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Osteoclasts Derive Predominantly from Bone Marrow–Resident CX3CR1⁺ Precursor Cells in Homeostasis, whereas Circulating CX3CR1⁺ Cells Contribute to Osteoclast Development during Fracture Repair

Sanja Novak, Emilie Roeder, Judith Kalinowski, Sandra Jastrzebski, Hector L. Aguita, Sun-Kyeong Lee, Ivo Kalajzic, and Joseph A. Lorenzo

Osteoclasts (OC) originate from either bone marrow (BM)–resident or circulating myeloid OC progenitors (OCP) expressing the receptor CX3CR1. Multiple lines of evidence argue that OCP in homeostasis and inflammation differ. We investigated the relative contributions of BM-resident and circulating OCP to osteoclastogenesis during homeostasis and fracture repair. Using CX3CR1EGFP/TRA tweTomato mice, we found CX3CR1 expression in mononuclear cells, but not in multinucleated TRAP⁺ OC. However, CX3CR1-expressing cells generated TRAP⁺ OC on bone within 5 d in CX3CR1CreERT2/Ai14 tdTomato reporter mice. To define the role that circulating cells play in osteoclastogenesis during homeostasis, we parabiosed TRAP tweTomato mice (CD45.2) on a C57BL/6 background with wild-type (WT) mice (CD45.1). Flow cytometry (CD45.1/45.2) demonstrated abundant blood cell mixing between parabionts after 2 wk. At 4 wk, there were numerous tdTomato⁺ OC in the femurs of TRAP tweTomato mice but almost none in WT mice. Similarly, cultured BM stimulated to form OC demonstrated multiple fluorescent OC in cell cultures from parabionts, but not from WT mice. Finally, flow cytometry confirmed low-level engraftment of BM cells between parabionts but significant engraftment in the spleens. In contrast, during fracture repair, we found that circulating CX3CR1⁺ cells migrated to bone, lost expression of CX3CR1, and became OC. These data demonstrate that OCP, but not mature OC, express CX3CR1 during both homeostasis and fracture repair. We conclude that, in homeostasis mature OC derive predominantly from BM-resident OCP, whereas during fracture repair, circulating CX3CR1⁺ cells can become OC. The Journal of Immunology, 2020, 204: 868–878.

B one homeostasis is a complex process, requiring the precise coordination between bone-forming and bone-resorbing cells. Osteoclasts (OC) are the only cells that can efficiently resorb bone. Pathologic regulation of OC formation and function contributes to the development of diseases, like inflammatory osteolysis and osteoporosis, which produce low bone mass and an increased risk of fragility fractures (1). OC-mediated bone resorption is critical for bone remodeling, which maintains the structural integrity of the skeleton by replacing worn or microfractured bone (1).

The OC is a unique, large, multinucleated cell, originating from a common mononuclear myeloid precursor that can also differentiate into macrophages and dendritic cells (2). Although the hematopoietic origin of the OC is well established (3, 4), the identification of its direct progenitor cell is not fully resolved (2, 5). In addition, it remains unclear whether OC in either pathologic or homeostatic conditions derive predominantly from a circulating monocyte precursor or a bone marrow (BM)–resident progenitor that commits to the OC lineage without circulating.

Our group characterized a population of murine OC progenitors (OCP) that were isolated from BM as CD45R− CD3⁻ CD11blow/low CD115⁺ , CD117high, intermediate, or low. These OCP are able to differentiate and mature in vitro into OC with >90% efficiency, as determined by single-cell cloning. In addition, others demonstrated that OCP in murine BM express the receptor CX3CR1 for the chemokine, fractalkine (CX3CL1) (6, 7). CX3CR1 is membrane bound and functions as both a chemo-attractant and adhesion molecule (8). CX3CL1 is produced by osteoblasts and has been shown to play a role in osteoclastogenesis through its ability to attract OCP to the resorption site (6, 7). In turn, this result suggests a role for CX3CR1, which is expressed on OCP (6, 9), in regulating OC migration. Initial studies of the persistence of CX3CR1 expression during OC maturation were performed in vitro and used murine BM cells (6, 7). These demonstrated an almost complete downregulation of CX3CR1 expression in mature OC. However, interpretation of the latter finding is complicated by the results of Ibáñez et al. (10), who described a separate origin for OC that were induced under
inflammatory conditions. These authors found that a proportion (20–30%) of inflammatory OC maintained expression of Cx3C1R1 throughout maturation. In contrast, they reported that expression of Cx3C1R1 on OC derived during noninflammatory conditions was less frequent (4–7%).

Currently, there is strong evidence that circulating cells are a source of OCP during states that “open” or perturb the integrity of the BM microenvironment. These include fracture (11), inflammation (5), enhanced resorption (12), osteoblast death (13), and defective OC function (14). However, the relative roles that BM-resident and circulating cells play in osteoclastogenesis during unperturbed or homeostatic conditions is controversial. Data implicating both BM-resident and circulating cells as the major source of OCP during homeostasis have been reported (12, 13, 15).

In this study, we used newer and more sensitive techniques than those employed previously to investigate the rate that OC matures from precursors in vivo and the role that circulating and bone-resident OCP play in the development of mature OC during homeostasis and fracture repair. We first evaluated how rapidly OCP-expressing Cx3CR1 differentiate into mature OC in vivo using CX3CR1CreER mice and injected these into the circulation of mice undergoing fracture repair throughout maturation. In contrast, they reported that expression of GFP in mature OC to identify circulating cells that shared-blood parabiosis system. This model employed TRAP tdTomato mice, which express high levels of the tdTomato fluorescent protein in mature OC to identify circulating cells that formed OC in bone. Finally, using dual transgenic CX3CR1-GFP/tdTomato mice, we isolated CX3CR11 BM cells and injected these into the circulation of mice undergoing fracture repair to determine if circulating CX3CR11 cells could incorporate into mature OC during this process.

Materials and Methods

**Experimental animals**

Female mice in a C57BL/6 background were used for all experiments, except for the adaptive transfer experiment, in which males were used. Unless otherwise stated, all mice were analyzed between 14 and 15 wk of age. CX3CR1CreER mice (16) were purchased from The Jackson Laboratory (stock no. 020940) and housed in the Center for Comparative Medicine at UConn Health under standard housing conditions. These mice have exon 1 of their Cx3cr1 gene replaced by a construct that expresses Cre-ER2. Because the construct was “knocked into” the Cx3cr1 gene, its expression is controlled by the endogenous Cx3cr1 gene regulatory elements. CX3CR1CreER mice were crossed with Ai14 Cre reporter mice from The Jackson Laboratory (stock no. 007914) to generate CX3CR1CreER/Ai14 mice. The Ai14 Cre reporter mouse line produces red fluorescent tdTomato protein in cells that express active Cre recombinase and their progeny.

TRAP tdTomato mice were a gift from Dr. M. Ishii (17) (Immunity Frontier Research Center, Osaka University, Osaka, Japan). This transgenic mouse contains a bacterial artificial chromosome construct that uses the endogenous mouse Acp5 regulatory sequence to drive expression of tdTomato protein.

CX3CR1-GFP mice were obtained from The Jackson Laboratory (stock no. 005582) (18). A C57BL/6 congenic strain that expresses CD45.1 rather than CD45.2, which is present on most C57BL/6 mouse lines, was obtained from The Jackson Laboratory (stock no. 002014, Pep Boy).

All animals used were heterozygous for the transgene used (CX3CR1-GFP or TRAP tdTomato).

The Institutional Animal Care and Use Committee of UConn Health approved all animal protocols.

**Flow cytometry and FACS**

The Abs used for flow cytometric analysis are all commercially available. These include anti-mouse CD117 (clone, ACK2, APC conjugated, catalog no. 17-1171-83; eBioscience, Carlsbad, CA) and anti-mouse CD115 (c-fms, clone: AFS98, biotinylated, catalog no. 13-1152-85; eBioscience), and anti-mouse CD45.1 (clone: A20, APC conjugated, catalog no. 558701; BD Biosciences, San Jose, CA), and anti-mouse CD45.2 (clone: 104, FITC conjugated, catalog no. 11-0458-85; eBioscience). Streptavidin PE (catalog no. 12-4317-87; eBioscience) was used to detect CD115. Dead cells were excluded by their ability to incorporate propidium iodide. The leukocyte gate was set by the forward and side scatter light properties of the cells and further analyzed for CD45.1 and CD45.2 expression. Unstained sample and single staining controls were used to set the gates for each fluorescent channel. Flow cytometric analysis was done on an LSR II flow cytometry system (BD Biosciences) and data analysis performed using FlowJo software (Tree Star) or FACS Diva v8 (BD Bioscience). Tibia and femur BM cells from CX3CR1-GFP mice were isolated and sorted by the expression of GFP+, GFP+, and GFP+ and further cultured. Sorting was performed on a FACSaria II (BD Bioscience) equipped with five lasers and 18 fluorescence detectors.

**BM cell cultures**

Mouse BM cells were isolated from the femur and tibia by a modification of published methods (19–21). Cells were then cultured (5 × 104 cells per well in 96-well plates) with complete αMEM medium (catalog no. 12571-063; Life Technologies, Paisley, U.K.) with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin–streptomycin (catalog no. 15140-122; Life Technologies) in the presence of mouse M-CSF (catalog no. M2000; ConnStem, Cheshire, CT) and/or mouse receptor activator of NF-κB ligand (RANKL) (catalog no. R2001, ConnStem). BM macrophage/monocyte (BMM) cells were prepared by incubating total BM cells overnight in complete αMEM on tissue culture plastic. Nonadherent cells were collected and mononuclear cells were separated using Ficoll-Hypaque (catalog no. GE17-1440-02; GE Healthcare, Sweden) density gradient centrifugation.

**Pit formation assay**

For analyzing resorptive activity of OCPs, pit formation assay was performed by culturing sorted CX3CR1-GFP+, GFP+, and GFP+ BM cells on UV-sterilized, devitalized bovine cortical bone slices, which we prepared. These were placed in 96-well plates. Cells were treated for 8 d with M-CSF and RANKL (both at 30 ng/ml) (2). Pit perimeter and area per OC were measured using a light microscope (BX53; Olympus Scientific, Waltham, MA) and images analyzed by CellSens software (Olympus, Tokyo, Japan).

**In vitro OC formation assay**

Mouse BMM were cultured at an initial plating density of 5500 cells per well in 96-well culture plates with M-CSF and RANKL (both at 30 ng/ml or dosage indicated). Mouse whole BM (50,000 cells per well of a 96-well plate), spleen cells (50,000 cells per well of a 96-well plate), and blood cells (20,000 cells per well of a 96-well plate) were cultured with M-CSF and RANKL (both at 30 ng/ml) for 6 d. OC precursor population were isolated as described (2) for an in vitro OC formation assay and cultured at 5000 cells per well in 96-well culture plates with M-CSF and RANKL. The medium was replenished every 3 d. At the completion of an experiment, cells were fixed with 2.5% glutaraldehyde in PBS for 15 min at room temperature prior to staining for TRAP using a commercial kit (catalog no. 387A-1KT; Sigma Aldrich, St. Louis, MO). TRAP-positive cells that contained more than three nuclei were counted as OC.

**CX3CR1 expression in mature TRAP+ OCs**

To determine expression of CX3CR1 in mature OCs, we crossed CX3CR1-GFP reporter mice with TRAP tdTomato mice (CX3CR1-GFP/ TRAP tdTomato). Animals were sacrificed at the age of 3 wk, femurs dissected, fixed for 3 d in 10% formalin, transferred to 30% sucrose-PBS overnight, embedded, and cut frozen on a cryostat (Leica, Germany). Slides were mounted with DAPI (catalog no. D-1306; Molecular Probes, Eugene, OR) to identify nuclei, coverslipped and scanned (Axio Scan; Carl Zeiss Microscopy, Germany). After scanning slides for identifying EGFP (green) and tdTomato (red) reporter expression and nuclear staining, sections were stained for hematoxylin and rescreened.

**Real time PCR**

Total RNA from BM cultures was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) and RNA isolated by the manufacturers protocol. RNA concentration and purity were assessed on a NanoDrop One (Thermo Fisher Scientific, Waltham, MA). One microgram of RNA was transcribed to cDNA using a reverse transcription kit (catalog no. 4368814; Applied Biosystems). Quantitative RT-PCR was performed with SYBR Green Master Mix (Thermo Fisher) and primers for Acp5, Csf1, and Acp5.
CX3CR1 and β-actin gene. Data are presented as relative gene expression to β-actin housekeeping gene, calculated using ΔΔ cycle threshold method.

Lineage tracing
CX3CR1CreER/Ai14 female mice at 14 wk of age were sacrificed at either 1 or 5 d after a single s.c. injection of tamoxifen (75 mg/kg body weight) (Sigma Aldrich) dissolved in corn oil (Acricons Organics, Geel, Belgium). Femurs were harvested and fixed for 4 d in 10% formalin, placed in 30% sucrose–PBS overnight before being cryo-embedded with Shandon Cryomatrix embedding resin (catalog no. 6769006; Thermo Fisher Scientific), and 7-μm sections cut on a cryostat (Leica). Sections were stained with anti-RFP Ab (catalog no. 600-401-379, 1:200; Rockland Immunocchemicals) and secondary Ab goat anti-rabbit Alexa 647 (1:500; Invitrogen), mounted with DAPI and coverslipped. Fluorescence for tdTomato and nuclei was imaged on a fluorescent microscope (Axio Scope; Carl Zeiss Microscopy). Afterwards, the coverslip was removed, and the sections were stained for tartrate resistant acid phosphatase using the leukocyte Acid Phosphatase Kit (catalog no. 3874A-1KT; Sigma Aldrich) according to the manufacturer’s protocol or an E197 substrate (catalog no. E6589; Molecular Probes). In brief, sections were prepared for fluorescent TRAP staining by incubating with TRAP buffer (112 mM sodium acetate, 50 mM sodium tartrate dibasic dihydrate, and 17 mM sodium nitrite [pH 4.1–4.3]) at room temperature for 10 min. TRAP buffer was removed and incubated with E197 substrate diluted 1:400 in TRAP buffer for 7 min under UV light (Spectroline XLE-1000) and washed two times with PBS for 5 min. OC were identified histologically as multinucleated, TRAP tdTomato positive, and positive by enzyme histochemistry for immunofluorescent TRAP. OC were scored for expression of fluorescent label if they were on trabecular bone in the primary spongiosa, adjacent to the growth plate.

Parabiosis
Isocronic anastomosis surgery was carried out using 10-wk-old female mice as described previously by Donskoy and Goldschneider (22). A TRAP tdTomato mouse (CD45.2) was surgically joined to a wild-type (WT; CD45.1; Pep Boy) mouse. Parabiosed pairs were maintained for 4 wk after surgery. Food pellets and water gel were provided on the floor of the cage to minimize stretching movement while the mice were adjusting to parabiosis. Establishment of a shared-blood circulation was confirmed at 2 wk after surgery in the blood of both mice by flow cytometry for cells expressing CD45.1 and CD45.2.

At 4 wk postparabiosis, animals were sacrificed, and cells from the spleen, blood and BM of each mouse were harvested for flow cytometry analysis to determine the relative expression of CD45.1 and CD45.2. Cells were also collected for culture in osteoclastogenic conditions. Femurs of WT and TRAP tdTomato mice were harvested, and processed for histology identically to that for lineage tracking.

CX3CR1-GFP cell transplantation in intact animals and animals with a femur fracture
CX3CR1-GFP mice were crossed with TRAP tdTomato mice to generate CX3CR1-GFP/TRAP tdTomato mice, and the heterozygous progeny for both transgenes were used as a source of donor cells for adoptive transfer into WT mice. BM cells were collected from the long bones of male 8-wk-old CX3CR1-GFP/TRAP tdTomato mice, and live CX3CR1-GFP cells that were negative for propidium iodide were isolated on an FACS Aria II system (BD Biosciences). These were used as donor cells for systemic transplantation into 8-wk-old male 8-wk-old C57BL/6 mice (The Jackson Laboratory). Eight mice were randomly divided into two groups or one-way ANOVA and the Bonferroni post hoc test when ANOVA demonstrated significant differences for comparison of multiple groups. All experiments were repeated at least twice and representative data are shown. Statistical analyses were performed using Graph Prism 6 software (GraphPad Software, San Diego, California).

Results
Downregulation of CX3CR1 in BM OCP with differentiation
To confirm that CX3CR1 can be used to identify BM OCP, we sorted GFP+ , GFPlow, and GFPhigh BM cells from CX3CR1-GFP reporter mice, which have enhanced GFP knocked into the Cx3cr1 gene (16) and cultured them with RANKL and M-CSF (30 ng/ml for both) for 5 d (Fig. 1a, 1b). TRAP staining demonstrated that GFP− cells did not differentiatate into TRAP+ OC, whereas GFPlow cells formed mature OC, but their maturation rate was slower than that of GFPhigh cells, because they demonstrated no OC in 4-d cultures and required 8 d to produce TRAP+ OC (Fig. 1a). In contrast, GFPhigh cells produced OC in vitro at both 4 and 8 d of culture. Measurement of bone-resorbing activity by pit assay of cells cultured for 8 d on bovine cortical bone found that GFPhigh cells produced abundant resorption. GFPlow cells had much less resorbing activity at this time point, whereas GFP− cells had none.

Fluorescence imaging of the cultures demonstrated that GFP+ cultures did not become GFP− with culture. In contrast, in the GFPhigh culture after 4 d, immature mononuclear GFP+ cells were present (Fig. 1b). Significantly, the expression of GFP in mature OC in 4-d cultures of GFPhigh cells was minimal to absent. This result is not unexpected, as CX3CR1 expression was previously demonstrated to decrease as OCP mature into OCs (6, 24). With 4 d of culture, GFPlow mononuclear cells expressed GFP at a level that was equal to GFPhigh cells at 4 d of culture. In addition, they were TRAP positive by enzyme histochemistry on day 8 of culture. These results argue that treatment with M-CSF and RANKL for 4 d induced a switch of GFP low cells to GFPhigh cells, which may partially explain the slower maturation of GFPlow cells into OC compared with GFPhigh cells.

Additionally, to determine expression of CX3CR1 in mature OC and confirm our in vitro result that CX3CR1 expression in OCPs was extinguished during their differentiation, we crossed CX3CR1-GFP reporter mice with TRAP tdTomato mice, which use the Acp5 regulatory regions to drive expression of tdTomato florescent protein (17). We cultured BM cells from CX3CR1-GFP/TRAPE197B tdTomato mice with M-CSF and RANKL, 5 d after the expression of the GFP and tdTomato reporters. We found that cells expressing CX3CR1 did not express TRAP, and vice versa (Fig. 2a). As expected, tdTomato was predominantly expressed in large multinucleated cells, whereas GFP was present only in mononuclear cells. Furthermore, to confirm downregulation of CX3CR1, we determine the time course of the expression of Acp5 (TRAP) and Cx3cr1 in cultured BMMs that were treated with M-CSF and RANKL (30 ng/ml for both). We found that as the BMMs differentiated, expression of Acp5 increased from undetectable to its highest levels on day 6. In contrast, Cx3cr1 expression decreased progressively from day 0 to day 6 (Fig. 2b).

Because mononuclear CX3CR1-expressing cells persisted in the culture (Fig. 2a), it was not unexpected that Cx3cr1 mRNA expression, through day 6 of culture, never fell to undetectable levels.

Similar to the results of BM cell cultures from CX3CR1-GFP/TRAPE197B tdTomato mice, we examined femurs from 3-wk-old animals with frozen section histology and observed tdTomato expression predominantly in large multinucleated cells adjacent to bone, whereas GFP expression was only observed in mononuclear cells in the trabecular bone and BM. We did find an occasional double-positive cell, which either represented an overlaying cell or a cell...
transitions from an OCP to an OC (arrow, Fig. 2c). Hence, both our in vitro and in vivo data argue that CX3CR1 is expressed in OCP, but not mature OC in homeostasis.

**Maturation rate of OCP in vivo during homeostatic condition**

We next examined bones from CX3CR1CreER/Ai14 mice. Ai14 mice are a Cre reporter line that expresses tdTomato red fluorescent protein in cells after Cre recombination. CX3CR1CreER mice have a mutated Cre recombinase inserted into the CX3CR1 gene. This ERT2Cre is essentially inactive until the mice are treated with tamoxifen. Frozen femur sections were cut and stained with an anti-RFP Ab to increase the sensitivity of tdTomato signal and examined microscopically by immunofluorescence. Importantly, bones from CX3CR1CreER/Ai14 mice, which were not treated with tamoxifen, exhibited only an occasional tdTomato-expressing cell (10.1 ± 2.7% tdTomato fluorescent red cells of all TRAP-positive cells in the primary spongiosa, lining bone surfaces) (Fig. 3). This result demonstrated the minimal “leakiness” of the Cre recombinase in CX3CR1CreER/Ai14 mice.

One day after a single tamoxifen injection, the CX3CR1CreER/Ai14 mouse femurs displayed significantly more tdTomato+ cells that were also TRAP+ (35.0 ± 4.6%) in the primary spongiosa and lining trabecular bone surfaces, and most of these cells were mononuclear (Fig. 3). Five days after a single tamoxifen injection, the number of CX3CR1 tdTomato TRAP double-positive cells was further increased (60.8 ± 3.4%) (Fig. 3). In addition, cells in 5-d cultures were larger than those on day 1, and most were multinuclear. We also observed numerous mononuclear cells expressing tdTomato in the BM after a single tamoxifen injection. However, those cells were TRAP negative, implying they were CX3CR1+ OCP or, more likely, monocytes of an alternate lineage.

**Evaluation of the circulating or BM residency of OCP**

OCP can circulate in the blood (12). Hence, mature OCs, under the homeostatic conditions of the previous experiment, could have originated from either a circulating cell or a cell that is BM resident and noncirculating. To test whether circulating cells contribute to OC development in vivo during homeostasis, we performed parabiosis. In these experiments, we created a shared-blood circulation system between female WT mice and TRAP tdTomato mice (both in a C57BL/6 background) of the same age (isochronic). To evaluate the shared circulation, isoforms of CD45 (CD45.1 and CD45.2) were used as markers of the origin of the cells. TRAP tdTomato mice (CD45.2) were parabiosed to WT mice that were congenic for CD45.1. Before surgery, blood samples from both mice were analyzed by flow cytometry. In both the WT and the TRAP tdTomato mice, >90% of the blood cells expressed their respective marker (Fig. 4a). To confirm the adequacy of parabiosis, we assessed the establishment of the shared-blood circulation by flow cytometry of blood cells 2 wk after surgery (Fig. 4a). The blood of both mice had a mixture of CD45.1
CX₃CR1 expression during OCP differentiation. (a) BMM from CX₃CR1-GFP/TRAP tdTomato mice cultured with M-CSF and RANKL for 5 d. Yellow arrows identify mononuclear cells expressing GFP only. There is also present red fluorescent mononuclear OCP that have committed to the OC lineage and red fluorescent multinuclear OC. Image to the far left is a composite of bright field, red and green fluorescent images. (b) BMM cultures were treated with M-CSF and RANKL (30 ng/ml for both) and relative mRNA expression of Acp5/β-actin and Cx₃cr1/β-actin determined on 0, 2, 4, and 6 d of cultures (n = 3–4). Data are presented as relative expression normalized to β-actin ± SEM. (c) Fluorescent light image of the metaphyseal area from a femur of a CX₃CR1-GFP/TRAP tdTomato mouse. The three bottom images are enlargements of the white box in the two upper images. These demonstrate, respectively, a merged, green and red fluorescence image (left) and the same section stained with hematoxylin and imaged in bright field. The lower three images are (from left to right) a merged (green and red), green-filtered, and red-filtered image of the same field. Only one yellow OC (dual positive for the expression of TRAP and CX₃CR1) could be detected anywhere in the bone. The yellow arrow indicates either a CX₃CR1 mononuclear cell overlying a mature OC or an OCP fusing and transitioning into a mature OC. In either case, the images demonstrate that levels of the GFP and tdTomato proteins were sufficient to identify additional yellow cells if dual expression were more frequent. Images in (c) (upper) were taken under original magnification ×10. Scale bar, 500 μm. Sections were scanned on a Slide Scanner Axio Scan (Zeiss) and tiles automatically stitched to create the final image in Zen Pro software (Zeiss). The bone slides were scanned first for DAPI, CX₃CR1-GFP, and TRAP tdTomato, and a second time for conventional TRAP staining.
FIGURE 3. Maturation of CX3CR1 OCP in vivo during homeostatic conditions. Images of sections from the femoral bone of a CX3CR1CreER/Ai14 mouse that was not injected with tamoxifen or injected 1 or 5 d prior to sacrifice and histological analysis. To determine multinucleated cells, the sections were stained for DAPI (blue color). After tamoxifen was injected into mice, CX3CR1-expressing cells were labeled with tdTomato (red). Sections were stained for TRAP with the fluorescent substrate Elf97 (yellow) and visualized by epifluorescence. All TRAP+ cells and double-positive cells for CX3CR1 and TRAP in the primary spongiosa were counted to determine percentage of mature OC differentiating from CX3CR1-expressing cells. We also determined monoclear CX3CR1+ expressing cells in BM. Images were taken under original magnification ×10 (n = 6 for non–tamoxifen-treated control, n = 12 for 1 d prior to tamoxifen, and n = 12 for 5-d tamoxifen group). Sections were scanned on a Slide Scanner Axio Scan (Zeiss) and tiles automatically stitched to create the final image in Zen Pro software (Zeiss). The bone slides were scanned first for DAPI, CX3CR1, and bright field and a second time for fluorescent TRAP, DAPI, and bright field. Two images of the same section were merged using Adobe Photoshop CS6 (version 6.0, Adobe Systems Inc., San Jose, CA) and analyzed in ImageJ. Scale bars, 500 μm. **p < 0.01, ***p < 0.001 (significantly different).

(WT, 48.5 ± 1.8%; TRAP tdTomato, 34.0 ± 2.2%)– and CD45.2 (WT, 35.1 ± 2.5%; TRAP tdTomato, 54.4 ± 2.7%)+–positive cells, proving that a shared circulation between parabiosed mice was established. Pairs were sacrificed 4 wk after parabiosis surgery. Blood, spleen, and BM cells were harvested for flow cytometric analyses. Blood and spleen samples (Fig. 4a, 4b) showed a similar mixture of CD45.1- and CD45.2-positive cells in both mice (Table I), whereas in the BM (Fig. 4c, Table I), there was a much lower level of engraftment of cells from the other parabiont (5.4 ± 1.8% CD45.1+– and CD45.2+– and CD45.2+–positive cells, 54.4% CD45.1+–CD45.2+– in WT mice and 6.8 ± 1.3% CD45.1+–CD45.2+– in TRAP-paired mice) (Fig. 4d).

We next cultured BM cells from the WT and TRAP tdTomato parabionts with M-CSF and RANKL for 5 d and examined the OC that formed in vitro under UV light to determine the number of multinucleated fluorescence cells per well. Consistent with the results of the flow cytometry for CD45.1 and CD 45.2 in the BM of the mice, we demonstrated only a few (6.6 ± 3.6 per well) fluorescent OC in the WT BM cultures. In contrast, there were many more (164.3 ± 1.6 per well) fluorescent OC in BM cultures from the TRAP tdTomato parabiont (Fig. 4d, 4e). Similar results were obtained when we cultured whole-BM cells with M-CSF and RANKL for 6 d (Fig. 4e). In these experiments, the total number of TRAP-positive OC per well, as determined by enzyme histochemistry, was similar in all groups (data not shown).

In vivo evaluation of the engraftment of tdTomato cells was performed after 4 wk of parabiosis in pairs of mice, which, at the time of sacrifice, were 14 wk old. In these studies, eight parabiont pairs were examined with identical results. Bones from the TRAP tdTomato mice at 14 wk of age contained numerous large cells on bone that were multinucleated, TRAP+ by enzyme histochemistry and tdTomato+ by Ab-enhanced epifluorescence (Fig. 5). Indeed, we found that in the TRAP tdTomato mice, all tdTomato-positive cells were also positive for TRAP, as measured by enzyme histochemistry. In contrast, bones from WT mice contained few, if any, cells that were both tdTomato+ and TRAP+ (Fig. 5). However, as expected, conventional TRAP enzyme histochemical staining demonstrated abundant TRAP+ OC in the WT mice.

CX3CR1-GFP cell transplantation
Circulating cells can be a source of OCP during states that open or perturb the BM microenvironment (5, 11–14). To examine the role that circulating cells play as a source of OCP during fracture repair and to confirm that expression of CX3CR1 labels OCP, we adapted a protocol from the work of Göthlin and Ericson (11). EGFP-expressing BM cells from CX3CR1-GFP/TRAP tdTomato mice were isolated by FACS and injected i.v. into WT recipients that either remained intact or underwent a femur fracture. Cells were injected on days 0 and 10 postfracture, and animals were sacrificed on day 18. Systemic transplantation of sorted CX3CR1-GFP cells from the BM of CX3CR1-GFP/TRAP tdTomato animals into C57BL/6 mice that were not fractured did not demonstrate any TRAP tdTomato-positive cells in the femurs. In addition, only a few CX3CR1-GFP+–positive cells were present in the BM of the femurs of these mice (Fig. 6).

In contrast, when the same cell population was transplanted into a C57BL/6 animal that had undergone a femur fracture, multinucleated TRAP tdTomato-positive cells were present at day 18 postfracture in the bone of the repair callus and in new bone that...
formed along the tract of the stabilizing pin insertion. These cells were on bone, multinucleated, and positive for TRAP as stained by fluorescent Elf97 substrate, confirming their osteoclastogenic phenotype (Fig. 7). In both Figs. 6 and 7, we also observed some nonspecific staining of muscle tissue by the anti-RFP Ab.

Discussion
Our results with CX3CR1-GFP mice demonstrated that the CX3CR1 promoter/enhancer sequences identify essentially all cells in the BM that have the potential to form OC in vitro. This conclusion is based on our finding that no mature OC formed in cultures from CX3CR12 murine BM cells. In contrast, there was rapid (within 4 d) OC formation in vitro and bone-resorbing ability (within 8 d) in cultures of cells expressing high levels of GFP. Cells that were GFPlow required a longer culture time to form mature OC, which suggests that murine BM CX3CR1-GFPlow cells represent a less-mature OCP compared with CX3CR1-GFPhigh cells. We expect that if cultured for longer than 8 d, CX3CR1-GFPlow BM cells would eventually form abundant resorption pits, although we did not test this hypothesis.

Our studies of CX3CR1-GFP/TRAP tdTomato mice on a C57BL/6 background in homeostatic conditions argue that CX3CR1 is expressed essentially only in OCP and not in mature OC. In vitro, we found that almost all cultured mononuclear cells from these mice expressed either the GFP or tdTomato marker, with few cells coexpressing both. In addition, gene expression of
OCP that were differentiating in BMM cultures demonstrated that Cx3cr1 was rapidly diminished, whereas Acp5 was reciprocally increased during OC differentiation. Similarly, in vivo, we found that tdTomato-positive OC were almost all negative for GFP, whereas almost all GFP+ OCP did not express tdTomato. We speculate that the small number of cells that were double positive (expressing both CX3CR1 [green] and TRAP [red]) either represent overlying cells or were transitioning from OCP to mature OC.

Similar to our results, Han et al. (25) examined sections of homeostatic mouse bone from CX3CR1-GFP mice and found

Table I. Frequency of CD45.1 and CD45.2 cells in parabiont mice at 2 and 4 wk of parabiosis

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time Point (wk)</th>
<th>Parabiosis Pair</th>
<th>CD45.1⁺ (%)</th>
<th>CD45.2⁺ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2</td>
<td>WT</td>
<td>48.5 ± 1.8</td>
<td>35.1 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>TRAP</td>
<td>34.0 ± 2.2</td>
<td>54.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>WT</td>
<td>62.4 ± 2.0</td>
<td>33.7 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>TRAP</td>
<td>41.7 ± 1.8</td>
<td>54.4 ± 1.4</td>
</tr>
<tr>
<td>BM</td>
<td>4</td>
<td>WT</td>
<td>71.6 ± 1.2</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>TRAP</td>
<td>6.8 ± 1.3</td>
<td>73.4 ± 1.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>4</td>
<td>WT</td>
<td>53.7 ± 2.0</td>
<td>32.6 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>TRAP</td>
<td>37.2 ± 2.3</td>
<td>50.3 ± 1.5</td>
</tr>
</tbody>
</table>

FIGURE 5. After 4 wk of parabiosis, circulatory OCP do not engraft in the primary and secondary spongiosa. Images of sections from the femoral bone of a WT mouse and TRAP tdTomato mouse that were parabiosed for 4 wk. In the WT mouse, arrows indicate cells that are TRAP positive by enzyme histochemistry but are negative for tdTomato. In the TRAP tdTomato mouse, arrows indicate cells that are double positive for the tdTomato reporter and TRAP as stained by enzyme histochemistry. The sections were also stained for DAPI to identify nuclei and then scanned to visualize multinuclear tdTomato-positive cells. After imaging under fluorescent light, sections were stained for TRAP by enzyme histochemistry and visualized under conventional light. Experiments were repeated eight times with similar results, and representative samples are shown. Images are taken under original magnification ×10. Scale bar, 500 µm. Sections were scanned on a Slide Scanner Axio Scan (Zeiss) and tiles automatically stitched to create the final image in Zen Pro software (Zeiss). The bone slides were scanned first for DAPI and TRAP tdTomato and a second time for conventional TRAP staining. Two images of the same section were merged using Adobe Photoshop CS6 (version 6.0, Adobe Systems Inc.).
FIGURE 6. Circulatory CX3CR1-GFP cells do not incorporate into mature OC in the bones of intact mice. Image of a section from the femoral bone of a C57BL/6 mouse that was transplanted with CX3CR1-GFP cells (sorted from CX3CR1-GFP/TRA1 tdTomato mice) on days 0 and 10 and sacrificed on day 18. We could not identify TRAP tdTomato + cells in the primary spongiosa of the intact femur (a'). Some CX3CR1-GFP cells were present in the BM, as indicated by green arrow (b') (n = 4). Sections were scanned on a Slide Scanner Axio Scan (Zeiss) and tiles automatically stitched to create the final image in Zen Pro software (Zeiss). The bone slides were scanned first for DAPI, TRAP tdTomato, CX3CR1-GFP, and bright field and a second time to scan sections for DAPI, fluorescent TRAP, and bright field. Two images of the same section were merged using Adobe Photoshop CS6 (version 6.0, Adobe Systems Inc.). Images were taken under original magnification ×10.

abundant fluorescence in mononuclear TRAP-negative cells and little fluorescence in multinucleated TRAP-positive OC. Similarly, Ibáñez et al. (10), using flow cytometry on cells that they isolated from mouse bones, found that, under homeostatic conditions, only a small percentage (4–7%) of OC expressed CX3CR1 protein.

However, Ibáñez et al. did find that under inflammatory conditions expression of CX3CR1 was maintained in a subpopulation of OCs. This result suggests that there are multiple origins of OC and that the conditions of inflammation maintain CX3CR1 expression in mature OC (10). Interestingly, in our studies of OCP differentiation from circulating cells during fracture repair, a state associated with inflammation (26), we did not see GFP + -OC, indicating expression of CX3CR1. However, the peak of the inflammatory response in fracture repair occurs earlier than day 18, so it is possible that, by this time, OC expression of CX3CR1 had been extinguished.

The data from our studies of CX3CR1CreER/Ai14 mice in vivo demonstrate the minimal leakiness of the CX3CR1CreER construct because, in the absence of tamoxifen, few, if any, tdTomato-positive cells were present in the bones of the mice. In contrast, 1 d after a single injection of tamoxifen, the femurs demonstrated a significant increase in tdTomato-positive and TRAP-positive cells in the mice. These tdTomato-positive cells were relatively small compared with mature multinucleated OC, TRAP negative as measured by enzyme histochemistry, and most likely represented mononuclear CX3CR1-expressing OCP or CX3CR1-expressing monocytes of other lineages. We would have expected that if mature OC expressed significant amounts of CX3CR1 protein under these conditions, a large number of multinucleated OC would be labeled with the tdTomato reporter at 1 d after tamoxifen injection, which we did not see. At 5 d posttamoxifen, there were abundant tdTomato-positive, large, multinucleated OC on bone in the primary spongiosa. Hence, similar to our in vitro results, our in vivo data argue that the Cx3cr1 promoter/enhancer sequences identifies OCP that rapidly (within 5 d) form mature OC in vivo.

The time of 5 d that it took for mature OC to form from OCP in vivo in CX3CR1CreER/Ai14 mice is similar to the time it takes for the most mature OCP to form OC in vitro (Fig. 1) (2, 27). Hence, it appears that this time period is inherent for the maturation of OC from OCP and not a unique characteristic of in vitro culture systems.

It has been known for over 40 y that OC can circulate and engraft into bones with a perturbed microenvironment (14). However, it has been unclear if circulating cells were a major component of the OC that form in bone under homeostatic conditions. Our parabiosis studies argue that under the conditions of these studies, production of OC from circulating precursors is a relatively rare event. Similar to our results, Boban et al. (13) showed that, during parabiosis between Col3.6GFP and Col2.3DTK mice, engraftment of OC between parabionts did not occur unless the mice were treated with ganciclovir, which killed osteoblast-lineage cells and opened up the BM microenvironment to engraftment. However, these studies used the Col 3.6 promoter to drive GFP expression in OC, and it can be argued that this off-target expression of GFP might not produce levels of the fluorescent marker in OC that were intense enough to be detected easily by epifluorescence.

Kotani et al. (12) also demonstrated minimal engraftment of circulating monocyte progenitors in the BM during normal homeostasis using CX3CR1-GFP mice to label cells. However, their approach also may have underrepresented the number of cells that migrate from the circulation to the bone and matured into OC. This is because our data and that of others (6, 7) demonstrate that the Cx3cr1 promoter, which they used to drive GFP expression, can be rapidly downregulated as OCP mature into OC.

In contrast, our studies used the strong Acp5 promoter/regulatory regions to drive high-level tdTomato expression in OC, as
demonstrated in vitro and in vivo. In addition, to further enhance the sensitivity of our detection assay, we used an Ab to tdTomato but still failed to find significant engraftment of OCP between parabionts. Based on our results from 4 wk of parabiosis and our finding in CX3CR1CreER/Ai14 mice that OCP differentiate into OC within 5 d, we conclude that circulating cells represent a minor source of OCP under the conditions of parabiosis that we studied.

There are several studies, which demonstrate that the BM environment can be opened to engraftment by circulating OCP after various perturbations. In a seminal study, Walker (14) found that osteopetrosis, because of defective OC in mice, could be cured by parabiosis of an osteopetrotic mouse with a normal mouse. Similarly, Göthlin and Ericsson (11) injected labeled cells into the blood of rats that had undergone a fracture and showed the incorporation of the labeled cells into OC in the resolving callus. Kotani et al. (12) found that there was good transfer of labeled circulating OCP into mature OC after stimulating bone resorption in mice by injecting them with RANKL, whereas Charles et al. (5) showed that circulating OCP could become OC in bone that was undergoing inflammation.

Our experiment, in which FACS-purified GFP+ BM cells from CX3CR1-GFP/TRA(TdTomato mice were transferred into mice with a repairing femur fracture, demonstrated that CX3CR1 marked BM cells, which, when released into the circulation, can become OC during fracture repair. These studies also confirmed the influence of environmental conditions on the ability of circulating OCP to engraft and differentiate into OC on bone in vivo. Finally, the failure of circulating CX3CR1+ OCP to engraft and incorporate into OC in the bones of mice that were not fractured further argue that, during homeostasis, the BM is relatively protected from engraftment by circulating OCP.

Additional support for this hypothesis comes from our previous studies of mice that had Cre recombinase knocked into the cathepsin K gene (28) and were crossed with Ai14 reporter mice (29). Using this mouse model as a source of OCP, we found that after a single injection of highly purified BM or spleen OCP from Cat-K-Cre/-Ai14 mice, there was no engraftment of cells in the BM or their maturation into OC in nonirradiated mice (29). In contrast, in irradiated mice (9 Gy), there was significant engraftment of BM or spleen cells, and these formed multinuclear mature OC that were TRAP+ in resorption lacuna at 15 d postirradiation and transplant (29). Our results with parabiosis are consistent with our adoptive transfer studies, as outlined above, and those of others (10, 30) that required some perturbation of the bone microenvironment for engraftment and maturation of circulating OCP into mature OC to occur in bone.

However, our results are in contrast to those of Jacome-Galarza et al. (15), who showed that, in parabiosed animals after 4–8 wk, essentially all OC lining the bone surface expressed both YFP (host) and tdTomato (donor) reporters, and these cells persisted for at least 24 wk after separation of the parabionts. Moreover, in their study, they showed successful engraftment of Ly-6C+ monocyte from Csf1rcre;Rosa26LSL-tdTomato mice into Csf1rcre;Rosa26LSL-YFP mice after adoptive transfer, without irradiating the recipient mice. Both we and Jacome-Galarza et al. (15) used the identical anti-RFP Ab to increase tdTomato signal detection in frozen sections. Hence, we believe that our ability to detect engrafted cells between parabionts was similar to theirs. We are at a loss to fully explain the contradictory results between our studies. Given the
long experience with adoptive transfer and the use of radiation or other perturbation to allow engraftment, it was unexpected that Jacome-Galarza et al. (15) would find significant BM engraftment of circulating OCP in homeostatic conditions. As a possible explanation for the discrepancy between our results, we postulate that Jacome-Galarza et al. (15) produced an inflammatory reaction by parabiosis mice with two different reporters. Stripeke et al. (31) showed that fluorescent reporters like GFP can induce an immune response, which is stronger in BALB/c mice than in C57BL/6. The study by Jacome-Galarza et al. (15) did not report the background of the animals used, but two different reporters used in parabiosed animals might cause sufficient inflammatory conditions, which enhanced engraftment of OCPs. Because in all our experiments we used C57BL/6 mice, which have lower immune responses to the reporter protein, we believe that the parabions in our study more closely resemble the homeostatic state of an intact mouse. Our demonstration of high-level engraftment of cells from one parabiont to the other in the spleens of the mice and the general health of the animals during parabiosis argues that we did not have an inflammatory reaction in our parabiosed animals that destroyed the cells in bone from the other mouse and resulted in an absence of detectable OCP transfer between the parabions. Our findings that i.v. injection of labeled OCP into mice with a repairing fracture [a state associated with inflammation (26)] produced engraftment and incorporation of labeled OCP into OC, whereas injection of labeled OCP into intact mice did not, further argues that inflammation itself is not the reason for our failure to find engraftment and maturation of circulating OCP into OC in our homeostatic models.

In summary, our results show that CX3CR1-expressing cells are OCP, which downregulate expression of this gene when they differentiate into mature OC both in vitro and in vivo in homeostasis and during fracture repair in mice. Furthermore, our studies of TRAP tdtomato–WT-parabiosed mice and our studies of adoptive transfer of circulating OCP during fracture repair argue that, under homeostatic conditions in adult mice, the BM is the principal reservoir of the OCP that form mature OC in the bone, whereas when the BM integrity is perturbed by fracture, circulating CX3CR1+ cells can become OC during fracture repair.

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Disclosures
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References