PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis

Hui Xie1,2, Zhuang Cui1,3, Long Wang1,4, Zhuying Xia1,2, Yin Hu1,2, Lingling Xian1, Changjun Li1, Liang Xie1, Janet Crane1, Mei Wan1, Gehua Zhen1, Qin Bian1, Bin Yu1, Weizhong Chang1, Tao Qiu1, Maureen Pickarski5, Le Thi Duong2, Jolene J Windle6, Xianghang Luo2, Eryuan Liao2 & Xu Cao1

Osteogenesis during bone modeling and remodeling is coupled with angiogenesis. A recent study showed that a specific vessel subtype, strongly positive for CD31 and endomucin (CD31hiEmcnhi), couples angiogenesis and osteogenesis. Here, we found that platelet-derived growth factor-BB (PDGF-BB) secreted by preosteoclasts induces CD31hiEmcnhi vessel formation during bone modeling and remodeling. Mice with depletion of PDGF-BB in the tartrate-resistant acid phosphatase–positive cell lineage show significantly lower trabecular and cortical bone mass, serum and bone marrow PDGF-BB concentrations, and fewer CD31hiEmcnhi vessels compared to wild-type mice. In the ovariectomy (OVX)-induced osteoporotic mouse model, serum and bone marrow levels of PDGF-BB and numbers of CD31hiEmcnhi vessels are significantly lower compared to sham-operated controls. Treatment with exogenous PDGF-BB or inhibition of cathepsin K to increase the number of preosteoclasts, and thus the endogenous levels of PDGF-BB, increases CD31hiEmcnhi vessel number and stimulates bone formation in OVX mice. Thus, pharmacotherapies that increase PDGF-BB secretion from preosteoclasts offer a new therapeutic target for treating osteoporosis by promoting angiogenesis and thus bone formation.

Bone size and shape is precisely modeled and remodeled throughout life to ensure the structure and integrity of the skeleton1–3. Primary factors in temporal and spatial regulation of bone remodeling have been characterized3–5. Angiogenesis is coupled with bone formation in these processes for proper bone homeostasis5,6. A recent study revealed that a specific vessel subtype, CD31hiEmcnhi vessels, couple angiogenesis and osteogenesis7,8. However, the cellular and molecular regulation of angiogenesis during coupling to osteogenesis remains elusive.

During bone remodeling, osteoclast bone resorption is coupled with osteoblast bone formation by endocrine and paracrine factors to induce migration and differentiation of the precursors of these cell types9,10. In response to stimulation with macrophage colony–stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), monocytes and macrophages first commit to the osteoclast lineage as tartrate-resistant acid phosphatase–positive (TRAP+) mononuclear cells (preosteoclasts)10. Preosteoclasts subsequently fuse to form TRAP+ multinuclear cells (osteoclasts)10,11. Growth factors such as transforming growth factor-β (TGF-β) and insulin-like growth factor type 1 (IGF-1) are released from the bone matrix during osteoclast bone resorption to induce migration and differentiation of mesenchymal stem cells (MSCs) into osteoblasts for new bone formation12,13. During bone modeling, osteoclast bone resorption and osteoblast bone formation occur independently1. The cell signaling mechanisms that regulate bone modeling are less well understood, although studies in humans and mice with osteopetrosis with impaired osteoclast function suggest osteoclasts may promote osteogenesis independent of resorptive activity14–17. There are abundant numbers of preosteoclasts on the periosteal bone surface, especially in regions associated with rapid growth18,19. Of note, v-ATPase V0 subunit D2–deficient mice with failure of fusion of preosteoclasts show increased bone formation16. Recently, F4/80+ macrophages on periosteal and endosteal surfaces, termed ‘OsteoMacs’, have also been shown to regulate in vitro mineralization of osteoblasts and are required for the maintenance of mature osteoblasts in vivo20,21.

An adequate blood supply is critical for bone health by transporting nutrients, oxygen, minerals and metabolic wastes essential for maintaining proper osteoblastic bone matrix synthesis and mineralization22,23. Cortical bone is enmeshed by capillaries that interact with a network of intracortical canals consisting of longitudinal Haversian canals and transversal Volkmann canals that link the periosteum and the endosteum24,25. During modeling associated with growth, endothelial cells invade the cartilage at the growth plate region to form a vascular channel for nutrient supply and serve as a scaffold for new bone formation24. Capillaries are also present at bone remodeling sites and help further couple bone resorption and bone formation24,26.
Angiogenesis involves migration and proliferation of endothelial cells, capillary tube formation and MSC stabilization of newly formed tubes. Of note, the interaction of MSCs with endothelial cells further regulates angiogenesis by secretion of angiogenic growth factors, cytokines and other signaling molecules. PDGF-BB stimulates migration and angiogenesis of endothelia progenitor cells (EPCs) and MSCs. PDGF-BB is believed to mobilize cells of mesenchymal origin, stabilize newly formed vessels and orchestrate cellular components for osteoblast differentiation. It has been reported that osteoclasts secrete PDGF-BB to induce migration of MSCs or osteoblasts.

In this study, we report that preosteoclasts secrete PDGF-BB during bone modeling and remodeling to induce angiogenesis and thus proper osteogenesis. Depletion of PDGF-BB in the TRAP+ cell lineage reduces angiogenesis in the bone marrow and periosteum with reduced bone formation. Inhibition of cathepsin K (CTSK) effectively reduces angiogenesis in the bone marrow and periosteum with proper osteogenesis. Depletion of PDGF-BB in the TRAP+ cell line-abolished the preosteoclast-conditioned medium–induced migration of CD31hiEmcnhi vessel and bone formation in OVX mice. Thus, PDGF-BB secreted by preosteoclasts stimulates angiogenesis, further supporting osteogenesis.

RESULTS

TRAP+ cell deficiency impairs cortical bone formation

To examine the role of osteoclast-lineage cells in bone formation, we analyzed colony-stimulating factor-1 (CSF-1)-deficient (Csf1−/−) mice, which have TRAP+ cell deficiency as CSF-1 is essential for the survival of monocyte-macrophage–lineage cells. Due to deficiency in osteoclast bone remodeling, a high volume of unmineralized bone was formed in Csf1−/− mice relative to their wild-type littermates (Fig. 1a,b). Notably, the cortical bones of Csf1−/− mice were extremely thin (Fig. 1a,b). The periosteal perimeter of femoral diaphysis in the Csf1−/− mice was significantly lower relative to that of their wild-type littermates (Fig. 1b). TRAP staining of femur sections confirmed that there were no TRAP+ cells on the periosteal bone surface in the Csf1−/− mice (Fig. 1c,d). Coimmunostaining of CD31 and Emcn confirmed that the number of CD31hiEmcnhi cells in the periosteum of Csf1−/− mice was significantly lower relative to that in their wild-type littermates (Fig. 1e,f).

Postnatal cortical bone grows quickly during puberty, and the growth decreases gradually when approaching adulthood. We examined the numbers of preosteoclasts on the periosteal bone surface during postnatal growth through adulthood in wild-type mice. The number of perosteal preosteoclasts was very abundant at day 15 after birth, decreased by 45% by 1 month of age and by 78% by 3 months and was rarely detectable by 6 months (Fig. 1g,h). Consistent with previous reports, periosteal TRAP+ cells were largely monocellular (Fig. 1c,g). These observations suggest a potential role of preosteoclasts in cortical bone formation during skeletal modeling.

PDGF-BB from preosteoclasts induces MSC and EPC migration

To examine the potential molecular mechanism by which preosteoclasts regulate trabecular bone remodeling and periosteal bone modeling, we cultured monocytes and macrophages to differentiate them into preosteoclasts and osteoclasts, as evidenced by TRAP-positive staining and the number of nuclei (Supplementary Fig. 1a). We collected the conditioned media of monocytes and macrophages, preosteoclasts and osteoclasts with or without bone slices. Conditioned medium from preosteoclasts induced significantly more MSC migration relative to control conditioned medium from monocytes and macrophages, and the migration was further stimulated when a bone slice was added to the culture (Fig. 2a). Conditioned medium from osteoclasts without the bone slice had very little effect on MSC migration, indicating that the unique factor for promoting MSC migration was secreted specifically in preosteoclast-conditioned medium (Fig. 2a). To identify this secreted factor, we tested neutralizing antibodies against TGF-β1, TGF-β2, TGF-β3, IGF-1, IGF-II, PDGF-AA and PDGF-BB, as well as noggin, in the conditioned media. Only the antibody against PDGF-BB abolished the preosteoclast-conditioned medium–induced migration (Fig. 2b). TGF-β1–neutralizing antibody inhibited the migration induced by the osteoclast bone resorption–conditioned medium (Fig. 2b), consistent with our previous report. ELISA analysis confirmed that preosteoclasts secreted PDGF-BB and adding a bone slice enhanced the secretion, whereas osteoclasts with or without bone slice

Figure 1  TRAP+ cell deficient mice exhibit reduced cortical bone.
(a) Representative microcomputed tomography (µCT) images of femora. Red arrows indicate cortical bone. Scale bars, 1 mm. n = 3 per group. (b) Quantitative µCT analysis of the trabecular bone fraction (Tb. BV/TV), cortical thickness (Ct. Th) and periosteal perimeter (Ps. Pm) of femora. n = 3 per group. (c,d) Representative TRAP staining images (c) and quantitative analyses of the number of preosteoclasts (N. POCs) on periosteal bone surface (BS) (d) of femoral diaphysis. Black arrowheads indicate preosteoclasts. Scale bars, 500 µm (top), 20 µm (bottom). n = 5 per group. (e,f) Representative confocal images of immunostaining of CD31 (red), and Emcn (green) and DAPI (blue) staining of nuclei (e), and quantitative analysis (f) of the number of CD31hiEmcnhi cells (yellow) in femoral diaphyseal periosteum. Dashed lines outline the bone surface. P, periosteum; CB, cortical bone. Scale bar, 50 µm. n = 5 per group. (g,h) Representative TRAP staining images (g) and quantitative analysis of the number of preosteoclasts on periosteal bone surface (h) of femoral diaphysis of wild-type male mice at different ages. Scale bar, 20 µm. n = 5 per group. Data shown as mean ± s.d. *P < 0.05, **P < 0.01 (b,d,f, Student's t-test; h, analysis of variance (ANOVA)).
Figure 2 Preosteoclasts secrete PDGF-BB to induce migration of MSCs and EPCs. (a) Transwell assays for the migration of MSCs using conditioned medium (CM) collected from different cell cultures with (+) or without (–) bone slices. (b) Transwell assays for the migration of MSCs using conditioned medium of preosteoclasts + bone slices (POC CM) with addition of individual neutralizing antibody (Ab), IgG or noggin, as indicated, or using conditioned medium of osteoclasts + bone slices (OC CM) with addition of individual neutralizing Ab or IgG. (c,d) ELISA analysis of expression of PDGF-BB (c) and CTX (d) in different conditioned media. (e) Immunoprecipitation (IP) and immunoblotting (IB) analysis of PDGF-BB levels in different conditioned media. Data are representative of three independent experiments. Platelet, mouse platelet lysate (positive control). (*) Transwell assays for the migration of EPCs using conditioned media from different cell cultures as indicated or conditioned medium of preosteoclasts + bone slices with addition of IgG or PDGF-BB neutralizing Ab. (f) Transwell assays for the migration of EPCs using conditioned media from different cell cultures as indicated or conditioned medium of preosteoclasts + bone slices with addition of IgG or PDGF-BB neutralizing Ab. (*P < 0.05, **P < 0.01 (ANOVA).

PDGF-BB from preosteoclasts induces bone formation in mice

We then examined the function of PDGF-BB secreted by preosteoclasts in bone remodeling and periosteal bone formation. We crossed Pdgfb-flox/flox mice (Pdgfb<sup>flox/flox</sup> mice) with TRAP-Cre mice to generate TRAP<sup>−/−</sup> mice. TRAP<sup>−/−</sup> mice were positive for PDGF-BB in both trabecular bone and periosseum of Pdgfb<sup>flox/flox</sup> mice, but we rarely observed such cells in Pdgfb<sup>−/−</sup> mice (Fig. 3c). The number of TRAP<sup>+</sup> cells on trabecular and periostial bone surfaces and serum CTX concentrations were not different in Pdgfb<sup>−/−</sup> mice relative to their Pdgfb<sup>flox/flox</sup> littermates (Fig. 3c,d), indicating that specific deficiency of PDGF-BB in TRAP<sup>+</sup> cells does not markedly affect osteoclast bone resorption. Of note, PDGF-BB concentrations in both bone marrow and peripheral blood were significantly lower and bone marrow vascular endothelial growth factor (VEGF) concentrations were also significantly lower in Pdgfb<sup>−/−</sup> mice than in their Pdgfb<sup>flox/flox</sup> littermates (Fig. 3e). The bone marrow PDGF-BB concentration was lowered by 72.6% in Pdgfb<sup>−/−</sup> mice relative to their Pdgfb<sup>flox/flox</sup> littermates (Fig. 3e) and Supplementary Fig. 4a). Endothelial cells also express PDGF-BB. We therefore further analyzed the relative contribution of PDGF-BB produced in the bone marrow by preosteoclasts and osteoblasts. We found that the PDGF-BB amount in sorted endothelial cells (CD31<sup>+</sup>CD45<sup>−</sup>Ter119<sup>−</sup> cells) constituted about 46.0% of total bone marrow PDGF-BB protein in Pdgfb<sup>−/−</sup> mice (Supplementary Fig. 4b). The results indicate that bone marrow PDGF-BB arises primarily from TRAP<sup>+</sup> cells (72.6%), with 12.6% from endothelial cells and 14.8% from the rest of the bone marrow cells (Supplementary Fig. 4c).

Microphil-perfused angiography showed that vessel volume and surface area were significantly lower in Pdgfb<sup>−/−</sup> mice than in their Pdgfb<sup>flox/flox</sup> littermates (Fig. 3f). Pdgfb<sup>−/−</sup> mice had significantly lower numbers of CD31<sup>hi</sup>Emcn<sup>hi</sup> endothelial cells in both bone marrow and periostium as compared to their Pdgfb<sup>flox/flox</sup> littermates (Fig. 3g,h). Pdgfb<sup>−/−</sup> mice had less proliferation of endothelial cells in metaphysis versus their Pdgfb<sup>flox/flox</sup> littermates (Fig. 3i). The lower osteocalcin<sup>+</sup> osteoblastic cell numbers on trabecular and periostial bone surfaces and lower serum osteocalcin concentration in Pdgfb<sup>−/−</sup> mice...
compared to their Pdgfbfl/fl littermates (Fig. 3j) further suggest that the lower degree of angiogenesis is related to less osteoblast bone formation. Taken together, these results suggest that PDGF-BB secreted by preosteoclasts stimulates angiogenesis and bone formation.

CTSK inhibition induces PDGF-BB secretion in preosteoclasts

Pycnodysostosis is a rare genetic osteopetrotic disease due to mutations in the CTSK gene. CTSK inhibition is under active investigation as a pharmacotherapy for osteoporosis. Preclinical and clinical data have noted that CTSK inhibition impairs osteoclast bone resorption but also increases numbers of preosteoclasts and osteoclasts and bone formation. Preosteoclasts and osteoclasts from Ctsk−/− mice lack normal apoptosis and senescence and exhibit overgrowth both in vitro and in vivo, which has been well characterized in a previous study. We therefore examined whether a CTSK inhibitor enhances secretion of PDGF-BB by preosteoclasts to promote angiogenesis and bone formation. We prepared conditioned media from preosteoclasts from Ctsk−/− mice and their wild-type littermates and examined their effects on migration of MSCs and EPCs by the transwell migration assay. The conditioned media from preosteoclasts with either inhibition of CTSK activity or gene deletion of Pdgfb had higher concentrations of PDGF-BB protein (Supplementary Fig. 5a) and induced significantly more MSC and EPC migration versus conditioned medium from vehicle-treated wild-type preosteoclasts, and the elevated cell migration was
abolished by a PDGF-BB–neutralizing antibody (Supplementary Fig. 5b,c). Coimmunostaining of TRAP and PDGF-BB in longitudinal femur sections revealed that the number of TRAP⁺PDGF-BB⁺ cells was higher in the trabecular bone and periosteum in Ctsk⁻/⁻ mice and wild-type mice treated with a CTSK inhibitor, L-006235 (L-235)44, than in vehicle-treated wild-type mice (Fig. 4a). PDGF-BB concentrations in both bone marrow and peripheral blood were significantly higher and bone marrow VEGF concentration was also higher in these mice compared to their vehicle-treated wild-type counterparts (Fig. 4b), suggesting more angiogenesis. Vessel volume and surface area in bone marrow, as well as CD31⁺Emcn⁺ cell numbers in both bone marrow and periosteum, were significantly higher in Ctsk⁻/⁻ mice and L-235–treated wild-type mice than in vehicle-treated wild-type mice (Fig. 4c–e). The trabecular bone volume and number, cortical bone thickness and periosteal perimeter were all higher in Ctsk⁻/⁻ mice and L-235–treated wild-type mice relative to vehicle-treated wild-type mice (Supplementary Fig. 6a,b). Calcium double labeling confirmed that Ctsk⁻/⁻ mice and L-235–treated wild-type mice had more bone formation in both trabecular and cortical bone versus vehicle-treated wild-type mice (Supplementary Fig. 6c). The higher osteocalcin⁺ cell numbers and serum osteocalcin concentration in Ctsk⁻/⁻ mice and L-235–treated wild-type mice than in vehicle-treated wild-type mice (Supplementary Fig. 6d,e) indicate more osteoblast bone formation.

To validate that CTSK inhibitor–stimulated bone formation was mediated by increased angiogenesis and PDGF-BB secretion by preosteoclasts, we treated Pdgfb⁻/⁻ mice with L-235. In Pdgfb⁻/⁻ mice, the CTSK inhibition did not increase PDGF-BB and VEGF concentrations in either bone marrow or peripheral blood (Fig. 4f). As expected, CTSK inhibition did not increase vessel volume and surface area, CD31⁺Emcn⁺ cell numbers or proliferation of metaphyseal endothelial cells in Pdgfb⁻/⁻ mice (Fig. 4g,h). CTSK inhibition did not increase cortical bone thickness and periosteal perimeter (Fig. 4i) and only moderately increased trabecular bone volume and number in Pdgfb⁻/⁻ mice (Fig. 4i). The inhibitor did not increase serum osteocalcin concentration in Pdgfb⁻/⁻ mice, whereas levels of CTX in serum were lower in Pdgfb⁻/⁻ mice and their wild-type littersmates (Fig. 4j). These results reveal that inhibition of CTSK increases secretion of PDGF-BB to enhance angiogenesis for bone formation.
PDGF-BB from preosteoclasts induces angiogenesis via focal adhesion kinase

To examine the signaling mechanisms of PDGF-BB in promotion of angiogenesis-coupled bone formation, we prepared conditioned media from preosteoclasts or monocytes and macrophages or control medium to culture EPCs, MSCs or both in co-culture. Preosteoclast-conditioned medium induced EPC tube formation. Co-culture with MSCs significantly enhanced the EPC tube formation. whereas addition of PDGF-BB–neutralizing antibody attenuated the effect (Fig. 5d). Independent inhibition of all components of this pathway was sufficient to block preosteoclast-conditioned medium–induced tube formation of MSC and EPC co-culture and migration of MSCs and EPCs (Fig. 5e,f). The results indicate that Akt-dependent activation of FAK signaling mediates PDGF-BB–induced cell migration and angiogenesis.

Sphingosine-1-phosphate (S1P), the product of phosphorylation of sphingosine by sphingosine kinase 1 (Sphk1), stimulates osteoblast differentiation and function. S1P and Sphk1 production is increased during RANKL-induced osteoclastogenesis. Moreover, deletion of Ctsk increases Sphk1 expression and S1P secretion in osteoclasts. S1P secretion and Sphk1 expression were lower in preosteoclasts of inhibitor AG1296 blocked phosphorylation of PDGFR and the rest of the downstream kinases, the PI3K inhibitor LY294002 blocked phosphorylation of PI3K, Akt and FAK, and the Akt inhibitor MK2206 blocked phosphorylation of Akt and FAK, whereas the FAK inhibitor Y15 blocked only phosphorylation of FAK (Fig. 5d). Independent inhibition of all components of this pathway was sufficient to block preosteoclast-conditioned medium–induced tube formation of MSC and EPC co-culture and migration of MSCs and EPCs (Fig. 5e,f). The results indicate that Akt–dependent activation of FAK signaling mediates PDGF-BB–induced cell migration and angiogenesis.
Figure 6  Increasing PDGF-BB stimulates CD31hiEmcnhi vessel formation and bone formation in ovariectomized mice. (a) PDGF-BB and VEGF concentrations by ELISA in serum and bone marrow (BM) in sham-operated (Sham) or ovariectomized (OVX) mice. (b) Quantification of vessel volume and surface area. (c) Representative images of TRAP (red) and PDGF-BB (green) immunostaining and quantification of TRAP+ and TRAP+PDGF-BB+ cells on trabecular (TB) and periosteal bone (PB) surfaces, respectively. (d) CD31 (red) and Emcn (green) immunostaining and quantification of CD31hiEmcnhi (yellow) cells in BM and periosteum. Scale bar, 50 µm. (e) PDGF-BB and VEGF concentrations by ELISA in serum and bone marrow of OVX mice treated with vehicle, PDGF-BB or L-235. (f) Angiography-based quantification of vessel volume and surface area. (g) Quantification of CD31hiEmcnhi immunostaining. (h) Quantification of mineral apposition rate (MAR) and bone formation rate (BFR) in TB and PB. (i) Serum osteocalcin and CTX concentrations by ELISA. (j) Model of PDGF-BB secreted by preosteoclasts to couple angiogenesis with osteogenesis. In periosteal bone modeling, preosteoclast secretion of PDGF-BB induces formation of CD31hiEmcnhi vessels and stimulates secretion of S1P to promote osteoblast differentiation. In trabecular bone remodeling, CD31hiEmcnhi vessels induced by preosteoclast secretion of PDGF-BB improves transport of nutrients, oxygen, minerals and metabolic wastes during bone remodeling. Dashed lines outline bone surface. Scale bar, 50 µm. Data shown as mean ± s.d. For a–d and h, n = 5 per group; For e–g and i, n = 10 per group. * P < 0.05, ** P < 0.01 (Student's t-test (a–d) and ANOVA (e–i)).

Pdgfb−/− mice than in preosteoclasts of age-matched Pdgfblox/fox mice (Fig. 5g), indicating S1P action is downstream of PDGF-BB. Indeed, Sphk1 expression and secreted S1P concentration were significantly higher in preosteoclasts and osteoclasts than in monocytes and macrophages (Fig. 5h), consistent with the previous report46. Moreover, Sphk1 and S1P production were significantly higher in L-235–treated wild-type preosteoclasts or preosteoclasts from Ctsk−/− mice than in vehicle-treated wild-type preosteoclasts (Fig. 5i). Preosteoclast-conditioned medium induced alkaline phosphatase activity and matrix mineralization of MSCs (Fig. 5j). The S1P1,3 antagonist VPC23019 reduced osteoblast differentiation (Fig. 5i). However, VPC23019 could not inhibit the preosteoclast-conditioned medium–induced migration of MSCs or EPCs (Supplementary Fig. 5b,c). Altogether, our results show that PDGF-BB induces Akt/FAK-dependent angiogenesis in coupling osteogenesis by S1P signaling.

Increasing PDGF-BB attenuates bone loss in OVX mice

Postmenopausal osteoporosis is associated with increased osteoclastic bone resorption and decreased angiogenesis49–51. To examine the role of PDGF-BB in postmenopausal osteoporosis, we generated OVX mice, which had significantly smaller uterus size and lower uterus weight (Supplementary Fig. 7a,b), as well as significantly lower trabecular bone volume, thickness and number and cortical thickness, compared to sham-operated (sham) mice (Supplementary Fig. 7c,d). PDGF-BB concentrations in both bone marrow and peripheral blood and the VEGF concentration in bone marrow were significantly lower in OVX mice than in sham mice (Fig. 6a). Microphile-perfused angiography demonstrated that vessel volume and surface area were significantly lower in OVX mice than in sham mice (Fig. 6b). As expected, OVX mice had significantly higher numbers of osteoclasts compared to sham mice (Fig. 6c). Of note, the osteoclasts were largely negative for PDGF-BB in the bone marrow from OVX mice, whereas periosteal osteoclasts were positive for PDGF-BB (Fig. 6c). Similarly, CD31hiEmcnhi cell numbers were significantly lower in marrow but higher in periostem in OVX mice compared to sham mice (Fig. 6d). There was also less proliferation of endothelial cells in metaphysis in OVX mice than in sham mice (Supplementary Fig. 7e).

We then examined whether increasing PDGF-BB abundance could be an effective potential therapy for postmenopausal osteoporosis. We treated OVX mice with either local injection of PDGF-BB in the femur or systemic delivery of L-235. PDGF-BB concentrations were significantly increased in bone marrow of OVX mice treated with either PDGF-BB or L-235, but increased only in peripheral blood after treatment with L-235, compared to vehicle-treatment concentrations.
Marrow VEGF concentrations were also increased in the PDGF-BB and L-235 groups compared to the vehicle group (Fig. 6e). Microphil-perfused angiography demonstrated that OVX mice treated with either PDGF-BB or L-235 had significantly increased vessel volume and surface area in bone marrow compared to vehicle-treated mice (Fig. 6f). Bone marrow injection of PDGF-BB significantly increased CD31hiEmcnhi cell numbers in bone marrow only, whereas the CTSK inhibitor L-235 significantly increased CD31hiEmcnhi cell numbers in both marrow and periosteum compared to vehicle treatment (Fig. 6g). There was also more proliferation of endothelial cells in metaphysis in the PDGF-BB and L-235 groups compared to the vehicle group (Supplementary Fig. 8a). Treatment of OVX mice with either PDGF-BB or L-235 substantially increased trabecular bone volume, thickness and number relative to vehicle treatment (Supplementary Fig. 8b). The cortical bone thickness was increased and endosteal perimeter decreased markedly with administration of either PDGF-BB or L-235 (Supplementary Fig. 8b). Calcein double labeling confirmed increased trabecular bone formation with administration of either PDGF-BB or L-235 (Fig. 6h). Periosteal bone formation and periosteal perimeter were increased with L-235, but not with PDGF-BB, probably owing to bone marrow local injection (Fig. 6h) and Supplementary Fig. 8b). Serum osteocalcin concentrations were higher with both treatments and CTX concentrations were lower with L-235 compared to vehicle treatment (Fig. 6i). Taken together, these results show that OVX-induced bone loss in mice results in a lower degree of PDGF-BB secretion and bone formation. Either local PDGF-BB administration or stimulation of periosteal CTSK secretion of PDGF-BB by systemic CTSK inhibition can temporally increase angiogenesis and spatially promote bone formation by coupling angiogenesis with osteogenesis.

**DISCUSSION**

Our study reveals that preosteoclasts secrete PDGF-BB to recruit EPCs and MSCs to promote angiogenesis during its coupling with osteogenesis. PDGF-BB secreted by preosteoclasts determines the temporospatial vessel formation needed for the subsequent bone resorption and new bone formation (Fig. 6j). Vessel formation influences the microenvironment for differentiation of osteoprogenitors. A recent study identified a specific CD31hiEmcnhi cell subtype with distinct morphological and molecular properties and location. The relative abundance of the CD31hiEmcnhi vessels is associated with bone formation or bone loss. We found that PDGF-BB secreted by preosteoclasts induces formation of the CD31hiEmcnhi vessel subtype to promote the coupling of angiogenesis with bone formation. Notably, concentrations of PDGF-BB are substantially decreased in both bone marrow and peripheral blood after OVX-induced bone loss in mice, and CD31hiEmcnhi vessels are also decreased in the bone marrow. Increasing maturation of osteoclasts from preosteoclasts reduces PDGF-BB abundance in ovariec-tomized mice, probably affecting bone formation. Knockout of Ctsk or injection of its inhibitor effectively increases the concentrations of PDGF-BB. In particular, treatment with a CTSK inhibitor increases angiogenesis, including CD31hiEmcnhi vessels, and bone formation in ovariec-tomized mice. Our finding of preosteoclast-induced angiogenesis represents a potential therapeutic target for bone loss, particularly for postmenopausal osteoporosis.

Cortical bone is a compact bone tissue that provides mechanical support for the body weight and makes up 80% of the weight of the human skeleton. In modeling, bone grows in both length and width, whereas in remodeling bone mass is homeostatically maintained. Longitudinal growth is achieved through endochondral ossification at the growth plate, as has been studied extensively. However, little is known about the mechanisms that regulate the width of bone, which is determined mainly by periosteal cortical bone formation. We found that periosteal preosteoclasts function as signaling cells by secreting PDGF-BB to promote periosteal bone formation. However, the periosteal TRAP+ cells expressing PDGF-BB are not F4/80+ OsteoMacs. Preosteoclasts have been observed previously in aquatic vertebrate skeleton and on the periosteal surface of cortical bone of mammals, and Preosteoclasts exhibit limited bone resorption but are present on the bone surface to direct osteogenesis.

The periosteum is a microvascularized connective tissue covering the outer surface of the cortical bone, a special microenvironment that could resemble a unique marrow for cortical bone modeling and growth. PDGF-BB from TRAP+ preosteoclasts residing on the bone surface is able to temporally and spatially coordinate angiogenesis during bone growth, modeling and remodeling. Preosteoclast-conditioned medium induces migration of MSCs and EPCs, and MSCs enhance tube formation in vitro assays. PDGF-BB from periosteal preosteoclasts stimulates secretion of S1P to also promote bone formation, further coupling angiogenesis to bone formation in the periosteal environment. Modeling of trabecular bone could also be modulated by preosteoclasts via a similar mechanism, whereas in trabecular bone remodeling, two distinct factors are employed: TGF-β1 activation during bone remodeling recruits MSCs for bone formation and PDGF-BB secreted by preosteoclasts prepares angiogenesis for subsequent bone formation in addition to recruitment of MSCs. Indeed, PDGF-BB has been widely used for bone regeneration and fracture healing and the ability of PDF-BB to direct cortical bone formation could be the cellular mechanism by which it achieves these clinical effects.

CTSK is a cysteine protease and is highly expressed in osteoclasts. It is responsible for bone matrix protein degradation during bone resorption. The selective CTSK inhibitors decrease osteoclastic bone resorption activity by preventing the degradation of bone matrix proteins. Deletion of Ctsk specifically in osteoclasts increases secretion of S1P for bone formation during bone remodeling. The CTSK inhibitor has also been shown to increase cortical dimension in mice, monkeys and humans and has been histomorphometrically demonstrated to stimulate periosteal bone formation of the long bones in preclinical models. Notably, in Ctsk−/− mice and wild-type mice treated with a CTSK inhibitor, the number of periosteal preosteoclasts and the levels of PDGF-BB in the periosteum are markedly increased. Taken together, the genetic and pharmacological findings demonstrate that stimulation of secretion of PDGF-BB by preosteoclasts may enhance the recruitment of EPCs and MSCs and is a potential therapeutic target for periosteal cortical defects and osteoporosis.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

H.X. conceived the ideas for experimental designs, conducted the majority of the experiments, analyzed data and prepared the manuscript. Z.C., L.W.,
X.Z. and Y.H. maintained mice and collected tissue samples, performed microcomputed tomography analyses, conducted immunohistochemistry and immunofluorescence, conducted cell culture and western blot experiments, and helped with manuscript preparation. L.Q., L.X., and W.C. maintained mice and helped with flow cytometry, cell culture and transwell migration assay. J.C., M.W., G.Z., Q.B., Y. and M.P. provided suggestions for the project and critically reviewed the manuscript. T.Q. performed confocal imaging. L.T.D. and J.J.W. provided mouse models. X.L. and E.L. participated in experimental design and helped compose the manuscript. X.C. developed the concept, supervised the project, conceived the experiments and wrote most of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mouse generation. We purchased the Csf1<sup>op</sup> and Pdgfb<sup>flx/lox</sup> mouse strains from Jackson Laboratory. We obtained the TRAP-Cre mouse strain<sup>61</sup> from J.J. Windle (Virginia Commonwealth University, Richmond, VA, USA). We obtained the Cisk knockout (Cisk<sup>−/−</sup>) mice<sup>63</sup> from Bone Biology Group of Merck Research Laboratories.

We generated Csf1<sup>−/−</sup> offspring and their wild-type littermates by crossing two heterozygote Csf1<sup>op</sup> strains. Hemizygous TRAP-Cre mice were crossed with Pdgfb<sup>flx/lox</sup> mice. The offspring were intercrossed to generate the following offspring: wild type mice, TRAP-Cre (mice expressing Cre recombinase driven by TRAP promoter), Pdgfb<sup>flx/lox</sup> (mice homozygous for Pdgfb flox allele) and TRAP-Cre; Pdgfb<sup>flx/lox</sup> (mice with Pdgfb conditional deletion in TRAP lineage cells are referred to as “Pdgfb<sup>−/−</sup>” in the text).

We generated Cisk<sup>−/−</sup> offspring and their wild-type littermates by crossing two heterozygote strains as described.<sup>62</sup> We analyzed male mice at 1 month of age, except as noted in specific experiments. We treated 4-week-old wild-type male mice and 6-week-old Pdgfb<sup>flx/lox</sup> and Pdgfb<sup>−/−</sup> male mice daily with either vehicle or 50 mg kg<sup>−1</sup> b.w. L-235 (Merck) for 30 d. L-235 was administered orogastrically in a 0.5% Methocel (w/v) suspension via a feeding tube.

We determined the genotype of the mice by PCR analyses of genomic DNA isolated from mouse tails using the following primers: Csf1<sup>op</sup> allele forward, 5′-TGTCAATCTCTGTTCTTCTG-3′ and reverse, 5′-GTGACCGGCTTGGTTG-3′; reverse, 5′-GTGACCGGCTTGGTTG-3′; and reverse, 5′-GGAAGGATTGTTGATAAGAG-3′ and reverse, 5′-ACAAGTGACATCTCCGTACC-3′.

For ovary removal surgery, 3-month-old C57BL/6 female mice were generally anesthetized and subjected to either a sham operation (Sham) or bilateral ovariectomy (OVX). We randomly divided mice into five groups: Sham, OVX, OVX + vehicle, OVX + PDGF-BB and OVX + I. 235. For OVX + PDGF-BB group, recombinant mouse PDGF-BB (1 μg; Pepro Tech) was delivered into the bone marrow cavity of the OVX mice from the medial side of the patellar tendon using 0.5-ml syringes with 27-gauge needles. The treatment was conducted once a month with the first injection at the same day of OVX. For OVX + L-235 group, L-235 (50 mg kg<sup>−1</sup> b.w.) was delivered orogastrically into OVX mice. The treatment was conducted daily for 2 months. All mice were sacrificed at the age of 5 months. Whole-blood samples were collected by cardiac puncture immediately after euthanasia, and serum samples obtained by centrifugation (1,500 r.p.m., 15 min) were stored at −80 °C before analyses. Uteri were isolated and weighed to confirm the effects of ovariectomy. Femora and tibia were also collected.

We maintained all animals in the Animal Facility of the Johns Hopkins University School of Medicine. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Johns Hopkins University, Baltimore, MD, USA.

μCT analysis. Femora were dissected from mice, fixed overnight in 70% ethanol and analyzed by high-resolution μCT (SkyScan 1172, Skyscan)<sup>13,63,64</sup>. The scanner was set at a voltage of 49 kV, a current of 200 μA and a resolution of 8.7 μm per pixel. We used image reconstruction software (NRecon v1.6), data analysis software (CTAn v1.9) and three-dimensional model visualization software (μCTVOL v2.0) in order to analyze the parameters of the diaphyseal cortical bone and the distal femoral metaphyseal trabecular bone. We established cross-sectional images of the femora to perform two-dimensional morphometric analyses of the cortical bone and three-dimensional histomorphometric analysis of the trabecular bone. Trabecular bone region of interest (ROI) was drawn starting from 5% of femoral length proximal to distal epiphyseal growth plate and extended proximally for a total of 5% of femoral length. The trabecular bone was segmented from the bone marrow and analyzed to determine the trabecular bone volume fraction (BV/TV), trabecular thickness (Th. Th), trabecular number (Th. N) and trabecular separation (Th. Sp). Diaphyseal cortical bone ROI was drawn starting from 20% of femoral length proximal to distal epiphyseal growth plate and extended proximally for a total of 10% of femoral length. We analyzed the cortical bone in order to determine the cortical thickness (Ct. Th), periosteal perimeter (Ps. Pm) and endosteal perimeter (Es. Pm).

Angiography. We used μCT for imaging bone vessels as previously described<sup>65,66</sup>. Briefly, after euthanization of mice, the thoracic cavity was opened, a needle was inserted into the left ventricle, the vasculature was flushed with heparinized saline (0.9% normal saline containing 100 U ml<sup>−1</sup> heparin sodium) and 10% neutral buffered formalin was injected for pressure fixation and then flushed from the vasculature using heparinized saline. Then, we injected a lead chromate-containing radiopaque silicone rubber compound (Microfil MV-122, Flow Tech.) into the vasculature. Mice were stored at 4 °C for 24 h and femora were then removed, fixed, decalcified and imaged by μCT. Vascular volume and surface area were measured.

Immunocytochemistry, immunofluorescence and histomorphometry. For immunocytochemical staining, we incubated cultured cells with primary antibody to mouse PDGF-BB (Abcam, ab21234, 1:50) at 37 °C for 2 h and subsequently used a horseradish peroxidase–strepavidin detection system (Dako) to detect the immunoreactivity, followed by TRAP staining using a staining kit (Sigma-Aldrich).

At the time of euthanasia, we dissected and fixed the femora with intact periosteum in 10% buffered formalin for 48 h, decalcified them in 10% EDTA (pH 7.4) (Amresco) for 21 d and embedded them in paraffin or optimal cutting temperature compound (Sakura Finetek). We processed four-micrometer-thick coronally (longitudinally) oriented sections of bone including the metaphysis and diaphysis for TRAP staining using a staining kit (Sigma-Aldrich).

We performed immunofluorescence analysis of the bone sections as described previously<sup>13,64</sup>. Briefly, we incubated bone sections with individual primary antibodies to mouse CD31 (Abcam, ab28364, 1:50), endomucin (Santa Cruz, V.7C7, 1:50), TRAP (Santa Cruz, sc-30833, 1:200), PDGF-BB (Abcam, ab21234, 1:50), Ki67 (Novus Biologicals, NB500-170, 1:50), osteocalcin (Takara, 1713, 1:200) and F4/80 (Biolegend, BM8, 1:25) overnight at 4 °C. Subsequently, we used secondary antibodies conjugated with fluorescence at room temperature for 1 h while avoiding light. We used isotype-matched controls, such as polyclonal rabbit IgG (R&D Systems, AB-105-C), polyclonal goat IgG (R&D Systems, AB-108-C) and monoclonal rat IgG2A (R&D Systems, 54447) under the same concentrations and conditions as negative controls. We counted the numbers of positively stained cells in the whole diaphyseal periosteum or four random visual fields of distal metaphysis per femur in five sequential sections per mouse in each group and normalized them to the number per millimeter of adjacent bone surface (N mm<sup>−1</sup>) in trabecular and cortical bone or per square millimeter of bone marrow area (N mm<sup>−2</sup>) in trabecular bone. We used Leica TCS SP1 or SP2 confocal microscope or an Olympus BX51 Microscope for imaging samples.

To examine dynamic bone formation, we subcutaneously injected 0.1% calcine (Sigma, 10 mg kg<sup>−1</sup> b.w.) in PBS into the mice 10 and 3 days before euthanization. We observed calcine double labeling in undecalcified bone slices under a fluorescence microscope. We measured periosteal bone formation at the site starting from 20% of femoral length proximal to distal epiphyseal growth plate and extended proximally for a total of 10% of femoral length. We measured trabecular bone formation in four randomly selected visual fields in distal metaphysis of femur.

Flow cytometry. For the analysis or sorting of CD31<sup>hi</sup>Emcn<sup>hi</sup> cells and total ECs in bone, after euthanization of 1-month-old male mice, we collected femora and tibiae, removed euthanasia at the end and the muscles and periosteum around the bone, and then crushed the metaphysis and diaphysis regions of the bone in ice-cold PBS. We digested whole bone marrow with collagenase at 37 °C for 20 min to obtain single-cell suspensions. After filtration and washing, we counted cells and incubated equal numbers of cells for 45 min at 4 °C with fluorescein isothiocyanate-conjugated endomucin antibody (Santa Cruz, V.7C7, 1:20) for 45 min, then washed cells, and further incubated them with peridinin chlorophyll protein (PerCP)-conjugated CD31 antibody (Biolegend, 390, 1:100) for 45 min at 4 °C. We also incubated cells for 45 min at 4 °C with phycoerythrin-PerCP- and allophycocyanin-conjugated antibodies to mouse CD31 (Biolegend, 390, 1:100), CD45 (Biolegend, 30-F11, 1:100) and Ter119 (Biolegend, TER-119, 1:100) and analyzed by flow cytometry.
Preparation of MSCs and EPCs. After euthanization, we collected bone marrow cells from 6-week-old wild-type mice and cultured them in α minimum essential medium (αMEM, Mediatech, Inc.) containing 100 U ml⁻¹ penicillin (Sigma-Aldrich), 100 µg ml⁻¹ streptomycin sulfate (Sigma-Aldrich) and 20% FBS (Atlanta Biologicals) at 37 °C in a 5% CO₂ humidified incubator. After 72 h, we removed non-adherent cells and cultured adherent cells for an additional 7 days with a media change. Then, we incubated cell aliquots for 20 min at 4 °C with fluorescein isothiocyanate- or phycocerythrin- or peridinin chlorophyll protein- and allophycocyanin-conjugated antibodies to mouse CD29 (Biolegend, HMB1-1, 1:50), Sca-1 (Biolegend, D7, 1:100), CD45 (Biolegend, 30-F11, 1:100) and CD11b (Biolegend, M1/70, 1:50). We performed acquisition on a FACS Aria model (BD Biosciences), and did the analysis using FACS DIVA software version 6.1.3 (BD Biosciences). We sorted CD31⁺CD45⁻Ter119⁻ cells as total endothelial cells, as well as total bone marrow cells, in an ELISA lysis buffer solution and stored them at −80 °C until ELISA analysis.

Preparation of conditioned media from preosteoclasts and osteoclasts. In vitro

In vitro assays for migration by EPCs, MSCs or co-culture. We plated Matrigel (BD Biosciences) in 96-well culture plates and incubated at 37 °C to polymerize for 45 min. We then seeded EPCs (2 × 10⁶ cells/well), MSCs (2 × 10⁵ cells/well), or combined MSCs (1 × 10⁵ cells/well) and EPCs (1 × 10⁴ cells/well) on polymerized Matrigel in plates. We cultured the cells with conditioned medium collected from preosteoclasts or monocytes and macrophages culture system. After incubation at 37 °C for 4 h, we observed tube formation by microscopy and measured the cumulative tube lengths. In some experiments, we added the neutralizing antibody for PDGF-BB described above to preosteoclast-conditioned medium. In another set of experiments, we preincubated the cells with AG1296 (20 µM), LY294002 (30 µM), MK2206 (10 µM) or Y15 (2 µM), specific inhibitors for PDGF-BB, PTK, Akt or FAK, respectively, for 1 h, before incubating them with the conditioned media.

Immunoprecipitation and immunoblotting. We measured PDGF-BB concentrations in conditioned media by immunoprecipitation and immunoblotting analysis as described. Briefly, for immunoprecipitation, we incubated conditioned media with PDGF-AA or PDGF-BB antibodies described below and then used protein G-Sepharose to absorb antigen–antibody complexes (Amersham Biosciences).

Measurement of S1P in conditioned medium. We processed conditioned media prepared as described above for mass spectrometry. We extracted S1P as previously described. Briefly, we added 100 µl 3 N NaOH into the medium (1 ml) with addition of C17-S1P (100 pmol; internal standard) for alkalization, then mixed with 1 ml CHCl₃, 1 ml CH₂OH and 9 µl HCl. We centrifuged the mixture at 300 g for 5 min to separate the alkaline aqueous phase containing S1P. We reextracted the organic phase with 0.5 ml methanol, 0.5 ml 1 N NaCl and 50 µl 3 N NaOH. We acidified the collected aqueous phases with 100 µl concentrated HCl and extracted the dephosphorylated sphingosine extracted twice with 1.5 ml CHCl₃ each. We then dried the combined CHCl₃ phases under vacuum and resuspended them in 200 µl CH₂OH. We performed sample analysis using Voyager DE-STR mass spectrometer (Applied Biosystems).

Assessment of osteogenic differentiation of MSCs. We seeded MSCs at a density of 5 × 10³ cm⁻² with serum-containing conditioned media from

ELISA analysis. We performed osteocalcin or CTX ELISA analysis of serum using a mouse osteocalcin EIA kit (Biomedical Technologies Inc.) or a RatLaps EIA kit (Immunodiagnostic Systems). We performed CTX ELISA analysis of conditioned media using a CrossLaps for Culture ELISA kit (Immunodiagnostic systems). We performed PDGF-BB or VEGF ELISA analysis of conditioned medium, serum or bone marrow supernatant using a Mouse/Rat PDGF-BB Quantikine ELISA kit or a Mouse VEGF Quantikine ELISA Kit (R&D Systems). We did all ELISA assays according to the manufacturers’ instructions.

Bone marrow supernatant collection. We exposed bone marrow of euthanized mice after cutting two ends of tibia and placed the samples for centrifugation for 15 min at 3,000 r.p.m. and 4 °C to obtain bone marrow supernatants, which we then stored at −80 °C until ELISA assay.
preosteoclast cultures. Serum-containing conditioned medium from monocyte and macrophage cultures served as a control. After 2 d of culture, we homogenized the cells and assayed ALP activity by spectrophotometric measurement of p-nitrophenol release using an enzymatic colorimetric ALP Kit (Roche). After 3 weeks of culture, we evaluated the cell matrix mineralization by alizarin red S staining (2% of alizarin red S (Sigma-Aldrich) dissolved in distilled water with the pH adjusted to 4.2). Alizarin red S staining was released by cetyl-pyridinium chloride (Sigma-Aldrich) and quantified by spectrophotometry. To normalize protein expression to total cellular protein, an aliquot of the cell lysates was measured with the Bradford protein assay.

Statistical analyses. All error bars are s.d. Data presented as mean ± s.d. We used unpaired, two-tailed Student’s t-tests for comparisons between two groups and one-way analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons. All data demonstrated a normal distribution and similar variation between groups. For all experiments, P < 0.05 were considered to be significant and indicated by ‘*’; P < 0.01 were indicated by ‘**’. All inclusion/exclusion criteria were preestablished and no samples or animals were excluded from the analysis. No statistical method was used to predetermine the sample size. The experiments were randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

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