BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation

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Bone morphogenetic protein (BMP) signaling is required for endochondral bone formation. However, whether or not the effects of BMPs are mediated via canonical Smad pathways or through noncanonical pathways is unknown. In this study we have determined the role of receptor Smads 1, 5 and 8 in chondrogenesis. Deletion of individual Smads results in viable and fertile mice. Combined loss of Smads 1, 5 and 8, however, results in severe chondrodysplasia. Smad1/5CKO (cartilage-specific knockout) mutant mice are nearly identical to Smad1/5CKO;Smad8–/– mutants, indicating that Smads 1 and 5 have overlapping functions and are more important than Smad8 in cartilage. The Smad1/5CKO phenotype is more severe than that of Smad4CKO mice, challenging the dogma, at least in chondrocytes, that Smad4 is required to mediate Smad signaling through BMP pathways. The chondrodysplasia in Smad1/5CKO mice is accompanied by imbalances in cross-talk between the BMP, FGF and Ihh/PTHrP pathways. We show that Ihh is a direct target of BMP pathways in chondrocytes, and that FGF exerts antagonistic effects on Ihh expression. Finally, we tested whether FGF exerts its antagonistic effects directly through Smad linker phosphorylation. The results support the alternative conclusion that the effects of FGFs on BMP signaling are indirect in vivo.

KEY WORDS: BMP, Smad, Growth plate, Chondrogenesis, Mouse

INTRODUCTION

Bone morphogenetic proteins (BMPs) and their receptors are required for chondrogenesis and are expressed throughout the growth plate and perichondrium (Retting and Lyons, 2006; Yoon and Lyons, 2004; Minina et al., 2005; Minina et al., 2001; Solloway et al., 1998; Yoon et al., 2006). BMPs are required at early stages for condensation, maintaining Sox9 expression and matrix production (Haas and Tuan, 1999; Hatakeyama et al., 2004). BMPs also promote proliferation and differentiation at later stages, and are required for induction of type II and X collagen (Fujii et al., 1999; Shukunami et al., 2000; Drissi et al., 2003; Leboy et al., 2001; Valcourt et al., 2002).

BMPs transduce signals by binding to complexes of type I and II serine/threonine kinase receptors. Ligand binding induces phosphorylation of the receptors, which then activate canonical signaling via receptor Smads (R-Smads) 1, 5 and 8 (Smad8 is also known as Smad9 – Mouse Genome Informatics) (Massague et al., 2005). R-Smads contain two domains connected via a linker region. R-Smads are phosphorylated at the C-terminus by the activated type I receptor. They then complex with Smad4, triggering nuclear translocation.

Overexpression of BMPs leads to increased chondrocyte proliferation and fused skeletal elements (Brunet et al., 1998; Duprez et al., 1996; Wijgerde et al., 2005). Conversely, mice lacking BMP receptors exhibit almost complete loss of cartilage (Yoon et al., 2005). In contrast to these severe phenotypes, cartilage-specific loss of Smad4 results in only mild defects (Zhang et al., 2005). These divergent phenotypes raise the possibility that canonical Smad signaling is largely dispensable in the growth plate, or that R-Smads signal independently of Smad4. BMPs trigger non-Smad (noncanonical) pathways in chondrocytes in vitro by inducing Tgfβ-activated kinase (Tak1; Map3k7), activating p38 MAPK (Mapk1). The relative roles of canonical versus noncanonical pathways, and whether they act independently, cooperate (Qiao et al., 2005; Reilly et al., 2005; Stanton et al., 2004) or antagonize (Hoffmann et al., 2005) each other in chondrocytes, are unknown.

FGFs inhibit chondrocyte proliferation (Ornitz, 2005; Murakami et al., 2004; Raucci et al., 2004; Sahni et al., 2001), and the BMP and FGF pathways antagonize each other in cartilage (Minina et al., 2005; Yoon et al., 2006). FGFs reduce Bmp4 and Ihh expression through undefined pathways (Chen et al., 2001; Naski et al., 1998). Phosphorylation of the Smad linker region represents one potential mechanism of FGF-mediated antagonism. The linker region contains consenus sites for phosphorylation by Erk1/2 (Mapk3/1), leading to inhibition of Smad activity (Fuentesalba et al., 2007; Kretzschmar et al., 1997; Peri et al., 2003; Sapkota et al., 2007).

The secreted factor Indian hedgehog (Ihh) is expressed in the prehypertrophic zone, and maintains chondrocyte proliferation by promoting Pthrp (Pthlh) expression in distal cells of the cartilage anlagen. PTHrP binds to the PTHrP receptor (PPR; Pthr1r) and negatively regulates Ihh expression in a feedback loop (Kronenberg, 2003). The Ihh/PTHrP pathway acts cooperatively with BMPs (Kronenberg, 2003; Minina et al., 2001; Pathi et al., 1999; Grimsrud et al., 2001; Pateder et al., 2000), and BMP receptor Smads can directly activate the Ihh promoter (Seki and Hata, 2004).

We show here that ablation of Smad1 and Smad5 in mice results in a nearly complete block in chondrocyte differentiation, and in imbalances in signaling cross-talk between the BMP, FGF and Ihh/PTHrP pathways. This is in marked contrast to the mild phenotype in mice lacking Smad4 in cartilage (Zhang et al., 2005). These results demonstrate that canonical Smad signaling is the major mechanism of BMP signal transduction in endochondral bone, and that Smad1 and Smad5 are key regulators of BMP canonical signaling in the growth plate. The data also demonstrate that Smad4 is not required for the majority of canonical BMP

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signaling. Finally, we provide evidence that linker phosphorylation of Smads represents a physiologically significant mechanism regulating BMP signaling in the growth plate, but that the inhibitory effects of EGFRs are likely to be mediated through different mechanisms.

MATERIALS AND METHODS

Generation of Smad1<sup>CKO</sup>-Smad5<sup>CKO</sup>-Smad8<sup>−/−</sup> mice

Smad1 (Huang et al., 2002) and Smad5 (Umans et al., 2003) floxed mice were crossed with Col2-Cre (Ovchinnikov et al., 2000) to generate Smad1<sup>fl/fl</sup>;Col2-Cre and Smad5<sup>fl/fl</sup>;Col2-Cre mice (referred to as Smad1<sup>CKO</sup> and Smad5<sup>CKO</sup>, respectively; CKO, cartilage-specific knockout), and intercrossed with Smad8<sup>−/−</sup> mice (a gift from Richard Behringer, M. D. Anderson Cancer Center, Houston, TX, USA). Smad1<sup>fl/fl</sup>;Smad5<sup>fl/fl</sup>;Smad8<sup>−/−</sup>;Col2-Cre mice were intercrossed to generate Smad1<sup>CKO</sup>;Smad5<sup>CKO</sup>;Smad8<sup>−/−</sup>;Col2-Cre mice (Smad1<sup>1CKO</sup>;Smad8<sup>−/−</sup>).

Histology

Skeletal preparations were generated as described (Ivkovic et al., 2003; Yoon et al., 2006). Alcian Blue/nuclear Fast Red staining was performed as described (Luna, 1992). Von Kossa staining was performed by incubation in 1% silver nitrate under UV light for 20 minutes and counterstaining with nuclear Fast Red. Safranin O staining was performed by staining in Weigert’s iron hematoxylin solution for 10 minutes, followed by Fast Green (0.001%) and Safranin O (0.1%) for 5 minutes each.

For immunofluorescence, sections were boiled for 15 minutes in citrate buffer (Ivkovic et al., 2003). Sections were blocked with 5% goat or donkey serum for 1 hour and incubated with primary antibody overnight at 4°C, followed by incubation with secondary antibody for 1 hour at room temperature, then with fluorophore for 30 minutes at room temperature. Primary antibodies were as follows: phospho-Smad1/5/8 and phospho-Smad1/5 (Cell Signaling Technology); type II collagen and Ptf1r (Abcam); type I collagen (Southern Biotech); type X collagen (a kind gift from Robin Poole, Shriners Hospitals for Children, Montreal, Quebec, Canada); aggrecan (Developmental Studies Hybridoma Bank, Iowa City, USA); Pena (Zymed); Fgf1 and Stat1 (Sigma); phospho-Smad1L (a kind gift from Eddy De Robertis, University of California, Los Angeles, CA, USA). Secondary antibodies were conjugated with AlexaFluor-555 and AlexaFluor-488. Sections were counterstained with DAPI (Vectorshield). For TUNEL staining, the fluoroscence In Situ Cell Death Detection Kit (Roche) was used according to the manufacturer’s protocol. In situ hybridization was performed as described (Song et al., 2007).

Limb culture

Embryos were harvested at 16.5 days of gestation (E16.5). Forelimbs were isolated and cultured as described (Minina et al., 2001; Minina et al., 2002). The contralateral limb was cultured in the presence of recombinant human Ihh and 994 bp Ihh promoters were gifts from Akiko Hata (Seki and Hata, 2004) and Toshihisa Komori (Yoshida et al., 2004). The Smad1 expression constructs were gifts from Eddy De Robertis (Pera et al., 2003). Cells were stimulated with recombinant human BMP2, FGF2 (R&D Systems), ERK inhibitor (PD98059, 10 μM; Calbiochem) or p38 inhibitor (SB202190, 10 μM; Calbiochem). BMP2 was used at 100 ng/ml. Unless otherwise stated, FGF2 was used at 10 ng/ml. Induction was measured by the dual luciferase assay. All experiments were performed in triplicate and repeated three times; representative experiments are shown. Statistical significance was assessed by Student’s t-test, *P<0.05.

Primary sternal chondrocytes were isolated as described (Lefebvre et al., 1994). Cells were seeded at 1×10<sup>4</sup> cells/well in 6-well plates, and cultured in MEMalpha supplemented with 10% FBS and pen/strep. For western blot and RT-PCR, cells were serum starved in MEMalpha containing 1% FBS overnight, then stimulated the next day with 50-100 ng/ml BMP2, 10 ng/ml FGF2, or 10 ng/ml noggin (R&D Systems) for 60 minutes. For immunocytochemistry, cells were trypsinized and seeded at 4×10<sup>4</sup> cells/well into an 8-well chamber slide overnight.

RESULTS

Loss of BMP receptor Smads leads to severe chondrodysplasia

Mice harboring cartilage-specific deletions of Smad1 and Smad5 (Smad1<sup>1CKO</sup> Smad5<sup>1CKO</sup>) were generated as described in the Materials and methods. Phosphorylated (activated) BMP receptor Smad (pSmad) staining was abundant in wild-type (WT) growth plates but was undetectable in mutants (Fig. 1A,B). Western blot analysis of extracts from WT and mutant growth plates demonstrated loss of total and activated Smad1 and Smad5 (Fig. 1C). When sternal chondrocytes were examined, pSmads 1 and 5 stained the nuclei of total and activated Smad1 and Smad5 (Fig. 1D,E). Thus, Smad1 and 5 are excised efficiently in cartilage.

Mice deleted for individual Smads, or harboring heterozygous allelic combinations, were recovered in Mendelian ratios and showed no abnormalities (Fig. 2). Mice harboring combined deletions of Smad1 and Smad8 (Smad1<sup>1CKO</sup>;Smad8<sup>−/−</sup>) and mice with only one functional allele of Smad5 were recovered in Mendelian ratios and showed no abnormalities (Fig. 2). RT-PCR and western analysis of growth plate cartilage RNA was extracted from proximal humeri using the RNeasy Kit (Qiagen). Synthesis of cDNA was performed with Superscript III (Invitrogen). Reverse transcriptase (RT)-PCR reactions comprised 35-42 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute. For western blotting, growth plate cartilage was isolated and homogenized in RIPA buffer. Whole-tissue lysates were run on 10-15% SDS-polyacrylamide gels.

Cell culture

Rat chondrosarcoma (RCS) cells were cultured and transfected as described (Yoon et al., 2006). The 1.8 kb fragment of the mouse Mx2 promoter has been described previously (Brugger et al., 2004). The mouse proximal 2HC8 and 994 bp Ihh promoters were gifts from Akiko Hata (Seki and Hata, 2004) and Toshihisa Komori (Yoshida et al., 2004). The Smad1 expression constructs were gifts from Eddy De Robertis (Pera et al., 2003). Cells were stimulated with recombinant human BMP2, FGF2 (R&D Systems), ERK inhibitor (PD98059, 10 μM; Calbiochem) or p38 inhibitor (SB202190, 10 μM; Calbiochem). BMP2 was used at 100 ng/ml. Unless otherwise stated, FGF2 was used at 10 ng/ml. Induction was measured by the dual luciferase assay. All experiments were performed in triplicate and repeated three times; representative experiments are shown. Statistical significance was assessed by Student’s t-test, *P<0.05.

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Fig. 1. Cartilage-specific excision of mouse Smad1 and Smad5. Smad1 and Smad5 are excised in cartilage of Smad1<sup>1CKO</sup> Smad5<sup>1CKO</sup> mutants. (A,B) Immunofluorescence analysis of C-terminal Smad phosphorylation (pSmad1/5) in wild-type (WT) (A) and mutant (B) cartilage, counterstained with DAPI. Arrows demarcate the border of the perichondrium. In the mutant, blood vessels autofluoresce red and delineate the location of the perichondrium. (C) Western blot analysis of microdissected WT and mutant growth plate lysates for total and phosphorylated forms of Smad1 and Smad5 (C-termius, pSmad1/5C; linker region, pSmad1L). (D,E) Immunofluorescence of pSmad1/5 in cultured WT (D) and mutant (E) primary sternal chondrocytes.
(Smad1CKO;Smad5CKO;Smad8−/−) were also normal (Fig. 2, and data not shown), demonstrating that a single allele of Smad5 is sufficient to transduce BMP signals in cartilage. The above results suggest functional redundancy between Smads. This was confirmed by the phenotypes of Smad1/5 double and Smad1/5/8 triple mutants (Fig. 2). Col2-Cre is expressed in the axial skeleton at E9.5 (Ovchinnikov et al., 2000). Skeletal preparations revealed the absence of an axial skeleton in double and triple mutants (Fig. 2). Vertebral bodies were replaced by fibroblasts and loose aggregates of chondrocytes (Fig. 3A,B), similar to the phenotype seen in mice lacking Bmpr1a and Bmpr1b (Yoon et al., 2005). MicroCT analyses of newborn mice demonstrated severely reduced mineralization. Consistent with the later onset of Col2-Cre expression in appendicular elements, chondrogenesis proceeded to a greater extent in long bones than in axial elements in mutants. Shortened long bones consisting of a thin cartilage rod surrounded by a thick bone collar were seen. Intramembranous elements (skull and clavicles) ossified normally, confirming that Smad1/5 deletion was restricted to the expression domain of Col2-Cre (Fig. 3C,D).

Skeletal preparations revealed no major differences between Smad1/5CKO double and Smad1/5CKO;Smad8−/− triple mutants (Fig. 2). Similarly, no additional defects were seen in Smad1/5CKO;Smad8−/− and Smad5CKO;Smad8−/− mice as compared with Smad1CKO and Smad5CKO mice (not shown). However, cartilage condensations were slightly smaller in Smad1/5CKO;Smad8−/− triple mutants as compared with Smad1/5CKO;Smad8+/− mice (Fig. 3E-G). Thus, although Smad8 may play a role in chondrogenesis, its contribution is minor.

Smad1 and Smad5 are required for limb development

Because Smad8 has a minor role in chondrogenesis, subsequent analysis focused on Smad1/5CKO mice. In WT embryos, concentric layers of elongated fibroblasts surround the cartilage (Fig. 4A,B). There were fewer layers around condensations in mutants, suggesting that the initial stages of condensation require canonical Smad signaling for cell recruitment. Long bones were shorter in E14.5 Smad1/5CKO mutants than in the WT. Cells in the center of the cartilage anlagen exhibited a hypertrophic morphology, but were smaller than in WT littermates (Fig. 4C,D), and DAPI staining (Fig. 4E,F) revealed that cells in mutant growth plates were more densely packed. This can be attributed to impaired type II collagen production. Moreover, a thicker type I collagen-producing perichondrium was seen in mutants (Fig. 4F).

Distinct zones of resting, columnar and hypertrophic chondrocytes can be seen in WT growth plates by E16.5 (Fig. 4G). Loss of Smad1/5 led to growth plate disorganization and loss of hypertrophic chondrocytes (Fig. 4H). It is likely that the hypertrophic chondrocytes seen at E14.5 in mutants are descendents of cells that were committed to differentiation prior...
to completion of Cre-mediated excision of all four alleles of Smad1 and Smad5, and by E16.5 these cells are cleared from the growth plate.

The bone collar forms in the perichondrium surrounding prehypertrophic and hypertrophic chondrocytes in E16.5 WT mice (Fig. 4I). A bone collar formed in mutants, but there was no sign of mineralization in the marrow cavity (Fig. 4J; see Fig. S1 in the supplementary material). Osteoblasts failed to invade mutant cartilage, leading to excessive bone formation in the collar. Consistent with a block in osteoblast recruitment into the cartilage, mutants exhibited an expanded domain of type I collagen-producing cells (Fig. 4K,L). These cells were randomly oriented, unlike the structure of the WT perichondrium, in which they are perpendicular to the growth plate. Moreover, type II collagen-producing cells, which are normally restricted to the growth plate, were embedded in the mutant periosteum (Fig. 4K,L; inset in Fig. 4L).

There was no evidence of stratification of chondrocytes in Smad1/5CKO mutants up to P0 (Fig. 4M,N). The lack of trabecular bone and vascular invasion persisted in mutants. Cortical bone extended into the marrow cavity and surrounded the rudimentary cartilage template (Fig. 4M,N). Hence, loss of canonical Smads leads to a failure in osteoblast invasion into the cartilage. Consistent with the presence of chondrocytes embedded in the thickened periosteum (Fig. 4L), ectopic cartilage formation at the edge of the bone collar in mutants (Fig. 4M,N).

BMP canonical Smad signaling is required for chondrocyte proliferation, survival and differentiation

Mutant chondrocytes were rounder and more densely packed in mutants than in WT littermates (Fig. 5A,B), and the hypertrophic zone was absent (Fig. 5C,D). PcnA staining revealed little proliferation in mutants (Fig. 5E,F), and was confined to the perichondrial cells. Histomorphometric analysis revealed no differences in the percentage of PcnA-positive cells in the perichondrium of WT and mutant littermates (data not shown). Apoptosis is normally confined to the hypertrophic zone (Fig. 5G). However, apoptosis was expanded in mutant cartilage (Fig. 5H). Thus, loss of BMP canonical Smad signaling leads to reduced chondrocyte proliferation and increased apoptosis.

Cartilage-specific extracellular matrix (ECM) proteins are required for growth plate organization (Gustafsson et al., 2003; Li and Schwartz, 1995; Watanabe and Yamada, 1999). Smad1/5CKO mutants exhibited no defects in proteoglycan production as assessed by Alcian Blue or Safranin O staining (Fig. 4; Fig. 5; Fig. 6A,B); however, whereas aggrecan is present in the WT growth plate (Fig. 6C), it was sporadic and intracellular in mutants (Fig. 6D). Smad1/5CKO mice also exhibited a severe reduction in type II collagen deposition, suggesting that mutant chondrocytes are not fully differentiated (Fig. 6E,F). Type X collagen is produced in hypertrophic chondrocytes (Fig. 6G), but little could be seen in Smad1/5CKO mutants (Fig. 6H), suggesting a defect in terminal differentiation.

The transcription factor Sox9 is required for chondrocyte survival and expression of ECM components (Bell et al., 1997; Bi et al., 1999; Lefebvre et al., 1997). RT-PCR analysis revealed decreased levels of Sox9 in mutant cartilage (Fig. 6I), and that the deficits in collagens II and X in mutants occur at the transcriptional level. The detectable, albeit decreased, expression of Col10a1 and Runx2 (Fig. 6I) suggests that at least a few cells with the characteristics of hypertrophic chondrocytes were present in mutants. However, these cells were not organized into a distinct layer (Fig. 6A,B). Expression of alkaline phosphatase was examined to test whether the paucity of hypertrophic chondrocytes in mutants is due to accelerated
conversion of these cells to late hypertrophic chondrocytes. The decreased level of alkaline phosphatase in mutants (Fig. 6I) argues against this possibility, and supports the alternative hypothesis that chondrocyte maturation is blocked. We tested this by examining \textit{Ucma} expression. \textit{Ucma} is a marker for upper (resting) chondrocytes (Tagariello et al., 2008; Surmann-Schmitt et al., 2008). \textit{Ucma} was expressed as robustly in mutant as in WT cartilage (Fig. 6I). Hence, \textit{Smad1/5CKO} chondrocytes are impaired in their ability to undergo terminal differentiation and retain characteristics of resting chondrocytes.

\textit{Smad1/5CKO} mice exhibit a thick bone collar with an expanded domain of type I collagen expression. In accordance, elevated levels of \textit{Col1a1} were detected in mutant cartilage by RT-PCR (Fig. 6I).

\textit{Smad1/5CKO} mutants also exhibited increased expression of osteocalcin (\textit{Bglap1/2}) (Fig. 6I). This is likely to be due to the excessive accumulation of osteoblasts in the mutant perichondrium as a result of their inability to invade the mutant cartilage. The defect in osteoblast invasion is likely to be a consequence of the block in cartilage differentiation in \textit{Smad1/5CKO} mutants.

Fig. 5. Growth plate disorganization and impaired chondrocyte survival in \textit{Smad1/5CKO} mutants. (A,B) Autofluorescence (green) and DAPI (blue) staining in E17.5 WT (A) and mutant (B) mouse proximal tibial growth plates. (C,D) Safranin O staining of E17.5 WT (C) and mutant (D) tibial growth plates. Double-headed arrows demarcate the borders of the perichondrium. (E,F) PcnA immunofluorescence for aggrecan. (G,H) Immunofluorescence for type II collagen in growth plates. Arrow indicates restricted type II collagen production in the mutant. (I,J) Images in C, E and G are adjacent sections; images in D, F and H are adjacent sections.

Loss of canonical Smad proteins disrupts expression of BMP signaling components

The \textit{Smad1/5CKO} phenotype is similar to that of \textit{Bmpr1aCKO;Bmpr1b–/–} mice (Yoon et al., 2005), suggesting that the canonical pathway is the major transducer of BMP signals in cartilage. Unexpectedly, the \textit{Smad1/5CKO} phenotype is markedly more severe than the \textit{Smad4CKO} phenotype (Zhang et al., 2005), even though \textit{Col2-Cre} was used to drive cartilage-specific excision in both models. These divergent phenotypes challenge the dogma that Smad4 is required to mediate canonical Smad signaling in chondrogenesis.

Smad4 and BMP receptor expression was examined in mutant cartilage to investigate the basis for this divergence (Fig. 6J). RT-PCR analysis indicated that \textit{Smad4} is expressed in mutant cartilage. Therefore, the severe chondrodysplasia in \textit{Smad1/5CKO} mice does not correlate with altered \textit{Smad4} expression. However, the expression of type II (\textit{Bmpr2}) and type I (\textit{Bmpr1a} and \textit{Bmpr1b}) BMP receptors was reduced. Thus, the similarity in the receptor-deficient and Smad-deficient cartilage phenotypes might be a consequence of reduced BMP receptor expression in \textit{Smad1/5CKO} mutants. The results reveal the presence of a positive-feedback loop...
involving BMP receptors and BMP receptor Smads. \textit{Acvr1}, the gene encoding the type I BMP receptor ActRI (Alk2), is expressed at apparently normal levels in mutants, but ActRI alone is unable to support chondrogenesis in vivo (Yoon et al., 2005).

BMP signaling can activate noncanonical MAPK pathways in vitro in chondrocytes. The best characterized MAPK is p38, which is activated via Tak1 and Mekk3 (Map3k3) (Kimura et al., 2000; Qiao et al., 2005). RT-PCR analysis revealed reduced Tak1 and Mekk3 expression in mutants (Fig. 6f). The basis for this decrease is unclear, but might be related to reduced BMP receptor expression (Fig. 6f). Regardless of the mechanism, the results suggest that loss of canonical signaling does not lead to compensatory upregulation of TAK-mediated noncanonical pathways.

The BMP canonical Smad pathway is required for the Ihh/PTHrP signaling loop in vivo

Ihh and PTHrP form a signaling loop in the growth plate that regulates chondrocyte proliferation and differentiation. This loop is modulated by BMP and FGF pathways; transgenic mice overexpressing FGFs in cartilage display decreased expression of \textit{Bmp4}, \textit{Ihh} and PTHrP receptor (PPR) (Chen et al., 2001; Naski et al., 1998). FGFs also inhibit BMP- and Ihh-mediated proliferation in limb cultures (Minina et al., 2002). We showed previously that Ihh signaling is positively regulated by BMPs in vivo (Yoon et al., 2006). We extended this analysis by examining Ihh signaling in primary chondrocytes and \textit{Smad1/5}\textsuperscript{CKO} mutants. RT-PCR analysis showed that BMPs induce both \textit{Ihh} and \textit{PPR} expression. By contrast, \textit{Pthrp} expression was only moderately increased by BMPs (Fig. 7a). We then tested whether the Ihh signaling loop is impaired in mutant cartilage. \textit{Pthrp} mRNA levels were only slightly reduced in \textit{Smad1/5}\textsuperscript{CKO} cartilage, but \textit{Ihh} and \textit{PPR} mRNAs were not detected (Fig. 7b). These results confirm that \textit{Ihh} is a BMP target in the growth plate, and suggest that \textit{PPR} is as well.

\textit{Pthrp} transcripts are present in chondrocytes underlying the articular surface in WT elements at E165, as reported previously (Chen et al., 2006) (Fig. 7g,h). \textit{Pthrp} mRNA was also seen in \textit{Smad1/5}\textsuperscript{CKO} mutants (Fig. 7l,j). \textit{Ihh} is expressed in the prehypertrophic zone of WT growth plates; however, no \textit{Ihh} mRNA was detectable in mutants (Fig. 7c-f). Retention of \textit{Pthrp} expression in the apparent absence of \textit{Ihh} in \textit{Smad1/5}\textsuperscript{CKO} mutants was unexpected, as it has been shown that Ihh is normally required to maintain \textit{Pthrp} expression (Vortkamp et al., 1996; Lanske et al., 1996; Chung et al., 1998; St Jacques et al., 1999; Chung et al., 2001). Hence, we examined \textit{Ihh} expression at E14.5 in mutants to test the possibility that \textit{Ihh} might be present at higher levels at earlier stages. Cells resembling prehypertrophic and hypertrophic chondrocytes were present in \textit{Smad1/5}\textsuperscript{CKO} mutants (see Fig. S2a-d in the supplementary material). Low levels of \textit{Ihh} expression were detectable in the E14.5 mutant tibia, and \textit{Pthrp} was expressed in periarticular cells (see Fig. S2c,d in the supplementary material). \textit{Ihh} mRNA was also detected in E14.5 WT and mutant digits (see Fig. S3 in the supplementary material). We conclude that \textit{Ihh} is expressed in mutants, but that the level of expression declines during development, most likely owing to the gradual loss of the prehypertrophic cells that had formed prior to complete Cre-mediated recombination. \textit{Ihh} expression might be too restricted and/or disorganized to detect at E16.5, but still present at a sufficient level to maintain \textit{Pthrp} expression. To test this latter possibility, we performed in situ hybridization for patched 1 (\textit{Ptch1}), a sensitive readout of \textit{Ihh} signaling. In WT growth plates, \textit{Ptch1} is expressed in proliferating chondrocytes, as well as in adjacent perichondrium (Fig. 7m,n). Consistent with decreased \textit{Ihh} signaling, \textit{Ptch1} mRNA was detectable, albeit at much lower levels, in the mutant growth plate (Fig. 7o,p).

\textit{PPR} was not expressed at detectable levels in \textit{Smad1/5}\textsuperscript{CKO} growth plates (Fig. 7k,l), but was readily detected in mutant osteoblasts. Hence, \textit{Ihh} and PPR, which are highly induced by BMPs in vitro (Fig. 7a), require canonical Smads for expression in cartilage in vivo. The greatly diminished expression of \textit{PPR} and \textit{Ihh}, which are expressed in late columnar and prehypertrophic chondrocytes (MacLean and Kronenberg, 2005), is consistent with a block in chondrocyte differentiation beyond the resting phase in mutants.

\textbf{DEVELOPMENT}
 Canonical Smad signaling is required for BMP and FGF antagonism in chondrocytes

FGF signaling is elevated when BMP signaling is blocked through ablation of Bmpr1a and Bmpr1b (Yoon et al., 2006). The domain of Fgfr1 expression was expanded in Smad1/5<sup>CKO</sup> cartilage (Fig. 8A,B), as is the case in Bmpr1a/b<sup>CKO</sup> mice (Yoon et al., 2006). FGF receptor expression was also analyzed by RT-PCR (Fig. 6J). Fgfr1 levels were increased in Smad1/5<sup>CKO</sup> cartilage, but Fgfr2 and Fgfr3 levels were decreased. Fgfr1 mRNA is expressed in resting/periaricular and hypertrophic chondrocytes, Fgfr2 is expressed in proliferating chondrocytes, and Fgfr3 is expressed in proliferating and prehypertrophic chondrocytes (Minina et al., 2005). Hence, the absence of Fgfr2 and Fgfr3 expression in Smad1/5<sup>CKO</sup> mutants is consistent with defective formation of proliferating, prehypertrophic and hypertrophic chondrocytes. Elevated expression of Fgfr1 mRNA and protein (Fig. 6J; Fig. 8B) and of Ucma mRNA (Fig. 6J) in mutant chondrocytes indicates that they retain characteristics of resting cells.

FGFs signal through Stat1 and Erk1/2 in chondrocytes (Legeai-Mallet et al., 2004; Sahni et al., 1999). Stat1 was observed in the hypertrophic zone and in the articular and lateral edges of the growth plate in WT mice (Fig. 8C). An expanded domain of Stat1 expression was seen in mutants (Fig. 8D). In its inactive state, Stat1 is excluded from the nucleus; activation by FGFs and other pathways leads to Stat1 phosphorylation and nuclear entry. In WT growth plates, Stat1 staining is localized primarily in the cytoplasm and at the cell membrane (Fig. 8E). By contrast, nuclear localization of Stat1 was apparent in mutants, consistent with activation of Stat1 signaling (Fig. 8F). In summary, FGF signaling is enhanced by loss of canonical Smad signaling in Smad1/5<sup>CKO</sup> mutants.

FGF signaling exhibits functional antagonism with BMP signaling through ERK-mediated pathways

BMPs and FGFs are antagonistic in the growth plate. To investigate potential mechanisms, we used a 1.8 kb Msx2 promoter fragment (Brugger et al., 2004) linked to luciferase (1.8 kb Msx2-luc) as reporter in chondrocytic RCS cells (Fig. 9A). Levels of BMP-mediated induction varied from 5-fold to 28-fold in different experiments. Various factors may contribute to this variability, including the passage number of the RCS cells and the batch of BMP used. BMP-mediated induction of the 1.8 kb Msx2 promoter was also antagonized by FGF2 in a dose-dependent manner (Fig. 9A). An ERK inhibitor (PD98059) had no effect on basal (not shown) or BMP-mediated expression of 1.8 kb Msx2-luc, but was able to block the ability of FGF2 to antagonize BMP2 induction of the promoter (Fig. 9B). In fact, treatment with PD98059 led to a synergistic increase in promoter activity in the presence of BMP2 plus FGF2 (55-fold) as compared with BMP2 alone (6.5-fold). The basis for this synergy is unknown, but might reflect an effect of PD98059 on other signaling pathways, and/or the ability of non-ERK/MAPK pathways activated by FGF2 to synergize with BMP pathways to induce the 1.8 kb Msx2 promoter. A p38 MAPK inhibitor (SB202190) had no effect on BMP2-mediated induction. These experiments indicate that FGFs can antagonize canonical BMP signals via ERK/MAPK pathways.

ERK/MAPK can phosphorylate the linker regions of Smads, leading to Smad degradation (Fuentesalba et al., 2007; Pera et al., 2003; Sapkota et al., 2007). We overexpressed constructs encoding a WT Smad1 (Smad1WT), or a version in which the Erk1/2 phosphorylation sites in the linker region have been mutated so that they cannot be phosphorylated (Smad1LM) (Fig. 9C), to test whether this mechanism might account for ERK/MAPK-mediated inhibition of Msx2 promoter activity (Fig. 9D). Smad1WT and Smad1LM enhanced BMP-mediated Msx2 promoter activity ~2-fold (Fig. 9D). FGF2 treatment led to a slight induction in the experiment shown, but in other cases no induction was seen (Fig. 9A,B, and data not shown). The presence of Smad1WT and Smad1LM did not alter the effects of FGF (Fig. 9D). As expected, both Smad1WT and Smad1LM led to enhanced responsiveness to BMP2, and no significant difference was noted in the ability of either Smad construct to enhance this responsiveness. Moreover, both Smad constructs antagonized the effects of FGF2 on BMP2-mediated induction of the Msx2 promoter. In fact, the presence of Smad1LM led to a synergistic activation of Msx2 promoter activity by FGF2 and BMP2 as compared with BMP2 alone (Fig. 9D). This result provides additional evidence that FGF-mediated pathways may have positive effects on Msx2 promoter activity when ERK-mediated effects on linker phosphorylation are prevented.

Ihh is a target of the BMP and FGF pathways in chondrocytes (Naski et al., 1998; Minina et al., 2002), and the Ihh promoter contains Smad binding sites (Seki and Hata, 2004; Yoon et al., 2006). We therefore examined whether Ihh induction might be antagonized by FGFs via Smad linker phosphorylation. A 430 bp Ihh proximal promoter fragment (2HC8-luc) that is Smad1/5-responsive in some cell types (Seki and Hata, 2004) was poorly responsive in primary and RCS chondrocytes (data not shown). However, a longer, 994 bp Ihh promoter was responsive to BMP2, and was antagonized by FGF (Fig. 9E). Cells expressing Smad1WT or Smad1LM showed enhanced BMP2-mediated induction. Higher Ihh promoter activity was seen in response to BMP in the presence of Smad1LM than Smad1WT, and, in the case of Smad1LM, this induction was resistant to FGF antagonism (Fig. 9E). Thus, BMP-mediated induction of Ihh might be negatively regulated by ERK/MAPK through Smad linker phosphorylation.
Smad proteins are phosphorylated at both the C-terminus and the linker region in chondrocytes

The above results suggest that Smad1/5 linker phosphorylation accounts for the antagonistic effects of FGFs. However, whether this occurs in the growth plate is unclear. Therefore, we analyzed whether FGFs antagonize BMP signaling at the level of Smad activity in cultured limbs. Levels of activated (C-terminal-phosphorylated) nuclear Smads were highest in proliferating chondrocytes and at the edges of the growth plate in unstimulated limbs (Fig. 10A,C). FGF18 treatment reduced C-terminal phosphorylation throughout the growth plate (Fig. 10B), whereas inhibition of endogenous FGF signaling using the FGF receptor antagonist SU5402 led to activation (C-terminal phosphorylation) of Smad1/5 (Fig. 10D). Thus, FGFs inhibit C-terminal phosphorylation of Smads.

Linker phosphorylation was examined using an antibody specific to phosphorylated serine residues in the Smad1 linker region (pSmad1L) (Fuentealba et al., 2007). pSmad1L was detected in the cytoplasm and nuclei of proliferating chondrocytes and perichondrium (see Fig. S4A in the supplementary material). In contrast to pSmad1/5, pSmad1L was not detected in epiphyseal and hypertrophic chondrocytes. Thus, there are differences in the relative levels of pSmad1/5 and pSmad1L in different regions of the growth plate. No change in linker phosphorylation was detected upon FGF18 stimulation (Fig. 10E,F; see Fig. S4 in the supplementary material). Furthermore, treatment with the FGFR inhibitor SU5402 did not reduce pSmad1L levels (Fig. 10G,H). These results indicate that the activation of Smads by C-terminal phosphorylation is negatively regulated in the growth plate by FGF pathways. However, levels of Smad linker phosphorylation in the growth plate are regulated independently of FGFs, or, alternatively, FGF-mediated phosphorylation requires the activity of other factors.

Western blot analysis of primary chondrocytes confirmed that FGF stimulation does not induce linker phosphorylation, even though Erk1/2 activity is elevated (Fig. 10I). Linker and C-terminal Smad1 phosphorylation was observed only after BMP stimulation (Fig. 10I). These data are consistent with recent demonstrations that BMP signaling directly induces Smad linker phosphorylation following C-terminal phosphorylation and nuclear translocation (Fuentealba et al., 2007; Sapkota et al., 2007).

If Smad linker phosphorylation is catalyzed primarily by BMP pathways in chondrocytes, then nuclear colocalization of C-terminal- and linker-phosphorylated Smads is expected. Consistent with this prediction, Smad1 linker phosphorylation occurred primarily in the nucleus (Fig. 10J). Collectively, these data indicate that Smad linker phosphorylation is primarily a consequence of BMP-mediated canonical pathway activation in cartilage, and most likely represents a feedback mechanism to control the duration of BMP signaling. Moreover, despite widespread pSmad1/5 localization, modulation of pathway activity by linker phosphorylation appears to be restricted to the proliferative zone.
DISCUSSION
Smad1 and Smad5 are functionally redundant and required for endochondral bone formation

The phenotype of mice lacking Smads 1, 5 and 8 in cartilage demonstrates that canonical BMP signaling is required for chondrogenesis. Whereas mice lacking individual Smads in cartilage appear normal, simultaneous loss of Smad1 and Smad5 (Smad1/5/8 CKO) leads to severe chondrodysplasia. Deletion of Smad8 alone, or in combination with Smad1 or Smad5, does not lead to apparent defects. These findings are consistent with other reports that the phenotypes of Smad1- or Smad5-null mice are not altered by deletion of Smad8 (Arnold et al., 2006). Thus, although the conservation of Smad8 in vertebrates indicates a function, mutational analyses have not yet revealed this role. Developing cartilage is one of the tissues in which Smad8 is most highly expressed (Arnold et al., 2006); the Smad1/5/8 triple knockout exhibits slightly smaller cartilage condensations than those in Smad1/5 CKO mutants. It is conceivable that Smad8 plays a more prominent role in cartilage in adults.

It is thought that Smad4 is required for the majority of canonical BMP signaling (Feng and Derynck, 2005; Ross and Hill, 2008). The severe and lethal growth plate phenotype of Smad1/5 CKO mice compared with the mild and viable phenotype of Smad4 CKO mice (Zhang et al., 2005) was thus unexpected. In vitro (Sirard et al., 2000; Subramaniam et al., 2004) and in vivo (Wisotzkey et al., 1998; Chu et al., 2004) studies have shown that some Tgfβ/Smad-dependent processes occur in the absence of Smad4. R-Smads can form homotrimers that do not contain Smad4 (Wu et al., 1997; Chacko et al., 2001), but the signaling capacity of these complexes is unknown. A recent report described a Smad2-Smad3-Tif1γ transcriptional complex that has a function distinct from Smad2-Smad3-Smad4 heterotrimers (He et al., 2006), but another report characterizes Tif1γ (Trim33) as a Smad4 E3 ubiquitin ligase (Dupont et al., 2005). Thus, mechanisms underlying Smad4-independent canonical signaling in Tgfβ pathways remain unknown.

Even less is known about the requirement for Smad4 in canonical BMP pathways. Genetic studies reveal that some BMP receptor Smad-dependent processes occur in the absence of Smad4 (Chu et al., 2004; Wisotzkey et al., 1998). BMPs induce R-Smad nuclear translocation in Smad4-null colon cancer cells (Liu et al., 1997). A recent report demonstrated that BMPs transduce signals in a Smad4-independent manner through interaction of Smad1 with the Drosha complex, which promotes microRNA (miRNA) processing (Davis et al., 2008). The roles of specific miRNAs in chondrogenesis are unknown, however, and loss of the major miRNA processor, dicer 1, in cartilage leads to a less severe chondrodysplasia than is observed in Smad1/5 CKO mice (Kobayashi et al., 2008), indicating that other mechanisms underlie the Smad4-independent effects in Smad1/5 CKO mice.

C-terminal-phosphorylated Smad1/5/8 staining is most prominent at the lateral edges of the growth plate (e.g. Fig. 10A,C,D). This is consistent with studies demonstrating lateral expression of Bmp2, 4 and 7, and medial expression of the BMP inhibitor noggin (Minina et al., 2005). Taken together, these findings suggest that BMP ligands produced in the perichondrium signal through canonical pathways in a lateral-to-medial gradient.

Antagonism between BMP and FGF signaling pathways

This study demonstrates that canonical Smad signaling is required for growth plate formation and cross-talk with FGF and Ihh/PTHrP pathways, and that FGFs target canonical pathways by affecting the phosphorylation status of Smads. C-terminal Smad1/5 phosphorylation is reduced by FGF stimulation and elevated by FGF inhibition in limb cultures (Fig. 10). We demonstrate that FGF
signaling can activate ERK/MAPK pathways, which antagonize canonical Smad signaling in chondrocytes. On the other hand, we found that Mst2 promoter activity can be activated by non-ERK/MAPK pathways downstream of FGF. Hence, the interaction between BMP and FGF pathways can be both positive and negative for some target genes. The Ihh promoter is also a target of canonical Smads, and mutation of Smad linker phosphorylation sites attenuated FGF-mediated antagonism of Ihh in vitro. However, we saw no difference in the levels or localization of linker-phosphorylated Smad1 upon FGF stimulation (Fig. 10G-I), suggesting that although ERK/MAPK-mediated linker phosphorylation limits BMP signaling in vitro, FGFs might antagonize canonical pathways through a different mechanism in the growth plate. For example, FGFs might inhibit expression of BMP ligands and/or receptors, or activate a phosphatase that inactivates C-terminal-phosphorylated Smads in the growth plate.

Recent findings have shown that BMPs induce sequential Smad phosphorylations, first at the C-terminus and then in the linker region (Fuentealba et al., 2007; Sapkota et al., 2007). BMP-induced linker phosphorylation occurs independently of MAPK, and localizes to the nucleus, in contrast to MAPK-induced linker phosphorylation, which is seen mainly in the cytosol (Sapkota et al., 2007). The predominantly nuclear localization of linker-phosphorylated Smads in the growth plate, and the lack of a clear effect on this localization in response to FGF stimulation or inhibition, thus suggest that the majority of linker phosphorylation in the growth plate is mediated directly by BMPs. The high levels of nuclear linker-phosphorylated Smad1 in the growth plate are thus most likely to reflect rapid Smad turnover associated with active BMP signaling.

**BMP canonical Smad signaling regulates the Ihh/PTHrP signaling loop**

The Smad1/5CKO phenotype demonstrates that loss of BMP receptor Smad signaling disrupts Ihh/PTHrP signaling in the growth plate. Although Ihh levels are reduced, Phch1 expression can be detected, revealing that Ihh signaling does occur in mutants. Moreover, Phch transcripts have been detected in Ihh-deficient mice generated using a tamoxifen-inducible Col2-Cre allele (Maeda et al., 2007). Our findings, along with those of Maeda et al., raise the possibility that although Ihh is required for induction of Phch expression, Ihh-independent mechanisms might participate in the maintenance of Phch expression.

Loss of Ihh leads to premature hypertrophy, growth plate disorganization and a lack of osteoblasts in endochondral elements (St Jacques et al., 1999). Loss of PPR is also associated with accelerated differentiation (Lanske et al., 1996). Although Smad1/5 mutants exhibit diminished Ihh expression and undetectable PPR, chondrocyte differentiation is impaired. We have shown previously that loss of Bmprra and Bmpr1b leads to defects in the transition of chondrocytes from a resting to a columnar proliferating state, and to an inability of hypertrophic chondrocytes to complete terminal differentiation (Yoon et al., 2006). Hence, the diminished expression of PPR in Smad1/5 mutants might reflect a role for BMPs in commitment to differentiation. In this scenario, the absence of PPR does not lead to accelerated differentiation, owing to an earlier requirement for BMPs to permit differentiation. Our results are also consistent with the possibility that loss of PPR expression contributes to the growth plate defects in Smad1/5 mutants. Although accelerated differentiation is seen in PPR+/− mice at E18.5, chondrocytes in PPR−/− mice become hypertrophic later than in WT mice (Lanske et al., 1999), consistent with the differentiation defect in Smad1/5 mutants at midgestation stages. Moreover, in PPR−/− mice, chondrocytes are replaced by invading blood vessels and osteoblasts more slowly than in WT mice (Lanske et al., 1999), but loss of PPR ultimately leads to excess bone formation (Chung et al., 1998; Lance et al., 1996), similar to the ossification phenotype in Smad1/5 mutants. A third possibility is that BMPs have selective effects on signaling pathways independently of PPR. It has been shown that phospholipase C (PLC)-dependent signaling restrains chondrocyte proliferation and stimulates hypertrophic differentiation; these actions are opposite to the responses mediated by the cAMP/PKA (Prkaca) pathway downstream of PPR (Guo et al., 2002). Hence, BMPs may impact the balance between PLC and PKA pathway activation in chondrocytes. Finally, we cannot rule out the possibility that there is a low level of PPR expression in Smad1/5 mutant growth plates.

The greatly diminished expression of PPR in the growth plate raises the possibility that canonical BMP pathways act directly on the PPR promoter. Previous studies indicate that the highly conserved P2 promoter is the major PPR promoter in skeletal tissues (Bettoun et al., 1997; McCuaig et al., 1995). This promoter region contains multiple putative Smad-binding elements (data not shown). Additional studies are needed to determine whether these sites are active in chondrocytes.

Collectively, these studies demonstrate that canonical Smad signaling is the major BMP signal transduction pathway in chondrogenesis. If noncanonical BMP pathways play a role, they must do so in the context of active canonical signaling. Smads 1 and 5 are required to transduce canonical signals, are functionally redundant, required for all aspects of chondrogenesis, and do not require Smad4. Finally, our studies provide evidence that differential phosphorylation of canonical BMP receptor Smads might be an essential point of regulation of pathway activity in the proliferative zone of the growth plate.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/7/1093/DC1

**References**


