Smad4 is required to inhibit osteoclastogenesis and maintain bone mass

Mayu Morita1,*, Shigeyuki Yoshida1,*, Ryotaro Iwasaki1,*, Tetsuro Yasui2, Yuiko Sato3,4,5, Tami Kobayashi3,4, Ryuichi Watanabe1, Takatsugu Oike3, Kana Miyamoto2, Masamichi Takami6, Keiko Ozato7, Chu-Xia Deng8, Hiroyuki Aburatani9, Sakae Tanaka2, Akihiko Yoshimura10, Yoshiaki Toyama3, Morio Matsumoto3, Masaya Nakamura3, Hiromasa Kawana1, Taneaki Nakagawa1 & Takeshi Miyamoto3

Bone homeostasis is maintained as a delicate balance between bone-resorption and bone-formation, which are coupled to maintain appropriate bone mass. A critical question is how bone-resorption is terminated to allow bone-formation to occur. Here, we show that TGFβs inhibit osteoclastogenesis and maintain bone-mass through Smad4 activity in osteoclasts. We found that latent-TGFβ1 was activated by osteoclasts to inhibit osteoclastogenesis. Osteoclast-specific Smad4 conditional knockout mice (Smad4-cKO) exhibited significantly reduced bone-mass and elevated osteoclast formation relative to controls. TGFβ1-activation induced expression of Irf8 and Bcl6, both of which encode factors inhibiting osteoclastogenesis, by blocking their negative regulator, Prdm1, in osteoclasts in a Smad4-dependent manner. Reduced bone-mass and accelerated osteoclastogenesis seen in Smad4-cKO were abrogated by Prdm1 deletion. Administration of latent-TGFβ1-Fc to wild-type mice antagonized LPS-induced bone destruction in a model of activated osteoclast-mediated bone destruction. Thus, latent-TGFβ1-Fc could serve as a promising new therapeutic agent in bone diseases marked by excessive resorption.

Bone is continuously resorbed and constructed, and bone volume is controlled as a balance of both activities, a process termed “coupling”1. The coupling system is also critical to determine regions for new bone production, which are often sites where resorption has occurred2. Coupling failure results in bone diseases such as osteoporosis, in which bone formation following resorption is less effective, reducing bone mass3. To date, drugs such as bisphosphonates have been launched as osteoporosis therapy; however, their use frequently causes multiple adverse effects, including osteonecrosis of the jaw due to excessive inhibition of osteoclastic activity beyond levels required for physiological bone turnover4. Thus, understanding the coupling system is required to better enable us to increase bone formation at required levels and at sites where resorption occurs.

Several factors have been proposed to function as coupling factors5. For example, osteoclast/osteoblast signaling is reportedly transduced by ephrin B2/EphB4, Ephrin A2/EphA2, and Semaphorin 4D/PlexinB1 interactions or by Semaphorin 3A secreted by osteoblasts6–9. In addition, mice deficient in genes encoding either dendritic

1Division of Oral and Maxillofacial surgery, Department of Dentistry and Oral Surgery, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-k, Tokyo 160-8582, Japan. 2Department of Orthopedic Surgery, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-k, Tokyo 113-8859, Japan. 3Department of Orthopedic Surgery, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-k, Tokyo 160-8582, Japan. 4Department of Musculoskeletal Reconstruction and Regeneration Surgery, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-k, Tokyo 160-8582, Japan. 5Department of Pharmacology, Showa University School of Dentistry, 1-5-8 Hatanodai, Sina-gawa-k, Tokyo 142-0033, Japan. 6Laboratory of Molecular Growth Regulation, Genomics of Differentiation Program, NICHD, National Institutes of Health, Bethesda, MD. 7Faculty of Health Sciences, University of Macau, Macau SAR, China. 8Genome Science Division, Research Center for Advanced Science and Technology (RCAST), The University of Tokyo, 4-6-1 Komaba, Meguro-k, Tokyo 153-8904, Japan. 9Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-k, Tokyo 160-8582, Japan. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to T.M. (email: miyamoto@z5.keio.jp)
cell-specific transmembrane protein or the v-ATPase V0 subunit d2 exhibit increased osteoblastic activity, reduced osteoclastic bone resorption, and increased bone mass 

Transforming growth factor beta (TGFβ), a member of the TGFβ superfamily, also serves as a coupling factor. This ligand family consists of TGFβ1–5 as well as bone morpho
genetic proteins (BMPs), however, TGFβ3 and TGFβ5 are expressed only in chick and Xenopus, respectively. TGFβ1 ligands signal by binding to specific receptors and activating receptor-regulated transcription factors called Smads (R-Smads), namely Smad2/3 (for TGFβ1–3) and Smad1/5/8 (for BMPs), which form oligomers with the common mediator Smad4. Thus, loss of Smad4 results in loss of both TGFβ3–Smad2/3 and BMPs-Smad1/5/8 signals (Fig. 2a–c). Interestingly, Smad4-deficient mice show altered bone formation (Fig. 2d–f). Latent-TGFβ1, which in turn upregulates Irf8 and Bcl6 expression, inhibiting osteoclast differentiation. Reduced bone mass and elevated osteostegogenesis in Smad4-cKO were abrogated in Smad4/Blimp1 doubly mutant mice. Latent-TGFβ1 converted to an active form by osteoclastic activity in cultured cells, and administration of latent-TGFβ1-Fc to wild-type mice blocked LPS-induced bone destruction. We conclude that following bone resorption, inhibition of osteostegogenesis by activated TGFβ1 via Smad4 expressed in osteostects is crucial to maintain bone mass.

Results
Osteostegogenesis is differentially regulated in osteostect progenitor cells by high concentrations of TGFβ3 or TGFβ3 in vitro. To assess expression of TGFβ3 factors in bone, we undertook analysis of transcripts encoding these factors in bone tissues of wild-type mice and identified TGFβ3, 2, 3 and BMP2 mRNAs (Fig. 1a). We also found that osteostegogenesis, as assessed by expression of osteostectic genes such as Cathepsin K (Ctsk) and NFATc1 following RANKL treatment of cultured Raw264.7 cells, was enhanced by co-incubation of cells with RANKL plus either TGFβ3 or 33 (Supplementary Fig. 1). Osteostegogenesis in bone marrow macrophages (BMMs) was also significantly inhibited by SB431542, a TGFβ3 inhibitor, in vitro (Supplementary Fig. 2a–c). Interestingly, in vitro osteostegogenesis in wild-type BMMs was stimulated at a lower concentration (0.016 ng/ml) of either TGFβ3 or TGFβ3, while differentiation was significantly inhibited at higher concentrations (0.4, 2 or 10 ng/ml) of either TGFβ3 or TGFβ33 dose-dependently (Fig. 1b–d, Supplementary Fig. 3a and Supplementary Fig. 4). In contrast, osteostegogenesis from wild-type BMMs was stimulated at high concentrations of either TGFβ3 (10 ng/ml) or BMP2 (200 ng/ml) (Fig. 1b,c). Osteostegogenesis, as evidenced by appearance of multi-nuclear TRAP-positive cells, was stimulated by either 40 or 200 ng/ml BMP2 but inhibited by 1,000 ng/ml of BMP2 (Supplementary Fig. 5). These results suggest that osteostegogenesis is regulated in a complex manner by TGFβ3 superfamily members in the bone microenvironment.

Smad4 is required to inhibit osteostegogenesis and maintain bone mass. As noted, lack of Smad4 results in abrogation of both TGFβ3 and BMP signaling. We detected Smad4 expression in osteostects (Fig. 2a). To assess roles of Smad4 and downstream signaling in regulating osteostegogenesis and bone mass in vivo, we generated osteostect-specific Smad4 conditional knockout mice (Smad4-cKO) using Ctsk-Cre mice (Fig. 2b). Based on DEXA analysis, Smad4-cKO mice exhibited significantly reduced bone mass with accelerated osteostegogenesis as analyzed by TRAP staining and bone morphometric analysis compared with controls in vivo (Fig. 2c–f). Osteostegogenesis was normal in Smad4-cKO mice, while osteostect formation was activated (Fig. 2d–f). Thus, reduced bone mass seen in Smad4-cKO mice is likely due to elevated osteostegogenesis in vivo.

TGFβ31 and β33 inhibit osteostect differentiation via Smad4. We next focused on identifying osteostect-inhibiting signals mediated by Smad4 in vitro (Fig. 3). Osteostegogenesis in wild-type BMMs as analyzed by formation of multi-nuclear TRAP-positive cells in vitro, was significantly inhibited in the presence of high concentrations of TGFβ3 or TGFβ33, and inhibition was significantly reversed in Smad4-cKO cells (Fig. 3a,b and Supplementary Fig. 6a,b). Expression of the osteostect differentiation markers Ctsk and NFATc1
Figure 1. TGFβ1/33 and BMP2/TGFβ2 have differential effects on osteoclastogenesis. (a) Expression of TGFβ1–3 and BMP2 transcripts in mouse humerus bone was confirmed. Osteoclast progenitors from wild-type mice were cultured with recombinant BMP2 (200 ng/ml), TGFβ1 (10 ng/ml), TGFβ2 (10 ng/ml) or TGFβ3 (10 ng/ml) in the presence of M-CSF (50 ng/ml; M) and RANKL (25 ng/ml; R) for five days and then assessed for osteoclast formation by TRAP staining (b), by counting the number of multi-nuclear TRAP-positive cells (c) and by expression of the osteoclast markers Ctsk and NFATc1 based on realtime PCR (d). Data represent mean Ctsk or NFATc1 expression relative to β-actin ± SD (n = 3). Bar = 100 μm. *P < 0.05; ***P < 0.001; NS, not significant. Representative data of at least three independent experiments are shown.

Figure 2. Smad4 is expressed in osteoclasts and required for osteoclast inhibition. (a) Electrophoresis gel images of RT-PCR (left panels) or quantitative real-time PCR (right panel) analysis of Smad4 expression in macrophages (M), osteoclasts (OC) and osteoblasts (OB). β-actin served as an internal control. Data represent mean Smad4 expression relative to β-actin ± SD. (b) Western blotting in osteoclasts to assess Smad4 deletion efficiency in Smad4 cKO (cKO) compared with control (Smad4^flx/flx) osteoclasts. (c) Bone mineral density (BMD) of femurs bisected equally longitudinally from control (Smad4^flx/flx) and Smad4 cKO (cKO) mice. *P < 0.05; **P < 0.01; ***P < 0.001. (d) Bone histomorphometrical analysis of femurs from control and Smad4 cKO (cKO) female mice. Osteoblast surface per bone surface (Ob.S/BS) was determined. NS, not significant. Tibial sections from control (Smad4^flx/flx or flx/+ ) and Smad4 cKO mice were stained with TRAP (e), and the number of TRAP-positive osteoclasts was scored (f). Bar = 100 μm. **P < 0.01. Representative data of at least two independent experiments are shown.
was significantly inhibited by treatment of wild-type osteoclasts with either TGFβ1 or TGFβ3 in vitro, an effect reversed in Smad4 cKO cells (Fig. 3c and Supplementary Fig. 6c).

Smad4 regulates Bcl6 and Irf8 expression. To define molecular mechanisms underlying TGFβ inhibition of osteoclastogenesis through Smad4, we analyzed expression of potential inhibitory factors following treatment of wild-type osteoclasts with TGFβs. Candidates included Bcl6 and Irf8, both transcriptional repressors and reported inhibitors of osteoclastogenesis39,40. Bcl6 and Irf8 mRNA expression was upregulated following stimulation of wild-type osteoclasts with TGFβ1 (Fig. 4a). Interestingly, Bcl6 and Irf8 upregulation was significantly blocked in Smad4 cKO cells (Fig. 4b), suggesting that such upregulation is dependent on Smad4. Thus, next we treated Bcl6-deficient BMMs with TGFβ1 or β3 and found that their inhibition of osteoclast formation was abrogated relative to wild-type cells (Fig. 4c,d, and Supplementary Fig. 7a,b). Likewise, decreased expression of the osteoclastic genes Ctsk and NFATc1 seen following TGFβ1 or β3 treatment in wild-type osteoclasts was significantly rescued in Bcl6-deficient osteoclasts (Fig. 4e, Supplementary Fig. 7c). Similarly, Irf8-deficient cells were resistant to inhibition of osteoclastogenesis and suppression of osteoclastic gene expression by either TGFβ1 or β3 (Fig. 4f–h, Supplementary Fig. 7d–f).

Blimp1 is a direct target of Smad4 in osteoclasts. Both Bcl6 and Irf8 expression in osteoclasts is reportedly negatively regulated by Blimp1, a transcriptional repressor encoded by Prdm139,40. Thus, we asked whether
Figure 4. TGFβ1 stimulates increases in Bcl6 and Irf8 expression in osteoclasts via Smad4 expressed.
(a) Osteoclast progenitors were isolated from wild-type mice and cultured in the presence or absence of M-CSF (M) and RANKL (R) with or without indicated concentrations of TGFβ1. Irf8 and Bcl6 expression was then determined by realtime PCR. Data represent mean Bcl6 or Irf8 expression relative to β-actin ± SD (n = 3).
(b) Osteoclast progenitors were isolated from control (Smad4floxflox or flox+/+) or Smad4 cKO (cKO) mice and cultured in the presence or absence of RANKL with or without 10 ng/ml of TGFβ1. Irf8 and Bcl6 expression was determined by realtime PCR. Data represent mean Bcl6 or Irf8 expression relative to β-actin ± SD (n = 3).
(c–h) Osteoclast progenitors were isolated from wild-type, Bcl6-deficient (c–e) or Irf8-deficient (f–h) mice and cultured in the presence or absence of M-CSF (M) and RANKL (R) with or without 10 ng/ml TGFβ1. Osteoclast formation was evaluated by TRAP staining (c,f), by the number of multi-nuclear TRAP-positive cells (d,g) and by Ctsk and NFATc1 expression as analyzed by realtime PCR (e,h). Irf8 and Bcl6 expression was determined in Bcl6 and Irf8-deficient mice, respectively, by realtime PCR (i). Data represent mean Ctsk or NFATc1 expression relative to β-actin ± SD (n = 3). Bar = 100 μm. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Representative data of at least two independent experiments are shown.
elevated Bcl6 and Irf8 expression seen following TGFβ1 or TGFβ3 treatment was accompanied by decreased Prdm1 expression. In accordance, Prdm1 mRNA expression was significantly inhibited by either TGFβ1 or TGFβ3 treatment of wild-type osteoclasts (Fig. 5a, Supplementary Fig. 8), but such Prdm1 inhibition was abrogated in Smad4 cKO cells (Fig. 5a, Supplementary Fig. 8). To assess whether Prdm1 is a direct target of Smad in

**Figure 5. Prdm1 deletion rescues bone loss in Smad4-cKO mice.** (a) Osteoclast progenitors from control (Smad4fl/fl or flox/+ or Smad4 cKO mice were cultured with or without 10 ng/ml TGFβ1 in the presence or absence of M-CSF and RANKL. Prdm1 expression was analyzed by real-time PCR. Data represent mean Prdm1 expression relative to β-actin ± SD (n = 3). (b) Osteoclast progenitor cells from wild-type mice were cultured in the presence of M-CSF and RANKL with 10 ng/ml TGFβ1, and chromatin immune precipitation-sequencing analysis was performed by using anti-Smad2/3 antibody. Smad2/3 bound to regions upstream of the Prdm1 gene. (c) Bone mineral density of femurs divided equally longitudinally from control, Smad4 cKO and Smad4/Prdm1 double-mutant (DcKO) female mice. (d,e) Tibial sections from control (Smad4fl/fl or flox/+), Smad4 cKO and DcKO mice were stained with TRAP (d), and the number of TRAP-positive osteoclasts was scored (e). Data represent mean number of TRAP-positive cells per section ± SD (n = 3). Bar = 100 μm. *P < 0.05; **P < 0.01; NS, not significant. Representative data of at least two independent experiments are shown.
osteoclasts, we employed chromatin immune precipitation sequencing (ChIP seq) analysis using anti-Smad2/3 antibodies, and observed that Smad2/3 bound to an upstream region of the Prdm1 gene in osteoclasts under TGFβ1 stimulation (Fig. 5b). When we generated osteoclast-specific Smad4/Prdm1 double knockout (DcKO: CtskCre/Prdm1−/−) mice, in which Prdm1 is deleted from Smad4 KO mice, we found that the significantly decreased bone mass seen in Smad4 cKO mice was reversed and rather increased in DcKO mice (Fig. 5c–e). These observations suggest that Smad4 is required for Prdm1 inhibition in osteoclasts and to maintain bone mass following stimulation with either TGFβ1 or TGFβ3.

We also found that Prdm1 expression was significantly upregulated by either TGFβ1 or TGFβ3 in Raw263.7 cells (Supplementary Fig. 9). Although, Bcl6 expression was rather upregulated, elevated Prdm1 expression may explain, at least in part, why osteoclastogenesis was stimulated in Raw263.7 cells by either TGFβ1 or TGFβ3. Osteoclast formation was stimulated by 200 ng/ml of BMP2, however, either β1-Fc or control CD4-Fc administration (Fig. 6c–f). TGFβ1-Fc or control CD4-Fc protein by injection into mice resulted in a decrease in bone mass and elevated osteoclast formation we report here in Smad4 cKO mice suggests that in this system inhibitory latent-TGFβ1 osteoclastogenesis was inhibited in secondary cultures treated with supernatants from osteoclasts cultured with latent-TGFβ1 (Fig. 6b). Based on these results, we concluded that administered latent-TGFβ1 is converted to an active form by osteoclast to inhibit osteoclast formation. To test this hypothesis, we administered latent-TGFβ1-Fc or control CD4-Fc protein by injection in vivo in a mouse model of bone destruction in which LPS was injected on wild-type mouse calvariae. We found that LPS-induced bone-resorption and osteoclast formation as analyzed by micro CT (μCT), and anti-Ctsk with anti-NFATc1 staining, respectively, were significantly inhibited by latent-TGFβ1-Fc compared with CD4-Fc administration (Fig. 6c–f). TGFβ1 signaling is known to promote differentiation of TH17 cells, a type of osteoclastogenic T cells implicated in bone destruction41,42. Indeed, in an LPS-induced model of bone destruction, we found that TH17 cell frequency significantly increased in mice treated with LPS together with latent-TGFβ1-Fc compared with control mice treated with PBS plus latent-TGFβ1-Fc (Fig. 6g). The fact that bone destruction was inhibited by latent-TGFβ1-Fc, even under elevated TH17 cell conditions, suggests that latent-TGFβ1-Fc could antagonize bone destruction in osteoclast-activating conditions.

Discussion
Numerous bone-regulating factors maintain bone homeostasis1,44. Among them, factors activating signals via Smad4, including TGFβ and BMP, reportedly support osteoblastic cell migration, proliferation, differentiation and bone formation in vivo and in vitro (Supplementary Fig. 12a,14,44). This study demonstrates that Smad4 mediates osteoprotective signals that are coupled with osteoclastic bone resorption and acts as part of a negative feedback mechanism (Supplementary Fig. 12b, c). Our findings suggest overall that Smad4 plays a role in both inhibiting bone resorption and activating bone formation (Supplementary Fig. 12). Here, we show that latent-TGFβ3 is activated by osteoclasts, which inhibits their activity (Supplementary Fig. 12b).

The activity of TGFβ3 superfamily members in osteoclasts reportedly varies28–32, and we show that TGFβ1/3 inhibits osteoclastogenesis, while TGFβ2/BMP2 stimulates it. However, the significant reduction in bone mass and elevated osteoclast formation we report here in Smad4 cKO mice suggests that in this system inhibitory signals via Smad4 are dominant over stimulators. Since Smad4 null mice exhibit embryonic lethality28, Smad4 function in osteoclasts and bones has not previously been characterized. The Cre/loxP system employed here did not completely abrogate Smad4 activity in osteoclasts, and some Smad4 function may remain. Nonetheless, it allowed us evaluate Smad4 function in osteoclastogenesis and bone at adult stages. Those signals via TGFβ3 result from conversion of latent-TGFβ3 to TGFβ3, which in turn blocks expression of Prdm1, a repressor of osteoclastogenesis. Loss of the repressor encoded by Prdm1 upregulates Bcl6 and Irf8, both of which repress osteoclast differentiation (Supplementary Fig. 12). Although, at present, molecular mechanisms underlying are not clear, we found that Irf8 or Bcl6 expression was significantly inhibited in Bcl6- or Irf8-deficient osteoclasts, respectively (Fig. 4i), suggesting that these factors regulate each other in osteoclasts.

TGFβ3 and BMP signaling is regulated in a complex manner in osteoblasts41. Indeed, TGFβ3 is reportedly required for osteoblastogenesis19,44, while it is also reported to inhibit osteoblastogenesis induced by BMP246. However, there is not decrease in bone mass seen in osteoblast-specific Smad4-deficient mice59, suggesting that Smad4 signals in osteoblasts positively regulate bone formation. Thus overall, although why high concentration of BMP2 (1,000 ng/ml) inhibited osteoclast formation was not clear, Smad4 signaling in both osteoclasts and osteoblasts results in increases in bone mass.

Recent advances in developing anti-osteoporosis drugs have resulted in both anti-resorptive agents such as bisphosphonate or anti-RANKL antibodies, and bone-forming drugs, such as teriparatide44–50. Both types have significant therapeutic effects in increasing bone mass and preventing fractures in osteoporosis patients51. However, the broad effects of anti-resorptive or bone-forming agents in inhibiting or promoting osteoclast differentiation/function, respectively, can cause adverse side effects such as jaw osteonecrosis, super suppressive bone turnover or osteosarcoma formation1,42. As alternatives, investigators are currently seeking novel reagents targeting specific sites where bone formation is required following resorption. Our data strongly suggests that the
Figure 6. Latent TGFβ1 is converted to an active form by osteoclastic activity. (a) Osteoclast progenitors from wild-type mice were cultured in the presence of M-CSF and RANKL with or without either active- or latent-TGFβ1 (10 ng/ml each) for primary culture. Quantitative real-time PCR analysis of osteoclastic mRNAs was then undertaken. Data represent mean Ctsk or NFATc1 expression relative to β-actin ± SD (n = 3). (b) Primary culture supernatants were collected from wild-type cells and transferred to secondary cultures of wild-type osteoclast progenitors, which were then treated with M-CSF and RANKL, and Ctsk and NFATc1 expression was analyzed by realtime PCR. Data represent mean Ctsk or NFATc1 expression relative to β-actin ± SD (n = 3). (c–g) LPS (50 mg/kg) was administered subcutaneously onto the skull of living 8-week-old female wild-type mice with or without 16 mg of latent-TGFβ1. Five days later, osteolysis in calvariae was analyzed by μCT (c, low magnification; d, high magnification). PBS injection served as a negative control. The number of resorption pits per calvariae was scored. (e). Data represent mean resorption pit number per calvariae ± SD (n = 5). Sections were stained with mouse anti-Ctsk and rabbit anti-NFATc1 antibodies, followed by Alexa488-conjugated goat anti-mouse Ig’ antibody, Alexa488-conjugated goat anti-rabbit Ig’ antibody and DAPI. Sections were then observed by fluorescence microscopy (f). Spleen cells were stained with anti-CD4 and anti-IL-17 antibodies, and the frequency of TH17 cells (CD4+IL-17+ cells) was analyzed by flow cytometry (g). Data represent mean TH17 cell frequency ± SD (n = 5). Bar = 100 μm. *P < 0.05; NS, not significant. Representative data of at least two independent experiments are shown.
TGFβ3/Smad4 system is specifically activated at such sites. Our observations therefore provide a molecular basis for developing agents that both inhibit bone-resorption and activate bone-formation.

**Methods**

**Mice.** Wild-type mice were purchased from Sankyo Labo Service (Tokyo, Japan). Ctskcre/+ Smad4f/f, Prdm1f/f, Bcl6-deficient and Irf8-deficient mice were prepared as previously described38-40,53. Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Keio University Institutional Animal Care and Use Committee, and animal protocols were approved by that committee. All animal studies were performed in accordance with the Guidelines of the Keio University Animal care committee.

**Analysis of skeletal morphology.** Ctskcre/+ Smad4f/f, Ctskcre/+ Smad4f/fPrdm1f/f and control littermates were necropsied, and their hind limbs were removed, fixed in 70% ethanol, and subjected to DEXA analysis to measure bone mineral density, and analysis of bone histomorphometric parameters. Bones were collected from 8-week-old female mice.

**In vitro osteoclast formation.** For in vitro analysis, bone marrow cells isolated from femurs and tibias were cultured for 72 h in MEM (Sigma-Aldrich Co.) containing 10% (vol/vol) heat-inactivated FBS (JRH Biosciences) and GlutaMax (Invitrogen Corp.) supplemented with M-CSF (50 ng/mL, Kyowa Hakko Kirin Co.). Subsequently, adherent cells were collected and cultured in 96-well plates (1 × 10⁵ cells per well) under indicated conditions containing M-CSF (50 ng/mL) and recombinant soluble RANKL (25 ng/mL, PeproTech Ltd.) with or without latent-TGFβ1 (10 ng/ml, R & D Systems), TGFβ3 (0.016–10 ng/ml, R & D Systems), TGFβ3 (0.016–10 ng/ml, R & D Systems) or BMP2 (40–1,000 ng/ml, Pepro Tech Ltd.). Medium was changed every 2 days. Osteoclastogenesis was evaluated by TRAP staining, and TRAP-positive multi-nuclear cells containing more than three nuclei were scored as osteoclasts.

For some experiments, supernatants from osteoclast culture for five days with or without latent-TGFβ3-Fc (10 μg/ml, R & D Systems) were added to secondary cultures, and osteoclastogenesis was evaluated by TRAP staining or expression of osteoclastic genes.

**Quantitative PCR analysis.** Total RNAs were isolated from bone marrow cultures using TRIzol reagent (Invitrogen Corp.), and cDNA synthesis was performed using oligo(dT) primers and reverse transcriptase (Wako Pure Chemicals Industries). Quantitative PCR was performed using SYBR Premix ExTaq II reagent and a DICE Thermal cycler (Takara Bio Inc.), according to the manufacturer’s instructions. β-actin (Actb) expression served as an internal control. Primers used for realtime PCR analysis were as follows.

| Gene      | Forward primer      | Reverse primer      |
|-----------|---------------------|---------------------|
| Ctsk      | 5′-TGGAGGGGAAATCGTGCGTGAC-3′ | 5′-CGCAGGACTCTCGTATAGT-3′ |
| Smad4     | 5′-AAGAAGGAGGGCCATGAGTT-3′ | 5′-GGGTAGTTACTTCTGAAAT-3′ |
| Nfatc1    | 5′-CAAGTCTTACACACGGCTCCTA-3′ | 5′-GCGTGGAGGTTCTATTCTCAGT-3′ |
| Smad4     | 5′-TACATCATAGGGGTTGTTCTCTCA-3′ | 5′-TCAAAATCTGGGTCTTGGTAGCT-3′ |
| Prdm1     | 5′-TTTCTTTTTGTGGATATTGTCCGGAGCTT-3′ | 5′-TTGGAACACTTCTTTGTTGGTAGTT-3′ |
| Bcl6      | 5′-AGAGGCAGTTGCAAAACCATAAAA-3′ | 5′-GCTCCCAAATTTGACATGGAG-3′ |
| Bcl6      | 5′-CAGATTACAATATAGCCAGGACTAG-3′ | 5′-AAATTATGCTTCCCTAGG-3′ |
| Irf8      | 5′-AGAGCTAGTTGCCATGAGCTG-3′ | 5′-AACATGGGCTGCTGAAAT-3′ |
| BMP2      | 5′-CTAGATCTGTACCAGGCACT-3′ | 5′-TCTTTCCCACCTACATTTGAGG-3′ |
| Smad3     | 5′-TTTGGGACTgTACTCTTCTCTGAGG-3′ | 5′-TTGGAACACTTCTTTGTTGGTAGTT-3′ |
| TGFβ1     | 5′-ACCCGTTGATACCTACCATTGCG-3′ | 5′-ACCGATGTTGCGTGGAGGAC-3′ |
| Smad3     | 5′-CAGACAAGGTTGGCATGATGCC-3′ | 5′-ATGAAAGAGGAGTTGAATATG-3′ |
| BMP2      | 5′-AGCCTGGAAGGATTTGCGGGATAC-3′ | 5′-AGCGGTAGTTGCTGTTGCTG-3′ |
| Smad3     | 5′-CCCTGGACACCATACTACTTGGAG-3′ | 5′-TCTTTCCACCTACATTTGCTCA-3′ |
| TGFβ1     | 5′-GGCGGCTGCTGAGGTTGAGT-3′ | 5′-GCTCGTGATTTGCTGTTGCTG-3′ |

**Western blot analysis.** Whole cell lysates were prepared from 8-week-old Smad4f/f (control) or Ctskcre/+ Smad4f/f mouse bone marrow cultures using RIPA buffer (1% Tween 20, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM Na3VO4, 5 mM NaF (Sigma-Aldrich Co.)). Equivalent amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane (EMD Millipore Corp.). Proteins were detected by using anti-Smad4 (9515, Cell Signaling) or anti-Actin (Sigma-Aldrich Co., St Louis, MO) antibody.

**Chromatin immune precipitation sequence (ChIP seq) assay.** Osteoclasts cultured with M-CSF + RANKL + TGFβ1 were harvested and ChIP-seq assay performed using anti-Smad2/3 antibody (BD biosciences, San Jose, CA, USA) as described37.

**In vivo osteolysis model.** 100 μl of PBS containing LPS (50 mg/kg) was injected with or without latent-TGFβ3-Fc onto the periosteal surface of calvariae in living 8-week old wild-type mice. Five days later, mice were euthanized,
and calvariae and spleen were harvested for micro-computed tomography (micro-CT) and flow cytometry, respectively. Micro-CT was performed using a (micro-CT) scan R_mCT2 system (Rigaku Corp., Tokyo, Japan). For flow cytometry, spleen cells were stained with anti-CD4 and anti-IL-17 antibodies, and analyzed by FACS Canto™ II (BD Biosciences, San Jose, CA, USA) as described. Spleen cells were collected from each group.

**Immunofluorescent staining.** Surgical sections of calvariae were stained with mouse anti-Cathepsin K (Ctsk) (1:100 Daichi Fine Chemical Co., Toyama, Japan) and anti-NFATc1 (NFATc1) (1:00 Santa Cruz Biotechnology) followed by Alexa488-conjugated goat anti-mouse Ig (1:200; Invitrogen, Carlsbad, CA). DAPI was used for a nuclear stain.

**Statistical analysis.** Results are expressed as the mean ± s.d. Differences between groups were examined for statistical significance using Student t test.

**References**

1. Sims, N. A. & Martin, T. J. Coupling the activities of bone formation and resorption: a multitudes of signals within the basic multicellular unit. *Bonekey Rep* 3, 481, doi: 10.1038/bonekey.2013.215 (2014).

2. Hattner, R., Epker, B. N. & Frost, H. M. Suggested sequential mode of control of changes in cell behaviour in adult bone remodelling. *Nature* 206, 489–490 (1965).

3. Zaidi, M. et al. Bone loss or lost bone: rationale and recommendations for the diagnosis and treatment of early postmenopausal bone loss. *Curr Osteoporos Rep* 7, 118–126 (2009).

4. Kuhl, S., Walter, C., Acham, S., Pfeffer, R. & Lambrecht, J. T. Bisphosphonate-related osteonecrosis of the jaws—a review. *Oral Oncol* 48, 938–947, doi: 10.1016/j.oraloncology.2012.03.028 (2012).

5. Matsuo, K. & Irie, N. Osteoclast-osteoblast communication. *Arch Biochem Biophys* 473, 201–209, doi: 10.1016/j.abb.2008.03.027 (2008).

6. Zhao, C. et al. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab* 4, 111–121, doi: 10.1016/j.cmet.2006.05.012 (2006).

7. Irie, N. et al. Bidirectional signaling through ephrinA2-EphA2 enhances osteoclastogenesis and suppresses osteoblastogenesis. *J Biol Chem* 284, 14637–14644, doi: 10.1074/jbc.M807598200 (2009).

8. Negishi-Koga, T. et al. Suppression of bone formation by osteoclastic expression of semaphorin 4D. *Nat Med* 17, 1473–1480, doi: 10.1038/nm.2489 (2011).

9. Hayashi, M. et al. Osteoprotection by semaphorin 3A. *Nature* 485, 69–74, doi: 10.1038/nature10000 (2012).

10. Lee, S. H. et al. v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. *Nat Med* 12, 1403–1409, doi: 10.1038/nm1514 (2006).

11. Xu, J., Cheng, T., Feng, H. T., Pavlos, N. J. & Zheng, M. H. Structure and function of V-ATPases in osteoclasts: potential therapeutic targets for the treatment of osteoporosis. *Histol Histopathol* 22, 443–454 (2007).

12. Takeshita, S. et al. Osteoclast-secreted CTHRC1 in the coupling of bone resorption to formation. *J Clin Invest* 123, 3914–3924, doi: 10.1172/JCI69493 (2013).

13. Assonian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M. & Sporn, M. B. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *J Biol Chem* 258, 7155–7160 (1983).

14. Wrann, M. et al. T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor-beta. *EMBO J* 16, 1633–1636 (1997).

15. Derynck, R. et al. A new type of transforming growth factor-beta, TGF-beta 3. *EMBO J* 17, 3737–3743 (1988).

16. Jakowlew, S. B., Dillard, P. J., Sporn, M. B. & Roberts, A. B. Nucleotide sequence of chicken transforming growth factor-beta 1 (TGF-beta 1). *Nucleic Acids Res* 16, 8730 (1988).

17. Roberts, A. B. et al. Mesoderm induction in Xenopus laevis distinguishes between the various TGF-beta isoforms. *Growth Factors* 7, 277–286 (1990).

18. Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425, 577–584, doi: 10.1038/nature02006 (2003).

19. Tan, X. et al. Smad4 is required for maintaining normal murine postnatal bone homeostasis. *J Cell Sci* 120, 2162–2170, doi: 10.1242/jcs.03466 (2007).

20. Zhang, L. et al. Smad4 is required for the normal organization of the cartilage growth plate. *Dev Biol* 284, 311–322, doi: 10.1016/j.ydbio.2005.05.036 (2005).

21. Canalis, E., McCarthy, T. & Centrella, M. Growth factors and the regulation of bone remodeling. *J Clin Invest* 81, 277–281, doi: 10.1172/JCI113318 (1998).

22. Urist, M. R. Bone: formation by autoinduction. *Science* 150, 893–899 (1965).

23. Hayden, J. M., Mohan, S. & Baylink, D. J. The insulin-like growth factor system and the coupling of formation to resorption. *Bone* 17, 935–985 (1995).

24. Xian, L. et al. Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. *Nat Med* 18, 1095–1101, doi: 10.1038/nm.2793 (2012).

25. Tang, Y. et al. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nat Med* 15, 757–765, doi: 10.1038/nm.1979 (2009).

26. Takai, H. et al. Transforming growth factor beta-activated WEEMOCF stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. *J Biol Chem* 273, 27091–27096 (1998).

27. Fuller, K., Lean, J. M., Bayley, K. E., Wani, M. R. & Chambers, T. J. A role for TGFbeta1 in osteoclast differentiation and survival. *J Cell Sci* 113 (Pt 13), 2445–2453 (2000).

28. Quinn, J. M. et al. Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. *J Bone Miner Res* 16, 1787–1794, doi: 10.1359/jbmr.2001.16.10.1787 (2001).

29. Weivoda, M. M. et al. Osteoclast TGF-beta1 Receptor Signaling Induces Wnt1 Secretion and Couples Bone Resorption to Bone Formation. *J Bone Miner Res* 31, 76–85, doi: 10.1002/jbmr.2586 (2016).

30. Itoh, K. et al. Bone morphogenetic protein 2 stimulates osteoclast differentiation and survival supported by receptor activator of nuclear factor kappaB ligand. *Endocrinology* 142, 3656–3662, doi: 10.1210/endo.142.8.8300 (2001).

31. Sotillo Rodriguez, J. E. et al. Enhanced osteoclastogenesis causes osteopenia in twisted gastrulation–deficient mice through increased BMP signalling. *J Bone Miner Res* 24, 1917–1926, doi: 10.1359/jbmr.090507 (2009).

32. Okamoto, M. et al. Conditional deletion of Bmp1a in differentiated osteoclasts increases osteoblastic bone formation, increasing volume of remodeling bone in mice. *J Bone Miner Res* 26, 2511–2522, doi: 10.1002/jbmr.477 (2011).

33. Kim, J. H. & Kim, N. Signaling Pathways in Osteoclast Differentiation. *Chonnam Med J* 52, 12–17, doi: 10.4068/cmj.2016.52.1.12 (2016).
or other third party material in this article are included in the article’s Creative Commons license, This work is licensed under a Creative Commons Attribution 4.0 International License. The images

Acknowledgements
Prdm1 flox mice were kindly provided by Dr. A. Sakamoto and Dr. T. Tokuhisa. CtskCreERT2 mice were provided by Dr. S. Kato. T. Miyamoto was supported by a grant-in-aid for Scientific Research in Japan and a grant from Japan Agency for Medical Research and Development. Y. Sato and K. Miyamoto were supported by a grant-in-aid for Scientific Research in Japan. This study was supported in part by a Grant-in-aid for Scientific Research and a grant from the Translational Research Network Program.

Author Contributions
Mayu Morita, S.Y., R.I. and Y.S. performed culture and animal experiments. A.Y. performed FACS experiments. T.Y., S.T. and H.A. performed Chip sequence experiments. T.K., M.T., K.O. and C.-X.D. prepared animals for experiments. R.W., T.O. and K.M. analyzed data. Y.T., Morio Matsumoto, M.N., H.K., T.N. and T.M. designed the study. T.M. wrote the manuscript with input from all authors. All authors discussed the results and commented on the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Morita, M. et al. Smad4 is required to inhibit osteoclastogenesis and maintain bone mass. Sci. Rep. 6, 35221; doi: 10.1038/srep35221 (2016).

© The Author(s) 2016