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Supplemental Information

Fate Distribution and Regulatory Role of Human Mesenchymal Stromal Cells in Engineered Hematopoietic Bone Organs

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Transparent Methods section

Isolation and culture of hMSCs

All experiments were conformed to the regulatory standards of the ETH Zurich, the University Hospital of Basel and the University of Zürich. hMSCs were isolated from human BM aspirates from the iliac crest, after ethical approval (EKBB, Ref. 78/07) and informed donor consent from patients. Bone marrow aspirates (20 mL volume) were harvested from healthy donors (N≥3, females and males 30 to 65 years old) using a biopsy needle inserted through the cortical bone, and immediately transferred into plastic tubes containing 15,000 IU heparin. After diluting the marrow aspirates with phosphate buffered saline (PBS) at a ratio of 1:4, nucleated cells were isolated using a density gradient solution (Histopaque, Sigma Chemical, Buchs, CH). Complete medium consisting in α-minimum essential Medium (αMEM) with 10% fetal bovine serum, 1% HEPES (1 M), 1% sodium pyruvate (100 mM) and 1% of Penicillin–Streptomycin– Glutamine (100X) solution (all from Gibco). Nucleated cells were plated at a density of 33×10^6 cells/cm3 in complete medium supplemented with 5 ng/ml of fibroblast growth factor-2 (FGF-2, R&D Systems) and cultured in a humidified 37 °C/5% CO_2 incubator. Medium was changed twice in a week. HMSCs were selected on the basis of adhesion and proliferation on the plastic substrate one week after seeding. In this study, donors were pre-selected based on their capacity to differentiate *in vitro* into the chondrogenic lineage.

Plasmid constructs

The lentivector VENUS-SDF1 was obtained by cloning the SDF1α gene extracted from the pBABE puro SDF-1 alpha plasmid (Addgene, plasmid #12270) was cloned by restriction digest into a third generation lentiviral backbone (Schambach et al., 2006) containing the VENUS transgene. The VENUS lentivector consisted in the same system without the SDF1α gene.

Virus production and hMSC transductions

Third generation vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentivirus was produced in human embryonic kidney (HEK) 293T cells and titrated using NIH-3T3 fibroblasts. The generation of VENUS and VENUS-SDF1α hMSC was performed by plating cells at 6000 cells/cm^2 in 60-mm dishes the day preceding the transduction. HMSC were transduced by overnight incubation with the corresponding lentivirus at a MOI of 20, expanded for up to

four passages and purified by flow cytometry based on their positive expression for the VENUS transgene.

Hypertrophic cartilage engineering

Two million hMSCs were seeded onto type I collagen meshes (cylinder of 6 mm in diameter, 2 mm thick; Ultrafoam, Davol) corresponding to a density of 3.5×10^3 cells/mm^3 to generate hyC. Tissues were cultured for 3 weeks in chondrogenic medium (DMEM supplemented with penicillin-streptomycin glutamine (Invitrogen), HEPES (Invitrogen), sodium pyruvate (Invitrogen), ITS (Insulin, Transferrin, Selenium) (Invitrogen), Human Serum Albumin 0.12% (CSL Behring), 0.1mM ascorbic acid (Sigma), 10^{-7}M dexamethasone (Sigma) and 10ng/ml TGF-β3 (Novartis)), followed by 2 weeks in a serum-free hypertrophic medium (supplemented with 50 nM thyroxine, 7 mM β-glycerophosphate, 10 nM dexamethasone, 0.25 mM ascorbic acid and 50 pg/mL IL-1β) (Scotti et al., 2013).

Gene expression analysis

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA), treated with DNAse and retrotranscribed into cDNA, as previously described (Frank et al., 2002). Real-time RT-PCR was performed with the ABIPrism 77000 Sequence Detection System (Perkin Elmer/Applied Biosystem, Rotkreuz, Switzerland) and expression levels of genes of interest were normalized to GAPDH. Primers and probe sets of osteogenic genes (Collagen type 2, Sox-9, RUNX2, Alkaline phosphatase, Osterix, Bone sialoprotein type 1, SDF1α) were designed and used as previously described (Frank et al., 2002).

Biochemical Stainings

After *in vitro* culture, constructs were fixed in 4% (vol/vol) paraformaldehyde; if necessary, decalcified with 7% (vol/vol) EDTA solution (Sigma); and embedded in paraffin. Sections (5 µm thick) were stained with H&E (Baker) or Alizarin red solution. Histological sections were analyzed using an Olympus BX-61 microscope.

Protein quantification

Protein levels were determined in supernatants collected during *in vitro* chondrogenic differentiation of hyC, and in the final hyC tissue retrieved after 5 weeks of *in vitro* culture.

Samples were analyzed for their content of a panel of growth factors, chemokines, and metalloproteinases, according to the manufacturer's instructions (R&D Immunoassay Kit) and by a Luminex device. Supernatant values were expressed as amounts produced per day per single hyC (pg/mL/day/construct). ECM values correspond to the total content per construct (pg/construct).

Human ossicles generation

In vitro engineered hyC were implanted in subcutaneous pouches of hMCSF / hTPO / hSIRP / -/-Rag2 -/-IL2rg mice, with a maximum of 4 implants per animal. Six weeks after ossicle implantation, 6-8x10^5 cord blood-derived CD34+ cells were pooled from a minimum of 5 donors and injected intravenously into sublethally irradiated immuno-compromised mice (400cGy) as previously reported (Rongvaux et al., 2014, 2011; Scotti et al., 2013). Humanized ossicles were retrieved 6 weeks post-transplantation for subsequent analysis. A minimum of 4 technical experimental replicates was performed. The human cord blood biopsies were approved by the Cantonal ethics committee of Zurich and obtained after informed donor consent.

Mice

Animals consisted in female RAG2−/−γc−/− mice humanized by insertion of human TPO, human M-CSF and human SIRPα. Human TPO and human M-CSF were inserted by knock-in replacement, performed using Velocigene Technology as reported previously (Rongvaux et al., 2011; Willinger et al., 2011). In addition, the Human SIRPα expression was achieved by transgenesis using a BAC in the same genetic background, as previously described (Strowig et al., 2011). All mice were maintained at the University Hospital Zurich animal facility according to the guidelines of the Swiss Federal Veterinary Office, and all the experiments were approved by the Veterinäramt of Kanton Zurich, Zurich, Switzerland (animal permit 187/2013).

Flow cytometry

In vitro samples; cells were retrieved from engineered hyC following digestion using a previously established protocol (Sittinger et al., 2012). Prior to intracellular and nuclear staining, cells were first fixed with fresh formaldehyde 4% and permeabilized by adding ice-

cold 100% methanol slowly to pre-chilled cells, while gently vortexing, to a final concentration of 90% methanol. Cells were then stained for the detection of Sox9 (Abcam), PPARg (Lifesciences), RunX2 (Cell Signaling) and Stro-1 (Biolegend) markers. Analysis were performed on a LSR Fortessa (BD Biosciences).

In vivo samples; Mice were euthanized with CO2 asphyxation and ossicles as well as mouse femur and fibia were removed. Explanted human ossicles and mouse bones were crushed using a mortar and pestle, digested at 37°C for 45 min in DMEM (Invitrogen), 10% FCS (Invitrogen), 10mM HEPES (Invitrogen), 0.4% collagenase II (Worthington) and 0.02% DNase I (Worthington) and washed with PBS containing 2% human Serum and then filtered on a 70 µm cell strainer. The resultant cells were blocked for nonspecific-antibody binding (human and mouse FcR Blocking Reagent, Miltenyi Biotec) and subsequently stained for the markers of interest. All samples were analyzed on a FACS Aria III (BD Biosciences) or LSR Fortessa (BD Biosciences). Antibodies used are listed in **Table S1**.

Colony-forming-unit assay (CFU)

For the colony-forming-unit assays, 1000 hCD45+hlin-hCD34+ cells from ossicle and femur bone marrow were sorted and plated in methylcellulose medium (StemCell Technologies) containing hIL-3 100 ng/ml, hIL-6 50 ng/ml, hIL-11 50 ng/ml, hSCF 50 ng/ml, hTPO 250 ng/ml, hEPO 20 U/ml, hGM-CSF 250 ng/ml and hFlt3L 50 ng/ml. Cultures were maintained at 37°C in 5% CO_2 and scored after 12-14 days.

Micro-computerized tomographic analysis

Microtomography was performed with *in vivo* retrieved ossicles. After fixation in formalin and storage in PBS, microcomputerized tomography data were acquired using a Phoenix nanotom m scanner (General Electric) with 0.5 mm aluminum filtered X-rays (applied voltage, 70 kV; current, 260 µA). Transmission images were acquired during a 360° scan rotation with an incremental rotation step size of 0.25°. Reconstruction was made using a modified Feldkamp algorithm at an isotropic voxel size of 2.5 µm. Threshold-based segmentation and 3D measurement analyses (bone mineral density and volume) were performed using the ImageJ software (ImageJ; National Institutes of Health) with the BoneJ (Meijer et al., 2007) and 3D Shape (Phinney et al., 1999) extensions. 3D rendering of the structures was performed using VGStudio MAX 2.2 software (Volume Graphics).

Immunofluorescence stainings

In vitro or in vivo samples were fixed in 4% (vol/vol) paraformaldehyde; if necessary, decalcified with 7% (vol/vol) EDTA solution (Sigma). Embedding was performed using 4% low-melting agarose (Sigma) and 150 to 250µm thick sections were cut using a Leica VT1200S vibratome with Endurium® low-profile ceramic injector blades (Cadence Inc.). For immunostainings, all steps were performed at room temperature with gentle rocking. Sections were blocked and permeabilized with TBS (final concentration 0.1M Tris, 0.15M NaCl, pH: 7.5) containing 0.05% Tween-20, 20% DMSO (both from Sigma) and 10% donkey serum (Jackson ImmunoResearch). This buffer was also used to dilute all primary antibodies, secondary detection reagents and blocking reagents. After blocking/permeabilization, endogenous avidins and biotins were block using the kit from Vector Labs, each step one hour followed by 30 minutes washes. Sections were then sequentially stained with primary, highly cross-absorbed secondary antibodies and streptavidins (when required), each overnight with 5x1h washes in between using TBS containing 0.05% Tween-20. For staining with two or more primary antibodies raised in the same species, sequential staining was performed with the following blocking steps between: 0.12-0.25mg/mL IgG of the same species as the antibody that needs blocking, 0.12-0.25mg/mL monovalent Fab fragments raised in donkey against the IgG species used in the previous step (both reagents from Jackson ImmunoResearch, both steps overnight), followed if required by additional avidin/biotin blocking steps. A list of primary and secondary antibodies is provided as **Table S2** and **Table S3** respectively.

Optical clearing and mounting of sections

Sections were optically cleared with graded series of 2,2'-thiodiethanol (TDE, Sigma) diluted in TBS until 100% TDE was reached. The final mounting solution consisted of 100% TDE with 0.1M N-propyl gallate (pH: 8.5, Sigma). The refractive index of this solution was measured using a handheld refractometer (Atago) and adjusted to 1.518 with TDE or TBS. Sections were mounted using custom-made silicone spacers (Grace Biolabs) on custom-made size 00, D263M borosilicate coverglass (RI: 1.518, Menzel-Gläser). Sections were mounted on size 1.5 coverslips.

Confocal microscopy

Confocal microscopy was performed on a Leica TCS SP5 equipped with three photomultiplier tubes, two HyD detectors, five lasers (405nm blue diode, argon [458, 476, 488, 496 and 514nm], and three helium neon [543, 594 and 633nm]) using type F immersion liquid (RI: 1.518) and a 20X multiple immersion lens (NA 0.75, FWD 0.680mm). All scans were acquired at 20-25oC, 400Hz, in the bidirectional mode, with z-spacing of 2.49mm (the optical slice thickness of the optics used was 2.69mm). Images were acquired either with a 1.1x to 2.2x optical zoom at a resolution of 512x512 or 1024x1024.

Statistics

Data are presented as means ± standard error of the mean and were analyzed using the GraphPad Prism software. Single comparison was performed using the non-parametric Mann Whitney t-test assuming a non-gaussian distribution of the values. Multiple comparisons were performed using the one-way ANOVA assuming a non-gaussian distribution of the values. Statistical significant differences were defined as: * =$p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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Supplementary Materials

Antibody	**Conjugated fluorophore**	**Specie**	**Company**	**Product number**
human lineage cocktail	PE Cy5	Mouse	Invitrogen	348807
human CD45	eFluor 450	Mouse	eBioscience	48-0459-42
human CD90	PE	Mouse	BD	555596
Human CD38-biotin	Streptatividin-Pacific orange	Mouse	Biolegend	303518
human CD34	PECy7	Mouse	BD	348811
human CD45RA	APC eFluor 780	Mouse	eBioscience	47-0458-42
mouse CD45	APC	Mouse	Biolegend	109814

Table 1. List of antibodies used for flow cytometry analysis, Related to Figure 3.

Primary antibody	Specie	Company	Product number
Collagen type 2	Mouse	Abbiotec	250484
SDF1α	Rabbit	eBiosciences	14-7992-81
Laminin	Rabbit	Novus BIologicals	NB300-144
Peripherin	Rabbit	Biolegend/Covance	PRB-576C
Alkaline Phosphatase	Goat	R&D systems	AF2910
CD34	Rabbit	Novus Biologicals	NBP2-38322
CD90	Sheep	R&D systems	AF2067
human CD45	Rat	AbD Serotec	MCA345GT
GFP	Chicken	Aveslab	GFP-1020

Table 2. List of primary antibodies used for confocal immunofluorescence analysis, Related to Figure 4.

Secondary antibody	Specie	Company	Product number
Anti-rat Alexa Fluor 488	Donkey	Thermofischer	A-21208
Anti-rabbit Alexa Fluor 555	Donkey	Thermofischer	A-31572
Anti-mouse Alexa Fluor 555	Donkey	Thermofischer	A-31570
Anti-rat	Donkey	Thermofischer	A-21096

Alexa Fluor 680			
Anti-rat CF633	Donkey	Biotium	20137
Anti-goat Alexa fluor 594	Donkey	Thermofischer	A-11058
Anti-rabbit CF633	Donkey	Biotium	20125
Anti-chicken	Donkey	Jackson Immunoresearch	703-545-155

Table 3. List of secondary antibodies used for confocal immunofluorescence analysis, Related to Figure 4.

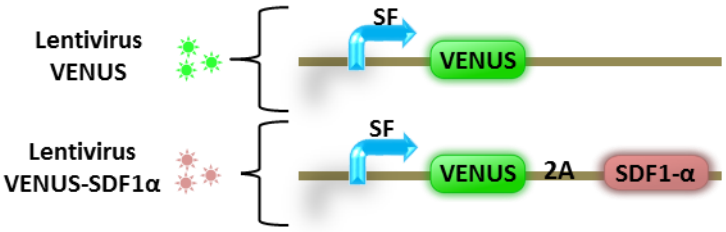


Figure S1. Lentivectors overview used for the generation of VENUS and VENUS-SDF1α hMSCs populations, Related to Figure 1.

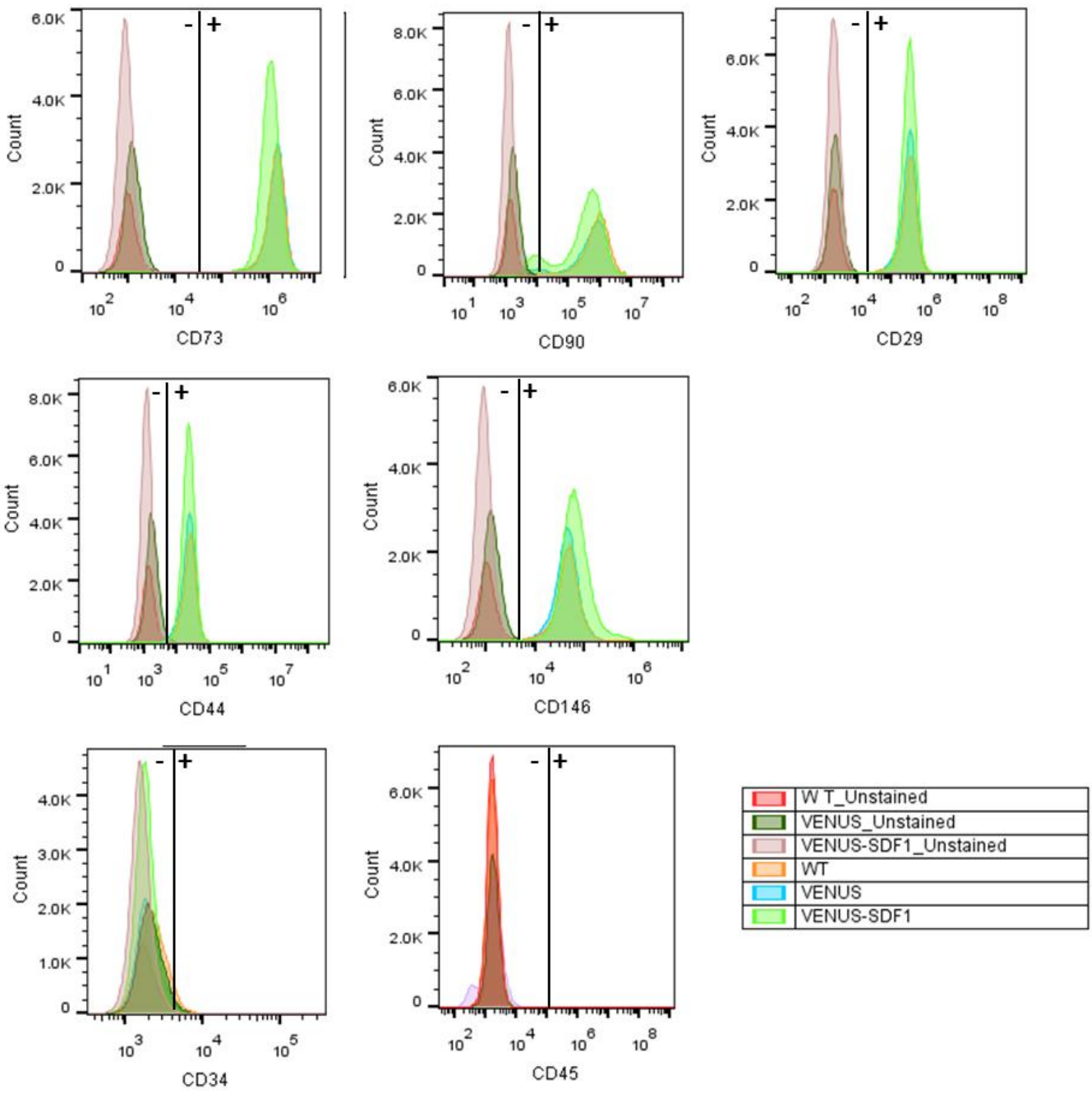


Figure S2. Comparable phenotypes of hMSCs untransduced (WT = primary hMSCs) and transduced with the VENUS or VENUS-SDF1α lentiviruses. All cells express CD73, CD90, CD29, CD44, CD146 but not CD34 and CD45. Data compiled from flow cytometry analysis, Related to Figure 1.

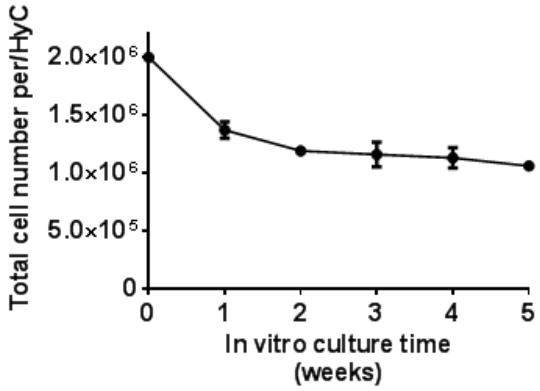


Figure S3. Quantification by DNA content of hMSC number in hyC over in vitro culture time, Related to Figure 1. n=3 biological replicates.

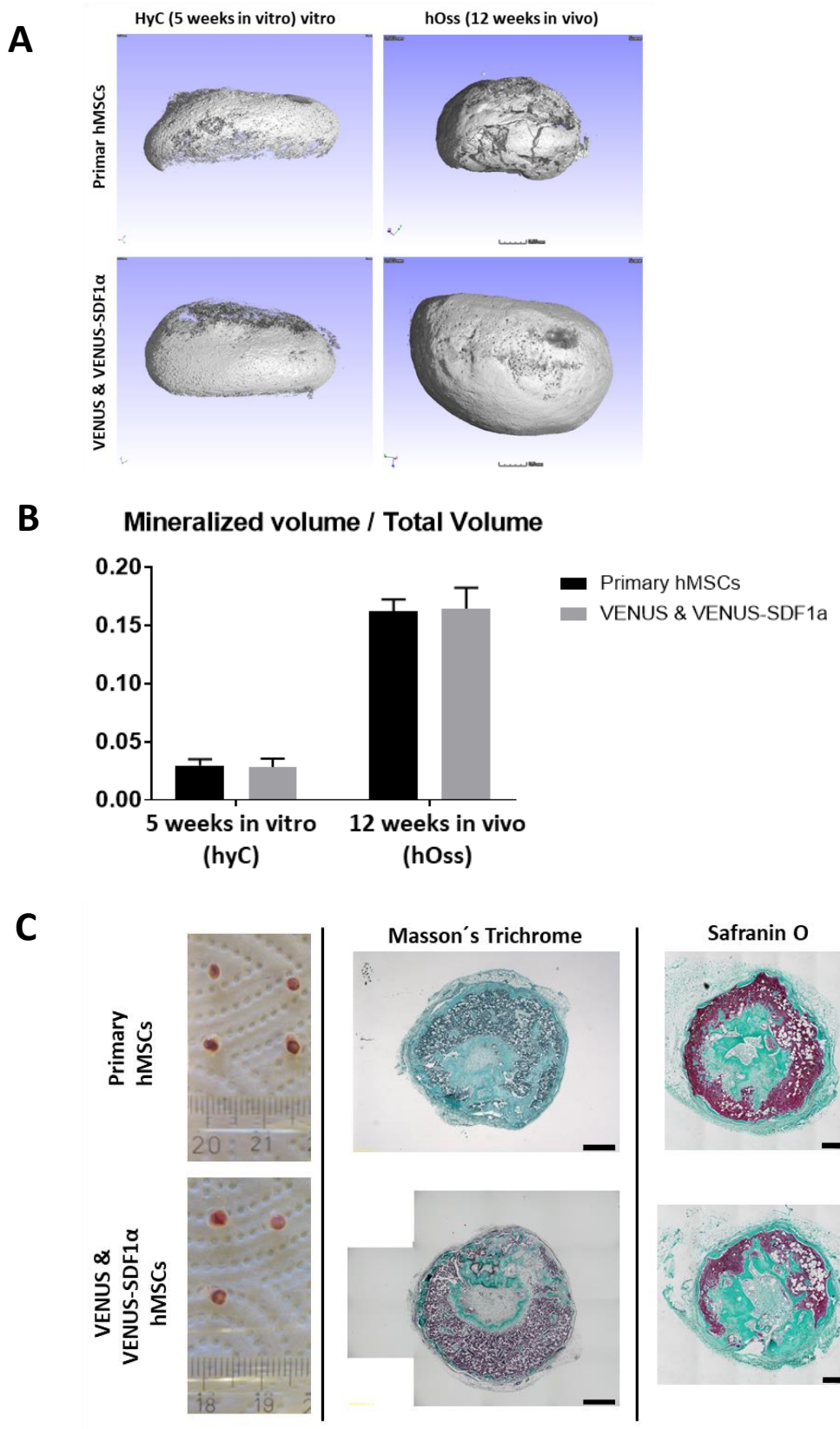


Figure S4. Histological and microcomputed analysis of engineered tissues, Related to Figure 3. (A) Representative MicroCT reconstitution of hyC and hOss generated from untransduced (primary hMSCs) or transduced hMSCs (VENUS & VENUS-SDF1α). (B) Quantification of the

mineral volume in the respective hyC and hOss. Data were then normalized to the average volume of the respective tissues, to display the final mineralized volume over total volume. n≥3. (C) Histological analysis of hOss generated from primary or transduced hMSCs (VENUS and VENUS-SDF1α). No differences could be observed between the two groups. hOss were explanted 12 weeks post-in vivo implantation. Scale bar = 500µm.

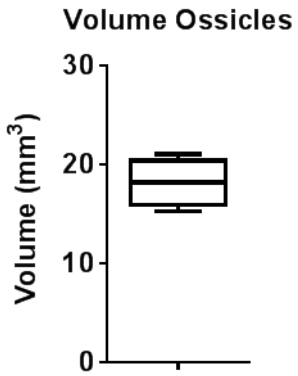
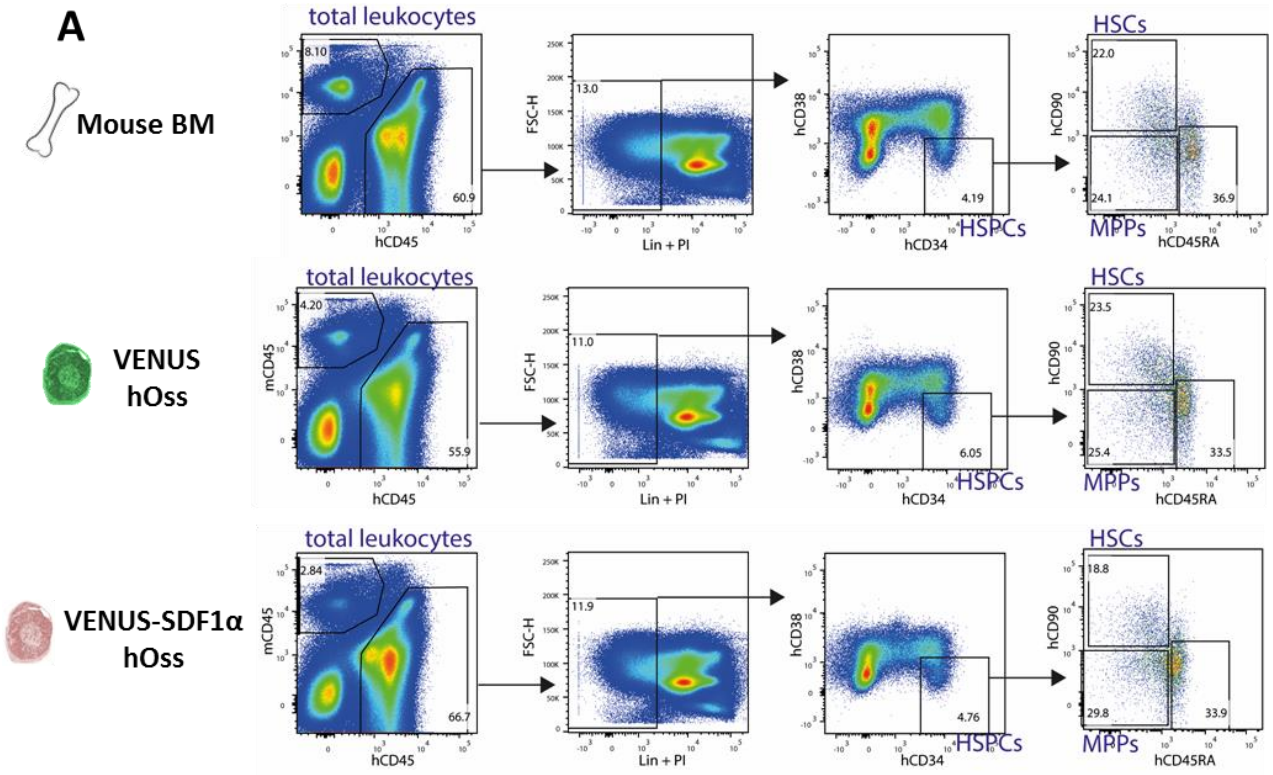
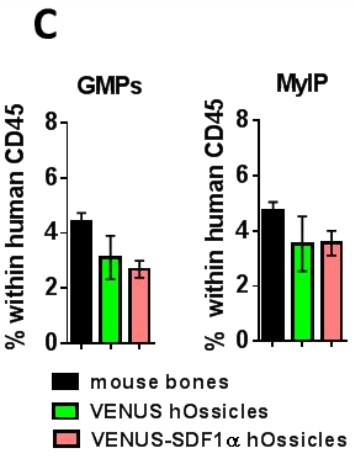


Figure S5. Mean volume of hOss (18 ±2.1 mm3) calculated by microtomography, Related to Figure 3. n=6 biological replicates. Data are represented as mean +/- SEM.



B

Population	Surface phenotype
HSPCs	Lin- CD34+ 38-
HSCs	Lin- hCD45+ CD34+ CD38- CD45RA- CD90+
MPPs	Lin- hCD45+ CD34+ CD38- CD45RA- CD90-
MylPs	Lin- hCD45+ CD34+ CD38- CD45RA+ CD90-
CMPs/MEPs	Lin- hCD45+ CD34+ CD38+ CD45RA-
GMPs	Lin- hCD45+ CD34+ CD38+ CD45RA+



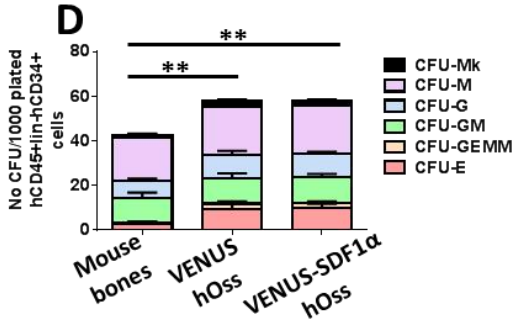


Figure S6. Bone marrow analysis of mouse bones and human ossicles, Related to Figure 3. (A) Gating strategy for analysis of HSPCs populations engrafted in mouse bones or humanized ossicles. (B) Phenotypic markers defining HSPCs, HSCs, MPPs, Myeloid progenitors (MylPs),

CMPs/MEPs and Granulocyte-monocyte progenitors (GMPs) populations. (C) Flow cytometry-derived frequencies of GMP amd MyLP populations in engineered hOss and mouse bones at the end of the 12 weeks *in vivo* period. n ≥12. (D) Human CD45+/CD34+ cells maintained in VENUS and VENUS-SDF1α ossicles displayed a superior capacity to form myeloid colonies *in vitro*. CFU: colony forming unit. GEmM: Colony-forming unit-Granulocyte, Erythroid, macrophage, Megakaryocyte. GM: Colony-forming unit-granulocyte and macrophage. E: colony forming unit-erythroid. G: colony forming unit granulocyte. M: colony forming unit macrophage. n ≥12 biological replicates. *p<0.05, **p<0.01. Data are represented as mean +/- SEM.

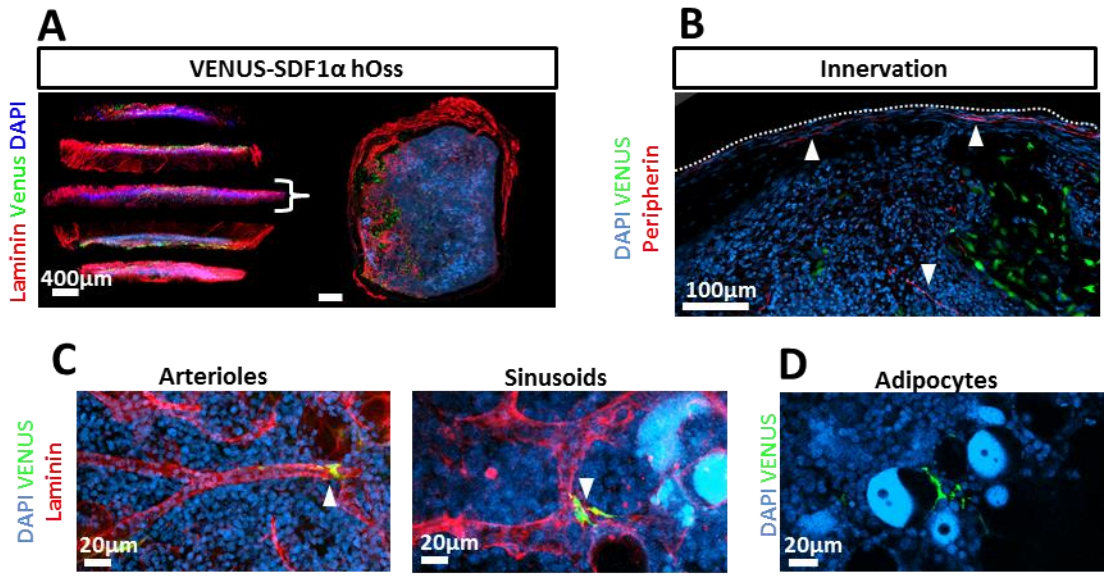


Figure S7. Confocal analysis of VENUS-SDF1α ossicles, Related to Figure 4. (A) Multidimensional confocal immunofluorescence imaging of VENUS-SDF1αhOss for 3D quantitative information retrieval (left). Top view of a transversal hOss section (right) illustrating the internal bone marrow cavity (DAPI) and intense peripheric vascularization (lamimin). Scale bar = 400µm. (B) Humanized ossicles display evidences of innervation (arrows), proving connection to the host nervous system. Dashline indicates the external border of the hOss. Scale bar = 100µm. (C) Implanted hMSC (VENUS positive) are detected in association with the established vasculature, which includes both arterioles (left) and sinusoids (right). Scale bar = 20µm. (D) Implanted hMSCs (VENUS positive) differentiate into adipocytes, as assessed by the presence of cytosolic lipid droplets autofluorescent in the blue channel (DAPI). Scale bar = 20µm.

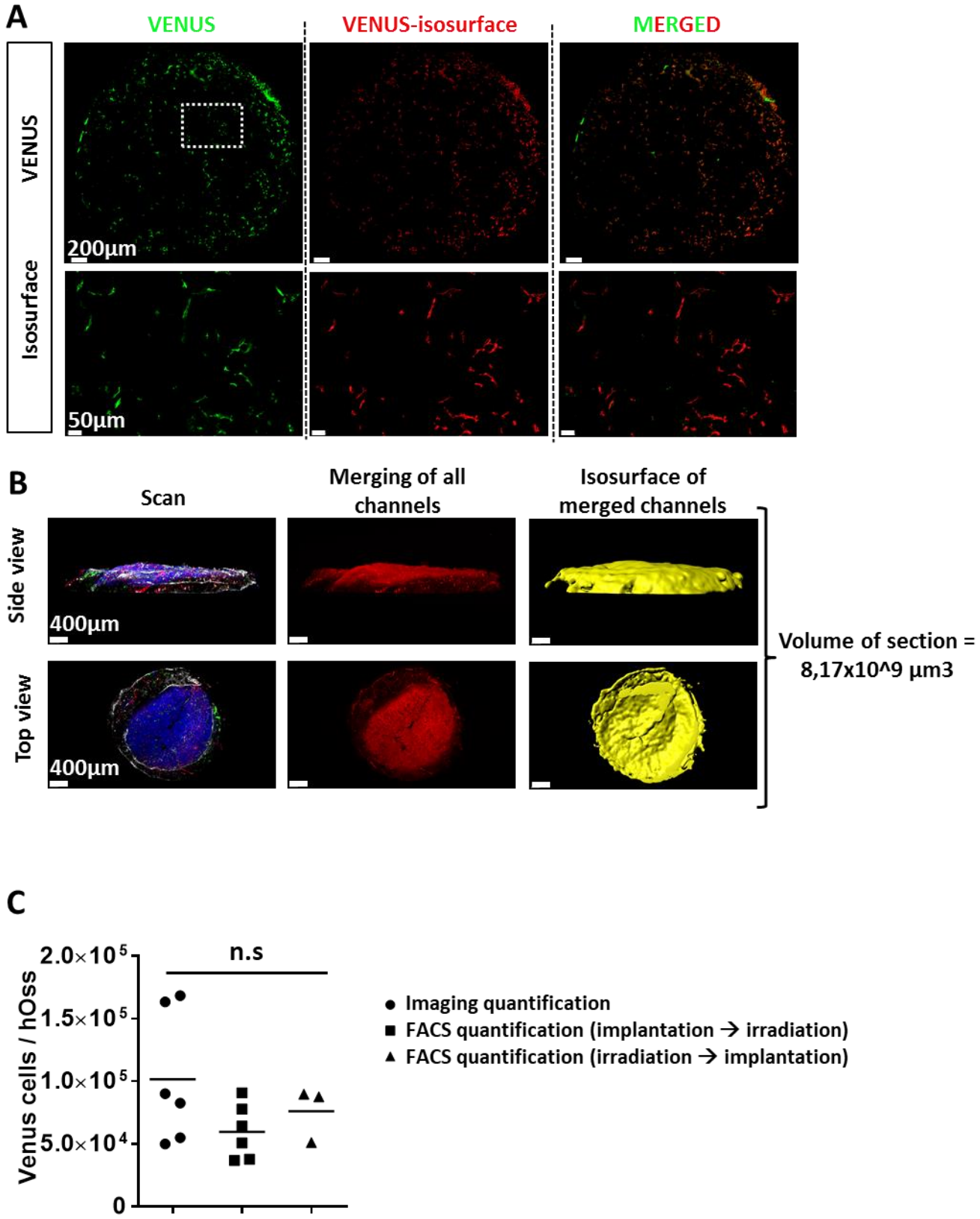


Figure S8. Imaging strategy for hMSCs quantification in humanized ossicles, Related to Figure 4. (A) Generation of isosurfaces for hMSC-VENUS fate determination/quantification using the

Imaris software. (B) Generation of isosurfaces of whole sections based on the combination of all channels using the Imaris software. This is used to derive the volume of section scanned and normalized the number of objects per volume of section. (C) Quantification of VENUS expressing hMSCs by segmentation of confocal scans and by flow cytometry based on the VENUS signal following digestion of hOss. One point represents one ossicle, the bar represents the mean. Significance was assessed by one-way ANOVA test.

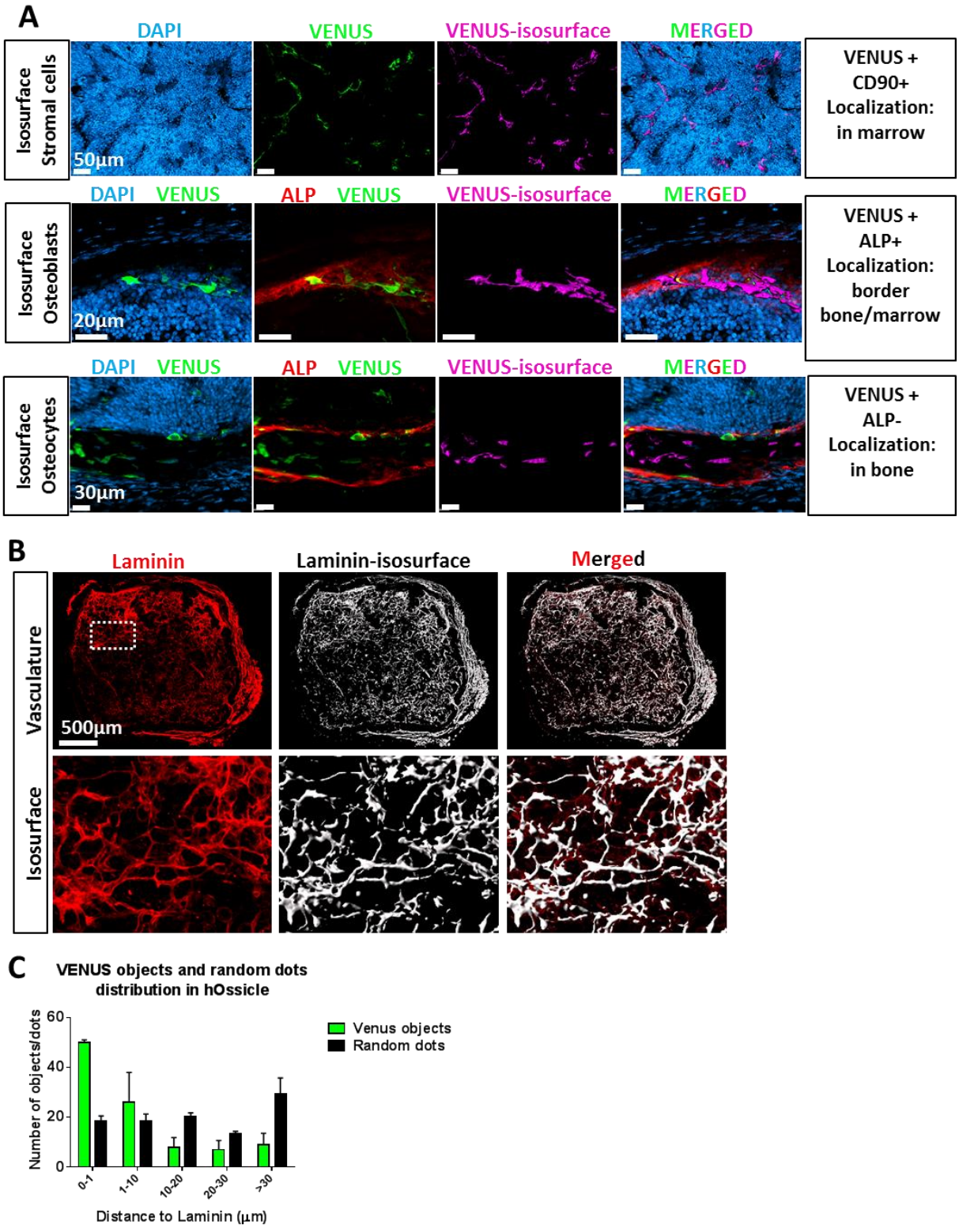


Figure S9. Isosurface strategies for the quantification of hMSCs fate in humanized ossicles, Related to Figure 4. (A) Rationale and representative example leading to the generation of isosurfaces for hMSC-VENUS fate determination/quantification. ALP: alkalyne phosphatase. (B) Generation of vasculature isosurfaces using laminin expression as a proxy for vasculature.

(C) Distance transform between VENUS objects and Laminin, and comparison with random dots distribution. Data are represented as mean +/- SEM.

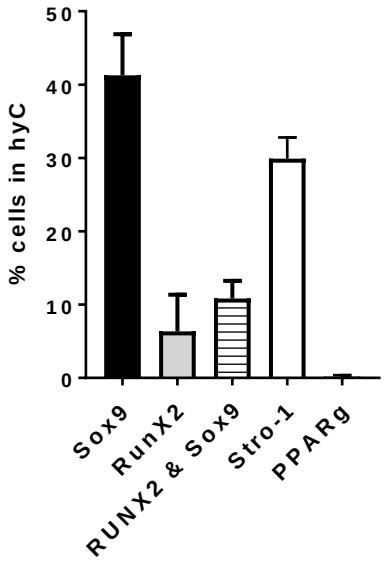


Figure S10. Intracellular staining of hMSCs retrieved from engineered hyC (5 weeks of *in vitro* differentiation), Related to Figure 4. Stainings for the transcription factors Sry-box 9 (Sox-9), Runt-related transcription factor 2 (RunX2), peroxisome proliferator-activated receptor gamma (PPARg) and Stro-1 were used as chondrocytic, osteoblastic, adipocytic or progenitor marker, respectively. Most of the hMSCs display lineage commitment, but some maintain the expression of the Stro-1 progenitor marker. n=2 independent experiments, N≥6 biological replicates. Data are represented as mean +/- SEM.

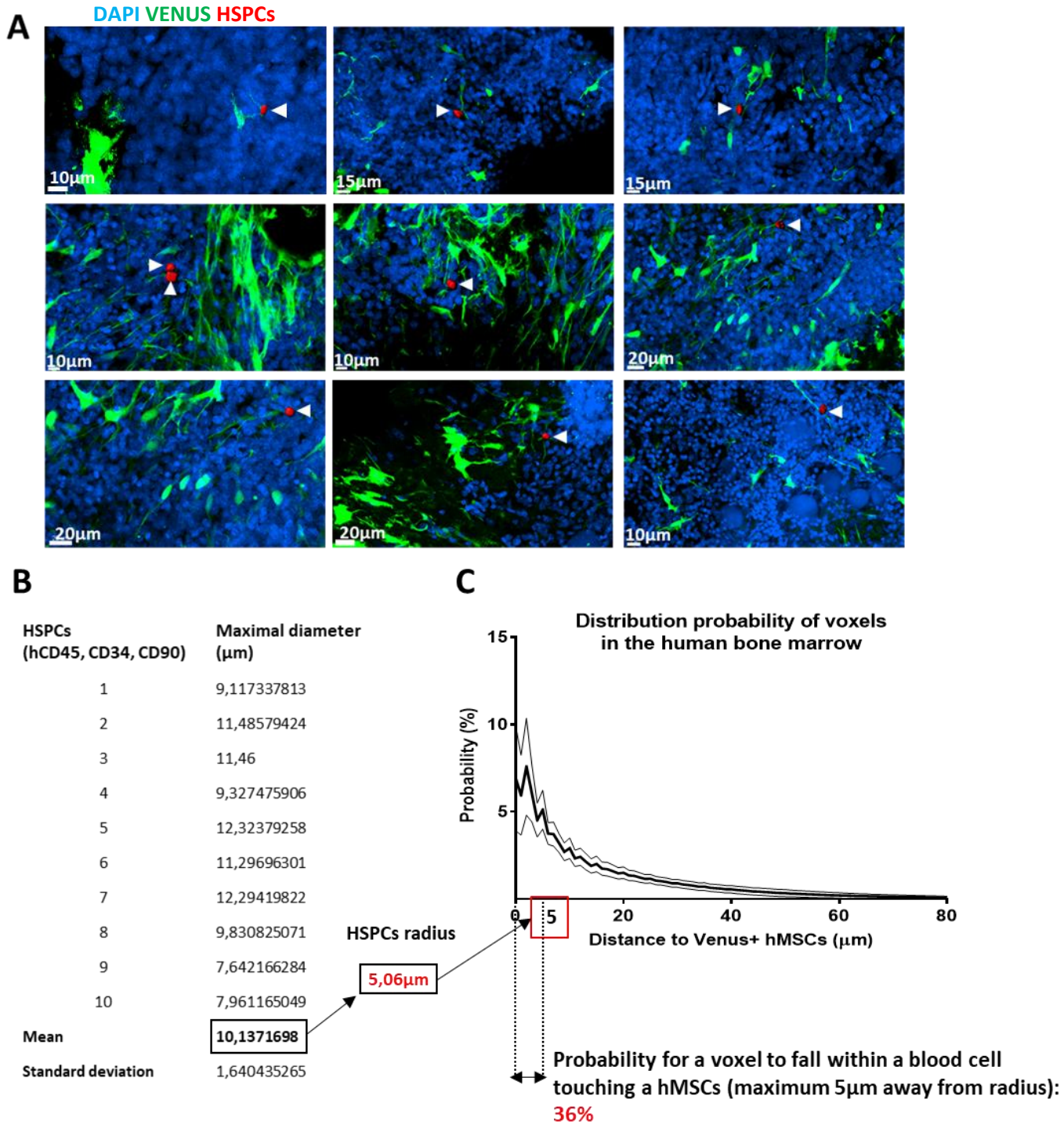


Figure S11. Probability assessment of voxels to randomly distribute in a HSPCs touching a hMSCs, Related to Figure 4. (A) Confocal images of detected HSPCs (red objects defined by hCD45+/CD34+/CD90+) touching hMSCs (VENUS). (B) Maximal diameter calculation of HSPCs (n=10) allowed to determine the HSPCs radius (5µm). Any blood cells in physical contact with a hMSC will thus have its center maximum 5µm away from this hMSC. (C) Distance probability between randomly distributed voxels and hMSCs within the human bone marrow volume.

This was calculated by distance transform using the segmented isosurface of hMSCs (VENUS signal) and of the human hematopoietic compartment (based on the human CD45 signal). The central line (thick pattern) represent the mean. The upper and lower lines represent the mean of maximal and minimal values respectively. All voxels falling within a 0 to 5µm distance (corresponding to the maximum HSPCs radius) would thus necessary be confined in a blood cells touching a hMSC. Thus, the probability that a voxel falls directly within a blood cell in physical contact with hMSCs is of 36%. Data were compiled out of n=4 confocal scans.