BMPs regulate multiple aspects of growth-plate chondrogenesis through opposing actions on FGF pathways

Byeong S. Yoon1, Robert Pogue2, Dmitri A. Ovchinnikov3, Isaac Yoshii1, Yuji Mishina4, Richard R. Behringer3 and Karen M. Lyons1,2,*

Bone morphogenetic protein (BMP) signaling pathways are essential regulators of chondrogenesis. However, the roles of these pathways in vivo are not well understood. Limb-culture studies have provided a number of essential insights, including the demonstration that BMP pathways are required for chondrocyte proliferation and differentiation. However, limb-culture studies have yielded contradictory results; some studies indicate that BMPs exert stimulatory effects on differentiation, whereas others support inhibitory effects. Therefore, we characterized the skeletal phenotypes of mice lacking Bmpr1a in chondrocytes (Bmpr1a<sup>CKO</sup>) and Bmpr1a<sup>CKO</sup>,Bmpr1b<sup>−/−</sup> (Bmpr1a<sup>CKO</sup>,Bmpr1b<sup>−/−</sup>) in order to test the roles of BMP pathways in the growth plate in vivo. These mice reveal requirements for BMP signaling in multiple aspects of chondrogenesis. They also demonstrate that the balance between signaling outputs from BMP and fibroblast growth factor (FGF) pathways plays a crucial role in the growth plate. These studies indicate that BMP signaling is required to promote Ihh expression, and to inhibit activation of STAT and ERK1/2 MAPK, key effectors of FGF signaling. BMP pathways inhibit FGF signaling, at least in part, by inhibiting the expression of FGFRI. These results provide a genetic in vivo demonstration that the progression of chondrocytes through the growth plate is controlled by antagonistic BMP and FGF signaling pathways.

KEY WORDS: Bone morphogenetic protein, Cartilage, Chondrogenesis, Fibroblast growth factor, indian hedgehog, BMP receptors, Mouse

INTRODUCTION

Endochondral ossification is the multistep process that forms the majority of the skeletal system. This process is initiated when mesenchymal cells condense and express SOX9. This transcription factor drives the commitment and differentiation of these cells into chondrocytes, resulting, ultimately, in the formation of cartilage growth plates (Kronenberg, 2003; Olsen et al., 2000). Longitudinal growth is driven by chondrocyte proliferation and enlargement. Once growth-plate chondrocytes exit the cell cycle, they form a transient pool of <i>indian hedgehog</i> (<i>Ihh</i>)-expressing prehypertrophic chondrocytes (St-Jacques et al., 1999). As they continue to differentiate, these cells upregulate expression of collagen X and undergo hypertrophy. Ultimately, terminally differentiated hypertrophic chondrocytes express osteopontin and matrix metalloproteinase 13 (MMP13) prior to undergoing apoptosis. Hypertrophic chondrocytes produce an extracellular matrix (ECM) that provides an environment conducive to bone mineralization and blood-vessel invasion (Kronenberg, 2003; Long et al., 2001).

Several signaling pathways play vital roles in the growth plate. IHH coordinates chondrocyte proliferation and differentiation, as well as osteoblast formation in the periostium (St-Jacques et al., 1999; Long et al., 2004; Chung et al., 2001). Parathyroid hormone-related protein (PTHrP) forms a feedback loop with IHH that regulates proliferation and the onset of hypertrophic differentiation (Karp et al., 2000; Kawashima et al., 1998; Lanske et al., 1999).

Fibroblast growth factor (FGF) signaling pathways negatively regulate proliferation and differentiation via several mechanisms, including activation of STAT and ERK1/2 MAPK (Sahni et al., 2001; Murakami et al., 2004).

Bone morphogenetic protein (BMP) signaling pathways are also crucial regulators of chondrogenesis. BMPs transduce signals through complexes of type I and type II serine/threonine kinase receptors. Upon BMP binding, type II receptors phosphorylate serine/threonine residues in type I receptors. Activated type I receptors phosphorylate, and thereby activate, receptor-regulated SMAD (R-SMAD)1, R-SMAD5 and R-SMAD8. Subsequently, these R-SMADs recruit and bind SMAD4. These SMAD complexes enter the nucleus and regulate transcription of target genes (ten Dijke et al., 2003; Massagué and Gomis, 2006). BMPs also signal by activating TGFβ-activated kinase 1 (TAK1). TAK1 activates p38 MAPK (Moustakas and Heldin, 2005; Qiao et al., 2005). Three type I receptors transduce BMP signals: BMP receptor type IA (BMPRIA, also known as ALK3), BMP receptor type IB (BMPRIB, also known as ALK6) and activin receptor type I (ACTRI, also known as ALK2) (ten Dijke et al., 1994; Rosenzweig et al., 2002). However, limb-culture studies have yielded contradictory results; some studies suggest that BMPs exert stimulatory effects on differentiation, whereas others support inhibitory effects. Whether these disparities arise as a result of differences in (a) utilization of signaling pathways at the different stages of development at which the studies were performed, (b) species-specific effects, and/or (c) divergent pathways in different skeletal elements, is unknown.

1Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA 90095, USA. 2Department of Orthopaedic Surgery, David Geffen School of Medicine at the University of California, Los Angeles, CA 90095, USA. 3Department of Molecular Genetics, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. 4Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA.

*Author for correspondence (e-mail: klyons@mednet.ucla.edu)

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Moreover, some of these studies involve application of BMPs at non-physiological levels and to all tissues, including the surrounding endothelial cells, perichondrium and osteoblasts, are exposed. The tissues surrounding cartilage affect chondrogenesis in the growth plate, raising the possibility of both direct and indirect effects (Colnot et al., 2004; Colnot et al., 2005). Moreover, these studies do not reveal the endogenous roles of different type I receptors.

A number of genetic approaches have been used to reduce BMP signaling in developing skeletal elements in the mouse. Mice lacking Gdf5 and Gdf6 exhibit defects in chondrogenesis and joint formation (Storm et al., 1994; Settle, Jr et al., 2003). Mice lacking Bmpr1b exhibit defects in chondrogenesis (Baur et al., 2000; Yi et al., 2000). However, in all of these cases, defects are restricted to a subset of skeletal elements, most probably as a result of functional redundancy (Yi et al., 2000; Settle, Jr et al., 2003). The idea that BMP signaling pathways play a much broader role than is suggested by these phenotypes has been confirmed by analysis of transgenic mice overexpressing noggin or Smad6 (Tsumaki et al., 2002; Horikii et al., 2004). All cartilages in these mice are hypoplastic and deficient in hypertrophic chondrocytes, suggestive of a requirement for BMP pathways in chondrocyte maturation. Recently, we demonstrated an essential role for BMP signaling at early stages of chondrogenesis by generating chondrocyte-specific Bmpr1a and Bmpr1b null mice (Yoon et al., 2005). Chondrogenesis in these double mutants is much more severely affected than in the noggin-overexpressing transgenics (Tsumaki et al., 2002).

We characterized the skeletal phenotypes of mice lacking Bmpr1a (Bmpr1aCKO) and Bmpr1bCKO;Bmpr1b+/− (Bmpr1aCKO;1b+/−) in cartilage in order to address the roles of BMP pathways in the growth plate. These mice displayed generalized chondrodysplasia but, unlike Bmpr1aCKO;1b−/− mice, in which chondrogenesis is so severely disrupted that growth plates never form (Yoon et al., 2005), all skeletal elements were present, permitting an assessment of the role of BMP pathways in the growth plate. This analysis demonstrated that BMP signaling is essential for multiple aspects of growth-plate chondrogenesis, including proliferation, and the completion of hypertrophic differentiation. We demonstrated that the balance between outputs from BMP and FGF pathways plays crucial roles in hypertrophic differentiation. Furthermore, these studies indicated that BMP signaling is required to promote Ihh expression and to inhibit FGFR1 expression. The increased level of FGF-receptor expression appears to have functional consequences, as activation of STAT and ERK1/2 MAPK, key effectors of FGF signaling, were elevated in Bmpr1aCKO and Bmpr1aCKO;1b−/− mutants. These results provide a genetic demonstration that the progression of chondrocytes through the growth plate is controlled by mutually antagonistic BMP and FGF signaling pathways.

MATERIALS AND METHODS

Generation of Bmpr1aCKO and Bmpr1aCKO;Bmpr1b+/− mice

Generation of Bmpr1b+/−, Bmpr1a floxed and Col2-Cre mice was described previously (Yi et al., 2000; Mishina et al., 2002; Ovchinnikov et al., 2000). To generate cartilage-specific Bmpr1a null mice, Bmpr1a+/−;Col2-Cre mice were intercrossed to generate Bmpr1a+/−;Col2-Cre (Bmpr1aCKO) mice. Bmpr1a+/−;Bmpr1b+/+;Col2-Cre mice were intercrossed to generate Bmpr1aCKO;Bmpr1b+/+ mice.

Skeletal preparation and histology

Skeletal preparations were performed as previously described (Ivkovic et al., 2003). Briefly, embryos were enucleated and fixed in 95% EtOH overnight at 4°C, followed by Alcian blue staining (30 mg/100 ml in 80% EtOH) at room temperature. Samples were then stained in Alizarin red (5 mg/100 ml in 0.5% KOH) and cleared in a series of graded KOH in glycerol.

For histology, embryos were fixed in 4% paraformaldehyde, decalcified and embedded in paraffin. Sections were stained with Hematoxylin and Eosin or Alcian Blue and Nuclear Fast Red (Luna, 1992).

Immunohistochemistry and in situ hybridization

For immunohistochemistry (IHC), sections were microwaved in citrate buffer for antigen denasking. Sections were blocked with 5% goat or donkey serum, incubated with primary antibody (BMPRIA and BMPRIB: Ori-bien; collagen II: Research Diagnostics; collagen X: gift from Robin Poole; osteopontin: Developmental Studies Hybridoma Bank; MMP13; Oncogene Research Products; phospho-SMAD1, phospho-SMADS and phospho-SMAD8, phospho-ERK1/2 MAPK, and p27 Kip1: Cell Signaling Technology; phospho-STAT1: Zymed; STAT 5a: Lab Vision; p16, p21 and PECAM: Santa Cruz Biotechnology; FGFR1: Sigma) overnight at 4°C, and then incubated with the appropriate secondary antibody at room temperature. Color was developed with DAB or the Zymed kit chromogen, and sections were counterstained with hematoxylin. For immunofluorescence, slides were washed and incubated overnight at 4°C with Alexa-Fluor-555-conjugated rabbit secondary antibody (Invitrogen).

In situ hybridization was performed with 32P-dUTP labeled RNA probes and carried out as previously described (Jamin et al., 2002).

Cell proliferation and TUNEL labeling

IHC for proliferating cell nuclear antigen (PCNA) was performed using anti-PCNA antibody (Zymed), as described above for IHC. For TUNEL labeling, the In Situ Cell Death Detection Kit, fluorescein (Roche), was used according to the manufacturer’s instructions. Cell proliferation was quantified as previously described (Yi et al., 2000).

Transient transfections and reporter assays

A 994 bp fragment of the mouse proximal Ihh promoter was a kind gift from Toshihisa Komori (Yoshida et al., 2004). 2HC8-luc contains 430 bp of the mouse proximal Ihh promoter and was a kind gift from Akiko Hata (Seki and Hata, 2004). The Smad expression construct was a kind gift from Eddy DeRobertis (Pera et al., 2003). RCS cells (Mukhopadhyay et al., 1995) were cultured in 10% FBS DMEM and transfected at 50-70% confluence using GeneJuice (Novagen), according to the manufacturer’s instructions, with 0.5 μg of 994-luc or 2HC8-luc, and either Smad1 or a lacZ control plasmid. Cells were serum-starved for 24 hours post-transfection and then treated with 60 ng/ml recombinant human BMP2 (R&D Systems). After an additional 24 hours, luciferase activity was measured with the Dual-Luciferase Promoter Assay System (Promega). Results were obtained in triplicate for each experiment and were normalized with Renilla luciferase. Statistical significance was assessed using a t-test for correlated samples.

RESULTS

BMPRIA and BMPRIB expression in the growth plate

The expression of BMPRIA and BMPRIB in growth plates was examined by indirect immunofluorescence on E16.5 femurs (Fig. 1). By E16.5, growth plates consisting of resting, proliferative, prehypertrophic and hypertrophic zones had formed. BMPRIA was expressed throughout the growth plate, but the highest levels were found in the postmitotic prehypertrophic and hypertrophic zones (Fig. 1A). Interestingly, chondrocytes near the junction between the resting and proliferative zones had higher BMPRIA expression levels than surrounding chondrocytes, suggesting a role in regulating the transition from the resting to the proliferative state. BMPRIB was also expressed throughout the growth plate (Fig. 1B), but the highest levels were observed near the epiphysial surface of the resting zone, suggesting a potential role in the formation of articular cartilage. The overlapping expression of the BMPRIA and BMPRIB proteins is consistent with the overlapping functions found during early chondrogenesis (Yoon et al., 2005). However, the differences in relative expression levels in different regions raise the possibility of a more predominant role of one receptor over the other in specific aspects of chondrogenesis.
IHC for phosphorylated BMP-specific R-SMADs (pSMAD1, pSMAD5 and pSMAD8) was performed to localize active BMP signaling. Activated SMAD signaling was found throughout the growth plate, consistent with the broad distribution of BMPRIA and BMPRIB (Fig. 1C). The percentage of cells responding to BMP signaling increased as chondrocytes progressed from the resting to the proliferative zone, suggesting a role for BMP signaling in this transition (Fig. 1D,E). Furthermore, levels of signaling increased steadily throughout the proliferative zone and peaked at the proliferative-prehypertrophic junction. Levels then appeared to decrease as cells became hypertrophic. These results indicate that proliferative and prehypertrophic chondrocytes are major targets of BMP signaling, and further suggest that canonical BMP pathways regulate the transition from proliferation to terminal differentiation.

**Bmpr1aCKO and Bmpr1aCKO;1b–/– mice develop generalized chondrodysplasia**

Mice carrying Bmpr1a floxed alleles were crossed with Col2-Cre transgenic mice to inactivate BMPRIA in chondrocytes, generating Bmpr1aCKO;Col2-Cre (Bmpr1aCKO) mice.

These mice were recovered at birth in Mendelian ratios, but died shortly afterwards as a result of respiratory failure caused by skeletal defects. Mutant ribs were shorter and thinner, resulting in a flattened, bell-shaped thoracic cavity. These defects reflect the generalized chondrodysplasia that occurs in Bmpr1aCKO mice (Fig. 2A,B).

Mice carrying various allelic combinations of Bmpr1aCKO and Bmpr1b alleles were generated in order to examine whether BMPRIA and BMPRIB exert overlapping functions. Bmpr1aCKO;Col2Cre;Bmpr1b–/– mice were viable and did not differ substantially from Bmpr1b–/– mice, indicating that a single copy of Bmpr1a is sufficient to sustain endochondral ossification (data not shown). Bmpr1b–/– mice developed severe skeletal defects in distal phalanges and minor defects in the radius/ulna and tibia/fibula (Yi et al., 2000; Baur et al., 2000). Bmpr1aCKO;1b–/– mice exhibited a generalized chondrodysplasia of considerably greater severity than that exhibited by Bmpr1aCKO mice. This finding is consistent with the overlapping expression patterns of BMPRIA and BMPRIB in the growth plate. In addition to rib malformations, axial defects were apparent along the entire vertebral column (Fig. 2C,D). The supraoccipital bone was significantly narrower in Bmpr1aCKO mice, but was completely absent in Bmpr1aCKO;1b–/– mice. Similarly, Bmpr1aCKO vertebrae did not fuse and the dorsal arches were broader; this phenotype was exacerbated in Bmpr1aCKO;1b–/– mice. Skeletal defects were also apparent throughout the appendicular skeleton (Fig. 2E,F). The scapula became increasingly hypoplastic as more alleles of Bmpr1a and Bmpr1b were lost. Appendicular elements were narrower and shorter in Bmpr1aCKO and Bmpr1aCKO;1b–/– mice, with the femur being most severely affected. Interestingly, malformations were more severe at the distal than at the proximal ends of mutant femurs and humeri, and at the proximal rather than distal ends of the tibia and fibula. Thus, the growth plates most severely affected were those that normally experience the greatest rates of cell division and differential growth (Wilsman et al., 1996a; Wilsman et al., 1996b; Pritchett, 1992).

**Bmpr1aCKO and Bmpr1aCKO;Bmpr1b–/– mice exhibit defects in the growth plate**

The above analysis reveals that, whereas chondrogenesis is essentially absent in Bmpr1aCKO;1b–/– mice (Yoon et al., 2005), a single intact copy of Bmpr1b (Bmpr1aCKO;1b+/–) restores endochondral development to the extent that a recognizable, albeit highly abnormal, skeleton forms. At E14.5, growth plates at the distal femurs of Bmpr1aCKO and Bmpr1aCKO;1b+/– mice displayed numerous histological defects (Fig. 3). Although the sizes of the resting zones were comparable in Bmpr1aCKO and wild-type mice, the proliferative zone was reduced in mutants (arrows in Fig. 3A,B). This reduction was intensified in Bmpr1aCKO;1b+/– mice. The severe...
constriction in the width of the femur at the junction of the resting and proliferative zones suggests that the initiation of column formation is impaired in the most severely affected growth plates of Bmpr1aCKO;1b+/– mice. Furthermore, the lengths of the chondrocyte columns were significantly reduced. These phenotypes persist throughout embryonic development, as they are also apparent at E16.5 and P0 (Fig. 3B, and data not shown). Bmpr1aCKO and Bmpr1aCKO;1b+/– mice also displayed defects in linear column formation. In wild-type growth plates, flattened proliferating chondrocytes intercalate with one another immediately after cell division. However, in Bmpr1aCKO mice, chondrocyte columns were disorganized. Proliferating chondrocytes did not intercalate properly, as many pairs of daughter cells remained juxtaposed (Fig. 3C). This phenotype became more exaggerated in Bmpr1aCKO;1b+/– mice. Very few chondrocyte columns formed in the distal femur and distal humerus, the most severely affected growth plates (Fig. 3C), and chondrocytes had a rounded morphology rather than the flattened shape observed in control littermates. By contrast, proliferative zones of mutants had a significant reduction in the rate of proliferation (Fig. 4A,C). TUNEL labeling was performed on E16.5 distal femurs (Fig. 4B,C) in order to examine cell survival. Apoptosis was undetectable in resting and proliferative zones in control growth plates. However, in mutants, there was a significant increase in apoptosis in both the resting and proliferative compartments. The highest levels were found at the junction between the epiphyseal and columnar regions. This area narrowed and bent in mutant femurs, raising the strong possibility that increased rates of apoptosis contribute to this defect in mutants.

Progression through the cell cycle is negatively regulated by cyclin-dependent kinase inhibitors (CKIs). Several CKIs are important regulators of proliferation in chondrocytes, including p16, p21 and p27Kip1 (Beier et al., 1999). In control growth plates, these CKIs were strongly expressed in the prehypertrophic and hypertrophic zones and only weakly in the proliferative zone. However, there was a significant induction of p16, p21 and p27 in the resting and proliferative zones in mutant growth plates (Fig. 4D). Thus, proliferation is inhibited in Bmpr1aCKO and Bmpr1aCKO;1b+/– mice, in part, because CKIs are upregulated.

BMP signaling regulates proliferation and apoptosis

We performed IHC for PCNA, and apoptosis assays in order to examine whether the reduced size of mutant proliferative zones is associated with reduced chondrocyte proliferation and/or survival (Fig. 4A,C). Levels of proliferation in resting zones of Bmpr1aCKO;1b+/– mice were indistinguishable from those in control littermates. By contrast, proliferative zones of mutants had a significant reduction in the rate of proliferation (Fig. 4A,C). TUNEL labeling was performed on E16.5 distal femurs (Fig. 4B,C) in order to examine cell survival. Apoptosis was undetectable in resting and proliferative zones in control growth plates. However, in mutants, there was a significant increase in apoptosis in both the resting and proliferative compartments. The highest levels were found at the junction between the epiphyseal and columnar regions. This area narrowed and bent in mutant femurs, raising the strong possibility that increased rates of apoptosis contribute to this defect in mutants.

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Defects in differentiation in mutant growth plates

Previous studies have shown that the onset of hypertrophic differentiation is delayed in mice lacking all alleles of Bmpr1a and Bmpr1b in cartilage (Yoon et al., 2005). By contrast, histological analysis indicated that hypertrophic chondrocytes were present in
Bmpr1a\(^{CKO}\) and Bmpr1a\(^{CKO},1b^{+/–}\) mice by E14.5. At E14.5, the lengths of the growth plates in the distal femur (presumptive joints to centers of cartilage condensations) and the lengths of the hypertrophic zones were smaller in Bmpr1a\(^{CKO}\) and Bmpr1a\(^{CKO},1b^{+/–}\) mice compared with wild-type. (C) H/E-stained sections through columnar zones of E16.5 wild-type, Bmpr1a\(^{CKO}\) and Bmpr1a\(^{CKO},1b^{+/–}\) littermates reveal a failure to adopt a flattened cell shape and intercalate in mutants. (D) H/E-stained sections through the hypertrophic zones of E16.5 wild-type, Bmpr1a\(^{CKO}\) and Bmpr1a\(^{CKO},1b^{+/–}\) littermates reveal the accumulation in mutants of cells with tightly packed nuclei (arrows), a characteristic feature of late hypertrophic chondrocytes. hyp, hypertrophic zone; ph, prehypertrophic zone; prol, proliferative zone; res, resting zone.

BMP signaling is required for terminal differentiation

By E16.5, additional defects in differentiation became apparent in Bmpr1a\(^{CKO}\) and Bmpr1a\(^{CKO},1b^{+/–}\) growth plates. Chondrocytes appeared to undergo premature hypertrophy in mutants (Fig. 3B), as evidenced by the shorter distance between the articular surface and the beginning of the hypertrophic zone. In addition, the hypertrophic zones of Bmpr1a\(^{CKO}\) and Bmpr1a\(^{CKO},1b^{+/–}\) femurs were expanded at this stage. The expansion observed in Bmpr1a\(^{CKO}\) mice was not exacerbated by the loss of one Bmpr1b allele, and is not observed in Bmpr1b\(^{–/–}\) mice (Yi et al., 2000). These results suggest a more prominent role for BMPRIA than for BMPR1B in hypertrophic chondrocytes, consistent with the high level of BMPRIA expression in this region. The expansion of the hypertrophic zone could conceivably be caused by a decreased rate of transit through the hypertrophic zone when BMP signaling is decreased, and/or by...
impaired apoptosis of terminal hypertrophic chondrocytes. Cells with tightly packed nuclei, a characteristic of terminally differentiated hypertrophic chondrocytes, accumulated in mutant hypertrophic zones (Fig. 3D) (Colnot et al., 2004). This finding suggests that the expanded hypertrophic zone may be a result, at least in part, of a delay in clearance of late hypertrophic chondrocytes through apoptotic mechanisms.

IHC analysis revealed an expanded zone of collagen X expression (Fig. 5B). The expression domains of osteopontin and MMP13 were also expanded in Bmpr1aCKO mice, suggesting that the expansion of the hypertrophic zone is, at least in part, due to an accumulation of terminally differentiated chondrocytes. This possibility is further supported by the finding that the region of the hypertrophic zone occupied by late hypertrophic chondrocytes was proportionately larger in mutants, as shown by a comparison of the zones of COLX and MMP13 expression.

Bmpr1aCKO growth plates exhibited aberrant angiogenesis in the hypertrophic zone (Fig. 6). In control mice, angiogenesis proceeded evenly across the chondro-osseous junction. However, in mutants, blood vessels invaded the hypertrophic zone, forming a disorganized chondro-osseous junction. This phenotype is possibly a consequence of the expansion in MMP13-expressing chondrocytes in mutants. MMP13 is a potent angiogenic factor that promotes growth-plate angiogenesis (D’Angelo et al., 2000; Johansson et al., 1997; Stickens et al., 2004).

BMP signaling is an important regulator of IHH signaling

The IHH/PTHrP pathway forms a negative-feedback loop that regulates the onset of hypertrophic differentiation. As in Bmpr1aCKO and Bmpr1aCKO;1b+/– mice, Ihh–/– growth plates initially exhibit smaller hypertrophic zones, followed by premature hypertrophic differentiation (St-Jacques et al., 1999). Therefore, we examined IHH/PTHrP pathway components in Bmpr1aCKO mice (Fig. 7A,B). Decreased expression of Ihh was observed in mutants, a finding consistent with the effects of the BMP antagonist NOGGIN in limb-culture studies (Pathi et al., 1999; Minina et al., 2001). Furthermore, Bmpr1aCKO growth plates had reduced expression of Ptc1, the receptor for IHH and a direct target of IHH signaling. No differences were found in expression of Pthr1, the receptor for PTHrP.

The existence of functional SMAD-responsive sites on the Ihh promoter (Seki and Hata, 2004), and the high levels of pSMAD1, pSMAD5 and pSMAD8 activation at the proliferative-prehypertrophic junction, suggest that IHH is a direct target of SMAD-mediated BMP signaling in the growth plate. Promoter assays using the Ihh promoter were performed in RCS chondrocytic cells to test this possibility (Fig. 7C). Exogenous BMP2 treatment and SMAD1 transfection resulted in an increase in promoter activity when a 994 bp Ihh promoter fragment was used (Yoshida et al., 2004), demonstrating that IHH is a target of BMP signaling in chondrocytes. The addition of BMP2 and overexpression of Smad1 also resulted in an increase in promoter activity when the Ihh SMAD-responsive element identified by Seki and Hata (Seki and Hata, 2004) was examined. However, the fold induction was considerably less than with the 994 bp fragment, suggesting that additional BMP-responsive sites on the Ihh promoter are utilized in chondrocytes.

Impaired BMP signaling leads to increased activation of FGF signaling pathways

Impaired BMP signaling in Bmpr1aCKO and Bmpr1aCKO;1b+/– mice leads to phenotypes resembling those observed in mouse models of FGF-induced achondroplasia. These observations prompted us to examine whether effectors of FGF signaling pathways are activated in BMP receptor mutants. At least two signaling pathways mediate FGF signaling in chondrocytes; STATs and ERK1/2 MAPK (Sahni et al., 2001; Murakami et al., 2004). STATs mediate the ability of FGFs to promote apoptosis and CKI expression (Sahni et al., 2001; Aszodi et al., 2003). Activation of ERK1/2 MAPK has been suggested to
mediate the ability of FGFs to prevent chondrocyte differentiation (Murakami et al., 2004). In control mice, activated phospho-STAT1 and STAT5a were predominantly localized to the prehypertrophic and hypertrophic zones (Fig. 8A, B, and data not shown). By contrast, there was a significant increase in activated STAT1- and STAT5a-positive chondrocytes in the proliferative zones in Bmpr1aCKO;1b+/– mice. Furthermore, a higher percentage of chondrocytes in mutant hypertrophic zones exhibited active STAT signaling.

Although ERK1/2 MAPK is a major mediator of FGF signaling in the growth plate, the localization of endogenous activated ERK1/2 has not been reported previously. In control mice, ERK1/2 MAPK signaling was restricted primarily to postmitotic regions of the growth plate: the periarticular and hypertrophic zones (Fig. 8C). There was a lateral expansion in ERK1/2 MAPK signaling in the periarticular region in Bmpr1aCKO;1b+/– mice. No differences in the percentage of cells undergoing ERK1/2 MAPK signaling could be observed between hypertrophic zones of Bmpr1aCKO and Bmpr1aCKO;1b+/– mice, but we cannot rule out quantitative differences. These results indicate that at least two functional components of FGF intracellular signaling, STAT and ERK1/2 MAPK, are hyperactivated when BMP signaling is impaired, and that this upregulation occurs in specific regions of the growth plate.

FGFR1 levels are increased in Bmpr1aCKO and Bmpr1aCKO;1b+/– growth plates

One mechanism that could account for the activation of multiple mediators of FGF signaling would be the elevated expression of FGF receptors. FGFR1 is expressed most highly in prehypertrophic and hypertrophic chondrocytes, regions where pSMAD1, pSMAD5, and pSMAD8 levels are also at their highest (Fig. 1) (Peters et al., 1992; Jacob et al., 2006). Therefore, we examined levels of FGFR1 expression (Fig. 9). The domain of expression of FGFR1 was expanded considerably in mutant growth plates at E16.5; in addition to expression in the enlarged hypertrophic zones of Bmpr1aCKO and Bmpr1aCKO;1b+/– mice, FGFR1 expression could now be detected throughout the columnar and ephiphyseal regions. This finding suggests that BMP pathways antagonize FGF pathways in part by inhibiting expression of FGF receptors.

**DISCUSSION**

**BMPRIA and BMPRIB in the growth plate**

BMPRIA and BMPRIB are coexpressed throughout the growth plate, and analysis of Bmpr1aCKO and Bmpr1aCKO;1b+/– mice demonstrates that they have overlapping functions in chondrogenesis. However, BMPRIA and BMPRIB have some unique roles. Bmpr1aKO mice develop an expanded hypertrophic zone, a phenotype that is not exacerbated by the loss of one Bmpr1b allele and is not observed in Bmpr1b+/– mice. BMPRIA is highly expressed in the hypertrophic zone, whereas BMPRIB is only weakly expressed there. These findings support the conclusion that BMPRIA plays a more prominent role in the hypertrophic zone than does BMPRIB. Conversely, BMPRIB has a more important role in periarticular cells, a region that will give rise to articular cartilage. BMPRIB is more highly expressed than BMPRIA in this area of the growth plate (Fig. 1). Furthermore, Bmpr1b+/– mice exhibit joint laxity and develop osteoarthritis (Yi
et al., 2000) (our unpublished data), phenotypes that could potentially arise from defects in the formation of the epiphysis and/or articular cartilage.

Loss of one Bmpr1a allele in the Bmpr1b null background results in a skeletal phenotype that is indistinguishable from that of Bmpr1b–/– mice (data not shown), suggesting that one functional Bmpr1a allele is sufficient for the formation of the majority of endochondral elements. By contrast, severe chondrodysplasia occurs in Bmpr1aCKO;1b+/– mice, in which only one functional Bmpr1b allele is present. These results suggest that BMPRIA is more important than BMPRIB in endochondral ossification. It is unclear whether this reflects differences in relative expression levels and/or qualitative differences between BMPRIA and BMPRIB. This will need to be resolved in future studies.

The contribution of the remaining type I receptor, ACTRI, in the growth plate remains to be addressed. Overexpression of CA-ACTRI delays differentiation and inhibits collagen X expression, effects opposite to those observed when growth plates are treated with BMPs. In agreement, CA-ACTRI induces expression of PTHrP, an inhibitor of hypertrophic differentiation (Zhang et al., 2003). These results suggest that BMPRIA is more important than BMPRIB in endochondral ossification. It is unclear whether this reflects differences in relative expression levels and/or qualitative differences between BMPRIA and BMPRIB. This will need to be resolved in future studies.

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BMP signaling regulates multiple aspects of differentiation in the growth plate

Immunostaining for phospho-SMAD1, phospho-SMAD5 and phospho-SMAD8 demonstrates a progressive increase in the percentage of cells responding to BMP signaling as they progress from the resting to the prehypertrophic zone, raising the strong possibility that BMPs play an important role in regulating the balance between proliferation and differentiation. In agreement, Bmpr1aCKO and Bmpr1aCKO;1b+/– mice develop shortened proliferative zones, probably due, in part, to defects in proliferation and survival. These findings are consistent with and extend previous limb-culture studies showing that exogenously applied BMPs...
increase proliferation (Pathi et al., 1999; Minina et al., 2001). We further demonstrate that BMPs regulate proliferation at least in part, by inhibiting CKIs, such as p16, p21 and p27. Interestingly, at the resting/proliferative junction, where the highest rates of proliferation are normally found (Smits et al., 2004), there is a significant increase in apoptosis in Bmpr1aΔCKO;1b−/− mice, suggesting that BMPs may regulate the decision to proliferate or die. In a recent study, ID3 was identified as an important regulator of this decision in neural-crest precursors (Kee and Bronner-Fraser, 2005). IDs are direct targets of BMP signaling in multiple cell types, and promote proliferation (Kawanetz et al., 2004). Consequently, BMPs, acting through IDs, may have a similar role at the resting/proliferative junction.

Analysis of Bmpr1aΔCKO and Bmpr1aΔCKO;1b−/− mice suggests that BMPs are important regulators of differentiation because of an essential role in IHH signaling. Ihh and its receptor Ptc1 are downregulated in mutants, consistent with previous work suggesting that BMPs function at least in part through an IHH-dependent pathway (Minina et al., 2001; Zhang et al., 2003). A high percentage of chondrocytes fail to intercalate and form columns in mutant growth plates, and in the more severely affected Bmpr1aΔCKO;1b−/− mice, fail to take on the characteristic flattened morphology. Similar features have been noted in Ihh−/− growth plates (St-Jacques et al., 1999), further strengthening the argument for a functional connection between BMP and IHH signaling.

As shown previously, loss of BMP signaling leads to a delay in the onset of hypertrophic differentiation (Yoon et al., 2005). At E14.5, Bmpr1aΔCKO;1b−/− mutant growth plates have reduced hypertrophic zones and have only just begun to express the late hypertrophic marker MMP13. This initial delay may be due to a reduced pool of cells as a result of impaired proliferation and increased apoptosis. This feature is also indicative of decreased signaling output from the PTHrP/IHH feedback loop (St-Jacques et al., 1999).

The relationships between this loop and BMP pathways have not been fully elucidated. Based on BMP-receptor overexpression studies in the chick, it was proposed that BMPs mediate the ability of IHH to induce PTHrP expression (Zou et al., 1997). However, in the mouse, it has been shown that BMPs do not directly upregulate PTHrP (Minina et al., 2001; Kobayashi et al., 2005). Similarly, some studies have suggested that BMP pathways induce Ihh expression in chondrocytes (Zhang et al., 2003), whereas other studies do not support this hypothesis (Kobayashi et al., 2005). We show that the Ihh promoter is responsive to SMAD1 in chondrocytes, suggesting that BMPs induce Ihh, and that this regulation may be direct.

By E16.5, Bmpr1aΔCKO and Bmpr1aΔCKO;1b−/− mice develop expanded hypertrophic zones. The expanded domains of osteopontin and MMP13 expression, and the accumulation of cells with tightly packed nuclei, a characteristic of late hypertrophic chondrocytes, suggest that the expansion of the hypertrophic zone is due, at least in part, to delayed differentiation and/or failure to complete terminal differentiation. Our conclusions differ somewhat from those found in limb-culture studies following inhibition of BMP pathways by treatment with NOGGIN (Minina et al., 2001). In those studies, exposure to NOGGIN led to an expanded domain of osteopontin expression; in contrast to our results, a reduced rather than an expanded domain of collagen X expression was observed. These results led the researchers to conclude that differentiation is accelerated when BMP signaling is inhibited (Minina et al., 2001). In Bmpr1aΔCKO and Bmpr1aΔCKO;1b−/− mice, although the length of the hypertrophic zone is expanded, the onset of ossification is delayed rather than accelerated, as would be predicted from the limb-culture models. The expansion of collagen X expression, accumulation of terminal hypertrophic chondrocytes and impaired ossification in Bmpr1aΔCKO and Bmpr1aΔCKO;1b−/− mice suggest that BMP signaling is required for completion of terminal hypertrophic differentiation. These different outcomes may reflect differences in responses to acute (as in limb-culture studies) versus chronic (as in
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CAN TRANSDUCE SIMILAR SIGNALS (REVIEWED IN ORNITZ, 2005). TO THE EXPANDED hypertrophic zone in (arrow), but is also seen in epiphyseal and columnar chondrocytes. A similar expansion of the domain of FGFR1 expression is observed in Bmpr1aCKO;1b+/– mice. Although the increased levels of FGFR1 expression suggest that functional redundancy and/or compensatory upregulation of other FGF receptors occurs in Fgfr1CKO mice. Third, the delays in maturation observed in Fgfr1CKO and Bmpr mutant mice may occur through distinct mechanisms. In support of this latter proposal, expression of OPN, MMP9 and MMP13 is reduced or unaltered in Fgfr1CKO mice, whereas the domains of expression of these markers are increased in Bmpr mutants (Fig. 8, data not shown). Clearly, additional in vivo studies are needed to clarify the nature of the interactions between BMP and FGF signaling pathways in distinct regions of the growth plate.

Our analysis of Bmpr1aCKO and Bmpr1aCKO;1b+/- mice also demonstrates that BMP signaling partially inhibits ERK1/2 MAPK. Although the increased levels of FGFR1 expression suggest that ERK1/2 levels may be elevated in Bmpr1aCKO and Bmpr1aCKO;1b+/- mice because of activation of FGF pathways, it is possible that additional pathways that transduce their signals through ERK1/2 are perturbed in mutants. In wild-type growth plates, ERK1/2 MAPK is activated in periarticular cells has not been fully elucidated. Our analysis of Bmpr1aCKO and Bmpr1aCKO;1b+/- mice demonstrates that BMPs antagonize FGF signaling by inhibiting at least two of the intracellular pathways activated by FGFs: STAT and ERK1/2 MAPK. One possible mechanism by which BMPs may inhibit STAT and ERK1/2 is by negatively regulating the expression of FGF signaling components. However, some limb-culture studies suggest that BMPs promote the expression of FGF signaling components in the growth plate, rather than inhibiting them. Future studies are needed to clarify the mechanisms by which BMPs inhibit FGF signaling, but our studies suggest that BMPs inhibit FGF pathways at least in part by limiting the domain of expression of FGFR1. Given the essential roles for FGFR3 and FGF18 in chondrogenesis (reviewed in Ornitz, 2005), examination of the expression levels of these signaling components will be of great interest in future studies.

Interestingly, loss of Fgfr1 in chondrocytes leads to a delay in terminal maturation (Jacob et al., 2006). This finding is seemingly at odds with our hypothesis that BMP and FGF pathways exert opposing functions in the growth plate, as loss of BMP signaling also leads to a delay in terminal maturation. There are several possible explanations for this apparent discrepancy. First, it is conceivable that BMP and FGF pathways have opposing roles in some aspects of chondrogenesis, but additive or synergistic roles in others. Thus FGF and BMP pathways may collaborate to promote aspects of hypertrophic chondrocyte maturation. Second, FGF1 and FGF3 exhibit some overlapping expression, and both receptors can transduce similar signals (reviewed in Ornitz, 2005). Thus, it is possible that functional redundancy and/or compensatory upregulation of other FGF receptors occurs in Fgfr1CKO mice. Third, the delays in maturation observed in Fgfr1CKO and Bmpr mutant mice may occur through distinct mechanisms. In support of this latter proposal, expression of OPN, MMP9 and MMP13 is reduced or unaltered in Fgfr1CKO mice, whereas the domains of expression of these markers are increased in Bmpr mutants (Fig. 8, data not shown). Clearly, additional in vivo studies are needed to clarify the nature of the interactions between BMP and FGF signaling pathways in distinct regions of the growth plate.

The percentage of cells engaged in ERK1/2 MAPK signaling is decreased and columnar chondrocytes. A similar expansion of the domain of FGFR1 expression is observed in Bmpr1aCKO;1b+/- growth plates.

**BMP and FGF signaling antagonize one another**

Activating mutations in FGFR3 lead to achondroplasia, the most common cause of dwarfism in humans. Expression of activated FGFR3 in mice leads to phenotypes that resemble those observed in humans (Naski et al., 1998). Growth plates of these mice exhibit both decreased proliferation and expression of Ihh, and increased expression of CKIs (Naski et al., 1998; Chen et al., 1999; Sahni et al., 1999), phenotypes recapitated in Bmpr1aCKO and Bmpr1aCKO;1b+/- mice. Limb-culture studies have shown that BMP and FGF signaling pathways have opposing functions in the growth plate (Minina et al., 2002). However, the mechanism of this antagonism has not been elucidated. Our analysis of Bmpr1aCKO and Bmpr1aCKO;1b+/- mice demonstrates that BMPs antagonize FGF signaling by inhibiting at least two of the intracellular pathways activated by FGFs: STAT and ERK1/2 MAPK. One possible mechanism by which BMPs may inhibit STAT and ERK1/2 is by negatively regulating the expression of FGF signaling components. However, some limb-culture studies suggest that BMPs promote the expression of FGF signaling components in the growth plate, rather than inhibiting them. Future studies are needed to clarify the mechanisms by which BMPs inhibit FGF signaling, but our studies suggest that BMPs inhibit FGF pathways at least in part by limiting the domain of expression of FGFR1. Given the essential roles for FGFR3 and FGF18 in chondrogenesis (reviewed in Ornitz, 2005), examination of the expression levels of these signaling components will be of great interest in future studies.

**Fig. 9. Expanded domain of FGFR1 expression in Bmpr1aCKO and Bmpr1aCKO;1b+/- growth plates.** Immunofluorescence staining for FGFR1 in (A) wild-type, (B) Bmpr1aCKO and (C) Bmpr1aCKO;1b+/- distal femurs at E16.5. Intense staining is restricted to the hypertrophic zone (arrow) in wild-type mice. In Bmpr1aCKO mice, intense staining for FGFR1 persists in the expanded hypertrophic zone in (arrow), but is also seen in epiphyseal and columnar chondrocytes. A similar expansion of the domain of FGFR1 expression is observed in Bmpr1aCKO;1b+/- growth plates.

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BMPs oppose FGFs in chondrogenesis

require to determined the extent to which alterations in each of these pathways contribute to the phenotypes observed when BMP signaling is decreased.

Taken together, our studies reveal that BMP pathways are essential for multiple aspects of chondrogenesis, and that BMPs regulate IHH levels at least in part via canonical SMAD pathways. To what extent do BMPs utilize canonical (SMAD-mediated) versus non-canonical pathways in cartilage remains an important unanswered question. Current models suggest that SMAD4 is required for transduction of canonical signals via BMP SMAD1, SMAD5 and SMAD8 (ten Dijke et al., 2003; Massagué and Gomis, 2006). Given the severity of the phenotypes seen in mice lacking Bmpr1a and Bmpr1b in cartilage (Yoon et al., 2005) (see also the results described here), it is thus surprising that mice lacking Smad4 in cartilage exhibit fairly normal skeletal development, prior to developing postnatal chondrodysplasia (Zhang et al., 2005). This disparity may reflect a more important role for non-canonical than canonical pathways in cartilage. Alternatively, several recent studies suggest that not all SMAD-mediated responses to TGF-β require SMADs (Yamashita et al., 2005) (see also the references described above). Analyses of the role of the Drosophila SMAD4 homolog Medea indicate that BMP SMADs can also function independently of SMAD4 (Wisotzkey et al., 1998). Analyses of mice lacking BMP SMADs in cartilage will thus be required to resolve whether the different phenotypes of mice lacking SMAD4 and those lacking BMP receptors reflect roles for non-canonical pathways, or canonical pathways independent of SMAD4.

References


