Smad4 is required for maintaining normal murine postnatal bone homeostasis

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Summary

Transforming growth factor β (TGFβ) is a multifunctional cytokine involved in skeletal development. Smad4 is the central intracellular mediator of TGFβ signaling. Our previous studies reveal that Smad4 is required for maintaining the normal development of chondrocytes in the growth plate. However, its biological function during postnatal bone remodeling is largely unknown. To investigate the role of Smad4 in maintaining bone homeostasis, we disrupted the *Smad4* gene in differentiated osteoblasts using the Cre-loxP system. The *Smad4* mutant mice exhibited lower bone mass up to 6 months of age. The proliferation and function of the mutant osteoblasts were significantly decreased. Bone mineral density, bone volume, bone formation rate and osteoblast numbers were remarkably reduced in *Smad4* mutants. Intriguingly, the trabecular bone volume in *Smad4* mutant mice older than 7 months was higher than that of controls whereas the calvarial and cortical bone remained thinner than in controls. This correlated with reduced bone resorption possibly caused by downregulation of TGFβ1 and alteration of the ligand receptor activator of NF-κB (RANKL)-osteoprotegerin (OPG) axis. These studies demonstrate essential roles of Smad4-mediated TGFβ signaling in coupling bone formation and bone resorption and maintaining normal postnatal bone homeostasis.

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Key words: Smad4, Osteoblast, Osteoclast, Bone mass, RANKL/OPG

Introduction

Bone remodeling is central to maintaining the integrity of the skeletal system, wherein the developed bone is constantly renewed by the balanced action of osteoblastic bone formation and osteoclastic bone resorption. The cellular growth and differentiation of osteoblasts and osteoclasts are precisely coordinated to maintain the bone mass homeostasis. Multiple signaling pathways, such as insulin-like growth factor, parathyroid hormone and transforming growth factor β (TGFβ) have been shown to couple bone formation to bone resorption (Harada and Rodan, 2003). However, much less is known about the intracellular control of osteoblastic function.

TGFβ is an important physiological regulator of osteoblast differentiation and function as coupling factors during bone remodeling (Martin and Sims, 2005). TGFβ is stored in large amounts in the bone matrix and subsequently activated by the acidic microenvironment created by bone-resorbing osteoclasts (Oreffo et al., 1989; Oursler, 1994). Once activated, TGFβ stimulates the proliferation of osteoblast precursor cells as well as osteoblast matrix production and induces bone formation in vivo (Noda and Camilliere, 1989; Yamada et al., 2000). Increasing evidence suggests that allelic variations at the *TGFB1* gene contribute to the development of osteoporosis (Yamada et al., 1998; Yamada et al., 2000; Keen et al., 2001). It has also been reported that bone loss in old male mice results from diminished activity and availability of TGFβ (Gazit et al., 1998). Consistently, decreased bone mass and bone elasticity have been shown in mice lacking the *Tgfb1* gene (Geiser et al., 1998). Osteoblastic overexpression of TGFβ2 results in a high-turnover osteoporosis owing to the increase in both bone resorption and bone formation (Erlebacher and Derynck, 1996), whereas transgenic mice expressing a truncated dominant negative TGFβ receptor II in osteoblasts show an age-dependent increase in trabecular bone owing to imbalanced bone formation and bone resorption (Filvaroff et al., 1999). These results suggest that TGFβ is a key mediator of the coupling of osteoblast differentiation to osteoclastic bone resorption. However, the molecular mechanism underlying the function of TGFβ during bone remodeling is still largely unknown.

Bone morphogenetic proteins (BMPs) were originally identified by their ability to induce ectopic bone formation (Wozney et al., 1988). BMPs are involved in embryonic and postnatal osteogenesis by stimulating the commitment of mesenchymal cells into osteoblastic lineage and promoting osteoblast differentiation (Canalis et al., 2003). However, the mechanism of BMP signaling on bone formation and postnatal bone metabolism remains unclear owing to the embryonic

lethality resulting from mutations of *Bmp2* and *Bmp4*, and their receptors (Mishina et al., 1995; Winnier et al., 1995). Recent studies have begun to reveal the necessary roles of BMP receptors in bone remodeling (Mishina et al., 2004). Postnatal osteoblast specific disruption of *Bmpr1a* results in low bone mass in relatively young mutants and an opposite phenotype in aged mice because of impaired bone formation and bone resorption (Mishina et al., 2004). The transgenic mice expressing a truncated dominant-negative *Bmpr1b* in osteoblasts exhibit osteopenia owing to impairment of bone growth and reduction of bone mineral density (Zhao et al., 2002). Transgenic mice overexpressing BMP antagonists, such as noggin and sclerostin, all exhibit low bone mass, which is due to decreased BMP signaling (Wu et al., 2003). It is noteworthy that BMP2 has been linked with osteoporosis in humans (Styrkarsdottir et al., 2003).

Smads are primary cytoplasmic signal transducers of TGFβ and BMPs. TGFβ activates Smad2 and Smad3, whereas BMP signaling is conveyed by Smad1, Smad5 and Smad8. Smad4 is the common mediator Smad shared by signaling pathways for BMPs and TGFβ/activin (Derynck and Zhang, 2003). It has been shown that the loss of Smad3 results in a lower rate of bone formation and osteopenia in addition to defects in chondrocyte differentiation (Borton et al., 2001; Yang et al., 2001). By contrast, recent studies revealed that a reduction in TGFβ signaling via Smad3 deletion enhanced the mineral concentration of the bone matrix and the bone mass (Balooch et al., 2005). In addition, Smad3 interacts with class IIa histone deacetylases to mediate TGFβ signaling, which inhibits osteoblast differentiation (Kang et al., 2005). As a common mediator Smad of TGFβ signaling, Smad4 is expressed in osteoblasts and mediates effects of both BMP and TGFβ signals in cultured human osteoblasts (Lai and Cheng, 2002). Several in vitro studies suggest that Smad4 plays an important role in osteoblasts by interacting with other molecules, such as Runx2 and Fos (Lee et al., 2000; Zhang et al., 2000; Lai and Cheng, 2002). However, it is difficult to access the in vivo function of Smad4 during bone remodeling owing to the early embryonic lethality of the *Smad4*-knockout mice (Sirard et al., 1998; Yang et al., 1998). To understand the role of Smad4-mediated TGFβ signals in postnatal bone formation and metabolism, we disrupted the *Smad4* gene specifically in differentiated osteoblasts using the Cre-loxP system. Our results suggest that Smad4-mediated TGFβ signaling is important for regulating postnatal bone homeostasis.

Results

Targeted ablation of *Smad4* in differentiated osteoblasts

To investigate the function of *Smad4* in osteoblasts, we generated a transgenic mouse strain (*OC-Cre*) that expressed the Cre recombinase under the control of the mouse osteocalcin (OC) promoter (Dacquin et al., 2002). Osteoblast-specific Cre activity was examined using *OC-Cre;ROSA26R* double transgenic mice. Although the Cre activity revealed by LacZ staining was barely detected in mandible bones of 1-day-old double transgenic mice (supplementary material Fig. S1A,B), it was observed in osteoblasts on trabecular bone (Fig. 1A), cortical bone (Fig. 1B), mandible bone (supplementary material Fig. S1C,D) and calvarial bone (data not shown) surfaces in 10-day-old and 6-week-old double transgenic mice.

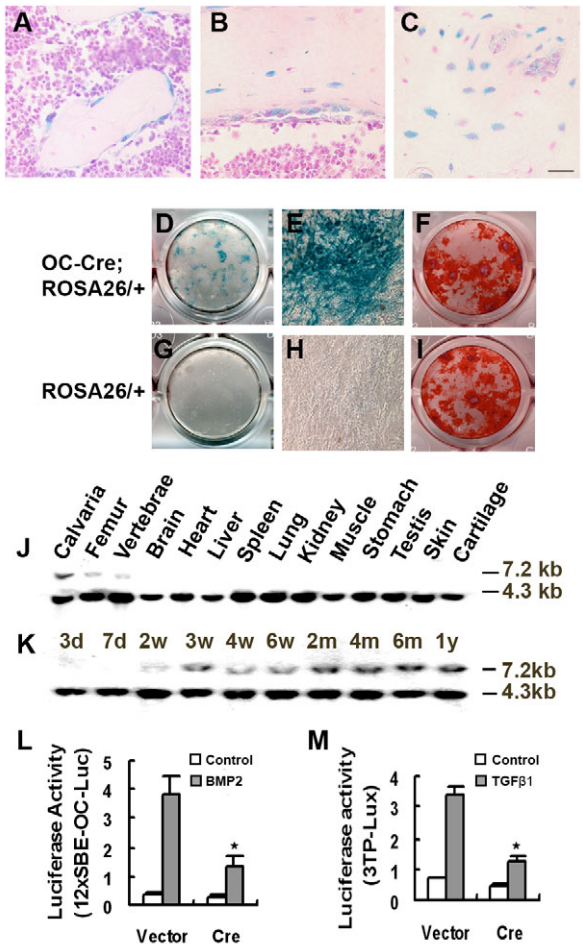

Fig. 1. Targeted disruption of *Smad4* in mouse osteoblasts. (A-I) Characterization of Cre activity in 6-week-old *OC-Cre;ROSA26* double transgenic mice. LacZ staining was detected in osteoblasts on trabecular bone (A), under the periosteum (B) and in osteocytes embedded in cortical bone (C). (D-I) Primary bone marrow cells of *OC-Cre/ROSA26* double heterozygous (D-F) and *ROSA26* (G-I) mice cultured for 12 days were subjected to LacZ staining (D,E,G,H) or Alizarin Red staining (F,I). E and H were higher magnification of areas in D and G, respectively. (J) Southern blot showing Cre-mediated recombination in bone tissues. The 4.3 kb fragment stems from the Smad4 conditional allele, the 7.2 kb fragment stems from the Smad4 allele after Cre-mediated recombination in osteoblasts. (K) Southern, using the probe previously described (Zhang et al., 2005), blot of *Smad4* mutant calvarial genomic DNA at different ages. (L-M) Blockage of BMP and TGFβ signaling. Primary $Smad4^{Co/Co}$ osteoblasts were transfected by *pCMV2* and *pCMV2-Cre* vector together with BMP reporter construct, *12xSBE-OC-Luc* (L) or TGFβ reporter construct, *3TP-Lux* (M), and then treated with 50 ng/ml BMP-2 (L) or 5 ng/ml TGFβ1 (M). Inactivation of *Smad4* blocked luciferase activity stimulated by BMP-2 and TGFβ1. **P*<0.01. Bar, 25 μm (A-C).

Most osteocytes derived from osteoblasts were stained positively (Fig. 1C). To examine the temporal expression of the *Cre* transgene under control of the mouse *osteocalcin* promoter, primary osteoblast cultures derived from bone marrow cells of double heterozygous and control mice were established and assayed at multiple time points for LacZ

staining. Positive staining was observed in osteoblasts when they started to mineralize (Fig. 1D-I).

Inactivation of *Smad4* in mature osteoblasts was achieved by breeding a mouse strain containing the *Smad4* conditional alleles (*Smad4*$^{Co/Co}$) (Yang et al., 2002) with the *OC-Cre* transgenic mice. Cre-mediated excision of exon 8 in different tissues isolated from a *Smad4*$^{Co/Co}$*;OC-Cre* mouse was detected by Southern blot. The results confirmed that Cre-mediated recombination occurred exclusively in calvaria, femurs and vertebrae, all of which are bone tissues containing osteoblasts (Fig. 1J). To follow the kinetics of *Smad4* deletion during the differentiation of the cultured primary osteoblasts, genomic PCR was used to detect the Cre-mediated excision. The results showed that the deletion was barely detectable in the early days of culture and gradually increased in later stages when mineralizing osteoblasts appeared (see supplementary material Fig. S1E). To examine when deletion of *Smad4* occurs in vivo, we performed a Southern blot on DNA extracted from calvarial cells at different ages. Cre-mediated DNA excision was easily detected in 2-week-old mice (Fig. 1K). All the data demonstrated that the *OC-Cre* transgenic mice could be used to achieve Cre-mediated recombination specifically in differentiated osteoblasts.

To determine the effect of *Smad4* deficiency on TGFβ and BMP signaling, reporter assays were performed in primary osteoblasts derived from calvaria of *Smad4*$^{Co/Co}$ mice. The *Smad4*$^{Co/Co}$ osteoblasts were transfected with a *12×SBE-OC-Luc* reporter construct with or without the *Cre* gene under the control of the *CMV* promoter. Addition of BMP2 stimulated luciferase activity of this reporter in *Smad4*$^{Co/Co}$ osteoblasts without the *CMV-Cre*. By contrast, co-transfection with the *CMV-Cre* in osteoblasts significantly inhibited the reporter activity, suggesting that BMP signaling is dramatically reduced in *Smad4*$^{Co/Co}$ osteoblasts after Cre-mediated recombination (Fig. 1L). TGFβ1-stimulated luciferase activity of *p3TP-Lux* was also inhibited by the Cre-mediated deletion of the *Smad4* gene in osteoblasts (Fig. 1M). These results indicated that ablation of *Smad4* in osteoblasts diminished the responsiveness of osteoblasts to both BMP-2 and TGFβ1.

Growth retardation and reduction of bone mineral density in *Smad4* mutant mice

The *Smad4*$^{Co/Co}$*;OC-Cre* mice were born normally and had similar body size and body weight to littermate controls (data not shown). They exhibited smaller body size as early as 3 weeks and more pronounced dwarfism at 6 weeks of age (Fig. 2A). The *Smad4*$^{Co/Co}$*;OC-Cre* mice were 50-70% smaller than wild-type controls at 6 weeks, and remained significantly smaller than their littermates throughout their lifetime. A quantitative measurement of femoral length indicated that the femurs of *Smad4* homozygous mutant mice were significantly shorter than wild-type controls at 3 (0.918±0.015 cm in male mutant vs 1.097±0.031 cm in controls, n=6, P<0.01), 6 (1.079±0.084 cm in male mutant vs 1.545±0.044 cm in controls, n=6, P<0.01) and 12 weeks (1.217±0.074 cm in male mutant vs 1.616±0.050 cm in controls, n=6, P<0.01). X-ray analysis of 6-week-old knockout mice showed shorter and more lucent femurs and tibias and suggested severe osteopenia (Fig. 2B). A dramatic decrease in bone mineral density (BMD) was evident at 6 and 12 weeks of age in both male and female *Smad4*$^{Co/Co}$*;OC-Cre* mice compared with the littermate controls (Fig. 2C).

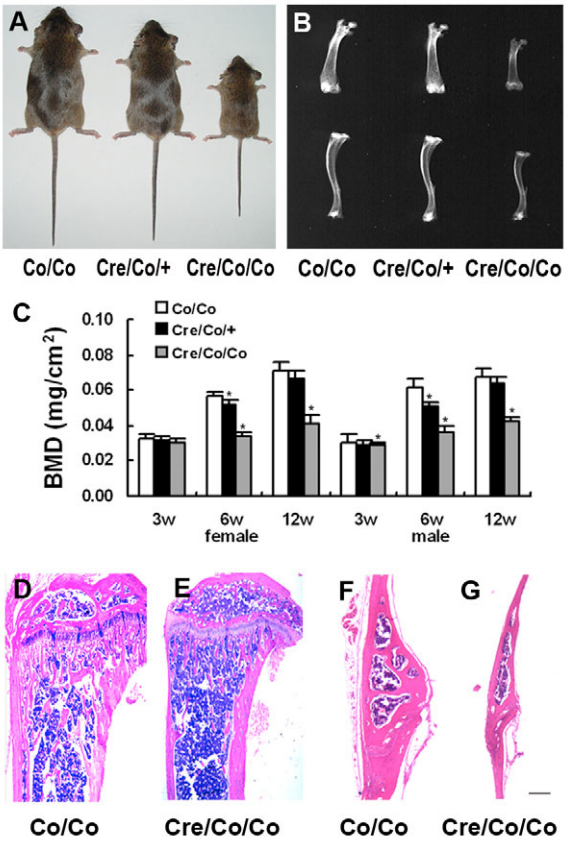

Fig. 2. Inactivation of *Smad4* in differentiated osteoblasts caused growth retardation and reduced bone density. (A) *Smad4* mutant (Cre/Co/+ and Cre/Co/Co) and control (Co/Co) mice at 6 weeks of age showing impairment of bone growth in *Smad4* mutants. (B) Soft X-ray images of femurs and tibias from 6-week-old female mice. (C) Significant loss of total bone density in *Smad4* mutant mice. (D-G) Histological analysis of tibias and calvarial bone in *Smad4* mutant and control mice. Sections of tibia (D,E) and lambda sagittal sutures (F,G) from 7-week-old *Smad4* mutant (E,G) and control mice (D,F). *P<0.01. Bar, 350 μm (D,E); 180 μm (F,G).

To characterize the skeletal abnormalities in the *Smad4*$^{Co/Co}$*;OC-Cre* mice in more detail, the proximal tibias and calvarial bones at different developmental stages were sectioned for histological analysis. At day 16, slightly decreased levels of trabecular bone were observed in knockout mice (data not shown). Histological sections of 7-week-old mutant tibias revealed that the number of trabecular bones was reduced significantly compared with the littermate controls (Fig. 2D,E). There was a marked decline in the amount of cortical bone in knockout mice, as assessed by cortical width of femoral midshaft at 8 weeks (103.9±15.5 μm in mutants vs 170.5±34.4 μm in controls, n=9, P<0.01). Calvarial thickness was decreased significantly in mutant mice compared with controls at this stage (57.1±10.0 μm in *Smad4* mutants vs 85.5±3.8 μm in controls, n=6, P<0.01). A loose connection of interparietal bones in the sagittal and coronal sutures was observed in *Smad4* mutants (Fig. 2F,G and data not shown). The growth plate of *Smad4* mutant mice was shorter than control littermates (Fig. 2E and see supplementary material Fig. S1), suggesting that endochondral ossification could be

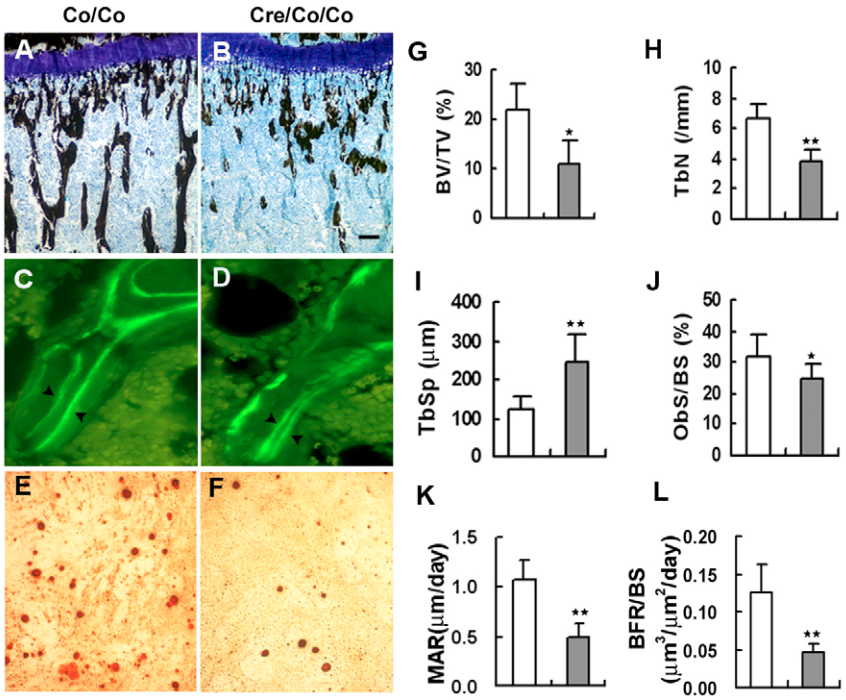

Fig. 3. Decreased bone formation in *Smad4* mutant mice. (A,B) Von Kossa staining of the proximal tibia sections of *Smad4* mutant (B) and control mice (A). (C,D) Representative images of the femora of 4-week-old male *Smad4* mutant and control mice labeled with sequential doses of calcein. (E,F) Formation of mineralized ECM in neonatal calvarial osteoblast cultures from *Smad4* mutant and control mice. Mineralized nodules were stained with Alizarin Red. (G-L) Quantitative histomorphometric measurements were performed on the secondary spongiosa at the distal femur of 8-week-old male mice. (G) BV/TV, bone volume/tissue volume; (H) TbN, trabecular number; (I) TbSp, trabecular separation; (J) ObS/BS, osteoblast surface/bone surface; (K) MAR, mineral apposition rate; (L) BFR/BS, bone formation rate/bone surface. All values are mean ± s.d. from six control (white bars) or *Smad4* mutant mice (grey bars). **P*<0.05; ***P*<0.01. Bar, 100 μm (A,B); 6.25 μm (C,D).

affected in the absence of *Smad4* in osteoblasts. Taken together, these observations reveal that targeted disruption of *Smad4* in osteoblasts causes osteopenia in young mutant mice.

Reduced osteoblast function in *Smad4*-deficient mice

To investigate the cellular basis underlying the bone mass loss in *Smad4* mutants, we performed static and dynamic bone histomorphometric analysis in sections of undecalcified distal femurs and tibias at 4 and 8 weeks. Von Kossa staining revealed significantly reduced bone volume in 7-week-old mutant mice (Fig. 3A,B). Calcein labeling analysis, a histomorphometric measurement of osteoblast activity in vivo, confirmed a remarkably decreased bone formation rate (BFR) associated with *Smad4* deficiency in 4- and 8-week-old animals (Fig. 3C,D and data not shown). Goldner trichrome staining revealed a 51% reduction in bone volume in 8-week-old mutant mice (Fig. 3G). Eight-week-old knockout mice also demonstrated a striking decrease in trabecular number (Fig. 3H), and an increase in trabecular separation (Fig. 3I) compared with normal siblings. Among bone formation parameters, the percentage of bone surface occupied by osteoblasts (Ob.S/BS) was significantly decreased by 27% in *Smad4* mutant mice compared with wild-type controls (Fig. 3J). Mineral apposition rate (MAR, an index of individual osteoblast activity) and bone formation rate/bone surface (BFR/BS is determined from the number and the function of osteoblasts) were markedly reduced in *Smad4* mutants (Fig. 3K,L). Consistently, formation of mineralized extracellular matrix (ECM) in neonatal calvarial osteoblast cultures from *Smad4* mutant mice was significantly delayed compared with that from control mice at day 18 of

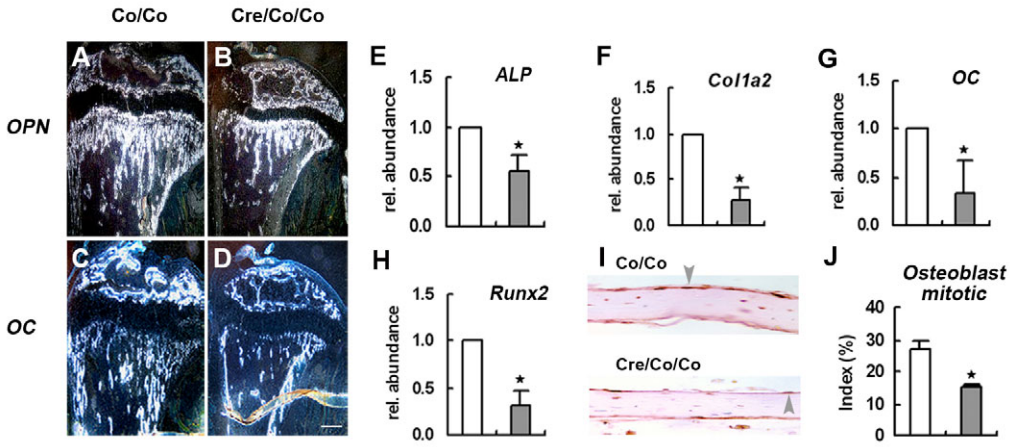

Fig. 4. Reduced production of bone extracellular matrix and decreased osteoblast proliferation in *Smad4* mutant mice. (A-D) In situ hybridization of proximal tibias from 4-week-old control (A,C) or mutant mice (B,D). The expression of (B) osteopontin (OPN) and (D) osteocalcin (OC) was decreased in *Smad4* mutant mice compared with controls (A,C). (E-H) Real-time PCR of *Akp1* (ALP), osteocalcin, *Col1a2* and *Runx2* from calvarial bone extracts of 7-week-old *Smad4* mutant (grey bars) and control mice (white bars). Values are presented as relative expression. (I) Sections of calvaria from 3-week-old mice showing a reduced number of BrdU-positive cells (arrowhead) in *Smad4* mutant mice compared with control littermates. (J) Osteoblast mitotic index (percentage of BrdU-positive cells per total cell number) in control (white bars) and *Smad4* mutant mice (grey bars). **P*<0.01. Bar, 400 μm (A-D); 26.5 μm (I).

culture (Fig. 3E,F). All these results indicate that the decreased bone density in *Smad4* mutant mice possibly results from a decrease in the bone-forming activity of osteoblasts.

We further examined the expression of bone ECM genes in *Smad4* mutants by in situ hybridization. The expression of osteopontin (*Spp1*, secreted phosphoprotein 1), an early osteoblast marker gene, was decreased in *Smad4* mutants at 4 weeks compared with controls (Fig. 4A,B). The expression of collagen I (*Col1a2*) and osteocalcin, late markers of osteoblasts, were also reduced in mutants (Fig. 4C,D and data not shown). Real-time PCR analyses confirmed that alkaline phosphatase (*Akp1*) (Fig. 4E), an early differentiation marker of committed osteoblasts, was also downregulated in bone extracts of *Smad4*-deficient mice. The mRNA levels of *Col1a2* and osteocalcin were all decreased (Fig. 4F,G). These data were consistent with the reduced number of osteoblasts described in the above histomorphometric measurements. We also found that the expression of *Runx2* was decreased (Fig. 4H). The mRNA levels of *Akp1*, *Col1a2,* osteocalcin and *Runx2* were all decreased in mutant calvarial cells that had been isolated under standard cultural condition for osteoblasts (supplementary material Fig. S2A,B and data not shown). The molecules in the Wnt/β-catenin signaling pathway were also changed. The mRNA levels of low-density lipoprotein-receptor-related protein 5 (*Lrp5*) and β-catenin were decreased, whereas lymphoid enhancer binding factor 1 (*Lef1*) was not changed. Levels of Dickkopf 2 (*Dkk2*), which blocks Wnt1-induced transcription of Lef1/Tcf target genes, were increased (see supplementary material Fig. S2C-F). To determine whether osteoblast proliferation was affected in *Smad4* mutant mice, we measured the BrdU-positive cells on calvarial periosteal surfaces. Significantly fewer labeled nuclei were detected in sections from *Smad4* mutant mice compared with those derived from control littermates (Fig. 4I,J). These results indicate that the proliferation of osteoblasts is decreased owing to the loss of *Smad4*.

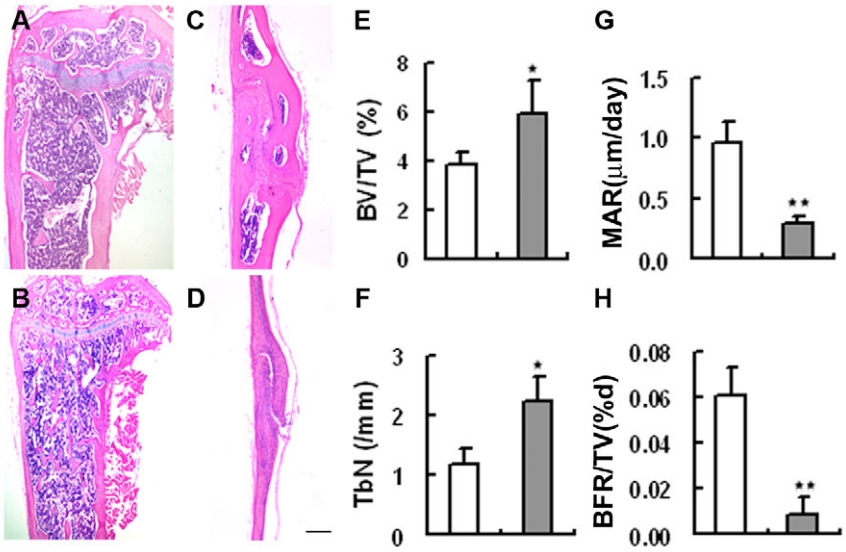

Fig. 5. Increased trabecular bone volume in old *Smad4* mutant mice. (A-D) Sections of tibias (A,B) and sagittal sutures (C,D) of 1-year-old *Smad4* mutant (B,D) and control mice (A,C). (E-H) Quantitative histomorphometric measurements were performed on the spongiosa at the proximal tibias of 11-month-old female mice. All values were mean ± s.d. of five control (white bars) or *Smad4* mutant mice (grey bars). **P*<0.05; ***P*<0.01. Bar, 350 μm (A,B); 100 μm (C,D).

Trabecular bone volume was increased in aged *Smad4* mutant mice

Although the *Smad4* mutant mice remained smaller than controls throughout their lifetime, the differences were reduced gradually in aged mice. The trabecular bone volume in knockout mice was increased when animals were older than 7 months, whereas the calvarial and cortical bone remained thinner than that of controls. This phenomenon was especially significant in 1-year-old mutant mice (Fig. 5A-D and data not shown). Histomorphometric measurements revealed increased trabecular bone volume (Fig. 5E), trabecular number (Fig. 5F) and a significantly decreased trabecular separation (data not shown) in 11-month-old mutant mice compared with normal siblings. Calcein labeling analysis detected markedly decreased mineral apposition rate (Fig. 5G) and bone formation rate (Fig. 5H) in aged *Smad4* mutants. All these data suggest that increased bone mass in aged *Smad4* mutants is possibly due to downregulation of osteoclast function.

Loss of *Smad4* in osteoblasts causes reduced osteoclast activity

A consequence of reduced osteoblast activity would be the reduction of osteoclast function. TRAP (tartrate-resistant acid phosphatase) staining on femur sections at 8 weeks revealed that this indeed was the case. A marked decrease in the number of TRAP-positive multinucleated osteoclasts demonstrated a decrease in bone resorption in *Smad4* mutant mice (Fig. 6A,B). This was confirmed by histomorphometric analyses. Bone resorption parameters, the percentage of bone surface covered by mature osteoclasts (OcS/BS) and the number of mature osteoclasts (OcN/BPm) were significantly decreased in *Smad4* mutant mice (Fig. 6C,D). Real-time PCR analysis revealed that the expression of TRAP and cathepsin K (*Ctsk*), lysosomal enzymes essential for osteoclastic bone resorption, were significantly reduced in calvaria of *Smad4* mutant mice (Fig. 6E,F). When a common osteoclast progenitor population derived from the wild-type spleens was co-cultured with primary cultured osteoblasts derived from *Smad4* mutant or control bone marrows, $TRAP^+$ cells were significantly reduced in co-cultures with *Smad4* mutant osteoblasts compared with controls (264.4±12.6 per well with mutants vs 169.25±7.8 per well with controls, n=5, P<0.001), indicating that the osteoclastogenic activity of the *Smad4* mutant osteoblasts was significantly decreased (Fig. 6G-I). These data suggest that increased trabecular bone volume in aged *Smad4* mutant mice is probably due to the decreased bone resorption.

The observation that the number of osteoclasts was reduced in *Smad4* mutant mice prompted us to check the expression of components of the RANKL-OPG axis by RT-PCR. The

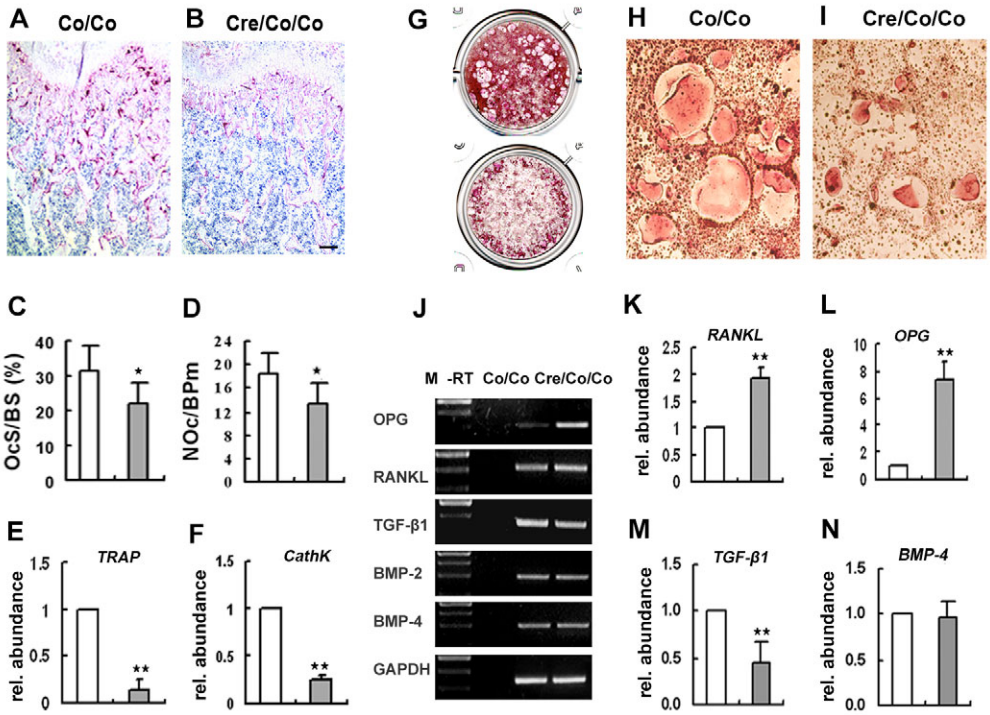

Fig. 6. Decreased bone resorption in *Smad4* mutant mice. (A,B) Representative images of TRAP-stained distal femur from 8-week-old control (A) and *Smad4* mutant mice (B). (C,D) Quantitative assessment by histomorphometric analysis of percentage of bone surface covered by mature osteoclasts (OcS/BS) and number of mature osteoclasts in bone perimeter (NOc/BPm) on the secondary spongiosa of distal femur in control (white bars) and *Smad4* mutants (grey bars). All values are mean ± s.d. of eight mice. (E,F) Real-time PCR of TRAP and cathepsin K (CathK) from calvarial bone extracts of 7-week-old *Smad4* mutant (grey bars) and control mice (white bars). (G-I) TRAP staining was performed following co-culture of osteoclast progenitor cells derived from the wild-type spleen with control (upper panel in G,H) or *Smad4* mutant (lower panel in G,I) osteoblast-like cells generated from bone marrow mesenchymal stem cells. (J) RT-PCR analysis to detect transcripts of OPG, RANKL, *Tgfb1*, *Bmp2* and *Bmp4* in calvarial bones of 7-week-old *Smad4* mutant and control mice. (K-N) Real-time PCR of OPG, RANKL, *Tgfb1* and *Bmp4* from calvarial bone extracts of 7-week-old *Smad4* mutant (grey bars) and control mice (white bars) **P*<0.05; ***P*<0.01. Bar, 100 μm (A,B).

transcripts of both RANKL and OPG genes were significantly increased in 7-week-old *Smad4* mutant calvaria (Fig. 6J). Real-time PCR showed that the expression of the RANKL gene was elevated twofold in the calvaria of *Smad4* mutants (Fig. 6K), whereas the expression of OPG was increased by a factor of seven (Fig. 6L). We also examined the expression of *Bmp2*, *Bmp4* and *Tgfb1*, which regulate coupling between osteoblasts and osteoclasts by RT-PCR and real-time PCR. The expression of *Tgfb1* was decreased (Fig. 6J,M), whereas changes in the expression levels of *Bmp2* and *Bmp4* were not significant (Fig. 6J,N and data not shown).

Discussion

Skeletal homeostasis is controlled by the intricate coordination of constituent cells including osteoblasts and osteoclasts (Karsenty and Wagner, 2002). The number and the function of cells present in the bone microenvironment determine skeletal homeostasis and are regulated by systemic hormones and local bone growth factors (Canalis et al., 2003). Ablation of *Smad4* in differentiated osteoblasts resulted in a growth retardation and decreased bone mass in young knockout mice. The *Smad4* mutants showed a decreased number and impaired function of osteoblasts, suggesting that Smad4 is required for control of osteoblast function and regulation of bone mass. Interestingly, the differentiated osteoblast specific disruption of *Smad4* resulted in an alteration of the RANKL/OPG axis and eventually led to decreased number and reduced activity of osteoclasts that caused higher trabecular bone volume in aged mutant mice. This indicates that responsiveness of osteoblasts to TGFβ signaling mediated by Smad4 plays an important role in the coupling of bone formation to bone resorption. Thus, the current study is the first direct evidence of an important function for Smad4 in maintaining bone homeostasis.

Previous studies have shown that the disruption of BMP signaling during embryogenesis affects skeletogenesis (Kingsley et al., 1992; Storm et al., 1994; Dudley et al., 1995; Luo et al., 1995; Thomas et al., 1997; Thomas et al., 1996; Katagiri et al., 1998; Solloway et al., 1998). Recently, tissue specific downregulation of BMP signals in mice has revealed an important function of BMP signaling in postnatal bone formation (Mishina et al., 2004). It is conceivable that the osteoblast-specific *Smad4*-knockout mutant mice largely copied the phenotypes observed in the osteoblast-specific *Bmpr1a*-knockout mutant mice and transgenic mice overexpressing a dominant-negative form of *Bmpr1b* in osteoblasts (Zhao et al., 2002; Mishina et al., 2004), given the fact that ablation of *Smad4* in osteoblasts impaired the responsiveness of osteoblasts to BMP2. Previous studies have shown that BMPs might stimulate osteoblast differentiation by inducing expression and activation of Runx2, and inhibition of BMP signaling disrupts the ability of Runx2 to stimulate osteoblast differentiation and gene expression (Chen et al., 1998; Zhao et al., 2002).

In this study, we showed that the *Smad4* mutant mice suffered from a decreased bone formation rate and a defect in bone mineralization, correlated with the downregulation of Runx2, providing in vivo evidence that Smad4 regulates osteoblast differentiation and maturation through Runx2. It has been shown recently that reduction of TGFβ enhanced the bone mass and the mechanical properties (Balooch et al., 2005). Although *Smad4* deletion also impaired the responsiveness of osteoblasts to TGFβ1, the phenotypes of the

Smad4 mutant mice were more similar to the mutants with reduced BMP signaling (Zhao et al., 2002; Wu et al., 2003; Mishina et al., 2004; Balooch et al., 2005) than those with diminished TGFβ signals (Balooch et al., 2005). This suggests that the differentiated osteoblasts at later stages might be less sensitive to TGFβ. Notably, in vitro reporter assays with TGFβ and BMP reporter-luciferase constructs revealed ~70% reduction in TGFβ and BMP responsiveness, suggesting that some Smad4-independent compensatory mechanisms might exist both in vitro and in vivo. Indeed, previous studies show that TGFβ signals regulate osteoblast differentiation and apoptosis through Smad-independent signaling pathways (Hay et al., 2001; Celil and Campbell, 2005).

We noted that ablation of *Smad4* in differentiated osteoblasts resulted in decreased proliferation and a reduced number of osteoblasts, which is different from the osteoblast-specific *Bmpr1a*-knockout mice which exhibit a normal number of osteoblasts (Mishina et al., 2004). This could be largely due to the downregulation of *Tgfb1* in *Smad4* mutant mice (Fig. 6J). Indeed, previous studies have shown that TGFβ1 increases the cell population that will differentiate into osteoblasts by inducing chemotaxis and proliferation (Janssens et al., 2005). The downregulation of TGFβ1 in *Smad4* mutants raised the possibility that Smad4 might mediate a positive feedback loop regulating TGFβ1 expression in osteoblasts.

It has been shown that TGFβ signals can function as coupling factors to maintain the bone formation and bone resorption and thus skeletal homeostasis (Harada and Rodan, 2003). A striking phenotype in osteoblast-specific *Smad4*-knockout mice is that the trabecular bone volume was significantly higher than that of controls at 7 months of age, because of reduced osteoclast bone resorption. This suggests that Smad4-mediated TGFβ signals in osteoblasts play an important role in the control of bone resorption. Previous studies have shown that TGFβ has both direct enabling effects on osteoclast formation (Fox et al., 2000; Fuller et al., 2000; Kaneda et al., 2000), and indirect effects through the regulation of osteoblastic RANKL and OPG expression (Murakami et al., 1998; Takai et al., 1998; Quinn et al., 2001; Thirunavukkarasu et al., 2001). Consistently, we found that deletion of *Smad4* in osteoblasts downregulated the expression of TGFβ1 and altered the RANKL/OPG ratio, and eventually caused a reduced rate of osteoclast differentiation. It is quite unexpected that the expression of OPG is greatly upregulated in *Smad4* mutant mice, because both BMP and TGFβ have been shown to be able to increase OPG production in vitro (Thirunavukkarasu et al., 2001). A recent study has suggested that in vitro effects of TGFβ1 on the RANKL-OPG axis and differentiation of osteoclasts depend greatly on TGFβ1 concentration (Karst et al., 2004). Our results indicated that the in vivo effects of TGFβ on RANKL-OPG axis expression are much more complicated than we appreciated. Nevertheless, the downregulation of TGFβ1 and the shift from the RANKL-OPG axis in favor of OPG suppressed osteoclast formation and prevented excessive bone loss.

Materials and Methods

Establishment of the osteoblast-specific *Smad4*-knockout mice

Mice that were homozygous for the floxed *Smad4* allele ($Smad4^{Co/Co}$) (Yang et al., 2002) were bred with transgenic mice in which the 1.3 kb mouse OC promoter (Dacquin et al., 2002) controls Cre recombinase expression (*OC-Cre*) to generate $Smad4^{Co/+}$;*OC-Cre* mice. $Smad4^{Co/Co}$;*OC-Cre* mice were obtained by breeding the $Smad4^{Co/+}$;*OC-Cre* mice with $Smad4^{Co/Co}$ mice. For routine genotyping, the *Smad4* locus and the *Cre* transgene were detected by PCR using primers described previously (Yang et al., 2005; Yang et al., 2002).

Staining for LacZ activity

To determine the specificity of Cre-mediated recombination, *OC-Cre* transgenic mice were bred with *ROSA26* reporter mice (Soriano, 1999). The bones were stained as previously described (Yang et al., 2005). Osteoblasts derived from the bone marrow cells of double heterozygous or control mice were also stained with X-gal as previously described (Yang et al., 2005).

Skeletal radiography

Radiographic analyses were carried out using a soft X-ray system (Contour Plus). BMD was measured from femurs by dual energy X-ray absorptiometry with a Piximus Mouse Densitometer (GE Lunar Medical System).

Histological and histomorphometric analyses

Tissues were fixed in 4% paraformaldehyde at 4°C overnight, decalcified in 5% EDTA-PBS and embedded in paraffin. The sections were stained with hematoxylin-eosin and TRAP by standard methods. For in vivo fluorescent labeling, 4-week-old animals were injected with calcein (20 mg/kg body weight) intraperitoneally at day 6 and day 3, whereas 8-week-old mice were injected at day 12 and day 2 before sacrifice. 5 μm plastic sections were stained using the modified Goldner trichrome technique (Gruber, 1992) and sections serial to the stained sections were left unstained for fluorescent analyses. An image analysis system (Osteometrics) was used for all histomorphometric analysis. Parameters for the trabecular bone were measured in an area 1.8 mm in length from 0.3 mm below the growth plate of the distal femur. For 11-month-old female mice, bone histomorphometric analysis was performed in an area 1.5 mm in length under the growth plate of the proximal tibias. All histomorphometric parameters are reported in accordance with the ASBMR nomenclature (Parfitt et al., 1987).

Primary cell culture

Calvarial cells from 3-day-old neonatal mice were established as previously described (Zhao et al., 2002). Bone marrow cells were inoculated at a density of 1.5×10^6 cells/well in 24-well plates, 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate were added at day 3 of culture. Osteoblast-osteoclast co-culture experiments were performed as described (Cao et al., 2003). Briefly, bone marrow cells were incubated for 7 days in differentiation medium and the osteoblast-like cells were plated at 2×10^4 per well in 24-well plates. Upon confluency, non-adherent monocyte/macrophage progenitor cells derived from wild-type spleen (5×10^5 per well) were then plated, and the cultures were complemented with 10^{-8} mol/L 1,25-dihydroxy vitamin D_3. TRAP staining was performed after 5 days of culture.

BrdU labeling

Mice at 3 weeks of age were injected intraperitoneally with 10 μl of 10 mM BrdU in PBS per gram of body weight, and sacrificed 24 hours later (Zhang et al., 2005). Calvarial sections were detected with an anti-BrdU antibody (Sigma). All BrdU-positive (brown) nuclei in an area of 0.3 mm in length from the sagittal suture were counted using Osteomeasure software. Five animals per genotype were used and statistical differences between groups were assessed using the Student's *t*-test.

Transient transfection and luciferase assay

The reporter construct, *12×SBE-OC-Luc* (Zhao et al., 2004) or *p3TP-Lux* (Chen et al., 1996) together with *pCMV-Cre* and *Renilla* control plasmids were co-transfected in osteoblasts isolated from calvaria of $Smad4^{Co/Co}$ mice using lipofectamine 2000 (GIBCO BRL). 24 hours later, the medium was replaced by fresh medium containing 1% FCS in the presence or absence of 50 ng/ml BMP2 or 5 ng/ml TGFβ1 (R&D Systems). Cells were lysed 48 hours after transfection, and firefly luciferase activity was assayed using a dual luciferase reporter assay system (Promega) and normalized by *Renilla* luciferase activity. Each experiment was performed using triplicate data points and performed three times.

In situ hybridization

In situ hybridization was performed using standard procedures. Probes were labeled with [^{35}S]UTP using the MAXIscript in vitro transcription kit (Ambion). Slides were dipped in emulsion (Amersham Pharmacia) and exposed for 2-10 days before developing.

Real-time PCR

Total RNA was isolated from calvarial bone of 7-week-old mice using the TRizol reagent (Invitrogen) according to the manufacturer's instructions. 5 μg total RNA was reverse transcribed to cDNA with the use of the first-strand cDNA synthesis kit (Invitrogen). Real-time PCR was performed using the LightCycler system (Roche) with the FastStart DNA Master SYBR Green. The standard curve method of quantification was used to calculate the expression of target genes relative to the housekeeping gene *Gapdh*. The wild-type expression level was set to 1 as described

previously (Glass, 2nd et al., 2005). Experiments were repeated at least three times. The following primers were used: OC, 5′-ACCCTGGCTGCGCTCTGTCTCT-3′ and 5′-GATGCGTTTGTAGGCGGTCTTCA-3′; *Col1a2*, 5′-CAGCGAAGAACTCATACAGCC-3′ and 5′-TTGGAGCAGCCATCGACTA-3′; *Akp1*, 5′-AGGGCAATGAGG TCACATCC-3′ and 5′-GCATCTCGTTATCCGAGTACCAG-3′; TRAP, 5′-AGACCCAGACCCTGAACACC-3′ and 5′-CGCCCAAGAAAGCTCTACCTAA-3′; *Ctsk*, 5′-CACGGCAAAGGCAGCTAAAT-3′ and 5′-CCATAGCCCACCACCAACAC-3′; OPG, 5′-ACGGACAGCTGGCACACCAG-3′ and 5′-CTCACACACTCGGTTGTGGG-3′; RANKL, 5′-CATGACGTTAAGCAACGG-3′ and 5′-AGGGAAGGGTTGGACA-3′; *Lrp5*, 5′-ATTGAAAGGGTCCACAAGGTC-3′ and 5′-GATAGCCACATCGTTGTTGTTAG T-3′; β-catenin, 5′-GCCATCTGTGCTCTTCGTC-3′ and 5′-ACACCCTTCTACTATCTCCTCC-3′; *Lef1*, 5′-TTCAGGTACAGGTCCCAGAATG-3′ and 5′-AGTCGGCGCTTGCAGTAGA-3′; *Dkk2*, 5′-CAGGGTAAACAATCAGTAGTCC-3′ and 5′-CAATGCCATTCCTTCACAA3′; *Gapdh*, 5′-TGCCCAGAACATCATCCCT-3′ and 5′-GGTCCTCAGTGTAGCCCAAG-3′. The primer pairs for *Tgfb1*, *Bmp4* and *Runx2* have been described previously (De Ranieri et al., 2005).

Statistical analysis

All results were expressed as mean ± s.d. All statistical analyses were performed using the SPSS software package for Windows release 11.0. $P<0.05$ was considered significant.

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