Supplemental Methods

Validation of Systemic Augmentation of Shh Levels

Shh was quantified in serum 5 days after vector administration using a mouse Shh ELISA kit (R&D Systems, Minneapolis, MN). To validate that Shh was functional, the mice were assessed for increased hair growth 18 days after vector administration as previously described [1,2].

Bone Histology

Femurs were fixed with 4% paraformaldehyde for 48 hr, decalcified, embedded in paraffin, sectioned, and stained with H&E (Histoserv, Germantown, MD). To measure trabecular bone area and perimeter, H&E stained slides were imaged by brightfield microscopy using a Nikon Microphot SA microscope equipped with a 10x PlanFluor objective and images were captured with an Olympus DP70 CCD camera. Five fields/femoral diaphysis were imaged and trabeculae were traced using Adobe Photoshop software (Adobe Systems, New York, NY) and area and perimeter calculated by integrated morphometry analysis using MetaMorph imaging software (Universal Imaging, Inc., Downingtown, PA). For each field, the values for trabecular area and perimeter were divided by the total bone marrow area (number of pixels in the entire field after excluding areas of cortical bone or large vascular spaces). To measure the tortuosity ratio of the endosteal surface (endosteal surface length divided by bone length), endosteal surface was traced from six fields/each H&E image and the surface length measured using MetaMorph software [3].

Osteocalcin ELISA

Osteocalcin was quantified in serum 18 days after vector administration using a mouse osteocalcin ELISA kit (Immuno-Biological Laboratories, Inc., Minneapolis, MN).

Immunofluorescence and Immunohistochemistry

For all stainings, femur slides were deparaffinized and rehydrated. To enhance staining, the sections were treated in citrate buffer solution at 90ºC (BD Biosciences Pharmingen, San Diego, CA).

For procollagen type I immunofluorescence, sections were stained with a mouse primary antibody for procollagen type I raised against cleaved procollagen peptide (SP1.D8; 1:100 dilution; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at +4ºC overnight, followed by incubation with a goat anti-mouse Cy5 conjugated AffiniPure F(ab')2 (1:100 dilution; Jackson Immunoresearch, West Grove, PA) and counterstaining with 4,6-diamidino-2-phenylindole (DAPI). Mouse IgG (Sigma Aldrich, St. Louis, MO) was used as an isotype control. Fluorescence microscopy employed an Olympus IX70 inverted microscope illuminated by a 100 w mercury arc lamp (Olympus America Inc., Center Valley, PA) and equipped with a x10 PlanFluor objective lens. Images were acquired with a Photometrix Quantix 57 cooled charge-coupled device camera with a 535 by 512, back-illuminated, UV-VIS coated chip operating at 3 MHz (Roper Instruments, Trenton, NJ). A threshold was set at 20% of the maximum dynamic range of the camera and the total area of brightly stained objects was counted with MetaMorph image analysis software (Universal Imaging). For each field, the area containing Cy5 signal (number of pixels above threshold) was divided by the bone marrow area. Five fields/femoral diaphysis were imaged. For each field, the value for area of Cy5 signals was divided by the area of bone marrow.

For N-cadherin immunohistochemistry, endogenous peroxidase activity was quenched using 0.3% H_2O_2 and blocking with normal goat serum to reduce background staining. Samples were incubated with a rabbit anti-human N-cadherin antibody (clone YS; 1:250 dilution;

Immuno-Biological Laboratories, Japan) at +4ºC overnight. The Vectastain Elite Rabbit ABC kit and 3, 3'-diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA) were used to visualize antibody binding and hematoxylin (Sigma Aldrich) to counterstain the sections. Brightfield microscopy was done using a Nikon Microphot microscope equipped with a 20x PlanFluor objective.

For osteopontin immunohistochemistry, endogenous peroxidase activity was quenched using 0.3% H_2O_2 and blocking with normal goat serum to reduce background staining. Samples were incubated with a goat polyclonal antibody raised against a peptide mapping near the N-terminus of osteopontin of mouse origin (1:2,000 dilution; Santa Cruz, Palo Alto, CA) at +4 ºC overnight in a humidified chamber. To assess the specificity of the osteopontin antibody binding, the osteopontin antibody was incubated with osteopontin peptide (Santa Cruz) at 23 ºC for 30 min to saturate binding sites before being applied to sample tissues. The Vectastain Elite Goat ABC kit (Vector Laboratories, Burlingame, CA) and 3, 3'-diaminobenzidine substrate kit (Vector Laboratories) were used to visualize antibody binding. The sections were counterstained with hematoxylin (Sigma Aldrich). Brightfield microscopy was done using a Nikon Microphot microscope equipped with a 20x PlanFluor objective. Images were captured with an Olympus DP70 CCD camera.

Labeling with Xylenol Orange and Calcein

To assess mineralization of the new bone, mice were injected intraperitoneally with 90 mg/kg of xylenol orange (Sigma Aldrich) 4 days before sacrifice and with 25 mg/kg of calcein (Sigma Aldrich) 1 day before sacrifice. Fluorescence microscopy was performed as described above. Pseudocolor images were formed by encoding xylenol orange fluorescence in the red channel, calcein fluorescence in the green channel, and autofluorescence in the blue

channel with Metamorph imaging software (Universal Imaging).

Quantitative PCR

Total tibial bone marrow was flushed out 18 days after vector administration and RNA extracted using TRIzol (Invitrogen, Grand Island, NY) and RNeasy MinElute Cleanup Kit (Invitrogen). and first strand cDNA synthesized using Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Gene expression assays for TaqMan real-time reverse transcriptase polymerase chain reaction (PCR) analysis included primers and probes for Runx2, osteopontin, angiopoietin-1, Jagged 1, Lef1, IL-2, IL-7, and SDF-1 (Applied Biosystems, Foster City, CA). Relative expression levels were calculated using the ΔΔCt method (Applied Biosystems), with 18S ribosomal RNA (rRNA) as the internal control (Eukaryotic 18S Ribosomal RNA Kit, Applied Biosystems), and average of AdNull samples, as the calibrator. The PCR reactions were run in an Applied Biosystems Sequence Detection System 7700.

Colony-Forming Unit (CFU) Assay for Hematopoietic Progenitors

To quantify the number of bone marrow hematopoietic progenitors, femoral bone marrow was harvested from PBS, AdNull, and AdShhN treated animals 18 days after vector administration, and $2x10^4$ nucleated cells were plated on 35 mm dishes in triplicate in Methocult methylcellulose medium containing methylcellulose in Iscove's MDM, fetal bovine serum, bovine serum albumin, recombinant human (rh) insulin, human transferrin (iron saturated), 2-Mercaptoethanol, L-glutamine, recombinant mouse (rm) stem cell factor, rm IL-3, rh IL-6, rh and erythropoietin, (M3434; StemCell Technologies, Vancouver, British Columbia, Canada). Colonies were scored by brightfield microscopy after 12 days incubation at 37 ºC and 5% CO_2.

Long-Term Culture-Initiating Cell (LTC-IC) Assay

The modified LTC-IC assay was performed as described in Stier et al.[4,5]. Feeder layers

were established as follows. Bone marrow was harvested from either naive or PBS, AdNull, and AdShhN treated animals 18 days after vector administration and cell suspensions were plated into Myelocult medium (StemCell Technologies), containing horse serum, fetal bovine serum, i-Inositol, folic acid, 2-Mercaptoethanol, L-glutamine, and MEM Alpha, and cultured at 33ºC and 5% CO_2. When cells reached approximately 80% confluency, they were irradiated with 1500 cGy from a ^{137}Cs-irradiation source and plated on 96-wells at a concentration of $2.5x10^4$ cells/well. Lin^- marrow cells were prepared as follows. Bone marrow of either PBS, AdNull, and AdShhN treated animals or naive mice were harvested and stained with APC-conjugated Lin antibody cocktail (BD Pharmingen Biosciences) followed by staining with anti-APC microbeads (Miltenyi Biotec, Auburn, CA) and applying the cells into a MACS magnetic separation column (LD column; Miltenyi Biotec) and collecting the flow-through containing Lin^- cells. Lin^- cells were then plated on top of feeder layers (naive Lin^- cells on feeder layers from PBS, AdNull, and AdShhN treated mice or Lin^- cells from PBS, AdNull, and AdShhN treated mice on naive feeder layers) in limiting dilutions from 6000 cells/well to 1500 cells/well in 8 wells each and cultured for 5 wk at 33 ºC and 5% CO_2 with weekly half-medium changes. Thereafter medium was replaced with 100 µl Methocult medium, containing methylcellulose in Iscove's MDM, fetal bovine serum, bovine serum albumin, recombinant human (rh) insulin, human transferrin (iron saturated), 2-mercaptoethanol, L-glutamine, recombinant mouse (rm) stem cell factor, rm IL-3, rh IL-6, rh and erythropoietin, and colonies were scored after 10 days incubation at 33ºC and 5% CO_2. LTC-IC frequencies were calculated using L-Calc software (Stem Cell Technologies).

Spleen analyses

For germinal center quantification, two low power (1.25X) images were taken to image the H&E stained spleen of each animal (n=3 per each group). Using NIH ImageJ program, the

total area of the spleen, the number of germinal centers, and the area of each germinal center were calculated.

For quantification of megakaryocytes, five high power (20X) images were taken to image the H&E stained spleens (n=3 per each group). Using NIH ImageJ program, the images of each spleen were combined and 10 random fields analyzed for the number of megakaryocytes.

Supplemental References

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