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Suppression of bone formation by osteoclastic expression of semaphorin 4D

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Most of the currently available drugs for osteoporosis inhibit osteoclastic bone resorption; only a few drugs promote osteoblastic bone formation. It is thus becoming increasingly necessary to identify the factors that regulate bone formation. We found that osteoclasts express semaphorin 4D (Sema4D), previously shown to be an axon guidance molecule, which potently inhibits bone formation. The binding of Sema4D to its receptor Plexin-B1 on osteoblasts resulted in the activation of the small GTPase RhoA, which inhibits bone formation by suppressing insulin-like growth factor-1 (IGF-1) signaling and by modulating osteoblast motility. *Sema4d*$^{-/-}$ mice, *Plxnb1*$^{-/-}$ mice and mice expressing a dominant-negative RhoA specifically in osteoblasts showed an osteosclerotic phenotype due to augmented bone formation. Notably, Sema4D-specific antibody treatment markedly prevented bone loss in a model of postmenopausal osteoporosis. Thus, Sema4D has emerged as a new therapeutic target for the discovery and development of bone-increasing drugs.

Bone is continuously renewed by a process called bone remodeling, in which the resorption phase is followed by the reformation phase[1–4]. Each of these phases and also the transition between them requires a fine regulation by humoral factors or molecules mediating the communication among bone cells. Typically these factors are either secreted by the bone cells, expressed on the membrane of the bone cells or released from the bone matrix[1,2,5–7]. For example, transforming growth factor-β (TGF-β) and IGF released during bone resorption are known to stimulate bone formation[2,5], thus being called 'classical' coupling factors. Despite a wealth of *in vitro* data on other candidate molecules that are crucial for osteoclast-osteoblast communication[6], *in vivo* evidence has been lacking.

Axon-guidance molecules are widely expressed outside the nervous system, where they control cell migration, the immune response, tissue development and angiogenesis[8–13]. Recent studies have suggested that axon-guidance molecules, such as the semaphorins and ephrins, are involved in the cell-cell communication that occurs between osteoclasts and osteoblasts[14–18]. Here we provide genetic evidence for the role of Sema4D derived from osteoclasts in the regulation of bone formation through its receptor Plexin-B1, which is expressed by osteoblasts.

RESULTS

Osteoclast-derived Sema4D inhibits bone formation

To gain insight into the involvement of axon guidance molecules in bone remodeling, we performed a comprehensive expression analysis of the semaphorin, ephrin, netrin and slit gene families in osteoclasts and osteoblasts in mice. We observed high expression of *Sema4d* in osteoclasts, but not in osteoblasts (**Fig. 1a** and **Supplementary Fig. 1a**). *Sema4d* expression increased during receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis. The Sema4D induction was markedly reduced in nuclear factor of activated T cells c1–knockout cells (**Supplementary Fig. 1b**).

The elevated expression of Sema4D in the osteoclast lineage led us to analyze its function in the skeletal system using *Sema4d*$^{-/-}$ mice. Bone volume and trabecular thickness were significantly greater in *Sema4d*$^{-/-}$ mice as compared to wild-type mice (**Fig. 1b**). The three-point bending test showed that bone strength was greater in the *Sema4d*$^{-/-}$ mice (**Fig. 1b**). Bone morphometric analyses revealed that both the osteoblast surface and bone formation rate markedly increased (**Fig. 1c**, **Supplementary Fig. 1c** and **Supplementary Table 1**), whereas the parameters for osteoclastic bone resorption were normal in the *Sema4d*$^{-/-}$ mice (**Fig. 1d**), suggesting an osteosclerotic phenotype. *In vitro* osteoclastogenesis in *Sema4d*$^{-/-}$ cells was also normal (**Supplementary Fig. 1d**,**e**). These results suggest that the high-bone-mass phenotype in the *Sema4d*$^{-/-}$ mice was caused by an increase in bone formation activity by osteoblasts, even though Sema4D is expressed exclusively by osteoclasts.

We performed an adoptive transfer of bone marrow cells including osteoclast precursor cells. Wild-type mice engrafted with Sema4D-deficient bone marrow cells had a high bone mass, whereas *Sema4d*$^{-/-}$ mice engrafted with wild-type bone marrow cells had a normal bone

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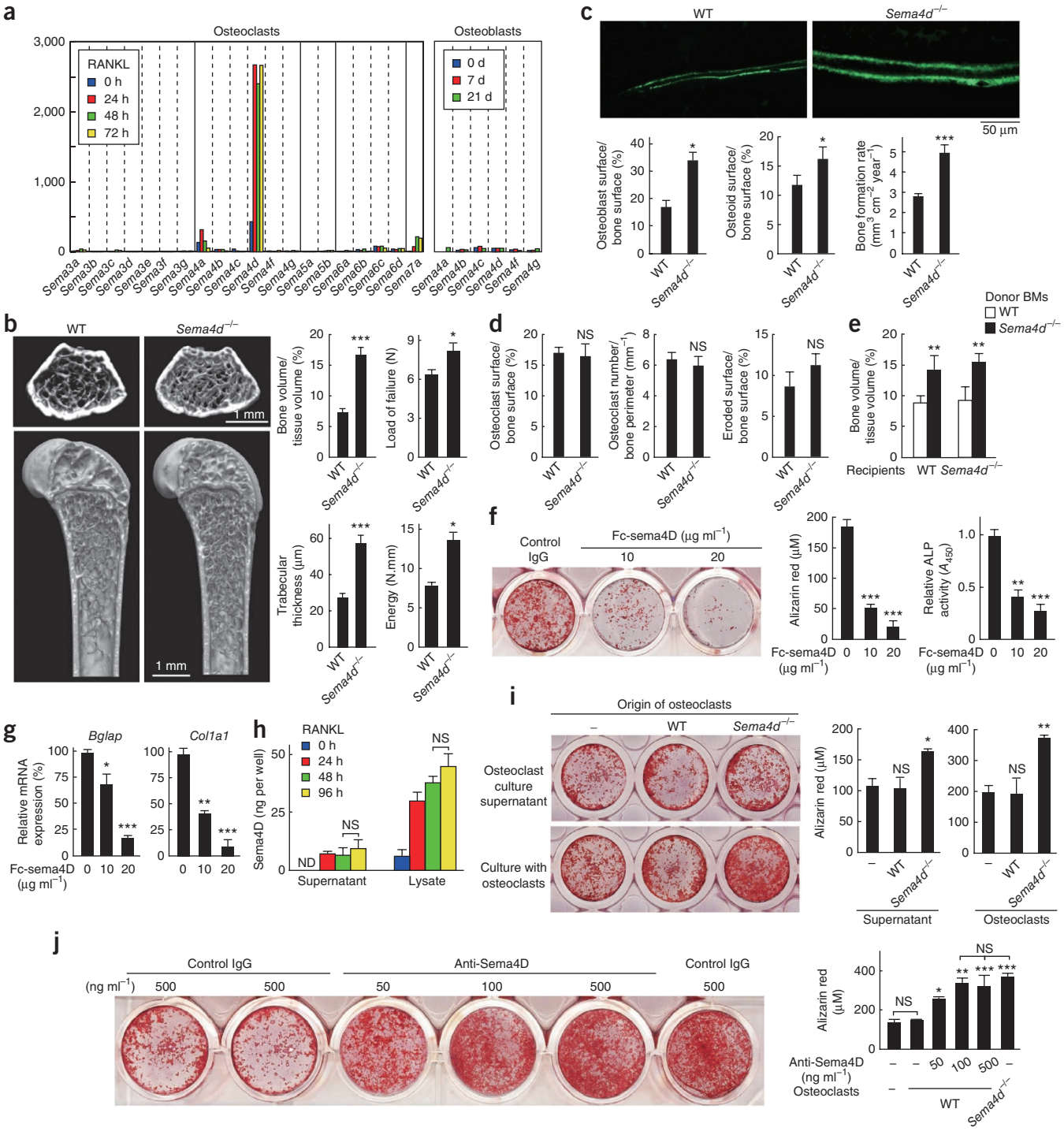

Figure 1 Inhibition of bone formation by osteoclast-derived Sema4D. (**a**) Genome-wide screening of mRNA for the semaphorin family proteins during differentiation of osteoclasts and osteoblasts. (**b**) Microcomputed tomography (μCT) of the proximal femur of the wild-type (WT) and *Sema4d*$^{-/-}$ mice (top left, axial view of the metaphyseal region; bottom left, longitudinal view). Bone volume and trabecular thickness were determined by μCT analysis (middle). Maximum load to failure and energy resorption were determined by the three-point bending test (right). (**c**) Bone formation, as observed by calcein double labeling at an interval of 4 d (top) and the parameters for osteoblastic bone formation, as determined by bone morphometric analysis (bottom). (**d**) The parameters for osteoclastic bone resorption, as determined by bone morphometric analysis. (**e**) Bone volume after adoptive transfer of wild-type or *Sema4d*$^{-/-}$ bone marrow cells (BMs) to wild-type (left) and *Sema4d*$^{-/-}$ (right) mice. (**f**) Effect of Fc-sema4D on bone nodule formation. Left, Alizarin red staining; middle, amount of alizarin red; right, effect of Fc-sema4D on ALP activity. (**g**) Effect of Fc-sema4D on the mRNA expression of *Bglap* and *Col1a1*. (**h**) The amount of Sema4D in the osteoclast supernatant and the cell lysate during osteoclast differentiation. The amount of Sema4D was analyzed using bone-resorbing osteoclasts 96 h after RANKL stimulation. (**i**) Effect of osteoclast culture supernatant or coculture with osteoclasts on bone nodule formation. Left, Alizarin red staining; right, amount of the alizarin red. (**j**) Effect of antibody to Sema4D (anti-Sema4D) on bone formation in wild-type osteoblasts cocultured with wild-type or *Sema4d*$^{-/-}$ osteoclasts. Left, Alizarin red staining; right, amount of alizarin red. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; NS, not significant; ND, not detected. Error bars show s.e.m.

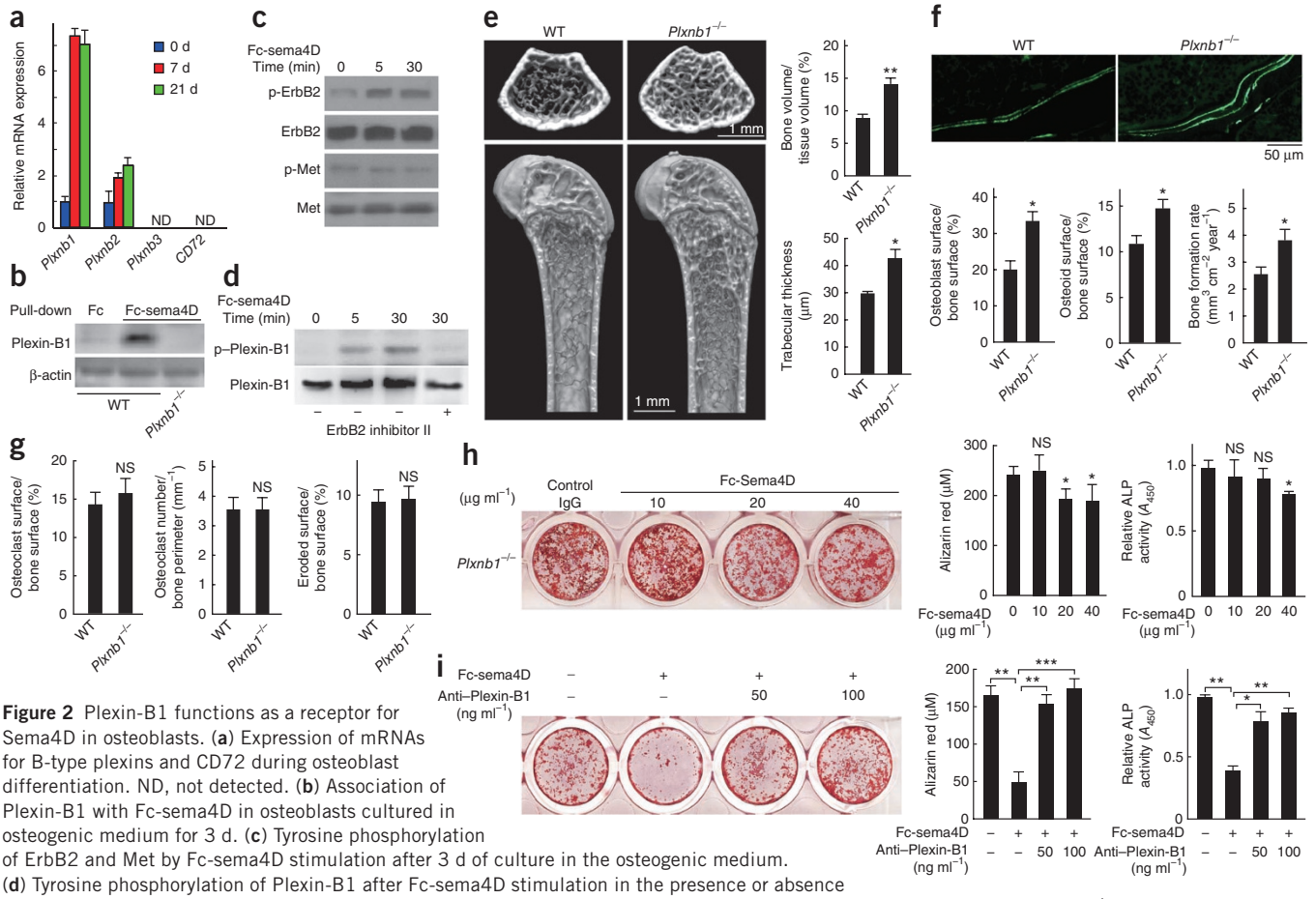

Figure 2 Plexin-B1 functions as a receptor for Sema4D in osteoblasts. (**a**) Expression of mRNAs for B-type plexins and CD72 during osteoblast differentiation. ND, not detected. (**b**) Association of Plexin-B1 with Fc-sema4D in osteoblasts cultured in osteogenic medium for 3 d. (**c**) Tyrosine phosphorylation of ErbB2 and Met by Fc-sema4D stimulation after 3 d of culture in the osteogenic medium. (**d**) Tyrosine phosphorylation of Plexin-B1 after Fc-sema4D stimulation in the presence or absence of 30 μM of an ErbB2 inhibitor (ErbB2 inhibitor II). (**e**) Microcomputed tomography of the femur of wild-type (WT) and *Plxnb1*$^{-/-}$ mice (see **Fig. 1b** legend for the details). (**f**) Bone formation, as observed by calcein double labeling and the parameters for osteoblastic bone formation determined. (**g**) The parameters for osteoclastic bone resorption were determined by bone morphometric analysis. (**h**) Effect of Fc-sema4D on bone formation in *Plxnb1*$^{-/-}$ osteoblasts. Left, bone nodule formation; middle, amount of alizarin red; right, ALP activity. (**i**) Effects of the antibody to Plexin-B1 (anti–Plexin-B1) on bone formation in Fc-sema4D–treated osteoblasts. Left, bone nodule formation; middle, the amount of alizarin red; right, ALP activity. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; NS, not significant; ND, not detected. Error bars show s.e.m.

mass (**Fig. 1e**), showing that the bone phenotype in the *Sema4d*$^{-/-}$ mice resulted from a defect in hematopoietic lineage cells including osteoclasts. Consistent with this, there was no obvious increase in the bone nodule formation of *Sema4d*$^{-/-}$ calvarial cells in the absence of osteoclasts (**Supplementary Fig. 1f–h**). These results suggest that the Sema4D expressed by osteoclasts inhibits osteoblastic bone formation and is a mediator of osteoclast-osteoblast communication.

To observe the inhibitory effect of Sema4D on osteoblasts, we added soluble Sema4D fused with the IgG1 Fc region (Fc-sema4D)[19] to the calvarial cells cultured under osteogenic conditions. Addition of Fc-sema4D suppressed bone nodule formation, alkaline phosphatase (ALP) activity and the expression of osteoblastic marker genes such as osteocalcin (*Bglap*) and type I collagen (*Col1a1*) in a dose-dependent manner (**Fig. 1f,g**). There was no difference in the number of mesenchymal stem cells and osteoblast precursor cells in bone marrow between wild-type and *Sema4d*$^{-/-}$ mice (**Supplementary Fig. 2**).

Although Sema4D is a transmembrane protein, it is proteolytically cleaved into a soluble form upon cellular activation[9,20]. We did detect soluble Sema4D in the osteoclast culture supernatant, but only a small portion was released as a soluble form, regardless of the osteoclast maturation stage (**Fig. 1h**), suggesting that membrane-bound Sema4D has a larger role in suppression of bone formation. To examine the contribution of osteoclast-derived Sema4D to the regulation of bone formation, we cultured calvarial cells in the presence of osteoclast culture supernatant or cultured osteoclasts. The addition of culture supernatant of the wild-type osteoclasts to calvarial cells or coculturing calvarial cells with wild-type osteoclasts did not influence bone nodule formation, ALP activity or the expression of osteoblastic marker genes; in contrast, these processes were markedly enhanced by either the culture supernatant of the *Sema4d*$^{-/-}$ osteoclasts or the coculture with the *Sema4d*$^{-/-}$ osteoclasts (**Fig. 1i** and **Supplementary Fig. 3a–d**). Consistent with this, bone formation in the presence of wild-type osteoclasts was increased by Sema4D-specific antibody in a dose-dependent manner (**Fig. 1j** and **Supplementary Fig. 3e–g**). We concluded that osteoclasts generate factors promoting bone formation[1,5], which is obvious only in the absence of Sema4D, but osteoclast-derived Sema4D has a dominant effect that antagonizes this bone-forming activity. Collectively, the evidence shows that osteoclasts suppress bone formation through the expression of Sema4D.

Plexin-B1 recognizes Sema4D and inhibits bone formation

To identify the Sema4D receptors expressed by osteoblasts, we analyzed the mRNA expression of B-type plexins and CD72, which are known to be Sema4D receptors preferentially expressed in nonlymphoid and lymphoid cells, respectively[9,21]. Plexin-B1 expression was markedly induced

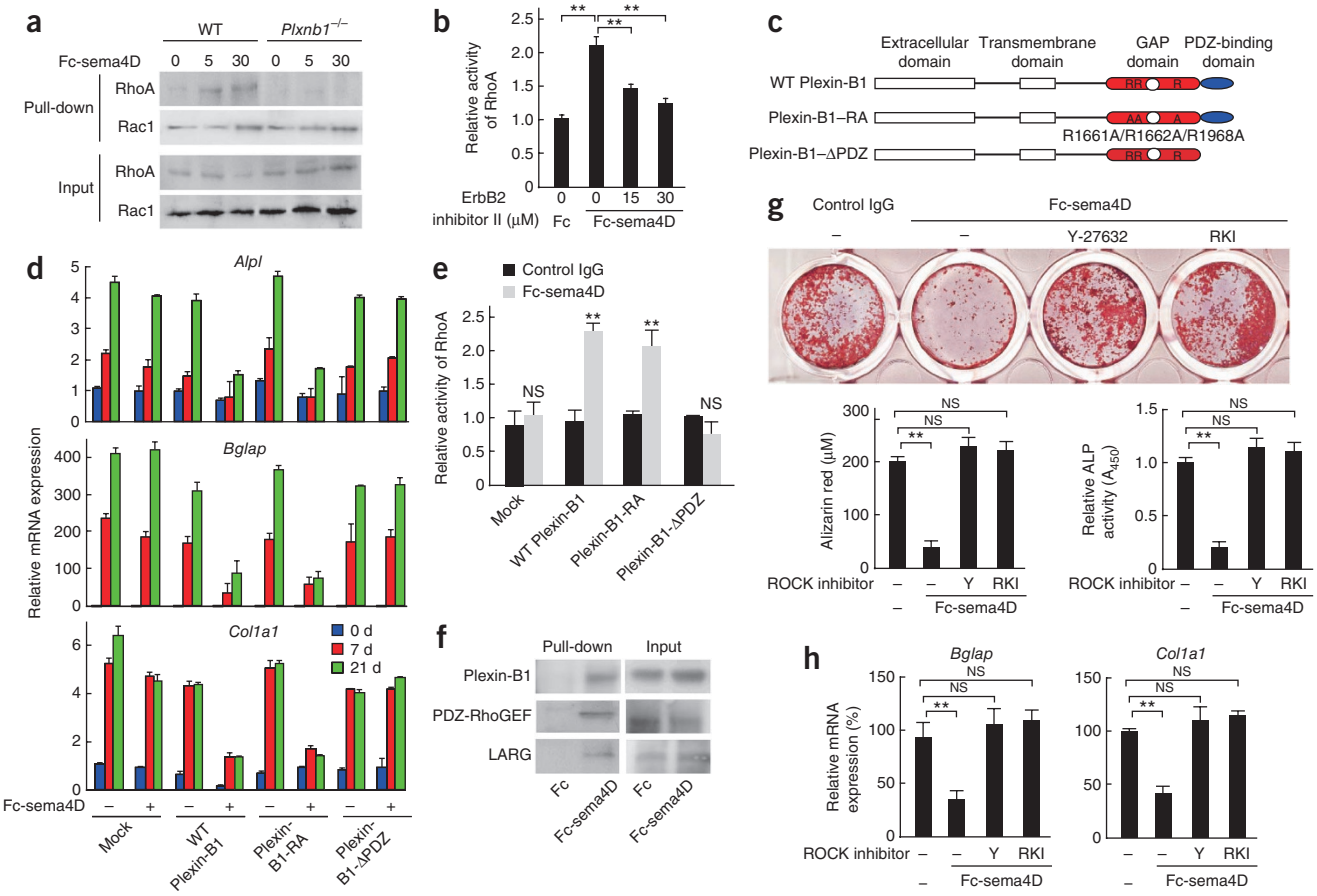

Figure 3 Inhibition of bone formation by the Sema4D–Plexin-B1–RhoA axis. (**a**) Amount of the GTP-bound form and total level of RhoA and Rac1 in wild-type (WT) and *Plxnb1*$^{-/-}$ osteoblasts cultured in osteogenic medium for 3 d. A pull-down assay was performed with GST-RBD (for RhoA) and GST-PAK1 (for Rac1) coupled to glutathione-Sepharose, followed by immunoblotting with antibody to RhoA (anti-RhoA) and antibody to Rac-1 (anti-Rac1), respectively. (**b**) Effect of the ErbB2 inhibitor on Fc-sema4D-induced RhoA activation in calvarial osteoblasts. (**c**) Schematic of wild-type Plexin-B1, a Plexin-B1-RA mutant harboring a mutation in the GAP domain (R1661A/R1662A/R1968A) and a truncated mutant of Plexin-B1 lacking the PDZ-binding domain (ΔPDZ). (**d**,**e**) Effect of retroviral expression of wild-type Plexin-B1 and the Plexin-B1 mutants in Fc-sema4D–treated *Plxnb1*$^{-/-}$ osteoblasts. The mRNA expression of *Alpl*, *Bglap* and *Col1a1* (**d**) and RhoA activation (**e**) were examined. (**f**) Association of Rho-specific GEFs, PDZ-RhoGEF and LARG with Plexin-B1 in osteoblasts. (**g**) Effect of the ROCK inhibitors Y-27632 and RKI on bone nodule formation in Fc-sema4D–treated osteoblasts. Top, Alizarin red staining; bottom left, amount of alizarin red; bottom right, ALP activity. (**h**) Effect of the ROCK inhibitors Y-27632 and RKI on mRNA expression of *Bglap* and *Col1a1*. $^{**}P < 0.01$; NS, not significant. Error bars show s.e.m.

during osteoblast differentiation (**Fig. 2a**), but not during osteoclast differentiation (data not shown). The induction of Plexin-B1 in osteoblasts was much higher than that of Plexin-B2, and Plexin-B3 and CD72 were almost undetectable (**Fig. 2a**). A pull-down experiment using Fc-sema4D indicated that Sema4D physically interacted with Plexin-B1 (**Fig. 2b**). Plexin-B1 forms a receptor complex with either erythroblastic leukemia viral oncogene homolog 2 (ErbB2) or hepatocyte growth factor receptor (Met), depending on the cell type[22]. Binding of Sema4D to Plexin-B1 results in the phosphorylation of the kinase (ErbB2 or Met) and Plexin-B1 (ref. 23). In osteoblasts, expression of ErbB2 was higher than that of Met (**Supplementary Fig. 4a**), and Sema4D stimulation induced the phosphorylation of ErbB2, but not Met (**Fig. 2c**). Sema4D induced the phosphorylation of Plexin-B1, and this phosphorylation was substantially lowered in the presence of the ErbB2 inhibitor (**Fig. 2d**), suggesting that Plexin-B1 functions as a receptor for Sema4D and ErbB2 serves as an associating kinase in osteoblasts.

Similar to *Sema4d*$^{-/-}$ mice, *Plxnb1*$^{-/-}$ mice had a high bone mass due to an increase in osteoblastic bone formation, and there was no obvious abnormality in osteoclastic bone resorption (**Fig. 2e–g** and **Supplementary Fig. 4b**,**c**). Fc-sema4D at 10 μg ml^{-1} did not inhibit bone formation in *Plxnb1*$^{-/-}$ osteoblasts but did cause strong inhibition in wild-type osteoblasts (**Fig. 2h** and **Supplementary Fig. 4d**), and the Plexin-B1–specific antibody effectively suppressed the inhibitory effect of Fc-sema4D on bone formation in wild-type cells (**Fig. 2i** and **Supplementary Fig. 4e**). In addition, similar to the *Sema4d*$^{-/-}$ osteoclasts, wild-type osteoclasts promoted bone formation in the *Plxnb1*$^{-/-}$ osteoblasts (**Supplementary Fig. 4f–h**). These results indicate that Plexin-B1 is mainly responsible for the osteoblast recognition of Sema4D. *Plxnb1*$^{-/-}$; *Sema4d*$^{-/-}$ mice, however, had a higher bone mass than did *Plxnb1*$^{-/-}$ mice (**Supplementary Fig. 5**), and a high concentration of Fc-sema4D slightly inhibited bone formation in *Plxnb1*$^{-/-}$ cells (**Fig. 2h** and **Supplementary Fig. 4d**), suggesting that the effect of Sema4D is partly mediated by other receptors, such as Plexin-B2.

RhoA mediates Sema4D-Plexin-B1 signaling in osteoblasts

The semaphorin-plexin system regulates cell morphology and migration by modulation of actin cytoskeletal rearrangement, primarily via Rho family small GTPases[23]. The amount of GTP-bound, activated form

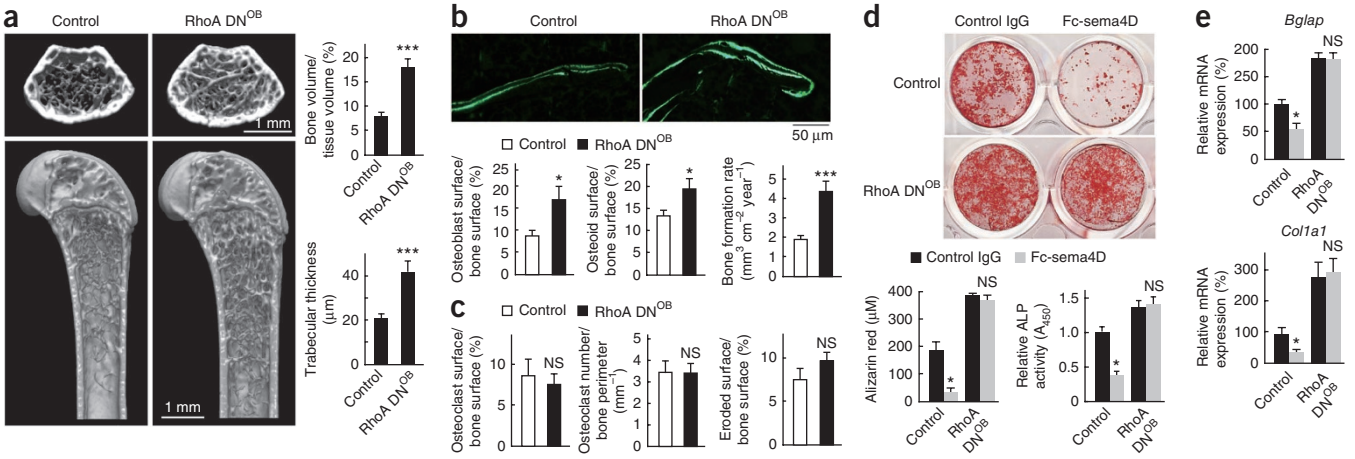

Figure 4 Osteoblas-spec st-specific loss of function of Rho GTPase leads to an osteosclerotic phenotype. (**a**) Microcomputed tomography of the femur in control and RhoA DN^{OB} mice (see **Fig. 1b** legend for the details). (**b**) Bone formation, as observed by calcein double labeling and the parameters for osteoblastic bone formation determined. (**c**) Parameters for osteoblastic bone formation and osteoclastic bone resorption, as determined by bone morphometric analysis. (**d**) Effect of Fc-sema4D on bone formation in calvarial cells derived from RhoA DN^{OB} mice. Top, alizarin red staining; bottom left, amount of alizarin red; bottom right, ALP activity. (**e**) Effect of Fc-sema4D on the mRNA expression of *Bglap* and *Col1a1*. $*P < 0.05$; $***P < 0.005$; NS, not significant. Error bars show s.e.m.

of RhoA in Sema4D-treated osteoblasts was notably lower in *Plxnb1*$^{-/-}$ cells as compared to wild type cells, whereas the level of GTP-bound Rac1 was not affected after Fc-sema4D stimulation (**Fig. 3a**). In addition, Sema4D-induced RhoA activation was reduced in the presence of an ErbB2 inhibitor (**Fig. 3b**). Adenoviral introduction of constitutively active RhoA (RhoA-V14) suppressed bone nodule formation in calvarial cells, whereas dominant-negative RhoA (RhoA-N19) promoted it (**Supplementary Fig. 6a–c**), suggesting that RhoA selectively mediates the inhibitory effect of Sema4D–Plexin-B1 on bone formation.

Plexin-B1 has two small GTPase regulatory domains, a GTPase-activating protein (GAP) domain and a PDZ-binding domain that binds Rho guanine nucleotide exchange factor (GEF)[24–27]. We generated two Plexin-B1 mutants: Plexin-B1-ΔPDZ, which is unable to activate RhoA[27], and Plexin-B1-RA, which does not elicit the GTPase activity of R-Ras[24] (**Fig. 3c**). We stimulated *Plxnb1*$^{-/-}$ calvarial cells overexpressing these mutants with Fc-sema4D and examined the effect on osteoblast differentiation. The inhibitory effect of Fc-sema4D on the mRNA expression of *Alpl* (encoding ALP), *Bglap*

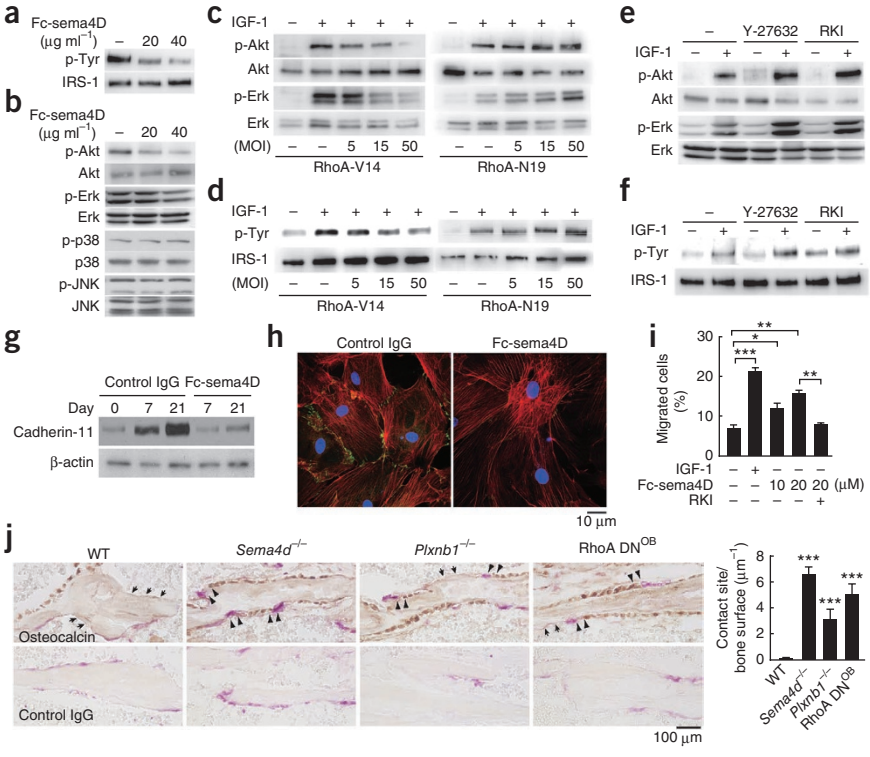

Figure 5 The mechanism of Sema4D–Plexin-B1–RhoA-mediated regulation of bone formation. (**a**) Effect of Fc-sema4D on the phosphorylation of IRS-1 during osteoblast differentiation. (**b**) Effect of Fc-sema4D on the phosphorylation of Akt, Erk, p38 and JNK during osteoblast differentiation. (**c**) Effect of constitutively active RhoA (RhoA-V14) or dominant-negative RhoA (RhoA-N19) on IGF-1–induced phosphorylation of Akt and Erk. MOI, multiplicity of infection. (**d**) Effect of RhoA-V14 or RhoA-N19 on IGF-1–induced phosphorylation of IRS-1. (**e**) Effect of the ROCK inhibitors Y-27632 and RKI on IGF-1–induced phosphorylation of Akt and Erk. (**f**) Effect of ROCK inhibitors on IGF-1–induced phosphorylation of IRS-1. (**g**) Effect of Fc-sema4D on the expression of cadherin-11 during osteoblast differentiation. (**h**) Immunofluorescence staining of cadherin-11 in osteoblasts. (**i**) Migration of osteoblasts in the presence of Fc-sema4D and the effect of RKI on Sema4D-induced cell motility. IGF-1 (100 ng ml^{-1}) was used as a positive control. (**j**) The spatial distribution of osteoblasts and osteoclasts on the bone surface of wild-type (WT), *Sema4d*$^{-/-}$, *Plxnb1*$^{-/-}$ and RhoA DN^{OB} mice. Osteoblasts and osteoclasts were detected as osteocalcin-positive (brown) and TRAP-positive cells (magenta), respectively. The osteoblast surface lies a certain distance from the osteoclast surface; arrows indicate the intervening quiescent surfaces. The number of sites in which osteoblasts are located within a 50-nm distance of osteoclasts (indicated by the arrowheads) were counted. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. Error bars show s.e.m.

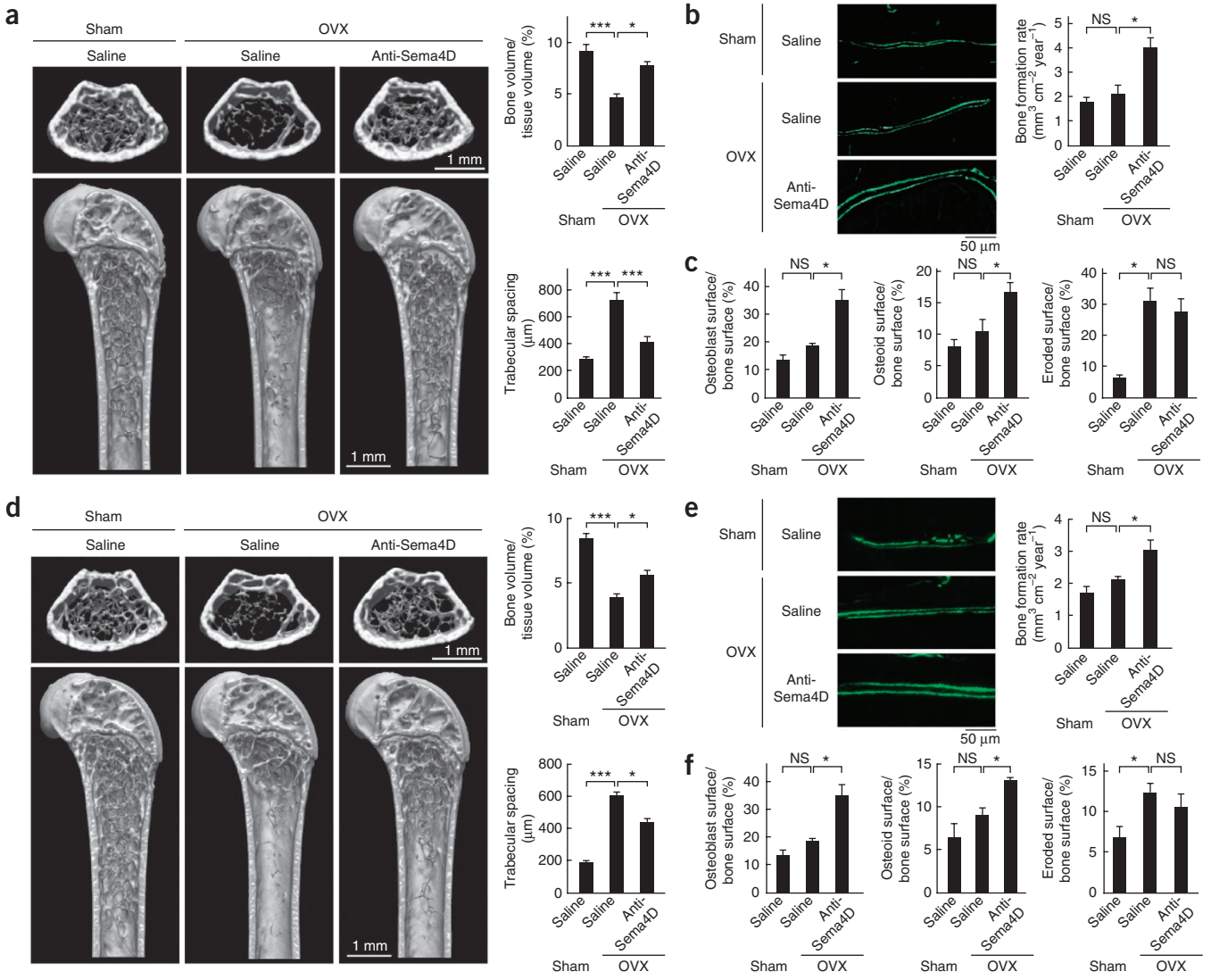

Figure 6 The bone-increasing effect of Sema4D–Plexin-B1 blockade. (**a**–**c**) The prophylactic effect of anti-Sema4D on ovariectomy (OVX)-induced bone loss. OVX mice were injected weekly with anti-Sema4D or saline from day 3 to week 8 after surgery. (**d**–**f**) The therapeutic effect of anti-Sema4D on OVX-induced bone loss. After 6 weeks of surgery, OVX mice were injected with anti-Sema4D or saline every 3 d for 3 weeks. In **a** and **d**, microcomputed tomography of the femur of the sham-operated (Sham), OVX and anti-Sema4D–treated OVX mice is shown (left; see **Fig. 1b** legend for the details). Bone volume and trabecular spacing were determined by microcomputed tomography analysis (right). In **b** and **e**, bone formation is shown by calcein double labeling and the bone formation rate. In **c** and **f**, the parameters for osteoblastic bone formation and osteoclastic bone resorption were determined by morphometric analysis using the tibia of mice. $^{*}P < 0.05$; $^{***}P < 0.005$; NS, not significant. Error bars show s.e.m.

and *Col1a1* in *Plxnb1*$^{-/-}$ cells was recovered by the overexpression of Plexin-B1-RA as well as wild-type Plexin-B1, but not by Plexin-B1-ΔPDZ (**Fig. 3d**), concomitantly with the restoration of RhoA activity (**Fig. 3e**). Plexin-B1 is known to bind two RhoGEFs, PDZ-RhoGEF (Arhgef11) and LARG (Arhgef12), through the PDZ-binding domain[25–27]. Osteoblasts expressed these RhoGEFs (**Supplementary Fig. 6d**), which interacted with Plexin-B1 (**Fig. 3f**), suggesting that these RhoGEFs are involved in the RhoA activation by Sema4D. RhoA activates various downstream effector molecules, including Rho-associated protein kinase (ROCK), whose inhibition, in turn, suppressed the inhibitory effect of Fc-sema4D on bone formation (**Fig. 3g**,**h**). These results suggest that the RhoA-ROCK pathway mediates the inhibition of bone formation by Sema4D.

We generated mice expressing dominant-negative RhoA specifically in osteoblasts (RhoA DN^{OB} mice) by crossing CAT-RhoA DN transgenic mice[28] with α1(I)-Cre transgenic mice[29]. RhoA DN^{OB} mice had a high bone mass due to enhanced osteoblastic bone formation, which recapitulates the bone phenotype of the *Sema4d*$^{-/-}$ and *Plxnb1*$^{-/-}$ mice (**Fig. 4a–c** and **Supplementary Fig. 6e**,**f**). Fc-sema4D did not inhibit bone formation in calvarial cells derived from RhoA DN^{OB} mice (**Fig. 4d**,**e**), indicating that RhoA regulates bone formation *in vivo* and may be a crucial mediator of bone mass modulation by Sema4D–Plexin-B1 signaling.

Sema4D controls osteoblast differentiation and migration via RhoA

What are the mechanisms by which Sema4D inhibits bone formation in osteoblasts? The RhoA-ROCK pathway mediates the phosphorylation of IRS-1, which is involved in IGF-1 signaling during embryogenesis[30]. In calvarial cells, Sema4D stimulation reduced the tyrosine phosphorylation of IRS-1 at the tyrosine residue required

for Akt and mitogen-activated protein kinase activation (**Fig. 5a**). Fc-sema4D reduced phosphorylation of Akt and extracellular signal-regulated kinase (Erk) in a dose-dependent manner, but not of p38 or JNK (**Fig. 5b**). The adenoviral introduction of RhoA-V14 reduced the IGF-1-induced phosphorylation of Akt and Erk, whereas either overexpression of RhoA-N19 or ROCK inhibitors markedly increased the phosphorylation of Akt and Erk as well as the tyrosine residue of IRS-1 (**Fig. 5c–f**). These results indicate that Sema4D-induced RhoA activation suppresses osteoblast differentiation at least in part through an attenuation of IGF-1 signaling.

Binding of Sema4D to the Plexin-B1–ErbB2 receptor complex is known to stimulate cell motility through RhoA activation[22]. In osteoblastic cells, Sema4D stimulation decreased the expression of cadherin-11 at the cell-cell contact region (**Fig. 5g,h**), suggesting that Sema4D stimulates cell motility through an impairment of cell-cell adhesion, which in turn results in the reduction in bone-forming activity. To test this hypothesis, we examined the effect of Sema4D on osteoblast motility. A Boyden chamber assay showed that Fc-sema4D induced an acceleration of spontaneous migration of osteoblasts, which was inhibited by a ROCK inhibitor (**Fig. 5i**), suggesting that Sema4D-induced RhoA activation promotes osteoblast motility.

In bone tissue, the osteoblast surface lies at certain distance from the osteoclast surface, and there is usually an intervening quiescent surface in between (**Fig. 5j**). It remains unclear how the quiescent surface is maintained, but it is possible that osteoclasts produce a molecule(s) that stimulates osteoblast motility and guides the osteoblasts to a proper site. In *Sema4d*$^{-/-}$ mice, as well as *Plxnb1*$^{-/-}$ and RhoA DN^{OB} mice, there were few quiescent surfaces, and we observed a cluster of osteoblasts in close proximity to bone-resorbing osteoclasts (**Fig. 5j**). Thus, osteoclast-derived Sema4D is required for the proper localization of osteoblasts and the maintenance of a quiescent surface.

Together, these data suggest that osteoclasts are involved in the spatial regulation of bone remodeling through Sema4D, which inhibits osteoblast differentiation in the proximity of osteoclasts and repels osteoblasts by increasing their motility.

Therapeutic effect of an antibody to Sema4D on bone loss

To test whether inhibition of Sema4D would be a useful approach to the treatment of osteoporosis, we examined the effect of a Sema4D-specific antibody on bone loss in an ovariectomized mouse model of postmenopausal osteoporosis. Ovariectomized 7-week-old mice were treated prophylactically with a weekly intravenous injection of the antibody starting 3 d after ovariectomy and continuing for 8 weeks, or osteopenic mice (6 weeks after ovariectomy) were therapeutically injected with the antibody once every 3 d for 3 weeks. Injection of the Sema4D-specific antibody was protective against bone loss after ovariectomy by promoting osteoblastic bone formation without affecting osteoclastic bone resorption in both prophylactic and therapeutic treatments (**Fig. 6** and **Supplementary Fig. 7**). Sema4D-specific antibody effectively increased bone formation in human osteoblastic cells in coculture with human osteoclasts or osteoclast supernatant (**Supplementary Fig. 8**). Along with the effect of Plexin-B1–specific antibody (**Fig. 2i** and **Supplementary Fig. 4e**), these results suggest that the blocking Sema4D–Plexin-B1interaction is a new and potentially effective strategy for increasing bone formation in humans.

DISCUSSION

The semaphorins have been implicated in repulsive axon guidance events in the developing nervous system. However, they are also widely expressed outside the nervous system and mediate diverse biological processes including organogenesis, vascularization, the immune response and tumor progression[31–35]. The semaphorins have also been suggested to be involved in the regulation of bone remodeling[15,16,18,36], although this function has not been clearly demonstrated *in vivo* in loss-of-function genetic models.

Sema4D, also known as CD100, was first identified in immune cells and has been extensively investigated in the immune system. Sema4D, constitutively expressed in T cells, regulates the activation of B cells[19] and dendritic cells[37] and inhibits monocyte migration[38]. In contrast to Sema3 family members, the function of Sema4D as an axon guidance cue has been less well understood, but a recent study provided genetic evidence that Sema4D positively regulates the migration of neurons during cortical development[39].

Bone remodeling is a cycle consisting of three phases: the initiation of bone resorption by osteoclasts; the transition into new bone formation by osteoblasts; and the synthesis of new bone and then termination[6]. In the initial phase, osteoblastic bone formation in the immediate vicinity of bone-resorbing osteoclasts or osteoblast recruitment to the bone resorption site needs to be suppressed until osteoclastic bone resorption is accomplished. The Sema4D expressed by osteoclasts may function as an inhibitor of bone formation in this phase by suppressing osteoblast differentiation and modulating osteoblast motility. Classically, coupling factors released during bone resorption activate bone formation, but the concept can be extended to include osteoclast-osteoblast communicating factors. These factors do not necessarily link bone resorption to formation but may dissociate formation from resorption, thus being crucial for specific phases in bone remodeling. In this study, we showed that osteoclasts control osteoblast localization through Sema4D regulation of osteoblast differentiation and motility, suggesting that Sema4D acts as a guidance molecule for bone cell positioning, which is analogous to the function of semaphorins in axon guidance.

We showed that the binding of Sema4D to Plexin-B1 results in the activation and autophosphorylation of ErbB2, which phosphorylates Plexin-B1. RhoA is subsequently activated by RhoGEFs including PDZ-RhoGEF and LARG, which associate with Plexin-B1. RhoA-ROCK inhibits the phosphorylation of IRS-1, which is the crucial step in IGF-1 signaling favoring osteoblast differentiation. Thus, we concluded that Sema4D inhibits osteoblast differentiation by RhoA activation.

Although the detailed mechanisms by which Sema4D regulates osteoblast motility remain obscure, we found that cadherin-11 expression was decreased by Sema4D stimulation. Cadherin-11 is a specific component of the osteoblast cell-cell adhesion machinery[40], and downregulation of cadherin-11 may be related to the higher osteoblast motility observed after Sema4D treatment. Consistent with this, mice lacking cadherin-11 show osteopenia, indicating that cadherin-11 is a positive regulator of bone formation[40]. Further study will be needed to understand the detailed function of cadherin-11 in Sema4D-mediated regulation of bone formation.

We confirmed that the Sema4D-specific antibody promotes bone formation in the coculture system of human osteoclasts and osteoblasts, suggesting that Sema4D is an auspicious therapeutic target in the treatment of human osteopenic disease (**Supplementary Fig. 8**). In the bone loss associated with inflammatory and neoplastic diseases, a decrease in bone formation is observed in addition to the enhanced osteoclastic bone resorption[41,42]. As Sema4D is expressed in T cells and certain types of cancer cells, Sema4D produced by these cell types may contribute to this reduction in bone formation. Intermittent parathyroid

hormone treatment is the only currently available strategy that has been demonstrated to increase bone formation. A Sost-specific antibody under development has attracted attention as a potential new bone-increasing agent among candidate strategies[43], and now the suppression of the Sema4D–Plexin-B1–RhoA signaling axis holds promise as a strategy for the design of new therapeutic approaches to bone and joint diseases, including osteoporosis, rheumatoid arthritis and bone tumors.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

T.N.-K. performed most of the experiments, interpreted the results and prepared the manuscript. M.S. performed the molecular analysis of the regulation of bone formation by RhoA and contributed to the GTPase analysis and manuscript preparation. N.K. performed the FACS analysis. H.B. generated adenoviruses and contributed to data interpretation. T.K. conducted the GeneChip analysis. R.H.F. generated *Plxnb1*$^{-/-}$ mice and contributed to data interpretation. H.T. directed the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and analysis of the bone phenotype. Generation of *Sema4d*$^{-/-}$, *Plxnb1*$^{-/-}$, CAT-RhoA DN and α(1)I-Cre mice was as previously described[28,29,44,45]. *Sema4d*$^{-/-}$ mice (RBRC no. 00753) were provided by the RIKEN BioResource Center. All mice were backcrossed with C57BL/6 mice more than eight times, born in the expected Mendelian ratio without apparent developmental abnormalities, and maintained under specific-pathogen–free conditions. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws. The femurs and tibiae of 12-week-old female mice ($n \geq 8$) were subjected to three-dimensional microcomputed tomography and histomorphometric analysis, respectively, as previously described[46]. Bone mechanical strength testing was performed using a three-point bending method, as previously described[47].

Cell culture. We described the *in vitro* osteoclast differentiation method previously[48]. The *in vitro* osteoblast differentiation method was also as previously described[49]. Briefly, cells derived from calvaria were cultured in osteogenic medium (50 μM ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate) and subjected to an analysis of the activity and mRNA expression of ALP (encoded by *Alpl*) (after 7 d), bone nodule formation and the expression of osteoblastic marker genes such as *Bglap* and *Col1a1* (after 21 d). Bone nodule formation was quantified as described[50]. Recombinant Sema4D (Fc-sema4D) (20 μg, unless otherwise indicated), neutralizing antibodies against Sema4D (BMA-12, MBL)[19,51] or Plexin-B1 (N-18, Santa Cruz)[52], or the ROCK inhibitors Y-27632 (10 μM, Calbiochem) and RKI (5 μM, Calbiochem), were added every 3 d. In **Figure 1i**, calvarial cells were cultured in osteoclast culture supernatant, which was collected from the culture of the wild-type and *Sema4d*$^{-/-}$ osteoclast precursor cells 2 d after RANKL stimulation. For the coculture of osteoblasts with osteoclasts, wild-type and *Sema4d*$^{-/-}$ osteoclasts were isolated by trypsinization 2 d after RANKL stimulation. Isolated osteoclasts (1×10^5 cells per well in a 24-well plate) were added every 3 d.

Immunoblot analysis. For the pull-down assay using Fc-sema4D, cell lysates were incubated with Fc-sema4D (500 ng) and analyzed by immunoblotting with antibodies to Plexin-B1, PDZ-RhoGEF and LARG. For analysis of the effect of Sema4D on intracellular signaling, calvarial cells were stimulated with Fc-sema4D after 3 d of culture in the osteogenic medium. Cells were stimulated with IGF-1 (10 nM, Sigma-Aldrich) after serum starvation for 8 h after 3 d of culture. Cells were pretreated with 30 μM of an ErbB2 inhibitor II (Santa Cruz), 10 μM Y27632 or 5 μM RKI for 1 h before stimulation with Fc-sema4D or IGF-1. Phosphorylation of Plexin-B1, Met, ErbB2 and IRS-1 was detected with a phosphotyrosine-specific antibody after immunoprecipitation with the specific antibodies. The antibodies used are listed in **Supplementary Table 2**.

Detection of RhoA activation. The pull-down assay to detect GTPase activity was performed as described[53]. Briefly, calvarial cells were cultured in the osteogenic medium for 3 d followed by incubation in serum-free α-MEM for 8 h, and then stimulated with Fc-sema4D. Cells were collected at the indicated time points and cell lysates were subjected to an incubation with 2 μg of GST-RBD (for RhoA) and GST-PAK1 (for Rac1) coupled to glutathione-Sepharose, followed by immunoblotting with RhoA-specific and Rac1-specific antibodies, respectively. For the ELISA of RhoA activity (**Fig. 3b**,**e**), GTP-bound RhoA was detected by G-LISA (Cytoskeleton) according to the manufacturer's protocol. To examine the effect of ErbB2, cells were pretreated with ErbB2 inhibitor II for 1 h before Fc-sema4D stimulation.

Ovariectomy-induced bone loss. This model of osteoporosis induced by ovariectomy has been described[54]. Briefly, 7-week-old female mice were ovariectomized or sham operated. More than eight mice were examined in each group. For the analysis of the prophylactic effect of the Sema4D-specific antibody, ovariectomized mice were intravenously injected with 20 μg of Sema4D-specific antibody (MBL) or saline via the tail vein weekly from day 3 to week 8 after surgery. To analyze the therapeutic effect of the Sema4D-specific antibody, after 6 weeks of surgery, we started an injection of the same amount of Sema4D-specific antibody and continued the injection every 3 d for 3 weeks. All of the mice were killed and subjected to microcomputed tomography and histomorphometric analyses 8 weeks (prophylactic model) or 9 weeks (therapeutic model) after surgery.

Motility assay. Cell migration was assessed using a modified Boyden chamber assay, as described[55]. Osteoblasts (3×10^4 cells) were added to the upper chamber. IGF-1 (100 ng ml^{-1}) or Fc-sema4D was added to the lower chamber. After 4 h, the cells that migrated to the lower surface of the membrane were counted. To analyze the effect of the ROCK inhibitor on Sema4D-induced cell motility, cells were pretreated with 5 μM RKI for 1 h and added to the upper chamber.

Statistical analysis. All data are expressed as the mean ± s.e.m. ($n = 5$). Statistical analyses were performed using the Student's *t* test analysis of variance followed by the Bonferroni test when applicable. Results are representative examples of more than four independent experiments.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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