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Hematopoietic derived cells do not contribute to osteogenesis as osteoblasts

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ABSTRACT

Despite years of extensive investigation, the cellular origin of heterotopic ossification (HO) has not been fully elucidated. We have previously shown that circulating bone marrow-derived osteoblast progenitor cells, characterized by the immunophenotype CD45−/CD44+/CXCR4+, contributed to the formation of heterotopic bone induced by bone morphogenetic protein (BMP)-2. In contrast, other reports have demonstrated the contribution of CD45+ hematopoietic derived cells to HO. Therefore, in this study, we developed a novel triple transgenic mouse strain that allows us to visualize CD45+ cells with red fluorescence and mature osteoblasts with green fluorescence. These mice were generated by crossing CD45-Cre mice with Z/RED mice that express DsRed, a variant of red fluorescent protein, after Cre-mediated recombination, and then crossing with Col2.3GFP mice that express green fluorescent protein (GFP) in mature osteoblasts. Utilizing this model, we were able to investigate if hematopoietic derived cells have the potential to give rise to mature osteoblasts. Analyses of this triple transgenic mouse model demonstrated that DsRed and GFP did not co-localize in either normal skeletogenesis, bone regeneration after fracture, or HO. This indicates that in these conditions hematopoietic derived cells do not differentiate into mature osteoblasts. Interestingly, we observed the presence of previously unidentified DsRed positive bone lining cells (red BLCs) which are derived from hematopoietic cells but lack CD45 expression. These red BLCs fail to produce GFP even under in vitro osteogenic conditions. These findings indicate that, even though both osteoblasts and hematopoietic cells are developmentally derived from mesoderm, hematopoietic derived cells do not contribute to osteogenesis in fracture healing or HO.

1. Introduction

Heterotopic ossification (HO), the process of bone formation outside of the skeleton, is known to be induced following traumatic injury or surgery, as well as in genetic disorders such as fibrodysplasia ossificans progressiva (FOP). The process of HO is initiated by an inflammatory phase similar to bone regeneration in fracture healing while normal skeletogenesis during development does not require inflammation, suggesting that a distinct cellular mechanism is involved in HO. It has recently been demonstrated that cells playing an important role in HO differ from those in normal skeletogenesis [1]. The extensive research on the type and source of cells which give rise to osteoblasts in HO has provided a variety of candidates including circulating mesenchymal progenitor cells [2–5], Tie2-expressing cells [6] and GLAST-expressing cells [1]. Previously, we have reported that circulating bone marrow-derived osteoblast progenitor cells are recruited to bone-forming sites and give rise to osteoblasts [3]. These circulating osteoblast progenitor cells are positive for CD44 and CXCR4 and negative for CD45 [4], suggesting that they were derived from non-hematopoietic bone marrow cells. However, others have reported that circulating CD45+/Collagen I+ osteogenic precursor cells contribute to HO in patients with FOP [5], and that hematopoietic derived fibroblast-like cells, so-called fibrocytes, also have the potential to become osteoblasts [2,7,8]. Moreover, it has been reported that hematopoietic stem cells can generate functional osteoblasts [9,10]. Considering these findings and given that osteoblasts and blood cells are both embryonically derived from mesoderm, CD45+ hematopoietic derived cells might lose their CD45 expression

Abbreviations: HO, heterotopic ossification; GFP, green fluorescent protein; BLC, bone lining cell; BMP, bone morphogenetic protein.

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postnatally and contribute to HO directly by differentiating into osteoblasts. To address whether hematopoietic derived cells give rise to osteoblasts in HO, it is necessary to trace their cell fate in vivo.

The Cre/loxP system makes it possible to trace cell lineages in vivo [11] by crossing tissue-specific Cre recombinase-expressing mouse strains with floxed fluorescent reporter mice. Several different Cre lines have been utilized in the study of HO [1,6,12] including Lyz-Cre (monocyte/macrophage lineage), CD19-Cre (B cell lineage), and LCK-Cre (T cell lineage) mice to study the hematopoietic lineages in HO models [12]; however, none of these lineages directly contribute to bone formation. Since these Cre lines label differentiated lineage cells instead of hematopoietic stem/progenitor cells, alternative Cre lines which cover a majority of hematopoietic cells including primitive stem cells are needed.

All blood cells in the adult hematopoietic system originate developmentally from definitive hematopoietic stem cells (dHSCs) in the embryonic hematopoietic system. In the mouse embryo, dHSCs, characterized by a VE-cadherin +/CD45 + immunophenotype, emerge in the aorta-gonad-mesonephros (AGM) by embryonic day 11 [13,14]. Since VE-cadherin is also expressed by endothelial cells, a VE-cadherin-Cre line is not ideal to specifically label hematopoietic cells. Conversely, CD45 is recognized as a pan-leukocyte marker and its expression is limited to hematopoietic cells including dHSCs but excluding mature erythrocytes, indicating that a CD45-Cre line would specifically label hematopoietic cells. Therefore, in this study, we used CD45-Cre mice with Z/RED reporter mice in conjunction with Col2.3GFP mice, which express GFP in mature osteoblasts, and tracked hematopoietic cells to examine whether they differentiate into functional mature osteoblasts in regular skeletogenesis, a bone morphogenetic protein-2 (BMP-2) induced HO model, or a tibia fracture model.

2. Materials and methods

2.1. Mice

CD45-Cre mice were kindly gifted by Dr. Eva Mezey and were produced as previously described [15]. Briefly, Cre recombinase cDNA was inserted into the 3′ end of exon 33 of the complete murine *CD45* gene with an internal ribosomal entry site (IRES) using a bacteria artificial chromosome (BAC). C57BL/6 wild type, Z/RED [16] and Col2.3GFP [17] mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Double transgenic F1 mice were developed by breeding CD45-Cre mice with Z/RED mice. Triple transgenic F2 mice were developed by crossing CD45-Cre/Z/RED F1 mice with Col2.3GFP mice. Colonies of these strains were maintained in the vivarium in The Research Institute at Nationwide Children's Hospital. All animal protocols were approved by the Institutional Animals Care and Use Committees of The Research Institute at Nationwide Children's Hospital.

2.2. Bone-associated cell isolation and culture

Femora and tibiae were harvested from mice. Muscle and soft tissues were removed from bones carefully under a stereomicroscope. After both ends of bone were removed, bone marrow cells were flushed with phosphate-buffered saline (PBS) using a 5-mL syringe and a 27G needle. Empty bones containing the diaphysis and a part of the metaphysis were placed in conical glass vials (REACTI VIALS; Thermo Fisher Scientific, Waltham, MA) and minced into small fragments in Dulbecco's modified Eagle medium-Ham's F12 mixture (DMEM/F12; Mediatech Inc., Manassas, VA, 3.151 g of glucose per liter). During this step, bone fragments were washed with DMEM/F12 several times and the supernatants were pooled for further cell isolation. Bone granules were digested on a shaker for 120 min at 37 °C with collagenase P (0.2 mg/mL; Roche Diagnostics, Mannheim, Germany) in DMEM/F12 supplemented with 100 U/mL penicillin/streptomycin (Mediatech Inc.) and washed with DMEM/F12. Following digestion, the collagenase P solution and washing media were combined with the above pooled supernatants, and were filtered through a 40 μm cell strainer (Corning, Corning, NY). Bone-associated cells were collected by centrifugation (500*g*, 5 min). The digested bone granules were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 50 μM L-ascorbic acid (Sigma-Aldrich, St. Louis, MO), and 100 U/mL penicillin/streptomycin as previously described [18]. Bone-associated cells, especially DsRed positive cells, grew significantly better in DMEM/F12 than in Minimum Essential Medium Eagle Alpha (αMEM) as shown in Supplemental Fig. 1.

2.3. In vitro differentiation

Osteoblastic differentiation was induced by switching the culture medium to DMEM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerol phosphate (Sigma-Aldrich), and 50 μM L-ascorbic acid. After 4 weeks of osteoblastic differentiation, cells were imaged for fluorescence on an AxioObserver A1 (Carl Zeiss, Thornwood, NY) using AxioVision 4.5SP1 software (Carl Zeiss). To isolate cells from mineralized extracellular matrix, Cells were then treated with 0.25% trypsin solution (Mediatech Inc.) and 173 U/mL collagenase type I solution (Worthington Biochemical Corporation, Lakewood, NJ) at 37 °C for 60 min. Cells were collected with a cell scraper followed by centrifugation (500*g*, 5 min). After fixation with 4% paraformaldehyde in PBS (4% PFA) at 4 °C for 45 min, cells were decalcified with 15% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) in PBS at 4 °C overnight. The cells were further dissociated by collagenase type I treatment at 37 °C overnight. Cells were filtered with a 70 μm cell strainer (Corning) prior to flow cytometric analysis. The cell recovery efficiency after the filtration was 88.2 ± 11.0% of initial cell population as assessed by measuring the quantity of DNA in the cells using a QIAamp DNA Mini Kit (Qiagen, Germantown, MD; Supplemental Fig. 2).

2.4. Flow cytometry

Peripheral blood was collected from the retro-orbital sinus. Following red blood cell (RBC) lysis, blood, bone marrow cells, and bone-associated cells were analyzed on a BD FACSCalibur and a BD LSRII (BD Biosciences, San Jose, CA) using allophycocyanin (APC) conjugated anti-mouse CD45 (30-F11; BD Biosciences). CD45 −/DsRed +/GFP − cells and CD45 −/DsRed −/GFP + cells were sorted from bone-associated cells isolated from the triple transgenic mice using a BD FACSAria. The DsRed positivity in CD45-positive cells was calculated with the following formula: DsRed positivity = the percentage of CD45 +/DsRed + cells / the percentage of CD45 + cells × 100. Data were analyzed using FlowJo software version 7.6 (Tree Star, Inc., Ashland, OR).

2.5. Immunofluorescent staining and analysis

Bones were fixed in 4% PFA at 4 °C for 24 h following the removal of soft tissues. After decalcification with 15% EDTA solution for 3 weeks, bones were embedded in paraffin and cut into 6 μm sections. After deparaffinization, sections were treated with 0.25% trypsin solution for 13 min at room temperature to retrieve antigens before blocking with normal serum. These sections were incubated at 4 °C overnight with polyclonal goat anti-GFP antibody (1:200, Novus Biologicals LLC., Littleton, CO), polyclonal rabbit anti-RFP antibody (1:200, Abcam, Cambridge, MA), monoclonal rat anti-mouse CD45 antibody (30-F11, 1:100, BD Biosciences), polyclonal rabbit anti-mouse osteocalcin antibody (1:1000, Takara Bio, Japan), monoclonal rat anti-mouse F4/80 antibody (A3-1, 1: 200, AbD Serotec, Raleigh, NC), polyclonal rabbit anti-rat Cathepsin K (1:100, Abcam), monoclonal rat anti-mouse Ter119 antibody (TER-119, 1:100, BD Biosciences), monoclonal rat anti-mouse CD3 (RM0027-3B19, 1:200, Abcam), monoclonal rat anti-mouse CD45R antibody (RA3-6B2, 1:200, BD Biosciences), monoclonal rat anti-mouse Gr-1 (RB6-8C5, 1:200, AbD Serotec) and/or monoclonal rat anti-mouse CD11b (M1/70, 1:50, Abcam). Subsequently, sections were stained

with the appropriate secondary antibodies such as Alexa Fluor 488 anti-goat IgG, Alexa Fluor 488 anti-rat IgG, Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 555 anti-rabbit IgG (1:200, Life Technologies) for 2 h at room temperature. Finally, sections were mounted with an antifade reagent containing 4′, 6-diamidino-2-phenylin-dole (DAPI) for nuclear staining (Life Technologies).

All images were acquired either with an Axiocam HRc camera or Axiocam MRm camera using AxioVision 4.5SP1 software (Carl Zeiss, Thornwood, NY) on Zeiss Axio Imager A1, or using ZEN software on Zeiss LSM 510 Meta (Carl Zeiss, Thornwood, NY).

2.6. Heterotopic ossification and fracture model

Heterotopic ossification (HO) was induced by implanting collagen discs containing 2 μg of recombinant human bone morphogenetic protein-2 (BMP-2; GenScript USA Inc., Piscataway, NJ) onto the backs of mice as previously described [3,4]. Briefly, BMP-2 was reconstituted in 5 mM HCl at a concentration of 100 μg/mL. 2 μg BMP-2 was diluted with 5 μL of PBS and loaded into a porous collagen disc (6 mm diameter, 1 mm thickness). The freeze-dried BMP-2 containing discs were implanted underneath the muscle fascia of the back of mice. Three weeks later, the discs were harvested and processed for histological analysis.

Closed transverse mid-diaphyseal tibial fractures were created on the left leg as previously described [19]. A 30G needle was introduced into the intramedullary canal of the tibia through a hole drilled above the tibial tuberosity. After closing the wound, closed mid-diaphyseal fractures were made by three-point bending.

2.7. RNA extraction and quantitative PCR

Total RNA was isolated from FACS-sorted cells using an RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative PCR was performed with 45 cycles on ABI 7500 Fast Real-Time Systems (Life Technologies) using TaqMan Gene Expression Assays (Life Technologies) for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), beta-2 microglobulin (*B2m*), cyclin-dependent kinase inhibitor 1B (*Cdkn1b*), ribosomal protein large P2 (*Rplp2*), actin beta (*Actb*), osteopontin (*Spp1*), runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (*Alp*), bone sialoprotein (*Bsp*), type I collagen (*Col1*), osteocalcin (*Bglap*), osterix (*Sp7*), CD45 (*Ptprc*) and F4/80 (*Emr1*). To determine and validate the most appropriate Cycle threshold (Ct) for low expressing genes, we titrated cDNA concentration for designated genes using one of the CD45 −/DsRed +/GFP − cell samples which had the least quantity of cDNA (Supplemental Fig. 3). 1× cDNA refers to the amount of cDNA that was used to perform the experiments shown in Fig. 5. The linearity of Ct values was maintained at 1/2× cDNA to Ct ≤ 38 ($R^2 > 0.99$) but deviated from linearity at lesser amounts of cDNA. Thus, we considered Ct values <38 as a valid indicator of gene expression. Data were analyzed using SDS 1.4 software.

2.8. Statistical analysis

Statistical analyses were performed by either paired or unpaired two-tailed *t*-test for comparison of two samples, and by one-way analysis of variance for multiple samples followed by Tukey's multiple comparisons test with Prism (Version 6; GraphPad Software Inc., La Jolla, CA). A minimum of three replicates were used for statistical analyses for each experiment. All statistical data are shown with mean ± standard error of the mean. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Analyses of CD45Cre × Z/RED F1 mice

In order to track the fate of hematopoietic cells, we utilized CD45-Cre mice, which express Cre recombinase under the promoter of the pan-leukocyte marker (CD45), crossed with Z/RED reporter mice, which express DsRed, a variant of red florescent protein, upon Cre mediated recombination. The CD45-Cre × Z/RED F1 mice allow us to trace the fate of hematopoietic cells as DsRed is expressed in all cells that have expressed CD45 at any point in their development even if they no longer express CD45. To evaluate the efficiency of Cre-mediated recombination, flow cytometric analyses were performed on peripheral blood cells and bone marrow cells following RBC lysis. DsRed was successfully detected in 91.7 ± 1.1% of CD45-positive cells in peripheral blood (Fig. 1A, P < 0.0001 vs. wild type control) and

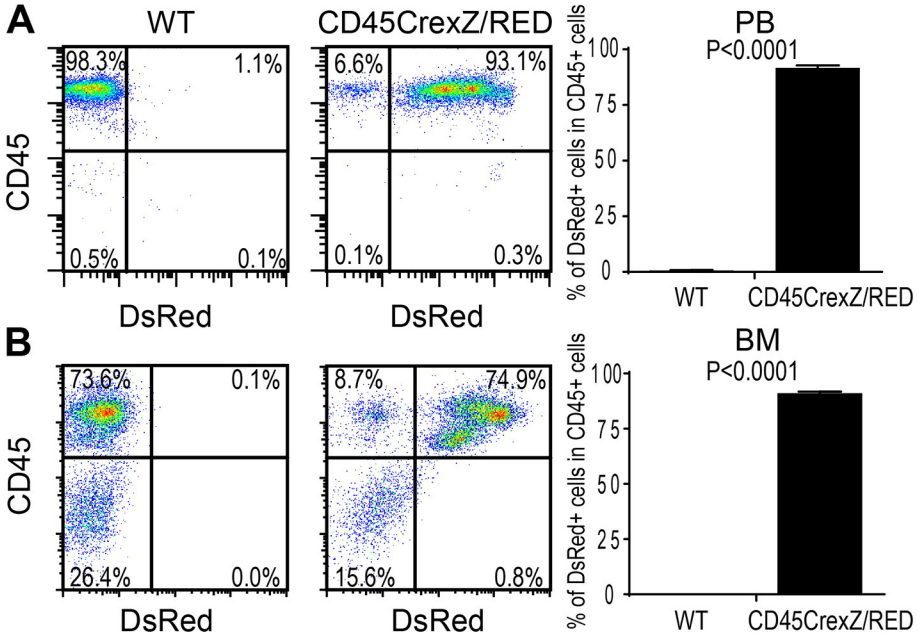


Fig. 1. Flow cytometric analyses of CD45Cre × Z/RED F1 mice. Peripheral blood (PB; A) and bone marrow (BM; B) from CD45Cre × Z/RED F1 mice and wild type mice (WT) as a control were analyzed by flow cytometry. The percentage of DsRed-positive cells in CD45-positive cells was shown in the bar graphs. All data were shown as mean ± SEM.

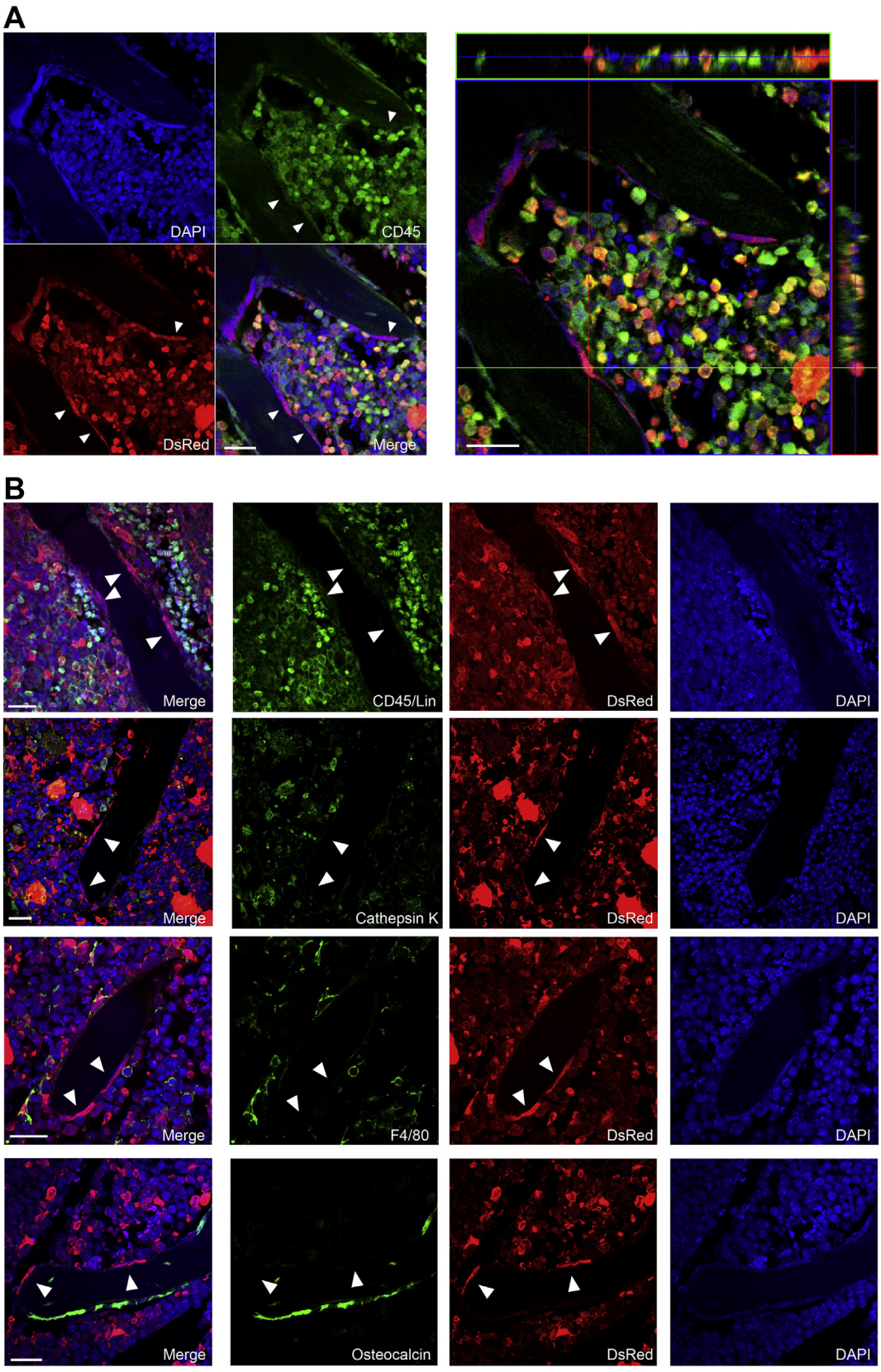


Fig. 2. Immunofluorescent staining of bone sections from CD45Cre × Z/RED F1 mice. Bone sections from CD45Cre × Z/RED F1 mice were stained for DAPI (blue), CD45 (green) and DsRed (red). Some of the bone lining cells (BLCs) were positive for DsRed but not for CD45 as shown by the white arrowheads. Confocal microscopy confirmed that the DsRed-positive BLCs were negative for CD45 (A). The sections were also stained for CD45 and hematopoietic lineages (Lin), cathepsin K, F4/80, or osteocalcin along with DsRed. The DsRed-positive BLCs were negative for CD45/Lin, cathepsin K, F4/80 and osteocalcin indicated by white arrowheads (B). Scale bars are 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

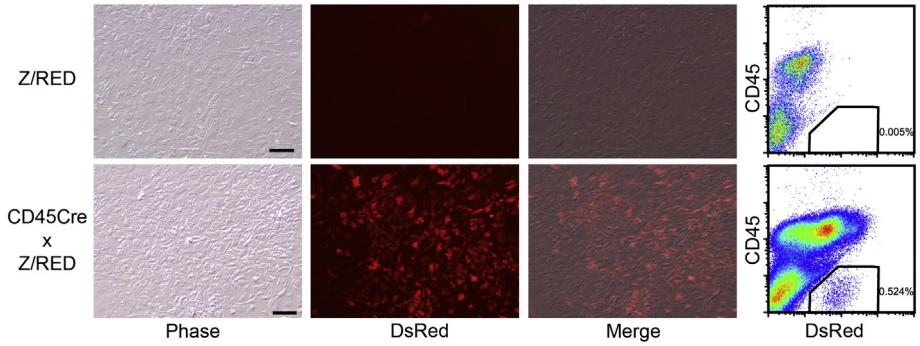


Fig. 3. Culture of BLCs. Bone-associated cells were isolated from CD45Cre × Z/RED F1 mice and Z/RED mice as a control and cultured in DMEM/F12 medium. DsRed-positive cells were successfully expanded in culture and flow cytometric analyses identified CD45-negative, DsRed-positive BLCs. Scale bars are 100 μm.

91.0 ± 0.8% of CD45-positive cells in bone marrow (Fig. 1B, $P < 0.0001$ vs. wild type), demonstrating a substantial level of recombination in our Cre/lox P system (Fig. 1A and B). DsRed positivity in hematopoietic lineage cells such as CD11b, F4/80 and Gr-1 populations was also over 90%, further supporting the reliability of this system (Supplemental Fig. 4).

Consistent with the results from flow cytometric analyses, immunofluorescent staining of bone sections showed that a majority of bone marrow cells were double-positive for DsRed and CD45 (Fig. 2A). Interestingly, we found some cells lining the endosteal bone surface that expressed DsRed but were negative for CD45 as well as hematopoietic lineage markers (Fig. 2A and B). These unique bone lining cells (BLCs) had a spindle shape similar to other BLCs including osteoblasts; however, these red BLCs were negative for osteocalcin, suggesting that they are not mature osteoblasts (Fig. 2B). Further staining also revealed that the red BLCs did not express F4/80 or Cathepsin K, indicating that the BLCs are less likely to be macrophages or osteoclasts (Fig. 2B).

3.2. Isolation and culture of the red BLCs

To isolate BLCs as well as bone cells, we first flushed out bone marrow and cut empty bones into small pieces followed by collagenase digestion as previously described [18]. During this grinding and digesting step, we collected bone-associated cells detached from bones and processed them for flow cytometry. Analyses of the CD45-negative fraction revealed that the red BLCs were positive for CD44 and CD29 but negative for hematopoietic markers including CD3, B220, CD11b, F4/80 and Gr-1 (Supplemental Fig. 5), which is consistent with our immunofluorescent staining. The digested bone fragments were then cultured in DMEM/F12 medium. DsRed positive cells adhered onto culture dishes and successfully expanded in culture (Fig. 3). Despite bone marrow depletion, flow cytometric analyses of the primary culture displayed contamination of CD45-positive hematopoietic cells. However, the red BLCs were clearly detected as the DsRed+/CD45− population as shown in Fig. 3.

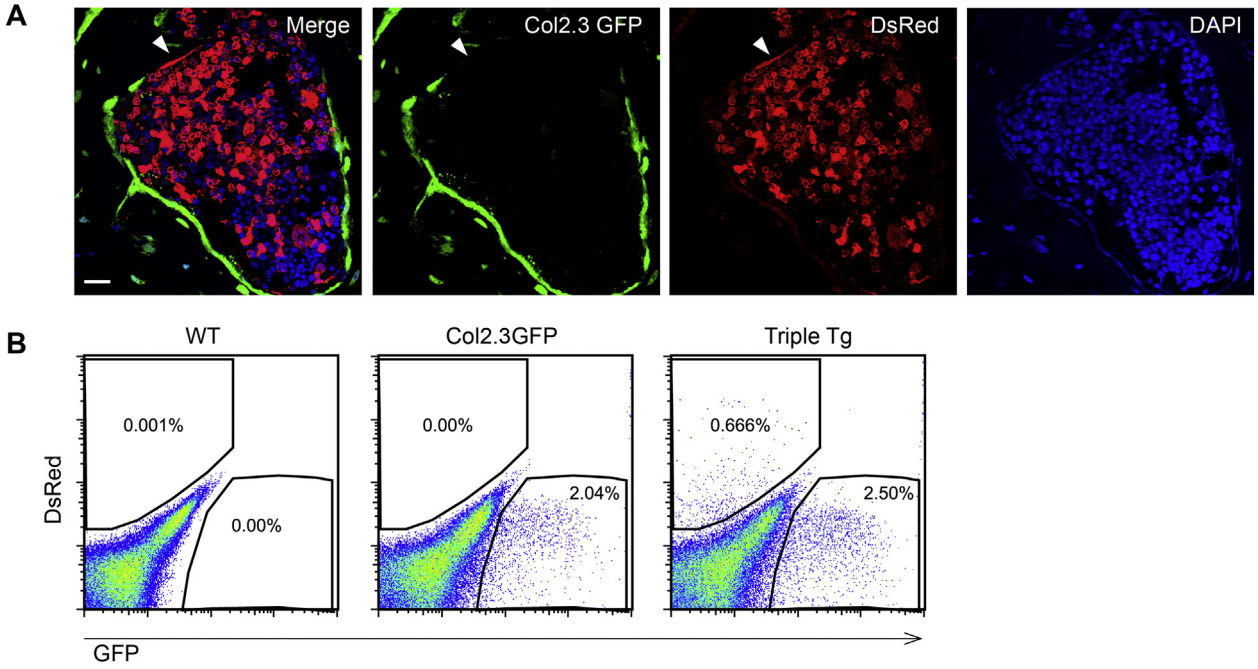
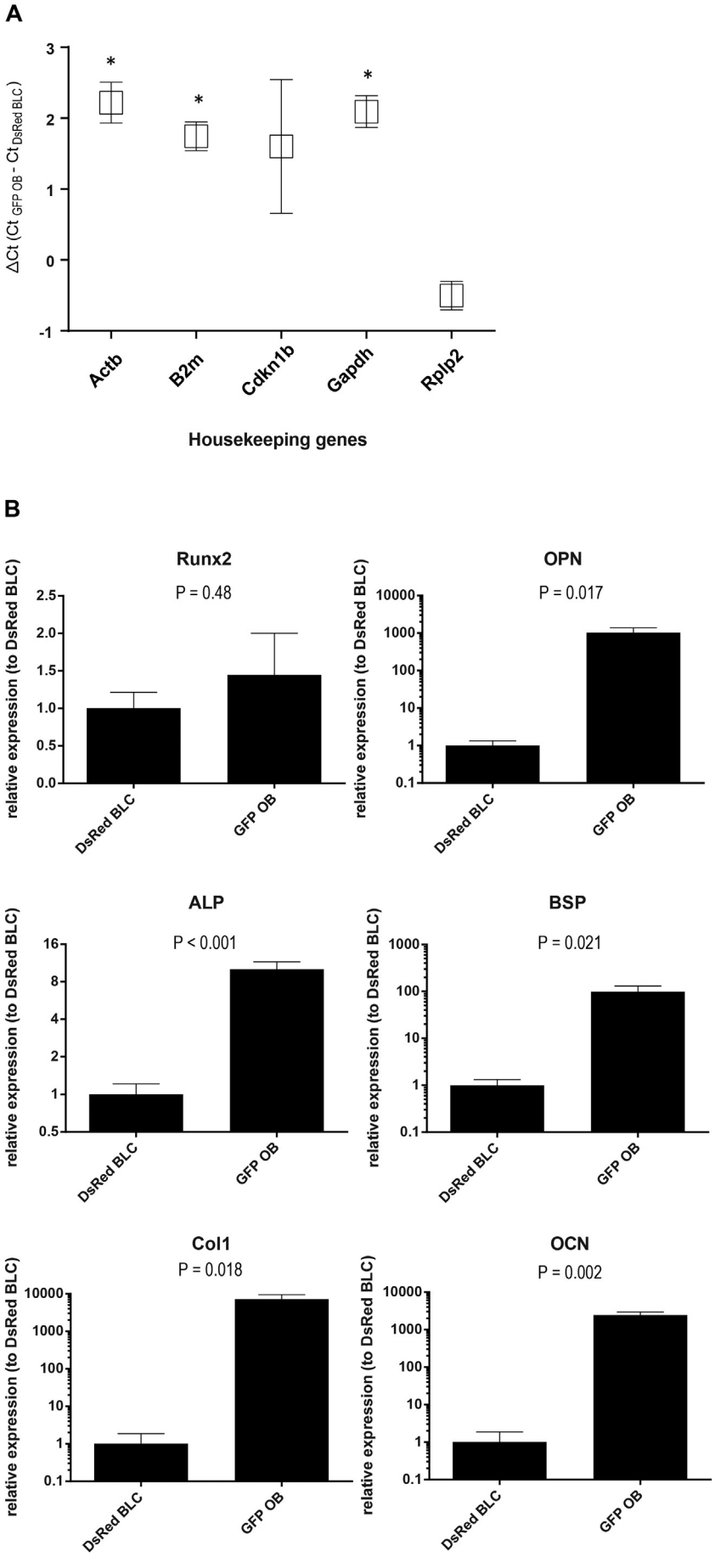


Fig. 4. Analyses of triple transgenic mice. Bone sections from the triple transgenic mice developed by crossing CD45-Cre mice, Z/RED mice and Col2.3GFP mice, were stained for GFP (green), DsRed (red) and DAPI (blue). DsRed-positive BLC (white arrowhead) did not express GFP (A). Bone-associated cells isolated from either wild type mice (WT), Col2.3GFP mice or the triple transgenic mice were analyzed by flow cytometry. When gated in CD45-negative fractions, single positive cells for either GFP or DsRed were detected. However, no double positive cells were found in the triple transgenic mice. Scale bar is 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)


A
ΔCt (Ct GFP OB - Ct DsRed BLC)
3
2
1
0
-1
*
*
*
Actb
B2m
Cdkn1b
Gapdh
Rplp2
Housekeeping genes
B
Runx2
P = 0.48
OPN
P = 0.017
ALP
P < 0.001
BSP
P = 0.021
Col1
P = 0.018
OCN
P = 0.002
relative expression (to DsRed BLC)
DsRed BLC
GFP OB

3.3. Development of triple transgenic mice

To examine whether the red BLCs have the potential to give rise to functional mature osteoblasts in normal skeletogenesis, we developed triple transgenic mice by crossing three transgenic strains, CD45-Cre mice, Z/RED mice and Col2.3GFP mice which express GFP in mature osteoblasts and osteocytes. In this triple transgenic model, osteoblasts/osteocytes produce GFP, and hematopoietic cells and their descendant cells express DsRed. If there are any mature osteoblasts and osteocytes derived from CD45+ cells, they will be detected as double positive for GFP and DsRed. Immunofluorescent staining of bone sections from the triple transgenic mice successfully showed GFP positive osteoblasts/osteocytes as well as red BLCs; however, none of these red BLCs co-expressed GFP (Fig. 4A). To confirm that the red BLCs were not positive for GFP in a more objective way, bone-associated cells including osteoblasts and BLCs were isolated from bones, stained with CD45 and analyzed on a flow cytometer. Analyses of the CD45-negative population detected single positive cells either for GFP or DsRed, but no double positive cells (Fig. 4B), which is consistent with our immunohistological analyses. These data suggest that red BLCs do not contribute to skeletogenesis in the development of osteoblasts.

3.4. Osteoblastic gene expression

To further examine the red BLCs, CD45−/DsRed+/GFP− red BLCs and CD45−/DsRed−/GFP+ mature osteoblasts were sorted from freshly isolated bone-associated cells for gene expression analyses. To identify appropriate housekeeping genes whose expression is maintained across samples, we first evaluated the expression level of five distinct housekeeping genes including Actb, B2m, Cdkn1b, Gapdh and Rplp2. The difference of Ct values between GFP-positive mature osteoblasts and the red BLCs were approximately 2 with less deviation in Actb, B2m and Gapdh compared to Cdkn1b and Rplp2, indicating that the expression of these three housekeeping genes is less variable across samples (Fig. 5A). Thus, we used Gapdh as a housekeeping gene for normalization to compare the expression level of osteoblastic genes such as Runx2, osterix (Osx), osteopontin (OPN), alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen 1a1 (Col1), and osteocalcin (OCN), between these two cell populations using quantitative reverse transcription PCR. As anticipated, all of the osteoblastic markers were highly expressed in mature osteoblasts. However, even though expression was detected for all genes but osterix, the red BLCs showed significantly reduced expression levels of all genes with the exception of Runx2 (Fig. 5B). Importantly, neither F4/80 nor CD45 were detectable in either cell population (Data not shown). These data indicate that these are two different populations and the red BLCs are not mature osteoblasts. Moreover, consistent with the immunofluorescent staining and flow cytometric analyses, the red BLCs are not osteal macrophages known as OsteoMacs, which are positive for F4/80.

3.5. In vitro osteogenic differentiation

The gene expression analyses showed that the red BLCs expressed significantly less osteoblastic genes compared to GFP-positive mature osteoblasts. However, signals from all of the genes in the red BLCs were measurable, raising the question whether the red BLCs are osteoblast progenitor cells that have the potential to become mature osteoblasts. To address this question, we cultured red BLCs and osteoblasts from bone fragments of the triple transgenic mice as shown in Fig. 3, and then induced osteogenic differentiation. After 4-weeks of culture in osteogenic medium, we evaluated the GFP expression in DsRed positive cells. As shown in Fig. 6A, culture in the osteogenic medium successfully induced osteoblast differentiation, resulting in GFP expression. Flow cytometric analyses demonstrated that DsRed-positive cells were distinct from GFP-positive cells, although the cellular autofluorescence was slightly higher presumably due to calcium deposition from osteoblasts (Fig. 6B). These results suggest that the CD45-derived cells including the red BLCs essentially lack the ability to differentiate into osteoblasts even under in vitro osteogenic conditions.

3.6. Contribution to heterotopic bone formation and fracture healing

Our findings demonstrated that CD45-expressing cells and their descendants were not able to differentiate into osteoblasts in vitro, and did not contribute to bone genesis as osteoblasts during development and homeostasis. To investigate if these cells have the potential to differentiate into osteoblasts in tissue regeneration, we made heterotopic bone by implanting a BMP-2 pellet, or a tibia fracture in the triple transgenic mice. Three weeks after the implantation or the induction of fracture, heterotopic bones and fractured tibiae were harvested and evaluated histologically. Immunofluorescent staining showed successful bone formation in both models; however, no cells double positive for GFP and DsRed were detected (Fig. 7A and B). These findings indicate that cells derived from CD45-positive cells do not differentiate into osteoblasts even though they localize along the endosteal bone surface.

4. Discussion

Although many efforts have been made, the cellular origin of osteoblasts responsible for HO is a long standing question which has yet to be solved [20]. Various candidate cells, both hematopoietic and non-hematopoietic, derived from different germ layers have been shown to potentially contribute to HO [1,3–7,12,21–24]. We have previously demonstrated that circulating osteoblast progenitor cells originating from bone marrow contributed to the development of heterotopic bone induced by BMP-2 [3,4]. These circulating osteoblast progenitor cells are negative for CD45, suggesting a non-hematopoietic origin of HO osteoblasts [4]. Contrary to our findings, others have reported that CD45 positive circulating cells formed HO [5,8,25,26]. Therefore, in this study, we traced hematopoietic cells using CD45-Cre mice and investigated whether hematopoietic derived cells contributed to skeletogenesis, HO and fracture healing. The advantage of using the Cre/loxP system is the ability to trace descendant cells derived from Cre-expressing cells, even if they discontinue expressing Cre. In our mouse model, cells that express CD45 at any developmental stage become DsRed positive and remain positive for DsRed even if CD45 expression turns off. In other words, all DsRed positive cells in our experimental animals originated from CD45 positive hematopoietic cells. Our results showed that over 90% of CD45 positive cells expressed DsRed, validating the efficiency of Cre-mediated recombination in our animal model. Additionally, we combined this Cre/loxP murine system with Col2.3GFP mice which are known to label mature osteoblasts with GFP [17], allowing us to evaluate if hematopoietic derived cells can give rise to mature osteoblasts. Our analyses of these triple transgenic mice demonstrated that hematopoietic derived cells did not differentiate into mature osteoblasts either in developmental skeletogenesis, in HO, or in bone regeneration after fracture. These results were supported by our findings that hematopoietic derived cells did not have the potential to become mature osteoblasts even under in vitro osteogenic conditions. Taken together, the results led us to

Fig. 5. Osteoblastic gene expression. The CD45−/DsRed+/GFP− BLCs and CD45−/DsRed−/GFP+ osteoblasts (OB) shown in Fig. 4 were sorted using a cell sorter followed by RNA isolation. The cycle threshold (Ct) values of Five housekeeping genes (Actb, B2m, Cdkn1b, Gapdh and Rplp2) were compared between GFP+ OB and DsRed + BLC (ΔCt = Ct value of GFP OB − Ct value of DsRed BLC) (A, *P < 0.05 the post-hoc analyses compared to Rplp2). Gene expression for Runx2, osteopontin (OPN), alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen 1a1 (Col1), and osteocalcin (OCN) were evaluated by quantitative PCR. The expression level of each gene was normalized to that of Gapdh (B). Gene expression for osterix is not shown since it was undetectable in DsRed+ BLCs. All data were shown as mean ± SEM.

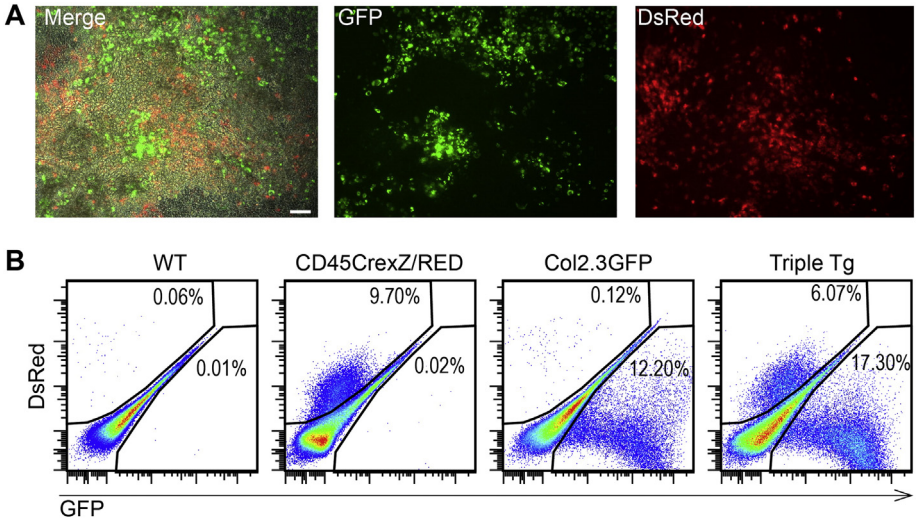


Fig. 6. In vitro osteogenic induction. Bone-associated cells isolated from either wild type mice (WT), CD45Cre × Z/RED mice, Col2.3GFP mice, or the triple transgenic mice were cultured and induced osteogenic differentiation for 4 weeks. Some of the bone-associated cells from the triple transgenic mice became mature osteoblasts expressing GFP (A). Flow cytometric analyses of these cells from osteogenic culture showed that DsRed-positive cells were negative for GFP (B). Scale bar is 100 μm.

conclude that hematopoietic derived cells are not responsible for heterotopic bone formation as osteoblasts in murine models. Possible explanations for the discovered inconsistencies with the previous findings by Suda et al. showing the contribution of CD45 positive circulating osteogenic precursor (COP) cells to heterotopic bone formation in patients with FOP [5] could be: 1) species differences between humans and mice; 2) our HO model induced by BMP-2 might not exactly reflect the pathogenesis and pathophysiology of patients with FOP; and 3) imperfection of Cre-mediated recombination, despite over 90% efficiency, might fail to label the CD45 positive COP cells, resulting in the failure to detect the contribution of these cells to HO.

Interestingly, we identified novel BLCs along the endosteal surface. They expressed DsRed, indicating that they were developmentally derived from CD45-expressing progenitors; however, they are currently negative for CD45. Since Cre is inserted downstream of the endogenous CD45 gene connected with an IRES using a BAC vector, its expression is tightly controlled by the endogenous CD45 promoter, suggesting that a leak of Cre expression into CD45 negative cells seems to be less likely as previously described [15]. The novel BLCs are spindle shaped like other endosteal cells, although they seem to be more flattened compared to mature osteoblasts. Using our triple transgenic mice with GFP-expressing mature osteoblasts, we found that these red BLCs did not co-express GFP in regular skeletogenesis, BMP-2 induced heterotopic ossification, and fracture repair, suggesting that they are not mature osteoblasts. Gene expression analyses also showed the red BLCs were immature for osteoblasts and were clearly distinct from mature osteoblasts. These red BLCs may not have potential to become mature osteoblast since they failed to differentiate even in osteogenic induction medium, although the hyperglycemic medium conditions may have reduced their osteogenic capacity [27]. It is well known that the endosteum contains osteoblastic cells in various differentiation stages. It has been thought that all endosteal immature osteoblastic cells are meant to be mature; however, our findings might provide novel insight that there is a unique endosteal cell population that does not have potential to become mature osteoblasts. Given that there is currently no way to distinguish the red BLCs from other preosteoblasts without using the CD45-Cre mice, further investigation is necessary to fully characterize them.

Importantly, the red BLCs are unlikely to be osteoclasts since they are negative for Cathepsin K, have a flattened shape, and are mononuclear making them distinct from multinucleated osteoclasts. Additionally, Chang et al. have previously reported that resident macrophages, designated as OsteoMacs, are intercalated bone lining cells [28]. They found that OsteoMacs are positive for F4/80 and localize in the endosteum forming a characteristic canopy structure over mature osteoblasts [28]. However, our red BLCs were negative for F4/80 as demonstrated by immunofluorescent staining, flow cytometry and quantitative PCR. Moreover, they did not form a canopy structure covering mature osteoblasts; rather they resided side by side with mature osteoblasts on the endosteal surface. Thus, these findings indicate that the red BLCs are unlikely to be OsteoMacs.

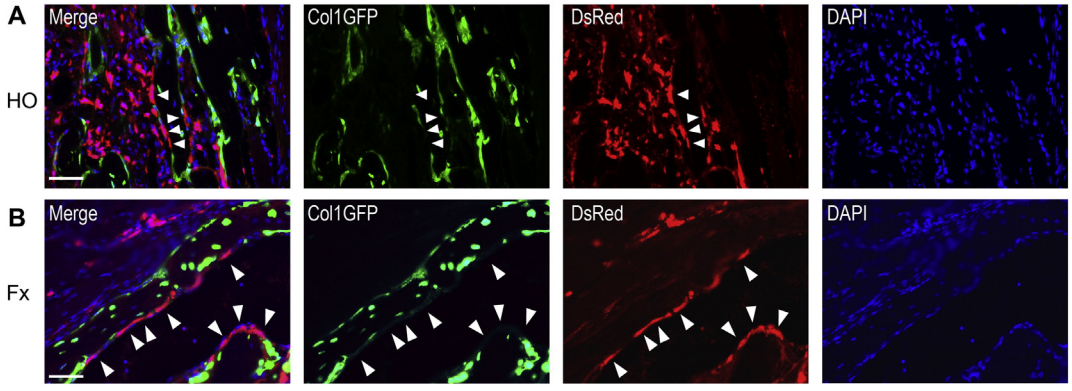


Fig. 7. Contribution to heterotopic bone formation and fracture healing. Sections from BMP-2 induced heterotopic ossification (HO) and fractured tibiae (Fx) were stained for GFP (green), DsRed (red) and DAPI (blue). DsRed-positive bone lining cells (white arrowheads) were found in both HO and Fx, however, they were negative for GFP. Scale bars are 50 μm.

Finally, given that the red BLCs were observed not only in the healing fractured bone but also in de novo bone marrow formed inside of heterotopic bone, the red BLCs might play an important role in osteogenesis and/or in bone marrow formation, even though they cannot give rise to mature osteoblasts. Further investigation should be performed to understand the true meaning and purpose of their existence in the endosteum.

In conclusion, we traced hematopoietic derived cells in fracture healing and HO using triple transgenic mice developed from CD45-Cre, Z/RED and Col2.3GFP transgenic mouse strains. We found that hematopoietic derived cells lacking CD45 expression were located along endosteal bone surface. However, these cells lacked any potential to give rise to mature osteoblasts and failed to directly contribute to osteogenesis in fracture healing and HO.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2016.10.003.

Conflicts of interest

All authors have no conflicts of interest.

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