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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. *Smad1/5^{CKO}* (cartilage-specific knockout) mutant mice are nearly identical to *Smad1/5^{CKO};Smad8$^{-/-}$* mutants, indicating that Smads 1 and 5 have overlapping functions and are more important than Smad8 in cartilage. The *Smad1/5^{CKO}* phenotype is more severe than that of *Smad4^{CKO}* mice, challenging the dogma, at least in chondrocytes, that Smad4 is required to mediate Smad signaling through BMP pathways. The chondrodysplasia in *Smad1/5^{CKO}* 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 consensus sites for phosphorylation by Erk1/2 (Mapk3/1), leading to inhibition of Smad activity (Fuentealba et al., 2007; Kretzschmar et al., 1997; Pera 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; Pth1r) 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 FGFs are likely to be mediated through different mechanisms.

MATERIALS AND METHODS

Generation of *Smad1^{CKO};Smad5^{CKO};Smad8$^{-/-}$* mice

Smad1 (Huang et al., 2002) and *Smad5* (Umans et al., 2003) floxed mice were crossed with *Col2-Cre* mice (Ovchinnikov et al., 2000) to generate *Smad1$^{fx/fx}$;Col2-Cre* and *Smad5$^{fx/fx}$;Col2-Cre* mice (referred to as *Smad1^{CKO}* and *Smad5^{CKO}*, respectively; CKO, cartilage-specific knockout), and intercrossed with *Smad8$^{-/-}$* mice (a gift from Richard Behringer, M. D. Anderson Cancer Center, Houston, TX, USA). *Smad1$^{fx/+}$;Smad5$^{fx/+}$;Smad8$^{+/-}$;Col2-Cre* mice were intercrossed to generate *Smad1$^{fx/fx}$;Smad5$^{fx/fx}$;Smad8$^{-/-}$;Col2-Cre* mice (*Smad1/5^{CKO};Smad8$^{-/-}$*).

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 Pth1r (Abcam); type I collagen (Southern Biotech); type X collagen (a kind gift from Robin Poole, Shriners Hospitals for Children, Montreal, Québec, Canada); aggrecan (Developmental Studies Hybridoma Bank, Iowa City, USA); Pcna (Zymed); Fgfr1 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 (Vectashield). For TUNEL staining, the fluorescein 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 FGF18 (10 ng/ml; Invitrogen) or the FGFR inhibitor SU5402 (10 μM; Calbiochem). In all cases, the right forelimb served as the untreated control. A total of six limbs were examined for each condition, in two separate experiments.

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 *Msx2* 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 DeRobertis (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 1×10^6 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 reseeded at 4×10^5 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* (*Smad1/5^{CKO}*) 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 WT cells, but were nearly absent in mutant chondrocytes (Fig. 1D,E). Thus, Smads 1 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^{CKO};Smad8$^{-/-}$*), and mice with only one functional allele of *Smad5*

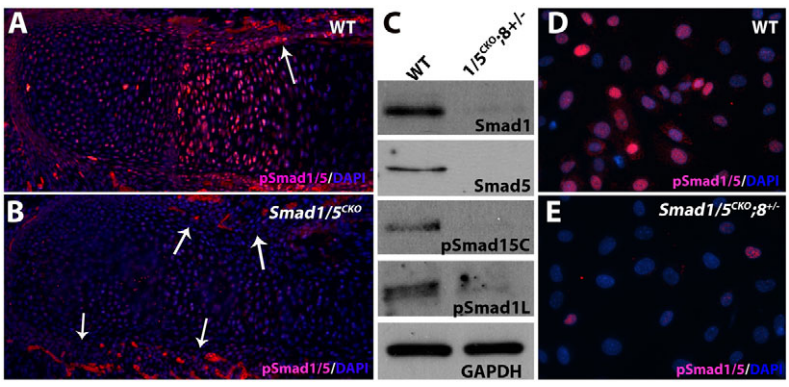

Fig. 1. Cartilage-specific excision of mouse *Smad1* and *Smad5*. *Smad1* and *Smad5* are excised in cartilage of *Smad1/5^{CKO}* mutants. (**A**,**B**) Immunofluorescence analysis of C-terminal Smad phosphorylation (pSmad1/5) in wild-type (WT) (A) and mutant (B) cartilage, counterstained with DAPI. 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-terminus, pSmad15C; linker region, pSmad1L). (**D**,**E**) Immunofluorescence of pSmad1/5 in cultured WT (D) and mutant (E) primary sternal chondrocytes.

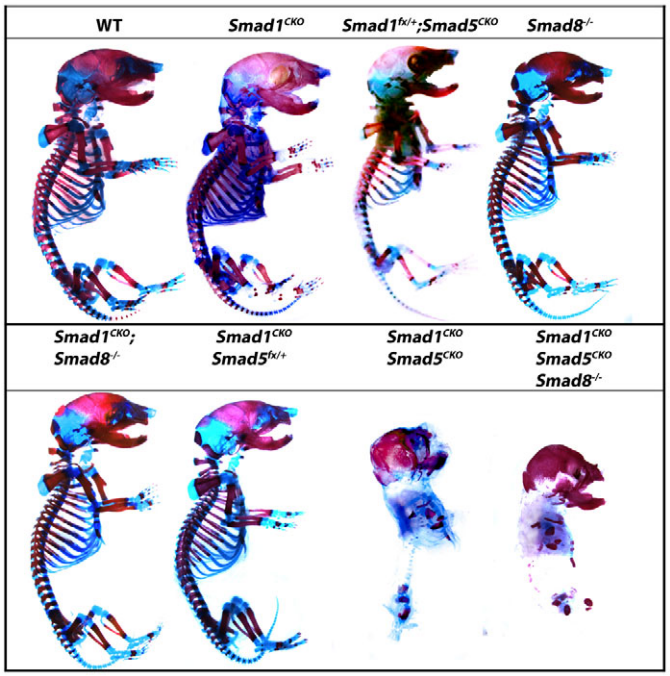

Fig. 2. Overlapping functions for Smad1 and Smad5 in cartilage. All cleared skeletal preps are of P0 mice. (Above) Mice lacking *Smad1*, *Smad5* or *Smad8* are viable and indistinguishable from WT littermates. (Below) Mice lacking *Smad1* and *Smad8* are viable and indistinguishable from WT littermates. Mice lacking *Smad1* and *Smad5* (*Smad1*^{CKO};*Smad5*^{CKO}) and *Smad1/5/8* triple mutants (*Smad1*^{CKO};*Smad5*^{CKO};*Smad8*$^{-/-}$) exhibit severe chondrodysplasia.

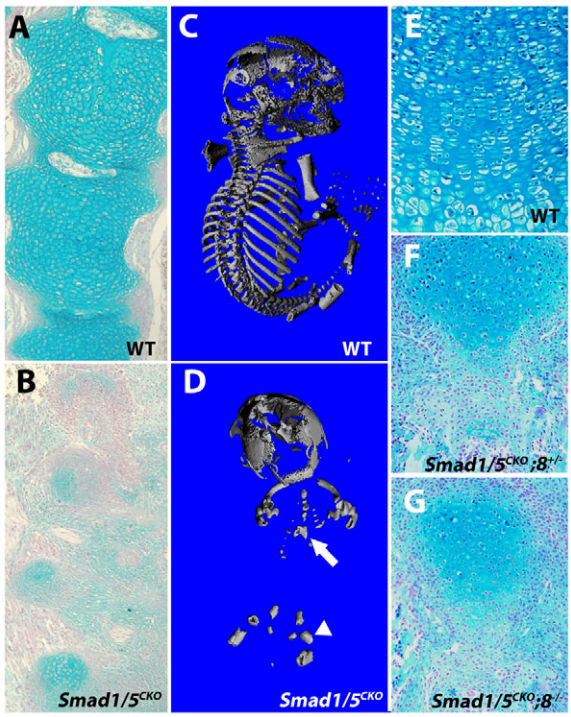

Fig. 3. Chondrodysplasia in *Smad1/5* double mutants and *Smad1/5/8* triple mutants. (**A**,**B**) Alcian Blue-stained sections through E16.5 vertebrae of WT and *Smad1/5*^{CKO} double-mutant littermate mice. (**C**,**D**) MicroCT analysis of P0 WT and *Smad1/5*^{CKO} littermates. Sternebrae are present, but malformed (arrow). Cortical bone is evident in appendicular elements of the mutant (arrowhead). (**E-G**) Alcian Blue-stained sections through E16.5 proximal tibiae. Sections through tibial condensations were smaller in triple mutants than in double mutants, but were otherwise indistinguishable.

(*Smad1*^{CKO};*Smad5*$^{fx/+}$;*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 *Smad1/5*^{CKO} double and *Smad1/5*^{CKO};*Smad8*$^{-/-}$ triple mutants (Fig. 2). Similarly, no additional defects were seen in *Smad1*^{CKO};*Smad8*$^{-/-}$ and *Smad5*^{CKO};*Smad8*$^{-/-}$ mice as compared with *Smad1*^{CKO} and *Smad5*^{CKO} mice (not shown). However, cartilage condensations were slightly smaller in *Smad1/5*^{CKO};*Smad8*$^{-/-}$ triple mutants as compared with *Smad1/5*^{CKO};*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 *Smad1/5*^{CKO} 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 *Smad1/5*^{CKO} 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

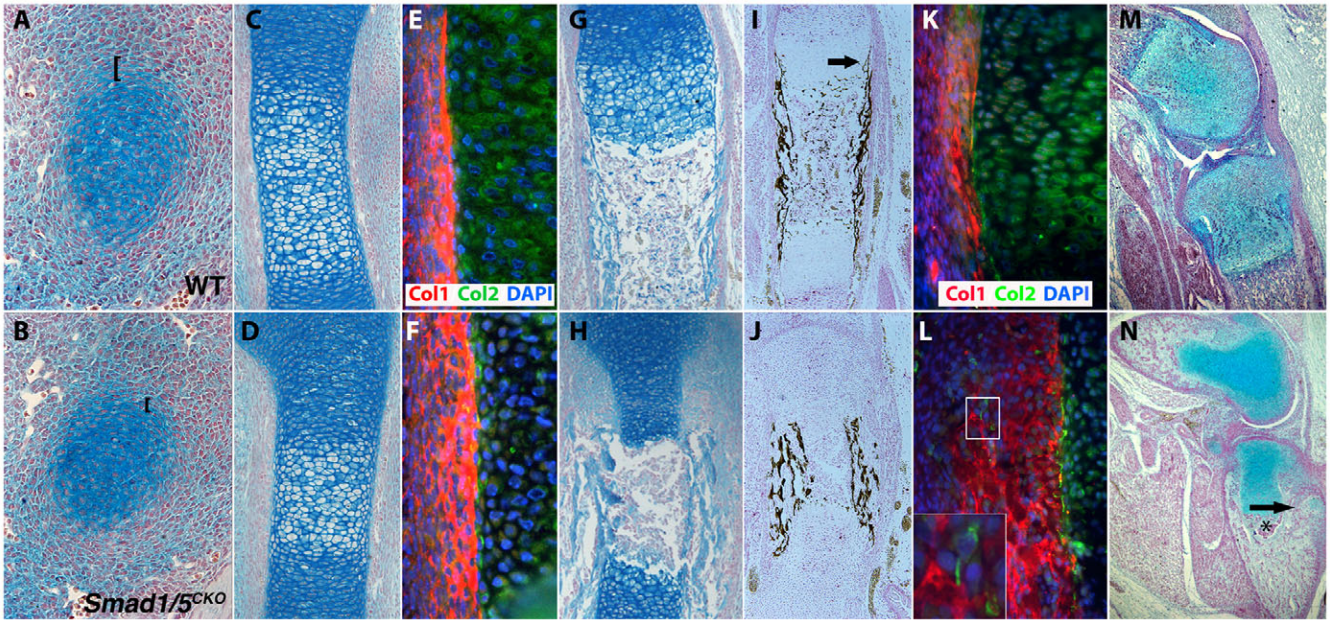

Fig. 4. Defective limb development in *Smad1/5*^{CKO} mutants. (**A**,**B**) WT (A) and mutant (B) Alcian Blue-stained E12.5 mouse limb cartilage. Brackets identify perichondrium. (**C**,**D**) WT (C) and mutant (D) proximal tibial growth plate at E14.5. (**E**,**F**) Immunofluorescence of type I collagen (red) and type II collagen (green) counterstained with DAPI (blue) in E14.5 WT (E) and mutant (F). (**G-J**) Alcian Blue and Von Kossa staining at E16.5 in WT (G,I) and mutant (H,J) proximal tibia, respectively. The arrows mark the bony collar in I,J. (**K**,**L**) Immunofluorescence of type I (red) and type II (green) collagen at E17.5 in WT (K) and mutant (L) proximal tibia. The inset in L is an enlargement of the boxed region showing type II collagen-producing cells embedded in the mutant type I collagen expression domain. (**M**,**N**) Alcian Blue staining of P0 WT (M) and mutant (N) knee joint. The arrow indicates ectopic cartilage formation in mutants; the asterisk demarcates a small marrow cavity.

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 *Smad1/5*^{CKO} 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 formed 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). *Smad1/5*^{CKO} 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). *Smad1/5*^{CKO} 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 *Smad1/5*^{CKO} 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

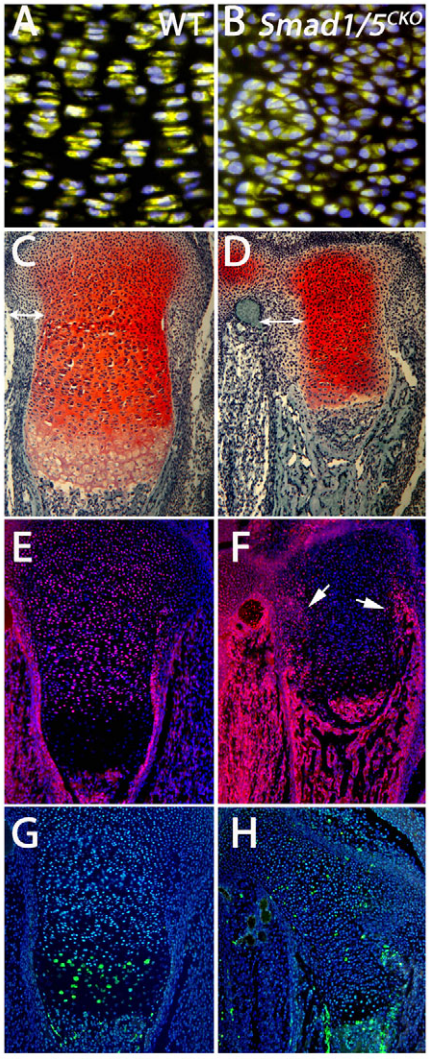

Fig. 5. Growth plate disorganization and impaired chondrocyte survival in *Smad1/5*^{CKO} mutants. (**A**,**B**) Autofluorescence (green) and DAPI (blue) staining in E17.5 WT (A) and mutant (B) mouse proximal tibial growth plates. (**C**,**D**) Safranin O staining of E17.5 WT (C) and mutant (D) tibial growth plates. Double-headed arrows demarcate the borders of the perichondrium. (**E**,**F**) Pcna immunofluorescence of WT (E) and mutant (F) proximal tibiae. Arrows indicate staining limited to perichondrium and lateral edges of the mutant cartilage element. (**G**,**H**) TUNEL staining in E17.5 WT (G) and mutant (H) proximal tibiae. Images in C, E and G are adjacent sections; images in D, F and H are adjacent sections.

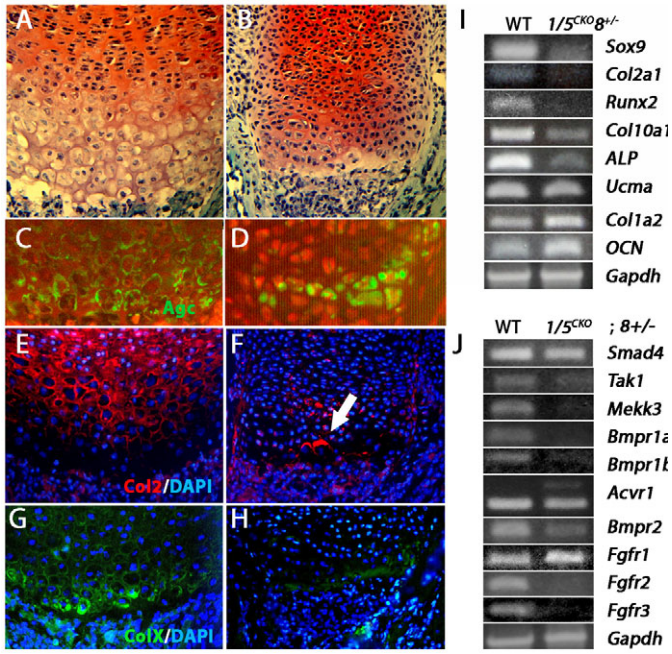

Fig. 6. Impaired matrix production and chondrocyte differentiation in *Smad1/5*^{CKO} mutants. (**A-H**) Adjacent sections of WT (A,C,E,G) and mutant (B,D,F,H) E17.5 mouse cartilage. (A,B) Safranin O staining. (C,D) Immunofluorescence for aggrecan. (E,F) Immunofluorescence for type II collagen in growth plates. Arrow indicates restricted type II collagen production in the mutant. (G,H) Immunofluorescence for type X collagen in growth plates. (**I**,**J**) RT-PCR analysis of gene expression using RNA isolated from microdissected WT and mutant cartilage.

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 *Ucma* expression. *Ucma* is a marker for upper (resting) chondrocytes (Tagariello et al., 2008; Surmann-Schmitt et al., 2008). *Ucma* was expressed as robustly in mutant as in WT cartilage (Fig. 6I). Hence, *Smad1/5*^{CKO} chondrocytes are impaired in their ability to undergo terminal differentiation and retain characteristics of resting chondrocytes.

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

Loss of canonical Smad proteins disrupts expression of BMP signaling components

The *Smad1/5*^{CKO} phenotype is similar to that of *Bmpr1a*^{CKO};*Bmpr1b*$^{-/-}$ mice (Yoon et al., 2005), suggesting that the canonical pathway is the major transducer of BMP signals in cartilage. Unexpectedly, the *Smad1/5*^{CKO} phenotype is markedly more severe than the *Smad4*^{CKO} phenotype (Zhang et al., 2005), even though *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 *Smad4* is expressed in mutant cartilage. Therefore, the severe chondrodysplasia in *Smad1/5*^{CKO} mice does not correlate with altered *Smad4* expression. However, the expression of type II (*Bmpr2*) and type I (*Bmpr1a* and *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 *Smad1/5*^{CKO} mutants. The results reveal the presence of a positive-feedback loop

involving BMP receptors and BMP receptor Smads. *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. 6J). The basis for this decrease is unclear, but might be related to reduced BMP receptor expression (Fig. 6J). 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 *Bmp4*, *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 *Smad1/5*^{CKO} mutants. RT-PCR analysis showed that BMPs induce both *Ihh* and *PPR* expression. By contrast, *Pthrp* expression was only moderately increased by BMPs (Fig. 7A). We then tested whether the Ihh signaling loop is impaired in mutant cartilage. *Pthrp* mRNA levels were only slightly reduced in *Smad1/5*^{CKO} cartilage, but *Ihh* and *PPR* mRNAs were not detected (Fig. 7B). These results confirm that *Ihh* is a BMP target in the growth plate, and suggest that *PPR* is as well.

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). *Pthrp* mRNA was also seen in *Smad1/5*^{CKO} mutants (Fig. 7I,J). *Ihh* is expressed in the prehypertrophic zone of WT growth plates; however, no *Ihh* mRNA was detectable in mutants (Fig. 7C-F). Retention of *Pthrp* expression in the apparent absence of *Ihh* in *Smad1/5*^{CKO} mutants was unexpected, as it has been shown that Ihh is normally required to maintain *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 *Ihh* expression at E14.5 in mutants to test the possibility that *Ihh* might be present at higher levels at earlier stages. Cells resembling prehypertrophic and hypertrophic chondrocytes were present in *Smad1/5*^{CKO} mutants (see Fig. S2A-D in the supplementary material). Low levels of *Ihh* expression were detectable in the E14.5 mutant tibia, and *Pthrp* was expressed in periarticular cells (see Fig. S2C,D in the supplementary material). *Ihh* mRNA was also detected in E14.5 WT and mutant digits (see Fig. S3 in the supplementary material). We conclude that *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. *Ihh* expression might be too restricted and/or disorganized to detect at E16.5, but still present at a sufficient level to maintain *Pthrp* expression. To test this latter possibility, we performed in situ hybridization for patched 1 (*Ptch1*), a sensitive readout of *Ihh* signaling. In WT growth plates, *Ptch1* is expressed in proliferating chondrocytes, as well as in adjacent perichondrium (Fig. 7M,N). Consistent with decreased Ihh signaling, *Ptch1* mRNA was detectable, albeit at much lower levels, in the mutant growth plate (Fig. 7O,P).

PPR was not expressed at detectable levels in *Smad1/5*^{CKO} growth plates (Fig. 7K,L), but was readily detected in mutant osteoblasts. Hence, *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 PPR and *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.

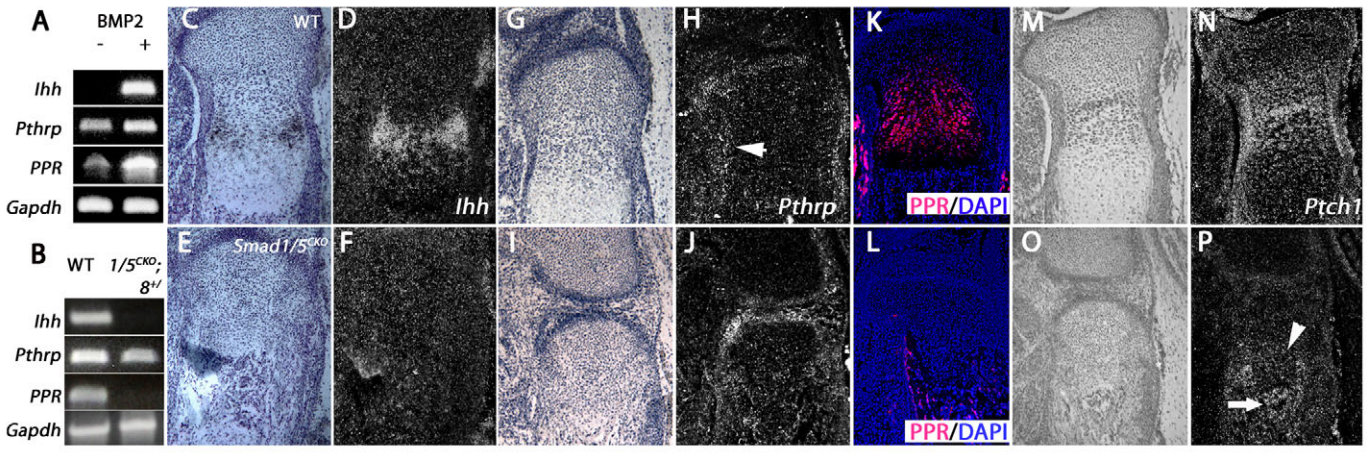

Fig. 7. The BMP canonical Smad pathway is required for the Ihh/PTHrP signaling loop. (**A**) RT-PCR analysis of WT mouse primary chondrocytes treated (+), or otherwise (–), with BMP2 for 2 hours. (**B**) RT-PCR analysis of RNA isolated from microdissected E18.5 WT and mutant growth plate cartilage. (**C-F**) In situ hybridization analysis of *Ihh* expression in E16.5 WT (C,D) and *Smad1/5*^{CKO} (E,F) proximal tibiae. (**G-J**) In situ hybridization analysis of *Pthrp* expression in WT (G,H) and *Smad1/5*^{CKO} (I,J) proximal tibiae. Arrowhead in H demarcates the perichondrium/periosteum. (**K**,**L**) Immunostaining for PPR in WT (K) and *Smad1/5*^{CKO} (L) proximal tibiae. (**M-P**) In situ hybridization for *Ptch1* expression, illustrating strong expression in WT proliferating chondrocytes, and a weaker signal in the mutant growth plate (P, arrowhead). Arrow in P highlights higher levels of *Ptch1* expression in mutant osteoblasts and periosteum.

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^{CKO}* cartilage (Fig. 8A,B), as is the case in *Bmpr1a/b^{CKO}* mice (Yoon et al., 2006). FGF receptor expression was also analyzed by RT-PCR (Fig. 6J). *Fgfr1* levels were increased in *Smad1/5^{CKO}* cartilage, but *Fgfr2* and *Fgfr3* levels were decreased. *Fgfr1* mRNA is expressed in resting/periarticular 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^{CKO}* 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. 6I) 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^{CKO}* mutants.

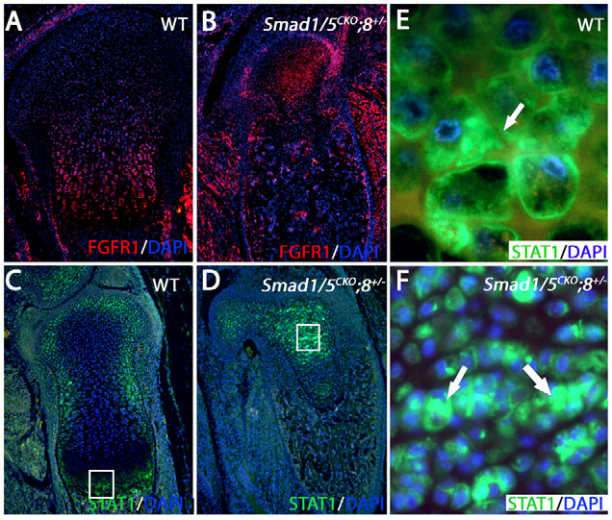

Fig. 8. Imbalance of BMP and FGF signaling in mutant cartilage. (**A**,**B**) Immunofluorescence staining for Fgfr1 in WT (A) and *Smad1/5^{CKO};Smad8$^{+/-}$* mutant (B) E17.5 mouse proximal tibiae. (**C**,**D**) Total Stat1 immunofluorescence in WT (C) and mutant (D) E16.5 proximal tibiae. (**E**,**F**) High-magnification images of the boxed regions from C and D showing subcellular localization of Stat1 (arrows) in WT (E) and mutant (F) chondrocytes.

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 (Fuentealba 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.

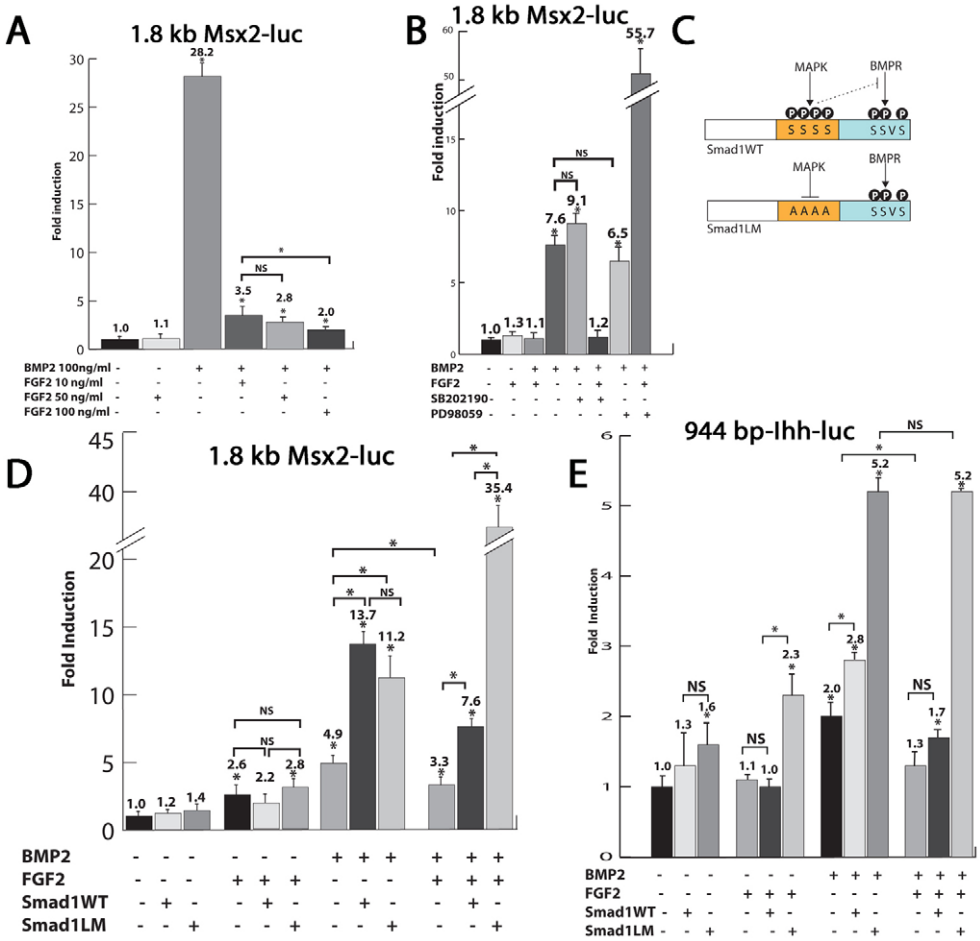

Fig. 9. FGF inhibits BMP activity in primary chondrocytes and RCS cells. (**A**) BMP2 induction of the mouse 1.8 kb *Msx2* promoter is inhibited by FGF2 in a dose-dependent manner in RCS cells. (**B**) An Erk1/2 MAPK inhibitor (PD98059) abrogates the inhibitory effects of FGF2 on BMP2 activity in RCS cells. (**C**) The phosphorylation (P) sites in the Smad constructs Smad1WT and Smad1LM. (**D**) BMP2 induction of the 1.8 kb *Msx2*-luc construct is enhanced by transfection of Smad1WT or Smad1LM. (**E**) The *Ihh* promoter is inhibited by FGF, and mutation of the Smad1 linker region can prevent these inhibitory effects. Each transfection experiment was repeated at least three times and a representative experiment is shown in each panel. The data represent an average from three wells with the indicated s.d. Brackets with an asterisk indicate significant differences between columns (Student's *t*-test; $P \leqslant 0.05$). All other asterisks indicate significant differences from the no treatment control. NS, not significant.

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.

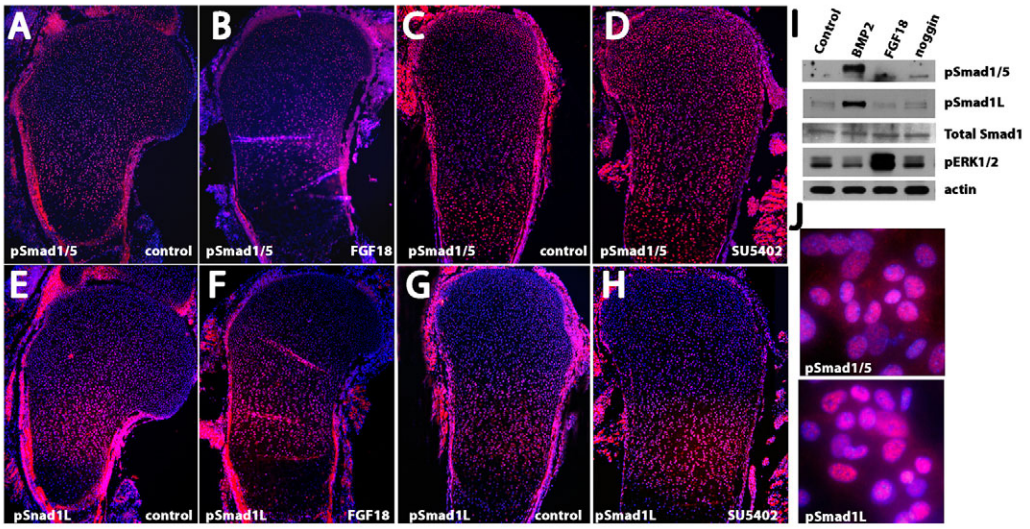

Fig. 10. C-terminal and linker phosphorylation of BMP receptor Smads in chondrocytes. (**A-H**) Immunostaining of radius and ulna of E16.5 cultured mouse limbs treated with FGF18 or the FGF receptor antagonist SU5402. For each experiment, the contralateral limb served as an untreated control. (A,B) Immunofluorescence images of phosphorylated (p) Smad1/5 expression in untreated control (A) and contralateral limb treated with FGF18 (B). (C,D) Immunofluorescence images of pSmad1/5 expression in untreated control (C) and contralateral limb treated with SU5402 (D). (E,F) Immunofluorescence images of pSmad1L expression in untreated control (E) and contralateral limb treated with FGF18 (F). (G,H) Immunofluorescence images of pSmad1L expression in untreated control (G) and contralateral limb (H). (**I**) Western blot showing that although FGF18 induces pErk1/2, it does not induce Smad1 linker phosphorylation. Rather, Smad1 linker phosphorylation is induced by BMP2. (**J**) Immunofluorescence images of pSmad1/5 and pSmad1L localization in primary chondrocyte cultures. Both forms of Smad1 are primarily nuclear.

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*^{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 *Msx2* 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 *Smad1/5*^{CKO} phenotype demonstrates that loss of BMP receptor Smad signaling disrupts Ihh/PTHrP signaling in the growth plate. Although *Ihh* levels are reduced, *Ptch1* expression can be detected, revealing that Ihh signaling does occur in mutants. Moreover, *Pthrp* 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 *Pthrp* expression, Ihh-independent mechanisms might participate in the maintenance of *Pthrp* 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 *Bmpr1a* 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; Lanske 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

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