Postnatal ablation of osteoblast *Smad4* enhances proliferative responses to canonical Wnt signaling through interactions with β-catenin

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Summary

Canonical Wnt (cWnt) signaling through β-catenin regulates osteoblast proliferation and differentiation to enhance bone formation. We previously reported that osteogenic action of β-catenin is dependent on BMP signaling. Here, we further examined interactions between cWnt and BMP in bone. In osteoprogenitors stimulated with BMP2, β-catenin localizes to the nucleus, physically interacts with Smad4, and is recruited to DNA-binding transcription complexes containing Smad4, R-Smad1/5 and TCF4. Furthermore, Tcf/Lef-dependent transcription, *Ccnd1* expression and proliferation all increase when Smad4, 1 or 5 levels are low, whereas TCF/Lef activities decrease when Smad4 expression is high. The ability of Smad4 to antagonize transcription of *Ccnd1* is dependent on DNA-binding activity but Smad4-dependent transcription is not required. In mice, conditional deletion of *Smad4* in osterix^{+} cells increases mitosis of cells on trabecular bone surfaces as well as in primary osteoblast cultures from adult bone marrow and neonatal calvaria. By contrast, ablation of *Smad4* delays differentiation and matrix mineralization by primary osteoblasts in response to Wnt3a, indicating that loss of *Smad4* perturbs the balance between proliferation and differentiation in osteoprogenitors. We propose that Smad4 and Tcf/Lef transcription complexes compete for β-catenin, thus restraining cWnt-dependent proliferative signals while favoring the matrix synthesizing activity of osteoblasts.

Key words: β-catenin, Smad4, Proliferation, Osteoblast

Introduction

Proliferation and production of bone matrix by bone-forming cells, osteoblasts, are regulated by interplay of canonical Wnt (cWnt) and bone morphogenetic protein (BMP) signaling. Canonical Wnt signals are mediated by β-catenin, which transactivates Tcf/Lef target genes downstream of heteromeric receptor complexes comprising Frizzled and Lrp5 or 6 (MacDonald et al., 2009). The role for β-catenin (*Catnnb1*) in osteoblasts is complex with distinct contributions to cell fate (Day et al., 2005; Hill et al., 2005; Ross et al., 2000), proliferation (Rodda and McMahon, 2006) and differentiation (Hu et al., 2005; Rodda and McMahon, 2006). Genetic loss of both Bmp2 and Bmp4 disrupts embryonic skeletal development (Bandyopadhyay et al., 2006), and BMP2 or BMP4 is sufficient to induce ectopic bone formation (Kang et al., 2004; Wozney et al., 1988). We have previously reported that β-catenin acts downstream of BMP2 through both Tcf/Lef-dependent and Tcf/Lef-independent mechanisms to favor osteoblast over adipocyte cell fate (Salazar et al., 2008). Furthermore, we have used *in vitro* and *in vivo* models of osteoblast differentiation and bone formation to show that the osteoblast activity induced by β-catenin can be synergistically enhanced by BMP2 or prevented by blockade of endogenously produced BMP2 and 4 (Mbalaviele et al., 2005; Salazar et al., 2008). These findings demonstrate that interactions between cWnt and BMP pathways contribute to osteoblast differentiation and thus, to bone formation. However, the mechanisms by which cWnt and BMP pathways interact to control specific stages of the osteoblast lifecycle, including lineage allocation, proliferation, differentiation and function, remain to be elucidated.

During differentiation, *Runx2*-expressing pre-osteoblasts proliferate to expand the osteoblast precursor pool. Subsequent expression of osterix (*Osx*) correlates with diminished proliferation and demarcates a transition where osteoprogenitors begin to acquire functional characteristics of matrix-producing, bone-forming cells (Aubin, 2008; Rodda and McMahon, 2006). *In vivo*, β-catenin is necessary for further differentiation of *Osx*$^{+}$ osteoprogenitors; and constitutive activation of *Catnnb1* in *Osx*$^{+}$ cells not only accelerates osteoblast maturation, but also stimulates proliferation (Rodda and McMahon, 2006). However, although BMP2 and 4 are required for osteoblast differentiation, their action on cell proliferation is not yet clear. Intriguingly, in both mice and humans, mutations that either activate canonical Wnt or deactivate *Bmpr1a*/*Smad4* result in increased mitogenesis (Kobielak et al.,

2003; Li et al., 2003; Mira et al., 2010; Qiao et al., 2006; Xu et al., 2000), indicating that BMP/Smad4 dysfunction have similar consequences on proliferation as Wnt hyperactivity. Indeed, both conditions cause pathological hyperplasia and increased predisposition to tumorigenesis (Anastas and Moon, 2013; Claes et al., 2011; Howe et al., 1998; Merg and Howe, 2004; Miyaki and Kuroki, 2003; Yang and Yang, 2010). Intriguingly, a similar relationship between BMP/Smad4 dysfunction and Wnt hyperactivity has been shown in flies, where forced expression of *Smad4* mutants that encode proteins unable to bind DNA causes phenotypes similar to those caused by activating mutations of wingless and armadillo, the fly orthologs of Wnt and β-catenin (Takaesu et al., 2005). Taken together, these findings thus lead us to hypothesize that BMP and Wnt intersect in a Smad4-dependent manner leading to opposing effects on proliferation.

β-catenin and Tcf/Lef family members can interact with Smad proteins (Shi and Massagué, 2003) to synergistically induce genes whose promoters contain both Tcf/Lef and Smad response elements. Examples include *gastrin* (Lei et al., 2004) and *c-Myc* (Hu and Rosenblum, 2005) in the intestine, *Xtwn* in the Spemann organizer of the frog (Nishita et al., 2000) or *Msx2* in osteoblasts (Hussein et al., 2003). However, it is conceivable that interaction between Smads and β-catenin may sequester β-catenin from the canonical Wnt transcriptional machinery, thereby reducing responsiveness to Wnt stimulation. In this view, such interaction would have negative consequences for expression of genes regulated primarily by Tcf/Lefs, such as the cell cycle regulator, cyclin-D1, resulting in altered cell proliferation. We tested this hypothesis in osteoblasts *in vivo* and *in vitro*. Results demonstrate that interactions between Smad4 and β-catenin negatively regulate Tcf/Lef-dependent transcription and proliferative responses to canonical Wnt signaling, and that this *Smad4*-mediated crosstalk between cWnt and BMP balances the proliferation and differentiation of bone-forming cells of the skeleton.

Results

BMP2 stimulates interaction between Smad4 and β-catenin on consensus Smad-binding elements

We first determined whether BMP2 alters β-catenin cellular distribution. Transfection of an EGFP–β-catenin fusion protein into C3H10T1/2 immortalized mouse embryonic fibroblasts resulted in a diffuse fluorescence signal throughout the cytoplasm in vehicle-treated cells (Fig. 1Ai). Treatment with lithium chloride (LiCl), a GSK-3β inhibitor, Wnt3a or BMP2 induced nuclear accumulation of the fusion protein with distinct punctate condensations (Fig. 1Aii–iv). In confluent MC3T3 immortalized mouse calvaria cells, endogenous β-catenin was found mostly at the periphery of the cell body (Fig. 1Bi). Treatment with either Wnt3a or BMP2 enhanced accumulation of β-catenin in the nucleus (Fig. 1Bii,iii). However, BMP2 had no effect on the activity of a Tcf/Lef reporter construct (TopFlash), and inhibited LiCl-induced TopFlash activity by >70% (Fig. 1C). Thus, BMP2 induces nuclear localization of β-catenin, but inhibits Tcf/Lef transcriptional activity, suggesting that β-catenin is being sequestered in the nucleus by BMP2 signals at the expense of interactions with Tcf/Lef transcriptional machinery. Co-immunoprecipitation and immunoblot analysis on C3H10T1/2 cell extracts revealed that β-catenin and Smad4 physically associate, and such association increased in response to BMP2 (Fig. 1D). Immunoblot analysis of cytosolic and nuclear extracts from confluent MC3T3s showed that β-catenin is present in both cytosolic and nuclear fractions, even in the

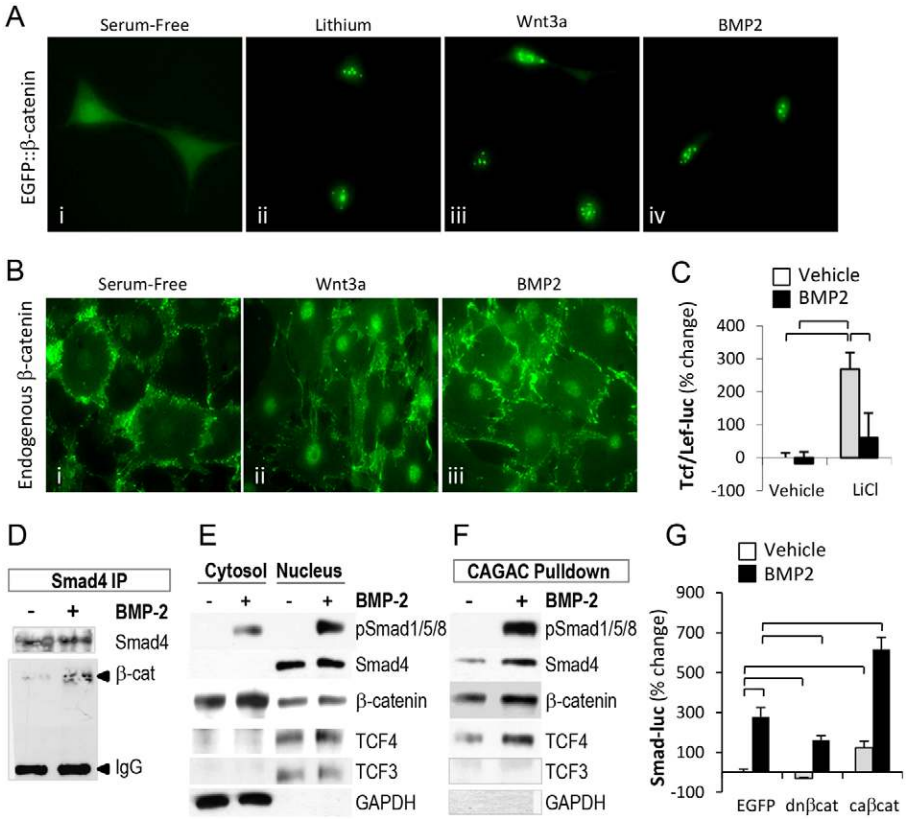

Fig. 1. BMP2 stimulates recruitment of β-catenin to transcriptionally active Smad4 protein complexes. (A) Fluorescence microscopy showing subcellular distribution of a chimeric EGFP–β-catenin protein expressed in subconfluent C3H10T1/2 cells, cultured overnight in serum-free or stimulated conditions. (B) Immunofluorescence of endogenous β-catenin in confluent MC3T3 cells, serum-starved overnight and treated for 90 minutes with Wnt3a or BMP2. (C) Tcf/Lef-dependent luciferase activity in C3H10T1/2 cells treated for 24 hours with lithium chloride and/or BMP2. (D) Immunoblot on Smad4 immunoprecipitates from C3H10T1/2 cells treated for 10 minutes with BMP2. (E) Immunoblots of cytosolic and nuclear fractions of confluent MC3T3 cells treated for 40 minutes with BMP2. (F) Nuclear proteins were mixed with biotinylated DNA oligonucleotides encoding Smad4-binding sequences (CAGAC). Pulldown products were immunoblotted for the indicated proteins. (G) Smad-dependent luciferase activity in C3H10T1/2 cells transfected with EGFP, dominant-negative (dn) or constitutively active (ca) β-catenin. Numerical data are means ± s.d. where $P<0.05$ (Student's *t*-test) is indicated by brackets.

absence of exogenous BMP2 (Fig. 1E). As expected, receptor-activated R-Smads 1, 5, and/or 8 (Smad1/5/8) are highly phosphorylated after stimulation with BMP2. Biotinylated DNA oligonucleotides encoding consensus Smad-binding elements (SBE) were used to pull-down DNA-binding nuclear proteins from MC3T3 cells. Immunoblot analysis of SBE-oligo precipitates revealed that BMP2 increased binding of Smad4, β-catenin, TCF4 as well as phosphorylated Smad1/5/8 to SBE sites, whereas TCF3 did not bind (Fig. 1F) despite its presence in the nucleus (Fig. 1E). Thus, BMP2 stimulates formation of a protein complex containing a β-catenin, TCF4, phosphorylated Smad1/5/8 and Smad4 that assembles on DNA sequences encoding the classic Smad recognition motif, CAGAC; a Tcf/Lef-binding motif is not required for complex formation. To further test whether BMP2 can recruit β-catenin to active Smad transcription complexes, we monitored the effect of constitutively active (caβ-cat) or dominant-negative (dnβ-cat) β-catenin mutants (Barth et al., 1997; Cong et al., 2003; Salazar et al., 2008) on Smad-dependent luciferase activity in C3H10T1/2 cells. caβ-cat weakly activated SBE-luc, but synergized with BMP2 in activating this reporter (Fig. 1G), as we previously reported (Salazar et al., 2008). dnβ-cat inhibited both basal and BMP2-induced SBE-luc activity. However, dnβ-cat did not completely prevent the BMP2 transcriptional response, indicating that β-catenin can modulate, but is not necessary for Smad-dependent transcription.

Smad4 antagonizes Tcf/Lef-dependent transcription, cyclin-D1 promoter activity and proliferation

We next determined the effect of BMP2 signaling on Tcf/Lef-dependent transcriptional activity in C3H10T1/2 cells. Transfection of C3H10T1/2 cells with *Smad4* small-interfering RNA (siRNA; Fig. 2A) or Smad4 expression plasmid (Fig. 2B) dose-dependently decreased or increased Smad4 levels, respectively. *Smad4* siRNA had no effect on *Smad5* expression. As expected, Smad-dependent luciferase activity was dose-dependently decreased by *Smad4* siRNA and dose-dependently increased by HA–Smad4 overexpression (Fig. 2C). Opposite results were obtained using TopFlash: Tcf/Lef transcriptional activity was increased by silencing of *Smad4* and decreased by overexpression of HA–Smad4 (Fig. 2D). To extend these results to a natural promoter context, we used a luciferase reporter driven by the human cyclin-D1 promoter (*CCND1-luc*), a β-catenin responsive promoter that contains multiple Tcf/Lef-binding elements (Tetsu and McCormick, 1999). *Smad4* siRNA

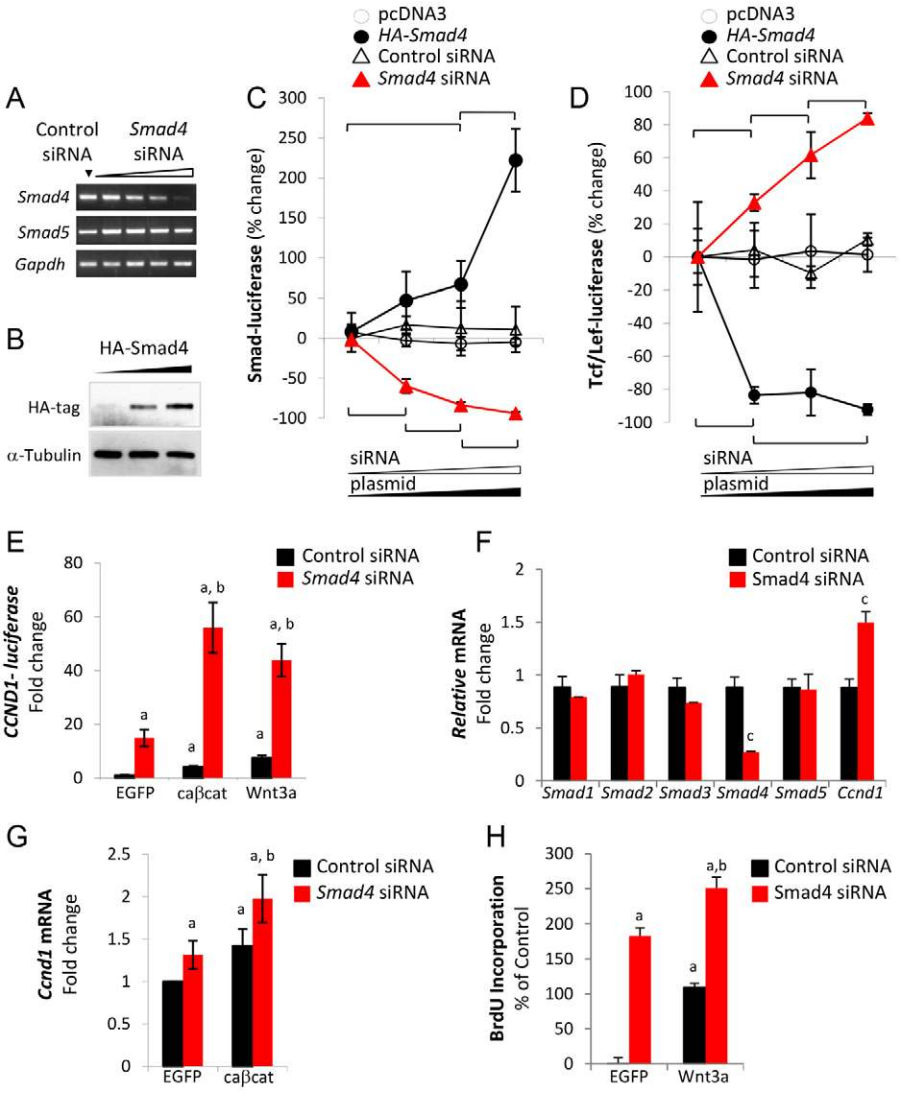

Fig. 2. Smad4 antagonizes Tcf/Lef-dependent transcription and cyclin-D1 promoter activity in C3H10T1/2 immortalized mouse embryonic fibroblasts. (A) RT-PCR showing *Smad4* knockdown by siRNA. (B) Immunoblot showing plasmid-based expression of HA-tagged Smad4. (C,D) Smad-dependent (C) or Tcf/Lef-dependent (D) luciferase activity. Cells were transfected with increasing amounts of control siRNA, *Smad4* siRNA, pcDNA3 or HA-Smad4. (E) Luciferase activity driven by the *CCND1* promoter. Cells were transfected with control or *Smad4* siRNA plus plasmids expressing EGFP, Wnt3a or constitutively active (ca) β-catenin. (F,G) QPCR on cells transfected with (F) control or *Smad4* siRNA plus (G) plasmids expressing either EGFP or caβ-catenin. (H) Proliferation of cells transfected with control or *Smad4* siRNA plus plasmids expressing either EGFP or Wnt3a. Numerical data are means ± s.d. where $P<0.05$ (Student's *t*-test) is indicated by brackets: 'a' versus EGFP + control siRNA; 'b' versus EGFP + Smad4 siRNA; 'c' versus control siRNA alone.

increased *CCND1-luc* basal activity 14.9-fold and enhanced responsiveness of *CCND1-luc* to constitutively active β-catenin (caβ-cat) (Barth et al., 1999; Salazar et al., 2008) or Wnt3a (Fig. 2E). Consistent with promoter upregulation, *Smad4* siRNA significantly ($P<0.05$) increased *Ccnd1* mRNA abundance, without affecting *Smad1*, *Smad2*, *Smad3* or *Smad5* mRNAs (Fig. 2F). Concordantly, endogenous *Ccnd1* mRNA abundance was significantly ($P<0.05$) increased by either *Smad4* siRNA or caβ-cat relative to EGFP expression, and increased additively in cells treated with *Smad4* siRNA plus caβ-cat (Fig. 2G). Furthermore, BrdU incorporation by C3H10T1/2 cells was approximately doubled by expression of Wnt3a, tripled by depletion of *Smad4*, and quadrupled by concomitant transfection of Wnt3a plasmid and *Smad4* siRNA, relative to cells expressing EGFP alone (Fig. 2H). Thus, *Smad4* expression inversely correlates with Tcf/Lef transcriptional activity and cell proliferation.

Smad1 and Smad5 antagonize Tcf/Lef-dependent transcription, cyclin-D1 promoter activity and proliferation

Because BMP2 stimulated recruitment of activated Smad1/5 to the complex containing Smad4 and β-catenin (Fig. 1F), a similar analysis was conducted after *Smad1* or *Smad5* silencing. MC3T3 cells were used to extend the previous findings to another osteoblast cell model. Transfection of MC3T3 cells with *Smad4*, *Smad1* or *Smad5* siRNAs selectively reduced their respective mRNAs by >70%, without affecting *Smad2* or *Smad3* mRNA (supplementary material Fig. S1A). Likewise, protein abundance of Smad1, Smad4 and Smad5 were selectively decreased by transfection of their respective siRNA, without affecting Smad1/5 phosphorylation or total β-catenin abundance (supplementary material Fig. S1B). In keeping with the results in C3H10T1/2 cells, transfection of MC3T3 cells with *Smad4*, but also with *Smad1* or *Smad5* siRNA enhanced basal Tcf/Lef transcriptional activity by 65%, 33% and 20%, respectively. Furthermore, the stimulatory effect of Wnt3a on Tcf/Lef activity was significantly ($P<0.05$) enhanced (more than twofold) in cells exposed to *Smad4*, *Smad1* or *Smad5* siRNAs (Fig. 3A). *CCND1* reporter activity was comparably enhanced in cells treated with Wnt3a, or transfected with *Smad4*, *Smad1* or *Smad5* siRNAs; and *CCND1* reporter activity was approximately doubled by Wnt3a in cells transfected with *Smad4*, *Smad1* or *Smad5* siRNAs (Fig. 3B). Notably, the increased *CCND1* promoter activity after *Smad4*, *Smad1* or *Smad5* siRNA transfection was significantly ($P<0.05$) reduced by introduction of dominant negative β-catenin (Fig. 3C). As expected, Wnt3a dose-dependently increased MC3T3 cell proliferation, determined by BrdU incorporation, and an effect of similar magnitude was seen with *Smad5* siRNA transfection; Wnt3a had a modest additive effect in the presence of *Smad5* siRNA (Fig. 3D). Intriguingly, transfection with *Smad4* or *Smad1* siRNA increased proliferation to a significantly ($P<0.05$) larger extent (about threefold) than with Wnt3a treatment, and in these conditions Wnt3a had no further effect (Fig. 3D). Thus, silencing of *Smad1* or *Smad5* has a similar effect as silencing of *Smad4* on Tcf/Lef transcriptional activity and cell proliferation.

Smad4 inhibits *CCND1* promoter activity through DNA-binding activity but not Smad-dependent transcription

To further define the mechanism by which Smad4 modulates Tcf/Lef-dependent transcriptional activity, we generated a library of Smad4 mutants containing an N-terminal HA tag (Fig. 4A). A single point mutant (R100T) reproduces an allele linked to human colorectal tumors (Schutte et al., 1996). This mutation lies outside the DNA binding motif, but causes conformational changes rendering the protein defective in DNA-binding activity (Kuang and Chen, 2004; Shi et al., 1998). All the other mutants were obtained by deletion of key Smad4 functional elements: the DNA-binding domain (ΔDBD) or MH1 domain (ΔMH1), both of which disrupt DNA-binding and transcriptional activity of

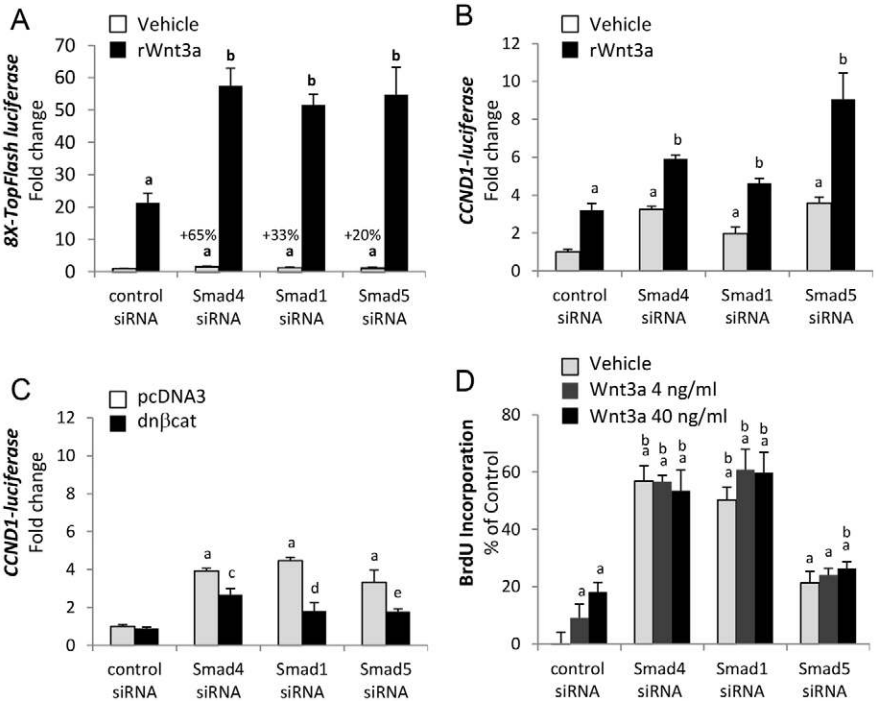

Fig. 3. *Smad1* and *Smad5* antagonize Tcf/Lef-dependent transcription and cyclin-D1 promoter activity. (A–D) Experiments in MC3T3 immortalized mouse calvaria cells transfected with control, *Smad4*, *Smad1* or *Smad5* siRNA (100 nM). (A) Tcf/Lef-dependent luciferase activity, and (B) *CCND1*-luciferase activity in cells transfected with the indicated siRNA and treated with vehicle or rWnt3a (40 ng/ml). (C) *CCND1*-luciferase activity in cells transfected with the indicated siRNA plus dominant-negative (dn) β-catenin. (D) Proliferation of cells transfected with the indicated siRNA and treated with vehicle, 4 ng/ml rWnt3a or 40 ng/ml rWnt3a. Data are means ± s.d. where $P<0.05$ for 'a' versus control siRNA, 'b' versus control siRNA + Wnt3a, 'c' versus *Smad4* siRNA, 'd' versus *Smad1* siRNA or 'e' versus *Smad5* siRNA (Student's *t*-test for unpaired samples).

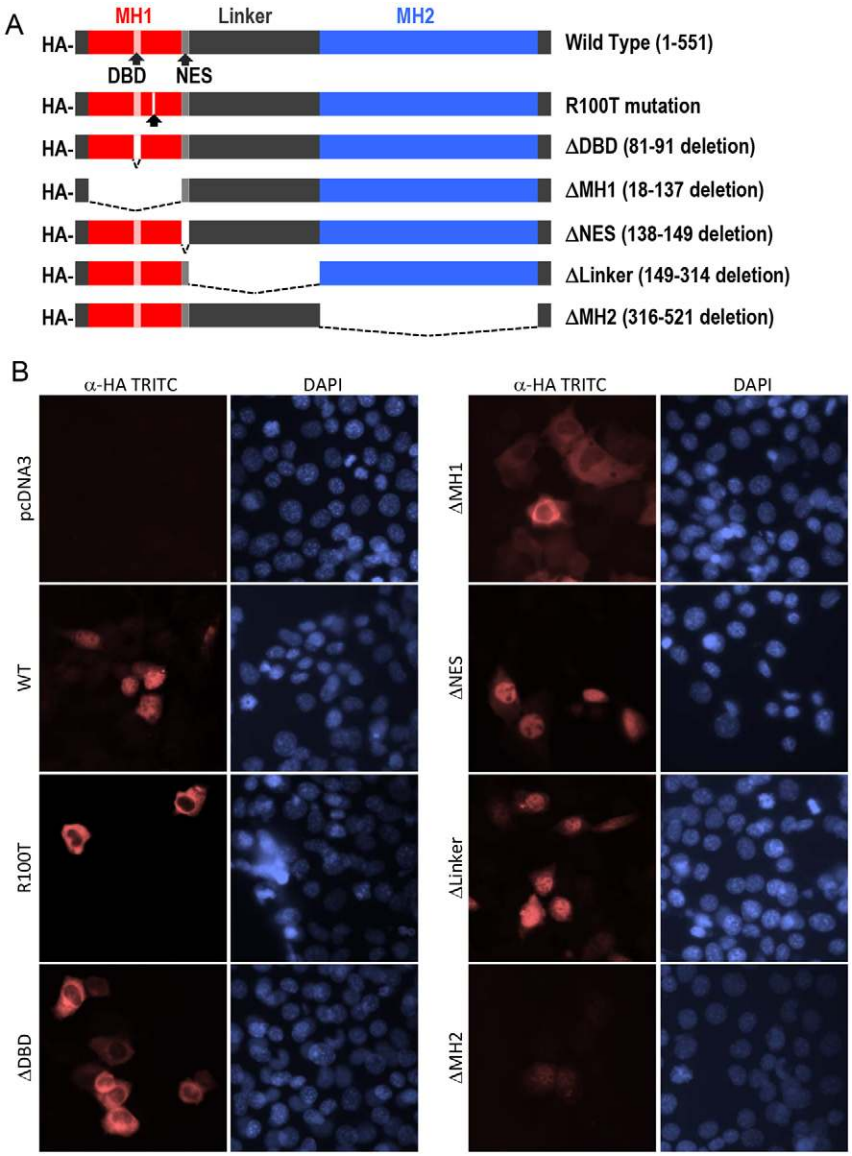

Fig. 4. Smad4 mutagenesis. (A) Schematic of mutations introduced into wild-type HA-tagged murine Smad4; DBD, DNA-binding domain; NES, nuclear export signal. Briefly, WT HA-Smad4 was used as a template for PCR reactions using Platinum High Fidelity Taq polymerase (Invitrogen); for details of the primers see Materials and Methods. (B) Subcellular localization of HA-Smad4 mutants in MLB13 immortalized mouse limb bud cells that were deprived of serum for 4 hours and treated with BMP2 (90 minutess, 200 ng/ml).

Smad4; the nuclear export signal (ΔNES); the central linker region (ΔLinker), which contains residues targeted for post-translational modifications (Verheyen, 2007); and the MH2 domain (ΔMH2), which mediates direct protein interactions with R-Smads. The NES was spared in the MH1 deletion mutant.

When expressed in MLB13 mouse limb bud cells, a skeletal progenitor line that has been shown to produce and respond to BMP2 (Rosen et al., 1993), wild type (WT), ΔNES and ΔLinker accumulated in the nucleus upon BMP2 exposure (90 minutes, 200 ng/ml), whereas the R100T, ΔDBD and ΔMH1 mutants remained largely in the cytoplasm; the signal was barely detectable in cells transfected with ΔMH2 (Fig. 4B). All Smad4 variants with the exception of ΔMH2 were easily expressed in MC3T3 cells, and migrated with the expected gel electrophoretic mobility (Fig. 5A). Notably, ΔMH1 produced a stronger band relative to all the other constructs. Differences in abundance of the various Smad4 mutants may be related to altered protein turnover, consequent to deletion of motifs involved in poly-ubiquitylation and targeting for proteosomal degradation (Dupont et al., 2009; Dupont et al., 2005; Wan et al., 2002; Wan et al., 2004). Expression of Smad4 mutants in MC3T3 cells caused no appreciable or reproducible difference in the abundance or phosphorylation of Lrp5/6 or GSK-3β, or expression of β-catenin or Tcf4 (Fig. 5A). Total β-catenin was also not changed by siRNA-mediated silencing of *Smad4*, *Smad1* or *Smad5* (supplementary material Fig. S1B). With the exception of the wild-type (WT) and the R100T construct, expression of all Smad4 deletion mutants decreased the abundance of total Smad2/3, although the effect was variable and had no impact on the abundance of phosphorylated (p) Smad2/3 (supplementary material Fig. S1C). Furthermore, the abundance of total Smad1, total Smad5 and pSmad1/5 was not affected by expression of Smad4 mutants, except for a decrease of total Smad1 in cells transfected with ΔDBD (supplementary material Fig. S1C).

Next, these Smad4 mutants were used to examine the domains required for physical interaction with β-catenin. Anti-HA immunoprecipitation was performed on non-denatured proteins extracted from MLB13 cells transfected with HA-Smad4 mutants and stimulated with BMP2 (40 minutes, 200 ng/ml). Because this experiment was strictly qualitative in nature, we chose to force

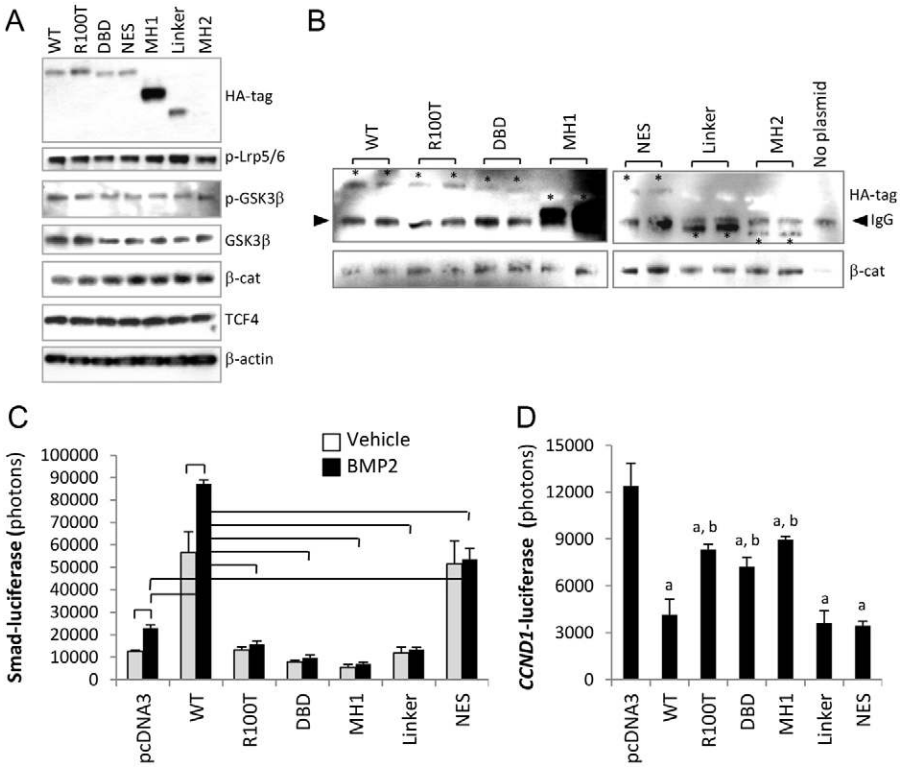

Fig. 5. DNA-binding activity is required for Smad4 to antagonize cyclin-D1 promoter activity. (A) Immunoblot of MC3T3 cells transfected with HA-Smad4 mutants, showing impact of HA-Smad4 mutants on the Wnt pathway. (B) HA–Smad4 mutants were immunoprecipitated from non-denatured lysates of MLB13 cells stimulated for 40 minutes with BMP2 (200 ng/ml). Immunoblotting was performed on complexes immunoprecipitated using a rat anti-HA antibody. Asterisks indicate various HA–Smad4 mutants precipitated during IP. The black arrowheads point to the heavy chain of anti-HA antibody precipitated during the IP. Experiment was performed in duplicate for each mutant. (C) Smad-dependent luciferase activity and (D) *CCND1*-dependent luciferase activity in MC3T3 cells transfected with HA-Smad4 mutants. Numerical data are means ± s.d. where $P<0.05$ (Student's *t*-test) is indicated by brackets, 'a' versus pcDNA3 and 'b' versus WT.

expression of ΔMH2 by transfecting cells with a fivefold higher amount of plasmid. Anti-HA immunoblotting showed that all mutants were efficiently immunoprecipitated. In extracts from cells not transfected with an HA-Smad4 plasmid, anti-HA IgG was efficiently precipitated from these extracts, however, only a negligible amount of β-catenin was detected in the immunoprecipitate (Fig. 5B), confirming that an HA–Smad4 protein is required to pull down β-catenin. Intriguingly, β-catenin was detected in immunoprecipitates from cells transfected with any of the HA-Smad4 plasmids (Fig. 5B), suggesting that the Smad4–β-catenin interaction domain falls in the N-terminal [amino acids (aa) 1–18] or C-terminal (aa 521–551) residues that were not targeted for mutagenesis in this study (Fig. 4A). As expected, WT Smad4 induced SBE-luc activity fivefold, an effect enhanced by BMP2 (Fig. 5C). The ΔNES mutant was equally able as WT Smad4 to transactivate SBE-luc, but was insensitive to BMP2, confirming previous observations (Xiao et al., 2001). By contrast, ΔMH1, ΔDBD, Δlinker and R100T mutants failed to stimulate SBE-luc and prevented the stimulatory effect of BMP2 (Fig. 5C). In particular, ΔMH1 and ΔDBD slightly inhibited basal SBE-luc activity, indicating they might be dominant negative for canonical Smad4 transcriptional activity. We next tested the effect of these Smad4 mutants on *CCND1*-luciferase activity. Co-transfection of WT Smad4 diminished *CCND1*-luc activity in MC3T3 cells by ~70% compared with control cells transfected with an empty expression plasmid (Fig. 5D). This suppressive effect was conserved in the ΔNES and ΔLinker Smad4 mutants, which reduced *CCND1*-luc by a magnitude comparable to the effect of WT Smad4. Notably, the ΔMH1, ΔDBD and R100T mutants were only partially inhibitory (27%, 41% and 33%, respectively) of basal *CCND1*-luc activity (Fig. 5D), suggesting that DNA-binding activity, and/or nuclear localization (Fig. 4B) are required for the antagonistic effect of Smad4 on *CCND1* promoter activity.

Acute ablation of *Smad4* in Osx$^+$ cells of adult mice enhances osteoblast proliferation and surpasses the mitogenic response to Wnt signaling *in vivo*

We next examined whether the anti-proliferative action of Smad4 associated with downregulation of Tcf/Lef signaling also occurs *in vivo*. *Osx-CreER* mice allow conditional and inducible gene ablation upon tamoxifen-dependent activation of Cre (Maes et al., 2007) at a stage in which osteoblasts can be induced to proliferate (Rodda and McMahon, 2006). Initial experiments in which *Osx-CreER* were mated with *ROSA26*$^{fl(lacZ)/+}$ reporter mice demonstrated that five consecutive doses of tamoxifen (100 mg/kg/day; supplementary material Fig. S2A) were sufficient to induce β-gal activity exclusively in *ROSA26*$^{fl(lacZ)/+}$*; Osx-CreER* femurs (supplementary material Fig. S2B). Histological examination of tibiae revealed strong β-gal staining in a high percentage of cells lining the endocortical and trabecular bone surfaces in tamoxifen-treated bones, whereas no β-gal staining was observed in the bone marrow of mutant mice, though positive osteocytes were occasionally seen (supplementary material Fig. S2C–F). The presence of the *Osx-CreER* allele and treatment with tamoxifen were both required for β-gal activity (supplementary material Fig. S2B–F). Hence, *Smad4*$^{flox/flox}$ and *Smad4*$^{flox/flox}$*; Osx-CreER* mice were treated with tamoxifen followed by a single BrdU injection 3 days after the last tamoxifen dose and 2 hours prior to sacrifice (supplementary material Fig. S2A). The number of BrdU$^+$ cells was dramatically higher in the femoral trochanter of *Smad4*$^{flox/flox}$*; Osx-CreER* relative to control *Smad4*$^{flox/flox}$ mice (supplementary material Fig. S2G–H). This accentuated cell proliferation following induction of *Smad4* ablation was evident in cells on the trabecular bone surfaces, the same cells that are targeted by tamoxifen-induced recombination.

To test whether increased osteoblast proliferation following *Smad4* ablation might be linked to interference with Wnt

signaling, we activated Wnt signaling *in vivo* by treating mice with an antibody that neutralizes Dkk1, a Wnt antagonist that causes endocytosis of Lrp5/6 from the cell membrane (Diarra et al., 2007). Because anti-Dkk1 antibody is administered systemically, we first determined that its action is at least in part directly on bone, since indirect effects on Lrp5 through duodenal serotonin secretion have been reported (Yadav et al., 2008). In our hands, *Dkk1* mRNA was highly expressed in bone but nearly undetectable in the duodenum (Fig. 6A). Furthermore, Lrp5 and β-catenin protein abundance in marrow-free bone extracts was dramatically upregulated within 15 minutes of a single dose of anti-Dkk1 (20 mg/kg body weight, intraperitoneally; i.p.), with the appearance of high molecular mass β-catenin species suggesting rapid changes in post-translational modification and/or degradation (Fig. 6B). These results are consistent with direct activation of Wnt–Lrp5 signaling in bone by anti-Dkk1. Therefore, we administered five consecutive daily doses of tamoxifen, alone or in combination with anti-Dkk1, to *Smad4*$^{flox/flox}$ and *Smad4*$^{flox/flox}$*; Osx-CreER* mice, followed by BrdU labeling 2 hours before sacrifice (Fig. 6C). Mitotic BrdU^{+} cells were visualized by immunofluorescence and counted in the subchondral bone of the primary spongiosa in the proximal tibia, where Cre-induced recombination was also apparent (Fig. 6D,E). Consistent with a mitogenic effect of Wnt signaling, BrdU staining was increased by anti-Dkk1 administration relative to saline-treatment of *Smad4*$^{flox/flox}$ mice (Fig. 6Ei,ii,F). However, a far larger increase in BrdU incorporation was evident in *Smad4*$^{flox/flox}$*; Osx-CreER* mice upon induction of *Smad4* ablation (Fig. 6Eiii,F); and importantly, anti-Dkk1 did not further increase the number of BrdU^{+} cells in these animals (Fig. 6Eiv,F). Thus, *Smad4* ablation surpasses the proliferative effect of Dkk1 inhibition on osteogenic cells *in vivo*.

To further prove that the mitotic stimulus following *Smad4* ablation does indeed target osteoblasts, we induced osteoblast differentiation of primary bone marrow stromal cells derived from *Smad4*$^{flox/flox}$*; Osx-CreER* or *Osx-CreER* mice. *Osx-CreER* mice were used as the control in these experiments to eliminate

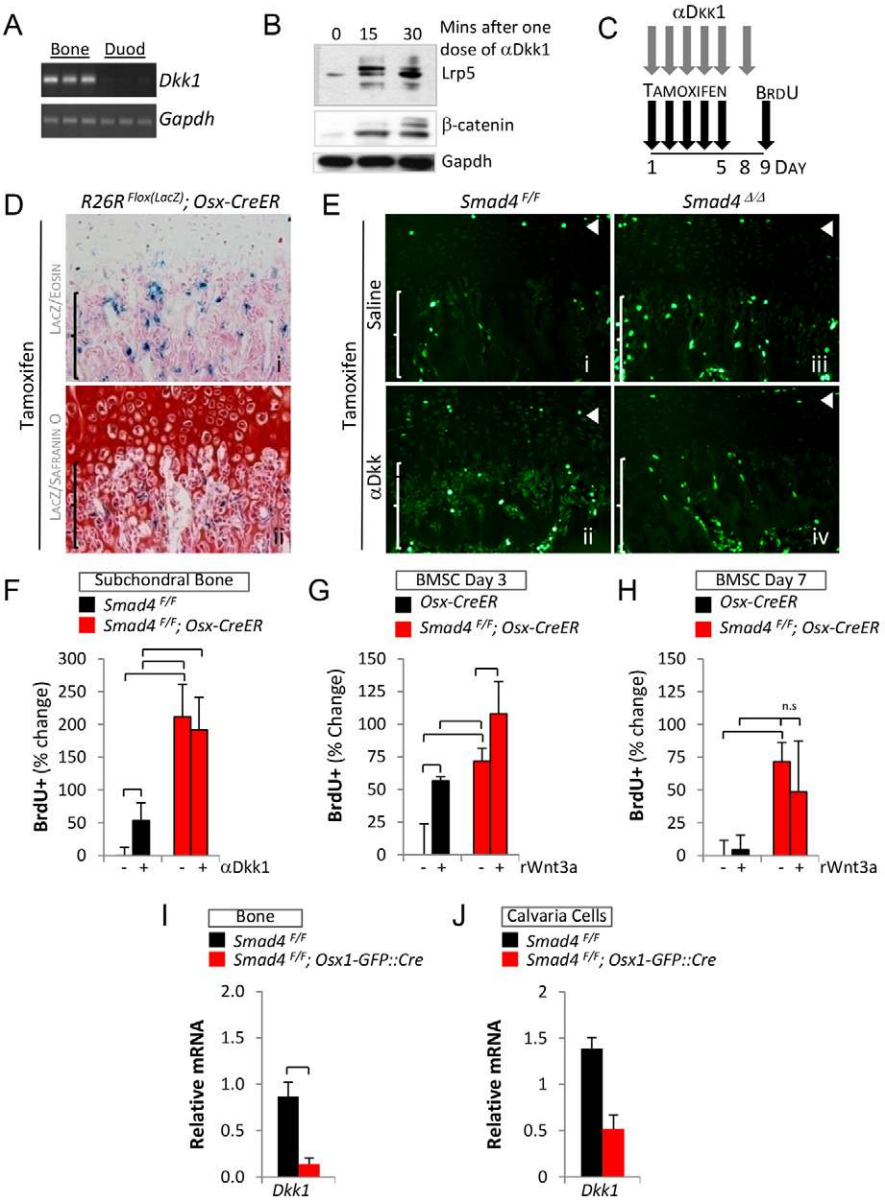

Fig. 6. The mitotic response to *Smad4* ablation exceeds the mitotic response to Dkk1 neutralizing antibody (αDkk1). (A) Reverse-transcription PCR for *Dkk1* in marrow-free bone or duodenum (n=3 mice). (B) Immunoblots of marrow-free bone from mice given one injection of αDkk1 (20 mg/kg, i.p.). (C) Treatment schema to compare osteo-proliferative effects of αDkk1 or *Smad4* ablation. (D) X-gal plus Eosin, or X-gal plus Safranin O staining on mid-sagittal sections from *R26R-lacZ; Osx-CreER* mice treated with tamoxifen. Black brackets indicate β-gal+ (recombined) cells in the subchondral bone below the proximal tibial growth plate. (E) *Smad4*$^{F/F}$ and *Smad4*$^{F/F}$*; Osx-CreER* mice were treated with tamoxifen, αDkk1 and BrdU as indicated in A. Mitotic cells at the proximal tibia were visualized in histological sections by BrdU immunofluorescence. White brackets correspond to the black brackets in D and highlight mitotic cells in the subchondral bone. White arrowheads indicate proliferative chondrocytes in the growth plate. (F) BrdU+ cells in the subchondral bone were counted (n≥3 mice per group). Proliferative chondrocytes in the growth plate (white arrowheads in E) were excluded from this quantification. (G,H) Primary bone marrow stromal cells from *Osx-CreER* or *Smad4*$^{F/F}$; *Osx-CreER* mice were differentiated under osteogenic conditions. To activate CreER *in vitro*, these differentiating osteoblasts were exposed to Tamoxifen (10 mM for 24 hours) on either (G) day 3 or (H) day 7. Cells were then labeled for 2 hours with BrdU in the absence or presence of Wnt3a (50 ng/ml). (I) QPCR on RNA extracts from marrow-free bone of 6-week-old *Smad4*$^{F/F}$ or *Smad4*$^{F/F}$; *Osx1-GFP::Cre* mice. Data are means ± s.e.m. for *Smad4*$^{F/F}$ (n=8) and *Smad4*$^{F/F}$; *Osx1-GFP::Cre* (n=5) mice. (J) Primary calvaria cells from newborn *Smad4*$^{F/F}$ or *Smad4*$^{F/F}$; *Osx1-GFP::Cre* littermate mice were seeded to confluence, cultured in osteogenic conditions. *Dkk1* mRNA was quantified by QPCR on day 8. Data are means ± s.e.m. for *Smad4*$^{F/F}$ (n=5 pups) and means ± s.e.m for *Smad4*$^{F/F}$; *Osx1-GFP::Cre* (n=3 pups). Unless otherwise indicated, numerical data are means ± s.d. and brackets indicate P<0.05 (Student's t-test).

any possible non-specific effects of *Osx-CreER*. To induce *Smad4* ablation, cells were treated with tamoxifen, after 3 or 7 days culture in osteogenic medium, by incubation with tamoxifen for 24 hours, followed by 2 hours of BrdU labeling. After 3 days in osteogenic medium, tamoxifen increased BrdU incorporation by 72% in *Smad4$^{flox/flox}$; Osx-CreER* cells relative to *Osx-CreER* cells. Wnt3a (50 ng/ml) increased the number of BrdU^{+} cells by 56% in control, *Osx-CreER* cultures, and to a significantly ($P<0.05$) greater extent (about 108%) in *Smad4$^{flox/flox}$; Osx-CreER* cultures (Fig. 6G). After 7 days in osteogenic medium, the stimulatory effect of tamoxifen-induced *Smad4* ablation on BrdU incorporation by *Smad4$^{flox/flox}$;Osx-CreER* bone marrow stromal cells was the same as at the earlier time point (about 72%). However, Wnt3a did not affect the number of BrdU+ cells of either genotype when added after 7 days in culture (Fig. 6H). Thus, the mitotic effect of Wnt3a wanes as osteoblasts differentiate. Furthermore, because all cells were exposed to tamoxifen, increased BrdU uptake was due to *Smad4* deletion rather than a spurious effect of tamoxifen. Finally, loss of *Bmpr1A* in osteoblasts can alter *Dkk1* expression (Kamiya et al., 2008), so we assessed *Dkk1* abundance in cells derived from mice in which the *Osx1-GFP::Cre* transgene drives *Smad4* ablation without the requirement of a pharmacological inducer (Rodda and McMahon, 2006). Indeed, ablation of *Smad4* in *Osx*+ cells significantly ($P<0.05$) decreased *Dkk1* mRNA abundance in primary osteoblasts from neonatal mice and in bone tissue from 6-week-old mice, relative to *Smad4$^{flox/flox}$* controls (Fig. 6I,J). Importantly, however, in C3H10T1/2 or MC3T3 samples that had been used earlier to monitor the abundance of mRNAs encoding Ccnd1 or various Smads (Fig. 2F,G), Dkk1 mRNA was undetectable by QPCR.

Increased proliferation in *Smad4*–deficient cells is associated with delayed osteoblast differentiation and a defect in mineralizing activity

We next looked at the effect of *Smad4* ablation on osteoblast differentiation. When cultured in mineralizing medium, bone marrow stromal cells (BMSC) from *Smad4$^{flox/flox}$* mice exposed to an adenovirus expressing GFP (adeno-GFP) produced abundant calcified matrix nodules that were positive for Alizarin Red staining, whereas *Smad4$^{flox/flox}$* BMSC exposed to adeno-Cre produced very few mineralized nodules. More to the point, Wnt3a stimulated formation of mineralized nodules in adeno-GFP-infected BMSC, but had no effect on nodule formation in cells exposed to adeno-Cre (Fig. 7A). Similarly, whereas BMP2 and Wnt3a potently stimulated alkaline phosphatase activity and deposition of mineralized matrix in BMSC cultures from 6-week old *Smad4$^{flox/flox}$* mice, no such response was observed in *Smad4$^{flox/flox}$; Osx-GFP::Cre* cells in the presence of either factor (Fig. 7B). Furthermore, although BMP2 stimulated expression of tissue non-specific alkaline phosphatase (TNAP) and Osx protein by neonatal calvaria cells relative to cultures kept in osteogenic medium alone, such stimulatory activity was substantially attenuated in *Smad4$^{flox/flox}$; Osx-GFP::Cre* cells. In fact, TNAP and Osx became readily

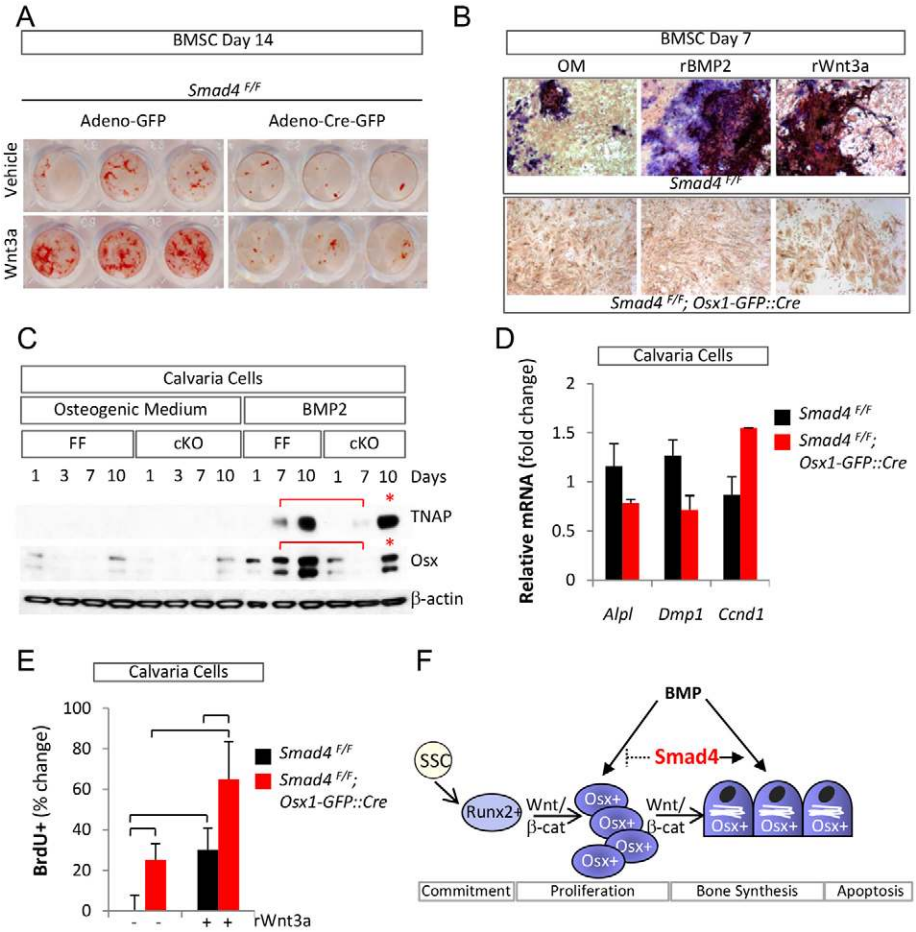

Fig. 7. Increased proliferation in *Smad4*-deficient cells is associated with delayed osteoblast differentiation and lack of mineralizing activity. (A) Primary bone marrow stromal cells from *Smad4$^{F/F}$* mice were transduced with either GFP or Cre-EGFP adenovirus, cultured for 14 days in osteogenic medium (OM) ± Wnt3a (40 ng/ml). Calcified matrix was stained with Alizarin Red. (B) Alkaline phosphatase activity and Alizarin-Red-positive nodules in bone marrow stromal cells from 6-week-old *Smad4$^{F/F}$* or *Smad4$^{F/F}$; Osx1-GFP::Cre* mice, seeded in equal numbers to peak confluency and cultured for 7 days in OM ± BMP2 (200 ng/ml) or Wnt3a (25 ng/ml). (C) Timecourse differentiation study using immunoblots of primary calvaria cells from newborn *Smad4$^{F/F}$* or *Smad4$^{F/F}$; Osx1-GFP::Cre* mice, seeded to peak confluency (day 0), and cultured in OM ± BMP2 (200 ng/ml). TNAP and Osx are expressed in WT but not Smad-null cells at day 7 (compare the two lanes at the end of each bracket). TNAP and Osx are expressed in Smad4-null cells by day 10 (asterisks). (D) qPCR on primary calvaria cells from newborn *Smad4$^{F/F}$* or *Smad4$^{F/F}$; Osx1-GFP::Cre* littermate mice were seeded to confluency and cultured for 8 days in OM. Data are means ± s.e.m. for *Smad4$^{F/F}$* ($n=5$ pups) and means ± s.e.m. for *Smad4$^{F/F}$; Osx1-GFP::Cre* ($n=3$ pups). (E) Primary calvaria cells were seeded to 70% confluency, serum-deprived overnight, and mitotic cells were labeled for 2 hours with BrdU in serum-free medium ± Wnt3a (40 ng/ml). Data are means ± s.d. and brackets indicate $P<0.05$ (Student's *t*-test). (F) *Smad4* acts in *Osx*+ cells of the osteogenic lineage to attenuate proliferative responses and instead promotes matrix-synthesizing responses to canonical Wnt signaling.

apparent by day 7 in *Smad4*$^{flox/flox}$ cells (Fig. 7C, compare the two lanes at the end of each red bracket), whereas they were not detectable until day 10 in *Smad4*$^{flox/flox}$*; Osx-GFP::Cre* cells (Fig. 7C, red asterisk). Reduced expression of *Alpl* and *Dmp1* (a marker of mature osteoblasts and osteocytes) was confirmed by QPCR on day 8 cultures of neonatal calvaria cells (Fig. 7D); and this was associated with increased *Ccnd1* mRNA expression (Fig. 7D), enhanced proliferation compared to *Smad4*$^{flox/flox}$ cells, and increased mitogenic response to recombinant Wnt3a (Fig. 7E).

Discussion

Our studies identified a novel role for Smad4, a transcription factor of the greater TGF-β/BMP superfamily, in modulating pro-mitotic canonical Wnt signals in osteogenic cells. We found that *Osx*+ cells residing on endosteal bone surfaces of adult mice are generally not mitotic, but can be stimulated to proliferate by acute *Smad4* ablation. Mechanistically, Smad4 antagonizes canonical Wnt signaling by recruiting β-catenin into transcriptionally active, Smad4-containing complexes, at the expense of Tcf/Lef-dependent transcription, resulting in attenuated expression of the pro-mitotic gene, *Ccnd1*. Antagonism of *CCND1* promoter activity by Smad4 correlates with the ability of Smad4 to accumulate in the nucleus and bind DNA, but it does not require Smad-dependent transcription. We propose β-catenin and Smad4 play opposing roles to control proliferation of differentiating osteoblasts.

We previously demonstrated that BMP2/4 signals are necessary for osteogenic action of β-catenin (Mbalaviele et al., 2005; Salazar et al., 2008). Here, we demonstrate that Smad4 is a key element linking BMP and cWnt signals at a critical stage of the osteoblast lifecycle, when cells commence expression of *Osx* and transition from a proliferative to a post-mitotic, matrix producing state. *Osx*$^{+}$ cells in adult mice are generally not mitotic under homeostatic conditions, but they retain the potential of re-entering the cell division cycle. For example, increased periosteal proliferation was previously observed following activation of β-catenin in *Osx*$^{+}$ cells (Rodda and McMahon, 2006). Here, we observe that loss of *Smad4* in *Osx*$^{+}$ cells also activates proliferation. Thus, both activation of β-catenin and loss of Smad4 lead to increased proliferation in the osteoblast lineage, supporting our initial hypothesis that β-catenin and Smad4 play opposing roles to control proliferation of differentiating osteoblasts. Interestingly, our data reveal that loss of *Smad4* provides an equal and often more powerful mitotic stimulus than Wnt activation, both *in vitro* and *in vivo*, and enhances the proliferative effect of cWnt signaling. By contrast, loss of *Smad4* prevents the stimulatory effect of Wnt3a on *in vitro* bone matrix production, consistent with our previous observation that BMP signaling is necessary for the osteogenic action of β-catenin (Mbalaviele et al., 2005; Salazar et al., 2008). Thus, Smad4 integrates BMP and Wnt signals in osteoblasts resulting in exit from the cell division cycle and acquisition of bone matrix synthesis function (Fig. 7F).

Based on the data reported here, we propose a competitive recruitment model whereby increased Smad4 expression and/or BMP2 stimulation reduces the abundance of β-catenin available to bind at Tcf/Lef promoter sites (Fig. 8A). Conversely, loss of Smad4 or loss of Smad4 DNA-binding activity alleviates such antagonism by making more β-catenin available for Tcf/Lef-dependent transcriptional activity. In *Osx*$^{+}$ pre-osteoblasts, this results in reactivation of the cell cycle and expansion of the progenitor pool, but resistance to stimulation of matrix synthesis by both BMP and Wnt signals (Fig. 8B). Several findings in our study support this model. First, BMP2 leads to accumulation of β-catenin in the nucleus while causing a decrease in Tcf/Lef-dependent transcriptional activity, associated with increased physical interaction between β-catenin and Smad4. Second, Smad-dependent and Tcf/Lef-dependent transcriptional activity are inversely affected by Smad4 abundance. We also show that activated Smad1 and Smad5 are present in BMP2-stimulated complexes containing Smad4 and β-catenin, and silencing either Smad1 or Smad5 increases Tcf/Lef activity and cell proliferation in a similar fashion to Smad4 silencing. Hence, a classic R-Smad–Smad4 heterotrimer is involved in the competition with Tcf/Lefs for β-catenin. Our structure–function analysis of Smad4 demonstrates that the MH1 and DNA-binding motifs are necessary for a full suppressive effect on Tcf/Lef transcriptional activity, whereas the nuclear export signal and linker domains are dispensable. Consistently, the R100T point mutation that disrupts Smad4 DNA-binding activity (Kuang and Chen, 2004) also removes the inhibitory action of Smad4 on Tcf/Lef activity. Since the MH1,

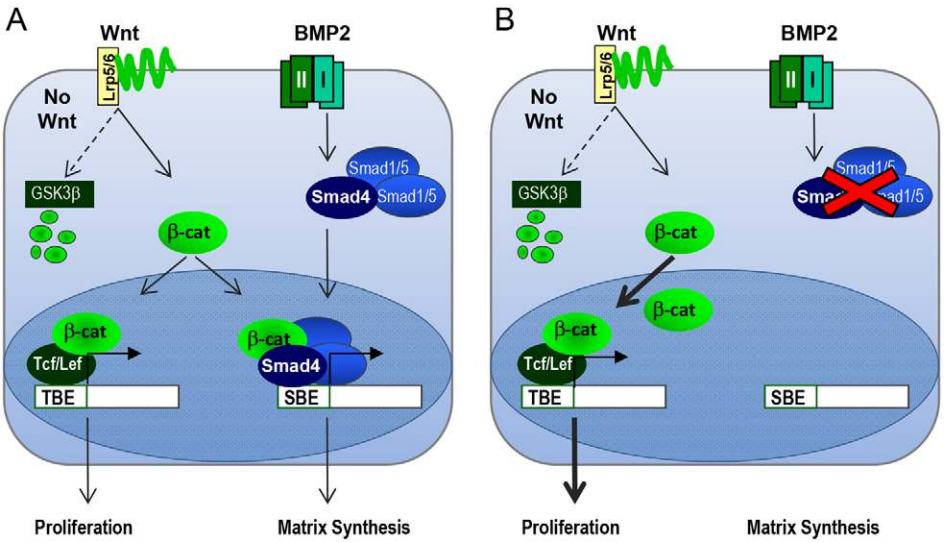

Fig. 8. Competitive recruitment of β-catenin by Smad4 regulates the balance between proliferation and mineralizing function of osteoblasts. (A) Smad4-replete osteoblast. BMP2 signaling stimulates incorporation of β-catenin into Smad transcription complexes, thereby restraining Tcf/Lef-dependent transcription and biological responses to canonical Wnt, such as proliferation. (B) Smad4-null osteoblast. Loss of Smad4 increases the availability of β-catenin to transactivate Tcf/Lefs, thereby increasing proliferation in response to canonical Wnt signaling but interrupting the matrix synthesizing function of the osteoblast.

DBD or R100T mutations each disrupt Smad4 DNA-binding activity and nuclear accumulation, Smad4 antagonism of Tcf/Lef activity is linked to its nuclear activities. Furthermore, deletion of the Smad4 linker region prevents Smad4 transcriptional activity but not antagonism on the *Ccnd1* promoter activity; thus, such antagonism cannot be mediated by Smad4-depedent gene transcription regulation, arguing against an indirect, autocrine/paracrine mechanism. To our surprise, our mutagenesis analysis suggests that the β-catenin interaction domain of Smad4 lies either at the extreme N-terminus (first 18 amino acids) or C-terminus (last 30 amino acids), as all the mutants we tested were able to interact with β-catenin. However, since other factors participate in this multimeric complex, including R-Smads and TCF4, a more in-depth analysis is required to dissect the details of Smad4–βcatenin interaction. Nonetheless, our proposed model of intracellular competitive recruitment represents a novel mechanism by which BMP–Smad signaling can interface with canonical Wnt–β-catenin activity.

It should be noted that this model does not preclude indirect crosstalk through regulation of *Dkk1*, and both mechanisms may be operative. Indeed, the lower than normal *Dkk1* mRNA abundance in *Smad4*-deficient bone, which is consistent with previous observations (Kamiya et al., 2010) may explain, at least in part, the lack of proliferative response to anti-Dkk1 in *Smad4* mutant mice. Nevertheless, the molecular analysis discussed above argues against an indirect effect through Smad4 transcriptional activity. And furthermore, since *Dkk1* mRNA is very low in MC3T3 and C3H10T1/2 cells, it is unlikely that *Dkk1* regulation contributes to the increased proliferation occurring upon *Smad4/1/5* silencing in these cells.

In contrast, cooperative interactions between β-catenin and Smad4 occur on the *c-Myc* promoter (Hu and Rosenblum, 2005). In *Xenopus*, β-catenin and Tcf/Lef factors form complexes with Smad4 and this interaction synergistically regulates expression of *Xtwn* during formation of the Spemann's organizer (Nishita et al., 2000). In murine ES cells, Smad4 forms complexes with Lef1 and β-catenin to activate the *Msx* promoter, and it contributes to Wnt activation of *Msx* transcription independently of BMP signaling (Hussein et al., 2003). Our proposed model of competitive recruitment accommodates these previously described cooperative interactions of Smad4, β-catenin and Tcf/Lefs that occur on promoters containing both Smad and Tcf/Lef binding sites. Notably, our model predicts that Smad4–β-catenin interaction would lead to transcriptional repression in promoters containing Tcf/Lef but not Smad-dependent response elements.

Although our study was conducted in cells of the osteogenic lineage, the results could be relevant for control of proliferation in non-skeletal tissues and in a variety of human cancers. In humans, deletion of *Smad4* (*DPC4*) is a leading cause of pancreatic cancer (Schutte et al., 1996), and loss of *BMPR1a* or *DPC4* is the leading cause of juvenile polyposis syndrome (Howe et al., 1998; Merg and Howe, 2004). In mice, conditional ablation of *Smad4* leads to gastric polyposis (Xu et al., 2000), intrahepatic cholangiocellular carcinoma (Xu et al., 2006) and skin tumors (Yang et al., 2005). More importantly, these models of tumorigenesis induced by loss of *Smad4* are associated with increased levels of *Ccnd1*, increased proliferation and tissue hyperplasia. Loss of *Bmpr1a* has also been shown to cause hyperplasia through interactions with β-catenin; although in that model, Bmpr1a antagonizes cWnt signaling through a mechanism involving PTEN, and so probably represents an additional level by which the BMP pathway can regulate the strength of cWnt signals. It is tempting to speculate that *Smad4* tumor suppressor function could at least in part be related to antagonism of pro-mitotic Wnt–β-catenin signals.

In summary, we propose that Smad4 competitively recruits β-catenin to Smad-containing complexes, thereby decreasing canonical Tcf/Lef transcriptional activity and mitotic responses to cWnt activity. Accordingly, *Smad4* ablation leads to increased osteoblast proliferation *in vivo* and *in vitro*; in addition *Smad4* ablation *in vitro* prevents the stimulatory effect of Wnt3a on osteoblast matrix synthesis. Thus, we conclude that Smad4 balances the pro-mitogenic and pro-mineralizing actions of β-catenin in *Osx*+ osteoblasts.

Materials and Methods

Materials

Rat monoclonal anti-mouse Dkk1 (clone 11H10), a kind gift from Dr William Richards, Amgen, Inc. (Thousand Oaks, CA), was administered at 20 mg/kg/day by i.p. injections (Diarra et al., 2007). All siRNAs were purchased from Sigma. Purified recombinant human BMP2 and mouse Wnt3a are from R&D Systems. p12X-SBE-Luc (SBE-luc) was a kind gift from Dr Di Chen (Rush University, Chicago, IL) and consisted of a luciferase open reading frame preceded by an osteocalcin minimal promoter and 12 tandem Smad binding elements. *CCND1*-luciferase (Tetsu and McCormick, 1999) was a kind gift from Dr Fanxin Long (Washington University in St. Louis). β-catenin cDNAs were previously described (Barth et al., 1997; Barth et al., 1999; Cong et al., 2003; Salazar et al., 2008). All other chemicals and reagents, unless specified otherwise, were obtained from Sigma.

Smad4 mutagenesis

Wild-type (WT) murine Smad4 with an N-terminal 3× HA tag in pCS2+ vector backbone was a kind gift from Dr V. Rosen (Harvard School of Dental Medicine). We used a previously described method (Byrappa et al., 1995) to generate in-frame deletion and point mutants of pCS2+ HA-Smad4. Briefly, WT HA-Smad4 was used as a template for PCR reaction using Platinum High Fidelity Taq polymerase (Invitrogen) and the following primers: (ΔDBD F) 5′-phospho-CATGTGATCT-ATGCCCGTC-3′ and (ΔDBD R) 5′-phospho-TCCATCCAATGTTCTCTGTAT-3′; (ΔNES F) 5′-phospho-AGTAATGCTCCAAGTATGTTA-3′ and (ΔNES R) 5′-phospho-GACAACCCGCTCATAGTG-3′; (ΔMH2 F) 5′-phospho-TGCTGGA-TTGAGATTCACCT-3′ and (ΔMH2 R) 5′-phospho-AGGATGATTGGAAATGG-GAG-3′; (ΔMH1 F) 5′-phospho-TCACCTGGAATTGATCTCTC-3′ and (ΔMH1 R) 5′-phospho-GCTCAGACAGGCATCGTT-3′; (ΔLinker F) 5′-phospho-CATCCT-GCTCCTGAGTAC-3′ and (ΔLinker R) 5′-phospho-CTGCAGTGTTAATCCTGA G-3′; (R100T F) 5′-phospho-ACGTGGCCTGATCTACACAAGAATG-3′ and (R100T R) 5′-phospho-CGTCCACAGACGGGCATAGATCAC-3′.

Cell culture

C3H10T1/2 cells were maintained in Eagle's Basal Medium (BME; Gibco), MC3T3 cells in ascorbic-acid-free α minimal essential medium (α-MEM; Invitrogen), and MLB13 clone 14 cells (Rosen et al., 1993) in DMEM. Media contained 10% FBS (Atlas Biologicals), 40 mM L-glutamine, 100 U/ml penicillin-G and 100 mg/ml streptomycin. Cells were incubated at 37℃ in a humidified atmosphere with 5% CO_2. To measure proliferation, transfected cells were serum-deprived overnight, labeled with BrdU for 2 hours in serum-free medium plus ligands, and detected with a Cell Proliferation ELISA kit (Roche).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 0.25% Triton X-100 and 5% goat serum. β-Catenin antibody (Cell Signaling, cat. no. 9581) or HA-tag antibody (Roche, 3F10) were prepared in PBS containing 0.25% Triton X-100. β-Catenin was visualized with goat anti-rabbit Alexa Fluor 488, HA with goat anti-rat TRITC.

Immunoprecipitation and immunoblotting

Whole-cell protein extracts were collected in 150 mM NaCl, 10 mM Tris at pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate and 5 mM EDTA. Subcellular proteins were prepared with a NE-PER kit (Pierce). Marrow-free bone extracts were prepared as previously described (Watkins et al., 2011). Non-denatured proteins for co-immunoprecipitation were collected in 150 mM NaCl, 20 mM Tris at pH 7.5, 1% NP40 and 5 mM EDTA. Immune complexes with primary antibody were formed overnight under agitation at 4℃, and precipitated using isotype-matched Dynabeads (Invitrogen). Proteins were separated by SDS-PAGE, transferred to PDVF, and probed with the following antibodies: β-catenin (BD Transduction Laboratories, cat. no. 610154), HA-tag (Roche, cat. no. 11867423001), Smad4 (Santa Cruz, cat. nos 7966, 7154), TCF4 (Millipore, cat. no. 05-512), β-actin (Sigma, cat. no. A5316), total GSK-3β

(Millipore, cat. no. 05-412) and HA tag (Roche, cat. no. 11-867-423-001). Cell Signaling antibodies: Smad1 (cat. no. 9743), Smad2 (cat. no. 3102), Smad3 (cat. no. 9523), Smad2/3 (cat. no. 3102), Smad5 (cat. no. 9517), pSmad1/5 (cat. no. 9516), pSmad2 (cat. no. 3101), pSmad3 (cat. no. 9520), Smad4 (cat. no. 9515), pGSK-3β (cat. no. 9336), pLrp5/6 (cat. no. 2568S).

Oligonucleotide pulldowns

Single-strand oligonucleotides encoding complementary Smad4 binding elements (SBE) and a 5′-biotin modification were resuspended in water and mixed in equal molar ratios. SBE forward 5′-AGTATGTCTAGACTGA-3′; SBE antisense 5′-TCAGTCTAGACATACT-3′. Single strand and 5′ biotinylated oligonucleotides were annealed into double-stranded DNA in saline-sodium citrate (SSC) buffer by heating to 95℃ and then cooling slowly to 25℃. Double-stranded oligonucleotides were bound to streptavidin-coated magnetic beads (Pierce), blocked with BSA and poly(dI-dC), and incubated with 10 µg of nuclear extracts for 45 minutes at 4℃ under agitation. Nucleoprotein complexes were pulled down on a magnetic rack (Dynal) and washed extensively prior to eluting precipitates in 1× Laemmli loading buffer.

Luciferase activity

Cells (4×10^4 cells per well of a 24-well plate) were transfected according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen) with the indicated combinations of luciferase reporter (0.4 µg/well), expression plasmid (0.4, 0.2 or 0.1 µg/well) or siRNA (100, 75, 50 or 25 nM). Luciferase activity was quantified using Bright-Glo (Promega).

RNA isolation and real-time quantitative PCR (qPCR)

Total RNA from cells was isolated using an RNeasy kit (Qiagen) or prepared from tissue as described previously (Watkins et al., 2011) and reverse transcribed using EcoDry Premix (Clonetech). Taqman® Gene expression assays (Applied Biosystems) were used as per the manufacturer's instructions.

Mice

Osx-CreER^{T2} mice express Cre::ERalpha fusion protein under control of the osterix promoter (Yu et al., 2003). *Rosa26R$^{flox\text{-}stop\text{-}flox(lacZ)}$* reporter alleles (*R26R^{F}*) are activated by Cre-mediated excision of a floxed stop codon upstream of a β-galactosidase cassette (Soriano, 1999). Both alleles were generous gift from Dr Henry Kronenberg, Harvard University). We examined β-galactosidase activity in bones of *Osx-CreER$^{Tg/0}$; R26R$^{F/+}$* mice to optimize the tamoxifen regimen sufficient to activate Cre-mediated recombination in bones of adult mice and to monitor efficiency and lineage specificity of *Osx-CreER^{T2}*. Conditional *Smad4* alleles (*Smad4^{F}*) are ablated by Cre-mediated excision of a floxed exon 8 (Yang et al., 2002). To induce acute ablation of *Smad4* in differentiating osteoblasts of adult mice, we generated *Osx-CreER$^{Tg/0}$; Smad4$^{Flox/Flox}$* (*Smad4$^{\Delta/\Delta}$*; tamoxifen-induced conditional knockout). *Smad4$^{Flox/Flox}$* (*Smad4$^{F/F}$*, conditional WT) and *Osx-CreER$^{Tg/0}$* (Cre only) were used as controls. *Osx1-GFP::Cre (Osx1-Cre)* transgenic mice express GFP−Cre in *Osx1+* cells (Rodda and McMahon, 2006). To achieve constitutive and tamoxifen-independent ablation of *Smad4*, we bred *Osx1-Cre$^{Tg/0}$; Smad4$^{F/F}$* (*Smad4$^{\Delta/\Delta}$* or cKO) and *Smad4$^{F/F}$* (wild-type control). The *Osx1-Cre* mouse model incorporates a Tet-OFF regulatory mechanism; however, tetracycline-mediated repression was not used in these studies. Mice were in a mixed C57BL/6-C129/J background, fed regular chow *ad libitum*, and housed at 25℃ with a 12 hour:12 hour light:dark cycle. Studies were approved by the Animal Studies Committee of Washington University in St Louis.

Genotyping

PCR was performed on genomic DNA extracted from mouse tails using the HotSHOT method (Truett et al., 2000). Primers available upon request.

Detection of *Rosa26R$^{flox(lacZ)}$* recombination

Intact bones were fixed for 1 hour in 54 µl of 10% neutral buffered formalin (NBF), 0.8 µl 25% glutaraldehyde per 1 ml PBS, decalcified for 7 days in 14% EDTA (free acid pH 8.0 with ammonium hydroxide), stained overnight at 30℃ in X-gal staining solution plus 100 mM galactose, and post-fixed in 10% NBF overnight at 4℃. Paraffin sections were counterstained with Eosin or Safranin O.

In vivo proliferation

Bromodeoxyuridine (BrdU, 100 mg/kg, i.p.) was administered to mice 2 hours prior to sacrifice. Bones were fixed for 12 hours in 10% neutral buffered formalin and decalcified for 7 days in 14% EDTA. BrdU^{+} cells were detected in paraffin sections using the BrdU Staining Kit (Invitrogen). Fluorescein-conjugated streptavidin (Amersham, cat. no. RPN 1232) was substituted for the DAB secondary detection reagents provided in the kit.

Harvesting bone marrow stromal cells (BMSC)

Marrow from femora and tibiae of 2-month-old mice was flushed by removing one of the epiphyses and centrifuging the bone at 9,000 rpm for 10 seconds. The marrow pellet was resuspended and incubated for 5 minutes on ice in red blood cell lysis buffer (Roche), washed with ice-cold PBS, filtered through a 70 µm cell strainer, and plated in ascorbic-acid-free α-MEM (Mediatech Inc., Herndon, VA) containing 20% fetal calf serum (FCS) and antibiotics. Non-adherent cells were removed.

Harvesting primary calvaria cells

Primary calvaria cells were harvested by chopping neonatal calvariae and collecting cells released by a 2-hour incubation in collagenase-A. Calvaria cells were washed with PBS, filtered through a 70 µm cell strainer, and plated in ascorbic acid-free α-MEM (Mediatech Inc., Herndon, VA) containing 10% FCS and antibiotics.

In vitro proliferation of bone marrow stromal cells

Cells were seeded at 30,000 cells per well in black-walled 96-well tissue culture plates and differentiated for 3–7 days. Cells were incubated for 24 hours in serum-free medium plus 10 µM tamoxifen (Sigma). Mitotic cells were labeled for 2 hours with BrdU and quantified with a Chemiluminescent Cell Proliferation ELISA kit (Roche).

Adenovirus-induced recombination

BMSC were seeded at 40,000 cells per well (96-well dish) and exposed to Ad-CMV-GFP or Ad-Cre-Ires-GFP adenovirus (1×10^7 PFU) for 7 hours.

Differentiation of BMSC

BMSC were seeded at 40,000 cells per well (96-well plate) and cultured for 10 days in osteogenic medium (10 mM β-glycerophosphate and 50 µg/ml ascorbic acid) ± 200 ng/ml rhBMP2 (Gibco) or 25 ng/ml rmWnt3a (R&D Systems).

Differentiation of primary calvaria cells

Calvaria cells were seeded at 800,000 cells per well (6-well plate) in osteogenic medium ± 200 ng/ml rhBMP2 or 25 ng/ml rmWnt3a (Gibco).

Statistics analysis

Data are expressed as the means ± s.d. or s.e.m., as indicated and statistical significance was determined using two-tailed Student's *t*-tests; $n\geq3$.

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Author contributions

V.S.S., G.M. and R.C. conceived, designed and interpreted this work. V.S.S. performed most of the experiments. N.Z. made Smad4 mutants. J.N., N.Z. and L.H. provided assistance with mouse husbandry, genotyping and injections. J.K., M.W. and S.B. performed immunoblots shown in supplementary material.

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