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Hematopoietic stem cells give rise to osteo-chondrogenic cells

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Abstract

Repair of bone fracture requires recruitment and proliferation of stem cells with the capacity to differentiate to functional osteoblasts. Given the close association of bone and bone marrow (BM), it has been suggested that BM may serve as a source of these progenitors. To test the ability of hematopoietic stem cells (HSCs) to give rise to osteo-chondrogenic cells, we used a single HSC transplantation paradigm in uninjured bone and in conjunction with a tibial fracture model. Mice were lethally irradiated and transplanted with a clonal population of cells derived from a single enhanced green fluorescent protein positive (eGFP⁺) HSC. Analysis of paraffin sections from these animals showed the presence of eGFP⁺ osteocytes and hypertrophic chondrocytes. To determine the contribution of HSC-derived cells to fracture repair, non-stabilized tibial fracture was created. Paraffin sections were examined at seven days, two weeks and two months after fracture and eGFP⁺ hypertrophic chondrocytes, osteoblasts and osteocytes were identified at the callus site. These cells stained positive for Runx-2 or osteocalcin and also stained for eGFP demonstrating their origin from the HSC. Together, these findings strongly support the concept that HSCs generate bone cells and suggest therapeutic potentials of HSCs in fracture repair.

Keywords

Hematopoietic stem cell; fracture; transplant; fracture repair

Introduction

There are approximately 6.5 million fractures in the United States per year, five to ten percent of which result in delayed union or non-union[1]. Therefore, methods to enhance

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and accelerate the fracture healing process are of significant clinical importance[2]. Bone healing is unique in that, once a fracture is sustained, the injured bone has the capacity to remodel and regenerate its original structure and integrity. Remodeling of skeletal bone requires the recruitment and proliferation of stem cells with the capacity to differentiate to functional osteoblasts that deposit and mineralize extracellular bone matrix. The potential of cell-based therapies in fracture repair, including stem cells, have recently been explored. Animal studies using culture-expanded mouse or human bone marrow (BM)-derived mesenchymal stem cells (MSCs) or adipose-derived stem cells have shown that these cells are able to enhance bone formation in critical size-defect models (reviewed in[3]). Patient studies have shown that treatment of bone defects with *ex vivo* expanded MSCs[4; 5] or percutaneous injection of autologous BM-derived buffy coat [6] resulted in improved bone healing. These studies have led to the development of clinical trials for testing BM-stem cell based (NCT00916981, NCT00512434), MSC-based (NCT01429012, NCT00250302, NCT01206179, NCT01435434) and hematopoietic stem cell (HSC)-based (NCT00632034) therapies for treatment of bone fracture, including non-union (www.clinicaltrials.gov).

Current dogma suggests that BM contains two types of stem cells, HSCs and MSCs, and that their repertoire of differentiation and reconstituting potentials are distinct and separate from each other. HSCs produce blood cells and some cells in the tissues such as mast cells and osteoclasts while MSCs are thought to generate a number of mesenchymal cells including fibroblasts, adipocytes, chondrocytes and osteocytes. During the last several years, however, it has become increasingly clear that hematopoiesis and the stromal environment are closely related and that a possible overlap between the two may exist. Simmons and Torok-Storb[7] reported generation of CFU-F from sorted CD34⁺ human BM cells, a population of cells enriched for HSCs. We have also observed CFU-F derived from HSCs[8]. Chen *et al*[9] have shown that the frequency of osteoblast progenitor cells is higher in CD34⁺ cells (approximately 1/5000) than in CD34⁻ population (1/33,000) of human BM. Murine transplantation studies have demonstrated that transplantation of 3000 side population (SP) cells that are highly enriched for HSCs generated osteoblasts *in vivo*[10]. In another study, Dominici *et al*[11] transplanted marrow cells that had been transduced with GFP-expressing retroviral vector and observed a common retroviral integration site in clonogenic hematopoietic cells and osteoprogenitors from each of the recipient mice. These studies provide compelling evidence for the existence of a common progenitor cell with both hematopoietic and osteocytic differentiation potentials in the non-adherent fraction of BM cells. Recent studies have also identified a population of circulating human osteoblastic cells which express osteocalcin or alkaline phosphatase and increase during pubertal growth and during fracture repair[12]. Studies also showed that these osteocalcin positive cells were able to form mineralized nodules *in vitro* and bone *in vivo*. This population was subsequently shown to be CD34⁺[13], suggesting that it is derived from the HSC. Using a transplantation model in which the BM of lethally irradiated recipient mice is reconstituted by a clonal population of cells derived from a single enhanced green fluorescent protein (eGFP⁺) HSC, we have documented that many types of tissue fibroblasts/myofibroblasts are derived from the HSC (reviewed in[14]). Based on this same model, we have demonstrated *in vitro* and *in vivo* that adipocytes are of HSC origin[15]. Recently, we have also shown that transplantation of 50 BM cells that are highly enriched for HSCs ameliorates bone pathologies in a mouse model of osteogenesis imperfecta[16]. Together, these studies challenge the current dogma that mesenchymal cell types, specifically bone cells, are derived solely from MSCs.

In the present study, we used our clonal cell transplantation model to test the ability of HSCs to give rise to osteo-chondrogenic cells in animals with and without non-stabilized tibial fractures. Our findings show that HSCs generate osteocytes and chondrocytes in long bones of clonally engrafted animals. This contribution is significantly enhanced during fracture

repair. Together these findings suggest that HSCs may serve as a novel source of osteochondrogenic cells during normal bone turnover and repair from injury.

Material and Methods

Mice

Breeding pairs of transgenic eGFP⁺ mice (C57BL/6-CD45.2) were kindly provided by Dr. Okabe[17] (Osaka University, Japan). Breeding pairs of congenic C57BL/6-CD45.1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were bred and maintained at the Animal Research Facility of the Veterans Affairs Medical Center. All aspects of animal research have been conducted in accordance with guidelines set by the PHS Policy on Humane Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of the Department of Veterans Affairs Medical Center.

Reagents

For lineage negative immunomagnetic selection, purified antibodies to murine B220/CD45R (RA3-6B2), Gr-1/Ly-6C (RB6-8C5), TER-119 (TER-119), CD4/L3T4 (GK-1.5), and CD8/Ly-2 (53.6.7) were purchased from BD Pharmingen (San Diego, CA). Sheep anti-rat IgG Dynabeads® were purchased from Invitrogen Dynal (Carlsbad, CA). Murine antibodies used for cell sorting including phycoerythrin (PE)-conjugated anti-Sca-1 (D7), allophycocyanin (APC)-conjugated anti-c-kit (2B8), biotin-conjugated lineage panel antibodies (B220, Gr-1, CD3ε, TER-119), and streptavidin-conjugated APC-Cy-7 were purchased from BD Pharmingen. Biotinylated murine anti-CD34 (RAM34) was purchased from eBiosciences (San Diego, CA). Appropriate isotypes were purchased from BD Pharmingen. PE-conjugated antibodies used for multi-lineage hematopoietic engraftment analysis including murine anti-B220/CD45R (RA3-6B2), Thy-1.2/CD90.2 (30-H12), Mac-1/CD11b (M1/70) and Gr-1/Ly-6G and Ly-6c (RB6-8C5) were purchased from BD Pharmingen. Murine anti-Runx-2 antibody and anti-green fluorescent protein (GFP) were purchased from Abcam (Cambridge, MA). Murine anti-osteocalcin antibody was purchased from Takara Bio Company (Clontech Labs, Madison, WI).

Cell Preparation for transplantation

Generation of mice exhibiting high level of multilineage engraftment from single HSC was conducted as previously described[8; 14; 18; 19; 20]. Briefly, ten to fourteen-week-old eGFP mice were used as donors. BM cells were flushed from tibiae and femurs of euthanized mice, pooled and washed with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Mononuclear cells (MNCs) were isolated by gradient separation using Lympholyte-M (Cedarlane Laboratories Limited, Ontario) then further enriched for lineage-negative (Lin⁻) cells by negative selection using antibodies to B220, Gr-1, CD4, CD8, TER-119, and Dynabeads® sheep anti-rat IgG beads. Lin⁻ cells were stained with PE-conjugated anti-Sca-1, APC-conjugated anti-c-kit, biotinylated anti-CD34 and biotinylated lineage panel antibodies (B220, Gr-1, CD3ε, TER-119) followed by streptavidin-conjugated APC-Cy7. The cells were then resuspended at 1×10^6 /ml in Ca²⁺-, Mg²⁺- free Hanks balanced salt solution (HBSS; Invitrogen, Gaithersburg, MD) containing 2% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 10mM HEPES, 1% penicillin/streptomycin and Hoechst 33342 (Sigma, St. Louis, MO; 5μg/ml) and incubated at 37°C for 60 min. Cells were then washed, stained with propidium iodide (1 μg/mL), resuspended in PBS/0.1% BSA, and kept on ice until sorting. Cell sorting was performed using a MoFlo Cell Sorter (DakoCytomation, Fort Collins, CO). Appropriate isotype-matched controls were analyzed. The gates used for side population (SP) cells were as previously described[16; 18; 20; 21] and corresponded to R3 and R4 fractions of Goodell, et al[22] and R1 and R2 populations of Matsuzaki, et al[23]. Individual Lin⁻ Sca-1⁺ c-kit⁺

CD34⁺ SP cells were deposited into wells of Corning U-bottom 96-well culture plates (Corning, NY) using the CloneCyt system (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Eighteen hours after single-cell deposition, wells containing single cells were identified. Incubation of these wells was continued for a total of 7 days at 5% CO₂, 37°C in media containing α -modification of Eagle's medium (ICN Biomedicals, Aurora, OH), 20% FBS, 1% deionized fraction V BSA, 1×10^{-4} mol/L 2-mercaptoethanol (Sigma), 100 ng/mL stem cell factor (SCF, R&D Systems, Minneapolis, MN), and 10 μ g/mL murine interleukin-11 (IL-11, R&D Systems). This combination of cytokines supports proliferation of primitive hematopoietic progenitors[24]. Because the majority of HSCs are dormant in cell cycle and begin cell division a few days after initiation of cell culture, we selected clones consisting of no more than 20 cells after incubation. This method significantly enhanced the efficiency of generating mice with high-level multilineage engraftment by a single HSC[16; 18; 20; 21; 25].

Transplantation and Engraftment Analysis

Recipient C57BL/6-CD45.1 mice were given a single 950-cGy dose of total-body irradiation using a 4×10^6 V linear accelerator. Contents of wells (~ 20 clonal cells) were injected via tail vein into lethally irradiated mice along with 500 CD45.1 bone marrow Lin⁺ c-kit⁺ Sca-1⁺ CD34⁺ radioprotective cells[18; 20; 21; 25]. These cells were shown to be effective radioprotective cells during the post radiation pancytopenia period[26]. After transplantation, the mice were fed an irradiated breeder's diet (Tekland Global Diets, Harlan Labs, Indianapolis, IN) and milliQ water *ad libitum*. Neomycin was added to the water in the first month after transplantation to prevent infection. For hematopoietic engraftment analysis, peripheral blood was obtained from the retro-orbital plexus of anesthetized mice two months after transplantation and red blood cells were lysed (1X PharM Lyse; BD Pharmingen). Donor-derived eGFP⁺ cells in T cell, B cell, granulocyte and monocyte/macrophage lineages were analyzed by staining with PE-conjugated anti-Thy-1.2, anti-CD45R/B220 and a combination of anti-Gr-1 and anti-Mac-1, respectively. Only those mice demonstrating high-level multilineage engraftment above 50% were used.

Non-stabilized Tibial Fracture

To induce fractures, mice were anaesthetized with isofluorane to a surgical plane. Tibial fractures were created by three-point bending using a modified fracture device based on that described by Hiltunen[27]. A tibial mid-diaphysis fracture was created by dropping a weight (~ 160 g) from a height of 14–20 cm. This created closed non-stabilized fractures from which the mice recovered without complication. Analgesia (Buprenorphine 0.1 mg/kg) was administered prior to recovery and then as needed per consultation with veterinarian (up to two deliveries on the next day at 8–10 hrs intervals). The contra-lateral intact tibia served as a control. Mice were euthanized by intra-cardiac perfusion of PBS followed by 4% paraformaldehyde at 1 week, 2 weeks and 2 months after fracture. Both tibia and surrounding tissues were removed and processed for tissue sectioning.

Bone Decalcification, Embedding and Sectioning

Decalcification was carried out using ethylenediaminetetra-acetic acid (EDTA) under microwave (Ted Pella Inc., Redding, CA) as previously described [28]. Bone in 0.12M EDTA was subjected to microwave heating at 42°C for 7–10 days with daily weighing of the sample. Decalcification end point was determined as the time at which a weight gain was noted. Samples were washed with water, dehydrated through serial ethanols (70%, 95%, 100%), infiltrated (using Histochoice in place of xylenes to preserve eGFP expression) in serial Histochoice:paraffin solutions (1:0, 3:1, 1:1, 1:3, 0:1) and embedded under vacuum (60 °C). Five micron sections were generated.

Masson's Trichrome Staining

Masson's trichrome staining was conducted per manufactures protocol (Sigma, St Louis, MO). Sections were deparaffinized, post-fixed in citrate buffer (1 h, 60°C), rinsed in water and stained in Weigert's iron hematoxylin (5 min). After washing, sections were stained in Biebrich scarlet solution (5 min), washed and sequentially placed in Phosphotungstic/phosphomolybdic acid, Aniline blue solution and 1% Acetic acid solution and rinsed in distilled water. The sections were then rehydrated in serial ethanols, coverslipped and imaged.

Immunohistochemistry

Sections were permeabilized in 0.02% Triton X-100/PBS (10 mins), blocked in 3% BSA/5% normal donkey serum/PBS (overnight, 4°C), and then incubated with appropriate primary antibodies diluted in PBS (2 hrs, 37°C). Sections were washed in PBS for 5 mins and incubated with appropriate fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA) diluted in PBS (1 hr, 37°C). Fluorochrome-conjugated secondary antibodies (Jackson) that could be readily separated from the emission spectrum of eGFP were used for these studies. For some studies, EGFP expression was detected using an antibody against GFP and antigen retrieval. For antigen retrieval, sections were deparaffinized, and treated with 10% formic acid (15 min, 37°C) before staining. This further ensured that the eGFP expression detected was not due to auto fluorescence, but to actual expression of eGFP protein.

Microscopy

Imaging was conducted using a Nikon 90i fluorescent microscope (Nikon Instruments, Melville, NY) capable of differential interference microscopy (DIC) and equipped with a high resolution DS-Fi1 digital color camera. GFP was imaged using a C-FL Endow GFP HYQ longpass (Lp) filter cube (Nikon). The wider and steeper filter spectra of the longpass filter cube (Ab 488, Em 509) allows for a clear differentiation between background noise, which appears as yellow, from the GFP signal which appears bright green, without a consequential increase in noise. Image processing was performed using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

Results

Generation of clonally engrafted mice

To investigate the potential contribution of HSCs to osteoblast differentiation and fracture healing, we first generated mice with high-level, multilineage hematopoietic engraftment by cultured clonal populations from single HSCs. As described in the Methods, Lin⁻Sca-1⁺CD34⁻SP cells were individually cultured for one week in the presence of SCF and G-CSF and clones consisting of 20 or fewer cells were transplanted into lethally irradiated recipients. Two months after cell transplantation, nucleated blood cells from these mice were analyzed for hematopoietic engraftment and mice exhibiting high-level multilineage engraftment by donor eGFP⁺ cells were selected for study. Figure 1 shows flow cytometric analysis of the nucleated blood cells from a representative recipient mouse exhibiting high-level multilineage engraftment. The majority of the granulocytes, macrophages, and B cells in this mouse were eGFP⁺, demonstrating an almost complete hematopoietic reconstitution by the transplanted clonal cell population derived from a single sorted cell. The slow turnover of T cells (i.e., memory T cells) results in the persistence of recipient EGFP⁻ T cells.

Identification of HSC-derived osteo-chondrogenic cells in steady state long bones

Examination of paraffin sections from bones harvested from a mouse two months after engraftment did not reveal eGFP⁺ osteocytes (data not shown). However, tibia from mice sacrificed 10 months after clonal transplantation and engraftment showed the presence of few eGFP⁺ cells with morphology of osteocytes as indicated by DIC microscopy (Figure 2). EGFP-expressing cells were observed embedded in the bone matrix (Figure 2A, arrows). It is important to note that the majority of cells with osteocyte morphology were eGFP⁻ (closed arrowheads). In addition, cells with morphology of chondrocytes were also seen in long bones harvested from clonally transplanted animals (Figure 2B, arrows). Again, these cells were extremely rare. The low incidences of newly generated (eGFP⁺) osteo-chondrocytes agree with the known slow turnover rates of bone cells and chondrocytes in the normal steady state [29]. To stimulate osteo-chondrogenesis, we used a tibia fracture model in which closed weight-induced impact/compression fractures were generated. In this model, both cartilage and bone remodeling at the site of injury (endochondral ossification) are stimulated. Bones from clonally engrafted animals with tibial fractures were then temporally examined.

Traffic of HSC-derived cells to the callus seven days after fracture

In the early process of fracture healing, a hematoma is formed and an inflammatory response occurs at the fracture site within the first 48 hours, as demonstrated by the invasion of macrophages, polymorphonuclear leukocytes, and lymphocytes. Osteo-chondrogenic progenitor cells are also recruited to the fracture site within the first week after fracture. Analysis of images taken from within the fracture callus of clonally engrafted animals seven days after fracture show a large infiltration of eGFP⁺ cells (Figure 3). At this stage of fracture repair, the eGFP-expressing cells within the fracture callus are an unorganized, mixed population as demonstrated by their heterogeneous morphology.

Contribution of HSC-derived cells to the fracture callus two weeks after fracture

During the two to three weeks post-fracture, the acute inflammation resolves, a soft callus with hypertrophic chondrocytes and osteoblasts can be appreciated and some of the cartilage forming the soft callus begins to calcify[30]. This stage of soft callus is depicted using Masson's Trichrome staining of sections from clonally engrafted animals two weeks post fracture (Figure 4A). To correlate cell morphology with cell origin, serial sections were imaged for Trichrome stain (Figure 4A–C) and expression of eGFP (Figure 4D–F). While serial sections do not allow for complete co-localization of cells, comparison of Trichrome stain and eGFP expression demonstrates the presence of a large number of eGFP⁺ cells within the fracture callus (Figure 4D). Examination of these cells at high magnification demonstrates that ~100% of hypertrophic chondrocytes, as identified by their morphology and Trichrome stain (B, C, open arrowheads), are eGFP⁺ (E, F, open arrowheads). Cells expressing eGFP⁺ were also found within the newly forming primary bone, embedded within the bone matrix (B, E, arrows), suggesting that they are early stage osteoblasts.

To exclude the possibility that artifactual autofluorescence accounted for the pattern of eGFP expression seen in the fracture callus, sections were stained using an antibody specific for GFP (Figure 5). Analysis of thin sections stained with antibodies confirmed the expression pattern seen with inherent eGFP expression. GFP⁺ cells were identified with the morphology (Figure 5A, E) of hypertrophic chondrocytes (closed arrowheads) and osteoblasts (arrows). Higher magnification analysis of cells within the soft callus reveal that the majority of cells with hypertrophic chondrocyte morphology are HSC-derived (Figure 5A–D). Together, these data demonstrate that HSCs generate cells with the morphological characteristics of osteoblasts and chondrocytes in the fracture callus two weeks after fracture.

Incorporation of HSC-derived osteoblasts and osteocytes into remodeled bone two months after fracture

To examine the long-term contribution of HSCs to fracture repair and bone remodeling, we examined tissues taken from a mouse two months after fracture (Figure 6). By this time, the soft callus is being replaced by trabecular bone to form a hard callus, which will eventually be replaced by compact bone that duplicates the original shape and structure of the bone. More specifically, hypertrophic chondrocytes in the callus secrete alkaline phosphatase for mineral deposition and calcification of the matrix. Once this calcification occurs, hypertrophic chondrocytes undergo apoptosis to create cavities in the bone, which are occupied by osteoprogenitor cells. Examination of paraffin sections at the fracture site reveals the presence of cells within the new bone matrix with morphology of mature osteoblasts and osteocytes that are both GFP⁺ and GFP⁻ (Figure 6A–C). We have shown that the majority of hypertrophic chondrocytes were derived from transplanted HSC in the two-week old fracture callus (Figures 4 and 5). This suggests that, with ossification, both HSC-derived (GFP⁺) and resident (GFP⁻) osteoprogenitors occupied the spaces left by apoptosis of hypertrophic chondrocytes. Slow turnover of osteoprogenitors may be one of the mechanisms of the long-term contribution of HSCs to fracture healing.

Expression of Runx-2 and osteocalcin (OCN) by the HSC-derived cells in the fracture callus

After demonstrating the presence of eGFP⁺ cells with hypertrophic chondrocyte, osteoblast, and osteocyte morphology in the fracture callus, we next sought to temporally characterize these HSC-derived cells based on marker staining (Figure 7). We used osteo-chondrogenic markers Runx-2, a marker of osteoblast differentiation that can be visualized during early fracture repair, and OCN, a marker of mature osteoblasts and osteocytes. Analysis of DIC images from paraffin sections through the fracture site taken at seven days (Figure 7A–E), two weeks (Figure 7F–J) and two months (Figure 7K–O) shows progressive callus remodeling after non-stabilized fracture. Staining with anti-GFP antibody shows the presence of HSC derived cells in the fracture callus at each time point after fracture (Figure 7C, H, M). Merged images of GFP and Runx-2 expression (taken at seven days and two weeks post-fracture, Figure 7E, J) or GFP and OCN expression (taken at two months post-fracture, Figure 7O) show the presence of GFP⁺ cells which expressed Runx-2 during early fracture repair and OCN at later stage remodeling. It is important to note that GFP⁺OCN⁺ cells can be found among GFP⁻OCN⁺ osteocytes (Figure 7O, asterisk). These findings confirm that HSCs generate hypertrophic chondrocytes, osteoblasts and osteocytes in the fracture callus and newly formed bone.

Discussion

There has been considerable interest in recent years in the use of stem cells for repair of a number of tissues including bone. Animal studies have shown some efficacy of ex vivo-expanded MSCs in enhancing bone repair when delivered locally at the site of injury in a variety of models [31; 32; 33; 34; 35; 36]. In human studies, treatment of fractures, segmental defects or non-union with culture expanded autologous MSCs alone [6] or in conjunction with porous hydroxyapatite scaffold [4; 5] resulted in complete fusion and integration of the graft. A recent xenograft study using human MSCs found that a combination of stem cells with bone morphogenetic protein-7 (BMP-7) resulted in a better osteoinductive graft than either the stem cells or BMP-7 alone [37]. Despite the emerging evidence that MSCs may have a utility in skeletal repair, the precise mechanism by which these cells enhance tissue regeneration remains unclear. Arthur et al. found that BrdU-labeled human MSCs when transplanted subcutaneously exhibited little or no proliferation *in vivo* [38], suggesting that expansion and subsequent differentiation into osteoblastic cells

may be limited. Prockop has proposed that tissue repair by MSCs is mediated only to some extent by differentiation into a specific functional cell and that the major mechanism by which these cells enhance tissue repair may be through paracrine secretions and cell-to-cell contact [39; 40]. Preclinical studies also suggested that the therapeutic effects afforded by MSC transplantation are short-lived and related to dynamic, paracrine interactions between MSCs and host cells [41]. Clinical improvement seen early after MSC transplantation in cases of osteogenesis imperfecta did not persist long term [42], raising questions as to the long term regenerative capacity of the donor-derived mesenchymal progenitors [43].

Given the perceived limitations of the MSC, it is apparent that additional sources of osteo-chondrogenic cells should be investigated for therapeutic use. Several studies have supported the hypothesis that osteoblasts can be derived from the HSCs [7; 9]. Murine bone marrow transplantation studies have demonstrated that bone marrow populations enriched for HSCs, including the non-adherent bone marrow population [11; 43] and the bone marrow side population [10], give rise to osteoblasts *in vivo*. Cells positive for CD34 and osteocalcin (“osteoblastic cells”) were found to line the cavities of the cartilage in the fracture site of rabbit tibial osteotomy model [44]. Transplantation of human CD34⁺ cells into immunocompromised rats with fracture non-union resulted in significant enhancement of functional bone healing [45]. The latter group also reported beneficial results of implantation of mobilized peripheral blood CD34⁺ cells in a collagen matrix at the fracture non-union site [46]. Our study based on single HSC transplantation establishes that chondrocytes, osteoblasts and osteocytes can be derived from HSCs and provides the experimental support for these earlier observations.

Our findings demonstrate an HSC origin for osteo-chondrogenic cells during both normal physiology and fracture repair. The clinical parallel of these findings is our recent study demonstrating that transplantation of an enriched population of HSCs ameliorates bone pathologies in a mouse model of osteogenesis imperfecta [16]. In the present study, the early contribution of HSC-derived cells in the fracture callus is striking. At one week post-fracture, the eGFP-expressing cells are abundant; however they are an unorganized, mixed population with heterogeneous morphology, likely representing many cell types including osteochondrogenic precursors as well as inflammatory cells. At the two week time point, the acute inflammatory response has resolved, leaving hypertrophic chondrocytes at the callus, the majority of which (~100%) express eGFP. At the final stages, the osteoblasts and osteocytes at the remodeled site are both eGFP⁺ (HSC-derived) and eGFP⁻ (resident). This may be due to the slow turnover of osteoprogenitors and/or radio-resistant progenitor cells, which leaves a population of none-GFP-expressing osteoprogenitors to contribute to fracture repair. In the treatment of a mouse model of osteogenesis imperfecta [16], quantifiable changes in the bone parameters were observable only at 3–6 months after HSC transplantation, also suggesting slow turnover of osteoprogenitors.

Using the same HSC transplantation method, we have shown that HSCs can give rise to additional mesenchymal, non-hematopoietic cells such as fibroblasts [8], tumor stromal cells [18; 20], interstitial cells of cardiac valves [19], glomerular mesangial cells [21], and adipocytes [15] (reviewed in [14; 47; 48]). In addition, studies from two groups based on single cell transplantation models have demonstrated that HSCs can give rise to skeletal muscle, another mesenchymal cell type [49; 50]. Together these findings suggest that the HSC contributes to mesenchymal tissues both in physiology and pathology.

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Figure1. Multilineage engraftment from a single eGFP⁺ HSC

Panels A and B show positive controls for CD45.1 (A) and eGFP (B) expression.

Representative peripheral blood analysis from a clonally engrafted mouse two months post-transplant shows that eGFP⁺ donor cells represent 70% of total nucleated cells (C). eGFP⁺ donor cells also represent 80% of granulocyte-macrophages (D), 85% of B-cells (E) and 50% of T-cells (F).

Figure 2. HSC derived osteocytes and chondrocytes in the long bone

When DIC images (A, E) of cells in the long bones from a clonally engrafted mouse were superimposed with fluorescent images of eGFP expression (B, F), eGFP⁺ cells with morphological characteristics of osteocytes (D, arrows) and chondrocytes (F, arrows) were observed. Cells with morphology of osteocytes and chondrocytes that did not express eGFP were also found within the bone (A–D, arrowheads). Nuclei are shown by Hoechst staining (C, G) and asterisk (A–D) indicates bone marrow. Bars= 25μM.

Figure 3. HSC-derived cells contribute to the callus seven days post non-stabilized fracture

Analysis of paraffin sections from clonally engrafted animal seven days after fracture reveals a large fracture callus with heterogeneous cell morphology as imaged by DIC (A). Nuclei are shown by Hoechst staining (B). Imaging for eGFP using the C-FL Endow GFP HYQ longpass filter cube allows for detection of eGFP expression (green) above background (yellow). Analysis shows a large number of eGFP⁺ cells in the callus (C), which can be appreciated at higher magnification in (D). Asterisks denote existing bone at the site of fracture. Bars A–C= 25μM, D= 25 μM.

Figure 4. Histological analysis of fracture callus two weeks post-non-stabilized fracture

Histological analysis of thin paraffin sections through fracture region two weeks post non-stabilized fracture in a clonally engrafted mouse shows the formation of a large fracture callus (asterisks, A, D). Serial sections were stained using Masson's Trichrome stain to identify bone and cartilage (blue in A–C) and imaged for fluorescent expression of eGFP to identify HSC-derived cells (D–F). Based on trichrome staining pattern, eGFP⁺ cells were identified as having morphology of hypertrophic chondrocytes (open arrowheads) and osteoblasts (arrows). Bars A, D = 100 μ M, B–C, E–F = 25 μ M.

Figure 5. HSC-derived cells in fracture callus two weeks post non-stabilized fracture
Thin paraffin sections from the two-week fracture callus were then analyzed for cell morphology and GFP-expression by staining to confirm the pattern of inherent eGFP expression. DIC images (A, E) and fluorescent expression of GFP as detected by immunofluorescent staining (C, G) identified HSC derived cells with the morphology of hyperthrophic chondrocytes (arrowheads) and osteoblasts (closed arrows). Higher magnification images are shown in D and H. Morphology (I), nuclear labeling (J) and secondary only control for anti-GFP stain (K, L), respectively, are depicted. Nuclei are shown by Hoechst staining (B, F, J), asterisks denote existing bone at the site of fracture and “fc” indicates fracture callus. Bars A–L = 25 μ M.

Figure 6. HSC-derived osteoblasts and osteocytes in callus two months post non-stabilized fracture

Paraffin sections through the fracture callus were examined two months after fracture in clonally engrafted mice. A representative DIC image (A) from the fracture callus shows GFP⁺ cells (C) with mature osteoblast/osteocyte morphology embedded within the newly forming bone matrix (A–C). Panels D–F show morphology, nuclear labeling and secondary only control for anti-GFP stain, respectively. Nuclei are shown by Hoechst staining (B, E). Bars A–F = 25μM.

Figure 7. HSC-derived EGFP⁺ cells in the fracture callus express Runx-2 and osteocalcin
 Paraffin sections through the fracture site were taken at seven days (A–E), two weeks (F–J) and two months (K–O) after non-stabilized fracture. DIC images (A, F, K) show morphology of cells within the fracture site. Staining with anti-GFP antibody (C, H, M) shows the presence of HSC derived cells in the fracture callus at each time point after fracture. When images of GFP (red) and Runx-2 (green, D, I) expression (7d, 2wk) or EGFP (red) and osteocalcin (OCN, green, N) expression (2mo) were merged, resulting images show the presence of a large number of GFP⁺ cells which stained positive for Runx-2 at seven days (E) and two weeks (J) and osteocalcin at two months (O). Control panels depict no primary antibody controls for GFP (R, W), Runx-2 (S, T) and osteocalcin (X, Y). Nuclei are shown by Hoechst staining (B, G, L, Q, V). Bars A–Y = 25μM.