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Human CD133-derived bone marrow stromal cells establish ectopic hematopoietic microenvironments in immunodeficient mice

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Abstract

Cultured adherent bone marrow stromal cells (BMSCs) are capable of forming ectopic hematopoietic microenvironments (HMEs) in immunodeficient mice. However, the cell surface phenotype of the native bone marrow stem/progenitor cell that gives rise to BMSCs that support hematopoiesis remains poorly defined. We recently reported the derivation of human BMSC-like cells (CD133BMSCs) by magnetic cell sorting against Prominin-1 (CD133), an epitope expressed by embryonic, fetal, and adult stem cells. Here we demonstrate that CD133BMSCs are capable of forming ectopic HMEs. Cultured adherent CD133BMSCs derived from sorted CD133-positive cells lacked CD133 expression, but were uniformly positive for CD146, an epitope recently described to identify self-renewing osteoprogenitor cells that could transfer the HME. CD133BMSCs were genetically-tagged by lentivirus, expanded, and seeded into HA/TCP/fibrin constructs that were implanted subcutaneously. After 60 days, CD133BMSCs produced human osteocytes, osteoblasts, adipocytes, and reticular cells that supported murine hematopoiesis. CD133BMSCs that were not transduced with lentivirus also formed HMEs. Control constructs seeded with human dermal fibroblasts formed connective tissue, but failed to form HMEs. Our data indicate that CD133 expression identifies a native human bone marrow stem/progenitor cell that gives rise to BMSCs capable of forming the HME.

Keywords

CD133; Stem cell; Bone marrow; Niche; Hematopoiesis; MSC

Introduction

Reciprocal signaling between non-hematopoietic cells that comprise hematopoietic stem cell niches and hematopoietic stem cells (HSCs) is critical for the development and maintenance of the hematopoietic system. Non-hematopoietic multipotent bone marrow stromal cells (BMSCs) contribute to both the sinusoidal and endosteal niche environments by producing cells that regulate HSC proliferation, mobilization, quiescence, and differentiation through paracrine and direct cell-cell signaling [1–5]. Cultured BMSCs are well-known to generate stromal fibroblasts, adipocytes, chondrocytes, and osteoblasts [6]. Cultured BMSCs

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transplanted *in vivo* have been shown to produce pericytes, reticular cells, adipocytes, chondrocytes, osteoblasts and osteocytes [7–11], and in some cases to establish ectopic hematopoietic microenvironments (HME) that are similar in structure to the natural bone marrow environment [8;11;12].

HSCs can be enriched and purified from adult murine or human bone marrow mononuclear cells by sorting against various combinations of cell surface epitopes [3;13]. In contrast, the cell surface phenotype of the native adult non-hematopoietic stem cell *in vivo* is poorly defined; this cell is likely to give rise to cultured adherent BMSCs. Sacchetti et al. (2007) described the CD146 epitope (Melanoma Cell Adhesion Molecule, MCAM) as an antigen associated with self-renewing human osteoprogenitors capable of HME formation when transplanted subcutaneously in hydroxyapatite/tri-calcium phosphate (HA/TCP)/fibrin constructs [11]. Generation of HMEs by BMSCs transplanted to ectopic sites suggests that such cells retain cell-autonomous information necessary for niche formation. Transplanted BMSCs that form HMEs also presumably secrete chemokines such as stromal-derived factor 1 alpha (SDF-1) that attract host-derived CD34-positive vascular and hematopoietic progenitors that then initiate hematopoiesis [14]. Several reports have demonstrated the prospective isolation of human BMSC-like cells by numerous cell surface epitopes such as STRO-1 [15], CD49b, CD73, CD90, CD105, CD130, CD146, CD200, integrin alphaV/beta5 [16], and also CD140b, CD340, and CD349 [17], but did not determine their ability to form HMEs *in vivo*.

CD133 (Prominin-1) is a pentaspanin glycoprotein localized to membrane protrusions of stem and progenitor cells of various origin [18–21] that display self-renewal and pluripotency [22] or multipotency for differentiation [23]. Here we show that adult bone marrow stem/progenitor cells prospectively isolated by sorting against CD133 generate BMSCs in culture that upon transplantation form ectopic HMEs in immunodeficient mice. Notably, our data indicate that the native adult human non-hematopoietic bone marrow stem cell expresses the CD133 antigen.

Materials and Methods

Isolation and preparation of CD133BMSCs

Isolation and preparation of human CD133BMSCs was performed as previously described [24]. CD133BMSCs were cultured in Nunclon Delta–coated 150 cm^2 dishes (Nunc; Thermo Fisher Scientific, Rochester, NY) with complete culture medium (CCM) containing alpha minimum essential medium (α-MEM, Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (lot selected for rapid growth of hMSCs; Atlanta Biologicals, Lawrenceville GA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L L-glutamine (Mediatech Inc., Herndon, VA). For cell tracking *in vivo*, CD133BMSCs were transduced with lentivirus to express DsRed2 targeted to mitochondria as previously reported [25]. For proliferation assays cell number was determined by dye binding of nucleic acids (CyQuant, Invitrogen).

Differentiation of CD133BMSCs

Differentiation of CD133BMSCs was performed as previously described [24]. Confluent cultures of cells were prepared by plating CD133BMSCs at 1,000 cells/cm^2 and incubating them in CCM for 5–7 days. The culture medium was then changed to either osteogenic or adipogenic medium. Osteogenic medium consisted of α-MEM containing 10% FCS, 1 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerol phosphate (Sigma-Aldrich, St. Louis, MO). After incubation in osteogenic medium for 3 weeks with changes of medium every 3 or 4 days, the cells were washed with PBS (pH 7.4), fixed (10% neutral-

buffered formalin, Fisher Scientific, Fair-lawn, NJ) for 20 min, and stained with 0.5% Alizarin Red S (pH 4.1; Sigma) for 20 min and washed three times for 5 min each with PBS. The adipogenic medium consisted of α-MEM containing 10% FCS, 0.5 μM hydrocortisone, 0.5 mM isobutylmethylxanthine, and 60 μM indomethacin (Sigma). After incubation in adipogenic medium for 3 weeks with media changes every 3 to 4 days, the cultures were washed with PBS, fixed with 10% formalin (Fisher Scientific), and stained with Oil Red O (Sigma) for 30 minutes and washed three times with PBS. The Oil Red O solution was prepared by diluting 3 parts of 0.5 % v/v stain in isopropanol with 2 parts water and clarified by filtration through a 0.2 μm PES filter (Nunc).

Phenotypic analysis by flow cytometry

Cell surface phenotyping of CD133BMSCs was performed as previously described [24]. Briefly, pellets of 1×10^5 to 5×10^5 cells were suspended in 0.5 ml PBS (pH 7.4) and were incubated for 30 minutes at 4 °C with fluorophore-conjugated monoclonal anti-human CD146 antibody that was pre-titered for flow cytometry (BD Biosciences, San Diego, CA). Non-specific antibody of the same isotype was used as negative control (BD Biosciences). After antibody labeling, cells were washed twice (PBS, pH 7.4) and analyzed by closed-stream flow cytometry (Epics XL; Beckman Coulter).

Hydroxyapatite tricalcium phosphate (HA/TCP) construct preparation

For each construct, an aliquot of 2×10^6 cells passage 5–6 in 100 μl of α-MEM was mixed with a previously autoclaved (sterile) aliquot of 40–50 mg of sintered hydroxyapatite/beta-tricalcium phosphate mixture (HA/TCP, ratio 20/80 by percent; 100–250 μm pore size; Himed Inc., Old Bethpage, NY). Fibrinogen (50 μl of 16mg/ml solution in α-MEM; final concentration of 3.2 μg/ml) and thrombin (100 μl of 50 units/ml in α-MEM with 2% $CaCl_2$) were added to the cell/HA/TCP mixture in 24-well plates (Nunc) and mixed thoroughly. Fibrinogen and thrombin were from Sigma. The resultant viscous mixture was incubated for 90 minutes in a 37 °C tissue culture incubator (Model 3130; Thermo Electron Corporation, Houston, TX) and then implanted into prepared subcutaneous pockets in immunodeficient mice.

Implant surgery

All animal work was approved by the University of Vermont College of Medicines' Office of Animal Care in accordance with American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. Female and male immunodeficient mice (NOD/SCID Interleukin-2-receptor gamma null, Strain name: NOD. Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, Jackson Laboratory, Bar Harbor ME) 9–12 weeks of age were anaesthetized with isoflurane (1–5%, to effect) and body temperature was maintained with a heated pad. The back/scapula area was shaved and sterilized by swabbing the area with topical ethanol followed by iodine three times (Sigma). A 15–20 mm longitudinal incision was made with fine scissors at the site of the scapula medial to the dorsal midline, and a 5–8 mm cavity between the dermis of the skin and the fascia underneath was made to accommodate the implant by spreading the fascia with forceps. Cellularized constructs were placed into the cavity with the use of sterile fine forceps. 1–4 sutures (6-0 monofilament nylon, Henry Schein, Melville, NY) and/or Vetbond (3M, St. Paul, MN) were used to seal the incision after each construct implantation. Sutures were removed 7 days after surgery. All mice received buprenorphine (0.05 mg/kg, s.c.) immediately and 24 hours after construct implantation.

Preparation of tissues for histological and immunohistochemical analysis

Cell-seeded HA/TCP constructs were removed from mice 60 days after implantation and fixed for two days (10% neutral buffered formalin, Fisher Scientific, Rochester NY) followed by decalcification (Decal®, Decal Chemical Corp. Tallman, NY) for 24 hours. Implants were mounted onto paraffin blocks and sectioned by microtome into 8 micron thick specimens on glass microscope slides (Superfrost Plus, Fisher Scientific, Rochester NY). After standard ethanol deparaffinization and hydration of sections, slides were stained with Hematoxylin and Eosin (H & E) as follows: 10 minutes in Harris Hematoxylin, rinse in tap water, 2 dips in acid alcohol (1% HCl in 70% ethanol), 1 minute in tap water, 2 dips in *blue in ammonia* (0.25% NaOH in distilled water), 5 minutes in tap water, 1.5 minutes in Eosin, and rinse in tap water. Slides were then dehydrated a standard gradient of ethanol solutions and 3 changes of xylenes. Sections were mounted with Permount (Fisher Scientific). Specimens on slides for IHC analysis were also deparaffinized and hydrated by through xylenes and a standard gradient of ethanol solutions. Two washes were performed with PBS (pH 7.4) for 5 minutes before and after each of the following treatments for specimens on *Superfrost Plus* microscope slides: 30 minutes in ice cold methanol (Sigma), antigen retrieval by 10 minutes of microwave treatment in 2X saline sodium citrate (SSC, Invitrogen), and 10 minutes protease digest in 50 μg/ml proteinase K (Invitrogen) in PBS at 37°C. Slides were blocked in 5% goat serum with 0.4% triton X-100 for 1 hr. The following antibodies were then diluted into blocking solution and incubated on the tissue sections: rabbit anti-human β-2-microglobulin (B2M, 1:1,000, Dako Corp., Carpinteria CA); rat anti-RFP (clone 5F8, 1:250, Chromotek, Planegg-Martinsried, Germany). Rabbit IgG was used as an isotype control for anti-B2M (1:1,000, 1 μg/ml). Rat IgG was used as an isotype control for anti-RFP (1:250, 4 μg/ml). After 3 × 5 min PBS washes, goat-anti-rabbit IgG Alexa-fluor 488 (1:500, 4 μg/ml) and goat-anti-rat IgG Alexa-fluor 594 (1:800, 2.5 μg/ml) (Molecular Probes, Invitrogen) were used as secondary antibodies. Following 3 additional washes in PBS, slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA).

Results

Cultured CD133BMSCs express CD146 and are multipotent

Cell surface phenotyping demonstrated that 100% of passage 3 CD133BMSCs were uniformly positive for the CD146 epitope (N=3 donors, Fig. 1A and data not shown). To assay growth potential, CD133BMSCs from 2 donors were subjected to a high density analysis of cell proliferation under both normoxic and hypoxic (1% oxygen) conditions. CD133BMSCs plated at 1,042 cells/cm^2 in 6-well plates were grown in CCM for 10 days. Two days after plating (day 0), some cultures were exposed to hypoxic conditions whereas replica cultures were maintained under normoxic conditions (21% oxygen). Cell numbers were then determined over 8 days. CD133BMSCs from both donors expanded equally well under normoxic and hypoxic conditions (Fig. 1B). To assay differentiation potential, CD133BMSCs from 3 donors were plated at 1000 cells/cm^2, expanded with CCM to 100% confluence, and then exposed to either osteogenic differentiation medium, adipogenic differentiation medium, or to fresh CCM (control) with medium changes every 3 days (Fig. 1C–F). After 3 weeks of culture in the various mediums, CD133BMSCs exposed to osteogenic medium displayed abundant calcium deposition that was detected by Alizarin Red S staining (Fig. 1C), while replica CD133BMSC cultures maintained in CCM showed no Alizarin Red S staining (Fig. 1E). CD133BMSCs cultured in adipogenic medium displayed adipocyte differentiation as detected by Oil-red-O staining for lipid deposition (Fig. 1D). Oil Red O staining was absent in replica control cultures grown in CCM (Fig. 1F).

Production and implantation of cell-seeded constructs

Based on previous reports describing HME transfer [11;26;27], we developed a protocol to produce human cell-seeded HA/TCP/fibrin constructs to assay ectopic HME formation in immunodeficient mice (Fig. 2). For cell tracking *in vivo*, CD133BMSCs from 1 donor were transduced with lentivirus to express a mitochondria-targeted DsRed2 reporter (Fig. 2B). To form constructs we combined aliquots of sterile HA/TCP sintered particles, 2×10^6 CD133BMSCs or fibroblasts (cell control), and clotting reagents ($CaCl_2$, thrombin, and fibrinogen) (Fig. 2C–E). The resultant mixture was placed into a 37 ºC tissue culture incubator for 90 minutes to increase construct viscosity. Within 90 minutes cells attached to HA/TCP particles of various shapes and sizes within the constructs (Figs. 2D and E). After 90 minutes of incubation, cell-seeded constructs were implanted into subcutaneous pockets in immunodeficient mice (Fig. 2F). At 60 days after implantation, constructs became solidified and vascularized (Fig. 2G).

HME formation by CD133BMSCs

Sixty days after implantation, constructs were removed, sectioned, and stained with H & E. CD133BMSCs created characteristic HMEs with gross structural similarity to native bone marrow compartments (Fig. 3A–C). CD133BMSCs from 2 out of 2 donors tested formed HMEs. One donor was transduced with lentivirus for lineage tracing and the other was not transduced to control for lentiviral infection. CD133BMSC seeded constructs contained all of the cellular requisites for blood formation including: osteocytes, osteoblasts, reticular cells, adipocytes, and endothelial cells (Figure 3). Both endosteal-like and sinusoidal-like niches were observed in the implants containing CD133BMSCs. Hematopoietic progenitors and blood cells at various stages of maturation were observed throughout the niches: promegakaryocytes, erythroblasts, erythrocytes, neutrophils, and others (Fig. 3A–C). Human dermal fibroblasts seeded into control HA/TCP/fibrin constructs formed connective tissue, but did not form HMEs (Fig. 3D).

Differentiation of CD133BMSCs into functional elements of the HME

Immunohistochemical analysis of the niches formed by CD133BMSCs revealed donor and host origin of non-hematopoietic and hematopoietic cells, respectively. The presence of human CD133BMSCs within HMEs was confirmed by immunostaining for human-specific β2-microglobulin (β2M) and for DsRed2-mito (genetic tag). Human β2M-positive osteocytes were observed in lacunae within human bone that surrounded islands of HA/TCP (Fig. 4A and B). In contrast, human osteocytes were not stained on sections that were incubated with isotype control antisera (Fig. 4C and D). Osteocytes within bone also stained positive for DsRed, which was appropriately localized to mitochondria in the human cells (Fig. 4E and F). Isotype control staining for DsRed in HMEs was negative (data not shown). DsRed2-positive inter-sinusoidal and adventitial reticular cells (ARCs) within the HMEs surrounded areas of active hematopoiesis. Adipocytes and fibrous connective tissues were also DsRed2-positive (Fig. 4G–J). Notably, all hematopoietic cells were DsRed2-negative (Fig. 4I and J). Staining with antisera specific to human von Willebrand Factor did not identify human endothelial cells in the HMEs (data not shown).

Discussion

Our data demonstrate that human CD133BMSCs seeded into HA/TCP/fibrin constructs can establish ectopic hematopoietic niches in immunodeficient mice. The presence of human cells in the HMEs was confirmed by staining for both human-specific β2M and a genetic tag (DsRed2-mito). We found that genetically-tagged CD133BMSCs differentiated into many non-hematopoietic cells known to contribute to HMEs including osteoblasts, osteocytes, adipocytes, and reticular cells. Notably, we found that CD133BMSCs contributed to both

endosteal-like niches as well as sinusoidal-like niches within ectopic HMEs. In agreement with previous reports, we found that the endothelial cells and hematopoietic cells within ectopic HMEs were host-derived [11;28].

Several different cell types present in bone marrow have been linked to the regulation of hematopoiesis. Osteoblasts that line endosteal HSC niches express the Notch receptor ligand Jagged 1 and regulate HSC activity [2], in part, through Notch-1 signaling [4]. In co-culture studies with HSCs, osteoblasts were reported to mediate long-term maintenance of HSCs (i.e. self-renewal) whereas short-term proliferation was influenced to a greater extent by sub-endosteal reticulocytes [29]. Sinusoidal endothelial cells also function in hematopoiesis [3;30], and have been shown to regulate thrombopoiesis from megakaryocytes [30]. Sub-endothelial adventitial reticular cells (ARCs) also express Jagged 1 and contribute both structurally and functionally to sinusoidal HSC niches [11]. Human ARCs have been localized *in vivo* by immunohistochemistry against the p75 low affinity nerve growth factor receptor (p75LNGFR) [31]. and CD146 (MCAM) [11]. Notably, these cell surface proteins are expressed by early passage cultures of human BMSCs and antibodies against either can be used to directly isolate BMSCs from aspirated bone marrow mononuclear cells [11;32].

Sacchetti et al. (2007) isolated self-renewing human osteoprogenitor cells on the basis of CD146 expression and demonstrated that clonal populations of the CD146-positive/CD133-negative cells were capable of forming ectopic HMEs [11]. CD146 is also expressed by endothelial cells, keratinocytes, smooth muscle cells, myofibroblasts, and lymphocytes in normal human tissues [33–35]. Previously, we demonstrated that magnetic-activated cell sorting against CD133 could be used to isolate a subpopulation of native human bone marrow stem/progenitor cells that gave rise to CD133-negative CD133BMSCs in culture [24]. Freshly-isolated adherent human CD133-positive cells were small, grew slowly, and had a distinct polyploid morphology with long, thin tenopodia and spoon-like lobopodia. In contrast, CD133BMSCs were larger, grew rapidly, and had the typical spindle-shaped morphology of cultured human BMSCs [24]. These observations were consistent with many reports showing that Prominin-1 (CD133) marks primitive stem cells (embryonic, fetal, and adult) and is downregulated during differentiation [36]. Whereas CD133 was lacking on CD133BMSCs, CD146 was uniformly and highly expressed through multiple passages. Pozzobon et al. (2009) reported that human bone marrow stem/progenitor cells isolated on the basis of CD133 expression were initially negative for CD146, but acquired its expression during expansion on cell culture plastic [37]. Taken together, the observations above indicate that a native $CD133^{+}/CD146^{-}$ stem/progenitor cell from human bone can generate $CD133^{-}/CD146^{+}$ BMSCs in culture that are capable of forming ectopic HMEs. Thus, it is clearly of interest to determine whether such a lineage relationship exists *in vivo* for a CD133-positive stem/progenitor cell and downstream CD133-negative components of HSC niches such as osteoblasts and ARCs.

Conclusion

CD133 identifies a native human bone marrow stem/progenitor cell that gives rise to CD133BMSCs in culture. CD133BMSCs are non-hematopoietic progenitor cells that form ectopic HMEs when transplanted into immunodeficient mice.

Research highlights

- CD133-derived bone marrow stem cells can form ectopic hematopoietic niches in mice.

- CD133 identifies bone marrow stem cells that lie upstream of CD146-positive cells.
- CD133 is likely to identify the native human stem/progenitor cell in bone marrow.

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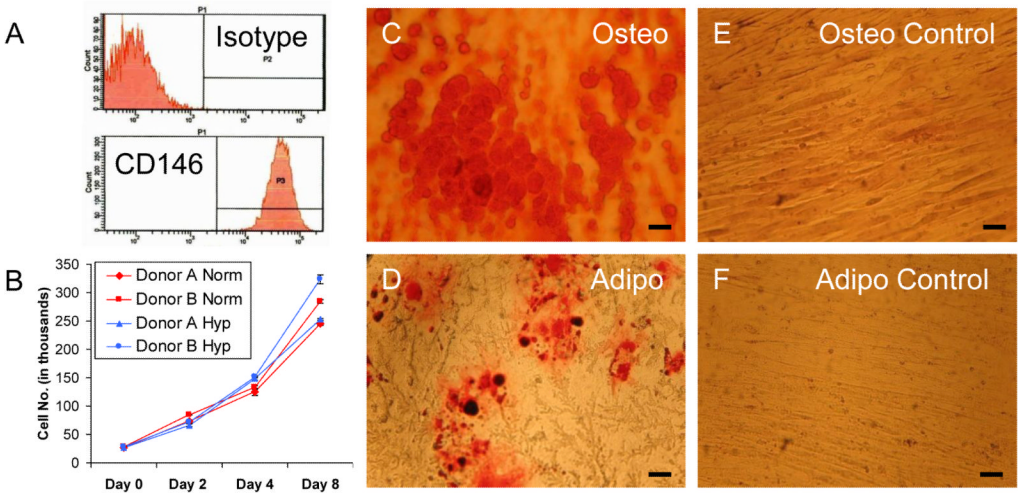

Fig. 1. Characterization of CD133BMSCs

A) Cell surface phenotyping for CD146 (MCAM). Note that isotype control is negative. **B**) High density growth of CD133BMSCs (N=2 donors) seeded at 1,042 cm2 in normoxic (21% oxygen; red lines) or hypoxic (1% oxygen; blue lines) conditions for 8 days. **C–F**) Phase contrast photomicrographs of cultured CD133BMSCs differentiated over 3 weeks into osteoblasts (Osteo) and adipocytes (Adipo). Calcium deposition was stained by Alizarin Red S (**C**) and lipid deposition was stained by Oil Red O (**D**). Alizarin Red S and Oil Red O staining was absent in CD133BMSCs treated with control growth medium **(E, F)**. C–F, 40X magnification.

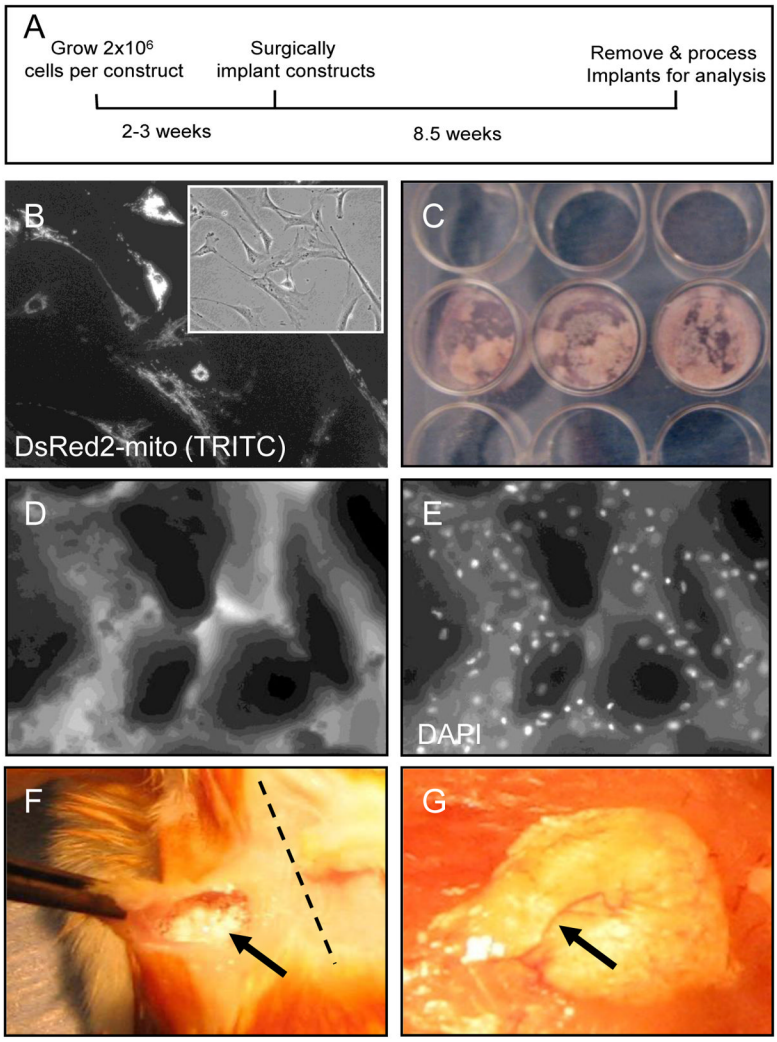

Fig. 2. Preparation and implantation of cell-seeded HA/TCP/fibrin constructs

A) Schematic of HME transfer protocol. **B**) Epifluorescent imaging of CD133BMSCs expressing DsRed2-mito reporter (20X magnification). Inset: phase contrast photomicrograph of CD133dMSCs pictured in (**B**). **C**) Picture of constructs prepared in 24-well plates and incubated for 90 minutes at 37ºC in a cell culture incubator prior to implantation. **D**) Phase contrast photomicrograph of HA/TCP particles incubated with cells and clotting factors (10X magnification). **E**) Epifluorescent image of (**D**). Note that CD133BMSCs are evenly dispersed throughout the construct. White: DAPI nuclear stain. **F**) At the time of surgery, gelatinous construct material was implanted into subcutaneous pockets (arrow) in immunodeficient mice at the scapula proximal or medial to the dorsal midline (dotted line). **G**) At 60 days after implantation, solidified constructs were integrated into host tissue and were vascularized (arrow).

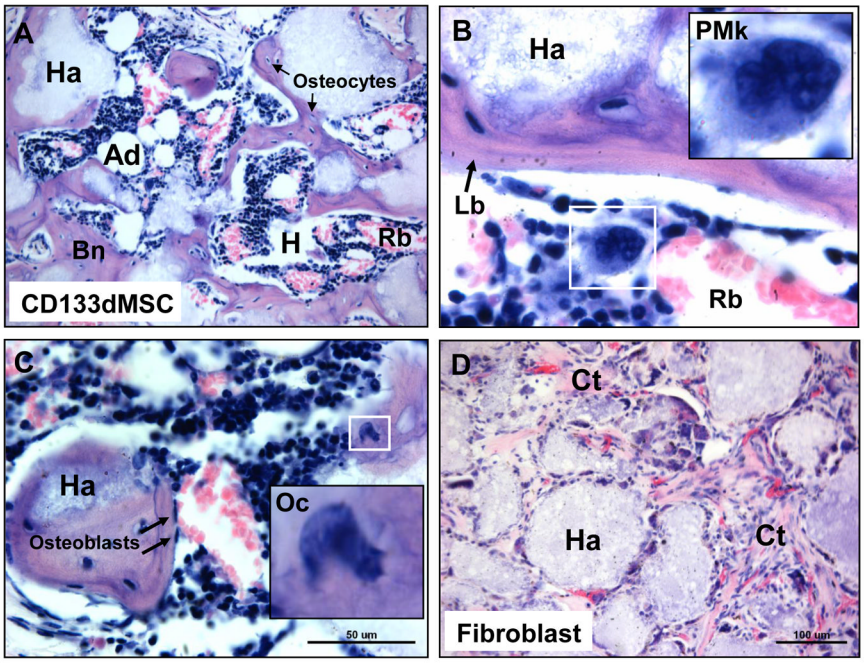

Fig. 3. Histology of cell-seeded constructs 60 days after implantation

A) H & E stain of CD133BMSC-seeded construct showing HME formation with blood forming hematopoietic cells (Hp), enucleated red blood cells (Rb), and adipocytes (Ad), that are interspersed throughout bone (Bn) (10X magnification). Note that bone surrounds the HA/TCP carrier (Ha). Osteocytes (Os) were observed residing within lacunae (arrows). **B**) Concentrically-layered lamellar bone (Lb, arrow) had formed on HA/TCP surfaces (Ha) (40X magnification). Inset: promegakaryocyte (PMk). **C**) Erythropoiesis at various stages was observed throughout the HMEs. Inset: osteoclast (Oc). Arrows: osteoblasts. **D**) HA/TCP/fibrin construct seeded with 2×10^6 human fibroblasts. Fibroblasts adhered to the HA/TCP matrix (Ha) formed connective tissue (Ct) containing microvasculature throughout the implant, but did not support hematopoiesis.

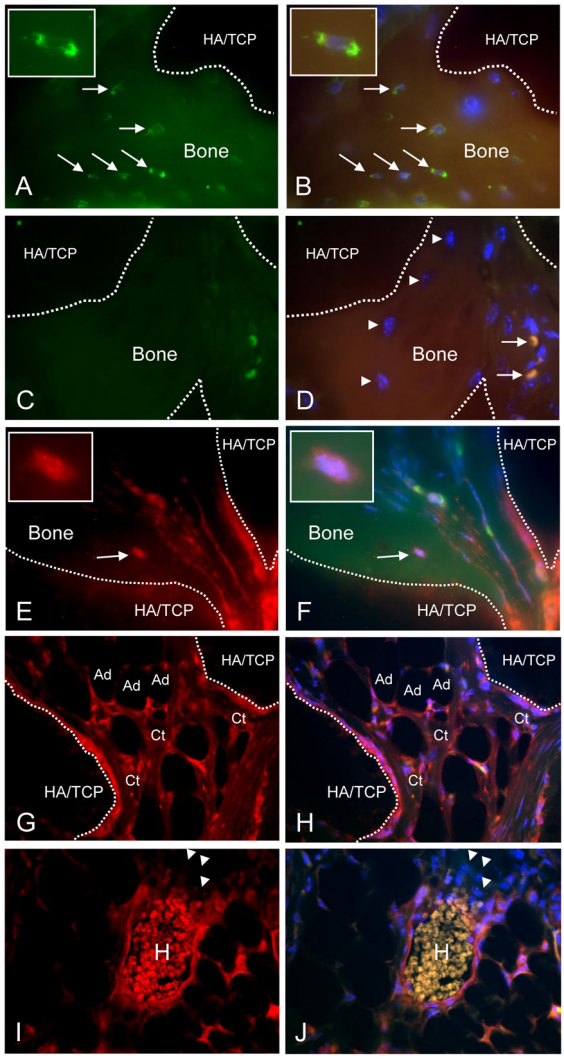

Fig. 4. Immunohistochemical evaluation of CD133dMSC-seeded constructs

A, B) Human-specific β2M staining (green, FITC channel) of osteocytes within bone formed from CD133BMSCs (arrows) (100X magnification). Dotted lines delineate bone (autofluorescent) and HA/TCP (dark areas). FITC/TRITC merge with DAPI (**B**). Insets: magnified view of an osteocyte. **C, D**) Isotype control staining (1 μg/ml IgG, 100X magnification). FITC/TRITC merge with DAPI (**D**). Arrowheads: osteocytes negative for staining by isotype. Arrows: strongly autofluorescent red blood cells. **E, F**) Staining for DsRed demonstrates characteristic punctate pattern of mitochondria within a bone-residing osteocyte (TRITC channel, **E**) and merge (**F**). Insets: DsRed-positive osteocyte. **G, H**) DsRed-positive inter-sinusoidal reticular cells, connective tissue (Ct), and adipocytes (Ad) in the implant. **I, J**) DsRed-positive peri-vascular reticular cells surrounding a sinus. **H**, Autofluorescent host-derived red blood cells. Arrowheads: Host-derived erythroblasts did not stain positive for DsRed. TRITC channel (**E–I**). TRITC/FITC merge with DAPI (**F–J**).