Stem Cell Reports, Volume *3*

Supplemental Information

Low/Negative Expression of PDGFR-α Identifies the Candidate Primary Mesenchymal Stromal Cells in Adult Human Bone Marrow

Hongzhe Li, Roshanak Ghazanfari, Dimitra Zacharaki, Nicholas Ditzel, Joan Isern, Marja Ekblom, Simón Méndez-Ferrer, Moustapha Kassem, and Stefan Scheding

Figure S1

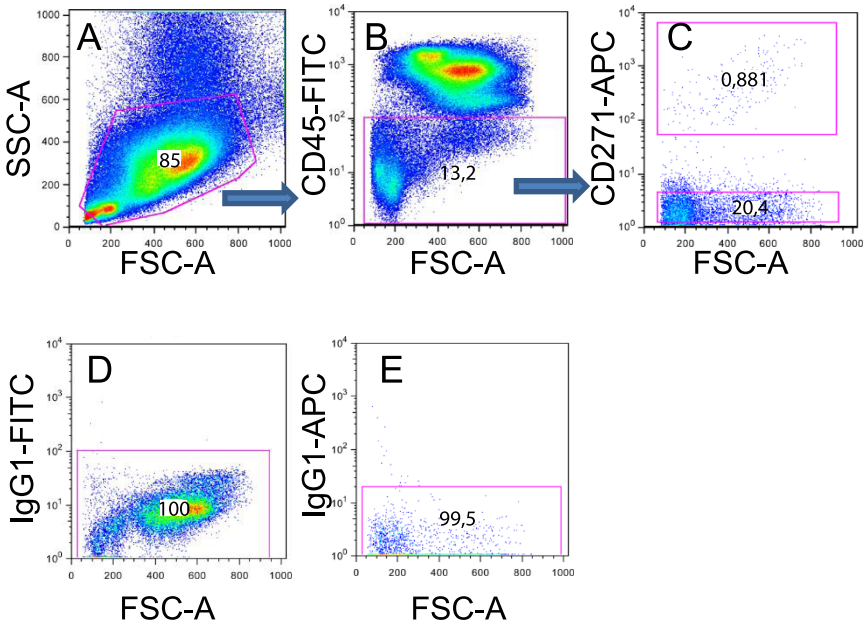

A
SSC-A
FSC-A
85
B
CD45-FITC
FSC-A
13,2
C
CD271-APC
FSC-A
0,881
20,4
D
IgG1-FITC
FSC-A
100
E
IgG1-APC
FSC-A
99,5

Figure S2

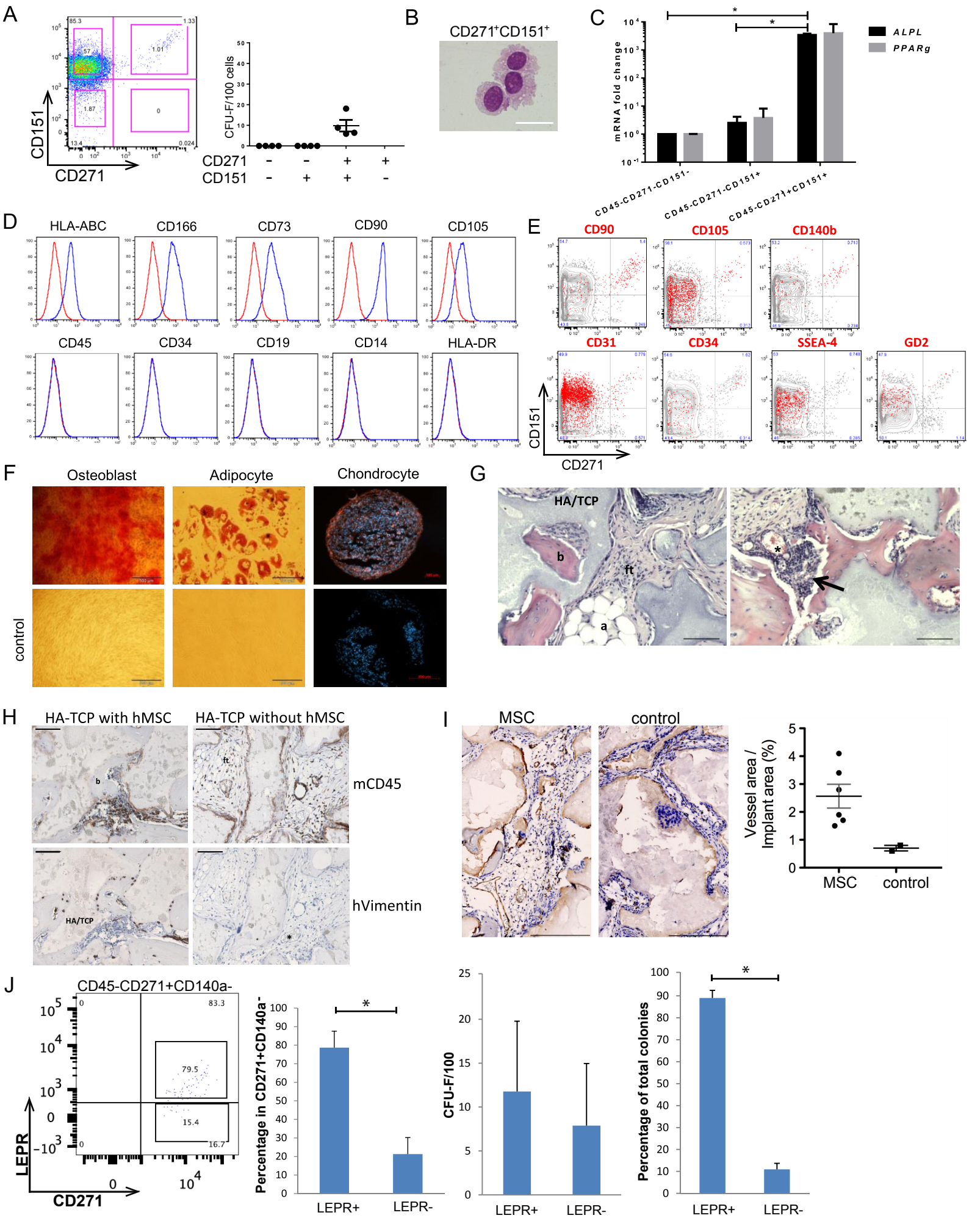

A
CD151
CD271
CFU-F/100 cells
CD271 - - + +
CD151 - + + -
B
CD271+CD151+
C
mRNA fold change
ALPL
PPARg
CD45-CD271-CD151-
CD45-CD271-CD151+
CD45-CD271+CD151+
D
HLA-ABC
CD166
CD73
CD90
CD105
CD45
CD34
CD19
CD14
HLA-DR
E
CD90
CD105
CD140b
CD31
CD34
SSEA-4
GD2
CD151
CD271
F
Osteoblast
Adipocyte
Chondrocyte
control
G
HA/TCP
b
ft
a
*
H
HA-TCP with hMSC
HA-TCP without hMSC
mCD45
hVimentin
HA/TCP
I
MSC
control
Vessel area / Implant area (%)
MSC
control
J
CD45-CD271+CD140a-
LEPR
CD271
Percentage in CD271+CD140a-
LEPR+
LEPR-
CFU-F/100
Percentage of total colonies

Figure S3

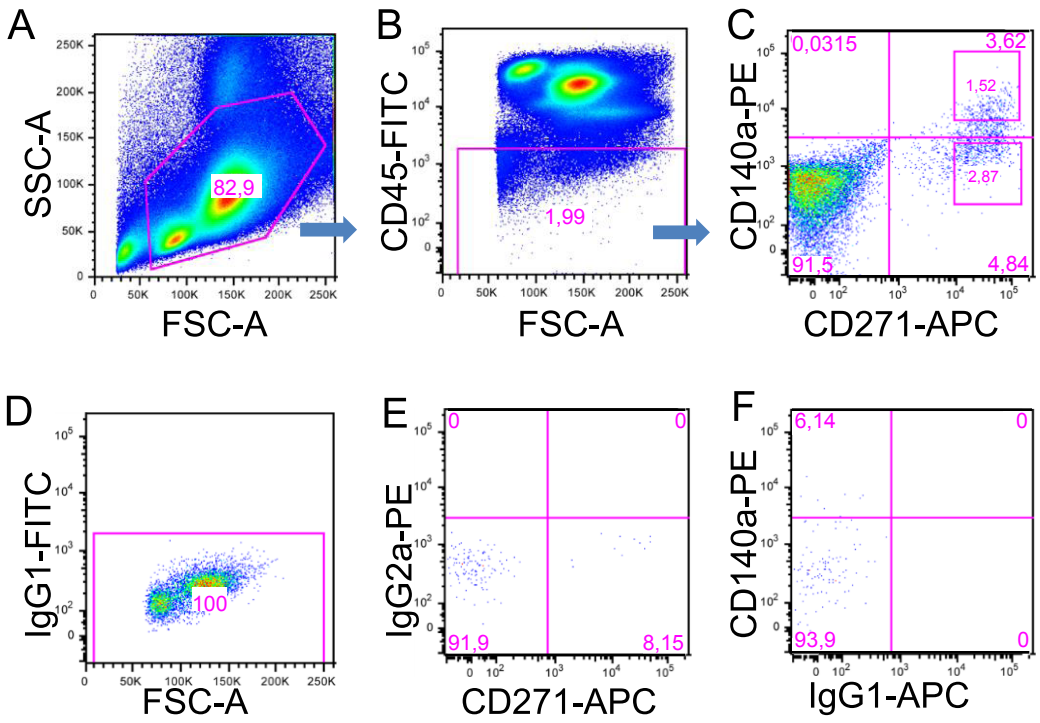
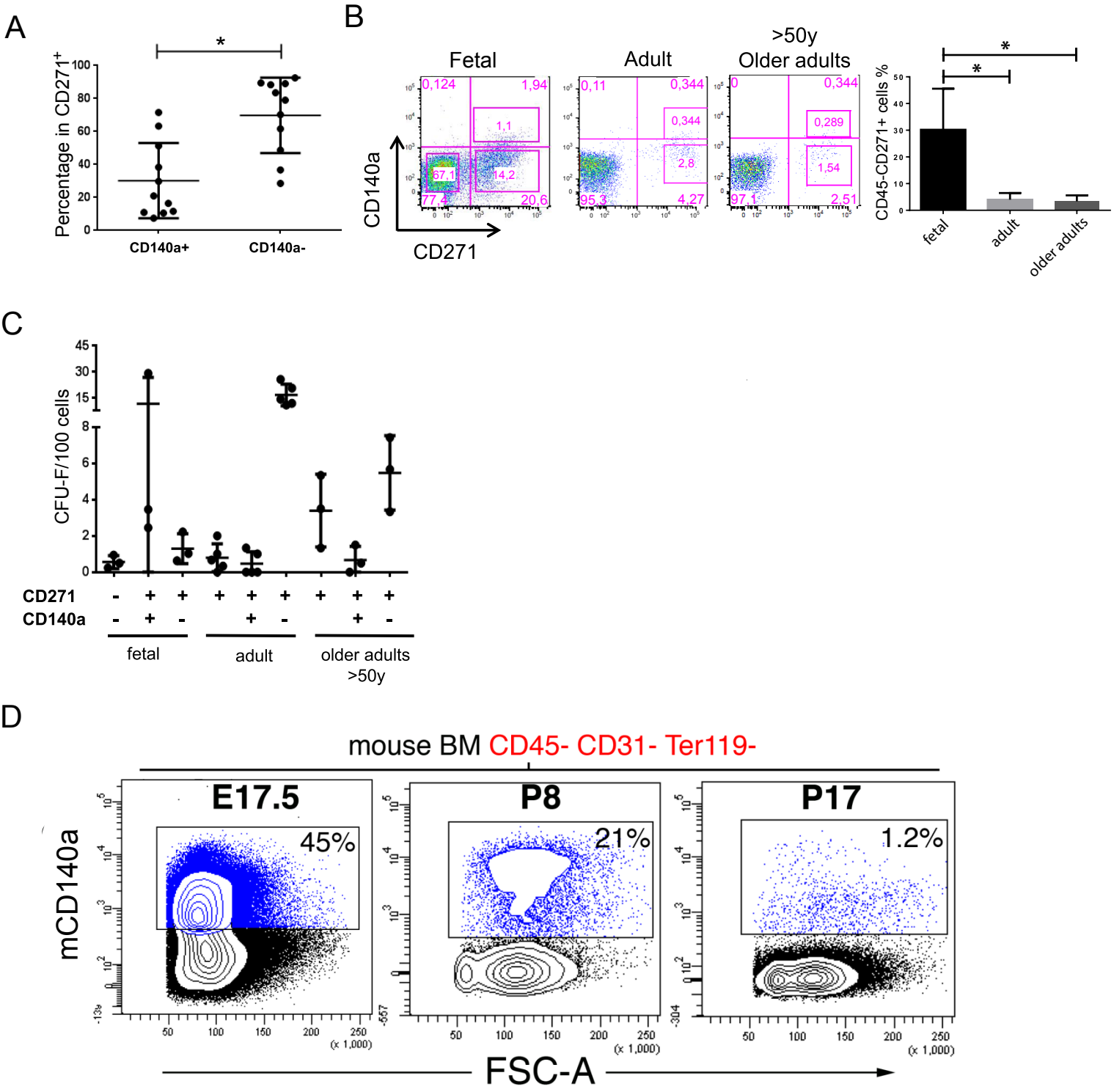

A
SSC-A
FSC-A
82,9
B
CD45-FITC
FSC-A
1,99
C
CD140a-PE
CD271-APC
0,0315
3,62
1,52
2,87
91,5
4,84
D
IgG1-FITC
FSC-A
100
E
IgG2a-PE
CD271-APC
0
0
91,9
8,15
F
CD140a-PE
IgG1-APC
6,14
0
93,9
0

Figure S4


A
Percentage in CD271+
CD140a+
CD140a-
B
Fetal
Adult
>50y
Older adults
CD140a
CD271
0,124
1,94
1,1
67,1
14,2
77,4
20,6
0,11
0,344
0,344
2,8
95,3
4,27
0
0,344
0,289
1,54
97,1
2,51
CD45-CD271+ cells %
fetal
adult
older adults
C
CFU-F/100 cells
CD271
CD140a
fetal
adult
older adults
>50y
D
mouse BM CD45- CD31- Ter119-
E17.5
45%
P8
21%
P17
1.2%
mCD140a
FSC-A
(x 1,000)

Supplemental Figure Legends

Figure S1, related to Figure 1. Gating strategy of isolating lin$^-$/CD45$^-$/CD271$^+$ and lin$^-$/CD45$^-$/CD271$^-$ MNCs for microarray analysis.

Freshly isolated lineage-depleted human BM-MNCs were stained with antibodies against CD271 and CD45 and lin$^-$/CD45$^-$/CD271$^+$ and lin$^-$/CD45$^-$/CD271$^-$ were sorted by FACS following (A) forward/side scatter gating, doublet and dead cell exclusion (7-AAD gating), and gating on CD45$^-$ cells (B). (C) Dot plot illustrating the sorting gates for CD271 expressing cells (upper gate) versus CD271$^-$ cells (C, lower gate). Gates for the CD45/CD271 sorts from lineage-depleted bone marrow were set according to the corresponding fluorescence-minus-one (FMO) control for CD45 (D) and CD271 (E). The figure shows a representative set of FACS plots.

Figure S2, related to Figure 1. MSC properties of lin$^-$/CD45$^-$/CD271$^+$/CD151$^+$ cells and lin$^-$/CD45$^-$/CD271$^+$/LEPR$^+$ cells.

(A) CFU-F frequencies of primary lin$^-$/CD45$^-$/CD271$^+$ bone marrow cell populations sorted on CD151 expression (left plot). Data are presented as individual data (dots) from bulk sorting (right figure, n= 4 independent experiments with at least three replicates for each experiment). (B) Cytospin preparation illustrating the morphology of CD45$^-$/CD271$^+$/CD151$^+$ cells (May-Grünwald/Giemsa staining). Scale bar indicates 20 µm. (C) Quantitative real-time PCR was performed on sorted lin$^-$/CD45$^-$/CD271$^-$/CD151$^-$, lin-/CD45$^-$/CD271$^+$/CD151$^+$, and lin$^-$/CD45$^-$/CD271$^-$/CD151$^+$ cells. Results are shown as fold mRNA change after standardizing with GAPDH levels. Data are given as mean ± standard deviation (SD) from 3 independent experiments. *:$p< 0.05$. (D) Flow cytometric analysis of cultured CD45$^-$/CD271$^+$/CD151$^+$-derived stroma cells stained with typical MSC markers (blue line). Red open histograms represent corresponding isotype controls. One representative set of histograms of a total of three experiments is shown. (E) Multicolor FACS analysis of primary, lineage-depleted BM-MNC after gating on CD45 negative cells. Events are plotted for CD271 (x-axis) against CD151 (y-axis). Red events in the plots indicate cells that co-expressed the marker listed on top of the plot, i.e. CD105, CD90, CD140b, STRO-1, CD31, CD34, SSEA4, and CD34, respectively. Grey events represent cells that did not co-express the listed marker. One representative set of FACS plots of a total of 3 experiments is shown. (F) *In vitro* differentiation capacity of cultured stromal cells generated from CD45$^-$/CD271$^+$/CD151$^+$ cells. Cultured cells were differentiated toward the osteoblastic, adipogenic, and chondrogenic lineage (upper panel). Chondrocyte control sections were stained with the secondary antibody only (lower panel). Scale bars indicate 500 µm (osteoblast), 100µm (adipocyte) and 200 µm (chondrocyte). Representative pictures from one of a total of 3 experiments are shown. (G) Multiclonal cultures generated from CD45$^-$/CD271$^+$/CD151$^+$ cells were transplanted subcutaneously (with HA/TCP particles) into immunodeficient mice. Representative sections 8 weeks after transplantation are shown. Bone (b), adipocytes (a), fibroblastic tissue (ft), and capillaries (*) are indicated. Dark brown areas indicate HA/TCP carrier particles

(magnification 10×). The arrow indicates invading hematopoietic cells. Scale bars indicate 100 µm. (H) Immunostaining of implants with antibodies against mouse CD45 and human vimentin. HA/TCP particles (with or without multiclonal cultures generated from $lin^-/CD45^-/CD271^+/CD151^+$ cells) were transplanted subcutaneously into immunodeficient mice. Representative sections 8 weeks after transplantation are shown. Bone (b), fibroblastic tissue (ft), capillaries (*) and HA/TCP particles are indicated. Dark brown dots indicate cells which were positive for mCD45 or hVimentin (magnification 10×). The empty control implant without MSC is negative for hVimentin and lacks bone formation. Scale bars indicate 100 µm. Sections of implants labeled for anti-mouse CD31 antibody and counterstained with hematoxylin. Scale bars indicate 250 µm (left). Vessel density is expressed as percentage of CD31 positive area relative to the total area of the implant (right). Data are shown as mean ± SD of three to six independent experiments, *:$p< 0.05$. (J) FACS plot showing the expression of LEPR in primary $lin^-/CD45^-/CD271^+/CD140a^-$ bone marrow cells in adult donors (left). Percentages of LEPR positive and negative cells in the $CD45^-/CD271^+/CD140a^-$ fraction (second from left). CFU-F frequencies of sorted LEPR positive compared to LEPR negative cells (second from right). Distribution of total colonies between LEPR positive and negative cells (right). Data are shown as mean ± SD of 3 independent experiments, *:$p< 0.05$.

Figure S3, related to Figure 2. Gating strategy for sorting of $lin^-/CD45^-/CD271^+/CD140a^+$ and $lin^-/CD45^-/CD271^+/CD140a^{low/-}$ MNC.

Freshly isolated lineage-depleted human BM-MNCs were stained with antibodies against CD271, CD45, and CD140a and $lin^-/CD45^-/CD271^+/CD140a^+$ and $lin^-/CD45^-/CD271^+/CD140a^{low/-}$ cells were sorted by FACS following (A) forward/side scatter gating (upper row, left plot) and (B) gating on $CD45^-$ cells . Gates for the CD45/CD271/CD140a sorts from lineage-depleted bone marrow were set according to the corresponding FMO controls for CD45 (D), CD140a (E), and CD271 (F). A representative set of FACS plots is shown.

Figure S4, related to Figure 2. CD140a expression on CFU-F in adult, fetal and older adult BM, and CD140a expression of murine non-hematopoietic BM cells over time.

(A) Percentage of $CD140a^+$ and $CD140a^-$ cells in $lin^-/CD45^-/CD271^+$ cells. Data are obtained from 11 independent experiments. *:$p< 0.05$. (B) FACS plots showing the expression of CD271 and CD140a in primary $lin^-/CD45^-$ bone marrow cell populations in fetal, adult and older donors. Human fetal BM sample, between 15-17 embryonic developmental weeks, were obtained from Novogenix Laboratories. Lower limbs were dissected and bone marrow cells were flushed with PBS containing 10% FBS and 1% AB/AM. Bones were crushed and rinsed with PBS to harvest the cells attached to the bone. After 1x Pharm Lyse™ treatment, BM cells were incubated with blocking buffer followed by FACS. In contrast to adult and older adult bone marrows, fetal bone marrow showed a higher percentage of $CD271^+$ cells (right plot). Data are obtained from 4 fetal, 4 adult and 3 elderly bone marrows. *:$p< 0.05$. (C) CFU-

F frequencies of primary $lin^-/CD45^-/CD271^+$, $lin^-/CD45^-/CD271^+/CD140a^+$, $lin^-/CD45^-/CD271^+/CD140a^-$, $lin^-/CD45^-/CD271^-/CD140a^-$ bone marrow cell from fetal, adult and older donors. In contrast to adult marrow, CFU-F were not only detected in $CD271^+$ cells, but also in the $CD271^-$ fraction. Data are presented as individual data (dots) from bulk sorting (n= 3-5 independent experiments). (D) Collagenase-digested mouse bone marrow samples from the indicated fetal and postnatal stages were stained with antibodies against mouse CD45, CD31, Ter119, CD140a and subjected to FACS analysis. Note the progressive down-regulation and the decrease of the stromal fraction expressing CD140a during postnatal times. In accordance with previously published data (Takashima et al., 2007), we found that the fraction of CD140a expressing cells in murine $CD45^-/CD31^-/TER119^-$ BM cells decreased from 45% at E17.5 (embryo day 17.5) to 21% at P8 (postnatal day 8) and 1.2% at P17. A representative set of FACS plots with the corresponding $CD140a^+$ frequency (within all stromal population), at each stage is shown. Primary anti-mouse antibodies used to stain murine BM samples were the following (all from BD Biosciences): CD45-biotin (clone 30-F11), CD31-biotin (clone MEC13.3), Ter119-biotin (clone TER-119) and CD140a-APC (clone APA5). For CD140a staining, murine bones were dissected at different stages, gently crushed and digested with collagenase type I (Stem cell technologies). Samples were stained with the aforementioned antibodies and mBMSC cells identified by negative gating on $CD45^-/CD31^-/Ter119^-$ population.

Table S2, related to Figure 1. Up-regulated surface marker genes in lin$^-$CD45$^-$CD271$^+$ cells

Gene name and description	Fold change*
VCAM1/CD106 : vascular cell adhesion molecule 1	52.23
ITGB5 : integrin beta-5	41.91
IL11RA : interleukin 11 receptor, alpha	27.62
GHR: growth hormone receptor	16.08
LEPR/CD295 : leptin receptor	15.40
PDGFRB/CD140b : beta-type platelet-derived growth factor receptor	14.60
CDH11: cadherin-11	14.45
CD81 : tetraspanin-28 (Tspan-28)	13.66
FGFR3/CD333 : fibroblast growth factor receptor 3	11.02
PDGFRA/CD140a: alpha-type platelet-derived growth factor receptor	10.93
TNFRSF19 : tumor necrosis factor receptor superfamily, member 19	8.74
TGFBR2 : transforming growth factor, beta receptor II	7.48
TNFRSF1A : tumor necrosis factor receptor superfamily member 1A	6.95
TMEM98 : transmembrane protein 98	6.77
TMEM119 : transmembrane protein 119	6.22
ITGB2/CD18 : integrin beta-2	6.10
IFNGR2 : interferon gamma receptor 2	5.90
CNTNAP2 : contactin-associated protein-like 2	5.74
ABCA8 : ATP-binding cassette sub-family A member 8	5.57
IL1R1 : interleukin 1 receptor, type I	4.84
TGFBR3 : transforming growth factor beta receptor III	4.61
CD151 : a member of the tetraspanin family	4.52
MME/CD10 : membrane metallo-endopeptidase, neprilysin	3.97
NTRK2 : neurotrophic tyrosine kinase, receptor, type 2	3.92
TMEM2 : transmembrane protein 2	3.75
BST2/CD317 : tetherin, bone marrow stromal antigen 2	3.61
FCRLA : Fc receptor-like A	3.13
EFNA1: Ephrin-A1	3.10

* Fold change represents the differences of the mean gene expression intensity in lin$^-$/CD45$^-$/CD271$^+$ compared to lin$^-$/CD45$^-$/CD271$^-$ cells. Gene expression analysis was performed on sorted cells from five donors.

Table S3, related to Figure 1-3. Primer sequences for qRT-PCR analysis*

GAPDH	F	5'- CACTCCACCTTTGACGC -3'
	R	5'- GGTCCAGGGGTCTTACTCC -3'
ACAN	F	5'- ACTCTGGGTTTTCGTGACTCT -3'
	R	5'- ACACTCAGCGAGTTGTCATGG -3'
ALPL	F	5'- AGCTGAACAGGAACAACGTG -3'
	R	5'- CAGCAAGAAGAAGCCTTTGG -3'
CD45	F	5'- ACCTTGAACCCGAACATGAG -3'
	R	5'- TCCTGGACTCCCAAAATCTG -3'
CD81	F	5'- TAACACGTCGCCTTCAACTG -3'
	R	5'- GAAGGAACATCAGGCATGCT -3'
CD151	F	5'- TCATCCTGCTCCTCATCATC -3'
	R	5'- TTGGTCATGGTGTCCTTCAG -3'
CD271	F	5'- CTGCAAGCAGAACAAGCAAG -3'
	R	5'- TCGCTGTGGAGTTTTTCTCC -3'
LEPR	F	5'- ACCTCTGGTTCCCCAAAAAG -3'
	R	5'- GTCGTTGAGTTTGGCTGTTG -3'
Nanog	F	5'- AACAATCAGGCCTGGAACAG -3'
	R	5'- GAGAATTTGGCTGGAACTGC -3'
Oct4	F	5'- GAGGATTTTGAGGCTGCTG -3'
	R	5'- TAGCCTGGGGTACCAAAATG -3'
PPARg	F	5'- TGCAGGTGATCAAGAAGACG -3'
	R	5'- GAAGGGAAATGTTGGCAGTG -3'
Sox-2	F	5'- AGAACCCCAAGATGCACAAC -3'
	R	5'- CGTCTCCGACAAAAGTTTCC -3'
VCAM1	F	5'- TCCGTCTCATTGACTTGCAG -3'
	R	5'- CATTCGTCACCTTCCCATTC -3'
CXCL12	F	5'- TGCCGATTCTTCGAAAGC -3'
	R	5'- ATCTGAAGGGCACAGTTTGG -3'
ANGPT1	F	5'- TCACATAGGGTGCAGCAATC -3'
	R	5'- ACAGTTGCCATCGTGTTCTG -3'
SPP1	F	5'- GAAGTTTCGCAGACCTGACAT -3'
	R	5'- GTATGCACCATTCAACTCCTCG -3'
DcR2	F	5'-TACCACGACCAGAGACACC -3'
	R	5'-CACCCTGTTCTACACGTCCG -3'
p21	F	5'-TGAGCCGCGACTGTGATG -3'
	R	5'- GTCTCGGTGACAAAGTCGAAGTT -3'
p16	F	5'- ATGGAGCCTTCGGCTGACT -3'
	R	5'- GTAACTATTCGGTGCGTTGGG -3'

*All primers were obtained from Life Technologies.

Supplemental Experimental Procedures

Antibodies

For FACS analysis and cell sorting the following antibodies were used: CD31-FITC (clone WM59), CD34-FITC (clone 581), CD45-FITC (clone 2D1), CD90-FITC (clone 5E10), HLA-DR-FITC (clone L243), SSEA4-FITC (clone MC813-70), CD10-PE (clone HI10a), CD14-PE (clone MϕP9), CD18-PE (clone 6.7), CD19-PE (clone SJ25C1), CD34-PE (clone 8G12), CD45-PE (clone HI30), CD73-PE (clone AD2), CD81-PE (clone JS-81), CD106-PE (clone 51-10C9), CD140a-PE (clone αR1), CD151-PE (clone 14A2.H1), CD166-PE (clone 3A6), HLA-ABC-PE (clone G46-2.6), CD106-APC (clone 51-10C9), CD45-APC-Cy7 (clone 2D1), purified GD2 (clone 14.G2a) (all from BD Bioscience, Erembodegem, Belgium), CD271-FITC, CD271-PE, CD271-APC (clone ME20.4-1.H4, Miltenyi Biotec, Bergisch Gladbach, Germany), purified STRO-1 (clone STRO-1), TGFBRII-PE (clone 25508), TGFBRIII-PE, FGFR3-PE (clone 136334), IFNGR2-APC, IL1R1-PE, TNFR1-PE (clone 16803), LEPR-PE (clone 52263) (all from R&D Systems, Abingdon, United Kingdom), CD105-FITC (clone SN6, AbD Serotec, Kidlington, UK), and PDGFR-beta-FITC (clone 7H36, US Biological, Swampscott, MA, USA). Matching isotype controls were from BD Bioscience and R&D Systems. For unconjgated primary antiobodies, goat anti-mouse IgG2a-FITC and goat anti-mouse IgM-FITC (Jackson ImmunoResearch Laboratories, Inc., Suffolk, UK) were used as secondary antibodies. For staining of cryo sections antibody against aggrecan was used followed by secondary antibody staining (donkey anti-goat IgG-Texas Red, Jackson ImmunoResearch Laboratories).

Microarray expression analysis

RNA from sorted cell populations was isolated, subjected to a two-round amplification and analyzed for gene expression by microarray analysis using Illumina Human HT-12 expression v4 BeadChips (Illumina, San Diego, CA). Pre-hybridization treatment, hybridization and post-hybridization washes were performed using the Illumina hybridization protocol (Manual 11322187 Revision A). Basic Illumina chip and Experimental Quality Analyses were performed using the GenomeStudio software V2011.1. 2. Probe summarization and data normalization were performed as described previously (Tusher et al., 2001). Signals were log2 transformed after probesets having no gene annotations or expired annotations were filtered out. SAM (significance analysis of microarrays) analysis was performed to identify significantly differentially expressed genes between groups following quantile normalization in the BioArray Software Environment (BASE). SAM was performed with Delta = 15.321 pursuing a false positive rate of 0%. Normalized data have been deposited in the GEO database (GSE57927).

Flow cytometry

Cultured cells were harvested, washed, and unspecific binding was blocked with human normal immunoglobulin. Cells were stained (30 min, 4˚C) with combinations of antibodies and samples were analyzed on a FACS Calibur (BD). Freshly isolated RosetteSep-depleted BM-MNCs were antibody stained (see Supplementary Materials) and analyzed on a LSR II flow cytometer (BD).

CFU-F assay and limiting dilution assay

FACS-sorted cells were cultured at plating densities of 1-10 cells/cm^2 when assaying CD45$^-$/CD271$^+$/CD151$^+$, CD45$^-$/CD271$^+$/CD140a$^{low/-}$ and CD45$^-$/CD271$^+$/CD140a$^+$ sorted cells, and 1000 cells/cm^2 in case of lineage-depleted unsorted cells. Colonies were counted after 14 days

(1% Crystal Violet, Sigma). Colonies containing ≥40 cells were counted as CFU-F. Generally, assays were set up in duplicates or triplicates.

For single cell CFU-F assays, cells were sorted into 96-well plates and cultured in MSC medium. Colonies were counted after 3 weeks, harvested and split for continued culture for in vitro differentiation assays.

Limiting dilution assays were performed with $CD271^{+}/CD45^{-}/CD140a^{low/-}$ cells seeded at plating densities of 1, 2, 3, 4, 5, 10 and 20 cells per well in 96-well plate in replicates of 8 wells per density level. Colony frequencies were calculated using Poisson distribution statistics (L-CalcTM software, StemCell Technologies).

Cell cycle analysis

BM mononuclear cells were stained with antibodies against CD45, CD271, and CD140a followed by fixation/permeabilization (Cytofix/Cytoperm kit, BD Biosciences) and intracellular staining with anti-KI67-FITC (BD) and DAPI (Sigma). Analysis was performed on a LSR II flow cytometer (BD).

***In vitro* differentiation assays – differentiation-induction media**

Osteogenesis induction medium contained of NH Expansion medium (Miltenyi Biotec), 1% Antibiotic/Antimicotic solution, 10 mM β-glycerophosphate, 0.1µM Dexamethasone (all from Sigma), 0.05 mM L-ascorbic acid (Wako chemicals, Richmond, VA, USA)]. Chondrogenesis-induction medium contained of DMEM-high glucose supplemented with 0.1 µM dexamethasone, 1 mM sodium pyruvate, 0.35 mM L-proline (all from Sigma), 0.17 mM ascorbic acid (Wako Chemicals, Richmond, VA, USA), 1% ITS+ culture supplements (BD Biosciences) and 0.01 µg/ml TGF-ß3 (R&D Systems, Abingdon, UK)].

Quantitative real-time PCR

RNA was isolated using QIAshredder Homogenizers columns (Qiagen) and RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. The concentration and purity of RNA was determined by Nanodrop (Thermo Fisher Scientific, Gothenburg, Sweden). cDNA was synthesized using SuperScript VILO cDNA synthesis kit (Life Technologies) on C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR analysis was carried out using Fast SYBR master mix (Applied Biosystems by Life Technologies) according to manufacturer's instructions. Primer sequences are listed in Table S3. The crossing point of each sample was measured and analyzed with StepOne Software v2.1 (Applied Biosystems). Each gene-specific mRNA was normalized to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA. The expression of each mRNA was determined using the $2^{-\Delta\Delta CT}$ threshold cycle method.

Immunohistochemistry and quantification of CD31-pos vasculature

Paraffin sections of implants were stained for CD31 (ab28364, Abcam) using standard immunohistochemistry methods, developed with DAB and counterstained with hematoxylin. Positive staining appeared as brown precipitate associated to vascular structures. High-resolution images of CD31-stained sections were acquired using a digital slide scanner (Hamamatsu), encompassing the whole implant. Digital images were processed using Image J analysis software (NIH). For calculation of the CD31-positive vasculature area, DAB-stained areas were measured after color deconvolution by adjusting the threshold levels in the brown component channel. Vessel density was expressed as percentage of $CD31^+$ area relative to the total implant tissue area.

Supplemental References

Takashima, Y., Era, T., Nakao, K., Kondo, S., Kasuga, M., Smith, A.G., and Nishikawa, S. (2007). Neuroepithelial cells supply an initial transient wave of MSC differentiation. Cell *129*, 1377-1388.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A *98*, 5116-5121.