Mimicking the functional hematopoietic stem cell niche *in vitro*: recapitulation of marrow physiology by hydrogel-based three-dimensional cultures of mesenchymal stromal cells

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Online Supplementary Data

Mice

The protocols used in animal experimentation have been approved by the institutional animal ethics committee. The NOD.CB17-Prkdcscid/J (NOD/SCID), C57BL/6J (CD45.2) and B6.SJL-PtprcaPepcb/BoyJ (Ptprc; CD45.1) mice (Jackson Laboratory, Bar Harbor, USA) were housed and bred in our experimental animal facility (EAF). NOD/SCID mice were kept in micro-isolators and fed with sterile food and acidified water. The CD45.1 mice were crossed with the CD45.2 mice to get F1 progeny; 8-12 week old mice were used for transplantation studies.

Cell culture

Human samples

The protocols used for human samples were approved by the Institutional Review Board. Ribs obtained as clinical waste in surgical procedures and placentas discarded after caesarean section delivery were collected in sterile containers after obtaining written informed consent of the patients.

The marrow mononuclear cells (MNCs) were obtained from the ribs. The rib pieces were digested with collagenase (Type IA, Sigma-Aldrich, St. Louis, USA). $CD34^+$ cells were isolated from the MNCs obtained using the $CD34^+$ progenitor cell selection system (Dynal Biotech ASA, Oslo, Norway; purity 97-98%; *Online Supplementary Figure S1A*). The MSCs were grown from the marrow or from a full-term placenta. Phenotypic analyses confirmed that the cells were positive for CD146, CD166, CD105, CD73, CD90, CD54, HLA-ABC, CD271 and negative for CD34, CD45 and HLA-DR (Becton-Dickinson, San Diego, CA, USA; *data not shown*).

Culture of placenta-derived MSCs

A piece from the central placenta lobules was washed with plain IMDM (Sigma) and mechanically minced before enzymatic digestion with 0.25% trypsin-EDTA (TPVG; Sigma) for 10 min at 37°C. The homogenate was passed through sterile muslin cloth to obtain a single-cell suspension. After washing with plain medium, the cells were suspended in growth medium (IMDM supplemented with 10% FBS; Stem Cell Inc.) and were seeded in 75 cm^2 flasks (BD). Non-adherent cells were removed after 72 h. Adherent cells were fed with fresh medium. The cells were serially passaged at confluence by trypsinization. The cells between 3-10 passage numbers were used for experiments.

Culture of marrow-derived MSCs

The $CD34^-$ fraction obtained after immuno-magnetic separation of $CD34^+$ cells was suspended in growth medium and seeded in culture flasks. Non-adherent cells were removed after 72 h. Adherent cells were fed with fresh medium. The cells between 3-5 passage numbers were used for experiments.

Trilineage differentiation of MSCs in 2D and 3D cultures

2D-/3D- MSCs were cultured in differentiation media (Invitrogen) for 20-25 days. Cultures were fed every 3rd day. The cells were fixed with 10% buffered formaldehyde for 20 min and then washed twice with distilled water. The cells were stained with Oil Red O solution for 30 min to detect adipocytes; with 2% Alizarin red for 30 min to detect osteoblasts and with 1% Alcian blue solution for 30 min to detect chondrocytes. The cells were washed and were imaged on an inverted microscope (Olympus) equipped with a digital camera (Olympus camedia C5060).

Murine cells

Lineage negative (Lin^-) cells were isolated from the murine bone marrow-derived MNCs by using biotin-labeled lineage cocktail (BD) and streptavidin-labeled magnetic beads (Invitrogen, Carlsbad, CA, USA). The whole bone marrow cells were seeded in the growth medium to obtain MSCs. After 72 h, non-adherent cells were discarded. Adherent MSCs were fed with fresh medium.

Colony-forming unit (CFU) assay

$1\text{-}2\times10^4$ cells harvested from 2D/3D culture were suspended in 1% methylcellulose (Sigma) along with a combination of growth factors; Stem Cell Factor, (SCF), 20ng/mL; granulocyte monocyte-colony stimulating factor (GM-CSF) 20 ng/mL; interleukin-3 (IL-3) 20 ng/mL; and erythropoietin (Epo) 2U/mL; Peprotech. The plates were incubated at 37°C in 5% CO_2 in a humidified atmosphere. Colonies belonging to blast-forming unit erythroid (BFU-E), granulocyte-monocyte (GM), and granulocyte-erythroid-monocyte-megakaryocyte (GEMM) were scored after 14 days according to standard morphological criteria.

Long-term culture initiating cell (LTC-IC) assay

M210B4 cells (ATCC) cells were seeded on collagen-coated wells of 24-well plate and after 18 h, the plate was γ-irradiated (8,000 rads). 1×10^5 sort-purified $CD45^+$ cells from 2D/3D cultures were seeded on the feeder layer in human myelocult (Stem Cell Tech) medium supplemented with freshly dissolved hydrocortisone (10^{-6}M). The cultures were maintained with weekly demi-defoliation. Each set was kept in triplicate. After five weeks, the cultures were harvested and further assayed for CFU assay as described earlier.

Migration assay

1×10^5 2D-/3D-$CD34^+$ output cells suspended in 100 μL migration medium (IMDM supplemented with 1% BSA) were seeded in the upper wells of migration chambers (8 microns, BD Falcon) and 700 μL of migration medium, with or without SDF1α (100ng/mL; Peprotech), was added in the bottom well. Migrated cells were counted after 5 h. A mean ± S.E.M. of triplicate readings was calculated.

Flow cytometry

The cells were stained with fluorescently-labeled antibodies (*Online Supplementary Table S1*) and acquired on FACS Canto II, Calibur or Aria (BD) after RBC lysis when required. The cells stained with isotype-matched antibodies were used as controls. Data were analyzed by FACS DIVA - version 5.0 or Cell Quest Pro software - version 5.1.2 (BD).

Cell cycle analysis

The harvested cells were stained with CD45-PE-Cy7, CD34-APC and lineage-cocktail-PE. After washing, the cells were suspended in cytofix/cytoperm (BD) for 20 min. They were then stained with an antibody to Ki67-FITC (BD) and DAPI (4′6-diamidino-2-phenylindole·2HCl, 30 μM, InVitrogen). Gated $CD45^+34^+$ Lin^- cells were analyzed for cell-cycle status.

Division history tracking

PKH-26-Red-labeled (Sigma) $CD34^+$ cells were seeded on 2D- or 3D-MSCs. An aliquot of labeled cells was acquired immediately to mark the base-level intensity of undivided cells. After seven days, the cells were harvested and analyzed on FACS Canto II (BD) after staining with CD45-PE-Cy7, CD34-APC and lineage cocktail-FITC antibodies. The division history of gated $CD45^+34^+Lin^-$ HSCs was tracked based on the 0 day PKH26-intensity.

SP cell analysis

The harvested cells were stained with Hoechst 33342 (Sigma), with or without 50 μM verapamil (Sigma), for 90 min at 37°C and were acquired on FACS Aria (BD) using UV laser (excitation λ: 350 nm, emission λ: 450nm - blue and 650nm - red).

Immunofluorescence staining

The cultures set on coverslips were fixed with freshly prepared 4% buffered paraformaldehyde and permeabilized using 0.1% Triton X-100 in PBS (pH 7.4) for 5 min. After blocking with 1% BSA in PBS, they were incubated with primary antibodies (*Online Supplementary Table S2*) first for 12–16 h at 4°C and then with appropriate fluorescently-tagged secondary antibodies for 2 h at room temperature in the dark. DAPI was used to demarcate the nuclei. Imaging was performed on confocal laser-scanning microscope (Carl Zeiss, Jena, Germany, LSM 510 META; 63X/oil/1.4NA objective) using AIM 4.2 software. The matrix was pre-tested for its possible non-specific interaction with antibodies or for interference with the washing steps. The cells stained with secondary antibodies alone were used to detect non-specific binding. For double-labeling experiments, the images were captured in sequential mode. Serial optical sections of 0.5 microns were taken. Image analysis was carried out using LSM-5 Image examiner.

In vivo studies

The harvested cells were transplanted by intravenous injections into sublethally irradiated (350 rads, Co^{60}) NOD/SCID mice. Mice were sacrificed 10-12 weeks post transplantation. Multi-lineage engraftment of human cells was assessed in the BM using hCD45-PE, hCD71-PE, hCD66b-FITC, hCD15-Alexa Fluor® 488, hCD19-PE-Cy5.5, hCD20-PE-Cy5.5, hCD34-APC antibodies, after blocking with Fc-Blocker at 4°C. Cells from irradiated un-infused animals were used to detect any non-specific staining. BM cells from primary recipients were infused into irradiated secondary recipients to estimate long-term repopulating HSCs (LT-HSCs).

The competitive repopulation ability of 2D- *versus* 3D-HSCs was assessed in a head-to-head competition assay using mouse chimera model. The multi-lineage engraftment of the CD45.1 *versus* CD45.2 cells was monitored after 4 and 16 weeks post transplantation. Peripheral blood was collected via the orbital plexus. The cells were stained with CD45.1-PE, CD45.2-FITC, CD45R (B220)-PB, CD3e-APC, CD11b-PE-Cy7, and Ly-6G-PE-Cy7 for 45 min and acquired on Canto II (BD).

Gene expression studies

mRNA was isolated using mRNA isolation kit (Dynal/InVitrogen) and was reverse transcribed using Superscript II (InVitrogen). An RT^{neg} set was kept to detect genomic contamination. The cDNA was subjected to real time or semi-quantitative PCR experiments (*Online Supplementary Table S3*).

Quantitative and semi-quantitative PCR

Real time PCR was carried out on ABI 7500 Fast Sequence Detection System (Applied Biosystems) using inventoried Taqman gene expression assays (assay on demand) or gene-specific primers using SYBR green (InVitrogen) chemistry to quantify gene expression. Primers were designed using Primer-3 tool V0.4.0 (*http://prodo.wi.mit.edu/primer3/*) and a melting curve analysis was carried out to validate specific amplification of genes. Ct values were normalized with those obtained from the amplification of GAPDH.

Semi-quantitative PCR experiments were carried out to study the expression of nestin and Tie-2 genes using an Eppendorf thermal cycler. cDNA was amplified using specific primers listed in the *Online Supplementary Table S3*.

PCR conditions were as follows:

Initial denaturation: 95°C for 2 min.

Denaturation: 94°C for 30 s;

Annealing: 60°C (GAPDH and nestin)/ 64 °C (Tek) for 30 s;
Extension: 72°C for 40 s;
25 cycles (GAPDH) or 30 cycles (nestin and tek)
Final extension: 72°C for 10 min.

Amplified PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Images were acquired on gel documentation system (Syngene) and densitometric analysis was carried out using Image J software.

Statistical analysis

Data were analyzed by one-way repeated measures analysis of variance (One-Way RM ANOVA; Sigma Stat; Jandel Scientific Software, San Rafael, CA, USA). $P \leq 0.05$ was considered significant. Error bars represent standard error of mean (SEM).

Online Supplementary Table S1. Antibodies used for cell sorting and flow cytometry.

Antigen HUMAN	Label	Clone	Distributor
MSC characterization			
CD45	PE	HI30	BD Biosciences
CD34	PE	8G12	BD Biosciences
CD166	PE	3A6	BD Biosciences
CD105	PE	166707	R&D Systems
CD73	APC	AD2	eBiosciences
CD90	PE	5E10	BD Biosciences
HLA-ABC	PE	G46-2.6	BD Biosciences
HLA-DR	PE	G46-6	BD Biosciences
CD146	PE	P1H12	BD Biosciences
CD271	PE	C40-1457	BD Biosciences
Human hematopoeitic lineage cocktail			
CD2	PE	RPA-2.10	BD Biosciences
CD3	PE	HIT3a	BD Biosciences
CD14	PE	MφP9	BD Biosciences
CD16	PE	3G8	BD Biosciences
CD19	PE	HIB19	BD Biosciences
CD56	PE	B159	BD Biosciences
CD61	PE	VI-PL2	BD Biosciences
CD 235a	PE	GA-R2 (HIR2)	BD Biosciences
Human lineage cocktail	FITC	22-7778	eBiosciences
hHSC characterization			
CD45	PE-Cy7	HI30	BD Biosciences
CD34	APC	8G12	BD Biosciences
CD133	PE	AC133	Miltenyi Biotech
CXCR4	PE	12G5	BD Biosciences
Ki67	FITC	B56	BD Biosciences
CD38	FITC	HB7	BD Biosciences
NOD/SCID engraftment			
CD45	PE	BRA-55	Sigma
CD71	PE	MEM75	Biolegend
CD66b	FITC	G10F5	Biolegend
CD15	Alexa Fluor® 488	H198	Biolegend
CD19	PE-Cy5.5	HIB19	Biolegend
CD20	PE-Cy5.5	2H7	Biolegend
CD34	APC	8G12	BD Biosciences

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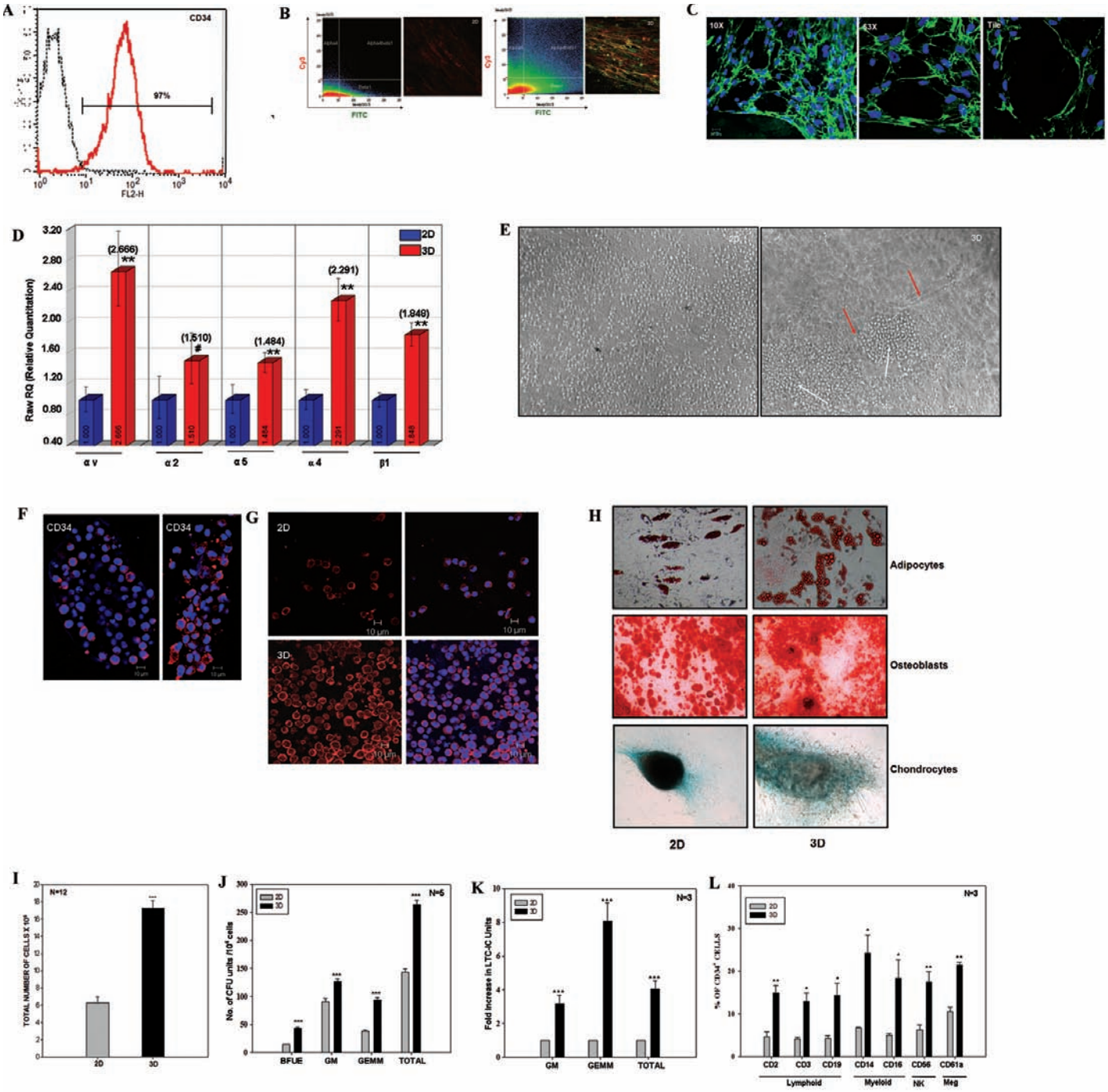
MOUSE			
Isolation of lineage negative cells			
Lineage cocktail	Biotin	559971	BD Biosciences
mHSC characterization			
Lineage cocktail	APC	558074	BD Biosciences
Sca-1	PE	D7	BD Biosciences
Ckit	PE-Cy7	2B8	BD Biosciences
CD34	FITC	RAM34	BD Biosciences
Head-to-head competition			
CD45.1	PE	A20	eBiosciences
CD45.2	FITC	104	eBiosciences
CD45R(B220)	eFluor 450	RA3-6B2	eBiosciences
CD3e	APC	145-2C11	eBiosciences
CD11b(Mac-1alpha)	PE-Cy7	M1/70	eBiosciences
Ly-6G (Gr-1)	PE-Cy7	RB6-8C5	eBiosciences

Online Supplementary Table S2. **Antibodies used for immunofluorescence.**

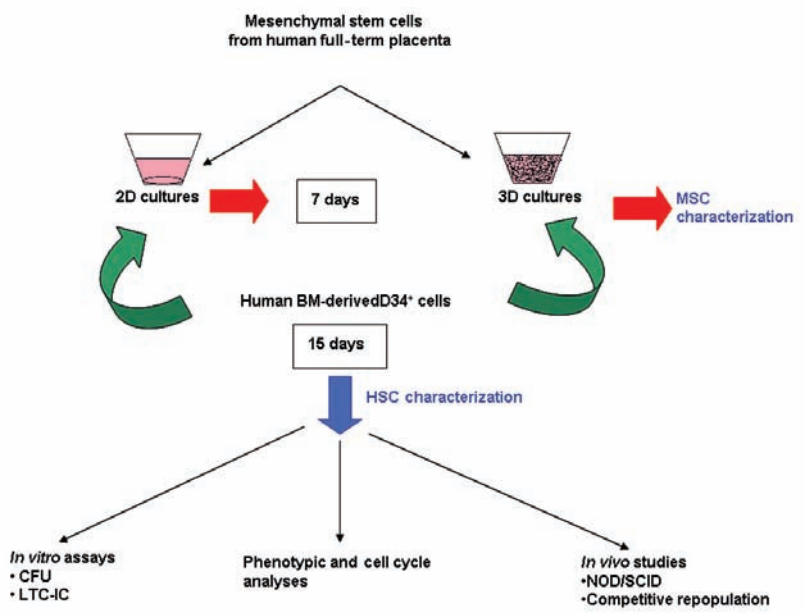
Antigen HUMAN	Clone/Cat#	Distributor
Cellular FN	FN-3E2	Sigma
Collagen IV	CIV 22	Neomarker
Vitronectin	VIT-2	Sigma
Laminin	LAM-89	Sigma
Alpha5beta1	JBS5	Chemicon
Alpha2beta1	Gi9	Biodesign
Alphavbeta3	LM609	Chemicon
CD34	9B10D4	Abcam
N-cadherin	EPR1792Y	Epitomics
SDF-1	79014.111	R&D systems
Nestin	10C2	Millipore
Hypoxy probe Mab	4.3.11.3	Chemicon
HIF1alpha	H-206	SCBT
Alpha4	H-210	SCBT
Beta1	6S6	Chemicon
Donkey anti mouse Cy3	AP192C	Chemicon
Goat anti rabbit Alexafluor488	A11034	Invitrogen
Donkey anti goat Alexafluor488	A11055	Invitrogen

Online Supplementary Table S3. List of Primers.

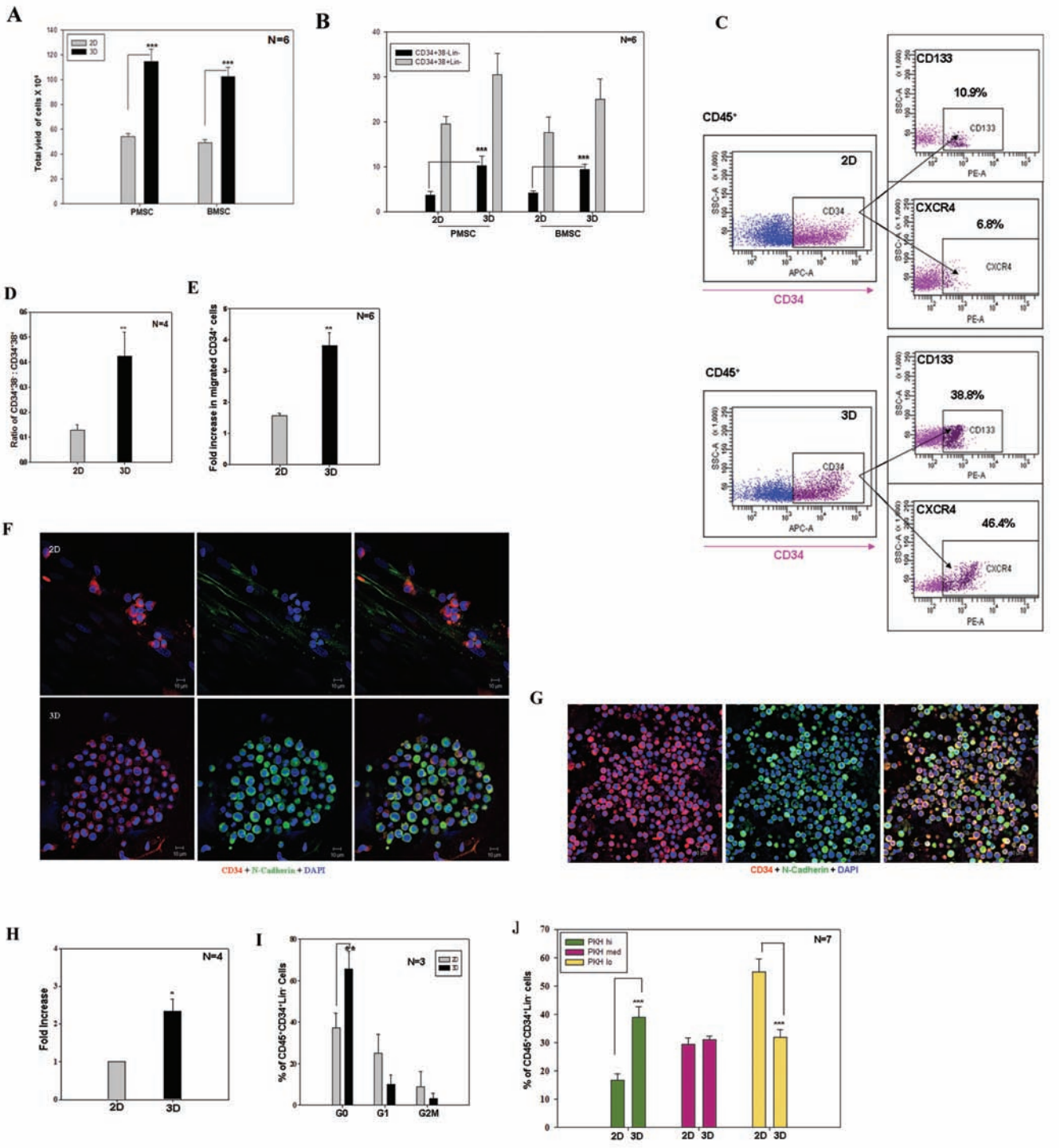
Name	Sequence	Description and GenBank accession number	Product size
GAPDH-F GAPDH-R	5' ACTGCCACCCAGAAGACTGT 3' 5' CCATGCCAGTGAGCTTCC 3'	Human glyceraldehyde-3-phosphate Dehydrogenase [NM_002046.3]	151
Osteopontin-F Osteopontin-R	5' GAAGTTTCGCAGACCTGACA 3' 5' ATTCAACTCCTCGCTTTCCA 3'	Homo sapiens secreted phosphor-protein 1 (SPP1) [NM_000582.2]	82
Angiopontin1-F Angiopontin1-R	5' CTTCAAGGCTTGGTTACTCG 3' 5' TGTGTCCATCAGCTCCAGT 3'	Homo sapiens angiopoietin 1 (ANGPT1), [NM_001146.3]	117
RUNX2-F RUNX2-R	5' TGACACTGCCACCTCTGACT 3' 5' ATGAAATGCTTGGGAACTGC 3'	Homo sapiens runt-related transcription factor 2 (RUNX2), [NM_004348.3]	116
SDF1α-F SDF1α-R	5' CCAACGTCAAGCATCTCAAA 3' 5'CTTTAGCTTCGGGTCAATGC 3'	Homo sapiens chemokine (C-X-C motif) ligand 12 [NM_199168.3]	107
Nestin-F Nestin-R	5' GGCTCCAAGACTTCCCTCAG 3' 5' TCAGGACTGGGAGCAAAGAT 3'	Homo sapiens nestin (NES) [NM_006617.1]	100
Tek-F Tek-R	5' GCCATTGCCAATAGCACCGCG 3' 5' GTCCTAGGCCGCTTCTTCAGC 3'	Homo sapiens TEK tyrosine kinase, endothelial [NM_000459.3]	599
Integrin β	Hs00236976_m1		
VEGF	Hs00173626_m1		
Integrin α2	Hs00158127_m1		
Integrin α2b	Hs01116228_m1		
Integrin αv	Hs00233808_m1		
Integrin α6	Hs00173952_m1		
GAPDH	Hs99999905_m1		
HIF1α	Hs00936368_m1		
Integrin α4	Hs00168433_m1		
Integrin α5	Hs01547673_m1		



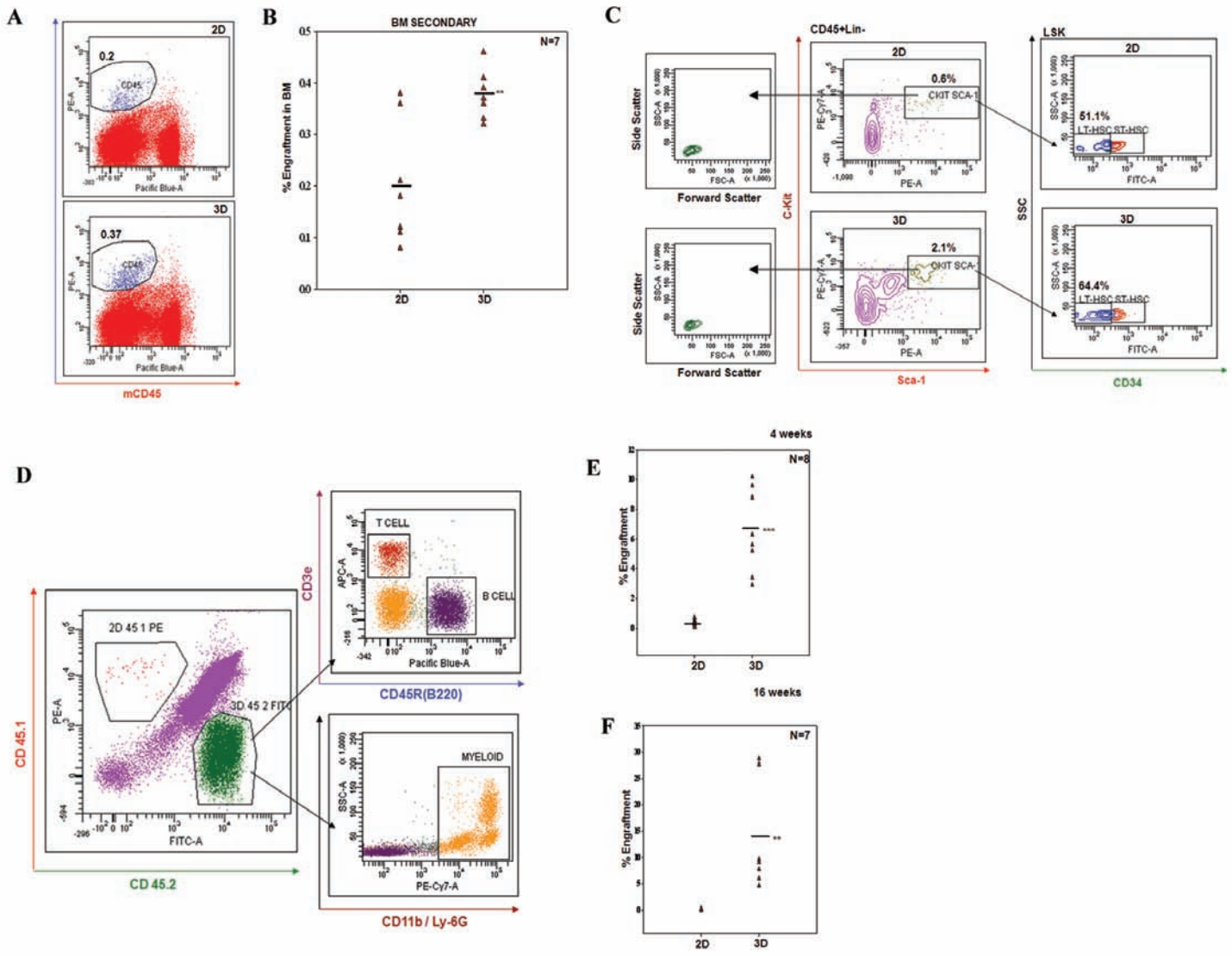
Online Supplementary Figure S1. (A) A representative flow cytometric analysis of human bone marrow-derived CD34⁺ cells isolated by immunomagnetic separation. The purity of these isolated cells was typically over 96%. (B) Presence of $\alpha 4\beta 1$ dimer (yellow) on the surface of the 3D-MSCs (right panel) is shown by co-localization analysis of the cells stained with a combination of antibodies to $\alpha 4$ (green) and $\beta 1$ (red). 2D-MSCs expressed very low levels of $\beta 1$ integrin. Only the signal for α 4 (red) is seen (left panel). LSM5 image examiner was used for the analysis. (C) Fibronectin⁺ MSCs (FITC) assume the 3D-matrix architecture. 10X, 63X and tile view of 3D-MSCs immunostained with an anti-fibronectin (cellular) antibody are shown. (D) Quantitative RT-PCR analysis of various integrin mRNAs in 2D- and 3D-MSCs. 3D-MSCs express high levels of integrin subunits compared to the 2D-MSCs (n=3; $^{**}P<0.01$; # not significant). (E) The phase contrast micrograph shows that in the hydrogel cultures (right panel) the MSCs grow along with the matrix fibers (red arrows) while the hematopoietic cells grow as tight clusters (white arrows) in the pockets formed. A scattered growth of hematopoietic cells over a monolayer of the MSCs is seen in the 2D-cultures (left panel). The images were captured on an inverted phase contrast microscope (Olympus) equipped with Olympus SC35 camera using 10X objective (N.A.0.30), 10X eye-piece and a 1.5X magnification slider. Original magnification 150X. (F) Confocal microscopy analysis of 3D co-cultures immunostained with an anti-CD34 antibody shows that the matrix pockets are populated by CD34⁺ (Cy3) cells. Images of two such pockets filled with CD34⁺ cells are shown. Nuclei are demarcated by DAPI (blue). (G) CD34⁺ cells proliferate extensively under 3D-conditions. Image captured on a confocal laser-scanning microscope shows a large expanse of CD34⁺ (Cy3) cells formed under the 3D conditions (lower panels) *versus* a scanty growth under the 2D conditions (upper panels). The nuclei are demarcated by DAPI (blue). (H) Tri-lineage differentiation capacity of the MSCs under 2D- and 3D-conditons is shown. The images were captured on an inverted phase contrast microscope (Olympus) equipped with an Olympus camera (camedia C5060) using 10X objective (N.A.0.30), 10X eye-piece and a 1.5X magnification slider. Original magnification 150X. Data show that the differentiation capacity of the MSCs was not affected by the growth in hydrogel. (I) 3D-MSCs support robust hematopoiesis. Quantification of the total number of hematopoietic cells revealed that the 3D-MSCs yield a significantly higher number of total hematopoietic cells compared to their 2D counterparts (n=12; $^{***}P<0.001$). (J) Colony formation assays performed on the output hematopoietic cells showed a significantly higher output of progenitors from the 3D-MSCs compared to the 2D ones (n=5; ***P<0.001). (K) LTC-IC assays performed on the CD45⁺ cells sorted from the harvested cells show that the 3D-MSCs harbor a significantly higher number of primitive progenitors compared to the 2D-MSCs (n=3; $^{***}P \leq 0.001$). (L) Flow cytometric analyses of the gated CD34⁺ population using lineage markers show that the 3D-cultures support a robust multi-lineage hematopoiesis (n=3; $^{*}P<0.05$ $^{**}P<0.01$).



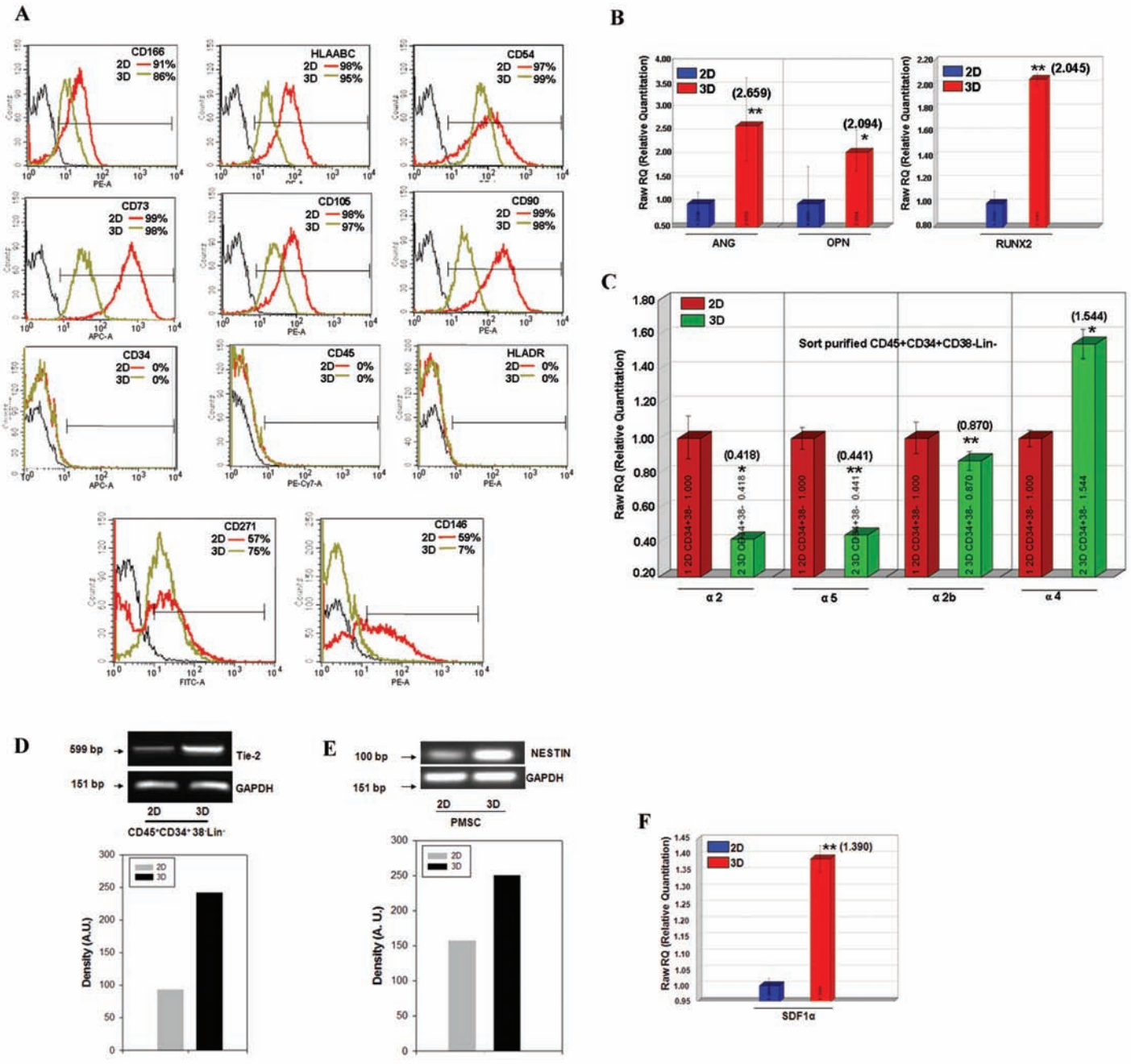
Online Supplementary Figure S2. Schematic representation of the experimental design.



Online Supplementary Figure S3. PMSC and BMSC provide equivalent HSC-support. The total hematopoietic cells (A) and $CD34^+CD38^-Lin^-$ HSCs (B) obtained from the 2D- and 3D-cultures of BMSC and PMSC are comparable (n=6; NS). The 3D-cultures of either MSCs consistently gave significantly higher yield compared to their 2D counterpart (n=6; ***P<0.001). (C) The flow panel shows that the $CD45^+CD34^+$ gated cells obtained from the 3D-MSCs contained a higher percentage of cells expressing primitive HSC-markers: CD133 (upper panel) and CXCR4 (lower panel). (D) A 3.5-fold higher ratio of $CD34^+38^-$:$CD34^+38^+$ cells obtained in the 3D-HSCs shows that the 3D-system favors the growth of a primitive population (n=4, **P<0.01). (E) Transmigration assays were set up in response to SDF1α. Quantification of the migrated cells showed a 4-fold higher migration of 3D-$CD34^+$ cells towards SDF1α compared to the 2D ones. ($4.817x10^4$±0.523 for 3D-CD34+ *vs.* $1.883x10^4$±0.119 for 2D-$CD34^+$; n=6; **P<0.01). (F) Confocal laser microscopy analyses show that most 3D-$CD34^+$ cells (Cy3) express N-Cadherin (FITC), albeit at variable levels (lower panel). The 2D-$CD34^+$ cells did not express N-Cadherin (upper panel). The nuclei are demarcated by DAPI (blue). (G) A large cluster of $CD34^+$N-Cadherin$^+$ cells growing in the 3D-MSCs is shown (tile mode, pixel size 2048 X 2048). DAPI (blue) demarcates the nuclei (blue). (H) 3D-MSCs harbor a higher percentage of SP cells compared to the 2D ones. Fold increase in the percentage of SP population data (mean±S.E.M.) obtained in 4 independent experiments is represented (n=4; *P<0.05). (I) HSC-quiescence is maintained in the 3D-MSCs. Cell cycle analysis of the gated $CD45^+34^+Lin^-$ population shows that a higher percentage of these cells were present in G0 state compared to the 2D ones. Cell cycle data obtained in 3 independent experiments (mean±S.E.M.) is shown (n= 3; **P<0.01). (J) Division history tracking of HSCs. Flow cytometric analyses of the PKH26-labeled $CD34^+$ cells after seven days of co-culture showed that a majority of the $CD45^+34^+Lin^-$ population from 3D-MSCs (green bars) did not divide during the culture period, whereas more than 70% of their 2D counterparts (yellow bars) had undergone proliferation. Analysis of data obtained in 7 independent division tracking experiments (mean±S.E.M.) showed that that the observed phenomenon was consistent and statistically significant (n=7; ***P≤0.001).



Online Supplementary Figure S4. (A) 3D-HSCs possess long-term reconstitution ability. The marrow cells harvested from the primary recipients were infused in the sublethally irradiated secondary recipients to assess their long-term reconstitution ability. Representative flow cytometric data show the percentage of human CD45$^+$ cells seen eight weeks after transplantation. (B) The scatter plot shows that the 3D-MSCs harbor a significantly higher number of LT-HSCs compared to the 2D ones. (n=7; $^{**}P<0.01$). (C) Validation of 3D-system in murine model. 2D- and 3D-cultures of murine MSCs were set up and used to co-culture Lin$^-$ cells. Representative flow panel shows the percentage of Lin$^-$Sca1$^+$cKit$^+$CD34$^-$ (LT-HSCs) and percentage of Lin$^-$Sca1$^+$cKit$^+$CD34$^+$ (ST-HSCs) in the output cells. 3D-cultures of murine MSCs yielded much higher proportion of LT-HSCs compared to those harvested from 2D-cultures. (D) 'Head-to-head' competitive repopulation assay. Equal numbers of sorted 2D-(CD45.1) and 3D-(CD45.2) output cells along with the freshly isolated marrow cells from the F1 mice were infused into lethally irradiated F1 recipients. Four weeks after transplantation, the peripheral blood cells were collected and the percentage of engraftment of the donor cells was analyzed by flow cytometry. The engrafted 3D-cells (green cluster) were further analyzed using myeloid- and lymphoid-specific markers (middle panels). The level of engraftment by the 2D-cells (red cluster) was too low for such an analysis. The scatter plots show that a significantly higher percentage of engraftment by 3D-HSCs at four weeks (E) (n=8; $^{***}P<0.001$) and at 16 weeks (F) (n=7; $^{**}P<0.01$) post transplant. Swapping cell types with culture conditions (CD45.1 grown in 2D or 3D and CD45.2 grown in 3D or 2D respectively) yielded similar results (*data not shown*).



Online Supplementary Figure S5. (A) Phenotypic characterization of placenta-derived MSCs under 2D- and 3D-conditions. The phenotype was not affected by the growth in hydrogel, except that a large percentage (59%) of the 2D-MSCs was CD146$^+$, while a vast majority (93%) of the 3D-MSCs does not express CD146. (B) Quantitative real time PCR analyses of angiopoietin-1, osteopontin and runx-2 mRNA show 2-2.5 fold upregulation of these genes in the 3D-MSCs compared to the 2D ones (n=3; $^{**}P<0.01$ $^{*}P<0.05$). (C) Real time PCR analyses of mRNA for various integrin subunits in 2D *versus* 3D sort-purified CD45$^+$34$^+$38$^-$Lin$^-$ HSCs is shown. Downregulation of α2 and α5 mRNA and upregulation of α4 mRNA was seen in the 3D-HSCs compared to the 2D ones (n=3; $^{**}P<0.01$, $^{*}P<0.05$). Expression level of α2b mRNA was similar in both populations. (D) Semi-quantitative PCR analysis of Tie2 mRNA shows that the 3D CD45$^+$34$^+$38$^-$Lin$^-$ cells express higher levels of Tie2, a ligand for angiopoietin-1, compared to their 2D counterpart. Densitometric analysis using GAPDH as the input control is shown (lower panel). (E) Semi-quantitative PCR analysis for nestin mRNA shows that nestin transcript is up-regulated in 3D-MSCs compared to 2D ones. Densitometric analysis using GAPDH as the input control is shown in the lower panel. (F) The 3D-MSCs possess chemokine-rich environment. Real time PCR analysis shows transcriptional upregulation of SDF1α mRNA in the 3D-MSCs compared to the 2D-MSCs (n=3; $^{**}P<0.01$).