METHODS

Mouse strains. *Nes-Gfp* (ref. 20), FVB-Tg(*Col1a1-cre*)1Kry/Mmcd (ref. 29), B6.Cg(SJL)-TgN(*Nes-cre*)1Kln (ref. 31), *Nes-Cre*^{ERT2} (ref. 32), *RCE:loxP* (ref. 33), C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J[39], B6.129S4-Gt*(ROSA)* 26Sortm1Sor/J[30] transgenic mice (Jackson Laboratories) and C57BL/6-CD45.1 mice (Frederick Cancer Research Center) have been used in these studies. Experimental procedures were approved by the Animal Care and Use Committee of Mount Sinai School of Medicine.

Cell isolation and culture. For isolation of primary cells, the bone marrow from the femora, tibia and humeri was flushed in FACS buffer prepared freshly as described[28] with Leibovitz's L-15 medium (Invitrogen) containing 1 mg ml^{-1} bovine serum albumin (BSA, Sigma), 10 mM HEPES (Sigma) pH 7.4 and 1% penicillin-streptomycin (Invitrogen). After erythrocyte lysis with 0.8% NH_4Cl, the bone marrow was processed as previously described for neural crest cells from the postnatal gut[28]. Cells were enriched by immunomagnetic depletion using anti-CD45 magnetic beads (Milteyi Biotec), following the manufacturer's recommendations. CD45$^-$GFP$^+$ and CD45$^-$GFP$^-$ cells were further purified using an automated cell sorter. For sphere formation, cells were plated at clonal density (<1,000 cells cm^{-2}) or by single-cell deposition in ultra-low adherent 35-mm dishes (StemCell Technologies) or 96-well plates (Corning). The growth medium was adapted from neural crest[26] and pericyte[27] culture and contained 15% chicken embryo extract, prepared as described[25], 0.1 mM β-mercaptoethanol, 1% non-essential amino acids (Sigma), 1% N2 and 2% B27 supplements (Gibco), fibroblast growth factor (FGF)-basic, insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and oncostatin M (OSM) (Peprotech) (20 ng ml^{-1}) in DMEM/F12 (1:1)/human endothelial (Gibco) (1:2). The cultures were kept at 37 °C with 5% CO_2 in a water-jacketed incubator and left untouched for 1 week to prevent cell aggregation in low density cultures. One-half medium changes were performed weekly.

***In vitro* differentiation.** Osteoblastic differentiation was induced by culturing the cells for 4 weeks with 50 μg ml^{-1} L-ascorbic acid 2-phosphate, 10 mM glycerophosphate (Sigma) and 15% FBS in α-MEM with penicillin-streptomycin (Invitrogen). Adipocyte differentiation was induced with 1 μM dexamethasone, 10 μg ml^{-1} insulin (Sigma) and 10% FBS in α-MEM with penicillin-streptomycin. Chondrogenic differentiation was induced in confluent CFU-F cultures prepared as described[18] and also in cell pellets with 10^{-7} M dexamethasone, 10^{-4} M L-ascorbic acid, 1 mM sodium pyruvate, non-essential amino acids, 1× ITS+1 (10 mg l^{-1} bovine insulin, 5.5 mg l^{-1} transferrin, 5 μg l^{-1} sodium selenite, 4.7 μg ml^{-1} linoleic acid, and 0.5 mg ml^{-1} bovine serum albumin; Sigma) and 10 ng ml^{-1} TGF-β3 (Peprotech) in DMEM (Invitrogen) over 4 weeks. All cultures were maintained with 5% CO_2 in a water-jacketed incubator at 37 °C, and half-medium changes will be performed weekly. To assess *in vitro* differentiation into mesenchymal lineages, cells were briefly washed with phosphate buffer saline (PBS) supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (modified PBS) and fixed for 10 min at room temperature with 3% paraformaldehyde in modified PBS (Sigma). For the detection of alkaline phosphatase activity, cells were washed twice with PBS and incubated for 20 min at room temperature with 50 μg ml^{-1} Naphthol AS-MX phosphate, 0.5% *N,N*-dimethylformamide and 0.6 mg ml^{-1} Fast Red Violet LB in 0.1 M Tris-HCl, pH 8.9. Bone nodule formation was evaluated by von Kossa staining. Briefly, cells were rinsed three times and stained with fresh 5% silver nitrate for 30 min. After rinsing three times, the reaction was developed with 5% sodium carbonate in 25% formalin for 5 min. After rinsing three times, the cells were fixed with 5% sodium thiosulphate for 2 min, and rinsed three times. Adipocytes were stained with Oil Red O as follows: cells were washed with 60% isopropanol and allowed to dry completely. Oil Red working solution was prepared as a 6:4 dilution in bidistilled water of a 0.35 g ml^{-1} Oil Red O solution in isopropanol (Sigma), and filtered 20 min later. Cells were incubated for 10 min with Oil red working solution and rinsed four times. To stain mucopolysaccharides associated with chondrocytic differentiation, fixed cultures and cell pellets were incubated for 30 min at room temperature with 1% Alcian blue 8GX (Sigma) in 3% acetic acid, pH 2.5, and rinsed four times.

Long-term culture-initiating cell. (LT-CIC) assay was performed as previously described[13]. Bone marrow HSC frequencies were estimated by the Newton-Raphson method of maximum likelihood and Poisson statistics (L-CalcTM software, StemCell Technologies) as the reciprocal of the number of test cells that yielded a 37% negative response.

Heterotopic bone ossicle assay. Ceramic porous cubes (~3 mm^3) composed of 65% calcium phosphate hydroxyapatite and 35% tricalcium phosphate (Ceraform) were washed with bidistilled water twice to discard small detached fragments, autoclaved and coated with 0.1 mg ml^{-1} fibronectin from bovine plasma (Sigma). To eliminate the air contained in the cubes and ensure proper coating of the pores, ossicles were placed in a tube containing the fibronectin solution and agitated for 1 min while negative pressure was applied by suction with a 60-ml syringe with a 21 gauge needle through the tube cap. The procedure was repeated once. Fibronectin-coated ossicles were allowed to dry overnight in a laminar flow hood. Freshly sorted cells were sucked into the ossicles using a similar procedure, or single spheres were gently deposited onto the ossicles and allowed to attach for 24 h in the incubator. The ossicles were implanted subcutaneously under the dorsal skin of 6–12-week-old anaesthetized recipient animals.

Histological analyses of ossicles. Two months after transplantation, anaesthetized recipient animals received 100 U sodium heparin (intraperitoneally, Sigma) to prevent coagulation and were intracardially perfused through the left ventricle with ~ 20 ml 2 mM $MgCl_2$ in cold PBS followed by 100 ml 2% paraformaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$ in PBS, pH 7.4, at 4 °C. The ossicles were harvested and post-fixed for 2 h at 4 °C with the same fixative. After two rinses with 2 mM $MgCl_2$ in cold PBS, the ossicles were washed for 10 min with 2 mM $MgCl_2$, 0.01% sodium deoxycholate (Sigma), 0.02% Nonidet P-40 (Roche) in PBS. X-gal staining of the ossicles was performed with 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate (Sigma), 1 mg ml^{-1} 5-Bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-gal, BioSynth AG), 0.02% Nonidet P-40 (Roche) in PBS overnight at 37 °C under agitation. Ossicles were partially decalcified with 0.25 M EDTA for 2–3 days, cryoprotected, sectioned (10 μm) with a tungsten carbide blade (Diamond Knives) and mounted onto 4× methacrylate-coated slides using the CryoJane tape transfer system (Instrumedics). Immunostaining for GFP was performed as previously described for tyrosine hydroxylase detection[18], using a polyclonal rabbit anti-GFP antibody (1:200, Invitrogen). Immunostaining procedures for the detection of SLAM markers have been described before[14].

***In vivo* treatments.** Isoprenaline or BRL37344 (2 mg kg^{-1}; Sigma) were injected intraperitoneally 2 h before harvesting; the adrenergic agonists (50 μM) were also present in the enzymatic digestion and sorting steps, that were carried out at 37 °C and room temperature respectively. G-CSF or vehicle was administered as described previously[16]. Parathormone or vehicle was administered as previously described[3].

Nestin$^+$ cell depletion experiments. *Nestin-cre*^{ERT2}*/iDTR* double- and *iDTR* single-transgenic mice, as a control, were administered tamoxifen (143 mg kg^{-1}, intraperitoneally, 3 times, every other day) and diphtheria toxin (4 μg kg^{-1}, intraperitoneally, 2 days after the last tamoxifen injection).

Homing of haematopoietic progenitors. *Nestin-cre*^{ERT2}*/iDTR* double-transgenic mice and control *iDTR* single-transgenic mice were treated with tamoxifen and diphtheria toxin and lethally irradiated (12 Gy, one dose) 16–20 h later. Five million nucleated cells from congenic C57BL/6 donor mice in 200 μl PBS were intravenously injected into each mouse. After 3 h, the bone marrow was harvested and transferred to colony-forming units in culture (CFU-C) assay. Bone marrow CFU-C content was calculated as previously described[42].

Isolation, labelling, transplantation and *in vivo* tracking of HSCs. Bone marrow samples from congenic C57BL/6 mice were stained with biotinylated antibodies for haematopoietic lineages (detected with Pacific Orange-conjugated streptavidin), APC-conjugated anti-c-kit, Pacific blue-conjugated anti-Sca-1, PE-conjugated anti-CD150 and FITC-conjugated anti-CD48. CD150$^+$CD48$^-$ LSK cells were sorted and stained with Vybrant DyD (Invitrogen). A number of 5,000–11,000 CD150$^+$CD48$^-$ LSK cells were intravenously injected into lethally irradiated *Nes-Gfp* transgenic mice as described previously[40]. Average shorter distances between Vybrant DyD$^+$ cells, bone marrow *Nes*-GFP$^+$ cells and the bone surface were measured 2, 48 and 96 h later. Bone matrix (blue) was visualized by second harmonic signal generated by bone collagen when illuminated by femtosecond titanium:sapphire laser pulses, as previously described[40].

RNA isolation and quantitative real-time RT–PCR. Sorted cells were collected in lysis buffer and RNA isolation was performed using the Dynabeads mRNA DIRECT Micro Kit (Invitrogen). Reverse transcription was performed using the Reverse Transcription System (Promega), following the manufacturer's recommendations. Quantitative real-time RT–PCR (Q-PCR) was performed as previously described[18]. The sequences of oligonucleotides for Q-PCR are included in Supplementary Table 2.

Gene chip. RNA samples from FACS-sorted cells were obtained using the RNAdvance Cell kit (Agencourt), amplified with the WT-Ovation Pico RNA amplification system and converted to sense transcripts using the WT-Ovation Exon Module (NuGen Technologies). The cDNA was fragmented and labelled with the FL-Ovation cDNA Biotin Module V2 (NuGen Technologies). Microarray was performed using Affymetrix GeneChips Exon 1.0 ST Array for the mouse.

Analyses of microarray experiments. The selected experiments were conducted on different Affymetrix platforms and include microarrays from human and murine cells. The raw data were first processed using the Affymetrix Expression Console V.1.1 (publicly available from the Affymetrix website). Data were normalized using Signal Algorithm MAS 5.0 and exported as text files.

Following the normalization step, the probes were matched to gene IDs for each chip using the associated reference annotation files published on the Affymetrix website. In the case of multiple probes per gene ID the mean value for the probes was used. To make the analysis portable across human and mouse, mouse homologues genes were matched to human genes using HomoloGene (http://www.ncbi.nlm.nih.gov/homologene). Because bone marrow CD45$^-$ *Nes*-GFP$^+$ gene expression profile was obtained using the GeneChip Mouse Exon 1.0 ST Array, genes across platforms were matched using gene IDs. The expression of 9,000 annotated matched genes, across all platforms, was ultimately compared. To compare gene expression patterns across multiple cell types from the data collected from CD45$^-$ *Nes*-GFP$^+$ cells and other relevant cells using different microarray platforms, a non-parametric approach was implemented. Each individual expression vector from each experiment was first sorted to rank genes in descending order based on absolute expression level. In the case that the expression levels of two genes were precisely equal, tied ranks were used. All ranked vectors for all experiments were converted to a matrix in which each row represents a gene and each column corresponds to an experiment. Hierarchical clustering was conducted on the most variable (top 10% = 948) ranked genes using the Manhattan (city block) metric and weighted average linkage function using the Matlab Bioinformatics Toolbox.

Gene Ontology analysis of genes differentially expressed in bone marrow CD45$^-$ *Nes*-GFP$^+$ cells. To identify genes that were differentially expressed in bone marrow CD45$^-$ *Nes*-GFP$^+$ cells by comparison with all the other experimental groups, the Kruskal–Wallis non-parametric ANOVA test was used. Genes that displayed a significant difference between bone marrow CD45$^-$ *Nes*-GFP$^+$ cells and at least one of the other experimental groups ($P < 0.01$) were selected for Gene Ontology (GO) analysis. Subsequently, ranks <3,000 and ranks >6,000 were used to separate up- versus downregulated genes.

Protein–protein interactions from up- and downregulated genes. The program Genes2Networks was used as described previously[43] to find direct protein–protein interactions based on the genes that were up- or downregulated in bone marrow CD45$^-$ *Nes*-GFP$^+$ cells, as compared to all other experiments. Enrichment of Gene Ontology terms for Biological Process for two up- or downregulated groups of genes was applied to find biological processes in which these were involved. The Fisher Exact Test was used to quantify the enrichment in the list of genes. Terms with $P < 10^{-7}$ were fixed to this maximum level for better visualization.

42. Katayama, Y. *et al.* PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. *Blood* **102,** 2060–2067 (2003).
43. Berger, S. I., Posner, J. M. & Ma'ayan, A. Genes2Networks: connecting lists of gene symbols using mammalian protein interactions databases. *BMC Bioinform.* **8,** 372 (2007).