Embryonic ablation of osteoblast Smad4 interrupts matrix synthesis in response to canonical Wnt signaling and causes an osteogenesis-imperfecta-like phenotype

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Summary
To examine interactions between bone morphogenic protein (BMP) and canonical Wnt signaling during skeletal growth, we ablated Smad4, a key component of the TGF-β–BMP pathway, in Osx1+ cells in mice. We show that loss of Smad4 causes stunted growth, spontaneous fractures and a combination of features seen in osteogenesis imperfecta, cleidocranial dysplasia and Wnt-deficiency syndromes. Bones of Smad4 mutant mice exhibited markers of fully differentiated osteoblasts but lacked multiple collagen-processing enzymes, including lysyl oxidase (Lox), a BMP2-responsive gene regulated by Smad4 and Runx2. Accordingly, the collagen matrix in Smad4 mutants was disorganized, but also hypomineralized. Primary osteoblasts from these mutants failed to mineralize in vitro in the presence of BMP2 or Wnt3a, and Smad4 mutant mice failed to accrue new bone following systemic inhibition of the Dickkopf homolog Dkk1. Consistent with impaired biological responses to canonical Wnt, ablation of Smad4 causes cleavage of β-catenin and depletion of the low density lipoprotein receptor Lrp5, subsequent to increased caspase-3 activity and apoptosis. In summary, Smad4 regulates maturation of skeletal collagen and osteoblast survival, and is required for matrix-forming responses to both BMP2 and canonical Wnt.

Key words: Bone, Collagen, Osteoblast, Smad4, BMP, Wnt, β-catenin

Introduction
Skeletal development and growth (bone modeling) depends upon the proliferation and differentiation of chondrocytes, the resorptive activity of osteoclasts, and the bone-forming function of osteoblasts. The coordinated actions of osteoblasts and osteoclasts further function to replace old bone in the mature skeleton (bone remodeling). Bone modeling and remodeling are controlled by molecular programs modulating proliferation, differentiation, function and survival of skeletal cells. Two key regulators of skeletal development and homeostasis are the bone morphogenic protein (BMP) and Wnt signaling systems. BMP2 and BMP4 are secreted ligands which are together necessary (Bandyopadhyay et al., 2006) and each sufficient (Kang et al., 2004; Wozney et al., 1988) to drive de novo bone formation by osteoblasts. Osteoblast-specific ablation of Bmpr1a or the downstream transcription factor Smad4 decreases both bone formation and resorption (Kamiya et al., 2008a; Kamiya et al., 2008b; Mishina et al., 2004; Tan et al., 2007). Wnt ligands are secreted factors that signal through heteromeric receptor complexes comprising a Frizzled receptor and one of the low density lipoprotein receptors Lrp5 or Lrp6 (Nusse, 2005). Gain- or loss-of-function mutations of LRP5 in humans result in high or low bone mass syndromes, respectively (Boyden et al., 2002; Gong et al., 2001; Little et al., 2002). In mice, the osteogenic effects of Lrp5 or Lrp6 activity derive largely, though not exclusively from β-catenin, which acts cell autonomously to specify osteoblast cell fate (Day et al., 2005; Hill et al., 2005; Ross et al., 2000), enhance osteoprogenitor proliferation (Rodda and McMahon, 2006), drive osteoblast differentiation and new bone formation (Hu et al., 2005; Rodda and McMahon, 2006), and regulate bone resorption through paracrine effects on osteoclasts (Glass et al., 2005).

Complex and sometimes contradictory interactions between BMP and Wnt/β-catenin signaling have been reported in the skeletal system. For example, BMPs can act upstream of canonical Wnt (Bain et al., 2003; Rawadi et al., 2003) in a manner that requires β-catenin (Chen et al., 2007; Hill et al., 2005). However, we and others have shown that osteogenic activity of β-catenin is suppressed by blocking BMP2 or BMP4, implying instead that BMPs act downstream of Wnt, or that the two pathways act cooperatively (Salazar et al., 2008; Winkler et al., 2005). Thus, simple epistatic models do not satisfactorily explain how BMP and Wnt/β-catenin signaling interact to drive osteogenesis. To further our understanding of the molecular mechanisms underlying BMP and Wnt/β-catenin interactions for skeletal growth and homeostasis, we generated mice with conditional ablation of Smad4, a key component of the greater TGF-β/BMP signaling system.
Using Osx1-Cre to drive Smad4 ablation in differentiating osteoblasts we find, somewhat unexpectedly, far more severe skeletal abnormalities than previously reported with ablation restricted to mature osteoblasts (Tan et al., 2007). Loss of Smad4 in Osx1+ cells causes a pleiotropic phenotype consisting of a combination of features seen in Wnt-deficiency syndromes (Gong et al., 2001; Gong et al., 1996), cleidocranial dysplasia (Otto et al., 1997) and osteogenesis imperfecta (Forlino et al., 2011). This phenotype is caused by production of an abnormal collagen bone matrix and increased apoptosis in Smad4-deficient osteoblasts. We also find that Smad4 deficiency renders bone cells resistant to matrix-mineralizing effects of the canonical Wnt pathway. Our study uncovers novel interactions between Smad4 with the Wnt/β-catenin system and Runx2, positioning Smad4 as a key modulator of multiple signaling pathways that control bone mass through the function of bone-forming cells.

Results
Severe growth retardation and spontaneous fracture in mice with deficiency of Smad4 in Osx1+ cells
Because pilot studies revealed that Osx1-driven Smad4 ablation results in a far more severe phenotype than previously reported for a more restricted osteoblast Smad4 deletion (Tan et al., 2007), an in-depth characterization of the skeletal phenotype was performed. At birth, Smad4+/D pups were slightly smaller than Smad4F/F (Fig. 1A), but were severely runted by postnatal day (P)28 (Fig. 1B). About 50% of Smad4D/D pups died by P14, and almost none survived to 8 weeks. Early lethality was not alleviated by access to paste-formula food or by keeping Smad4D/D pups with the mother. Smad4+/D and Smad4D/D mice developed oral malocclusion (Fig. 1C,D), a trait observed in Osx1-Cre hemizygous mice (http://jaxmice.jax.org/strain/006361.html). However, dental abnormalities were far more severe in Smad4D/D mice, which exhibited yellow or even black dental discoloration typical of enamel hypoplasia (Fig. 1D). On whole-body 3D high-resolution microcomputed tomography (µCT) reconstructions at P28, Smad4D/D mice exhibited underdeveloped incisors (Fig. 1E, blue asterisk). And despite the presence of intact skeletal tissues observed during necropsy (Fig. 5D), µCT further revealed that the craniofacial and axial skeleton were severely under mineralized (Fig. 1E, red asterisks). Rib number was normal but the thoracic cavity was small. Multiple rib fractures, some with callus formation, were evident on plain radiographs at 8 weeks of age (Fig. 1F, red asterisks).

In the appendicular skeleton, Smad4D/D tibiae were small but morphologically normal (Fig. 2A,B). Trabecular bone, which is normally restricted to primary and secondary ossification centers, populated the entire diaphysis of Smad4D/D bones (Fig. 2B). In Smad4D/D mice at P28, tibias were 22.1±7.4% shorter (Fig. 2C), and bone mineral density (calculated as mineral content per unit volume of cortical bone) was reduced 20±3.8% relative to Smad4+/D mice (Fig. 2D). Trabecular bone volume as a function of trabecular tissue volume (BV/TV) in the metaphysis (first 30 slices below the growth plate) was not different between genotypes (Fig. 2E); but, when trabecular bone was instead quantified from the growth plate to the tibia-fibular junction, BV/TV was almost threefold higher in Smad4D/D than in Smad4+/D or Smad4+/D mice (Fig. 2F). Thus, trabecular architecture at the metaphysis is appropriate for the smaller bone size of Smad4D/D mice, but their diaphyseal medullary space is inappropriately

![Fig. 1. Skeletal phenotype of Smad4+/D; Osx1-Cre Mice.](image-url)
populated by an excessive amount of trabecular bone. Increased diaphyseal trabeculation was observed when deleting Smad4 in Bglap+ cells, and this was attributed to decreased osteoclastogenesis (Tan et al., 2007). Indeed, TRAP-positive osteoclasts were very sparse on the residual trabeculae, endocortical and periosteal surfaces of mutant Smad4D/D bones (supplementary material Fig. S3). Smad4D/D tibias were narrow at the mid-diaphysis with total cross-sectional tissue area reduced 43.6% relative to Smad4F/F and modestly reduced in Smad4+/D mice (Fig. 2G). Relative to Smad4F/F, cortical thickness (calculated as the percentage of total diaphyseal tissue area occupied by cortical bone) was also decreased in Smad4+/D tibias (15.1%, Fig. 2H). Cortical thickness was 23% higher in Smad4+/-, but this was the only abnormality evident in Smad4+/D mice. Notably, trabecular structures in the diaphysis of Smad4+/D tibiae were Safranin O negative and Fast Green positive (Fig. 2I), thus they are bone and not cartilage left behind during resorption of endochondral cartilage template. Goldner trichrome stain revealed that Smad4+/D cortical bone was primarily woven rather than lamellar (Fig. 2J). And, as evident from hematoxylin and eosin staining (Fig. 2K), osteocyte density was significantly increased in the cortex of Smad4+/D relative to Smad4+/F bones, whereas no differences were seen in cancellous bone (Fig. 2L).

The Osx1-Cre transgene has been reported to cause skeletal abnormalities (Davey et al., 2012). At 4 weeks of age, Osx1-Cre tibiae were morphologically normal (supplementary material Fig. S2A,B) with a modest (~7%) decrease in length relative to wild-type littermates (supplementary material Fig. S2C). Bone mineral density measured by μCT was not altered (supplementary material Fig. S2D), and trabeculation was restricted to primary and secondary ossification centers as in wild-type mice (supplementary material Fig. S2B). Trabecular bone volume (BV/TV) in the metaphysis (first 30 slices below the growth plate) was reduced by ~36% in Osx1-Cre bones, although this did not reach statistical significance (supplementary material Fig. S2E). However, Osx1-Cre tibiae were about 25% narrower at the mid-diaphysis relative to wild-type mice (supplementary material Fig. S2F) and cortical thickness was increased by about 14.5% (supplementary material Fig. S2G). These changes are inconsistent with the more severe phenotype evident in Smad4+/D mice.

Smad4 modulates osteoblast responses to BMP and TGF-β
loss of Smad4 in Osxl+ cells had no effect on the abundance of R-Smad mRNAs in marrow-free bone extracts (Fig. 3A); however, it sharply decreased the abundance of phosphorylated Smad1, Smad5 and Smad2 proteins (Fig. 3B). Smad3
phosphorylation was below detection in all genotypes (not shown). Total Smad1 and Smad2, but not Smad3 or Smad5 proteins, were sharply reduced in Smad4<sup>F/F</sup> bones (Fig. 3B) despite normal steady-state mRNA levels (Fig. 3A). Because both Smad1–Smad5 and Smad2–Smad3 signaling were affected by loss of Smad4, primary calvaria cells from neonatal mice were used to determine how Smad4 regulates osteoblast differentiation, proliferation and survival in response to BMP2 or TGF-β. BMP2 increased BrdU incorporation by about 30%, and this effect was enhanced fourfold in the absence of Smad4, whereas TGF-β did not affect calvaria cell proliferation (Fig. 3C). Whereas basal levels of tissue non-specific alkaline phosphatase (ALP) activity were similar in Smad4<sup>F/F</sup> and Smad4<sup>A/A</sup> calvaria cells, the response to BMP2 stimulation of ALP activity was reduced about 50% in Smad4<sup>A/A</sup> cells. By contrast, ALP activity was inhibited about 70% by TGF-β in Smad4<sup>F/F</sup> cells and about 90% in Smad4<sup>A/A</sup> cells (Fig. 3D). In calvaria cells, TGF-β and BMP2 both reduced the number of TUNEL<sup>+</sup> and DAPI<sup>+</sup> nuclei (about 40 and 60%, respectively), which act as markers of apoptosis. Although loss of Smad4 alone had no effect on <em>in vitro</em> apoptosis, it abolished the pro-survival effect of BMP2 and reduced that of TGF-β by ~50% (Fig. 3E).

### Abnormal collagen matrix and collagen-modifying enzymes in Smad4<sup>A/A</sup> bones

Growth retardation, hypomineralization of bone and teeth, propensity to fracture and increased osteocyte density are hallmarks of osteogenesis imperfecta (Forlino et al., 2011), raising the possibility that Smad4<sup>A/A</sup> osteoblasts either do not differentiate or that they do mature but are unable to secrete a normal bone matrix. Despite the report of an osteoblast differentiation defect upon Bglap-driven ablation of Smad4 (Tan et al., 2007), we found that mRNA levels of early osteoblast genes, Runx2 and Osx1, as well as genes prevalently expressed in osteocytes, Dmp1 and Phex were unaltered in Smad4<sup>A/A</sup> bones (Fig. 4A). Likewise, genes involved in osteoblast maturation and bone matrix production, such as <em>Alp1</em> (tissue non-specific alkaline phosphatase), <em>Spp1</em> (osteopontin) and <em>Ibsp</em> (bone sialoprotein) were also unaffected <em>in vivo</em> by Smad4 deletion. Expression of both <em>Col1A1</em> and <em>Bglap</em> (osteocalcin) was 32% lower in Smad4<sup>A/A</sup> relative to Smad4<sup>F/F</sup>, although the difference did not reach statistical significance (Fig. 4B). Nonetheless, histologic sections of Smad4<sup>A/A</sup> tibiae from 6-week-old littermate mice stained with Picrosirius Red and examined under polarized light emitted a red/yellow light (Fig. 4Ciii,iv) compared with the prevalently green color emitted by trabecular and cortical bone of Smad4<sup>F/F</sup> (Fig. 4Ci,ii), indicating that thickness or packing density of collagen fibers is abnormal (Dayan et al., 1989). Together, these data indicate that differentiation of Smad4-deficient osteoblasts was delayed (Fig. 3D), but not blocked (Fig. 4A,B), and furthermore, that Smad4-deficient osteoblasts did not function properly, depositing abnormal collagen into the skeletal extracellular matrix. Accordingly, three collagen-processing enzymes, prolyl 4-hydroxylase, alpha subunit III (<em>P4Ha3</em>), procollagen-lysine-2-oxoglutarate 5-dioxygenase 3 (<em>Plod3</em>) and lysyl oxidase (<em>Lox</em>) were sharply downregulated in Smad4<sup>A/A</sup> mice (Fig. 4D). Plod3 and P4Ha3 are required for collagen triple helix formation, and Lox mediates covalent crosslinking between collagen fibrils, a crucial step required for the structural integrity of a collagen matrix (Trackman, 2005).

### Smad4 and Runx2 regulate Lox transcription

Among the three collagen-processing enzymes noted above, only <em>Lox</em> mRNA was acutely upregulated in MC3T3s by overnight stimulation with BMP2; both <em>Plod3</em> and <em>P4Ha3</em> were significantly upregulated by TGF-β, although the induction was modest (supplementary material Figs S4A–C). Thus, we narrowed our focus to <em>Lox</em>. <em>In vivo</em>, Lox protein was significantly diminished in conditional heterozygous Smad4<sup>A/A</sup> bones, and it was almost undetectable in Smad4<sup>A/A</sup> bones. Osterix and β-actin were unchanged across genotypes (Fig. 5A). To determine whether...
induction of \textit{Lox} by BMP2 is dependent on \textit{Smad4}, we transfected MC3T3 calvaria cells with \textit{Smad4} siRNA, which reduced \textit{Smad4} expression \textasciitilde70\% (Fig. 5B) and inhibited BMP2 responsiveness of a luciferase reporter containing 12 tandem \textit{Smad}-binding elements (\textit{Smad}-luciferase; supplementary material Fig. S4D). Both basal and BMP2-stimulated (\textasciitilde5.5-fold) \textit{Lox} mRNA expression was significantly inhibited (about 50\% and 80\%, respectively) by \textit{Smad4} siRNA (Fig. 5B). Because the murine \textit{Lox} promoter contains \textasciitilde9 putative \textit{Smad}-binding elements (supplementary material Fig. S5), we determined whether \textit{Smad4} binds to the \textit{Lox} promoter by performing chromatin immunoprecipitation (ChIP) on MC3T3 nuclear extracts. Using qPCR primers targeted to the \textasciitilde252 bp 5' region of the \textit{Lox} promoter, we found that \textit{Smad4} antibody
precipitated 99-times more Lox promoter fragments from the chromatin of BMP2-treated cells relative to untreated cells (Fig. 5C), demonstrating that Smad4 is recruited to the Lox promoter in response to BMP2.

Intriguingly, Smad4+/−/ mice exhibited hypomineralized interparietal bones and clavicle hypoplasia, features evident at birth by whole-mount skeletal preparations (Fig. 5D) and by μCT of 4-week-old littersmates (supplementary material Fig. S4E). This combination of features is a hallmark of cleidocranial dysplasia, a condition linked to Runx2 haploinsufficiency (Mundlos et al., 1997; Otto et al., 1997). Because Runx2 mRNA was expressed at normal levels in Smad4+/−/ bones (Fig. 4A), we considered the possibility that at least part of the phenotype of Smad4+/−/ mice arises from disruption of Runx2 transcriptional activity. In fact, the Lox promoter contains ≥8 putative Runx2-binding elements interspersed with Smad4 binding elements (supplementary material Fig. S5); thus, we tested whether BMP2 acts through both Smad4 and Runx2 to regulate expression of Lox in osteoblasts. We first used the OSE2-Luc reporter, which contains six Runx2 responsive elements (Ducy and Karsenty, 1995) to demonstrate that Runx2 transcriptional activity in MC3T3s is sensitive to BMP2 (supplementary material Fig. S4F). qPCR showed that Runx2 mRNA is upregulated ~twofold in MC3T3s by BMP2. We decreased Runx2 mRNA ~53% in control cells and ~71% in BMP2-treated cells by treating with Runx2 siRNA. Runx2 siRNA diminished steady-state levels of Lox mRNA by ~42%, and attenuated BMP2 stimulation of Lox expression by ~80% (Fig. 5E). In ChIP assays, Runx2 antibody precipitated ~55-times more Lox promoter fragments from nuclear extracts of BMP2-treated cells than from untreated cells (Fig. 5F). Neither Smad4 nor Runx2 antibodies immunoprecipitated promoter fragments of Rpl30, a control gene promoter (supplementary material Fig. S4G). Therefore, both Smad4 and Runx2 are recruited to the Lox promoter in response to BMP2. Loss of either Smad4 or Runx2 is sufficient to impair Lox expression upon BMP2 stimulation.

**Smad4 maintains the mineralizing response to canonical Wnt signaling**

Although defective collagen processing can explain some of the osteogenesis-imperfecta-like features, it is probably not the only cause of the severe skeletal growth and delayed ossification defects present in Smad4+/−/ mice. As noted, the BMP and Wnt/β-catenin pathways intersect at different levels in bone cells (Chen et al., 2007; Rawadi et al., 2003), and we and others find that BMP2 and BMP4 signaling is permissive for the osteogenic effect of β-catenin (Salazar et al., 2008; Winkler et al., 2005). Hence, we tested whether loss of Smad4 affects how osteoblasts respond to Wnt/β-catenin signaling. Consistent with normal expression of Alp1 in bones from 4-week-old Smad4+/−/ mice (Fig. 4B), bone marrow stromal cells (BMSCs) from Smad4+/−/ mice developed normal levels of alkaline phosphatase activity when grown for 28 days in osteogenic medium; but, in contrast to Smad4+/F cells, no further increase was observed after exposure to Wnt3a in the mutant cells (Fig. 6A). More to the point, whereas BMP2 and Wnt3a potentiated stimulation of mineralized matrix in control BMSCs, no such response was observed in Smad4+/−/ cells in the presence of either factor (Fig. 6B). To determine whether insensitivity of Smad4+/−/ cells to Wnt3a reflects in vivo resistance to Wnt signaling, we used a Dkk1 neutralizing antibody (zDkk1) that has been shown to induce bone formation when given systemically to mice with rheumatoid arthritis (Diarra et al., 2007). Treatment with zDkk1 (20 mg/kg/day, i.p. three times/week, 4 weeks) produced about 40% and 50% increments of trabecular bone mass in female Smad4+/F and Smad4+/−/ mice, respectively, whereas no accrual of bone mass occurred in one Smad4+/−/ female that survived the 4 week treatment to 3 months of age (Fig. 6C). Abundant double calcein labels were present in Smad4+/F tibiae after zDkk1 treatment (Fig. 6Ei,ii), whereas only a few labeled surfaces with

**Fig. 6. Smad4 is required for mineralizing responses to BMP2 and Wnt3a.** (A) Alkaline phosphatase activity and (B) Alizarin-Red-positive nodules in bone marrow stromal cells cultured for (A) 28 days or (B) 10 days in osteogenic medium (OM) ± BMP2 (200 ng/ml) or Wnt3a (25 ng/ml). (C) Effect of anti-Dkk1 (20 mg/kg/day, i.p. three times/week, 4 weeks) on trabecular bone mass at the proximal tibia of female mice, assessed before and after treatment by in vivo μCT. n=7 for Smad4+/F, n=6 for Smad4+/−/ and n=1 for Smad4−/−. Data are means ± s.e.m., where brackets indicate P<0.05. (D) Goldner tetrachrome stain and (E) calcein labeling on serial sections of the tibia from Smad4+/F (left) or Smad4+/−/ (right) mice after 4 weeks of anti-Dkk1 treatment. (i) and (iii) are 4× magnification; (ii) and (iv) are 40× magnification, representing boxed areas in (i) and (iii). Open arrowheads indicate endosteal surface, closed arrowheads show periosteal surface. Note lack of second calcein label on endosteal surface of Smad4+/−/ [open arrowhead in (iv)].
no double labels were observed in the Smad4^+/A tibia (Fig. 6Eiii,iv). Goldner trichrome stain was used for orientation on serial sections (Fig. 6D). This was recapitulated in a pair of male Smad4^+/A and Smad4^F/F littermate mice (supplementary material Fig. S6).

To understand the molecular mechanisms of Wnt resistance in Smad4^+/A mice and cells, components of the Wnt pathway were analyzed by immunoblot on marrow-free bone extracts (Fig. 7A). Lrp5 was barely detectable in Smad4^+/A relative to Smad4^F/F and Smad4^+/+ bones, whereas Lrp6 abundance was normal. Total and phospho-GSK3β were unaffected by loss of Smad4, as was phosphorylation of GSK3β-target residues on β-catenin. However, an antibody against the β-catenin N-terminus revealed fainter immunoreactive bands (82–115 kDa) in Smad4^+/A relative to Smad4^F/F and Smad4^+/+ bone extracts. Likewise, multiple bands were also revealed in Smad4^F/F and Smad4^+/A bone using an antibody recognizing the C-terminus of β-catenin; however, these bands were barely detectable in the Smad4^+/A lane, where a faster migrating band became far stronger (Fig. 7A). Truncation of β-catenin C-terminus deactivates its transcriptional activity (Cong et al., 2003); indeed, mRNAs of the β-catenin target genes EphB4 (Batlle et al., 2002) and Ccnd1 (Tetsu and McCormick, 1999) were downregulated, 70% and 50%, respectively in Smad4^+/A bone. However, the abundance of mRNAs for Lrp5, Lrp6 and Catnb were unaffected by Smad4 genotype (Fig. 7B). The discrepancy between mRNA and protein data suggests that embryonic ablation of Smad4 triggers a GSK3β-independent mechanism of β-catenin degradation. As shown in Fig. 7C, two Caspase-3 cleavage sites reside within the C-terminal transactivation domain of β-catenin; three sites are in the N-terminal regulatory domain (Hunter et al., 2001). Indeed, Smad4^+/A bones contain high levels of activated (cleaved) Caspase-3 and Caspase-3, relative to Smad4^+/F and Smad4^+/F bone. Substantiating increased caspase activity, cleaved PARP, a known substrate of Caspase-3, was far more abundant Smad4^+/A bones relative to controls (Fig. 7D). Consistent with the earlier observation (Fig. 7A), when intact femurs from 4-week-old Smad4^+/A male mice are cultured ex vivo in osteogenic medium, the abundance of full-length β-catenin was decreased relative to Smad4^+/F bone, whereas the abundance of faster-migrating bands increased. Importantly, the intensity of these slower-migrating bands reactive to the C-terminus β-catenin antibody was significantly reduced in the presence of the caspase-3 inhibitor, Z-DEVD-fmk (Fig. 7E, red arrowhead), suggesting these are in fact cleavage products of activated Caspase-3. In vivo, there were abundant TUNEL+ osteoblasts and osteocytes in focal aggregates on the diaphysis of Smad4^+/A bones (Fig. 7F).
vitro, exposure to Wnt3a had no effect on the percentage of Smad4−/− BMSC that underwent apoptosis after serum deprivation. However, Wnt3a increased the percentage of TUNEL+ Smad4−/− BMSC to almost 50% (Fig. 7G), providing a likely explanation as to why Smad4−/− BMSCs differentiated for 2–4 weeks in the presence of Wnt3a appeared less dense, despite being initially seeded at peak confluence and in equal number to Smad4+/− cells (Fig. 6A).

**Discussion**

This work reveals novel and unexpected functions of Smad4 in bone. We show that Smad4 is necessary in Osx+ cells for skeletal development, longitudinal bone growth and production of normal bone matrix. These diverse functions of Smad4 are linked to its ability to interface with Runx2 and to regulate canonical Wnt signaling. Specifically, loss of Smad4 in Osx+ bone cells disrupts expression of an essential Runx2 gene target and causes β-catenin cleavage associated with caspase-3 activation.

The phenotype resulting from Smad4 ablation in Osx+ cells represents a combination of features present in different genetic disorders. Severe growth defects, propensity to fracture, dental enamel defects, fibrillar collagen abnormalities and increased osteocyte density are features of osteogenesis imperfecta (Forlino et al., 2011). Although Col1A1 mutations are responsible for most cases of the disease, absence of collagen-processing enzymes has been linked to recessive forms (Forlino et al., 2011). P4Ha3, Plod3 and Lox are all severely deficient in Smad4−/− mice. Although none of these genes has been linked to osteogenesis imperfecta, Plod3 mutations have been found in subjects with low bone mass and features of Ehlers-Danlos syndrome, a condition also characterized by connective tissue matrix defects (Salo et al., 2008). Furthermore, Ehlers-Danlos type VI, with kyphoscoliosis and tissue fragility, is caused by loss-of-function mutations of Plod1 (Beighton et al., 1998). Hence, it is conceivable that the compound deficiency of multiple collagen-processing enzymes accounts for the osteogenesis-imperfecta- and Ehlers-Danlos-like features of Smad4−/− mice. Notably, Lox-null mice exhibit normal skeletal patterning at birth; however, the skeleton is extremely fragile, collagen fiber size is altered, and Lox-null osteoblasts do not mineralize well (Pischon et al., 2009), conditions also present in Smad4−/− mice. Because Lox-null mice die perinatally, it is not yet clear whether other lysyl-oxidase-like enzymes can compensate for Lox, or whether Lox deficiency alone can retard growth (Nishioka et al., 2012). Interestingly, the growth defect in Smad4−/− mice is far more severe than reported in Smad4−/− mice (Tan et al., 2007). Since Osx-Cre has been shown to recombine in prehypertrophic chondrocytes of the growth plate (Maes et al., 2010), the longitudinal growth defect in Smad4−/− mice is likely to be the result of Smad4 ablation in the growth plate (Retting et al., 2009; Zhang et al., 2005). Indeed, BrdU histology reveals a more than 90% decrease in proliferation of growth plate chondrocytes of 4-week-old Smad4−/− mice relative to Smad4+/− littersmates (data not shown).

We focused on Lox to study the molecular action of Smad4 in osteoblasts because Lox is acutely regulated by BMP2, is a key enzyme of the collagen biosynthetic pathway and collagen abnormalities represent a novel feature of Smad4 deficiency. We found that Lox is regulated directly by Smad4 and Runx2, and that each transcription factor is required but not sufficient in the absence of the other for induction of Lox in osteoblasts by BMP2. Thus, Smad4 and Runx2 act cooperatively to regulate Lox transcription. Consistent with the contribution of Runx2 to Smad4 activity, Smad4−/− mice also exhibit skeletal features reminiscent of cleidocranial dysplasia (skull hypomineralization, clavicle hypoplasia), a condition caused by Runx2 haploinsufficiency (Otto et al., 1997). Such features are not present in TGF-β mutant mice, but are reported in mice with osteoblast-specific ablation of BMP2 and BMP4 (Bandyopadhyay et al., 2006) or TAK1 and p38 (Greenblatt et al., 2010), a pathway used by BMPs to activate Runx2 transcriptional activity. Altogether, these data support the notion that BMP2 and BMP4 act through Smad4 and Runx2 to regulate collagen crosslinking during skeletal development.

We also made the novel observation that Smad4 is required for the mineralizing stimulus of canonical Wnt signaling. Interactions between BMP and Wnt/β-catenin pathways have been reported, sometimes with divergent findings (Bain et al., 2003; Rawadi et al., 2003; Salazar et al., 2008; Winkler et al., 2005). Our earlier data show that BMP signaling is required for induction of osteoblast differentiation and mineralization by β-catenin (Salazar et al., 2008). Here, Smad4−/− osteoblasts failed to mineralize in response to not only BMP2, but also Wnt3a; and Smad4−/− mice failed to activate bone formation in response to systemic Wnt activation by Dkk1 antibody. The mechanisms for such insensitivity of Smad4-deficient cells and animals to Wnt are complex, but the major abnormalities are downregulation of Lrp5, and β-catenin cleavage by activated caspase-3. Interruption of canonical Wnt signaling probably contributes to the very low bone mineral content of Smad4−/− mice, compounding Smad4- and Runx2-dependent defects in collagen processing. Importantly, analysis of Smad4−/− bones shows that although osteoblast survival and collagen processing are defective, bone-forming cells retain their ability to undergo full differentiation. Thus, whereas Smad4 is dispensable in Osx+ osteoblasts for further progression along their lineage, osteoblasts in Smad4−/− bones do not properly use Runx2 signaling to drive collagen matrix maturation, undergo caspase-3 mediated cleavage of β-catenin and are susceptible to apoptosis. Consequently, by 4 weeks of age, canonical Wnt signals are dampened following embryonic ablation of Smad4, leaving Smad4−/− osteoblasts resistant to the osteogenic stimulus of both BMP2 and Wnt proteins (Fig. 8). Because Smad4 is deleted in Osx+ cells, loss of β-catenin occurs after the crucial Runx2–Osx1 transition point for which β-catenin is absolutely required (Rodda and McMahon, 2006), explaining why osteoblasts differentiate in these Smad4−/− mice despite β-catenin cleavage.

In the absence of tetracycline repress, the primary recombination event in Osx-Cre models occurs between embryonic day (E)14.5 and birth (Rodda and McMahon, 2006). Since we did not delay recombination with tetracycline, the bone extracts we analyzed from 4- to 6-week-old mice are expected to be composed largely of mature osteoblasts and osteocytes derived from progenitors that lost Smad4 during embryonic skeletogenesis. Thus, our western blot and qPCR data reflect a homeostatic state achieved several weeks after the loss of Smad4, and might be distinct from the effects of acute or postnatal recombination of Smad4. Indeed, time-course cultures of osteoblasts made from calvaria of newborn mice indicate that some molecular events such as depletion of β-catenin commence over time, not immediately (data not shown).
A potential limitation of our study is that the Osx1-Cre transgene affects cortical structure by itself, and could account for the modest decrease in tibia length and mild cortical abnormalities observed in mice, with evidence of Caspase-3-associated cleavage of β-catenin. Protein levels of the Wnt receptor Lrp5 are also critically low in Smad4+/- bones. Consequently, anabolic responses to canonical Wnt signaling are dampened following constitutive embryonic ablation of Smad4, leaving Smad4+/- osteoblasts resistant to the osteogenic stimulus of both BMP2 and Wnt proteins.

In summary, Smad4 is a key regulator of skeletal development and homeostasis, affecting the function and survival of bone-forming cells. Genetic ablation of Smad4 in Osx1+ cells results in a complex and severe phenotype characterized by growth retardation, dental hypoplasia, skeletal hypomineralization, abnormalities of collagen matrix and spontaneous fractures. Our findings suggest that dysfunction of BMP signaling through Smad4 might be involved in the pathogenesis of some forms of osteogenesis imperfecta or Ehlers-Danlos syndromes. We also uncover novel interactions between Smad4 and crucial osteoblast signaling systems, specifically Runx2 and Wnt/β-catenin, demonstrating that Smad4 in osteoblasts is necessary for the osteogenic actions of both BMP2 and canonical Wnt proteins.

Materials and Methods

Materials

Rat monoclonal anti-mouse Dkk1 (clone 11H10, Lot 14073109), a gift from Dr William Richards, Amgen (Thousand Oaks, CA), was prepared in sterile saline and administered at 20 mg/kg/day by intra-peritoneal injections, three times a week for 4 weeks. Mice were different, far more severe than in Smad4+/- mice. Consequently, anabolic responses to canonical Wnt signaling are dampened following constitutive embryonic ablation of Smad4, leaving Smad4+/- osteoblasts resistant to the osteogenic stimulus of both BMP2 and Wnt proteins. However, Smad4-deficient osteoblasts do not properly drive collagen matrix maturation, exhibit reduced mineralizing activity, and are more susceptible to cell death. By 4 weeks of age, Smad4+/- mice exhibit extensive apoptosis of osteoblasts in bone, and severe phenotype characterized by growth retardation, dental hypoplasia, skeletal hypomineralization, abnormalities of collagen matrix and spontaneous fractures. Our findings suggest that dysfunction of BMP signaling through Smad4 might be involved in the pathogenesis of some forms of osteogenesis imperfecta or Ehlers-Danlos syndromes. We also uncover novel interactions between Smad4 and crucial osteoblast signaling systems, specifically Runx2 and Wnt/β-catenin, demonstrating that Smad4 in osteoblasts is necessary for the osteogenic actions of both BMP2 and canonical Wnt proteins.

A potential limitation of our study is that the Osx1-Cre transgene affects cortical structure by itself, and could account for the modest decrease in tibia length and mild cortical abnormalities observed in conditional heterozygous Smad4+/- mice, which also carry the Osx1-Cre transgene. However, the changes observed in Smad4+/- mice are different, far more severe than in Smad4+/-/mice, and also affect cancellous bone; thus, they probably represent a true effect of Smad4 ablation. Skeletal abnormalities are not reported in Smad4+/- mice in the absence of a Cre allele (Tan et al., 2007; Zhang et al., 2005) and structural µCT parameters of Smad4+/- bones fall within expected values for wild-type mice on a mixed strain background (Di Benedetto et al., 2010).

Histology

lacz
Whole bones were fixed for 1 hour in 54 µl of 10% NBF and 0.8 µl of 25% glutaraldehyde per ml of PBS, decalcified in 14% EDTA pH 8.0 for 9 days, stained at 30°C in a standard X-gal solution supplemented with 100 mM D-galactose, post-fixed overnight in 10% neutral formalin. Paraffin sections were counterstained with eosin.

Picrosirius Red
Decalculated sections of decalcified bone were stained for 1 hour in 0.1% solution of Sirius Red F3B in saturated picric acid and washed in 0.5% (v/v) glacial acetic acid.

Calcine
Intra-peritoneal injections were given 11 and 3 days before euthanasia (15 mg/kg). Non-decalcified bones were embedded in methylmethacrylate.

TUNEL
Decalculated sections were processed according to manufacturer’s directions using In Situ Cell Death Detection Kit with Fluorescein (Roche). Sections were mounted with Cytoseal (Thermo Scientific) or Prolong Gold Antifade (Invitrogen). Brightfield and fluorescence microscopy were captured at room temperature with a Nikon Eclipse E600 using an Oimaging ExcilBlue camera and Qcapture software; Picrosirius Red with Olympus BX51P polarized light microscope; and whole mounts with a digital camera. Objectives: Nikon 4× /0.13, 10× /0.45 and 40× /0.75.

Samples from 4-week-old male mice (n=5 per genotype), embedded in 2.0% agarose, were scanned post-mortem with a µCT40 (Scanco Medical AG) using 16 µm voxel size, 45 kVp (whole body) or 55 kVp (tibia) of radiation energy, 87 µA (whole body) or 144 µA (tibia) intensity and 300 microsecond integration time. For in vivo analysis, female mice were anesthetized with isoflurane, and tibiae were scanned using a Viva µCT40 set to 70 kVP and 110 µA. Unless specified otherwise, the first 30 slices below the growth plate were used for trabecular analysis and cortical measurements were assessed using 10 slices at the mathematical midpoint of the tibia.

Preparation of marrow-free bone tissue and BMSCs
Soft and connective tissue were removed from ribs and femurs of 4-week-old mice and kept in cold PBS during necropsy. A scalpel was used to remove the distal tibia and the proximal femur such that the marrow cavity was exposed. Bones were loaded with cut ends facing down into sterile 0.7 ml microfuge tubes where the bottom of the tube had been pierced with a sterile 18-gauge needle. Bones in 0.7 ml tubes were stacked into sterile 1.5 ml tubes and the multiplex tube assembly was centrifuged at 9000 rpm for 10 seconds at 4°C. Marrow-free bones in 0.7 ml tubes were immediately snap frozen in liquid nitrogen and stored at −80°C. Bone marrow cell pellets in the outer 1.5 ml tubes were used for culture of BMSCs. The marrow pellet was resuspended and incubated for 5 minutes on ice in red blood cell lysis buffer (Roche), washed with ice-cold PBS, resuspended in ascorbic-acid-free α-MEM (Mediatech, Herndon, VA), filtered through a 70 µm cell strainer, and finally resuspended in α-MEM containing 20% fetal calf serum (FCS) and antibiotics. Non-adherent cells were removed by vigorous washing after 3 days of culture.

Fig. 8. Smad4 is dispensable in Osx1+ cells for the formation of fully differentiated Dmp1/Phex+ osteoblasts in bone. However, Smad4-deficient osteoblasts do not properly drive collagen matrix maturation, exhibit osteoblasts and osteocytes, but not bone marrow cells (supplementary material Fig. S1A). To conditionally ablate Smad4, Osx1-CreF/F mice were crossed to Smad4+/- mice. Osx1-CreF/F, Smad4+/- males were crossed to Smad4+/- females to generate Osx1-CreF/F, Smad4+/- (Smad4+/-) or cKO) and Smad4+/- (wild-type control). The Osx1-Cre mouse model incorporates a Tet-OFF regulatory mechanism. However, tetracycline-mediated repression was not used in these studies. Demonstrating efficient gene recombination, Smad4+/- mice show Smad4 immunohistochemical staining in cells adherent to trabecular structures in the primary spongiosa, whereas there is no detectable Smad4 staining around the trabecular structures in Smad4+/- (supplementary material Fig. S1B). Some visible signal appears immediately below the growth plate, and might represent Smad4 in osteoclasts, which are abundant in subchondral bone and are not targeted by Osx1-Cre. Smad4 protein is nearly undetectable in marrow-free bone extracts of Smad4+/- versus Smad4+/- (supplementary material Fig. S1C). Mice were in a mixed C57BL/6-C129/J background, fed regular chow ad libitum, and housed at 25°C with 12 hour light/dark cycles. All studies were approved by the Animal Studies Committee of Washington University in St Louis.

Materials

Materials

Rat monoclonal anti-mouse Dkk1 (clone 11H10, Lot 14073109), a gift from Dr William Richards, Amgen (Thousand Oaks, CA), was prepared in sterile saline and administered at 20 mg/kg/day by intra-peritoneal injections, three times a week for 4 weeks. All chemicals and reagents, unless specified otherwise, were obtained from Sigma Aldrich. Antibodies are listed in supplementary material Table S1.

Mice

Osx1-GFP: Cre (Osx1-Cre) transgenic mice express GFP::Cre in Osx1+ cells (Rodda and McMahon, 2006). Rosa26RCreERT2 reporter alleles (R26R) express β-galactosidase in Cre+ cells (Soriano, 1999). Conditional Smad4 alleles (Smad4+) undergo Cre-mediated excision of a floxed exon 8. Osx1-Cre hemizygous mice (Osx1-Cre(8)) were mated with Rosa26RCreERT2 homozygous mice to verify by lacZ staining that the Osx1-Cre transgene targets cortical and trabecular
Immunoblot and Real-Time quantitative PCR

Frozen, marrow-free bones were pulverized at 2000 rpm for 20 seconds with a Braun Mikrodismembrator, 7 ml shaking flasks and 10 mm chromium steel grinding balls (Sartorius BBI Systems). Shaking flasks and grinding balls were washed in RNase-away soap and were pre-cooled in liquid nitrogen prior to sample processing. For immunoblotting, RIPA buffer (500 µl/bone) was used to collect freshly pulverized bone from shaking flasks. Proteins were separated by SDS-PAGE. Blots represent trends in n = 3 for Smad4D14 and n = 4 for Smad4D4. For qPCR, Trizol (Invitrogen, 500 µl/bone) was used to collect freshly pulverized bone from shaking flasks. RNA from cultured cells was extracted with RNeasy Plus Kit (Qiagen). Following DNA digestion, RNA was reverse transcribed with oligo dT plus random hexamer primers using EcoDry Premix (Clontech). Data were normalized to Cyclophilin, analyzed using the ΔACT method and expressed relative to the average of control cells or Smad4D4F samples. n = 7 for Smad4D4F, n = 6 for Smad4D14 and n = 4 for Smad4D4. Primers are available upon request.

Cell culture

Cells were incubated at 37°C in a humidified atmosphere with 5% CO2 in basic medium [ascorbic-acid-free α-MEM (Invitrogen) plus 10% FBS, 40 mM L-glutamine, 100 U/ml penicillin-G and 100 mg/ml streptomycin], plus osteogenic cocktail (50 µg/ml ascorbic acid, 10 µM α -glycerophosphate) as indicated. MC3T3 mouse calvaria cells are subclone 4 (ATCC). The adherent fraction of bone marrow cells were seeded onto 48-well cell culture plates and cultured for 10–21 days in osteogenic medium [ascorbic-acid-free osteogenic basemedia supplemented with 100 ng/ml rhBMP2 (Gibco) or 25 ng/ml rmWnt3a (R&D Systems). Primary calvaria were harvested by chopping neonatal mice, Dr Vicki Rosen (Harvard University) for support during preparation of this manuscript, Drs Deborah Novack and Little, R. D., Carulli, J. P., Del Mastro, R. G., Dupuis, J., Feng, J. Q. and Mishina, Y. (2003). Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene Ther. 10, 1312-1320.


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Characterization of Osx1-Cre Activity in Postnatal Bone.  
(A) Eosin and LacZ on mid-sagittal sections of the tibia from 6 week-old mice (20X).  
(B) Smad4 immunohistochemistry on mid-sagittal sections of the tibia from 4 week-old mice (20X).  
(C) Immunoblot on marrow-free bone.
Bone structure of Osx1-Cre Mice. (A-B) 3-D µCT reconstructions of tibiae of 4 week-old littermates, shown to scale. (C-G) Quantitative µCT analysis of the tibiae (N ≥ 4 males/genotype). Data are Mean ± SEM, where p values are calculated using student’s T-test vs. mice without Osx1-Cre. (C) Tibia length. (D) Mineral content per unit volume of cortical bone at the mid-diaphysis. (E) Volume of trabecular bone per volume of the trabecular compartment, calculated in the first 30 slices below the proximal growth plate. (F) Total peripheral cross-sectional area occupied by the diaphysis at mid-shaft. (G) Percent of cross-sectional area occupied by cortical bone.
Decreased Osteoclastogenesis in Smad4^{F/F}; Osx1-Cre Mice. (A) TRAP stain on mid-sagittal sections of the tibia (ventral, 20X).
Lysyl Oxidase is a BMP2 Responsive Gene Regulated by Smad4 and Runx2. (A-C) QPCR on MC3T3s ± 24 hr BMP2 (200ng/ml) or TGF-β (10ng/ml). Data are Mean ± SEM, brackets indicate p<0.05, n=3. (D) Smad-dependent luciferase activity using MC3T3 cultures transfected with either non-targeted or Smad4 siRNA and treated 24 hr with BMP2 (200ng/ml). Data are Mean ± SEM, where p<0.05 vs. control siRNA and p<0.05 vs. control siRNA + BMP2, n=9. (E) 3-D μCT reconstructions of skeletal structures in 4 week-old littermates. Bars=5 mm. (F) Runx2-dependent luciferase activity (OSE-luc) using MC3T3s treated 24 hr with BMP2 (200ng/ml). Data are Mean ± SEM where p<0.05, n=3. (G) Rabbit, Histone H3 (HH3), Smad4, or Runx2 IgG was used to perform chromatin immunoprecipitation on MC3T3s treated 72 hr ± BMP2 (200ng/ml). Pulldowns were assessed by QPCR for abundance of the Rpl30 promoter. Data are expressed as the median result of ChIP on two independent cultures where error bars show distribution.
**Lysyl Oxidase Promoter**

NCBI Reference Sequence: NM_010728.2.

Chromosome 18 D1:18 29.0cM (52,517,238-52,529,708): NC_000084.6

**Putative Runx2 binding motif**

**Putative Smad4 binding motif**

Region targeted by ChIP primers

```
ACCCTGGAGAGGCGGGAAGCAGGAGGTGGGGAGAGTGGTGGGGGCCAAAC -1951
TGTGTGCTCTGGATGGGGGAGGTTGTGACTAAGGCTTATGCTTAAAGAA -1901
ACCAGCAGCAGATATGGCTGATTGATGATGAGATGAGATGAGATGAGATGCTGT -1851
CTTAAATAGCAGCAGCTTGGCATCAGAGTTGACTAAGGCTTATGCTTAAAGAA -1801
GACCTCAAGCAGCTTGGCATCAGAGTTGACTAAGGCTTATGCTTAAAGAA -1751
```

Figure S5

Putative Binding Sites for Smad4 and Runx2 in the Lysyl Oxidase (Lox) Promoter. (A) The 5’ proximal 2 kb region upstream of the murine Lox locus contains abundant consensus binding sequences for Smad4 and Runx2, which are highlighted in red and blue, respectively.
Figure S6

*Smad4* is Required for Mineralizing Responses to Dkk1 neutralizing antibody. New bone formation in the femurs of 2 male littermate mice following treatment with αDkk1 (20 mg/kg/day, i.p. 3 times/week, 4 weeks). (A) Calcein labeling in longitudinal sections of the femur from Smad4<sup>F/F</sup> (left) or Smad4<sup>Δ/Δ</sup> (right) mice after 4-weeks αDkk1. Visualization of entire femur was accomplished by assembling 4X images. Femurs are shown to scale. Note lack of calcein labels in Smad4<sup>Δ/Δ</sup>. 

**A**

- **Smad4**<sup>F/F</sup> 
  - 20 mg/kg αDkk1

- **Smad4**<sup>Δ/Δ</sup> 
  - 20 mg/kg αDkk1
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