3 Results

3.1 Limb culture system

3.1.1 Concentration- and time-courses in the limb culture system

Limb culture system represents a good *in vitro* system to analyze the effects of various growth factors in chondrocyte proliferation and differentiation. In contrast to chondrocyte cell culture the limb culture system allows to test the effects of applied growth factors in intact tissues, where the local interactions between different cell types stay unaltered. It has been shown that in cultured skeletal elements of limb chondrocyte proliferation and differentiation take place similar to *in vivo* conditions (Chen et al., 2001; Haaijman et al., 1999). Although the perichondrium surrounding the terminal hypertrophic chondrocytes can differentiate into osteoblast-forming periosteum, the replacement of cartilage by bone does not occur in this system. To ensure sufficient diffusion of nutrients and supplemented growth factors into the cartilage structure, the skin and muscles were thoroughly removed from the limb explants. To investigate the specific roles of growth factors on chondrocyte development in this study an organ culture system for embryonic limb explants was established. Moreover, the double treatment experiments were set up to analyze the epistatic relationship of different signaling pathways. A critical step in analyzing the specific effect of a growth factor on cartilage development is defining its optimal concentration. The concentration should be high enough to show a specific effect on chondrocyte proliferation and differentiation, and simultaneously as low as possible. In addition, the time of treatment should be short enough to reveal the first changes in gene expression. To define the optimal concentration of BMP2, limbs of mouse embryos at stage E14.5 were treated with different concentrations of BMP2 and photographed before and after culture (Fig. 9). After 4 days of culture untreated limb showed a distinct increase in size (Fig. 9A,B). On molecular level chondrocyte differentiation and proliferation proceeded normally, as can be seen by the normal pattern of the *ColX* expression, demarcating hypertrophic chondrocytes, and BrdU-labeled proliferating chondrocytes (Fig. 9 G,H and data not shown). After treatment with BMP2 no difference was found in size between the control limbs and limbs, treated with 5 ng/ml or 50 ng/ml of BMP2 (Fig. 9B-D). However, limbs treated with BMP2 at 150 ng/ml and at 500 ng/ml were slightly bigger than untreated control limb (Fig. 9 B,E,F). In addition, limbs treated with BMP2 at 150 ng/ml and 500 ng/ml, revealed an increased amount of hypertrophic chondrocytes expressing *ColX* compared to untreated control limbs (Fig. 9H,K,L). As the BMP2-induced specific effect on chondrocyte proliferation and differentiation was always

reproducible with a concentration of 500 ng/ml, this concentration was considered as optimal for further treatments.

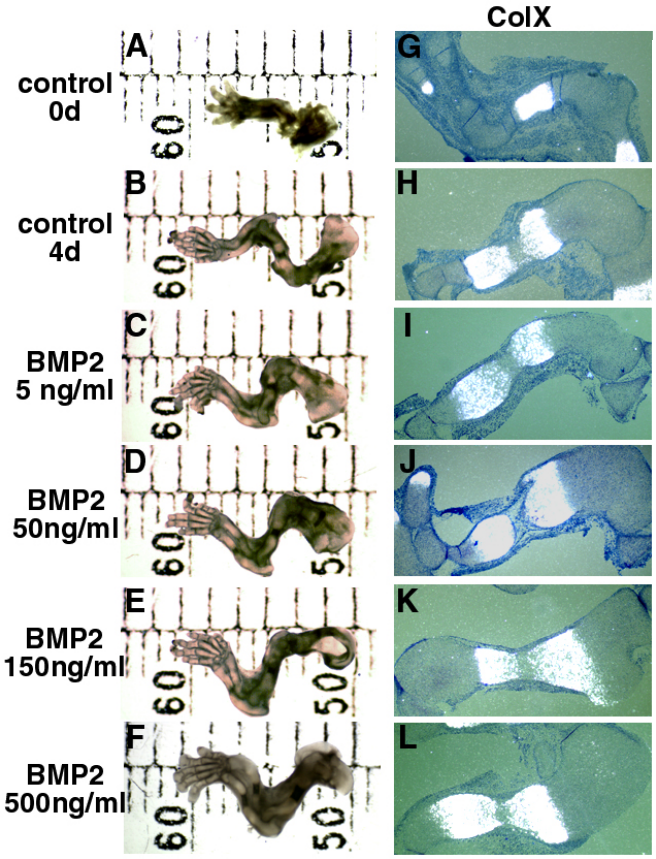

Fig. 9 The concentration course for BMP2 treatment

Forelimbs of E14.5 were cultured for 4 days and photographed before (A) and after culture (B-F). The limbs were sectioned and hybridized with antisense riboprobe for *ColX* (G-L). The skeletal elements grow during culture (A,B) and undergo hypertrophic differentiation as can been seen by the normal expression of *ColX* (G,H). Treatment with BMP2 at concentrations 5 ng/ml or 50 ng/ml does not influence the size of skeletal elements (B-D). Treated limbs reveal normal expression of *ColX* similar to that of untreated limbs (H-J). Treatments with BMP2 at 150 ng/ml and 500 ng/ml result in larger skeletal elements (E,F) and increased *ColX* expression domain (K,L), compared to that in untreated control limbs (B,H). (A-F) Ruler indicates relative size units.

To define the optimal culture time for BMP2 treatment, limbs were cultured with BMP2 for 12 hours, 24 hours, 48 hours (2 days) and 96 hours (4 days) and photographed before and after culture (Fig. 10). After culture limbs were sectioned and analyzed for changes in chondrocyte proliferation and differentiation by BrdU-labeling and *in situ* hybridization with *ColX*, respectively. After 48 hours limbs treated with BMP2 showed a slight increase in size compared to untreated control limbs (Fig. 10). After 96 hours the BMP2-treated limbs were markedly bigger than control limbs (Fig. 10). BrdU-labeling of these limbs showed an increased amount of proliferating chondrocytes (data not shown). The first sign of a change in chondrocyte differentiation was seen after 48 hours in limbs treated with BMP2, which revealed an increased zone of *ColX*- expressing cells. This zone was significantly bigger in limbs treated with BMP2 for 96 hours compared to that in untreated control limb (Fig. 9 H,L). Therefore, 48 hours of treatment with BMP2 was considered as an optimal time to see an effect on gene expression, and 96 hours was considered as the optimal time to investigate morphological changes of the cartilage structure.

For the other growth factors similar concentration- and time-courses were performed in culture of limbs at different stages (summarized in Table 3). To analyze a role of FGF signaling on cartilage development limbs at stages E14.5 and E16.5 were treated with FGF2. Surprisingly, treatment with 50 ng/ml FGF2 for limbs of E16.5 embryos was sufficient to give the specific effect on chondrocyte proliferation and differentiation, whereas for limbs of E14.5 embryos treatment with 250 ng/ml of FGF2 was necessary (Table 3). The optimal concentrations of other factors used in this study for all analyzed stages were $3x10^{-7}$ M Pthlh, 5 µg/ml Shh, 500 ng/ml Noggin and 10 µM cyclopamine (Table 3).

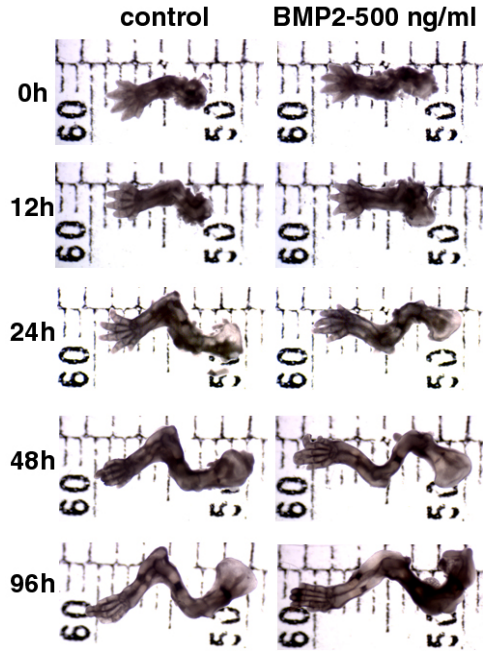

Fig. 10 The time course for BMP2 treatment to define the optimal culture period

Forelimbs at E14.5 were photographed before culture (0h) and after 12 hours (12h), 24h, 48h and 96h of treatment with BMP2 at 500 ng/ml (right panel) or without treatment (left panel). The increase in size of BMP2 treated limbs is first seen after 48h and is obvious after 96h. In all panels the left and right limbs are from the same embryo. In all panels the ruler indicates relative size units.

Factor	BMP2	FGF2	FGF2	Noggin	Pthlh	Cyclopa-mine
Optimal concentration	500 ng/ml	250 ng/nml	50 ng/ml	500 ng/ml	$3x10^{-7}$ M	10 mM
Mouse age	E14.5 E16.5	E14.5	E16.5	E14.5 E16.5	E14.5 E16.5	E14.5 E16.5

Table 3. Optimal concentrations of various factors in the limb culture system

Limbs of E14.5 and E16.5 mouse embryos were treated with BMP2, FGF2, Noggin, Pthlh and cyclopamine (first row) at different concentrations. A minimal concentration of the applied factor, showing effect on chondrocyte proliferation and differentiation, was considered as the optimal concentration (second row). The optimal concentrations for each factor were tested at least for two mouse stages, E14.5 and E16.5 (third row).

3.2 Ihh/Pthlh pathway

3.2.1 Ihh overexpression results in delayed chondrocyte differentiation

To analyze the epistatic relationship of the Ihh/Pthlh system with other signaling pathways we used a transgenic mouse system allowing overexpression of the chicken *Ihh* gene under the *ColII* promoter (Long et al., 2001). As direct expression of Ihh under the *ColII* promoter resulted in neonatal lethality (A.Vortkamp and A. McMahon, personal communication), two transgenic mouse lines were used. One mouse line misexpresses the yeast transcriptional activator *Gal4* under the *ColII* promoter (*ColII-Gal4*). The second mouse line carries the chick *Ihh* gene downstream of the yeast Gal4 binding site *UAS* (*UAS-Ihh*). Mating the two strains results in 25% embryos misexpressing *Ihh* under the *ColII* promoter (*ColII/Ihh* embryos) (Long et al., 2001). Transgenic embryos die shortly after birth and display severe defects in the developing skeleton. In the limbs the skeletal elements are broadened and joints of elbow and phalanges are missing or severely malformed. Chondrocyte differentiation in *ColII/Ihh* embryos was analyzed by *in situ* hybridization with *Ihh* and *ColX*, markers for prehypertrophic and hypertrophic chondrocytes, respectively, and with *Pthlh*, as target gene of Ihh signaling (Fig. 11 and data not shown). The endogenous Ihh message was analyzed using the probe specific for mouse *Ihh* gene. The mouse *Ihh* expression was reduced in the developing cartilage elements of *ColII/Ihh* embryos compared to that in limbs of wild type littermates (Fig. 11A,B). As expected, *Pthlh* was highly expressed in the periarticular region, compared to *Pthlh* expression in limbs of wild type littermates (Fig. 11C,D). In addition, the distance between the joint region and the onset of hypertrophic differentiation, demarcated by the distal end of the *Ihh* expression domain, was bigger than that in wild type embryos,

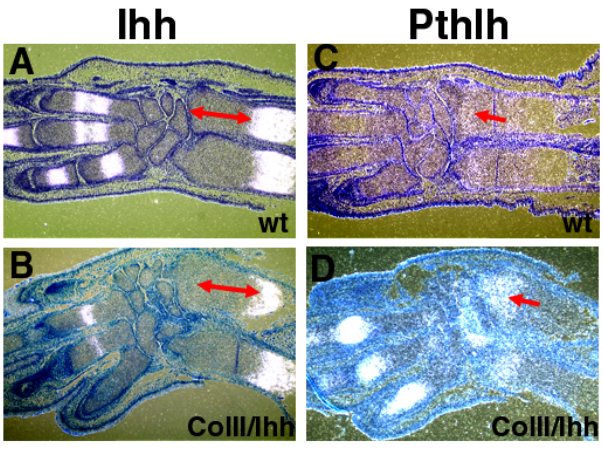

Fig. 11 Ectopic activation of Ihh delays hypertrophic differentiation of chondrocytes

Sections of forelimbs of *ColII/Ihh* transgenic (B,D) and wild type (A,C) mice at E16.5 were hybridized with antisense riboprobes for *Ihh* (A,B) or *Pthlh* (C,D). The ectopic activation of Ihh in *ColII/Ihh* mice results in downregulation of endogenous *Ihh* expression (B) and an enlarged distance between *Ihh* expressing cells and the joint region (double arrow), compared to that of wild type littermates (A). Additionally, *Pthlh* expression is upregulated in *ColII/Ihh* mice compared to that in wild type mice (C,D, arrows).

indicating a delay in the onset of hypertrophic differentiation (Fig. 11C,D) (Long et al., 2001). All single transgenic *ColII-Gal4* and *UAS-Ihh* or wild type littermates appeared

normal by expression analysis (data not shown). In addition to the role of Ihh in regulating chondrocyte differentiation, the *Ihh* knockout phenotype revealed a role for Ihh in regulating chondrocyte proliferation (Karp et al., 2000; St-Jacques et al., 1999). Long et al. have found an increased chondrocyte proliferation in *ColII/Ihh* mice compared to wild type littermates at E14.5 and E16.5 stages (Long et al., 2001). Therefore, ectopic upregulation of *Ihh* expression in cartilage delays chondrocyte differentiation and simultaneously promotes chondrocyte proliferation.

3.2.2 Modulation of Ihh signaling by Pthlh or cyclopamine in a limb culture

Previous studies have shown that Pthlh acts downstream of the Ihh signal, as a treatment with Shh protein could delay the advanced onset of chondrocyte differentiation in limbs of wild type mice but not Pthr -/- or Pthlh -/- knockout mice (Lanske et al., 1996; Vortkamp et al., 1996). In addition to analyzing *ColII/Ihh* embryos, functions of the Ihh/Pthlh were modulated in limb culture system by treatment of wild type limbs with Pthlh. Treatment with Pthlh delays chondrocyte differentiation resulting in reduced expression of *Ihh*, and in an increased distance between the joint region and the *Ihh*-expressing cells (Fig. 12A,C).

As targeted deletion of *Ihh* gene in mice disrupts cartilage development at the initial differentiation stage, the normal pattern of the various zones of chondrocyte differentiation in the growth plate is never established properly (St-Jacques et al., 1999). The interaction of signaling systems regulating the later stages of chondrocyte differentiation is thus difficult to address in these mice. Recently it has been demonstrated that the alkaloid cyclopamine inhibits the Hedgehog signaling pathway in various organisms, acting on the target cells of the Hedgehog signaling (Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000). To test if cyclopamine can block Ihh signaling in the limb culture system, limbs were treated with cyclopamine. Treatment of limbs with cyclopamine resulted in an increased amount of hypertrophic chondrocytes as indicated by the expanded expression domains of *Ihh* and *ColX* (Fig. 12A,B,G,H). In addition, *Pthlh* expression was lost after cyclopamine treatment (Fig. 17G). To test whether the cyclopamine induced effect on chondrocyte differentiation was a specific result of the blocking Ihh signaling, limbs were co-treated with cyclopamine and Pthlh. Double treatment of limbs resulted in reduced *Ihh* and *ColX* expression domains, similar to that after Pthlh treatment only (Fig. 12C-H). Additionally, limbs treated with cyclopamine and Pthlh showed an enlarged distance between the joint region and the *Ihh*-expressing cells compared to that in limbs treated with cyclopamine only (Fig. 12E,G). These results indicate that Pthlh can rescue the cyclopamine-induced defects on chondrocyte

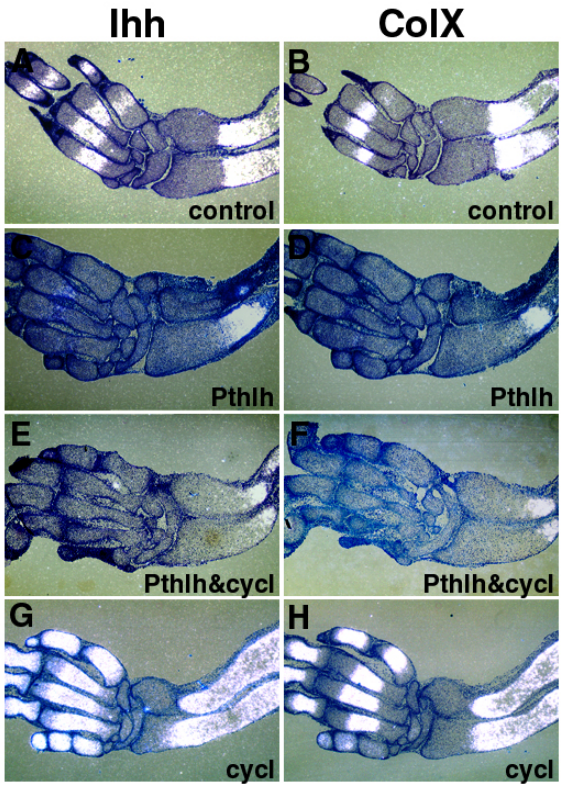

Fig. 12 Pthlh and cyclopamine modulate Ihh signaling

Forelimbs of E14.5 embryos were cultured for 4 days in control medium (A,B) or in medium supplemented with Pthlh (C,D), Pthlh and cyclopamine (E,F) or cyclopamine (G,H). Sections of these limbs were hybridized with antisense riboprobes for *Ihh* (A,C,E,G) or *ColX* (B,D,F,H). (A-D) Treatment with Pthlh results in a delay of hypertrophic differentiation as seen by the reduced expression of *Ihh* (A,C) and *ColX* (B,D) and the increased distance between the *Ihh* expression domain and the joint region. Treatment with cyclopamine results in an advanced onset of hypertrophic differentiation as can be seen from enlarged domain of *Ihh* and *ColX* expression (G,H). (E,F) Pthlh can rescue the advanced onset of hypertrophic differentiation in explants co-treated with Pthlh and cyclopamine. cycl: cyclopamine.

differentiation and therefore cyclopamine specifically interferes with Ihh signaling in the limb culture system.

3.3 Interaction of BMP signaling and the Ihh/Pthlh pathway

3.3.1 BMP signaling regulates chondrocyte differentiation and proliferation

It has previously been demonstrated that BMP signaling is required for proper bone development (Hogan, 1996; Kingsley, 1994b). However its precise function in regulating cartilage development was poorly understood. To analyze the effect of BMP signaling on chondrocyte differentiation, limb explants were cultured in the presence of BMP2 or Noggin protein, which is a potent antagonist of BMP signaling. After 4 days of culture, BMP2 treated limbs showed an increase in length if compared to untreated cultures (Fig. 13A-D). Additionally after treatment with BMP2, *Ihh* and *ColX* were expressed in expanded domains compared to untreated control limbs (Fig. 13G,K,H,L). In contrast, treatment with Noggin resulted in an inhibition of limb growth in culture and in decreased domains of *Ihh* and *ColX* expression compared to that in untreated control limb (Fig. 13B-K). However, despite the smaller size of the cartilage elements, *ColX* expression indicated that the chondrocytes have undergone hypertrophic differentiation. In addition the two zones of *ColX* expressing

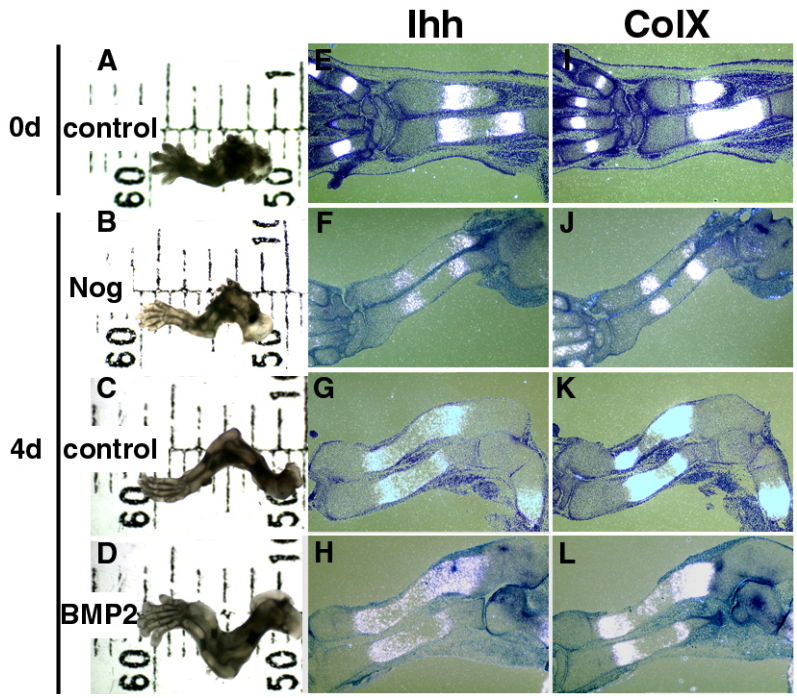

Fig. 13 BMP signaling regulates chondrocyte differentiation

(A-D) Forelimbs of E14.5 mouse embryos were cultured for 4 days and photographed before (A) and after culture (B-D). Serial sections of these limbs were hybridized with antisense riboprobes for *Ihh* (E-H) or *ColX* (I-L). Uncultured limbs (A) display the characteristic expression domains of *Ihh* in prehypertrophic (E) and *ColX* in hypertrophic chondrocytes (I). Limbs cultured for 4 days increase in size (C) but display the normal distribution of *Ihh* (G) and *ColX* (K) expressing cells. Treatment with BMP2 results in a further enlargement of the cartilage elements (D) and an increased size of the *Ihh* (H) and *ColX* (L) expression domains. In contrast, Noggin treatment blocks limb growth (B) and results in reduced expression of *Ihh* (F) and *ColX* (J). (A-D) Ruler indicates relative size units. In panels (E-L) ulna is up and radius is down. Nog: Noggin

hypertrophic cells were clearly separated from each other (Fig. 13J). They flanked a distinct zone of terminal hypertrophic cells (Fig. 20E,F). Noggin treatment also resulted in reduced distance between the *Ihh* expression domain and the joint region, indicating an advanced onset of hypertrophic differentiation (Fig. 13F). These effects of Noggin could be antagonized by BMP2 in cultures double treated with both factors (n=4, data not shown). The different lengths of the cartilage elements in BMP and Noggin treated limbs indicated that BMP signaling also regulates chondrocyte proliferation. To investigate this hypothesis, the limbs, treated with BMP2 or Noggin, were labeled with BrdU. After treatment with BMP2, the zones of proliferating chondrocytes were increased, whereas Noggin treatment resulted in a block of chondrocyte proliferation (Fig. 19A,B,E). Thus, by coordinating proliferation and hypertrophic differentiation of chondrocytes, BMP2 promotes the uniform expansion of these zones in the developing skeletal elements.

3.3.2 *Bmp* expression is upregulated in *Ihh*-overexpressing mice

It has previously been shown in chicken embryos that ectopic misexpression of *Ihh* in forelimbs results in upregulation of *Bmp2* and *Bmp4* expression in perichondrium of skeletal elements (Pathi et al., 1999). To test if *Bmp* expression is similarly regulated in mice, the expression of different *Bmp* genes was analyzed in *ColII/Ihh* transgenic embryos. At stage E14.5 *Bmp3*, *Bmp4* and *Bmp7* were strongly upregulated in the perichondrium of *ColII/Ihh* embryos, compared to wild type embryos (Fig. 14B-D,G-H). In addition, *Bmp4* and *Bmp7* expression was increased in proliferating chondrocytes (Fig. 14C,D,G,H). A similar upregulation of *Bmp* expression was found at E16.5 in *ColII/Ihh* embryos (data not shown). Therefore, like in chicken embryos overexpression of *Ihh* in mice leads to an upregulation of *Bmp* expression. Because BMPs regulate chondrocyte differentiation and proliferation, the increased expression of *Bmp* genes after ectopic *Ihh* expression implicates a possible interaction between the two pathways during cartilage development.

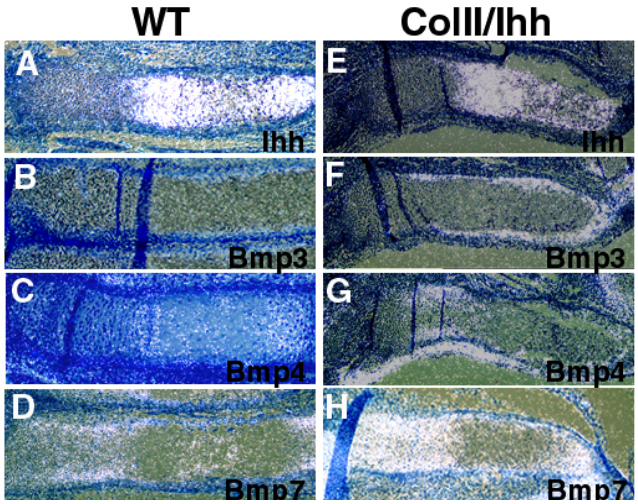

Fig. 14 Ectopic activation of Ihh signaling results in upregulation of *Bmp* genes

Forelimbs of wild type (A-D) and *ColII/Ihh* mouse embryos (E-H) at stage E14.5 were sectioned and hybridized with antisense riboprobes for *Ihh* (A,E), *Bmp3* (B,F), *Bmp4* (C,G) or *Bmp7* (D,H). In limbs of *ColII/Ihh* mice endogenous *Ihh* expression is reduced compared to that in the wild type mice (A,E). *Bmp3* (F), *Bmp4* (G) and *Bmp7* (H) are upregulated in perichondrium of *ColII/Ihh* mouse limbs compared to that of wild type limbs (B-D). In addition, in *ColII/Ihh* transgenic mice *Bmp4* (C,G) and *Bmp7* (D,H) are upregulated in proliferating chondrocytes. All panels show sections through the radius.

3.3.3 BMPs are not mediators of Ihh signaling in regulating hypertrophic differentiation

It has been proposed that BMPs act as a secondary signal downstream of Ihh signaling in regulating the onset of hypertrophic differentiation of chondrocytes (Zou et al., 1997). To test

whether BMP signaling is necessary for the observed delay in hypertrophic differentiation induced by *Ihh* overexpression, limbs of *ColII/Ihh* embryos were treated with Noggin protein to block BMP signaling. If BMP signaling acts as a secondary factor in the Ihh pathway one would expect an acceleration of hypertrophic differentiation. Surprisingly, hybridization with the hypertrophic marker *ColX* revealed that Noggin treatment of limbs from *ColII/Ihh* embryos results in a further reduction of hypertrophic chondrocytes in cultures of both, E14.5 and E16.5 limbs (Fig. 15D,F and data not shown). Consistent with this observation, hybridization with *Ihh* indicates a further reduction of the endogenous *Ihh* expression (Fig. 15C,E).

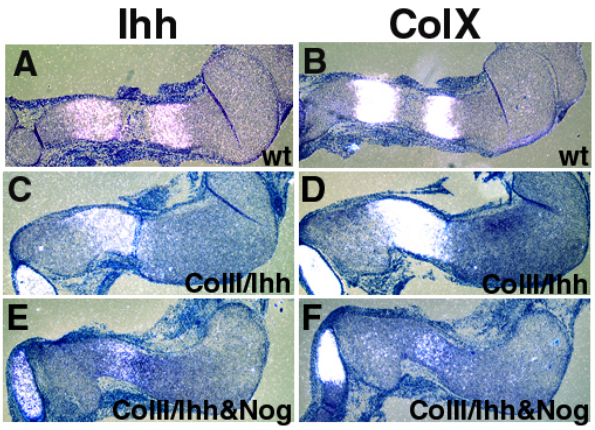

Fig. 15 A block of BMP signaling cannot overcome the Ihh-induced delay in hypertrophic differentiation

Forelimbs of E14.5 embryos from wild type (A,B) or *ColII/Ihh* transgenic mice (C,E,D,F) were cultured for 4 days in control medium (A,C,B,D) or treated with Noggin protein (E,F). Serial sections of these limbs were hybridized with antisense riboprobes for *Ihh* (A,C,E) or *ColX* (B,D,F). (A-D) Untreated limbs of *ColII/Ihh* embryos display a reduced expression domain of *Ihh* (C) and *ColX* (D) compared to untreated limbs of wild type embryos (A,B). (E,F) Forelimbs of *ColII/Ihh* embryos treated with Noggin show reduced expression of *Ihh* (E) and *ColX* (F). All panels show sections through the humerus. Nog: Noggin

Conversely, to test whether in limbs, where Ihh signaling was blocked by cyclopamine, BMPs can substitute the Ihh signals and delay the advanced onset of hypertrophic differentiation, limbs were co-treated with cyclopamine and BMP2. Double treated limbs revealed an extended zone of *ColX* expressing hypertrophic chondrocytes comparable to that of limbs treated with cyclopamine only (Fig. 16B,D,F). Surprisingly, the expression domain of *Ihh* in double treated cultures extended more towards the joint region than that after treatment with either factor alone (Fig. 16C,E,G; 2/6 and 10/10 in 2 and 4 days cultures, respectively). Summarizing, these experiments strongly indicate that BMP signaling does not act downstream of Ihh in regulating the onset of hypertrophic differentiation.

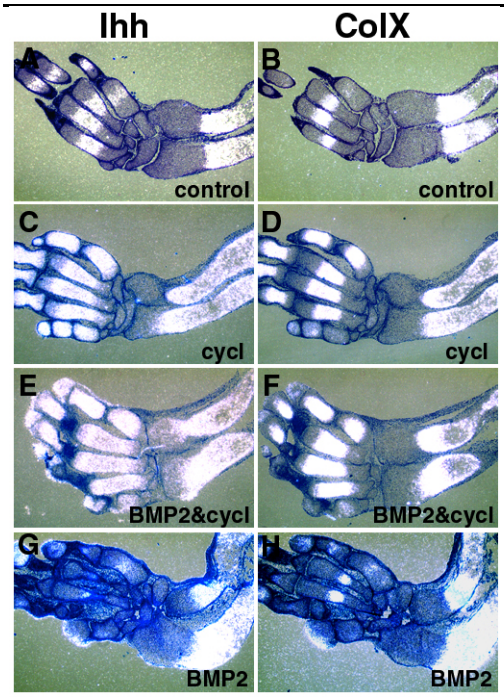

Fig. 16 BMP2 does not rescue the advanced onset of hypertrophic differentiation induced by a loss of Ihh signaling

Forelimbs of E14.5 embryos were cultured for 4 days in control medium (A,B) or in medium supplemented with cyclopamine (C,D), BMP2 and cyclopamine (E,F), or BMP2 (G,H). Serial sections were hybridized with antisense riboprobes for *Ihh* (A,C,E,G) or *ColX* (B,D,F,H). Co-treatment with BMP2 and cyclopamine does not rescue the advanced onset of hypertrophic differentiation induced by cyclopamine, as can be seen from more extended *ColX* expression domain than that in untreated control limbs (F,B). Double treated limbs also reveal a further enlargement of the *Ihh* expression domain (E) compared to that in limbs treated with either cyclopamine (C) or BMP2 (G). Limbs treated with cyclopamine or BMP2 plus cyclopamine were derived from the same embryo. In all panels ulna is up and radius is down. cycl: cyclopamine.

3.3.4 *Pthlh* expression is not regulated by BMP signaling

Previous studies have been demonstrated that the effect of Ihh signaling on chondrocyte differentiation is mediated by the induction of *Pthlh* expression in the periarticular region (Vortkamp et al., 1996). Misexpression of constitutively activated *Bmp-1a* raised the possibility that BMP signaling mediates the upregulation of *Pthlh* expression by Ihh (Zou et al., 1997). To test this hypothesis the limbs treated with different factors or their combination were analyzed for the expression of *Pthlh*. After treatment with BMP2 wild type limbs showed normal expression of *Pthlh* similar to that in untreated cultures, however treatment with Noggin resulted in a slight decrease in *Pthlh* expression (Fig. 17A,B,E). To differentiate whether this decrease was an effect of the reduced Ihh signal in these cultures (see Fig. 13F) or a direct effect of blocking BMP signaling, limbs of *ColII/Ihh* embryos were treated with Noggin protein. In these cultures *Pthlh* still was strongly expressed in the joint region comparable to that of untreated cultures of mutant embryos (Fig. 17C,D). Conversely, double treatment with cyclopamine and BMP2 did not rescue the expression of *Pthlh* in wild type limbs compared to that in limbs after cyclopamine treatment only (Fig. 17F). These results therefore strongly suggest that BMP signaling is not mediating the Ihh signal to induce *Pthlh* expression.

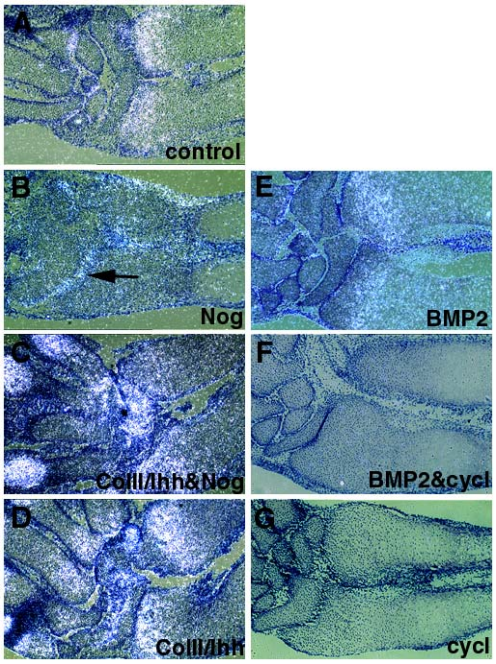

Fig. 17 BMP2 signaling does not act as a secondary signal of Ihh to induce *Pthlh* expression

Forelimbs of E14.5 mouse embryos were cultured for 4 days in control medium (A,D), or in medium supplemented with Noggin (B,C), BMP2 (E), BMP2 and cyclopamine (F) or cyclopamine (G). Limbs in (A,B,E,F,G) were derived from wild type embryos, limbs in (C,D) from *ColII/Ihh* embryos. Sections were hybridized with an antisense riboprobe for *Pthlh*. (A,B) Treatment of wild type limbs with Noggin results in a reduction of *Pthlh* expression (B, arrow) compared to untreated control limbs (A). (C,D) High expression of *Pthlh* in limbs of *ColII/Ihh* embryos (D) is not reduced by Noggin treatment (C). (E) BMP2 treated limbs show normal expression of *Pthlh*. (F,G) Cyclopamine treatment results in a block of *Pthlh* expression (G), which cannot be rescued by BMP2 in double treated cultures (F). In all panels ulna is up and radius is down. cycl: cyclopamine, Nog: Noggin.

3.3.5 BMPs and Ihh interact similarly in chick and in mouse

The experiments that first indicated a possible role for BMPs in regulating *Pthlh* expression were carried out by viral misexpression of an activated *Bmpr-Ia* in chick embryos (Zou et al., 1997). Thus, one possible explanation for the contradictory conclusions revealed in those earlier studies could be that the signaling systems interact in different ways in mouse and in chick. Therefore the interaction between Ihh and BMP signaling was examined using cultures of chick limbs at stage HH 32, which correspond to stage E14.5 of mouse embryonic development. To induce Ihh signaling its homologue Sonic hedgehog (Shh) was used. Shh has been shown to act through the same receptors and can substitute treatment with Ihh protein in limb culture conditions (Lanske et al., 1996). Treatment with Shh protein resulted in a delay of chondrocyte differentiation, whereas cyclopamine accelerated hypertrophic differentiation as assayed by the expression of the prehypertrophic and hypertrophic markers, *Ihh* and *ColX* (Fig 16A,G,M and data not shown). Limbs treated with a combination of Shh and Noggin protein did not reveal increased chondrocyte differentiation compared to treatment with Shh alone (Fig. 18C,E,G). In addition, *Pthlh* was strongly expressed in the periarticular region in spite of the block in BMP signaling (Fig. 18D,F,H). Conversely, cultures treated with a combination of cyclopamine and BMP2 displayed the same acceleration of hypertrophic differentiation seen after cyclopamine treatment only (Fig. 18I,K,M). Expression of *Pthlh* was not induced by BMP treatment in the absence of Ihh signaling (Fig. 18J,L,N). Therefore, in chick as well as in mice, BMP2 does not appear to mediate the effect of Ihh signaling on *Pthlh* expression and hypertrophic differentiation.

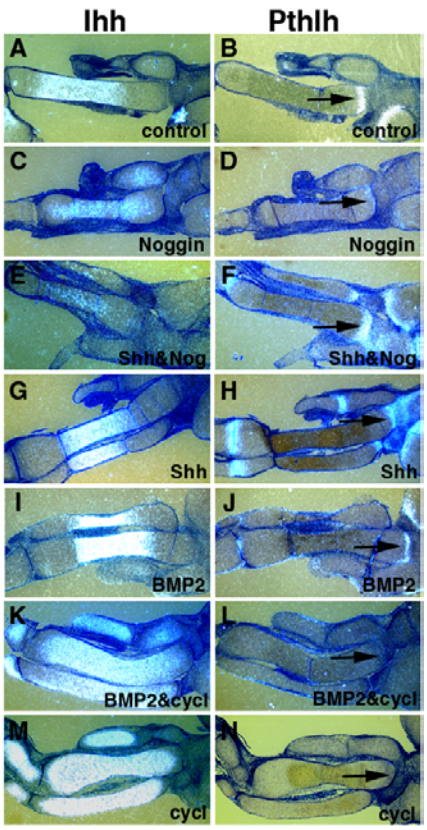

Fig. 18 Interaction of BMP and Ihh signaling in chick embryos

Wings of HH32 chick embryos were cultured for 2 days in control medium (A,B) or medium supplemented with Noggin (C,D), Shh and Noggin (E,F), Shh (G,H), BMP2 (I,J), BMP2 and cyclopamine (K,L) or cyclopamine (M,N). Parallel sections were hybridized with antisense riboprobes for *cIhh* (A,C,E,G,I,K,M) or *cPthlh* (B,D,F,H,J,L,N). Noggin treatment results in small cartilage elements showing a reduced level of *Ihh* (C) and *Pthlh* (D) expression compared to untreated cultures (A,B). Treatment with Shh leads to a delay in hypertrophic differentiation as seen by the smaller domain of *Ihh* expression and the increased distance between the *Ihh* expression domain and the joint region (G) if compared to untreated explants (A). Shh treated limbs show high expression of *Pthlh* in periarticular chondrocytes (H). Co-treatment of limbs with Shh and Noggin leads to a further reduction of *Ihh* expression (E) and does not block the expression of *Pthlh*(F). BMP2 treated limbs show high expression of *Ihh* (I) and *Pthlh* (J). Treatment with cyclopamine results in an advanced onset of hypertrophic differentiation as seen by the expanded domain of *Ihh* expression and reduced distance between *Ihh* expression domain and the joint region (M) compared to control limbs (A). Furthermore, *Pthlh* expression is blocked by cyclopamine treatment (N). Both effects cannot be rescued by BMP2 in explants co-treated with BMP2 and cyclopamine (K,L). Arrows point to the *Pthlh* expression domain. All panels display sections through metacarpals. cycl: cyclopamine, Nog: Noggin.

3.3.6 BMPs induces chondrocyte proliferation independent of Ihh

Limbs of *ColII/Ihh* embryos show a high level of chondrocyte proliferation demonstrating a continuous role of Ihh in regulating this process (Fig. 19D) (Long et al., 2001). To reveal the epistatic relationship between the Ihh and BMP signaling systems, double treated limbs were analyzed. Like treatment of wild type limbs, Noggin treatment of limbs that overexpress *Ihh* resulted in a block of chondrocyte proliferation (Fig. 19B-D). Therefore, Ihh does not act downstream of BMP signals. In the reverse experiment, limbs were treated with cyclopamine and BMP2. In double treated limbs chondrocyte proliferation was blocked similarly as in cultures treated with cyclopamine alone (Fig. 19E-G). Thus, BMP2 cannot overcome the block of chondrocyte proliferation induced by loss of Ihh signaling and hence BMPs do not act downstream of Ihh. Taken together these experiments indicate that the two signaling systems act in parallel pathways in regulating chondrocyte proliferation.

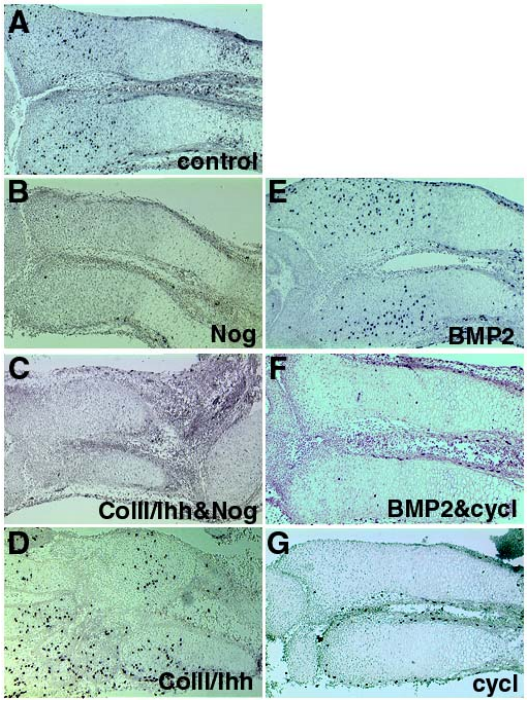

Fig. 19 BMP and Ihh signals regulate chondrocyte proliferation in parallel pathways

Forelimbs of E14.5 mouse embryos were cultured for 2 days in control medium (A,D) or in medium supplemented with Noggin (B,C), BMP2 (E), BMP2 and cyclopamine (F) or cyclopamine (G). Limbs in (A,B,E,F,G) were derived from wild type mice, limbs in (C,D) from *ColII/Ihh* embryos. Proliferating cells were labeled with BrdU and detected by antibody staining. (A,B) Noggin treatment results in a block of chondrocyte proliferation (B) compared to untreated limbs (A). Explants from *ColII/Ihh* embryos show a high level of chondrocyte proliferation (D), which is blocked after Noggin treatment (C). (E-G) BMP2 treatment results in an increased zone of proliferating cells (E) but cannot overcome the cyclopamine induced block of chondrocyte proliferation (G) in explants double treated with BMP2 and cyclopamine (F). In all panels radius is up and ulna is down. cycl: cyclopamine, Nog: Noggin.

3.3.7 BMP signaling negatively regulates terminal hypertrophic differentiation

As endochondral differentiation progresses, hypertrophic cells further differentiate into terminal hypertrophic chondrocytes, which are replaced by bone. At E14.5 the distance between the *ColX* expressing hypertrophic zones in the center of the skeletal elements reflects the rate of terminal hypertrophic differentiation. Although after Noggin treatment the zones of *Ihh* and *ColX* expression were significantly decreased in size, the *ColX* expressing hypertrophic domains were distinctly separated from each other (Fig. 20A,G and Fig. 13E,F,I,J). To analyze if this could reflect an additional effect of BMP signaling on terminal hypertrophic differentiation, limbs treated with Noggin were hybridized with osteopontin (Secreted phosphoprotein 1, *Spp1*), a gene expressed in terminally differentiated chondrocytes (Gerstenfeld and Shapiro, 1996; Nakase et al., 1994). *Spp1* expression was strongly upregulated in these cultures compared to control limbs, indicating a role of BMP signaling in regulating the differentiation process itself (Fig. 20B,H). To analyze the role of BMP signaling during terminal hypertrophic differentiation in relation to that of Ihh signaling, limb cultures were co-treated with cyclopamine and Noggin. Double treated limbs showed a strong reduction of the *ColX* expression, which resembles that in limbs treated with Noggin only (Fig. 20E,G). Importantly the expression of *Spp1* was induced between these two domains of *ColX*- expressing cells (Fig. 20F,H). In contrast, after cyclopamine treatment the expression of *Spp1* resembles that of untreated cultures (Fig. 20B,D). Therefore, BMP

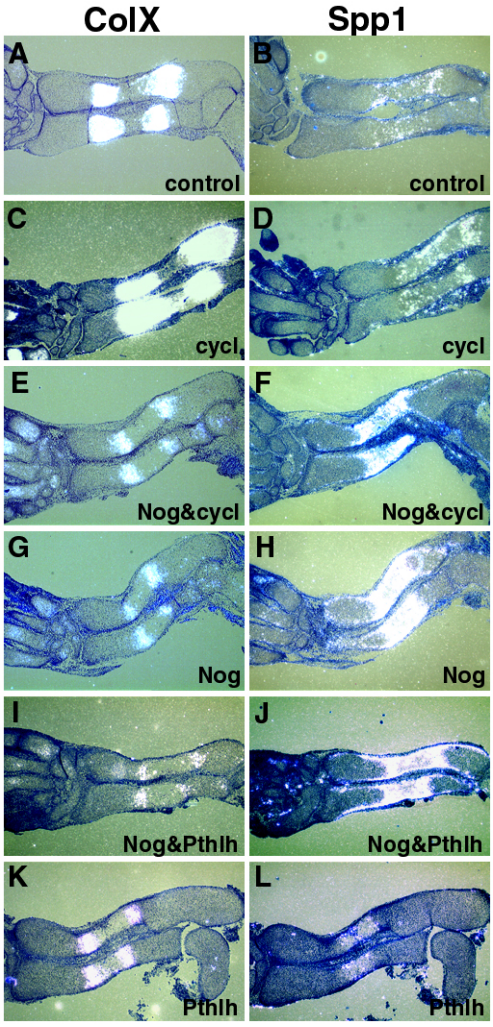

Fig. 20 BMP signaling delays terminal hypertrophic differentiation independent of the Ihh/Pthlh system.

Forelimbs of E14.5 mouse embryos were cultured for 2 days in control medium (A,B) or in medium supplemented with cyclopamine (C,D), Noggin and cyclopamine (E,F), Noggin (G,H), Noggin and Pthlh (I,J) or Pthlh (K,L). Serial sections were hybridized with antisense riboprobes for *ColX* (A,C,E,G,I,K) or *Spp1* (B,D,F,H,J,L). (A-D) Cyclopamine treated limbs show increased expression of *ColX* (C) but normal expression of the marker for terminally differentiated cells, *Spp1* (D) compared to untreated limbs (A,B). (E-H) Noggin treatment results in reduced expression of *ColX* (G) and significantly increased expression of *Spp1* (H) similar to co-treatment with Noggin and cyclopamine (E,F). (I-L) Limbs treated with Pthlh show reduced expression of *ColX* (K), which is further reduced in explants double treated with Noggin and Pthlh (I). The low expression of *Spp1* after Pthlh treatment (L) is increased in limbs co-treated with Noggin and Pthlh (J). In all panels ulna is up and radius is down. cycl: cyclopamine, Nog: Noggin.

signaling seems to negatively regulate terminal hypertrophic differentiation independent of Ihh signaling.

To confirm this result limbs treated with a combination of Noggin and Pthlh were analyzed. Treatment with Pthlh resulted in a delayed onset of hypertrophic differentiation as indicated by the reduced expression of *ColX* and *Ihh* and by a consequent enlargement of the distance between the *Ihh* expression domain and the end of the skeletal elements. This reduced *Ihh* and *ColX* expression could be recognized after 2 days (Fig. 20A,K) and was more obvious after 4 days of Pthlh treatment (Fig. 12A-D). As expected, *Spp1* was downregulated and was expressed only in a small region in the center of the cartilage elements (Fig. 20L). Cultures double treated with Noggin and Pthlh showed a strong reduction of *Ihh* and *ColX* expression already after 2 days and a complete loss of their expression after 4 days of culture (Fig. 20I and data not shown). Nevertheless, *Spp1* was upregulated in the center of the skeletal elements (Fig. 20J). Such a phenotype would be expected if the onset of hypertrophic differentiation is delayed by Pthlh and simultaneously, hypertrophic cells present at the beginning of the culture undergo accelerated differentiation into terminal hypertrophic

chondrocytes in response to Noggin treatment. Together these results strongly suggest that BMP signaling negatively regulates the process of terminal hypertrophic differentiation independent of the Ihh/Pthlh system.

3.4 Interaction of FGF signaling with the Ihh/Pthlh pathway

3.4.1 FGF signaling regulates the chondrocyte differentiation and proliferation

Fibroblast growth factors (FGF) are another family of signaling factors regulating bone development. Three forms of human dwarfism syndromes, hypochondroplasia, achondroplasia and thanatophoric dysplasia result from the activated mutations in the *Fgfr3* gene (Bellus et al., 1995; Rousseau et al., 1994; Shiang et al., 1994; Tavormina et al., 1995). Various mouse models for achondroplasia were created during the past six years by inserting the human mutations in the *Fgfr3* gene. These mice are characterized by severe shortening of the appendicular skeletal elements due to reduced zones of proliferating and hypertrophic chondrocytes (Chen et al., 1999; Chen et al., 2001; Iwata et al., 2001; Li et al., 1999; Naski et al., 1998; Segev et al., 2000; Wang et al., 1999). To further investigate the role of FGF signaling during chondrocyte development and its interactions with other signaling pathways, the limbs of wild type mice were treated with FGF2. Treated limbs

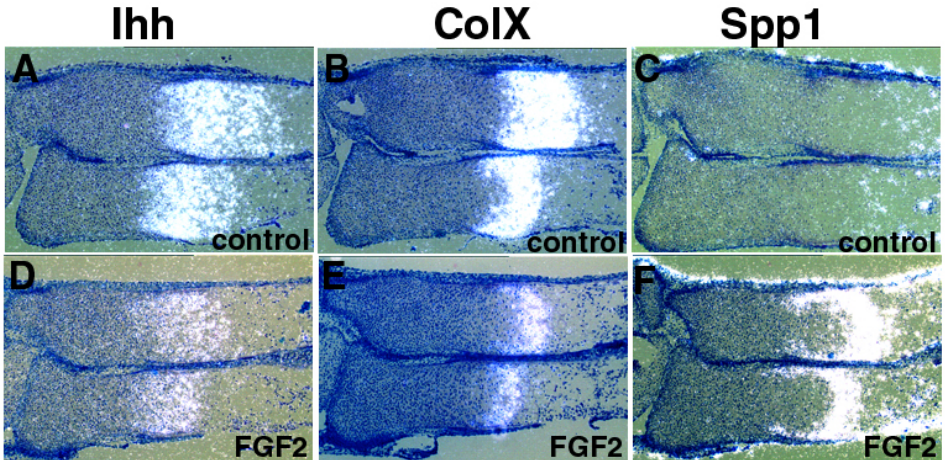

Fig. 21 FGF signaling advances the hypertrophic differentiation of chondrocytes

Forelimbs of E16.5 mouse embryos were cultured for 2 days in control medium (A-C) or in medium supplemented with FGF2 protein (D-F). Serial sections were hybridized with antisense riboprobes for *Ihh* (A,D), *ColX* (B,E) and *Spp1* (C,F). Treatment with FGF2 results in a reduced expression of *Ihh* (A,D) and *ColX* (B,D) in hypertrophic cells and increased expression of *Spp1* (C,F) in terminal hypertrophic chondrocytes. In all panels ulna is up and radius is down.

resulted in severe shortening of the skeletal elements compared to non-treated control limbs (data not shown). On molecular level limbs treated with FGF2 showed smaller domains of *Ihh* and *ColX* expression than that in untreated limbs thus mimicking the achondroplasia phenotype (Fig. 21A,B,D,E). Interestingly, the distance between joint region and *Ihh*-expressing chondrocytes was reduced after FGF2 treatment (Fig. 21A,D and Fig. 23A,B,M,N). This reduced distance may indicate either advanced onset of hypertrophic differentiation or reduced chondrocyte proliferation. BrdU-labeling of limbs after FGF2 treatment revealed a reduced rate of chondrocyte proliferation (Fig. 25A,B). Therefore, the defects induced by FGF treatment in limb cultures strongly resemble the FGFR3-mediated achondroplasia phenotype.

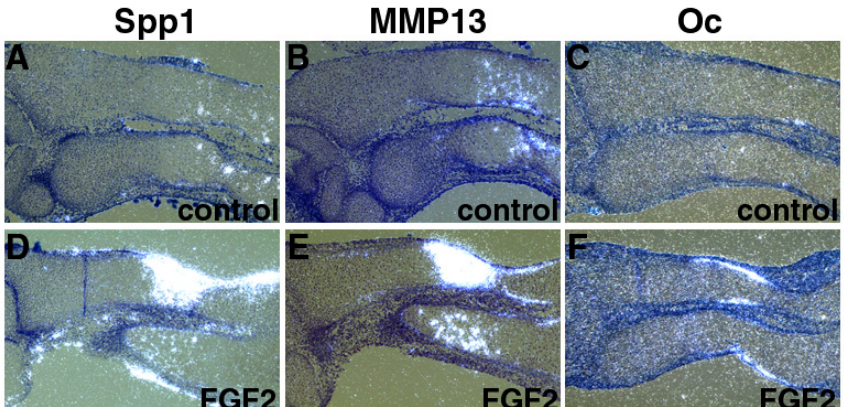

Fig. 22 FGF signaling accelerates terminal hypertrophic differentiation

Forelimbs of E14.5 mouse embryos were cultured for 2 days in control medium (A-C) or in medium supplemented with FGF2 protein (D-F). Serial sections were hybridized with antisense riboprobes for *Spp1*(A,D), *MMP13*(B,E) and *Oc* (C,F). Treatment with FGF2 induced the expression of *Spp1*(A,D) and *MMP13* (B,D) in terminal hypertrophic chondrocytes. In addition, the expression of *Oc* was induced in periosteum flanking terminal hypertrophic chondrocytes (C,F). In all panels radius is up and ulna is down. Oc: osteocalcin.

In addition to the reduced domains of *Ihh* and *ColX* expression, two regions of hypertrophic cells were distinctly separated from each other similar to that observed after Noggin treatment. Hybridization with *Spp1* revealed that these cells highly expressed *Spp1*, indicating the differentiation into terminal hypertrophic chondrocytes (Fig. 21C,F and Fig. 22A,B). Similarly, hybridization with matrix metalloproteinase 13 (MMP13), another gene expressed in terminal hypertrophic chondrocytes and osteoblasts, was upregulated by FGF2 (Fig. 22B,E). To differentiate between terminal hypertrophic cells and osteoblasts some sections of FGF treated limbs were hybridized with *osteocalcin*, which is exclusively expressed in osteoblasts. A strong expression of *osteocalcin* was found in the periosteum flanking the hypertrophic regions but no expression could be detected in the chondrocytes

expressing *Spp1* (Fig. 22C,F). FGF2 treatment therefore seems to accelerate chondrocyte differentiation into terminal hypertrophic cells, but does not induce differentiation into osteoblasts.

3.4.2 FGFs advance hypertrophic differentiation by downregulating *Ihh* expression

Decreased expression of *Ihh* as well as the reduced distance between the *Ihh* expression domain and the periarticular region after FGF2 treatment implicates FGF signaling in regulating the onset of hypertrophic differentiation of chondrocytes. FGF signaling could control the onset of hypertrophic differentiation either directly in parallel to the Ihh/Pthlh system or indirectly by reducing the *Ihh* expression. If the advanced onset of hypertrophic differentiation after FGF2 treatment is mediated by the Ihh/Pthlh system, simultaneous treatment with Pthlh and FGF2 should delay hypertrophic differentiation. To analyze this hypothesis, limb cultures were co-treated with FGF2 and Pthlh. Double treated limbs revealed an increased distance between the *Ihh* expression domain and the joint region compared to the limbs treated with FGF2 alone, indicating a delay in chondrocyte differentiation (Fig. 23G,I). Thus Pthlh acts downstream of FGF signaling in setting up the onset of hypertrophic differentiation.

To test, if Ihh itself can rescue the FGF induced phenotype, limbs of *ColII/Ihh* embryos at E16.5 and E14.5 were treated with FGF2 (Fig. 24 and data not shown). As in these mice *Ihh* expression is independent of the endogenous *Ihh* promoter, FGF2 treatment should not influence the onset of hypertrophic differentiation, if FGF signaling acts upstream of Ihh. Similar to wild type limbs, FGF2 treatment of limbs of *ColII/Ihh* embryos resulted in reduced expression of the endogenous *Ihh*, compared to untreated control limbs (Fig.21A,C). However, FGF treatment did not result in a reduction of *Pthlh* expression nor in a decreased distance between the *Ihh* expression domain and the periarticular region in mutant embryos, indicating that FGF signaling acts upstream of Ihh (Fig. 24B,D).

To test whether FGF2 could further advance the onset of hypertrophic differentiation independent of the Ihh signal, limbs were cultured with FGF2 and cyclopamine. Double treated limbs did not further accelerate the onset of hypertrophic differentiation compared to limbs treated with cyclopamine only (Fig. 23M,P). As cyclopamine effectively blocks Ihh signaling and therefore *Pthlh* expression, this result indicated that the FGF2 can not advance hypertrophic differentiation in the absence of the Ihh/Pthlh system. Taken together these

results strongly indicate that FGF signaling acts upstream of the Ihh/Pthlh system in regulating the onset of hypertrophic differentiation.

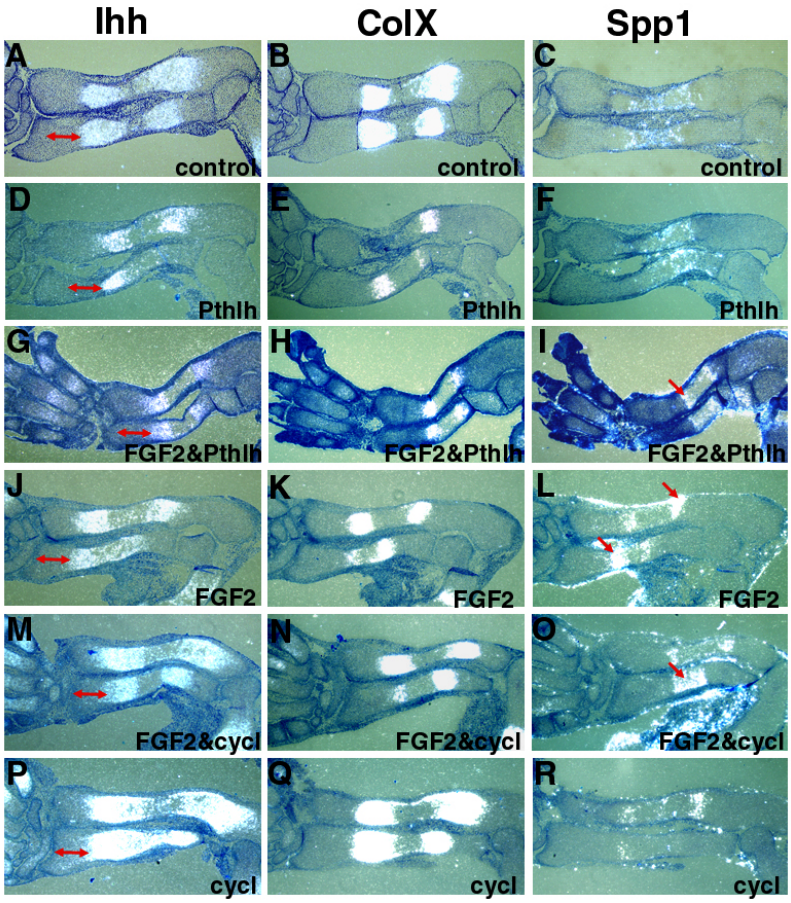

Fig. 23 Interaction of FGF signaling and the Ihh/Pthlh system in regulating hypertrophic differentiation

Forelimbs of E14.5 mouse embryos were cultured for 2 days in control medium (A-C) or in medium with Pthlh (D-F), FGF2 and Pthlh (G-I), FGF2 (J-L), FGF2 and cyclopamine (M-O) or cyclopamine (P-R). Serial sections were hybridized with antisense riboprobes for *Ihh* (A,D,G,J,M,P), *ColX* (B,E,H,K,N,Q) or *Spp1* (C,F,I,L,O,R). Treatment with FGF2 results in the reduced distance between the *Ihh* expression domain and the joint region demonstrating an advanced onset of hypertrophic differentiation (A,J). Pthlh treatment results in increased distance compared to that in untreated control limbs (A,D). Co-treatment with FGF2 and Pthlh results in the increased distance between the *Ihh* expression domain and the joint region compared to treatment with FGF2 only (G,J). Co-treatment with cyclopamine and FGF2 shows the same short distance between the *Ihh* expression domain and the joint region as that after treatment with cyclopamine alone (P,M). The terminal hypertrophic differentiation is stimulated by FGF2 treatment, as can been seen from smaller domain of *ColX*-expressing cells and induced *Spp1* expression (B,K,C,L). Although Pthlh delays and cyclopamine advances the hypertrophic differentiation, demarkated by *ColX* expression (B,E,Q), both treatments do not influence the terminal hypertrophic differentiation and show normal *Spp1* expression (C,F,R). Co-treatment with FGF2 induces terminal hypertrophic differentiation in both, FGF2&Pthlh and FGF2&cycl treated limbs (I,O). Double arrow shows the distance between the *Ihh* expression domain and the joint region after FGF2 treatment. Single arrow shows the upregulation of *Spp1* expression (C,F,I,L,O,R). In all panels ulna is up and radius is down. cycl: cyclopamine.

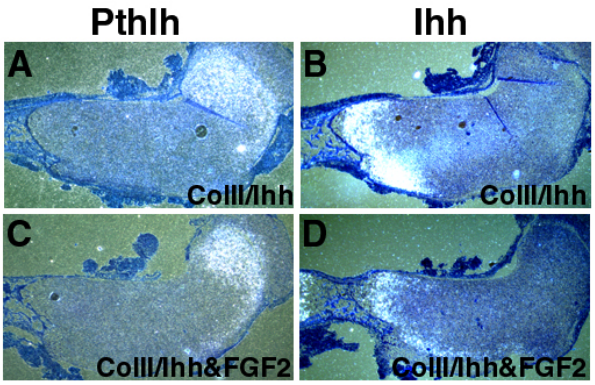

Fig. 24 FGF signaling acts upstream of Ihh in regulating *Pthlh* expression

Forelimbs of E16.5 *ColII/Ihh* embryos were cultured for 2 days in control medium (A,B) or with FGF2 (C,D). Serial sections were hybridized with antisense riboprobes for *Pthlh* (A,C) and *Ihh* (B,D). FGF2 treatment does not reduce expression of *Pthlh* in limbs of *ColII/Ihh* embryos (A,C) and cannot advance the onset of hypertrophic differentiation demarcated by the distance between the *Pthlh* and *Ihh* expression domains (B,D). However FGF2 reduces endogenous *Ihh* expression as it does in wild type limbs (compare (B,D) with Fig. 19A,D).

3.4.3 FGF signaling regulates terminal hypertrophic differentiation independent of the Ihh/Pthlh system

In addition to the regulation of the onset of hypertrophic differentiation FGF signaling also induces the differentiation of chondrocytes into terminal hypertrophic cells, expressing *Spp1* (Fig. 21C,F and Fig. 23C,L). This function of FGF signaling is very similar to the induction of terminal hypertrophic differentiation by Noggin. To test if FGF signaling, like Noggin, acts independent of Ihh signaling in regulating this process limbs were co-treated with FGF2 and cyclopamine. As expected cyclopamine treatment increased the region of *ColX*-expressing hypertrophic chondrocytes, but did not induce the differentiation of terminal hypertrophic cells, expressing *Spp1* (Fig. 23A,Q,C,R). In contrast, after double treatment with cyclopamine and FGF2 the region of *ColX*-expressing cells became smaller and *Spp1* was highly expressed (Fig. 23N,O). This result demonstrated that FGF signaling induces terminal hypertrophic differentiation independent of Ihh.

Analyzing limbs, which were co-treated with FGF2 and Pthlh, supported this result. As described before, Pthlh rescued the FGF2 induced delay of hypertrophic differentiation (Fig. 23D,G,J). Nevertheless, Pthlh failed to delay terminal hypertrophic differentiation, since limbs double treated with Pthlh and FGF2 showed strongly upregulated *Spp1* expression (Fig. 23F,I,L). In addition the *Ihh* and *ColX* expression domains were remarkably smaller in double treated limbs than those in limbs treated with either factor alone (Fig. 23 D,E,G,H,J,K). A possible explanation for the observed phenotype might be that Pthlh

delayed hypertrophic differentiation and simultaneously the hypertrophic chondrocytes that were present at the start of the culture underwent accelerated hypertrophic differentiation in response to FGF signal, similar as in limbs double treated with Pthlh and Noggin (Fig. 20G-L). Therefore differentiation of chondrocytes into terminal hypertrophic state is regulated by FGF and BMP signaling independent of the Ihh/Pthlh pathway.

3.4.4 FGF signaling reduces chondrocyte proliferation independent of Ihh/Pthlh

FGF signaling reduces chondrocyte proliferation, as it was shown in this and in previous studies (Naski et al., 1998), whereas Ihh has been shown to upregulate chondrocyte proliferation (Fig. 25) (Long et al., 2001). To investigate if similar to the regulation of the onset of hypertrophic differentiation FGF2 acts upstream of Ihh in regulating chondrocyte proliferation, limb explants of *ColII/Ihh* embryos were treated with FGF2. BrdU labeling revealed a reduced amount of proliferating cells restricted in a smaller domain than that in untreated limbs (Fig. 25C,D). Therefore, FGF signaling regulates chondrocyte proliferation independent of Ihh.

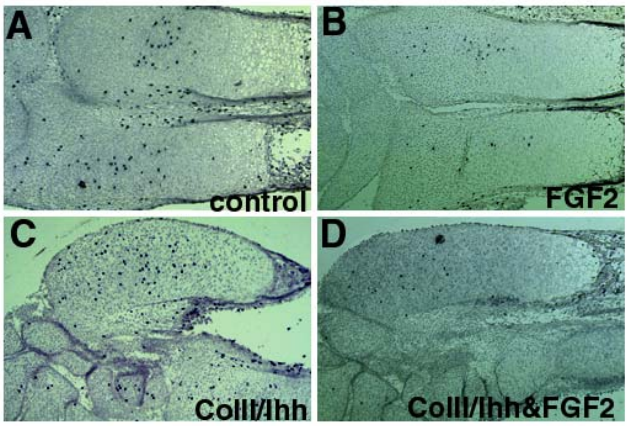

Fig. 25 FGF signaling regulates chondrocyte proliferation independent of Ihh signals

Forelimbs of E16.5 wild type or *ColII/Ihh* embryos were cultured for 2 days in control medium (A,C) or in medium supplemented with FGF2 (B,D). The proliferating cells were labeled with BrdU and detected by antibody staining. FGF2 treatment reduces the rate of chondrocyte proliferation in wild type (A,B) and in *ColII/Ihh* embryos (B,D). In all panels ulna is up and radius is down.

3.5 Interaction of FGF and BMP signaling

3.5.1 FGF signaling antagonizes BMP signal

The experiments analyzing the interaction between FGF signaling and the Ihh/Pthlh system revealed three roles for FGF signaling: downregulation of *Ihh* expression, negative regulation of chondrocyte proliferation and acceleration of terminal hypertrophic differentiation. Interestingly, blocking BMP signals by Noggin treatment resulted in a phenotype very similar to that after FGF-treatment (Fig. 20G,H and Fig. 23K,L). Thus, BMP and FGF signals seem to have opposite functions during chondrocyte development. To investigate the epistatic relationship between FGF and BMP signals limb explants were co-treated with FGF2 and BMP2. Double treated limbs revealed an increased rate of chondrocyte proliferation compared to FGF2 treatment only (Fig.23D,G). Similarly, BMP2 treatment enhanced the rate of *Ihh* expression, which was reduced after FGF2 treatment (Fig. 26 H,F). Last but not least, the FGF2-induced acceleration of terminal hypertrophic differentiation was reduced by

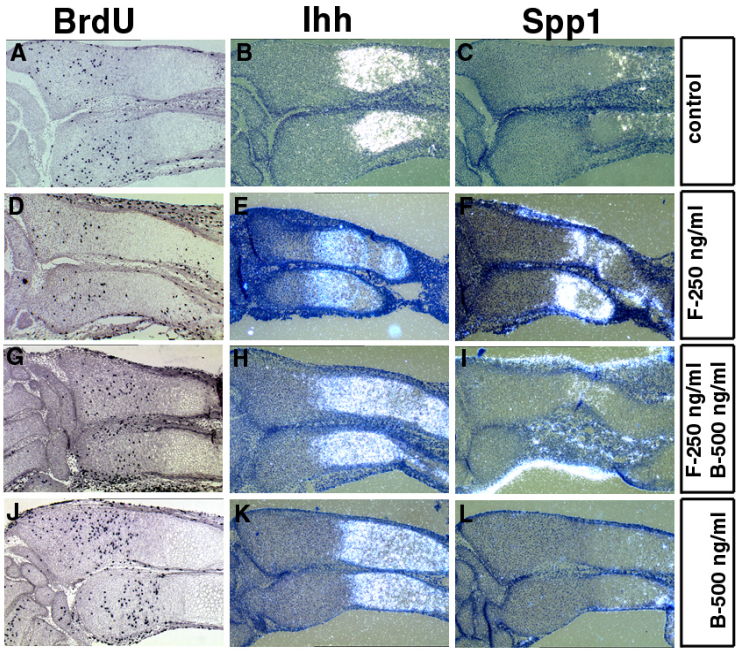

Fig. 26 BMP and FGF signaling have antagonistic effects on chondrocyte development

Forelimbs of E14.5 embryos were cultured for 4 days in control medium (A-C) or with FGF2 (D-F), FGF2 with BMP2 (G-I) or BMP2 (J-L). Serial sections were hybridized with antisense riboprobes for *Ihh* (B,E,H,K) and *Spp1* (C,F,I,L). In addition, proliferating cells were labeled with BrdU and detected by antibody staining (A,D,G,J). The co-treatment with BMP2 increases the rate of chondrocyte proliferation, reduced by FGF2 (A,D,G,J). Double treatment with BMP2 and FGF2 results in increased *Ihh* expression (B,E,H,K) and rescued onset of hypertrophic differentiation, as can be seen from increased distance between the *Ihh* expression domain and the joint region (F,H). In addition, BMP2 antagonizes the FGF2-induced terminal hypertrophic differentiation and results in reduced *Spp1* expression in double treated limbs (C,F,I,L). In all panels radius is up and ulna is down.

co-treatment with BMP2 (Fig. 26 F,I). Interestingly the effect of BMP2 treatment on chondrocyte differentiation in double treated cultures did not resemble the BMP-induced phenotype hence BMP signaling does not act downstream of FGF. Vice versa, FGF signaling does not act downstream of BMPs because BMP2 treatment could partially rescue the FGF2 effects (Fig. 26). Therefore FGF and BMP signals seem to act in parallel antagonistic pathways.

To further support the idea that FGF antagonizes the effects of BMPs, limbs were co-treated with the optimal concentration of FGF2 used in the previous experiments and with varying concentrations of BMP2 (data not shown). Vice versa limbs were treated with the optimal concentration of BMP2 and varying concentrations of FGF2 (Fig.24). The expression level of *Ihh* was decreased in these cultures with increasing concentrations of FGF2 (Fig. 27B,E,H). Furthermore the distance between the periarticular region and the *Ihh* expressing chondrocytes was also decreased in double treated limbs with increasing the FGF2 concentration (Fig. 27B,E,H). In contrast, in cultures with a constant FGF2 concentration the level of *Ihh* expression as well as the distance between the joint region and *Ihh* expressing chondrocytes were determined by the amount of BMP2 (data not shown).

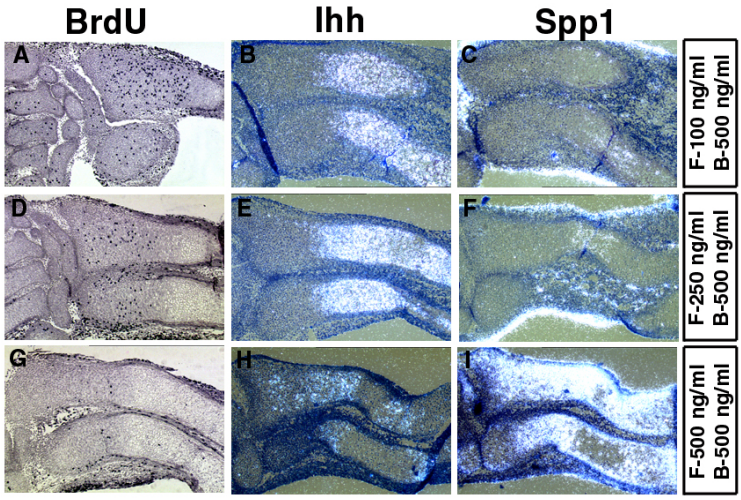

Fig. 27 Chondrocyte development is regulated by the balance of FGF and BMP signals

Forelimbs of E14.5 embryos were co-treated for 4 days with BMP2 at 500 ng/ml and FGF2 at different concentrations: 100ng/ml (A-C), 250 ng/ml (D-F) or 500 ng/ml (G-I). Serial sections were hybridized with antisense riboprobes for *Ihh* (B,E,H) and *Spp1* (C,F,I). In addition, proliferating cells were labeled with BrdU and detected by antibody staining (A,D,G). The rate of chondrocyte proliferation (A,D,G), as well as *Ihh* expression (B,E,H) were reduced with the increasing concentration of FGF2 in double treated limbs. In addition, distance between the *Ihh* expression domain and the joint region is reduced by increased concentration of FGF2 (B,E,H). FGF2 at 500 ng/ml can overcome the delay in terminal hypertrophic differentiation induced by BMP2 and results in upregulation of the *Spp1* expression (C,F,I). In all panels radius is up and ulna is down.

Similarly, terminal hypertrophic differentiation in the double treated cultures, defined by the expression of *Spp1*, was dependent on the variable concentrations of factors. Treatment of limbs with optimal concentration of BMP2 and increasing concentration of FGF2 resulted in an increased *Spp1* expression. Conversely, double treated limbs with constant optimal concentration of FGF2 and increasing concentration of BMP2 showed reduced expression of *Spp1* (Fig. 27 C,F,I and data not shown). Additionally chondrocyte proliferation was found to be regulated by the balance of the two signaling systems with BMPs increasing and FGF signals reducing chondrocyte proliferation (Fig. 27A,D,G). Overall these experiments strongly support an antagonistic interaction of FGF and BMP signaling in regulating chondrocyte development.

3.5.2 BMPs rescue FGF-induced defects in achondroplasia mice

The finding that BMP signaling can antagonize the FGF2 induced effects on chondrocyte development led to the question whether BMP2 treatment can rescue chondrocyte proliferation and hypertrophic differentiation in a mouse model for achondroplasia. For these experiments a mouse line was used that misexpresses *Fgfr3*, carrying one of the human

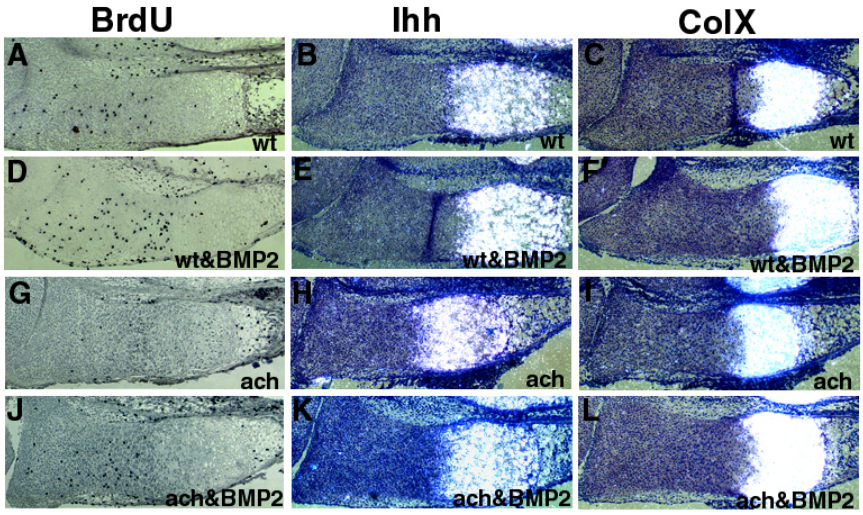

Fig. 28 BMP signaling rescues the achondroplasia phenotype in a mouse model

Forelimbs of E16.5 wild type (A-F) and FGFr3ach (G-L) embryos were cultured for 2 days in control medium (A-C, G-F) or in medium supplemented with BMP2 (D-F, J-L). Serial sections were hybridized with antisense riboprobes for *Ihh* (B,E,H,K) and *ColX* (C,F,I,L). In addition, proliferating cells were labeled with BrdU and detected by antibody staining (A,D,G,J). Similar as in wild type limbs BMP2 treatment can increase the expression of *Ihh* (B,E,H,K) and *ColX* (C,F,I,L) in hypertrophic cells of FGFr3ach mice. In addition the treatment with BMP2 leads to a delay in the onset of hypertrophic differentiation, as can be seen from the increased distance between *Ihh* expressing cells and joint region (H,K). Furthermore BMP2 treatment increases the reduced chondrocyte proliferation in FGFr3ach mice (G,J). All panels show sections through the radius. ach: FGFr3ach.

achondroplasia mutations (G380R), under the ColII promoter (FGFR3ach mouse) (Naski et al., 1998). Limb explants of E16.5 FGFR3ach mice were cultured for 2 days with BMP2**.** Similar to the results of Naski et al. untreated limbs of FGFR3ach mice displayed a reduced rate of chondrocyte proliferation and reduced domains of *Ihh* and *ColX* expression compared with control limbs of wild type littermates (Fig. 28 A-C,G-I). Correspondingly the distance between the *Ihh* expression domain and the joint region was shortened indicating an advanced onset of hypertrophic differentiation (Fig. 28 B,H). Treatment of FGFR3ach mouse limbs with BMP2 resulted in increased expression of *Ihh* and *ColX*, and an enlarged distance between the *Ihh* expression domain and the joint region (Fig. 28H,I,K,L). Finally BMP2 treatment resulted in increased chondrocyte proliferation compared to untreated limbs of FGFR3ach mice (Fig. 28G,J). Taken together these experiments show that BMP signaling can rescue the cartilage defects, produced by activated FGF signaling in the mouse model for achondroplasia. This result supports the idea of an antagonistic role of both signaling systems in regulating proliferation and differentiation of chondrocytes.

3.5.3 BMP and FGF signaling reciprocally regulate their expression

Previously Naski et al. have demonstrated that FGF signaling regulates the *Bmp4* expression, thus implicating the direct control of BMP signaling on the expression level (Naski et al., 1998). To address the question whether FGF signals regulate the expression of members of the BMP family, limbs were treated with FGF2 and the expression pattern of *Bmp4* and *Bmp7* were analyzed (Fig. 29). *Bmp4* was slightly upregulated, whereas *Bmp7* was strongly expressed in the proliferating chondrocytes (Fig. 29A-D). Interestingly in addition to a slight upregulation of *Bmp7* expression, the domain of *Bmp7* expression is markedly shifted towards the joint region (Fig. 29C,D). Thus, chondrocytes of the upper proliferating region, that normally express only low amounts of *Bmp7* have differentiated into *Bmp7* expressing chondrocytes normally found in the lower proliferating region. To analyze if BMPs conversely can regulate FGF expression, limbs treated with BMP2 were hybridized with *Fgf18*, which recently has been found to act as a ligand of FGFR3 (Liu et al., 2002). Limb explants treated with BMP2 showed a slight upregulation of *Fgf18* in the perichondral region surrounding the cartilage elements (Fig. 29E,F). Therefore, the results implicate a direct regulation of gene expression of *Fgfs* by BMP signaling and vice versa.

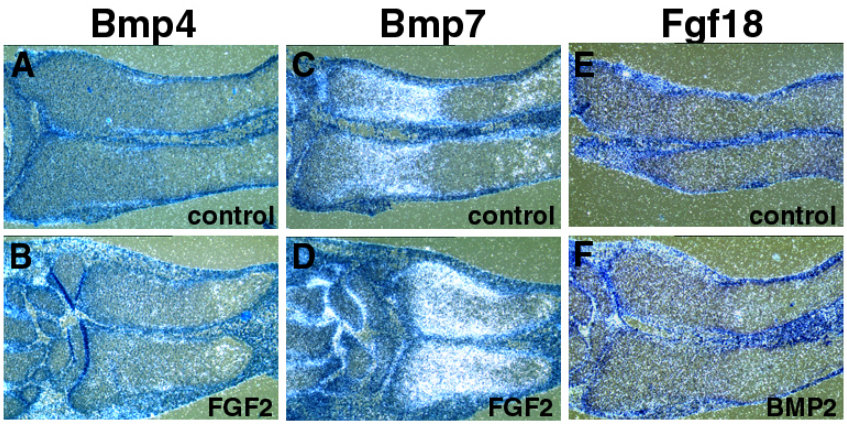

Fig. 29 BMP and FGF signaling positively regulate the expression of each other

Forelimbs of E14.5 wild type embryos were cultured for 2 days in control medium (A,C,E) or in medium supplemented with FGF2 (B,D) or BMP2 (F). Serial sections were hybridized with antisense riboprobes for *Bmp4* (A,B), *Bmp7* (C,D) and *Fgf18* (E,F). Treatment with FGF2 slightly upregulates the *Bmp4* expression in perichondrium and terminal hypertrophic chondrocytes (A,B) and strongly upregulates the *Bmp7* expression in proliferating chondrocytes (C,D). Importantly, the *Bmp7* expression domain is shifted towards the joint region (C,D). Treatment with BMP2 slightly upregulates the *Fgf18* expression in the perichondrium (E,F). In all panels ulna is up and radius is down.