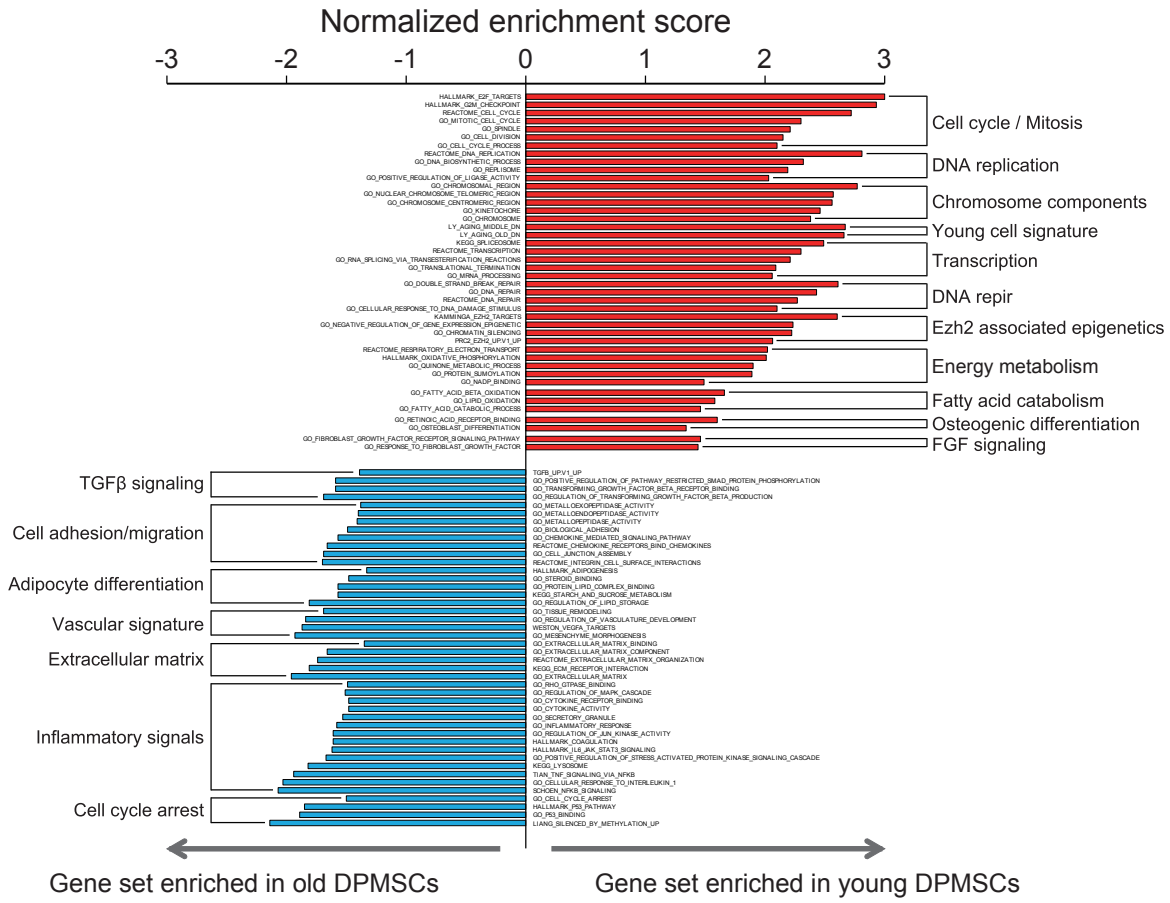

A
Feeder cells
CD271+SSEA-4+ (DPMSCs)
CD271+SSEA-4- (CD271 SP MSCs)
CD271-SSEA-4- (DN MSCs)
Co-cultures of CD34+CD38-CD133+ cells
Absolute nos. of CD45+ cells
Absolute nos. of CD34+ cells
Week
Young
Old
Stroma-free
B
Fold increase of CFCs
C
CFC capacities of co-cultured hematopoietic cells
Co-culture at 1 Week
Co-culture at 2 Weeks
% of CFCs
Stroma-free
Young DPMSCs
Old DPMSCs
Young CD271 SP
Old CD271 SP
Young DN
Old DN
CFU-Mix
BFU-E
CFU-GM

Figure S6

A Gene set enrichment analysis



B

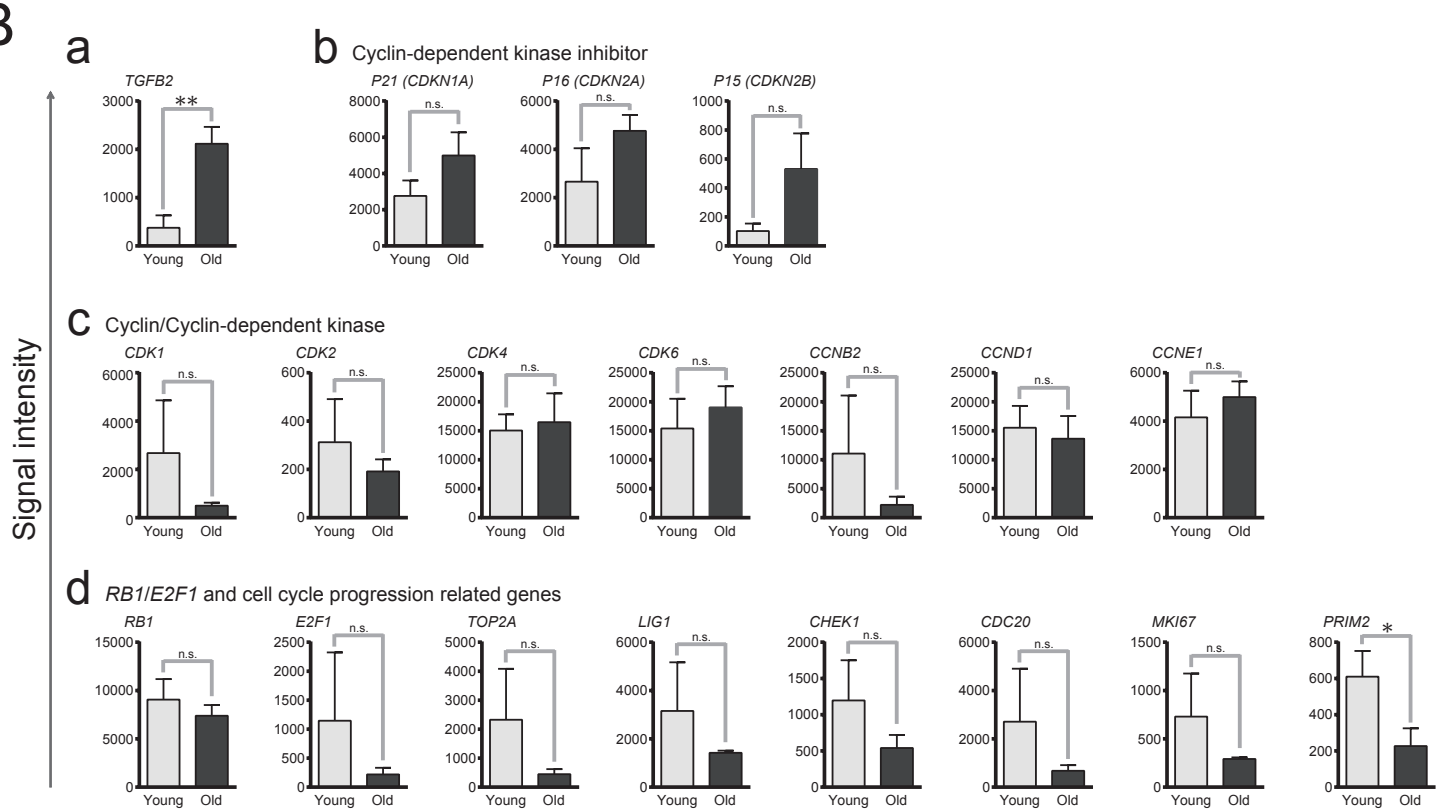
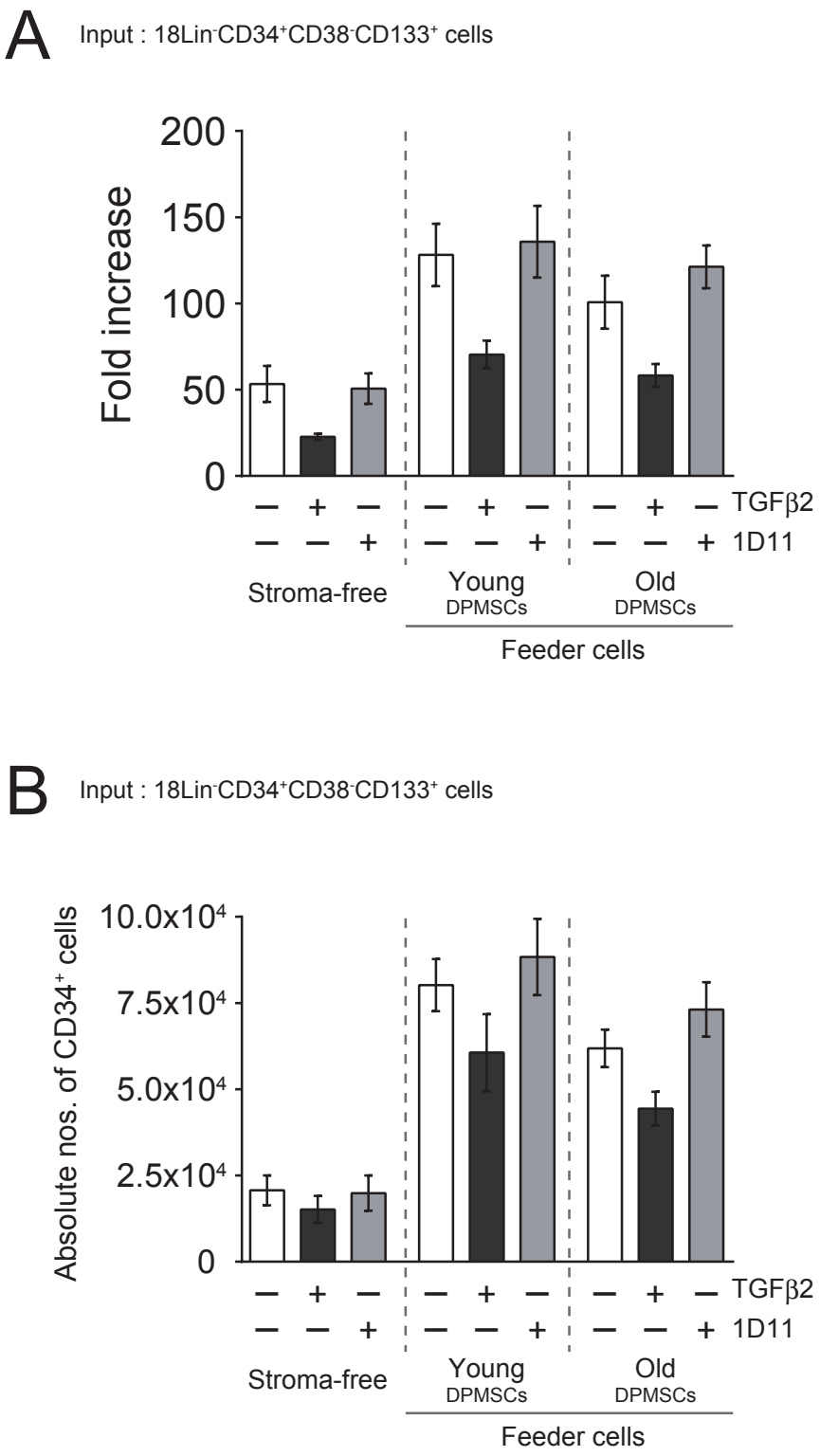


Figure S7


A
Input : 18Lin-CD34+CD38-CD133+ cells
Fold increase
200
150
100
50
0
TGFβ2
1D11
Stroma-free
Young DPMSCs
Old DPMSCs
Feeder cells
B
Input : 18Lin-CD34+CD38-CD133+ cells
Absolute nos. of CD34+ cells
10.0x10⁴
7.5x10⁴
5.0x10⁴
2.5x10⁴
0
TGFβ2
1D11
Stroma-free
Young DPMSCs
Old DPMSCs
Feeder cells

Supplemental Figure Legends:

Figure S1. A flowcytometric analysis of the proportion and anatomical distribution of 11Lin$^-$CD45$^-$CD271$^+$SSEA-4$^+$ cells in patient bone tissue. Related to **Figure 1**

(A) A comparison of proportions of 11Lin$^-$CD45$^-$CD271$^+$SSEA-4$^+$ cells obtained from bone tissue (femoral neck)- and bone marrow (BM)-derived nucleated cells in a 61-year-old female patient. Representative FCM profiles of the femoral neck bone tissue-derived cells **(a)** and BM-derived cells **(b)**. The percentages of R5-gated 11Lin$^-$CD45$^-$CD271$^+$SSEA-4$^+$ cells in the 7-AAD$^-$ living cell gate (R2) are depicted in each figure. **(B)** Representative FCM profiles of the bone-derived nucleated cells from a 76-year-old female patient. These cells were collected from various anatomical positions: **(a)** the femoral neck, **(b)** the femoral head and **(c)** the acetabular. The percentages of R5-gated 11Lin$^-$CD45$^-$CD271$^+$SSEA-4$^+$ cells in the 7-AAD$^-$ living cell gate (R2) are depicted in each figure. In these experiments, the R1 gate was set in order to exclude cell debris. The R2 gate was set for 7-AAD$^-$ living cells. Eleven hematopoietic lineages (CD2, CD3, CD4, CD14, CD16, CD19, CD24, CD41, CD56, CD66c, and CD235a)- and CD45-positive cells were gated out (R3). The R3-gated cells were further subdivided according to their expression of CD271 and SSEA-4 (R4 to R7).

Figure S2. Isolation and purification of mesenchymal stromal/stem cells from human bone tissues. Related to **Figures 1** and **S1**

(A) (a) A schematic diagram of the human femoral neck bone tissue. The bone tissue was resected from patients during surgery, and the yellow-colored part of the bone was trimmed and used in this study. **(b, c)** Representative photos of the human bone tissue used in this study. The bone tissue from a young female patient (28 years old) is indicated as "Young patient (28F)", and that from an elderly female patient (80 years old) is indicated as "Elderly patient (80F)". **(b)** The upper panel shows freshly isolated bone tissue (untreated) and **(c)** the lower panel shows bone tissue treated with enzymes (treated). The periosteal sides of the bone tissue are depicted as "p". Cancellous bone, cortical bone and osteophyte are indicated as Cn, Cb and Op, respectively. **(B)** Representative FCM profiles of the isolation of CD271$^{+/-}$SSEA-4$^{+/-}$ subpopulations from the young female patient (28F) bone-derived nucleated cell. The R1 gate was set in order to exclude cell debris. The R2 gate was set for living cells (7-AAD$^-$). Eleven hematopoietic lineages (CD2, CD3, CD4, CD14, CD16, CD19, CD24, CD41, CD56, CD66c, CD235a)- and CD45-positive cells were gated out (R3). The R3-gated cells were further subdivided according to their CD271 and SSEA-4 expression (R4 to R7). **(C)** Representative phase-contrast images of cultured MSCs at passage 4 are shown. These MSCs were established from **(b)** CD271$^+$SSEA-4$^+$ (R5), **(c)** CD271$^-$SSEA-4$^-$ (R6) and **(d)** CD271$^+$SSEA-4$^-$ (R7) fractions of cells. In contrast, adherent fibroblastic-cells could not be established from the **(a)** CD271$^-$SSEA-4$^+$ fraction (R4). Scale bars (500 μm) are depicted in each figure.

Figure S3. The surface marker expression of DPMSCs established from young and elderly patients' bone tissue. Related to **Figures 2** and **3**

(**A**) Representative FCM profiles of cell-surface markers expressed on young (Young, upper panels) and old (Old, lower panels) DPMSCs at passage 4. The expression of cell surface molecules/antigens **(a)** MSC, HSC and endothelial cell markers, including CD29, CD31, CD34, CD41, CD44, CD45, CD56, CD73, CD90, CD105, CD271, SSEA-4, HLA-ABC and HLA-DR, and **(b)** perivascular cell markers, such as CD146, NG2, PDGFRα and PDGFRβ, were analyzed. Solid and open histograms represent specific antibodies and isotype controls, respectively. (**B**) The expression of CD271 was downregulated during the passaging (P0 to P2) of young as well as old DPMSCs.

Figure S4. The expression of HSC-supportive and perivascular cell marker-related genes in young and old DPMSCs. Related to **Figures 4** and **5**

We used three young and three old DPMSC lines. The gene expression of established young and old DPMSCs was estimated by a qRT-PCR. The gene expression was normalized by *GAPDH*. The relative expression levels of each HSC-supportive gene, including *IGF-2*, *WNT3A*, *JAG1*, *ANGPT1*, *TGFB1*, *TGFB3*, *CXCL12*, *FOXC1* and *FGF2*, and perivascular cell marker-related genes, including *CSPG4*, *MCAM*, *PDGFRA*, *PDGFRB*, *ACTA2* and *NES*, were compared between young and old DPMSCs. The bars represent the mean ± S.D. of three independent measurements (each measurement contained 3 tubes per line). The data were evaluated by Welch's t-test. * $P < 0.05$; n.s., not significant.

Figure S5. The *in vitro* co-cultures of human hematopoietic stem/progenitor cells with three types of bone tissue-derived young and old MSCs. Related to **Figure 4**

(**A**) Three thousand cord blood (CB)-derived 18Lin$^-$CD34$^+$CD38$^-$CD133$^+$ cells were co-cultured with or without young (open circles with red solid lines) or old (open squares with blue dotted lines) CD271$^+$SSEA4$^+$ (DPMSCs, **a** and **d**), CD271$^+$SSEA4$^-$ (CD271 SP MSCs, **b** and **e**) or CD271$^-$SSEA4$^-$ (DN MSCs, **c** and **f**) bone-derived MSCs for 2 weeks in the presence of a cocktail of cytokines (TPO, SCF, FL, G-CSF, IL-3 and IL-6). Hematopoietic cells recovered from the co-cultures were analyzed after 1 and 2 weeks to determine (**A-a** to **c**) the absolute number of CD45$^+$ hematopoietic cells and (**A-d** to **f**) the absolute number of CD34$^+$ cells. We used three young and two old DPMSC lines, one young CD271 SP MSC line, one old CD271 SP MSC line and one young DN MSC line and one old DN MSC line. The data of stroma-free control are indicated with open triangles with green dotted lines. We used three culture dishes for each young or old MSC line. The bars represent the mean ± S.D. of the absolute numbers of CD45$^+$ and CD34$^+$ cells in culture dishes in each independent experiment. The data were evaluated by a two-way ANOVA with Tukey's multiple comparison. * $P < 0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$; n.s., not significant. (**B**) The expansion of colony-forming cells (CFCs) in co-cultures. We used three young and two old DPMSC lines, one young CD271 SP cell line and one old CD271 SP cell line, one young DN cell line and one old DN cell line. Co-cultures with each young and old MSC line were performed in quadruplicate. Hematopoietic cells were recovered from co-cultures with or without young (open circles with red solid lines) or old (open squares with blue dotted lines) MSCs, including (**a**) DPMSCs, **(b)** CD271 SP MSCs and (**c**) DN MSCs, after 1 and 2 weeks of co-culturing. Hematopoietic cells recovered from stroma-free cultures (open triangles with green dotted lines) were used as controls. These cells were then used for colony-forming cell (CFC) assays. These line graphs show the fold increase in CFCs in these three types of cultures. The bars represent the mean ± S.D. of data from 4 to 12 culture dishes in each independent experiment. The data were evaluated by a two-way ANOVA with Tukey's multiple comparison. * $P < 0.05$, **** $P<0.0001$; n.s., not significant. (**C**) The percentages of the types of colonies obtained after 1 week (**a** to **d**) and 2 weeks (**e** to **h**) of co-culturing. The data of stroma-free controls (**a** and **e**), co-cultures with DPMSCs (**b** and **f**), co-cultures with CD271 SP MSCs (**c** and **g**) and co-cultures with DN MSCs (**d** and **h**). Bright gray, open and dark gray bars represent CFU-GM, BFU-E and CFU-Mix, respectively. The bars represent the mean ± S.D. of three types of colonies in each independent experiment. The data were evaluated by a two-way ANOVA with Bonferroni correction. * $P < 0.05$, ** $P<0.01$, **** $P<0.0001$; n.s., not significant.

Figure S6. A comparison of the gene expression profiles of young and old DPMSCs, as determined by a microarray. Related to **Figures 2** and **6**

We used three young and three old DPMSC lines. We performed a microarray analysis once for each young or old DPMSC line (a total of three times). These data were uploaded at GEO (GSE101694). **(A)** The gene expression profiles of young and old DPMSCs were compared by a Gene Set Enrichment Analysis (GSEA) using the data from the microarray analysis. The gene sets enriched in young DPMSCs (red bars) and those in old DPMSCs (blue bars) with a Normalized Enrichment Score of < -1 or > 1 were selected and presented. Detailed lists of the genes in each gene set depicted in the figure can be found in the GSEA Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb /index.jsp). **(B)** A comparison of the gene expression profiles of the cell cycle-related genes between young and old DPMSCs. The gene expression profiles of *TGFB2* and the cell cycle-related genes of young and old DPMSCs were analyzed by a microarray. We extracted the cell cycle-related gene sets from the cell cycle arrest gene sets enriched in old DPMSCs, as shown in **Figure S6A**. In the cell cycle-related gene sets, we extracted the gene expression levels of (**a**) *TGFB2* and representative cell cycle progression- and/or cellular senescence-related genes (van Deursen J.M. 2014, Lossaint et al. 2011), such as (**b**) cyclin-dependent kinase inhibitors, (**c**) cyclin/cyclin-dependent kinases and (**d**) cell cycle progression-related genes (*RB1*, *E2F1* and others). The vertical axis of each graph represents the mean signal intensity of the mRNA expression obtained from a microarray analysis. The data are presented as the mean ± S.D. from three independent experiments. The data were evaluated using Welch's *t*-test. * $P < 0.05$, ** $P < 0.01$; n.s., not significant.

Figure S7. The effects of TGF-β2 and anti-TGF-β antibodies on the proliferation of 18Lin$^-$CD34$^+$CD38$^-$CD133$^+$ cells co-cultured with young and old DPMSCs. Related to **Figures 4** and **7**

We used two young and two old DPMSC lines. Three thousand 18Lin$^-$CD34$^+$CD38$^-$CD133$^+$ cells were co-cultured with young and old DPMSCs in the presence of 6 cytokines. Each culture was performed in triplicate. Recombinant human TGF-β2 (100 ng/ml) or anti-TGF-β antibody (1D11) (10 μM/ml) was added to these co-cultures. **(A)** The fold increase in the total numbers of cells, and **(B)** the absolute number of CD34$^+$ cells are shown. The bars represent the mean ± S.D. of six culture dishes in two independent experiments. The data were evaluated by a two-way ANOVA with Tukey's multiple comparison.

Table S1. The patient characteristics and the frequency of 11Lin$^-$CD45$^-$ cells expressing CD271 and/or SSEA-4*

Group	Age	Sex	Disease		Immunophenotypes of the 11Lin$^-$CD45$^-$ cells (Figure 1)				DPMSC established
					CD271$^-$SSEA-4$^+$ (R4, SSEA-4 SP)	CD271$^+$SSEA-4$^+$ (R5, DPMSCs)	CD271$^-$SSEA-4$^-$ (R6, DN)	CD271$^+$SSEA-4$^-$ (R7, CD271 SP)	
Young	28	Female	Osteonecrosis	Frequency of cells in 11Lin$^-$CD45$^-$ cells (%)	7.72	2.27	70.60	19.50	Yes
	31	Female	Osteonecrosis		0.61	1.77	74.50	23.70	Yes
	31	Male	Osteonecrosis		1.15	2.22	79.20	17.40	Yes
				Average (%)	3.16 ± 3.96	2.09 ± 0.62	74.77 ± 4.65	20.20 ± 3.55	
Elderly	80	Female	Osteoarthritis	Frequency of cells in 11Lin$^-$CD45$^-$ cells (%)	1.93	0.97	89.60	7.46	Yes
	83	Male	Osteoarthritis		1.47	0.01	98.00	0.34	No
	84	Female	Osteoarthritis		0.95	2.83	77.70	18.60	Yes
	91	Female	Osteoarthritis		0.49	0.69	50.50	48.30	Yes
	92	Female	Femur neck fracture		0.14	0.00	99.80	0.06	No
	96	Female	Femur neck fracture		0.62	0.01	98.50	0.88	No
	97	Female	Femur neck fracture		21.70	0.08	77.50	0.78	No
	97	Male	Femur neck fracture		0.18	0.00	99.70	0.15	No
				Average (%)	3.44 ± 7.41	0.57 ± 0.98	86.41 ± 17.28	9.57 ± 16.92	

*This table is related to Figure 1.

Table S2. Summary of the studies on the primary and secondary transplantation of 18Lin$^-$CD34$^+$CD38$^-$CD133$^+$ cells co-cultured with or without young and old DPMSCs*

(A) Primary Transplantation

Feeder cells	Input cells	No. of cells transplanted/mouse	Incidence of engraftment			Chimerism (% of hCD45+ cells)	% of hCD34$^+$ cells in hCD45$^+$7AAD$^-$ cells	% of hCD19$^+$ cells in hCD45$^+$7AAD$^-$ cells	% of hCD33$^+$ cells in hCD45$^+$7AAD$^-$ cells	% of hCD11b$^+$ cells in hCD45$^+$7AAD$^-$ cells	% of hCD14$^+$ cells in hCD45$^+$7AAD$^-$ cells		% of hCD3+ cells in hCD45$^+$7AAD$^-$ cells
Stroma-free	**18Lin$^-$CD34$^+$CD38$^-$CD133$^+$**	5.8 x 10^4	5 / 5	Left tibia (Injection site)	Range	**0.9 -7.4**	**0.0-10.0**	**6.2-72.7**	**1.4-96.0**	**0.0-19.8**	**1.2-69.0**	Thymus	**0.0-79.5**
					Median	**3.7**	**5.9**	**29.4**	**56.9**	**6.5**	**3.2**		**0.0**
				Peripheral blood	Range	**0.0 -1.0**	**0.0**	**0.0-54.5**	**0.0-66.7**				
					Median	**0.0**	**0.0**	**25.0**	**6.2**				
Young DPMSCs	**18Lin$^-$CD34$^+$CD38$^-$CD133$^+$**	18.1 x 10^4	5 / 5	Left tibia (Injection site)	Range	**3.7 – 69.2**	**1.6-19.1**	**26.4-76.9**	**9.9-60.6**	**1.2-16.7**	**0.6-4.1**	Thymus	**0.0-42.9**
					Median	**43.1**	**10.1**	**53.6**	**30.3**	**9.9**	**1.2**		**5.6**
				Peripheral blood	Range	**0.8 – 24.8**	**0.7-4.6**	**27.7-56.6**	**8.0-45.6**				
					Median	**4.2**	**2.0**	**45.1**	**8.8**				
Old DPMSCs	**18Lin$^-$CD34$^+$CD38$^-$CD133$^+$**	8.8 x 10^4	5 / 5	Left tibia (Injection site)	Range	**0.6 -73.2**	**0.1-9.2**	**4.5-50.7**	**2.7-50.1**	**0.5-16.7**	**0.2-17.1**	Thymus	**0.0-94.9**
					Median	**10.5**	**3.1**	**30.7**	**17.9**	**5.3**	**0.7**		**50.0**
				Peripheral blood	Range	**0.4 – 69.6**	**0.0-4.3**	**1.2-43.8**	**0.1-12.5**				
					Median	**11.7**	**0.0**	**34.9**	**6.5**				

(B) Secondary Transplantation

Feeder cells	No. of BM cells transplanted/mouse	No. of human cells transplanted/mouse	No. of hCD34$^+$CD38$^-$ cells transplanted/mouse	Incidence of engraftment		Chimerism (% of hCD45+ cells)	% of hCD34$^+$ cells in hCD45$^+$7AAD$^-$ cells	% of hCD19$^+$ cells in hCD45$^+$7AAD$^-$ cells	% of hCD33$^+$ cells in hCD45$^+$7AAD$^-$ cells
Stroma-free	3.7 x 10^6	6.5 x 10^5	2.8 x 10^3	0 / 10	Range	N.D.	N.D.	N.D.	N.D.
					Median				
Young DPMSCs	4.3 x 10^6	11.6 x 10^5	9.8 x 10^3	5 / 10	Range	**0.01-0.6**	**0.41-68.60**	**0.00-87.80**	**4.28-100.00**
					Median	**0.04**	**14.30**	**27.90**	**72.10**
Old DPMSCs	3.7 x 10^6	14.5 x 10^5	13.7 x 10^3	5 / 10	Range	**0.01-0.05**	**0.00-8.33**	**0.00-40.50**	**14.30-100.00**
					Median	**0.03**	**0.00**	**15.40**	**80.00**

N.D.: not detected

* This table is related to Figure 5.

Table S3. List of genes extracted for heat map analysis in Figure 6

Gene symbol	Mean intensity ratio (Old / Young)
IFI27	8.84021
KRT17	6.680124
KRT18P55	6.301351
MAMDC2	6.288721
ZIC4	6.040934
KRT18	5.859687
ICAM1	5.640284
TGFB2	5.629785
SYNPO2L	5.546092
LINC00667	5.475641
KRT14	5.101518
KRT16P2	5.053703
GDF15	4.698106
PTPRB	4.395882
LOC101927359	4.329124
HRK	4.263218
PADI2	3.979843
GLIPR1L2	3.948475
LOC100507033	3.922629
MEOX2	3.860051
HYAL1	3.843229
OLFM1	3.786124
AGMO	3.6774
GAFA2	3.572124
TMIE	3.410612
lnc-SMARCAL1-3	3.384621
VLDLR	3.37288
FNDC5	3.334098
DEPTOR	3.326151
KSR1	3.323417
LOC100506797	3.270868
ATF5	3.25731
XLOC_l2_000324	3.228129
LINC00702	3.187917
LAMA1	3.154369
RAC3	3.149702
FERMT3	3.134171
LINC01569	3.112384
LOC100132891	3.001603
TRANK1	2.996436
LINC01349	2.986266
TSPAN12	2.966868
lnc-VSTM2B-7	2.894141
CARNS1	2.890513
lnc-FANCF-1	2.87968
ZP3	2.871678
CMAHP	2.810669
RAB3IP	2.751574
DMD	2.704527
SNORD37	2.66214
lnc-TASP1-4	2.628355
MGAM	2.576375
KRTAP5-AS1	2.544869
AHNAK2	2.529532

Gene symbol	Mean intensity ratio (Old / Young)
HSPB6	2.521661
LOC101929911	2.519222
LOC100507487	2.502076
lnc-LRRC17-1	2.492794
SERPINA3	2.429912
KRT42P	2.42311
CLEC19A	2.421753
MFAP3L	2.404384
STOX2	2.380072
TEKT4P2	2.339243
lnc-EBF3-1	2.284163
SEP6	2.241952
LYPLAL1-AS1	2.236482
NFIA-AS2	2.202509
LNX1	2.186224
SH3BGRL2	2.184897
LOC101927963	2.134729
TGFBR3L	2.126126
ABCA1	2.1125
LOC389906	2.108863
TGFB2-AS1	2.104582
KRTAP3-1	2.079155
ITPR3	2.075866
LINC00592	2.048944
MICB	2.037303
lnc-ZNF71-3	2.006863
MEST	2.006162
OPRL1	2.002517
BPHL	0.498373
NAPRT	0.494898
LINC00211	0.494224
EBF3	0.491558
MGST1	0.487756
ZNF44	0.477091
RPL27A	0.475822
lnc-LRRC61-2	0.465719
lnc-SPATA9-1	0.460128
LOC100996654	0.447543
LOC101927260	0.440554
LOC100130992	0.434931
lnc-DUSP4-4	0.432408
NFE2	0.428391
SNORD114-10	0.412669
COTL1	0.392801
LEKR1	0.391181
SNHG23	0.386608
LOC400692	0.383592
PLIN2	0.380479
PRIM2	0.372586
RUNX2	0.370288
FRMD6-AS1	0.369802
LINC01268	0.369576
NQO1	0.363098
SPIN4	0.357584

Gene symbol	Mean intensity ratio (Old / Young)
MIR17HG	0.346573
SNORD114-27	0.345225
LAMA4	0.342012
SELENBP1	0.336248
PRR29	0.333373
ALS2CR11	0.332901
SMAD6	0.323898
KAZALD1	0.31179
XPNPEP3	0.306081
CCDC68	0.302524
GMFG	0.298287
LOC100132077	0.292433
PARPBP	0.289042
STEAP1	0.281018
METTL7A	0.277072
LOC100130417	0.26866
HAS2	0.267256
WFDC1	0.254521
STEAP1B	0.254289
RTN4RL1	0.254085
PDE7B	0.241355
C11orf87	0.241203
PDE4B	0.225207
ADCY4	0.218998
ID2	0.186066
SLC14A1	0.089483
RAB27B	0.050695

The genes which are specifically depicted in **Figure 6** (*TGFB2*, *SMAD6* and *ID2*) were highlighted in red.

Table S4. Monoclonal antibodies for flow cytometry used in this study*

Antibody	Flurochrome	Distributor	Clone	Dilution
CD2	FITC	DAKO	MT910	1:25
CD3	FITC	Beckman Coulter	UCHT1	1:25
CD4	FITC	eBioscience	RPA-T4	1:25
CD4	PE	eBioscience	RPA-T4	1:100
CD7	FITC	Beckman Coulter	8H8.1	1:25
CD8a	FITC	eBioscience	RPA-T8	1:100
CD10	FITC	Beckman Coulter	ALB1	1:25
CD11b	FITC	Beckman Coulter	Bear1	1:25
CD14	FITC	eBioscience	61D3	1:25
CD14	PE	Beckman Coulter	RMO2	1:25
CD16	FITC	DAKO	DJ130c	1:25
CD19	FITC	BD Biosciences	HIB19	1:25
CD20	FITC	Beckman Coulter	H299	1:25
CD24	FITC	DAKO	SN3	1:25
CD29	FITC	BioLegend	TS2/16	1:20
CD31	FITC	DAKO	5.6E	1:50
CD33	FITC	Beckman Coulter	HIM3-4	1:25
CD33	PE	Beckman Coulter	D3HL60.251	1:25
CD34	APC	BD Biosciences	581	1:50
CD34	Brilliant Violet 421	BioLegend	581	1:50
CD38	PE-Cy7	BioLegend	HIT2	1:100
CD41	FITC	Beckman Coulter	P2	1:25
CD41	PE	Beckman Coulter	P2	1:50
CD45	Pacific Blue	BioLegend	HI30	1:25
CD45	Brilliant Violet 510	BioLegend	HI30	1:50
CD45RA	FITC	SouthernBiotech	MEM 56	1:50
CD56	FITC	BD Biosciences	NCAM16.2	1:25
CD66c	FITC	Beckman Coulter	KOR-SA3544	1:25
CD73	PE	BD Biosciences	AD2	1:20
CD90	PE	BD Biosciences	5E10	1:25
CD105	PE	eBioscience	MJ7/18	1:20
CD127	FITC	eBioscience	eBioRDR5	1:25
CD133/1	APC	Miltenyi Biotec	AC133	1:25
CD140a (PDGFRα)	PE	BioLegend	16A1	1:20
CD140b (PDGFRβ)	APC	BioLegend	18A2	1:20
CD146	PE-Cy7	BD Biosciences	P1H12	1:25
CD235a	FITC	DAKO	JC159	1:25
CD271	PE	BD Biosciences	C40-1457	1:25
HLA-ABC	FITC	Beckman Coulter	B9.12.1	1:400
HLA-DR	PE	Beckman Coulter	Immu-357	1:20
NG2	FITC	R&D Systems	LHM-2	1:20
SSEA-4	APC	R&D Systems	MC-813-70	1:10
Aniti-mouse CD45.1	PE-Cy7	Beckman Coulter	A20	1:200

* This table is related to Experimental Procedures.

Table S5. Nucleotide sequences of the primers used in this study*

Amplicon		Nucleotide sequence
P16	Forward	5'- GAGGCCGGGATGAGTTGGGAGGAG -3'
	Reverse	5'- CAGCCGGCGTTTGGAGTGGTAGAA -3'
P21	Forward	5'- CCCTGTAGGACCTTCGGTGA -3'
	Reverse	5'- GAAGGTCCCTCAGACATCCC -3'
RUNX2	Forward	5'- AACCCACGAATGCACTATCCA -3'
	Reverse	5'- CGGACATACCGAGGGACATG -3'
PPARG2	Forward	5'- ATTGACCCAGAAAGCGATTCC -3'
	Reverse	5'- CACCTAGAGAGGCATTACCTTCT -3'
SOX9	Forward	5'- GCCAGGTGCTCAAAGGCTA -3'
	Reverse	5'- TCTCGTTCAGAAGTCTCCAGAG -3'
IGF2	Forward	5'- CCTCCAGTTCGTCTGTGGG -3'
	Reverse	5'- CACGTCCCTCTCGGACTTG -3'
WNT3A	Forward	5'- TGCAGTGACACGCTCATGTG -3'
	Reverse	5'- CAAACTCGATGTCCTCGCTAC -3'
JAG1	Forward	5'- TCAGTTCGAGTTGGAGATCCT -3'
	Reverse	5'- CCTTGAGGCACACTTTGAAGTA -3'
ANGPT1	Forward	5'- GTTCAGAACCACACGGCTACC -3'
	Reverse	5'- GCTTCTCTAGCTTGTAGGTGGA -3'
TGFB1	Forward	5'- CAAGCAGAGTACACACAGCAT -3'
	Reverse	5'- TGCTCCACTTTTAACTTGAGCC -3'
TGFB3	Forward	5'- TCAGCCTCTCTCTGTCCACTT -3'
	Reverse	5'- CATCACCGTTGGCTCAGGG -3'
CXCL12	Forward	5'- ATGAACGCCAAGGTCGTGGTC -3'
	Reverse	5'- CTTGTTTAAAGCTTTCTCCAGGTACT -3'
FOXC1	Forward	5'- CAGCCCAAGGACATGGTGA -3'
	Reverse	5'- ACTCGTTGAGCGAGAGGTTG -3'
FGF2	Forward	5'- AGAAGAGCGACCCTCACATCA -3'
	Reverse	5'- GTGACTTTGCTTGACCCGTCA -3'
CSPG4	Forward	5'- CTCACACAGAGGAACCCTGGA -3'
	Reverse	5'- AAGAAGTGTCCGGGGGAAGT -3'
MCAM	Forward	5'- CCAAGGCAACCTCAGCCATGTC -3'
	Reverse	5'- TCAGCAGGGTCTGACACTTCAGCTC -3'
PDGFRA	Forward	5'- ATCCGGCGTTCCTGGTCTTA -3'
	Reverse	5'- GGGTAATGAAAGCTGGCAGAGG -3'
PDGFRB	Forward	5'- CGGAGACCCGGTATGTGTCA -3'
	Reverse	5'- TTAGCTCCAGCACTCGGACA -3'
ACTA2	Forward	5'- TCGTTACTACTGCTGAGCGTG -3'
	Reverse	5'- ACTTGCCCATCAGGCAACTC -3'
NES	Forward	5'- TGGCTCAGAGGAAGAGTCTGA -3'
	Reverse	5'- TCCCCCATTTACATGCTGTGA -3'
GAPDH	Forward	5'- ACCACAGTCCATGCCATCAC -3'
	Reverse	5'- TCCACCACCCTGTTGCTGTA -3'

* This table is related to Experimental Procedures.

Supplemental Experimental Procedures

Preparation of human bone tissue-derived cells

The bone tissue specimens were isolated from the patients' femoral neck, femoral head and acetabular during surgery. Bone marrow (BM) cells residing in the above-mentioned bone tissues were collected after vigorous washing twice with Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS^-, Nacalai Tesque, Kyoto, Japan). The BM cells was immersed in hemolysis buffer (BD Pharm Lyse, BD Biosciences, San Jose, CA, USA) for 5 to 10 minutes to lyse the red blood cells, and the BM-derived nucleated cells (BMNCs) were collected by centrifugation at 300 *g* for 8 minutes at room temperature. Washed bone specimens were incubated in cell dissociation buffer containing α-modified minimum essential medium (α-MEM, Nakalai Tesque) supplemented with 5 % fetal bovine serum (FBS, BioWest, Kansas City, MO, USA) containing 1.5 mg/mL collagenase type I (Thermo Fisher Scientific, Waltham, MA, USA) and 2 mg/mL dispase (Thermo Fisher Scientific) at 37 °C for 1 hour with gentle shaking. The suspension was filtered with a 70-μm pore cell strainer (BD Falcon, Franklin Lakes, NJ, USA) to remove debris and collected by centrifugation at 300 *g* for 8 minutes at room temperature. Part of the precipitated bone-derived cells and BMNCs were used for cytometry (FCM). Then, the remaining precipitated cells were re-suspended and cryopreserved in cell culture freezing medium (Cell Reservoir One, Nacalai Tesque) at -80 °C until FCM and fluorescence-activated cell sorting (FACS).

Senescence associated β-gal assay

The senescence-associated-β-galactosidase (SA-β-gal) assay was performed using a Senescence Detection Kit (BioVison, Mountain View, CA, USA) in accordance with manufacturer's instructions. The DPMSCs established from young and elderly patients' bone tissue were plated onto six-well cell culture plates (BD Biosciences). When these cultured cells reached confluence, the cells were fixed and stained with staining solution containing X-Gal. The stained specimens were observed under an inverted microscope, and multiple fields on each well were pictured at random. The ratio of SA-β-gal positive cells in the pictured fields of microscopy was calculated and the cellular senescence was evaluated.

Analyses of the differentiation potentials of established human bone-derived DPMSCs

The osteogenic, adipogenic and chondrogenic differentiation capacities of young and old DPMSCs were assessed using a Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, MN, USA) according to the manufacturer's instructions. Then, calcified nodules (osteogenic differentiated cells), oil droplets (adipogenic differentiated cells) and cellular aggregations (chondrogenic differentiated cells) were detected by staining with alizarin red S solution (Sigma-Aldrich, St. Louis, MO, USA), Oil Red O solution (Sigma-Aldrich) and toluidine blue solution (Wako Pure Chemical, Osaka, Japan) respectively. For the quantitative evaluation of osteogenic and adipogenic differentiation potentials, DPMSCs were cultured with osteogenic and adipogenic differentiation induction media supplemented with fluorescent dye for three weeks. For the evaluation of osteogenic differentiation, 1 μg/mL of calcein (Dojindo Laboratories, Kumamoto, Japan) was used for calcium deposition staining. For the evaluation of adipogenic differentiation, 0.5 μM of boron-dipyrromethene (BODIPY, Thermo Fisher Scientific) was used for lipid droplets staining. The mean intensity of calcein and BODIPY fluorescence of differentiated DPMSCs in the pictured fields were analyzed by

fluorescence microscopy.

Flow cytometric analyses for cell-surface markers of DPMSCs

The cultured DPMSCs established from young and elderly patients' bone tissue were dissociated using Cell Dissociation Buffer (enzyme-free, PBS; Thermo Fisher Scientific). The collected cells were pre-incubated with PBS^--containing 2% FBS and 5 mg/ml human γ-globulin for 20 minutes at 4 °C and then stained for 30 minutes at 4 °C with the following mAbs: FITC, PE, APC, PE-Cy7 or PB-conjugated anti-CD29, CD31, CD34, CD41, CD44, CD45, CD56, CD73, CD90, CD105, CD271, SSEA-4, HLA-ABC, HLA-DR, CD146, NG2, PDGFRα and PDGFRβ mAbs and 7AAD. The FCM analysis was performed using a FACSCanto II flow cytometer (BD Biosciences) and the FCM data were processed using the FlowJo software program. The monoclonal antibodies used for FCM analyses are listed in **Table S4.**

Purification of human 18 lineage-negative $CD34^+CD38^-CD133^+$ cells from human cord blood

Previously, we reported the isolation method for highly-purified human $CD34^{+/-}$ HSCs residing in cord blood (CB) using a CD133 mAb (Takahashi et al., 2014). Using this method, human CB-derived 18 lineage-negative ($18Lin^-$) $CD34^+CD38^-CD133^+$ cells were sorted by FACSAria III cell sorter (BD Biosciences). These highly purified cells were used for subsequent *in vitro* and *in vivo* experiments.

The co-culturing of $18Lin^-CD34^+CD38^-CD133^+$ cells with three types of MSCs (DPMSCs, CD271SP MSCs and DN MSCs)

DPMSCs at passage 4 were plated onto 24-well culture plates (BD Biosciences) at a density of 2×10^4 cells per well. After 1 week, the DPMSCs were irradiated using a ^{137}Cs-γ irradiator at 12 Gy (Gammacell 40 Exactor; Best Theratronics, Kanata, ON, Canada). Purified $18Lin^-CD34^+CD38^-CD133^+$ cells were co-cultured with irradiated DPMSC layers at a density of 3×10^3 cells per well in StemPro34 serum-free medium (Thermo Fisher Scientific) in the presence of a cocktail of cytokines (SCF, FL, TPO, IL-3, IL-6 and G-CSF), as previously reported (Ishii et al., 2011, Takahashi et al., 2014, Matsuoka, Nakatsuka et al., 2015, Matsuoka, Sumide et al., 2015, Matsuoka et al., 2016, Matsuoka et al., 2017). After one week of co-culturing, the collected cells were evaluated for the cell number and the percentage of $CD34^+$ cells among the living $CD45^+$ cells by FCM. To disturb the cell-cell contact in the co-culture system, polyester membrane cell culture inserts (Transwell culture insert [pore size: 0.4 μm] Corning, Tokyo, Japan) were added onto 24-well culture plates and the independent culture condition was brought into contact with the feeder cell layer. Then, purified $18Lin^-CD34^+CD38^-CD133^+$ cells were seeded onto cell culture inserts and cultured in the same manner as described above. In one set of experiments, we co-cultured 3×10^3 $18Lin^-CD34^+CD38^-CD133^+$ purified cells with irradiated MSC layers (three types of MSC layers were used: DPMSCs, CD271SP MSCs and DN MSCs) in StemPro34 serum-free medium for 2 weeks. Each week, we evaluated the number of cells and the percentage of $CD34^+$ cells among the living $CD45^+$ cells by FCM, and the colony-forming cell (CFC) capacities in the methylcellulose cultures.

An in vitro lineage tracking analysis of co-cultured HSCs

To analyze the differentiation potentials of co-cultured 18Lin$^-$CD34$^+$CD38$^-$CD133$^+$ cells *in vitro*, we performed hematopoietic CFC assays. The CFC assays were performed according to the protocol for methylcellulose cultures as previously reported (Wang et al., 2003, Kimura et al., 2007, Kimura et al., 2010, Ishii et al., 2011, Takahashi et al., 2014, Matsuoka, Nakatsuka et al., 2015, Matsuoka, Sumide et al., 2015, Matsuoka et al., 2016, Matsuoka et al., 2017). The collected cells that were co-cultured with DPMSC feeders were reseeded onto a 35-mm culture dish (Corning) at a density of 200 cells per dish in the presence of a cocktail of cytokines (SCF, IL-3, granulocyte-macrophage colony- stimulating factor [GM-CSF], G-CSF and erythropoietin [EPO]) plus 30 % FBS. After 7 to 10 days of culture, hematopoietic cell-derived colonies were counted under an inverted microscope. The colony types identified in situ were granulocyte-macrophage colony (CFU-GM), erythroid burst (BFU-E) and erythrocyte-containing mixed colony (CFU-Mix).

Animals

Six-week-old female NOD/Shi-scid/IL-2Rγc^{null} (NOG) mice were purchased from the Central Institute of Experimental Animals (Kawasaki, Japan) (Ito et al., 2002). NOG mice were handled under sterile conditions and maintained in germ-free isolators located in the Central Laboratory Animal Facilities of Kansai Medical University. The animal experiments were approved by the Animal Care Committees of Kansai Medical University.

Primary and secondary SCID-repopulating cell (SRC) assays of co-cultured HSCs

SCID-repopulating cell (SRC) assays were performed using a previously reported method (Wang et al., 2003, Kimura et al., 2007, Kimura et al., 2010, Ishii et al., 2011, Takahashi et al., 2014, Matsuoka, Nakatsuka et al., 2015, Matsuoka, Sumide et al., 2015, Matsuoka et al., 2016, Matsuoka et al., 2017). The co-cultured cells were recovered, and 1/9 of the collected cells was transplanted by intra-BM injection (IBMI) into the left tibia of sublethally-irradiated (250 cGy using Gammacell 40 Exactor) 8-week-old NOG primary recipient mice (n=5/group). The mice were euthanized at 20 weeks after transplantation, and bone marrow (BM) cells in the left tibia, peripheral blood-derived nucleated cells and thymus (Thy)-derived nucleated cells were collected. To evaluate the repopulation of human hematopoietic cells in transplanted animals, 1/5 of the collected BM cells and PB and Thy-derived nucleated cells were stained with mAbs: PB-conjugated anti-human CD45 mAb, PE-Cy7-conjugated anti-mouse CD45.1 mAb, and 7AAD. The cells were also stained with FITC-conjugated anti-human CD19 (BM, PB), CD11b (BM) and CD3 (Thy) mAbs; PE-conjugated anti-human CD33 (BM, PB) and CD14 (BM) mAbs, and APC-conjugated anti-human CD34 (BM, PB) mAb for the detection of human stem/progenitor, B-lymphoid, T-lymphoid and myeloid/monocytic hematopoietic cells. These cells were analyzed using a FACSCanto II flow cytometer and the FCM data were processed using the FlowJo software program. The remaining BM cells from each experimental groups (stroma-free, and cocultures with young DPMSCs and old DPMSCs) were cryopreserved for secondary transplantation. After thawing, we checked the proportion of 18Lin$^-$CD34$^+$CD38$^-$ cells in part of the recovered cells from the three experimental groups. Then 1/10 of the remaining cells from the three experimental groups was transplanted by IBMI into secondary recipient NOG mice (n=10/group), as described above. Human hematopoietic repopulations in the mouse BMs

was analyzed at 6 weeks after transplantation by the BM aspiration method (Wang et al., 2003, Kimura et al., 2007, Kimura et al., 2010, Ishii et al., 2011, Takahashi et al., 2014, Matsuoka, Nakatsuka et al., 2015, Matsuoka, Sumide et al., 2015, Matsuoka et al., 2016). The aliquots of BM aspirates were stained with a Pacific Blue (PB)-conjugated anti-human CD45 mAb, PE-Cy7-conjugated anti-mouse CD45.1 mAb, FITC-conjugated anti-human CD19 mAb, PE-conjugated anti-human CD33 mAb, APC-conjugated anti-human CD34 mAb and 7-AAD and were analyzed using a FACSCanto II flow cytometer; the FCM data were processed using the FlowJo software program.

Total RNA preparation and real-time RT-PCR

Total RNAs were isolated from DPMSCs with the RNeasy Plus Micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The concentration and purity of the collected RNAs were evaluated by spectrophotometer (Nanodrop; Thermo Fisher Scientific). Complementary DNA was synthesized using the iScript DNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) and real-time RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) in a real-time PCR cycler (Rotor-Gene Q; Qiagen), with the data processed by the comparative CT method. The primer sets used in the PCR reactions are listed in **Table S5.**

Microarray analyses

Young and old DPMSCs at passage 4 were plated onto 6-well culture plates and cultured. After these cells reached confluence, total RNAs were extracted using an RNeasy Plus Micro kit (Qiagen). The RNA quality was assessed using an Agilent Model 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Complementary DNAs were synthesized using extracted total RNAs and hybridized to a gene expression microarray (SurePrint G3 Human GE 8x60K, Agilent Technologies) and the signals of each sample were compared (Takara Bio, Shiga, Japan). A comparison analysis of the gene expression data was processed by a gene set enrichment analysis (GSEA) software program (http://software.broadinstitute.org/gsea/index.jsp) in accordance with previous reports (Subramanian et al., 2005). To create a heat map of mRNA expression, the difference in the signal value between the young and old groups was examined using a two-tailed Student's t-test and Welch's t-test, and the data with a confidence level of < 0.05 were extracted. The data with a ratio of mean signal values between two groups over two-fold **(Table S3)** were extracted and shown in the heat map.

The effects of TGF-β2 and 1D11 on the proliferation and/or differentiation of young and old DPMSCs

Various doses (1, 10 or 100 ng/mL) of recombinant human TGF-β2 (Cell Signaling Technology, Danvers, MA, USA) were added to DPMSC cultures. The effects on the proliferation of young DPMSCs were then analyzed. In addition, 100 ng/mL of recombinant human TGF-β2 (Cell Signaling Technology) or 10 μM/mL of 1D11 (R&D Systems) was added to the differentiation cultures. The differentiation potentials of young DPMSCs was analyzed using the above-described methods. In another set of experiments, 100 ng/mL of recombinant human TGF-β2 (Cell Signaling Technology) or 10 μM/mL of 1D11 (R&D Systems) was added to the co-cultures to analyze the effects on the proliferation of co-cultured hematopoietic cells.

Cytokine assay

To measure the amount of TGF-β2 secreted by DPMSCs *in vitro*, the supernatants of the culture media were collected, and centrifuged at 1100 g for 5 minutes at 4 °C, and then the supernatants were preserved at -80 °C. The cytokine concentrations in thawed supernatants were measured using Quantikine ELISA Human TGF-β2 Immunoassay (R&D Systems) according to the manufacturer's instructions.

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