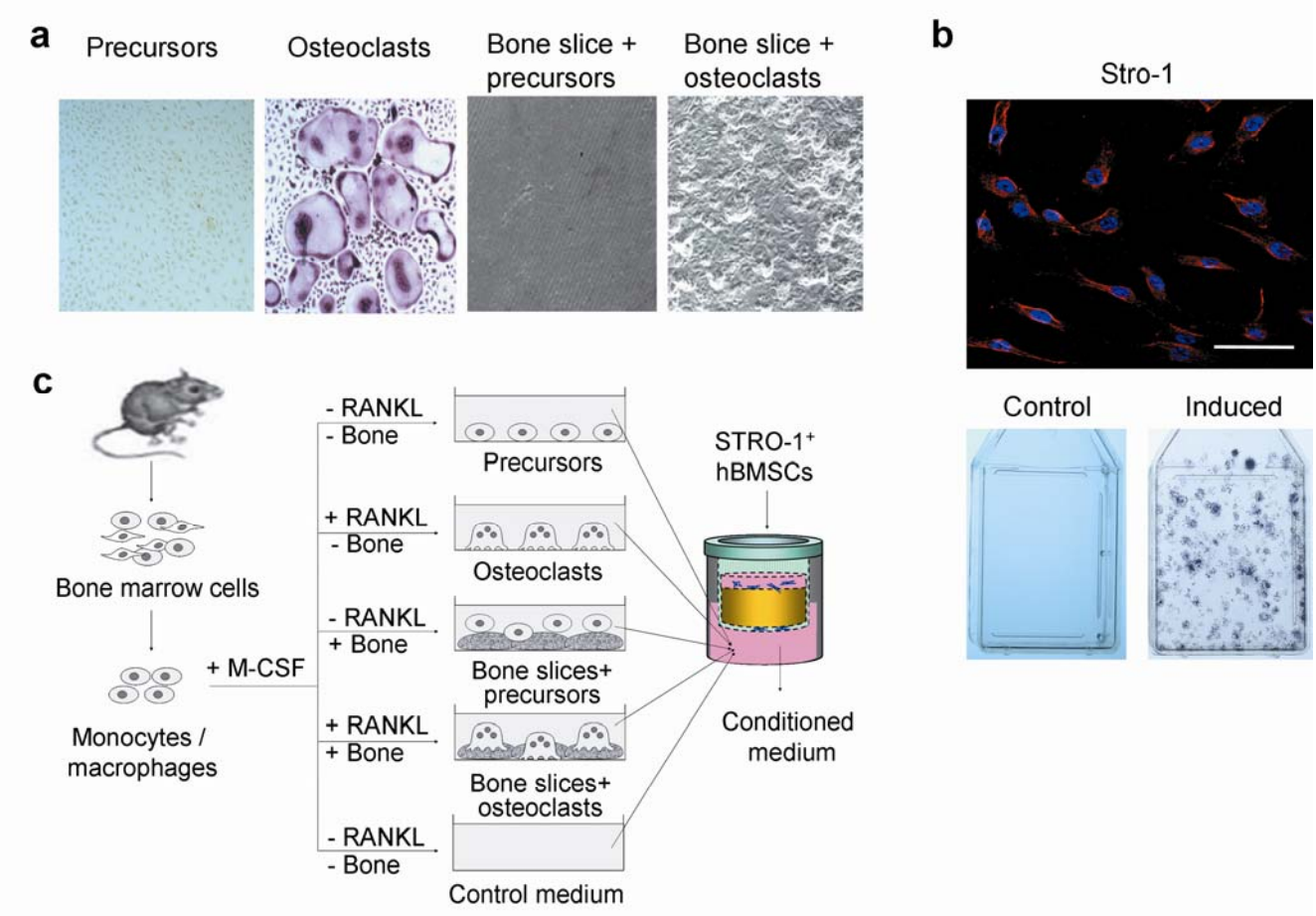
TGF-β1-induced Migration of Bone Mesenchymal Stem Cells Couples Bone Resorption and Formation

Yi Tang, Xiangwei Wu, Weiqi Lei, Lijuan Pang, Chao Wan, Zhenqi Shi, Ling Zhao, Timothy R. Nagy, Xinyu Peng, Junbo Hu, Xu Feng, Wim Van Hul, Mei Wan & Xu Cao

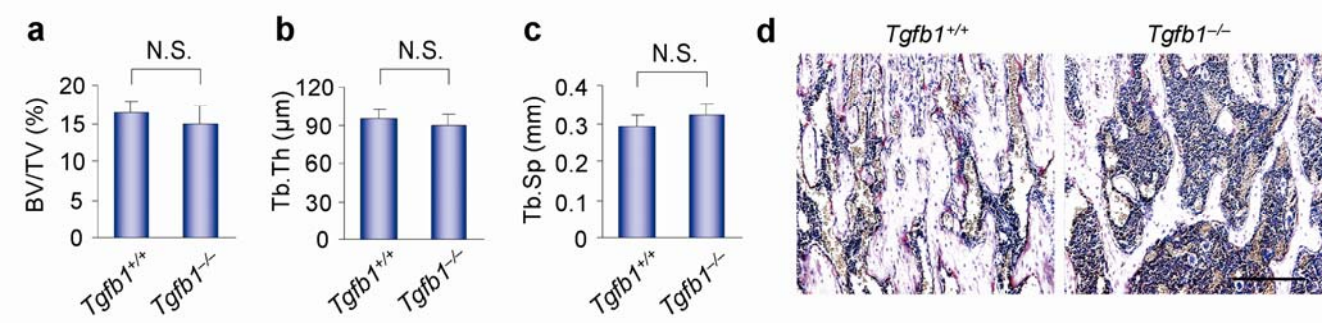
Supplementary Figure 1



Supplementary Figure 1. Preparation of osteoclastic bone resorption conditioned medium. (**a**) Macrophages/monocytes isolated from bone marrow of four to eight week-old mice, which were cultured in the presence of M-CSF (22 ng ml^{-1}) as osteoclastic precursors and negative for TRAP staining, or with both M-CSF and RANKL (100 ng ml^{-1}), which induced osteoclast formation as indicated by TRAP staining (left two panels). Scanning electronic microscopy revealed that the precursors did not resorb bovine bone slices after being cultured for seven days whereas the osteoclasts exhibited active bone resorption (right two panels). (**b**) Immunostaining of human bone marrow stromal cells or bone mesenchymal stem cells (BMSCs) with antibody to STRO-1 (R&D Systems), More than 90 percent of MSCs are STRO-1-positive ($STRO\text{-}1^+$) (upper panel). The BMSCs were purified from the second passage of human BMSCs using immunomagnetic beads with mouse antibody to STRO-1. $STRO\text{-}1^+$ BMSCs can form colony formation unit-fibroblasts (CFU-F), and can be induced into alkaline phosphotase (AP) positive osteogenic cells (lower two panels). Scale bar, 50 μm. (**c**) Conditioned medium from different cultures as indicated was harvested for transwell migration assay.

Nature Medicine: doi:10.1038/nm.1979

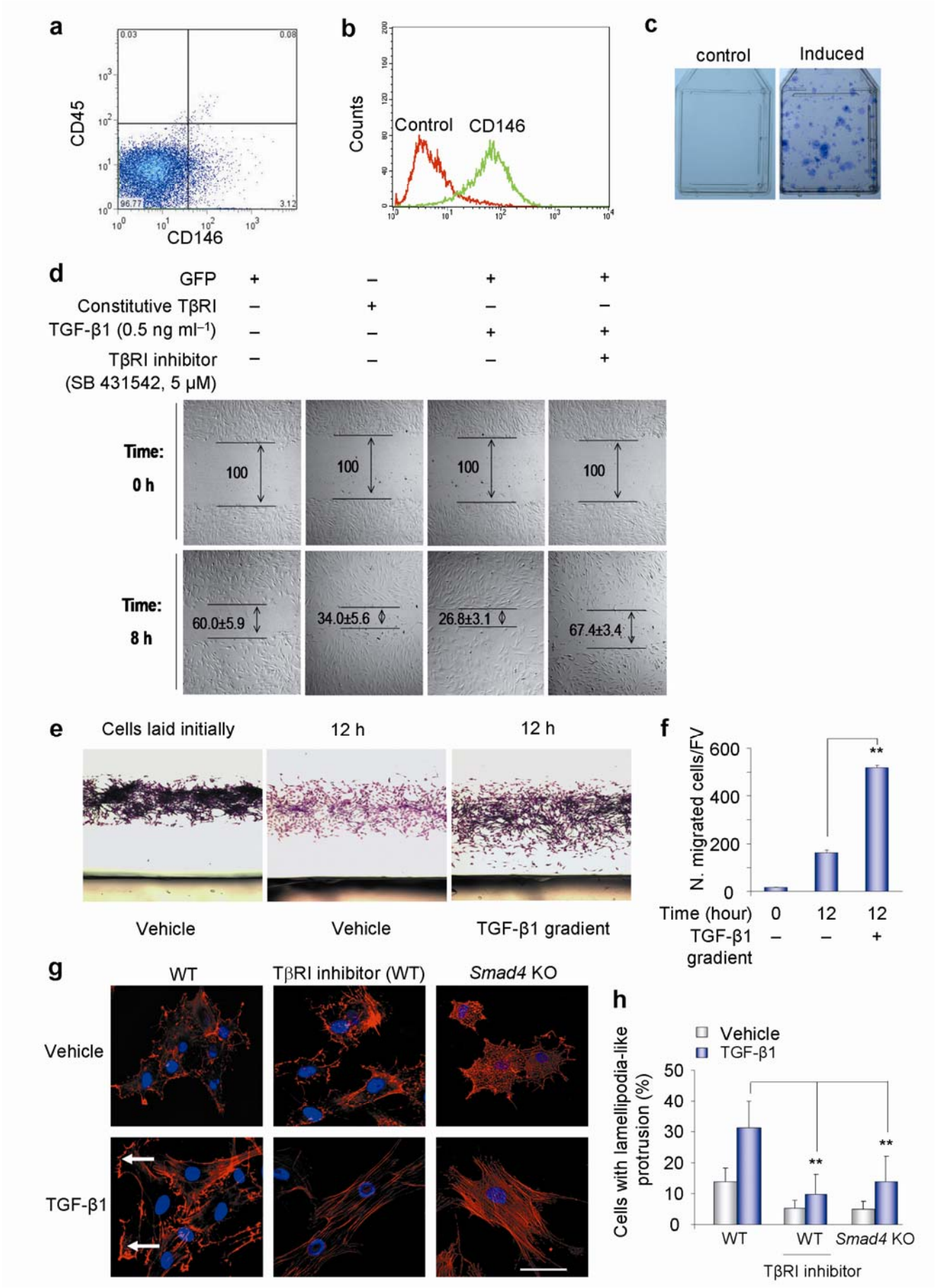
Supplementary Figure 2



Supplementary Figure 2. Bone phenotype of *Tgfb1*$^{-/-}$*Rag2*$^{-/-}$ mice. (**a**–**c**)Trabecular bone volume fraction (BV/TV, **a**), thickness (Tb.Th, **b**) and separation (Tb.Sp, **c**) in the tibia from one month-old *Tgfb1*$^{-/-}$*Rag2*$^{-/-}$ mice and their wild type littermates (*Tgfb1*$^{+/+}$*Rag2*$^{-/-}$) were measured by μCT. *n* = 5. N.S., not significant. (**d**) Light micrographs of TRAP-staining of trabecular bone sections from three month-old *Tgfb1*$^{-/-}$*Rag2*$^{-/-}$ mice or their WT littermates (*Tgfb1*$^{+/+}$*Rag2*$^{-/-}$). Scale bar, 200 μM.

Nature Medicine: doi:10.1038/nm.1979

Supplementary Figure 3

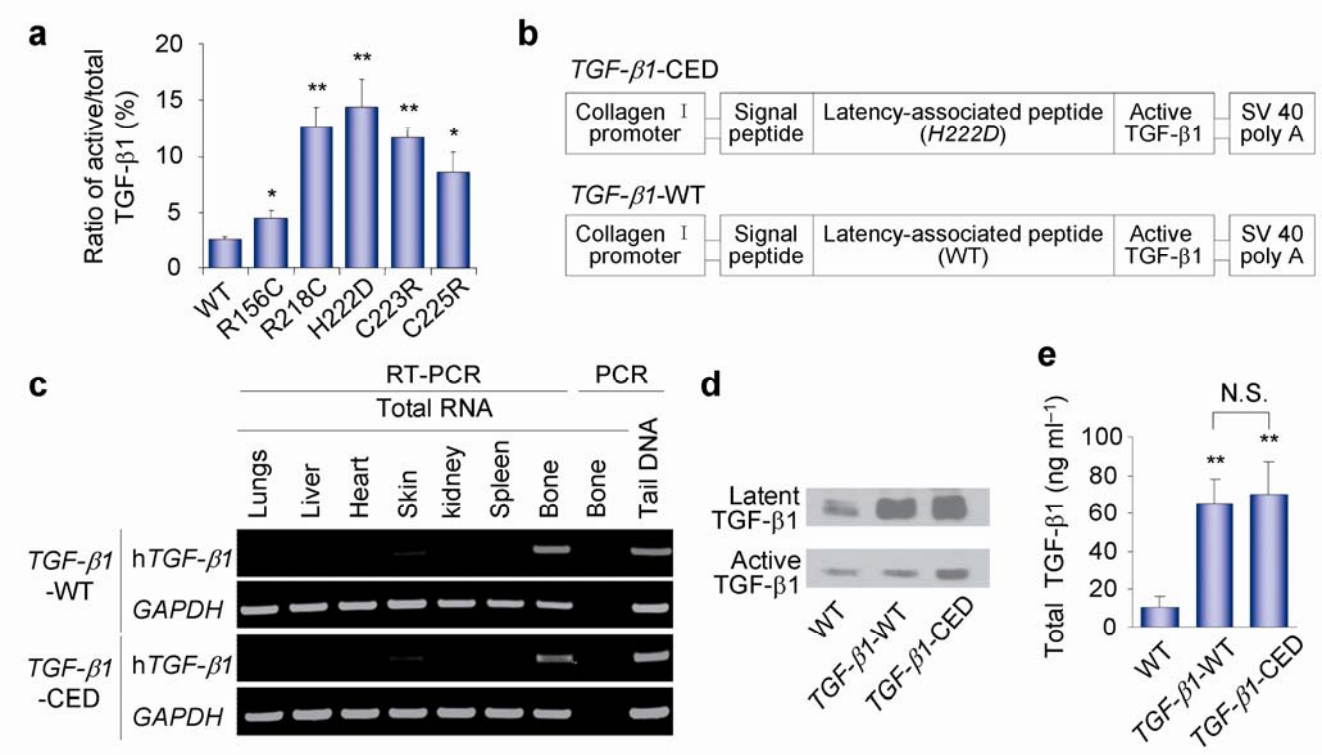


Nature Medicine: doi:10.1038/nm.1979

Supplementary Figure 3. Smad signaling mediates TGF-β1-induced migration of BMSCs. (**a**) Fluorescence activated cell sorting (FACS) of $CD146^{+}CD45^{-}$ cells from human BMSCs. The sorted cells were enriched and further confirmed by flow cytometry (**b**). (**c**) The sorted cells formed CFU-F and were osteogenic as shown with AP positive staining after being induced with osteogenic medium and the non-induced culture as control. (**d**) A scratch cell migration assay of human STRO-1^{+} BMSCs with retrovirus-mediated expression of GFP or constitutive active TβRI on type I collagen pre-coated 24-well tissue culture plates. The cells were also treated with mitomycin C (10 μg ml^{-1}) for two hours to prevent cell proliferation. *n* = 3. h, hour. (**e**,**f**) TGF-β1 gradient directs migration of BMSCs. A human natural TGF-β1 gradient (highest concentration of TGF-β1 = 50 ng ml^{-1}) was created in filter paper placed to one side of human STRO-1^{+} BMSCs in the culture dish as indicated. The BMSCs migrated specifically toward the gradient as shown in (**e**). The number of BMSCs that migrated passed the line of the cells laid initially toward the TGF-β1 gradient was counted from three random fields. The average of BMSCs migrated is presented (**c**). *n* = 3. $^{**}P < 0.01$ versus vehicle (12 hours). h, hour. (**g**,**h**) Inhibition of SMAD signaling blocks formation of lamellipodia-like protrusions by BMSCs. Staining of stress fibers using phalloidin showed that the STRO-1^{+} BMSCs migrating through the TGF-β1 gradient exhibit lamellipodia-like protrusions. Addition of TβRI kinase inhibitor (SB 431542) eliminates TGF-β1-induced formation of the lamellipodia-like protrusions by BMSCs, as does deletion of *Smad4* in primary BMSCs isolated from *Smad4* floxed mice by adenovirus-mediated expression of Cre**.** White arrows indicate the lamellipodia-like protrusion. Scale bar, 25 μm. The number of BMSCs with lamellipodia-like protrusions was counted from five random fields. The percentages of BMSCs with lamellipodia-like protrusions are presented (**h**). *n* = 3. $^{**}P < 0.01$ versus WT with TGF-β1.

Nature Medicine: doi:10.1038/nm.1979

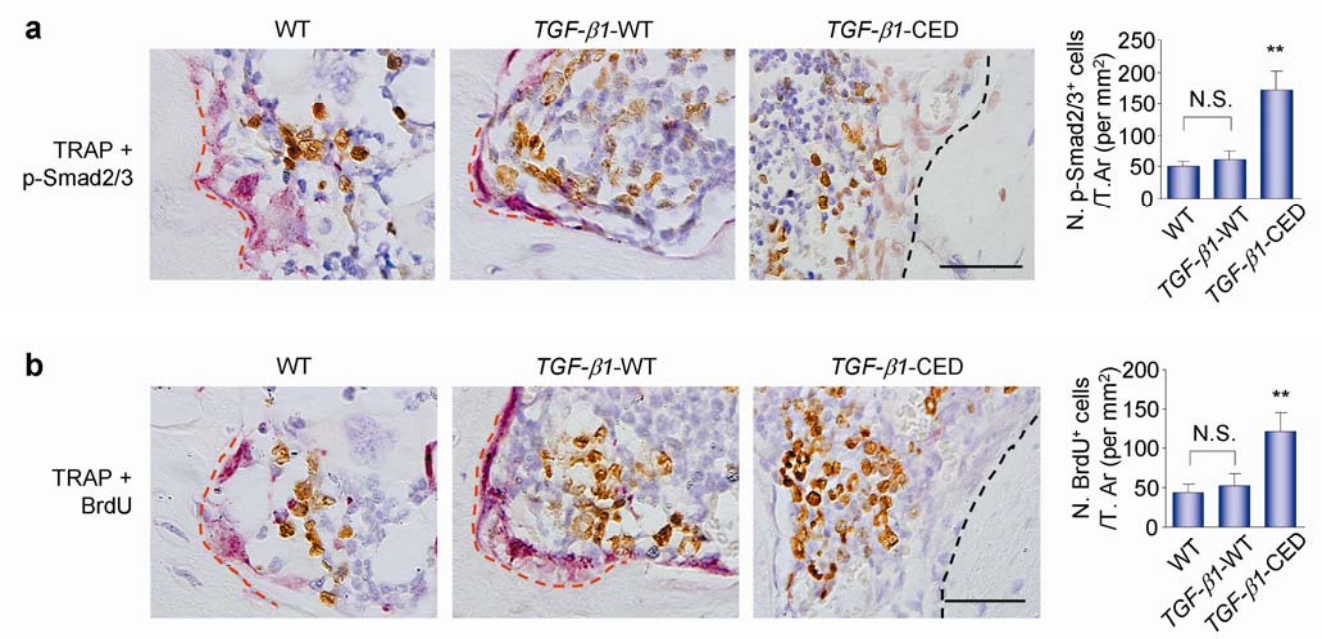
Supplementary Figure 4



Supplementary Figure 4. Osteoblast-specific expression of WT or CED *TGF-β1* in mice. (**a**) The ratio of active/total TGF-β1 in conditioned media from (**Fig. 5a**) was measured by ELISA. $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$ versus WT. (**b**) Schematic shows the strategy for generating mice expressing *TGF-β1* wild type (*TGF-β1*-WT) and CED mutant *TGF-β1* (*TGF-β1*-CED) exclusively in bone driven by bone-specific expressional promoter of 2.3 kb type I collagen. (**c**) Tissue specific expression of *TGF-β1*-WT and *TGF-β1*-CED in transgenic mice. Total RNA extracted from different tissues of transgenic mice as indicated was subjected to RT-PCR. PCR were performed using total RNA extracted from bone as template to confirm no DNA contamination. Genomic DNA extracted from tails of transgenic mice was used as a positive control. As a RNA extraction and reverse transcription control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified by RT-PCR. (**d**) Western blot analysis of active and latent TGF-β1 in the bone marrow under non-reducing conditions. (**e**) The amount of total TGF-β1 in the bone marrow was measured by ELISA. $n = 10$. $^{**}P < 0.01$ versus WT. N.S., not significant.

Nature Medicine: doi:10.1038/nm.1979

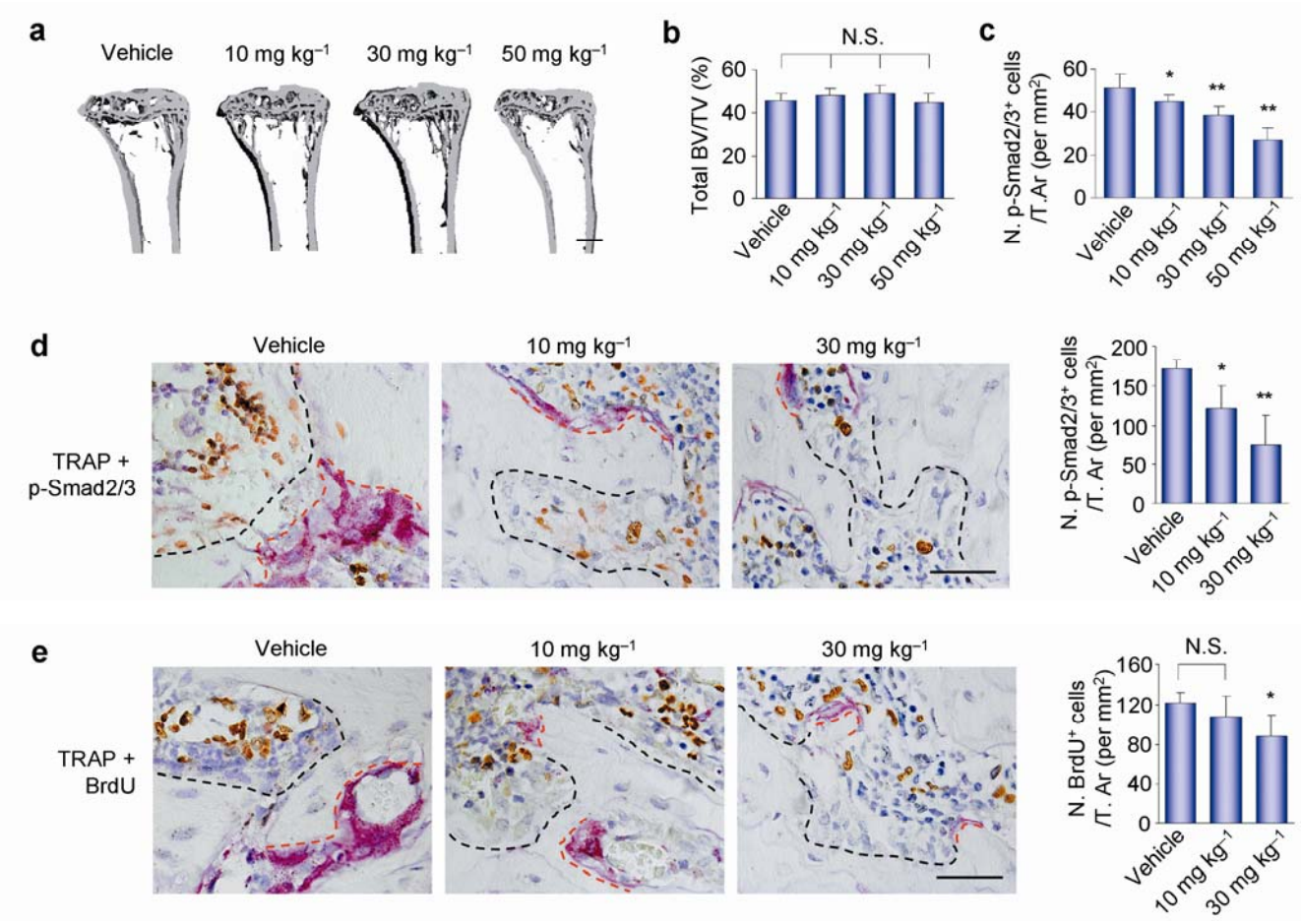
Supplementary Figure 5



Supplementary Figure 5. High levels of active TGF-β1 in the bone marrow microenvironment of CED mice. (**a**,**b**) Tibiae sections of three month-old WT, *TGF-β1*-WT, and *TGF-β1*-CED mice were immunostained for p-Smad2/3 (**a**) and BrdU (**b**) with co-staining of TRAP for osteoclasts. Red dotted lines indicate osteoclastic surface and black dotted lines indicate osteoblastic surface. Scale bar, 25 μm. Bone histomorphometrical analysis of the diaphysis of tibiae from different treatment groups. N. p-Smad2/3^{+} cells/T.Ar, number of p-Smad2/3 positive cells per tissue area. N. BrdU^{+} cells/T.Ar, number of BrdU positive cells per tissue area. n = 10. $^{**}P < 0.01$ versus WT. N.S., not significant.

Nature Medicine: doi:10.1038/nm.1979

Supplementary Figure 6



Supplementary Figure 6. Effect of TβRI inhibitor on bone. Two month-old male WT mice were injected every day intraperitoneally with either vehicle or SB-505124, a TβRI inhibitor, at different dosages (0.0, 10.0, 30.0, and 50.0 mg kg^{-1}) in vehicle for seven weeks. (**a**) Three-dimensional μCT images of proximal tibiae from the indicated treatment groups after treatment. Scale bar, 2 mm. (**b**) μCT analysis of total BV/TV of the whole tibiae from the indicated treatment groups after treatment. *n* = 5. N.S., not significant. (**c**) Tibiae sections from the indicated treatment groups after treatment were immunostained for p-Smad2/3. N. p-Smad2/3$^+$ cells/T.Ar were given. *n* = 5. **P* < 0.05; ***P* < 0.01 versus vehicle. (**d,e**) *TGF-β1*-CED mice treated with SB-505124 at different dosages (0.0, 10.0, and 30.0 mg kg^{-1}) in vehicle for seven weeks. Tibiae sections from the indicated treatment groups after treatment were immunostained for p-Smad2/3 (**d**) and BrdU (**e**) with a co-staining of TRAP for osteoclasts. Red dotted lines indicate osteoclastic surface and black dotted lines indicate osteoblastic surface. Scale bar, 25 μm. N. p-Smad2/3$^+$ cells/T.Ar and N. BrdU$^+$ cells/T.Ar were given. *n* = 10. **P* < 0.05; ***P* < 0.01 versus vehicle. N.S., not significant.

Nature Medicine: doi:10.1038/nm.1979

Supplementary Table 1. Histomorphometric analysis of diaphysis in tibiae of *TGF-β1*-CED mice with or without treatment of TβRI inhibitor.

Parameter	WT	*TGF-β1*-WT	*TGF-β1*-CED	*TGF-β1*-CED treated with TβRI inhibitor		
				Vehicle	10 mg kg^{-1}	30 mg kg^{-1}
Cortical porosity (%)	0.65±0.12	0.71±0.23	3.23±0.98 *	3.06±0.83	2.56±0.55	1.03±0.43 #
Cortical thickness (mm)	0.27±0.03	0.25±0.02	0.89±0.14 *	0.76±0.11	0.69±0.28	0.58±0.12
Ob surface/mm Es.S (%)	0.87±0.07	0.73±0.14	3.12±0.97 *	3.51±0.79	3.23±0.67	2.95±0.69
Oc surface/mm Es.S (%)	0.12±0.07	0.19±0.17	2.56±0.32 *	2.62±0.54	2.11±0.32	1.06±0.22 #
Ob surface/mm pore surface (%)	3.13±0.23	2.78±0.32	10.28±2.01 *	9.56±1.35	7.56±0.88	4.54±0.51 #
Oc surface/mm pore surface (%)	0.76±0.11	0.79±0.13	6.01±1.66 *	5.78±1.58	4.13±0.73	2.18±0.76 #
BFR/BS Es.S ($mm^3/mm^2/yr$)	120.39±9.61	128.51±11.52	163.32±35.26	160.21±25.78	156.72±29.12	142.88±31.79

Ob, osteoblast; Oc, osteoclast; Es.S, endosteal surface; BS, bone surface. $n = 10$. *$P < 0.05$ versus WT. #$P < 0.05$ versus vehicle.

Nature Medicine: doi:10.1038/nm.1979

Supplementary Methods

Scratch assay. We cultured BMSCs in 24-well plates coated with collagen type I (50 μg ml^{-1}) to confluence, and scraped the cell monolayer. After being washed three times with serum free medium, the cells were incubated for another eight hours. Photographs were taken and the average size of the gaps was quantitated as described previously[1].

***In Vitro* assays for migration of BMSCs.** For preparation of osteoclastic bone resorption-conditioned medium (BRCM), we isolated macrophages from bone marrow of four to eight week-old mice, cultured overnight in α-MEM containing 10% heat-inactivated FBS, and subjected to Ficoll-Hypaque density gradient centrifugation. Cells at the gradient interface were collected and cultured in suspension in Teflon beakers for two days in the presence of 10 ng ml^{-1} recombinant M-CSF (R&D Systems Inc.), then plated on bone slices of bovine cortical bone, *Tgfb1*$^{-/-}$ or *Tgfb1*$^{+/+}$ mouse bone in 24-well tissue culture plates ($1x10^5$ cells per well) and cultured in the presence of 10 ng ml^{-1} recombinant M-CSF and 100 ng ml^{-1} of RANKL[2]. Under these conditions, osteoclasts begin to form and resorb bone at days six–seven. The cultured cells were stained for TRAP activity at days eight–ten using a commercial kit (Sigma-Aldrich). The resorption of the bovine cortical bone slices was determined by scanning electronic microscopy. The conditioned media from the osteoclast-mediated resorption were harvested at days eight-ten by brief centrifugation. In some experiments, the neutralizing antibodies for certain factors including TGF-β1, TGF-β2, TGF-β3, IGF-I, IGF-II, PDGF (BD Biosciences) were added to the conditioned media or the conditioned media (BRCM) was depleted of active TGF-β1 by three cycles of immunoprecipitation using a monoclonal antibody specific for TGF-β1 adsorpted to protein G-Sepharose. Noggin (BD Biosciences) was added to a final concentration of 100 ng ml^{-1}.

Cell migration was assessed in Transwells (Corning, Inc.) essentially as described previously[3]. The eight μm pore membrane between the chambers was pre-coated with 0.5 μg ml^{-1} type I collagen (BD Biosciences). BMSCs were plated in the upper chambers and undiluted conditioned media from the osteoclastic bone resorption system were added to the lower chambers. After five hours-incubation, cells were fixed with 10% formaldehyde for four hours, and then the cells remaining on the top of the polycarbonate membrane were removed with cotton swabs. The cells that had migrated through the pores to the lower surface were stained with crystal violet (Sigma-Aldrich). Four fields at 200 × magnification were selected at random, photomicrographic images obtained and the cells in each image counted. Experiments were performed in triplicate.

TGF-β1 used in this manuscript was purchased from Sigma-Aldrich Inc, and it is the human natural TGF-β1 protein (Cat# T 1654, Sigma-Aldrich). Before use, the product was treated with 4 mM HCL containing 1 mg ml^{-1} BSA to make a stock solution and activate the protein.

Migration of cells in TGF-β1 gradient. We also assessed the migration of human STRO-1^+ BMSCs in TGF-β1 gradient by the method as described previously[4]. For actin staining, BMSCs were seeded on coverslips and incubated for two hours. After treatment with 2 ng ml^{-1} TGF-β1 for one hour, BMSCs were fixed using 4% paraformadehyde in PBS for one hour at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS. Rhodamine-conjugated phalloidin (Invitrogen) was added and incubated for 30 minutes at room temperature. Fluorescence localization was analyzed by confocal microscopy (Leica TCS SP2) with LCS software.

ELISA. We determined the amount of active and total TGF-β1 in the conditioned media or bone marrow

Nature Medicine: doi:10.1038/nm.1979

with the DuoSet ELISA Development kit (R&D Systems) according to the manufacturer's instructions. As the antibody used in the kit recognizes only active TGF-β1, the measurement of total TGF-β1 was carried out after the activation of the latent TGF-β1. Latent TGF-β1 in conditioned media was acidified with 1 N HCl for ten minutes and neutralized with 1.2 N NaOH/0.5 M HEPES.

Extraction of genomic DNA, total RNA and RT-PCR. We cut tail tips from pups at three weeks of age and digested in homogenizing solution (10 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA, 1% SDS and 0.5 mg ml^{-1} proteinase K) at 55°C for 12 hours. Genomic DNA was then extracted with phenol/chloroform. To determine expression of the transgene as well as the bone marker genes, femora and tibiae were isolated from the mice, wrapped with aluminum foil and immediately inserted into liquid nitrogen. Bones were pulverized using a hammer in the presence of liquid nitrogen. The frozen powder was placed in TRIzol reagent (Invitrogen) and homogenized with a cell homogenizer. Total RNA was extracted according to the manufacturer's instruction. To examine the expression of Col-*TGF-β1*-WT and Col-*TGF-β1*-H222D, RT-PCR was performed using total RNA isolated from bone or other tissues following the protocol provided by the manufacturer (Access RT PCR System; Promega). The forward primer was 5'-CCGCT GCTGC TACCG CTGCT G-3' and the reverse primer was 5'-ACCTC GGCGG CCGGT AGTGA A-3'. The primers were designed from human *TGF-β1* sequence (ACCESSION NM_000660 Homo sapiens transforming growth factor, beta 1 (*TGF-β1*), mRNA), which are different from mice *Tgfb1* sequence (ACCESSION NM_011577 Mus musculus transforming growth factor, beta 1 (*Tgfb1*), mRNA), to distinguish the transgene from endogenous mouse *Tgfb1*, whereas PCR was carried out using RNA samples to confirm no DNA contamination. As a control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified from the RNA samples by RT-PCR.

Manipulation of SMAD protein expression of SMADs. SiRNA of *GFP*, human *SMAD2*, *SMAD3*, and *SMAD4* were purchased from Santa Cruz Biotechnology Inc. The knockdown of these genes in human STRO-1^+ BMSCs was performed according to the manufacturer's directions. The viability of BMSCs was determined by counting of 0.4% (W/V) Trypan blue-stained cells using a dual-chamber hemocytometer. Greater than 95% of the BMSCs were viable after transfection with siRNAs. For overexpression, *SMAD7*, *TβRI* or green fluorescent protein (*GFP*) was cloned into the retrovirus *pMSCV* vector (BD, Bioscience). BMSCs were overlaid with the recombinant retroviruses for four hours at 37 °C, after which the medium was replaced with Dulbecco's modified Eagle's medium and cultured for another 48 hours to allow for expression of the transfected genes. The infection rate was assessed and was usually ~ 90%.

Western blot analysis. We lysed BMSCs with radioimmune precipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (10 mM sodium orthovanadate, and 50 mM sodium β-glycerophosphate)[5]. Lysates were centrifuged and the supernatants were separated by SDS-PAGE, blotted onto a PVDF (Bio-Rad Laboratories) membrane, analyzed with specific antibodies, and visualized by enhanced chemiluminescence (ECL Kit; Amersham Biosciences). To examine active and latent forms of the TGF-β1 protein, Western blot analysis was performed using antibodies recognizing TGF-β1 or LAP (latency associated peptide, R&D Systems) under non-reducing conditions.

Histochemistry, immunohistochemistry, and histomorphometric analysis. At the time of sacrifice, we resected and fixed the tibiae or femura in 10% buffered formalin for 48 hours, decalcified in 10% ethylenediamine tetraacetic acid (EDTA) (pH 7.0) for 20 days and embedded in paraffin. four-μm-thick

Nature Medicine: doi:10.1038/nm.1979

longitudinally oriented sections of bone including the metaphysis and diaphysis were processed for hematoxylin and eosin (H&E) staining. TRAP staining was performed using standard protocol (Sigma-Aldrich). BrdU staining was performed using standard protocol (Invitrogen). Immunostaining was performed using standard protocol. Sections were incubated with primary antibodies to mice Runx2 (M-70) (Santa Cruz Biotechnology Inc., diluted 1:100), human RUNX2 (27-k) (Santa Cruz Biotechnology Inc., diluted 1:100), GFP (Cell Signaling technology Inc., diluted 1:800), human leukocyte antigen A (HLA-A) (Santa Cruz Biotechnology Inc., diluted 1:100), osteocalcin (Santa Cruz Biotechnology Inc., diluted 1:100), and p-Smad2/3 (Santa Cruz Biotechnology Inc., diluted 1:50) for one hour at room temperature or overnight at 4°C. An HRP-streptavidin detection system (Dako) was subsequently used to detect the immunoactivity followed by counterstaining with hematoxylin (Dako) or methyl green (Sigma-Aldrich). For the HLA-A and RUNX2 double immuno-staining, we used a standard technique with a commercial kit (EnVision™ G|2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red), Dako). Isotype-matched negative control antibodies (R&D Systems) were used under the same conditions. Sections were microphotographed to perform histomorphometric measurements on the interesting areas of the bone displayed on the digitalized image. Quantitative histomorphometric analysis was conducted in a blinded fashion with OsteoMeasure$_{XP}$ Software (OsteoMetrics, Inc., Decatur, GA, USA). Two-dimensional parameters of trabecular bone were measured in a two-mm square, one-mm distal to the lowest point of the growth plate in the secondary spongiosa. Parameters of cortical bone were measured on both sides in the diaphysis (at the midshaft femur/tibia). To label mineralization fronts, mice were given subcutaneous injections of calcein (Sigma, 15 mg kg^{-1}) in 2% sodium bicarbonate solution ten days and three days before death. To examine cell proliferation *in vivo*, mice were injected with BrdU (Zymed, 1 ml per 100 g bodyweight) intraperitoneally two hours before sacrifice. Number of osteoblasts per tissue area (N.Ob/T.Ar, per mm^2), number of osteoclasts per tissue area (N.Oc/T.Ar, per mm^2), number of Runx2 positive cells on bone surface per bone perimeter (N.Runx2$^+$ cells/B.Pm, per mm), number of GFP positive cells on bone surface per bone perimeter (N.GFP+ cells/B.Pm, per mm), number of GFP positive cells in bone matrix per tissue area (N. GFP$^+$ cells in bone matrix/T.Ar, per mm^2), number of HLA-A positive cells on bone surface per bone perimeter (N.HLA$^+$ cells/B.Pm, per mm), number of HLA-A and RUNX2 double positive cells on bone surface per bone perimeter (N.HLA$^+$/RUNX2$^+$ cells/B.Pm, per mm), cortical porosity (%), cortical thickness (mm), bone volume per tissue volume (BV/TV, %), osteoblast surface per endosteal surface (Ob surface/mm Es.S, %), osteoclast surface per endosteal surface (Oc surface/mm Es.S, %), osteoblast surface per pore surface (Ob surface/mm pore surface, %), osteoclast surface per pore surface (Oc surface/mm pore surface, %), endosteal bone formation rate, surface referent (BFR/BS Es.S, $mm^3/mm^2/yr$), number of p-Smad2/3 positive cells per tissue area (N. p-Smad2/3$^+$ cells/T.Ar, per mm^2), and number of BrdU positive cells per tissue area (N. BrdU$^+$ cells/T.Ar, per mm^2) in four randomly selected visual fields per specimen, in five specimens per mouse in each group were measured.

X-ray analysis. Mice were exposed at 26 kV, 10 sec in a FAXITRON X-ray machine (FAXITRON, Wheeling, Illinois, USA). An interrupted continuity of the bone was defined as fracture.

Microcomputed tomography analysis. Tibiae and femora obtained from mice were dissected free of soft tissue, fixed overnight in 70% ethanol and analyzed by a high resolution microcomputed tomography (μCT) imaging system (MicroCT40; Scanco Medical AG, Basserdorf, Switzerland). The scanner was set at a voltage of 55 kVp and a current of 109 μA. The resulting two-dimensional longititude-sectional images or three-dimensional whole bone images are shown in gray scale. Three-dimensional structural parameter, total BV/TV: total bone volume (contains both trabecular and cortical bone) of per tissue volume, BV/TV:

Nature Medicine: doi:10.1038/nm.1979

trabecular bone volume per tissue volume, Tb. Th: trabecular thickness and Tb. Sp: trabecular separation were given.

Supplementary Methods References

1. Bandyopadhyay,B. *et al.* A "traffic control" role for TGF beta 3: orchestrating dermal and epidermal cell motility during wound healing. *Journal of Cell Biology* **172**, 1093-1105 (2006).

2. Liu,W. *et al.* Functional identification of three receptor activator of NF-kappa B cytoplasmic motifs mediating osteoclast differentiation and function. *Journal of Biological Chemistry* **279**, 54759-54769 (2004).

3. Sander,E.E. *et al.* Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *Journal of Cell Biology* **143**, 1385-1398 (1998).

4. Wong,K. *et al.* Signal transduction in neuronal migration: Roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. *Cell* **107**, 209-221 (2001).

5. Tang,Y., Liu,Z.Y., Zhao,L., Clemens,T.L. & Cao,X. Smad7 stabilizes beta-catenin binding to E-cadherin complex and promotes cell-cell adhesion. *Journal of Biological Chemistry* **283**, 23956-23963 (2008).

Nature Medicine: doi:10.1038/nm.1979