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Interaction of FGF, Ihh/Pthlh, and BMP Signaling Integrates Chondrocyte Proliferation and Hypertrophic Differentiation

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Summary

Mutations in fibroblast growth factor (FGF) receptor 3 lead to the human dwarfism syndrome achondroplasia. Using a limb culture system, we have analyzed the role of FGF signaling and its interaction with the Ihh/Pthlh and BMP pathways in regulating chondrocyte differentiation. In contrast to previous suggestions, we demonstrate that FGF signaling accelerates both the onset and the pace of hypertrophic differentiation. We furthermore found that FGF and BMP signaling act in an antagonistic relationship regulating chondrocyte proliferation, *Ihh* expression, and the process of hypertrophic differentiation. Importantly, BMP signaling rescues the reduced domains of proliferating and hypertrophic chondrocytes in a mouse model for achondroplasia. We propose a model in which the balance of BMP and FGF signaling adjusts the pace of the differentiation process to the proliferation rate.

Introduction

During embryonic development, endochondral ossification starts with the aggregation of mesenchymal cells, which differentiate into chondrocytes forming cartilage elements. In parallel, cells peripheral to the condensation differentiate into a fibroblastic cell layer, the perichondrium, surrounding the cartilage elements. Initially, all chondrocytes proliferate. At later stages starting from the center of the cartilage element, chondrocytes undergo a multistep program, differentiating into prehypertrophic, hypertrophic, and finally terminal hypertrophic chondrocytes. The region of terminally differentiated chondrocytes is subsequently invaded by blood vessels followed by osteoclasts and osteoblasts, which start to replace the cartilage with bone and bone marrow. In parallel, cells from the perichondrium flanking the hypertrophic chondrocytes differentiate into bone-producing osteoblasts, forming the periosteum (Erlebacher et al., 1995; Hinchcliffe and Johnson, 1980). Pre- and postnatal longitudinal growth of the skeletal elements depends on the prolongation of chondrocyte proliferation and hypertrophic differentiation. As hypertrophic chondrocytes are constantly replaced by bone, proliferation and hypertrophic differentiation have to be tightly controlled to ensure proper bone development.

Signaling by fibroblast growth factor (FGF) receptors plays a critical role in controlling chondrocyte differentiation. This is exemplified by three inherited human dwarfism syndromes, hypochondroplasia, achondroplasia, and thanatophoric dysplasia, which are caused by missense mutations in the FGF receptor 3 gene (*Fgfr3*; Bellus et al., 1995; Rousseau et al., 1994; Shiang et al., 1994; Tavormina et al., 1995). These mutations lead to different levels of receptor activation, which correlates well with the severity of the human phenotypes (Naski et al., 1996). Several mouse models mimicking the human achondroplasia phenotype have been created by expressing mutated forms of *Fgfr3* in the developing cartilage anlagen (*Fgfr3ach* mice; Chen et al., 1999; Iwata et al., 2000, 2001; Naski et al., 1998; Segev et al., 2000). These mice display a severe shortening of the appendicular skeletal elements due to reduced regions of proliferating and hypertrophic chondrocytes. In contrast, mice carrying a targeted deletion of *Fgfr3* are characterized by increased regions of proliferating and hypertrophic chondrocytes (Colvin et al., 1996; Deng et al., 1996). These studies have led to the conclusion that FGF signaling is a negative regulator of chondrocyte proliferation and differentiation. The expression of *Fgfr3* in proliferating and early hypertrophic chondrocytes correlates well with the bone phenotype in *Fgfr3* mutant mice.

Several lines of evidence have demonstrated that two other signaling factors, parathyroid hormone-like peptide (Pthlh; formerly PTHrP), expressed in periarticular chondrocytes (Lee et al., 1995), and Indian hedgehog (Ihh), expressed in prehypertrophic chondrocytes (Bitgood and McMahon, 1995), interact in a negative feedback loop to regulate the onset of hypertrophic differentiation (Vortkamp et al., 1996). First, overexpression of *Ihh* (Vortkamp et al., 1996) or *Pthlh* (Weir et al., 1996) or constitutive activation of the Pthlh receptor (Pthr; formerly PTH/PTHrP receptor; Schipani et al., 1997) results in a delayed onset of hypertrophic differentiation of chondrocytes. Second, overexpression of *Ihh* leads to increased expression of *Pthlh* (Vortkamp et al., 1996), whereas *Ihh* knockout mice do not express *Pthlh* (St-Jacques et al., 1999). Third, activation of Ihh signaling inhibits hypertrophic differentiation of chondrocytes in wild-type mice but not in *Pthlh* or *Pthr* mutant mice (Lanske et al., 1996; Vortkamp et al., 1996). These results place Ihh upstream of Pthlh and lead to a model in which Ihh serves as a measure for the number of chondrocytes undergoing hypertrophic differentiation, whereas the downstream factor Pthlh prevents chondrocytes from initiating the

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differentiation process. The level of Ihh and Pthlh signaling thus regulates the distance from the joint region at which hypertrophic differentiation takes place and indirectly determines the domain of proliferating chondrocytes (Chung et al., 2001; Vortkamp et al., 1996). In addition, targeted deletion of *Ihh* in mice revealed that Ihh directly regulates chondrocyte proliferation and is necessary for the ossification of endochondral bones (Karp et al., 2000; St-Jacques et al., 1999).

Bone morphogenetic proteins (BMP), a subgroup of the transforming growth factor β family (TGF-β), are a third group of secreted signaling factors regulating bone formation (reviewed in Massague, 2000; Massague et al., 2000). Several in vitro and in vivo studies have shown that BMP signaling induces chondrocyte proliferation leading to enlarged skeletal elements (Zou et al., 1997), whereas a loss of BMP signaling by overexpressing the BMP antagonist *Noggin* (Capdevila and Johnson, 1998; Pathi et al., 1999) or dominant-negative forms of the BMP receptors results in reduced bone growth (Zou and Niswander, 1996). In previous studies, we have demonstrated that Ihh and BMP signals interact at several stages to regulate chondrocyte development. We found that Ihh signaling induces the expression of several *Bmp* genes and that both signals act in parallel to regulate chondrocyte proliferation. Conversely, BMPs induce the expression of *Ihh* in cells that are released from the range of Pthlh signaling, thus indirectly regulating the onset of hypertrophic differentiation. In addition, the process of hypertrophic differentiation is negatively regulated by BMP signaling independently of the Ihh/Pthlh system (Minina et al., 2001).

In this study, we have investigated the epistatic relationship between the three signaling pathways. We found that FGF signaling regulates chondrocyte proliferation, the level of *Ihh* expression, and the rate of terminal hypertrophic differentiation. One important finding to come out of this analysis is that FGF signaling actually acts to promote hypertrophic differentiation instead of delaying it as previously suggested. The apparent decrease in the amount of hypertrophic chondrocytes is thus a secondary consequence of the accelerated differentiation process in combination with the reduced proliferation rate. This new interpretation has important implications for understanding the etiology of dwarfism syndromes. As activation of FGF signaling in the limb culture system resembles a loss of BMP signals, we have analyzed the interaction of FGF and BMP signaling. We found that both pathways act in antagonistic rather than epistatic relationships regulating chondrocyte proliferation, *Ihh* expression, and the process of hypertrophic differentiation. Accordingly, we have shown that BMP treatment rescues the reduced domains of proliferating and hypertrophic chondrocytes in a mouse model for achondroplasia.

Results

FGF Signaling Regulates Chondrocyte Proliferation and Differentiation

Activated FGF signaling in *Fgfr3ach* mice results in reduced zones of proliferating and hypertrophic chondrocytes (Naski et al., 1998). To further investigate the role of FGF signaling during chondrocyte development and its interactions with other signaling pathways, we have used a limb explant culture system (Minina et al., 2001). Treatment of limbs from E14.5 or E16.5 mouse embryos with FGF2 resulted in severe shortening of the skeletal elements compared to untreated control limbs (Figure 1A). To analyze the effect of FGF2 treatment at the molecular level, we hybridized sections of these limbs with *Ihh* and *type X collagen* (*ColX*), markers of prehypertrophic and hypertrophic cells, respectively. Limbs treated with FGF2 displayed smaller domains of *Ihh* and *ColX* expression than limbs from untreated cultures (Figures 1B, 1C, 1N, and 1O). In addition, BrdU labeling revealed a reduced rate of chondrocyte proliferation after FGF2 treatment (Figures 2I and 2J). Therefore, the molecular changes induced by FGF2 strongly resemble the *Fgfr3ach* phenotype.

In addition to the reduced domains of *Ihh* and *ColX* expression, we found an increased distance between the two *ColX*-expressing domains in E14.5 limb explants. Hybridization with *osteopontin* (*secreted phosphoprotein 1*; *Spp1*) and *matrix metalloproteinase 13* (*MMP13*), markers for terminally differentiated hypertrophic chondrocytes and osteoblasts, revealed that at both stages, E14.5 and E16.5, FGF2 treatment results in an increased expression of *Spp1* and *MMP13* (Figures 1D and 1P and data not shown). To differentiate between terminal hypertrophic cells and osteoblasts, we hybridized some of the FGF2-treated limbs with *osteocalcin* (*Oc*), which is exclusively expressed in osteoblasts. We found strong expression of *Oc* in the periosteum flanking the hypertrophic regions, but no expression could be detected in the cartilage core in cells expressing *Spp1* and *MMP13* (data not shown). FGF2 treatment therefore seems to accelerate chondrocyte differentiation into terminal hypertrophic cells. Interestingly, *MMP13* was upregulated at postnatal day 7 (P7) in *Fgfr3ach mice*, carrying one of the human achondroplasia mutations (*G380R*) under the *Collagen-II (ColII)* promoter (*Fgfr3ach (G380R)* mice; Naski et al., 1998). No significant upregulation could be detected at E14.5 or E16.5 (Figures 4H and 4K and data not shown; n = 2 for each stage).

FGF Signaling Acts Upstream of Ihh in Regulating the Onset of Hypertrophic Differentiation

The Ihh/Pthlh signaling system has been demonstrated to control the onset of hypertrophic differentiation of chondrocytes, demarcated by the distance between the *Ihh* expression domain and the joint region (Vortkamp et al., 1996). As described above, FGF2 treatment leads to reduced expression of *Ihh*. In addition, the distance between the *Ihh* expression domain and the periarticular region is reduced after FGF2 treatment, indicating an advanced onset of hypertrophic differentiation (Figures 1B and 1N; Table 1). To address the question of whether FGF signals directly regulate the onset of hypertrophic differentiation in parallel to the Ihh/Pthlh system or whether the phenotype is a secondary consequence of the reduced expression of *Ihh* after FGF2 treatment, we attempted to rescue the FGF2-induced effect by cotreatment with Pthlh. Compared to limbs treated with FGF2 double treatment with FGF2 and Pthlh resulted in an increased distance between the *Ihh* expression

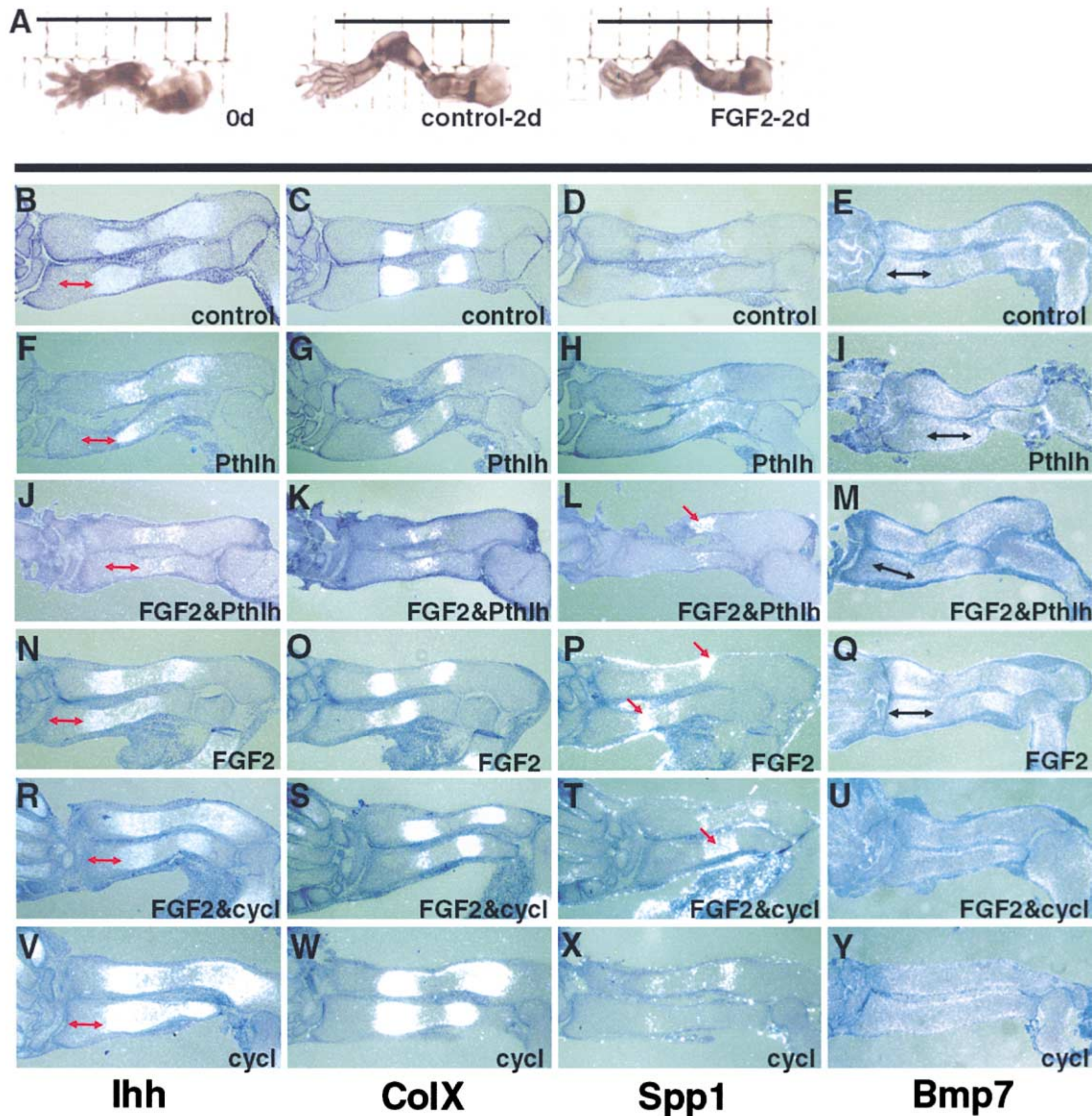

Figure 1. Interaction of FGF Signaling with the Ihh/Pthlh System

(A) Forelimbs of E14.5 mouse embryos were cultured for 2 days with (FGF2-2d) or without (control-2d) FGF2 and compared to uncultured limbs (0d). Rulers demarcate relative units, and the black lines indicate the size of the FGF2-treated explant.

(B–Y) Forelimbs of E14.5 mouse embryos were cultured for 2 days in control medium (B–E) or treated with Pthlh (F–I), FGF2 and Pthlh (J–M), FGF2 (N–Q), FGF2 and cyclopamine (R–U), or cyclopamine (V–Y). Serial sections were hybridized with antisense riboprobes for *Ihh* (B, F, J, N, R, and V), *ColX* (C, G, K, O, S, and W), and *Spp1* (D, H, L, P, T, and X). *Bmp7* (E, I, M, Q, U, and Y) was hybridized to sections of parallel cultures. Treatment with FGF2 results in an advanced onset of hypertrophic differentiation demarcated by the reduced distance between the *Ihh* expression domain and the joint region (B and N) and a reduced *Bmp7* expression domain (E and Q). In addition, FGF2 reduces the expression of *Ihh* and *ColX* and increases the expression of *Spp1* (B–D and N–P). Pthlh delays the onset of hypertrophic differentiation and increases the *Bmp7* expression domain but does not upregulate *Spp1* expression (B–I). Cotreatment with FGF2 and Pthlh results in a delay of the onset of hypertrophic differentiation compared to treatment with FGF2 (J, M, N, and Q) and in an increased expression of *Spp1* compared to treatment with Pthlh (H and L). Blocking of Ihh signaling by cyclopamine advances the onset of hypertrophic differentiation but does not regulate *Spp1* expression (V–Y). Cotreatment with cyclopamine and FGF2 does not accelerate the onset of hypertrophic differentiation compared to treatment with cyclopamine alone but results in upregulation of *Spp1* expression (R–Y). Double-sided arrows demarcate the distance between the *Ihh* expression domain and the joint region (red) or the size of the *Bmp7* expression domain (black) after FGF2 treatment. Red arrows point to upregulated *Spp1* expression. In all panels, ulna is up and radius is down. cycl, cyclopamine.

domain and the periarticular region, indicating a delay in chondrocyte differentiation. However, due to the reduced proliferation rate (see below), this distance does not reach that of Pthlh-treated explants (Figures 1B, 1F, 1J, and 1N; Table 1). To further support these results, we have analyzed the expression of *Bmp7* (n = 2, each), which is expressed at a low level in the distal population of proliferating chondrocyte. Strong expression is found in the central region of proliferating chondrocytes flanking the *Ihh* expression domain. FGF treatment results in a reduced size of the *Bmp7* expression domain compared to untreated explants (Figures 1E and 1Q). This

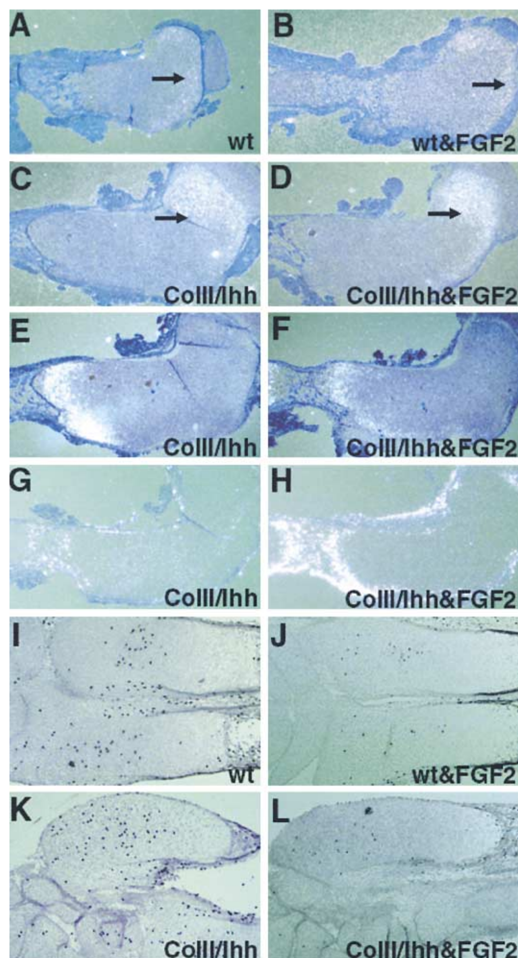

Figure 2. FGF Signaling Acts Upstream of Ihh in Regulating the Onset of Hypertrophic Differentiation but Independently in Regulating Terminal Hypertrophic Differentiation and Chondrocyte Proliferation

Forelimbs of E16.5 wild-type mice (A, B, I, and J) and *ColII/Ihh* embryos (C–H and K and L) were cultured for 2 days in control medium (A, C, E, G, I, and K) or treated with FGF2 (B, D, F, H, J, and L). Serial sections were hybridized with antisense riboprobes for *Pthlh* (A–D) and *Ihh* (E and F). *Spp1* (G and H) was hybridized to limbs from parallel cultures. Proliferating cells were labeled with BrdU and detected by antibody staining (I–L). The endogenous expression of *Ihh* is reduced in *ColII/Ihh* mice by FGF2 treatment (E and F). However, FGF2 treatment does not result in significantly reduced expression of *Pthlh* in wild-type or mutant limbs (A–D). In addition, FGF2 treatment does not advance the onset of hypertrophic differentiation demarcated by the distance between the *Pthlh* and *Ihh* expression domains (compare [C and E] with [D and F]). In contrast, FGF2 treatment accelerates terminal hypertrophic differentiation independently of Ihh (G and H) and reduces chondrocyte proliferation both in wild-type (I and J) and *ColII/Ihh* embryos (K and L). Black arrows indicate the *Pthlh* expression domain. (A)–(H) display the proximal end of the humerus and (I)–(L) show the distal ends of ulna (up) and radius (down).

phenotype can be rescued by cotreatment with Pthlh (Figure 1M), which on its own results in an increased domain of *Bmp7*-expressing chondrocytes (Figure 1I). Taken together, Pthlh acts downstream of FGF signaling in regulating the onset of hypertrophic differentiation.

To test whether Ihh itself can rescue the FGF2-induced phenotype, we treated limbs of mice overexpressing the chicken *Ihh* gene under the *ColII* promoter (*ColII/Ihh* embryos; Long et al., 2001) with FGF2. As in wild-type limbs, we found reduced expression of the endogenous *Ihh* message in *ColII/Ihh* mice after FGF2 treatment (Figures 2E and 2F). Corresponding to the reduced expression of the endogenous *Ihh* gene, slight reduction of *Pthlh* expression was found in limb explants of wild-type and *ColII/Ihh* embryos (Figures 2A–2D). However, FGF2 treatment did not result in significantly reduced *Pthlh* expression in *ColII/Ihh* embryos (Figures 2C and 2D) nor in a decreased distance between the *Ihh* expression domain and the periarticular region, indicating that FGF signaling acts upstream of Ihh (compare Figures 2C and 2E with 2D and 2F).

As a third experiment, we tested whether FGF2 could further advance the onset of hypertrophic differentiation in limbs where the Ihh signal was blocked. As we have demonstrated before, cyclopamine effectively blocks Ihh signaling, resulting in an upregulation of *Ihh* expression and an advanced onset of hypertrophic differentiation (Figures 1B, 1C, 1V, and 1W; Table 1; Minina et al., 2001). Double treatment with cyclopamine and FGF2 did not further accelerate the onset of hypertrophic differentiation compared to treatment with cyclopamine alone (Figures 1B, 1N, 1R, and 1V; Table 1). Corresponding to earlier results indicating that Ihh signaling upregulates the expression of *Bmp7* (Minina et al., 2001), treatment with cyclopamine blocks the expression of *Bmp7* in an FGF-independent way (Figures 1U and 1Y). Taken together, these experiments strongly suggest that FGF signaling acts upstream of the Ihh/Pthlh system in regulating the onset of hypertrophic differentiation.

FGF Signaling Regulates Chondrocyte Proliferation Independently of Ihh

Limb explants treated with FGF2 display a reduced rate of chondrocyte proliferation (Figures 2I and 2J), whereas Ihh has been shown to upregulate chondrocyte proliferation (Long et al., 2001). Similar to the onset of hypertrophic differentiation, Ihh might thus mediate the effect of FGF signaling on chondrocyte proliferation. To test this hypothesis, we treated limb explants of *ColII/Ihh* embryos with FGF2 and analyzed the proliferation rate by BrdU labeling. We found a reduced rate of chondrocyte proliferation compared to untreated cultures (Figures 2K and 2L), indicating that FGF signaling negatively regulates chondrocyte proliferation independently of Ihh.

FGF Signaling Regulates Terminal Hypertrophic Differentiation Independently of the Ihh/Pthlh System

In addition to regulating the onset of hypertrophic differentiation, we found that FGF signaling induces the differentiation of terminal hypertrophic cells expressing *Spp1* (Figures 1D and 1P). In previous experiments, we did not detect a role for Ihh in regulating the process of hypertrophic differentiation itself (Minina et al., 2001). To confirm that FGF signaling acts independently of Ihh during this process, we cotreated limbs with FGF2 and cyclopamine. As expected, cyclopamine treatment increased the region of *ColX*-expressing hypertrophic chondrocytes but had no effect on the differentiation of

Table 1. Onset of Hypertrophic Differentiation

Treatment	Age	N	Length (%)	±SD	P Value
F/control	E14.5	9	83.15	5.21	<0.001[a]
F/control	E16.5	7	90.60	4.24	0.004[a]
FP/F	E14.5	5	126.24	12.07	0.011[a]
FP/P	E14.5	5	76.95	7.19	0.012[a]
P/control	E14.5	6	133.14	15.25	0.004[a]
FC/F	E14.5	4	85.82	10.29	0.086[a,c]
C/FC	E14.5	5	88.01	13.45	0.149[a,c]
C/control	E14.5	5	72.94	5.59	0.003[a]
contr/B	E14.5	7	88.42	6.76	0.007[a]
F100B500/B	E14.5	5	87.77	7.21	0.054[a]
F250B500/B	E14.5	5	81.50	5.44	0.002[a]
F500B500/B	E14.5	4	71.45	5.93	0.005[a]
F250B250/F	E14.5	5	119.87	9.74	0.011[a]
F250B500/F	E14.5	8	135.97	13.53	<0.001[a]
F250B1000/F	E14.5	5	145.11	34.76	0.055[a]
ach/wt	E16.5	5	91.88	6.84	0.040[b]
ach and B/ach	E16.5	6	114.14	6.09	0.004[a]
wt and B/wt	E16.5	5	116.91	7.45	0.009[a]

[a] Paired, two-sided Student's t test.
[b] Unpaired, two-sided Student's t test.
[c] No significant difference, indicating a similar onset of hypertrophic differentiation.
B, BMP2; C, cyclopamine; F, FGF2; P, Pthlh; ach, *Fgfr3ach(G380R)* mice; wt, wild-type mice.

terminal hypertrophic cells expressing *Spp1* (Figures 1C, 1D, 1W, and 1X). In contrast, after double treatment with cyclopamine and FGF2, the region of *ColX*-expressing cells became smaller and *Spp1* was highly expressed (Figures 1O, 1P, 1S, 1T, 1W, and 1X), demonstrating that FGF signaling induces terminal hypertrophic differentiation independently of Ihh.

This result was supported by explants cotreated with FGF2 and Pthlh. As described earlier, FGF2 induces an advanced onset of hypertrophic differentiation, which is delayed by cotreatment with Pthlh. Nevertheless, in limbs double treated with Pthlh and FGF2, we found an upregulation of *Spp1* expression (Figures 1D, 1L, and 1P). In addition, the *Ihh* and *ColX* expression domains were severely reduced compared to limbs treated with either factor (Figures 1F, 1G, 1J, 1K, 1N, and 1O). Such an effect would be expected if hypertrophic differentiation was delayed by Pthlh and, simultaneously, the hypertrophic chondrocytes that were present at the start of the culture underwent accelerated hypertrophic differentiation. Similarly, treatment of limbs overexpressing *Ihh* with FGF2 resulted in an upregulation of *Spp1* expression (Figures 2G and 2H), supporting the idea that FGF signaling regulates terminal hypertrophic differentiation independently of the Ihh/Pthlh system.

FGF Signaling Is Antagonized by BMP Signals

In previous studies, we have shown that BMP signaling upregulates the expression of *Ihh*, increases the rate of chondrocyte proliferation, and delays the differentiation of terminal hypertrophic chondrocytes. In contrast, blocking BMP signals by Noggin treatment results in a phenotype very similar to that of FGF2 treatment (Minina et al., 2001). Thus, BMP and FGF signals seem to have opposite functions during chondrocyte development. To investigate the epistatic relationship between the two signaling systems, we cotreated limb explants with FGF2 and BMP2. Double-treated limbs revealed an increased rate of chondrocyte proliferation compared to FGF2-treated limbs and a reduced proliferation rate compared to BMP2 treatment (Figures 3A, 3D, 3G, and 3J). Similarly, cotreatment with BMP2 enhanced the reduced domain of *Ihh* expression after FGF2 treatment. However, *Ihh* expression was still reduced compared to BMP2 treatment alone (Figures 3B, 3E, 3H, and 3K). Correspondingly, the distance between the *Ihh* expression domain and the joint region increased when comparing BMP2 treatment to cotreatment with FGF2 and BMP2, or to FGF2 alone (Figures 3B, 3E, 3H, and 3K; Table 1). Finally, the FGF2-induced expression of *Spp1* was inhibited by cotreatment with BMP2 but remained higher than after BMP2 treatment alone (Figures 3C, 3F, 3I, and 3L). Interestingly, double-treated cultures did not resemble the BMP2 or FGF2 phenotype, as would be expected if one pathway acted downstream of the other in an epistatic relationship. We therefore speculated that FGF and BMP signals act in parallel, antagonistic pathways.

To further support this idea, we cotreated limbs with the optimized concentration of BMP2 used in previous experiments and varying concentrations of FGF2. We found that the expression domain of *Ihh* in these cultures decreased with increasing concentrations of FGF2 (Figures 3B, 3K, 3N, 3Q, and 3T). Correspondingly, the distance between the articular region and the *Ihh*-expressing chondrocytes was reduced in relation to the FGF2 concentration (Figures 3B, 3K, 3N, 3Q, and 3T; Table 1). Similarly, increasing concentrations of FGF2 resulted in an increase in *Spp1* expression (Figures 3C, 3L, 3O, 3R, and 3U) and a reduced proliferation rate (Figures 3A, 3J, 3M, 3P, and 3S). In contrast, in cultures treated with a constant FGF2 concentration and increasing amounts of BMP2, the level of *Ihh* expression, its distance from the joint region, the rate of chondrocyte proliferation, and the expression of *Spp1* were determined by the

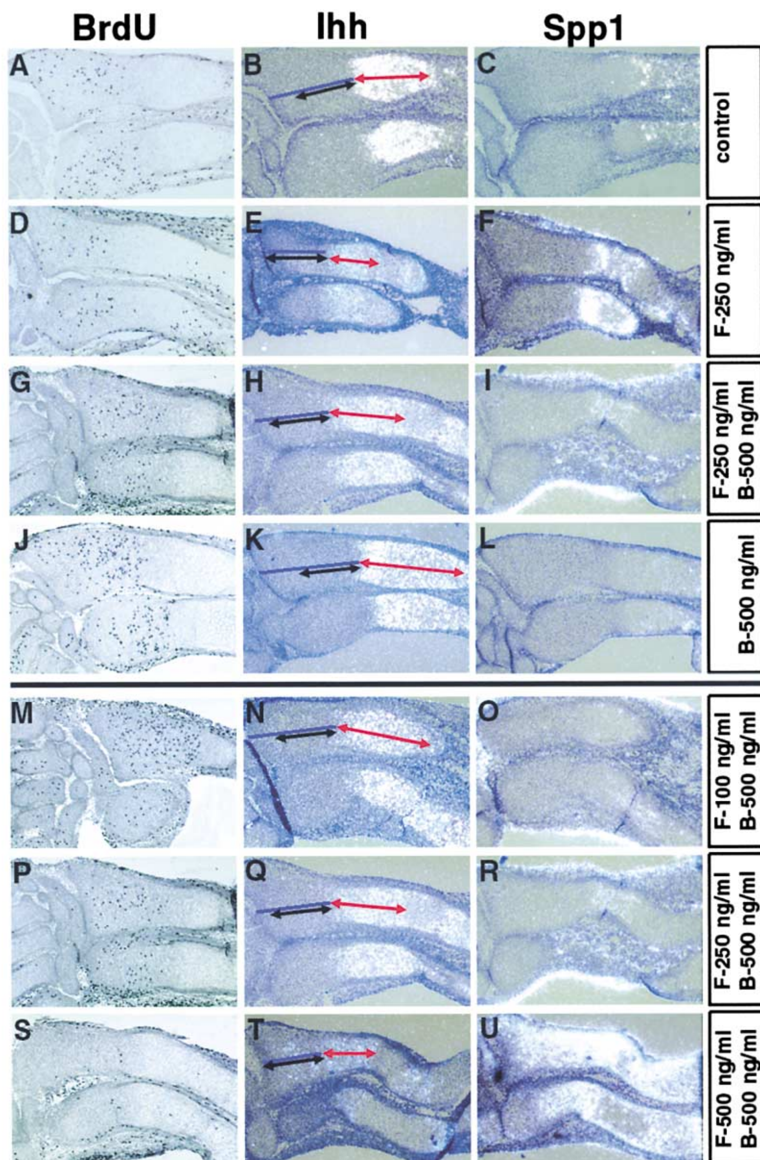

Figure 3. BMP and FGF Signaling Have Antagonistic Effects on Chondrocyte Development

Forelimbs of E14.5 embryos were cultured for 4 days in control medium (A–C) or in medium supplemented with FGF2 (D–F), FGF2 and BMP2 (G–I), BMP2 (J–L), and with 500 ng/ml BMP2 and FGF2 at different concentrations: 100 ng/ml (M–O), 250 ng/ml (P–R), or 500 ng/ml (S–U). Proliferating cells were labeled with BrdU and detected by antibody staining (A, D, G, J, M, P, and S). Serial sections were hybridized with antisense riboprobes for *Ihh* (B, E, H, K, N, Q, and T) and *Spp1* (C, F, I, L, O, R, and U).

(A–L) Limbs cotreated with BMP2 and FGF2 display an intermediate rate of chondrocyte proliferation and intermediate domains of *Ihh* and *Spp1* expression compared to limbs treated with either BMP2 or FGF2. In addition, the distance between the *Ihh* expression domain and the joint region has an intermediate length compared with one after either BMP2 or FGF2 treatment.

(M–U) In cultures with a defined concentration of BMP2, chondrocyte proliferation and the size of the domain of *Ihh* and *Spp1* expression depend on the concentration of FGF2. Double-sided arrows demarcate the size of the *Ihh* expression domain (red) or the distance between the joint region and the *Ihh* expression domain after treatment with 250 ng/ml FGF2 (black) compared to this distance in each experiment (blue line).

amount of BMP2 (Table 1 and data not shown). Taken together, these experiments strongly support an antagonistic function of FGF and BMP signaling in regulating chondrocyte development.

BMPs and FGFs Regulate the Expression of Genes from the Opposing Family

To address whether BMP and FGF signals regulate the expression of other members of the FGF and BMP family, we have treated limbs with FGF2 and analyzed the expression of *Bmp4* and *Bmp7*. We found that *Bmp4* is slightly upregulated in the proliferating and terminal hypertrophic chondrocytes (Figures 4A and 4D). Interestingly, in addition to an upregulation of *Bmp7*, the domain of *Bmp7*-expressing cells was markedly shifted toward the joint region (Figures 4B and 4E). Thus, the cells of the distal region of proliferating chondrocytes that normally express only low amounts of *Bmp7* have differentiated into *Bmp7*-expressing chondrocytes normally found in the medial proliferating region. Similarly in *Fgfr3ach(G380R)* mice, *Bmp7* expression is upregulated at E14.5 and E16.5 (Figures 4G and 4J and data not shown; n = 2 for each stage). Conversely, treatment of limb explants with BMP2 resulted in a slight upregulation of *Fgf18* in the perichondral region surrounding the cartilage elements (Figures 4C and 4F). Thus, BMP and FGF signals positively regulate the production of growth factors from the opposing pathways.

BMP Signaling Rescues the Achondroplasia Phenotype in a Mouse Model

As BMP signaling can antagonize the FGF2-induced effects on chondrocyte development, we asked whether BMP2 treatment would rescue the reduced rate of chondrocyte proliferation and the reduced size of the hypertrophic region in *Fgfr3ach(G380R)* mice. Limb explants

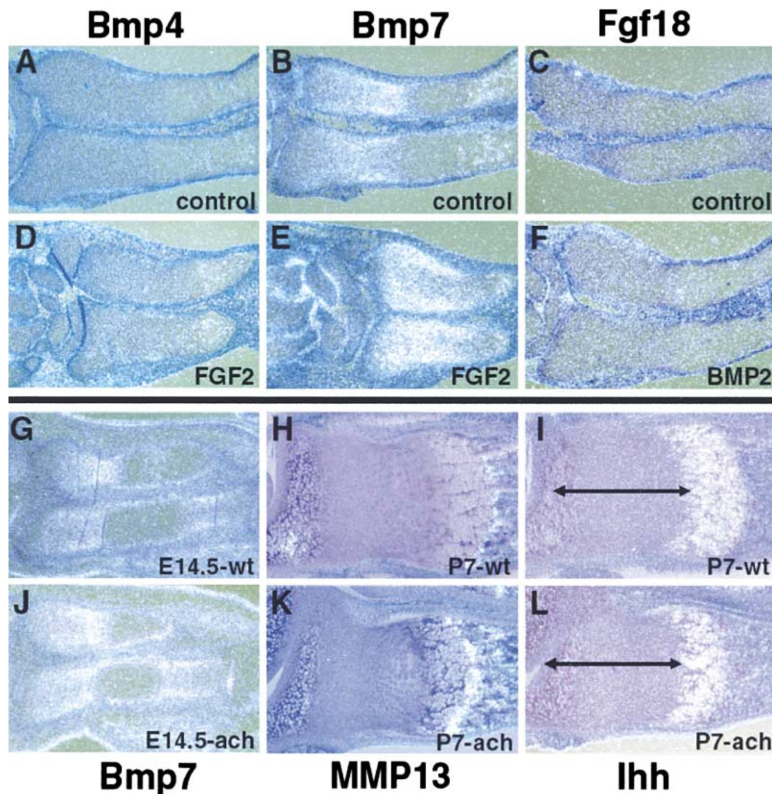

Figure 4. BMP and FGF Signaling Positively Regulate Gene Expression of the Opposite Pathways

(A–F) Forelimbs of E14.5 wild-type embryos were cultured for 2 days in control medium (A–C) or in medium supplemented with FGF2 (D and E) or BMP2 (F). Serial sections were hybridized with antisense riboprobes for *Bmp4* (A and D), *Bmp7* (B and E), and *Fgf18* (C and F). Sections of forelimbs of E14.5 (G and J) and P7 (H, I, K, and L) *Fgfr3ach(G380R)* mice were hybridized with antisense riboprobes for *Bmp7* (G and J), *MMP13* (H and K), and *Ihh* (I and L). Treatment with FGF2 results in slight upregulation of *Bmp4* expression in the perichondrium and in terminal hypertrophic chondrocytes (A and D) and strong upregulation of *Bmp7* expression in proliferating chondrocytes (B and E). Similarly, *Bmp7* is upregulated in E14.5 *Fgfr3ach(G380R)* mice (J) compared to wild-type littermates (G). Treatment with BMP2 increases *Fgf18* expression in the perichondrium (C and F). At P7, *Ihh* expression in *Fgfr3ach(G380R)* mice and the distance from the *Ihh* expression domain to the joint region are reduced (I and L). In addition, *MMP13* is upregulated in mutant mice compared to wild-type (H and K). Double-sided black arrows demarcate the distance between the *Ihh* expression domain and the joint region of *Fgfr3ach(G380R)* mice. In (A–G) and (J), ulna is up and radius is down, and (H), (I), (K), and (L) display sections through the radius. ach, *Fgfr3ach(G380R)* mice; wt, wild-type.

of E16.5 transgenic mice were cultured for 2 days. Similar to the results of Naski et al. (1998), we found that limb explants of *Fgfr3ach(G380R)* mice displayed a reduced rate of chondrocyte proliferation (Figures 5A and 5G) and reduced domains of *Ihh* and *ColX* expression compared to limbs of wild-type littermates (Figures 5B, 5C, 5H, and 5I). In addition, the distance between the *Ihh* expression domain and the joint region was shortened, indicating an advanced onset of hypertrophic differentiation (Figures 4I and 4L, uncultured; Figures 5B and 5H, after culture; Table 1). Treatment with BMP2 resulted in increased expression of *Ihh* and an enlarged distance between the *Ihh* expression domain and the joint region (Figures 5B, 5E, 5H, and 5K). In addition, the *ColX* expression domain was enlarged after BMP2 treatment (Figures 5C, 5F, 5I, and 5L). Finally, BMP2 treatment led

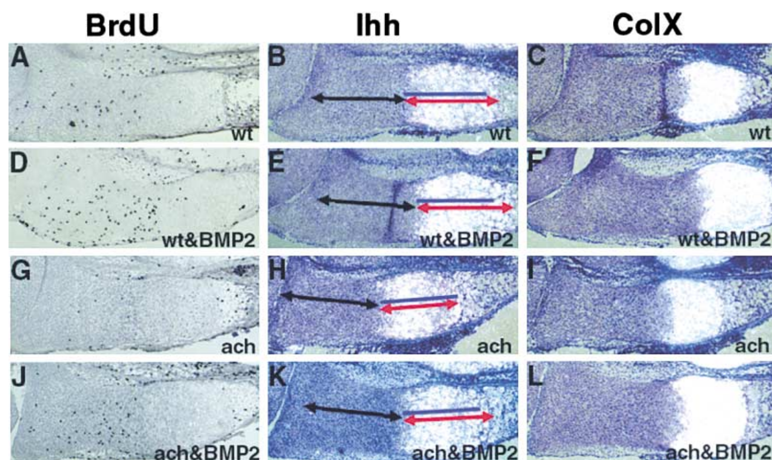

Figure 5. BMP Signaling Rescues the Achondroplasia Phenotype in a Mouse Model

Forelimbs of E16.5 wild-type (A–F) and *Fgfr3ach(G380R)* (G–L) embryos were cultured for 2 days in control medium (A–C and G–I) or in medium supplemented with BMP2 (D–F and J–L). Serial sections were hybridized with antisense riboprobes for *Ihh* (B, E, H, and K) or *ColX* (C, F, I, and L). Proliferating cells were labeled with BrdU and detected by antibody staining (A, D, G, and J). As in wild-type limbs, BMP2 treatment increases the expression of *Ihh* (B, E, H, and K) and *ColX* (C, F, I, and L) in hypertrophic cells of *Fgfr3ach(G380R)* mice. In addition, treatment with BMP2 leads to a delay in the onset of hypertrophic differentiation, as can been seen from the increased distance between *Ihh*-expressing cells and the joint region (H and K). Furthermore, BMP2 treatment increases the reduced rate of chondrocyte proliferation in *Fgfr3ach(G380R)* mice (G and J). Black arrows demarcate the distance between the *Ihh* expression domain and the joint region in untreated *Fgfr3ach(G380R)* mice. Red double-sided arrows demarcate the *Ihh* expression domain, compared to the *Ihh* expression in untreated *Fgfr3ach(G380R)* mice (blue line). All panels show sections through the radius. ach, *Fgfr3ach(G380R)* mice; wt, wild-type.

to an increase of chondrocyte proliferation compared to untreated limbs of *Fgfr3ach(G380R)* mice (Figures 5G and 5J). These experiments demonstrate that BMP signaling can improve the cartilage phenotype resulting from activated FGF signaling in a mouse model for achondroplasia, supporting the idea of an antagonistic relationship between these signaling systems.

Discussion

FGF Signaling Accelerates Hypertrophic Differentiation

In this study, we have used an organ culture system for embryonic limb explants to investigate the interaction of FGF signaling with that of the Ihh/Pthlh and BMP signaling systems. First, we demonstrated that treatment of limb explants with FGF2 mimics the phenotype of activated FGFR3 signaling in *Fgfr3ach(G380R)* mice (Naski et al., 1998) by reducing the rate of chondrocyte proliferation and the domain of *Ihh*- and *ColX*-expressing hypertrophic chondrocytes. In addition, our studies revealed that the distance between the domain of *Ihh* expression and the joint region is reduced by FGF signals. As this distance demarcates the onset of hypertrophic differentiation, the effect of FGF2 can be interpreted as an advanced onset of chondrocyte differentiation. Another signaling system regulating the onset of hypertrophic differentiation is the Ihh/Pthlh signaling system. Cotreatment of limb cultures with Pthlh and FGF2 (our study) or treatment of metatarsal from achondroplasia or *Fgfr3*$^{-/-}$ mice with Pthlh (Chen et al., 2001) results in a delay of chondrocyte differentiation. Similarly, activation of FGF signaling in limb explants of mice overexpressing *Ihh* cannot overcome the delay in hypertrophic differentiation. These experiments strongly support the hypothesis that FGF signaling acts upstream of the Ihh/Pthlh system in regulating the onset of hypertrophic differentiation. As activated FGF signaling in addition results in reduced expression of *Ihh* in limb cultures and in *Fgfr3ach* mice, FGF signals seem to regulate the onset of hypertrophic differentiation by directly regulating *Ihh* expression. Interestingly, *Bmp7* expression, which is normally found in the medial proliferating zone, is shifted toward the joint region by activated FGF signaling, indicating advanced chondrocyte differentiation even in the pool of proliferating chondrocytes.

Surprisingly, treatment of limb explants with FGF2 resulted in an increased region of *Spp1*-expressing terminal hypertrophic chondrocytes, indicating an acceleration of the process of hypertrophic differentiation. By cotreatment experiments, we have shown that this acceleration occurs independently of the Ihh/Pthlh system. Based on our results, we reinterpret the role for FGF signaling in regulating chondrocyte development in the following model. Instead of delaying hypertrophic differentiation of chondrocytes, as has been suggested in previous studies, our experiments show that FGF signaling accelerates hypertrophic differentiation, both by inducing the onset of hypertrophic differentiation and by accelerating the differentiation process itself. In concert with the reduced rate of chondrocyte proliferation, this acceleration leads to the decreased domains of hypertrophic chondrocytes seen in *Fgfr3ach* mice. By regulating both processes, FGF signaling seems to keep the pace of chondrocyte differentiation in phase with the development of the embryo. The Ihh/Pthlh system regulates the distance from the joint region at which cells switch from proliferating into differentiating chondrocytes. If FGF signals would only downregulate chondrocyte proliferation, it would take a longer developmental time to establish the Pthlh-determined distance for hypertrophic differentiation. Instead, by simultaneously downregulating proliferation and the Ihh/Pthlh feedback loop, FGF signaling adjusts the initiation of hypertrophic differentiation to the proliferation rate and allows chondrocytes to differentiate closer to the joint region. Similarly, the region of hypertrophic cells must be regulated by specific, not yet identified signaling systems. By accelerating terminal hypertrophic differentiation, FGF signaling might fulfill a similar function, namely adapting the differentiation process to the reduced proliferation rate.

FGF Signaling and Chondrocyte Proliferation

Different laboratories have investigated the negative role of FGF signaling on chondrocyte proliferation and found that FGF signaling activates STAT1 and possibly other members of the STAT family of transcription factors. Activation of STAT1 in turn leads to the upregulating of the cell cycle inhibitor p21$^{WAF1/CIP1}$, thereby inhibiting chondrocyte proliferation (Li et al., 1999; Sahni et al., 1999, 2001).

Ihh signaling has been shown to positively regulate chondrocyte proliferation (Long et al., 2001). Here we show that in contrast to the regulation of the onset of hypertrophic differentiation, both signaling pathways act independently of each other in regulating chondrocyte proliferation. This result is supported by recent experiments showing that activation of Ihh signaling results in an upregulation of Cyclin D1, a positive regulator of the cell cycle (Long et al., 2001). Although more experiments will be necessary to reveal the interaction of the two signal pathways on the molecular level, they seem to act on different regulators of the cell cycle.

Signaling through FGF Receptors

The expression of *Fgfr3* in proliferating and early hypertrophic chondrocytes implicates a role for this receptor in regulating chondrocyte proliferation and *Ihh* expression. In contrast, terminal hypertrophic differentiation might be mainly regulated by *Fgfr1* (Delezoide et al., 1998), which is strongly expressed in hypertrophic chondrocytes. The *ColII* promoter, which is used to express the activated *Fgfr3* in the achondroplasia mouse model, is only weakly expressed in hypertrophic chondrocytes. Nevertheless, we have found an upregulation of *Spp1* expression in these mice. Thus, low expression of *Fgfr3ach(G380R)* in hypertrophic chondrocytes seems to be sufficient to accelerate hypertrophic differentiation. Interestingly, this acceleration was found postnatally, indicating an increasing sensitivity of bone development to FGF signaling with age. As *Fgfr3* is expressed at a similarly low level in hypertrophic chondrocytes as *ColII*, activation of signaling through FGFR3 is likely to accelerate terminal hypertrophic differentiation in achondroplasia patients. The age-dependent increasing sensitivity to FGF signaling would thus add to the increasing severity of the achondroplasia phenotype with age.

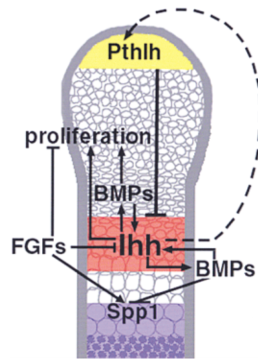

Figure 6. Integration of FGF, BMP, and Ihh/Pthlh Signaling into a Common Control Network

For details, see text.

A question not addressed in our study is which members of the FGF family regulate the specific steps during chondrocyte differentiation in vivo. *Fgf2*, *Fgf9*, *Fgf8*, and *Fgf17* (see references in Liu et al., 2002) have been shown to be expressed in the developing cartilage anlagen; however, inactivation of these *Fgf*s in mice does not result in skeletal dysmorphologies. Recently, mice have been created carrying a targeted deletion of *Fgf18* (Liu et al., 2002; Ohbayashi et al., 2002). Interestingly, these mice display an increased rate of chondrocyte proliferation and an elongated hypertrophic region similar to $Fgfr3^{-/-}$ mice, indicating that FGF18 signals through FGFR3. In addition, reduced expression of *Spp1* and delayed bone formation has been found in these mice. Thus, FGF18 may also signal through other receptors, for example FGFR1, to regulate terminal hypertrophic differentiation and thus the ossification process.

FGF and BMP Signaling Act as Antagonists during Cartilage Development

In previous studies, we have investigated the role of BMP signaling and its interaction with the Ihh/Pthlh system. We found that BMPs regulate chondrocyte proliferation in parallel to Ihh. Furthermore, BMP signaling upregulates the expression of *Ihh* in cells that are released from the range of the Pthlh signal. Last but not least, BMP signaling delays the differentiation of terminal hypertrophic chondrocytes (Minina et al., 2001). Thus, BMP and FGF signals seem to regulate the same stages of chondrocyte development, mediating however opposite effects. We have used double treatment with BMP2 and FGF2 to analyze the epistatic relationship between the two signaling systems. All three parameters tested—proliferation, *Ihh* expression, and terminal hypertrophic differentiation—showed an intermediate phenotype relative to treatment with either FGF2 or BMP2, alone. Furthermore, by varying the concentrations of the two factors in cotreatment experiments, we found that the balance of the concentrations was critical for the resulting phenotype. In contrast to an epistatic relationship, where the downstream signal should determine the phenotype, our experiments strongly indicate that FGF and BMP signals act in independent pathways having antagonistic effects on chondrocyte development. This is supported by the expression of at least some members of the *Bmp* and *Fgf* family such as *Bmp7* and *Fgf18*, which are up- and not downregulated, as would be expected in an epistatic relationship. Such an upregulation of antagonistically acting signals might serve to compensate variations in gene expression or signal transduction and thus stabilize proper chondrocyte differentiation. The observed upregulation of *Bmp4* is in contrast to the results of Naski et al. (1998) and might reflect an age-dependent difference. In contrast, *Bmp7* is clearly upregulated after FGF2 treatment and in *Fgfr3ach(G380R)* mice at embryonic stages.

As FGFs and BMPs signal through different types of kinase receptors and intracellular mediators, it will be important to investigate at which point the pathways converge. Both signaling systems could, for example, regulate the same promoters. Alternatively, both signaling pathways could regulate distinct downstream genes, which might then convey the antagonistic functions. A third possibility is that both signaling systems interact cytoplasmically at the level of the signal transduction cascade. BMPs signal through serine/threonine kinase receptors that upon phosphorylation activate transcription factors of the Smad family (Massague, 2000). FGFs signal through tyrosine kinase receptors, which in turn can activate at least two intracellular pathways, the extracellular signal-regulated kinase (Erk) pathway and the phosphatidylinositol-3-OH kinase (PI[3] kinase) pathway. Interestingly, Kretzschmar et al. have analyzed Erk-mediated signaling of the epidermal growth factor (EGF) and its interaction with BMP signals in cell cultures. They found that activation of the Erk pathway can inhibit BMP-induced phosphorylation of Smad1, thus preventing transport of the Smad1-Smad4 complex into the nucleus (Kretzschmar et al., 1997). It will be interesting to investigate whether FGF and BMP signals interact in comparable ways to regulate chondrocyte proliferation and differentiation.

BMP Signaling and Achondroplasia

Achondroplasia, the most common form of inherited human dwarfism, results from an activation of FGF signaling (Naski et al., 1996). In addition to their reduced body size, achondroplasia patients often suffer from severe pain as a result of bone and joint malformations. No successful treatment for achondroplasia has been found to date. In our study, we have started to unravel the interaction of FGF signaling with that of the BMP and Ihh/Pthlh signaling system. We demonstrate that BMP signaling can rescue the achondroplasia phenotype on the molecular level by increasing the domains of proliferating and hypertrophic chondrocytes. These experiments demonstrate the importance of deciphering the precise integration of different signaling systems to understand the molecular origins of dwarfism diseases. Such an understanding will be a prerequisite in order to develop specific treatment strategies. Ihh and Pthlh signaling, for example, can rescue the FGF-induced advanced onset of hypertrophic differentiation. However, in combination with elevated FGF signals, treatment with either factor would further reduce the region of hypertrophic chondrocytes, thereby most likely enhancing the bone phenotype. In contrast, BMP

signaling seems to act at very similar stages of chondrocyte development, as does FGF signaling. Therefore, manipulating the BMP signaling pathway would be a more promising approach to improve the achondroplasia phenotype. Further in vitro and in vivo studies will, however, be necessary to understand the molecular mechanism of their interaction.

Interaction of FGF, BMP, and Ihh/Pthlh Signaling

Based on the results of our study and previous investigations, we suggest the following model of how FGF signals interact with the Ihh/Pthlh and the BMP signaling pathways to control chondrocyte development (Figure 6). As previously described, Ihh, which is expressed in the prehypertrophic chondrocytes, induces the expression of various *Bmp*s in the flanking perichondrium/periosteum and the proliferating chondrocytes (Minina et al., 2001). Ihh and BMP signals act in parallel to induce chondrocyte proliferation, whereas FGF signaling inhibits chondrocyte proliferation independently of either signaling system. Ihh, in addition, regulates the expression of *Pthlh* in the periarticular region. The range of Pthlh signaling determines the distance from the joint at which the onset of hypertrophic differentiation takes place, thereby indirectly regulating the pool of chondrocytes that are competent to proliferate. Chondrocytes, which are released from the range of Pthlh signaling, change their competence and react to BMP signals with the upregulation of *Ihh* expression. In contrast, FGF signals act as antagonists of BMP signaling and negatively regulate *Ihh* expression. By acting upstream of Ihh, the balance of FGF and BMP signals regulates the distance from the joint region at which hypertrophic differentiation takes place. Furthermore, whereas FGF signaling accelerates the rate of terminal hypertrophic differentiation, BMPs have been shown to hinder this process. By simultaneously regulating proliferation, *Ihh* expression, and the rate of terminal hypertrophic differentiation, the balance of FGF and BMP signals seems to adjust the process of hypertrophic differentiation to the proliferation rate.

Experimental Procedures

Cultures of Mouse Limb Explants

Forelimbs of mouse embryos were cultured as described in Minina et al. (2001). Limbs were treated with the minimal concentrations of different growth factors necessary to induce a specific effect on chondrocyte differentiation. Limb explants of NMRI, *ColII/Ihh*, and *Fgfr3ach(G380R)* mice were treated with 250 ng/ml FGF2 (FGFb; Sigma) at embryonic day 14.5 (E14.5) and 50 ng/ml at E16.5. All other factors were applied at constant concentrations at E14.5 and E16.5: 500 ng/ml recombinant human BMP2 (Genetics Institute); 3×10^{-7} M human 1–34 Pthlh (Peninsula); 10 μM cyclopamine (Incardona et al., 1998). If not otherwise mentioned, each combination of growth factors was repeated at least five times with comparable results. For single-treatment experiments, the right limb was cultured in medium supplemented with the specific growth factor and compared to the left one cultured in control medium. For all cotreatment experiments, double-treated limbs were compared to contralateral limbs treated with each of the single factors. For each growth factor combination, at least one limb was hybridized with *ColII*, a general marker of chondrocytes, to confirm that the cells were still alive after culture.

Wild-type mice (NMRI) were derived from Charles River (Sulzfeld, Germany). *Fgfr3ach(G380R)* mice and *ColII/Ihh* mice were identified by PCR of tail DNA as previously described (Naski et al., 1998; Long et al., 2001).

Analysis of Limb Explants

Limb explants were fixed overnight in 4% paraformaldehyde at 4°C and embedded in paraffin. Serial sections of 5 μm were processed for radioactive in situ hybridization using [P^{33}]UTP-labeled antisense riboprobes. Hybridization was carried out at 70°C in 50% formamide as previously described (Vortkamp et al., 1996). Sections were counterstained with toluidine blue (Sigma). Probes for in situ hybridization were as follows: *rColII* (Kohno et al., 1984), *mColX* (Jacenko et al., 1993), *mIhh* (Bitgood and McMahon, 1995), *mSpp1* (Kim et al., 1999), *rPthlh* (Karaplis et al., 1990), *mBmp4* and *mBmp7* (Bitgood and McMahon, 1995), *mFgf18* (Xu et al., 2000), and *mMMP13* (Yamagiwa et al., 1999). For proliferation analysis, limb explants were labeled with 5-bromo-2-deoxyuridine (BrdU) and proliferating cells were detected by antibody staining according to the manufacturer's procedure (Minina et al., 2001).

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