4 Discussion

4.1 Analyzing of signaling systems using the limb culture system

Organ culture systems are widely used to study developmental mechanisms in skin, lung, bones and other tissues (Botchkarev et al., 1999; Chen et al., 2001; Weaver et al., 2000). Treatment of culture with different growth factors allows the activation of their signaling systems in tissue, because they can diffuse through the intercellular space. Cells of the treated tissue react to the specific factor relating their threshold of sensitivity, with stimulating or inhibiting cell functions, or without any response. In this study a limb culture system was used. To facilitate the diffusion of various factors and nutrients into the cartilage in the limb culture system, limbs were stripped out of skin and muscles and only skeletal elements were cultured. Although the limb culture system is an *in vitro* model for developmental studies, it competes well with *in vivo* models, such as mouse models with gain or loss of function, or viral misexpression approaches in chick embryos, for many reasons. First, the limb culture system allows analyzing stage-specific functions of signaling factors. Second it is appropriate to investigate the epistatic relationships of the signaling systems by co-treatment experiments or by treatment of limbs of mouse mutant. Last but not least, it takes only 2-4 days. In addition the limb culture system has particular advantages to cell culture approaches, as the intercellular organization and contacts stay intact in the cultured limb tissue. It allows cells to undergo the normal maturation process and interact with their neighbours in a way as they would do during normal development. Altogether, the regulation of proliferation and differentiation of chondrocytes can be easily investigated using the limb culture system.

In the present study the specific functions of the Ihh/Pthlh, the BMP and the FGF signaling pathways during cartilage development have been analyzed. In a first step the optimal concentration and time of treatment were defined for each signaling factor used in this study. Under optimal culture conditions the specific functions of each growth factor were analyzed and compared with the previous studies. As BMP proteins can substitute each other in experimental systems (Murtaugh et al., 1999; Vesque et al., 2000), in this study BMP2 protein was used to mimic the role of several BMPs. Activation of BMP signaling by treatment of limb cultures with BMP2 resulted in increased cartilage growth similar to that in *Noggin* -/- mutant mice (Brunet et al., 1998). Similarly activation of FGF signaling by supplementing the limb cultures with FGF2 induced similar defects that were found in transgenic mouse models, characterized by activated FGFR3 (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 analyze the Ihh/Pthlh pathway the *Ihh*-overexpressing transgenic mice, *ColII/Ihh* mice, were used. In addition, the limbs of wild type mice were treated with Pthlh. Activated Ihh signaling by Pthlh treatment of limb cultures resulted in similar delay in chondrocyte differentiation as that in limbs of *ColII/Ihh* transgenic mice.

To investigate the epistatic interactions between signaling pathways, inhibitors of BMP and Ihh signaling were used. To block BMP signaling limbs were treated with Noggin protein that has previously been demonstrated to be a potent inhibitor of at least BMP2, BMP4 and BMP7 (Kawabata et al., 1998; Zimmerman et al., 1996). Noggin treated limbs showed reduced growth of the skeletal elements similar to that found in some of the mouse mutants like *Bmp5* or *Bmp7* mutants (Jena et al., 1997; King et al., 1994). Since in limb cultures Noggin inhibits several BMPs the observed phenotype was much more severe than in single *Bmp* mutants (discussed below).

Since in *Ihh -/-* embryos the normal pattern of the various zones 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. To mimic loss of Ihh signaling limb explants were treated with cyclopamine, which is a potent inhibitor of the Hedgehog signaling pathway (Incardona et al., 1998). Cyclopamine treatment led to a block in the expression of the Hedgehog receptor *Ptc* (M.Wenzel and A.Vortkamp, personal communication) and to a block in *Pthlh* expression, similar to that in *Ihh -/-* mice (St-Jacques et al., 1999). Furthermore, the effect of cyclopamine on chondrocyte differentiation can be rescued by Pthlh co-treatment confirming that the cyclopamine-induced phenotype is a specific consequence of the loss of Ihh signaling.

4.2 Interaction of BMP signaling and the Ihh/Pthlh pathway

4.2.1 BMPs do not act as secondary signals of Ihh in regulating the onset of hypertrophic differentiation

It has previously been demonstrated that two secreted factors, Ihh from the prehypertrophic chondrocytes and Pthlh from the periarticular region, interact in a negative feedback loop regulating the onset of hypertrophic differentiation (Karp et al., 2000; St-Jacques et al., 1999; Vortkamp et al., 1996). These interactions require the transport of both signals through the growth plate. Pthlh has been shown to travel through the growth plate and signal directly

through Pthr to block hypertrophic differentiation of chondrocytes (Chung et al., 1998; Chung et al., 2001; Lanske et al., 1996; Lee et al., 1995; Tsukazaki et al., 1995). Ihh was recently demonstrated to travel over several cell diameters in the developing skeletal elements of limbs (Gritli-Linde et al., 2001). However *Patched (Ptc)*, a direct target of Hedgehog signaling (Marigo et al., 1996), is not expressed in the periarticular region suggesting that Ihh does not directly regulate *Pthlh* expression. Misexpression of constitutively activated *Bmpr-Ia* in the developing cartilage of chick embryos results in a block of hypertrophic differentiation and an increase in *Pthlh* expression. Therefore it has been hypothesized that BMPs act downstream of Ihh in mediating the induction of *Pthlh* expression and in the delay of hypertrophic differentiation (Zou et al., 1997).

To test this hypothesis limb explants of E14.5 or E16.5 mouse embryos were treated with combinations of inducers (BMP2, Ihh and Pthlh) and inhibitors (Noggin, cyclopamine) of the two signaling pathways. Treatment with Noggin, a potent inhibitor of BMP signaling (Smith, 1999; Zimmerman et al., 1996), could not antagonize the Ihh-induced delay of hypertrophic differentiation or the upregulation of *Pthlh* in limb cultures of mice overexpressing *Ihh* under the ColII promoter. Correspondingly, BMP2 could not rescue the cyclopamine-induced advanced onset of hypertrophic differentiation or induce the expression of *Pthlh*, which was lost by blocking the Ihh signaling pathway. Therefore BMP signaling, at least that of BMP2, does not act downstream of Ihh to regulate *Pthlh* expression and the onset of hypertrophic differentiation. Other members of the BMP family such as BMP7, which is expressed in the proliferating chondrocytes between *Ihh* and *Pthlh*, might serve as mediators of the Ihh signal. However, as BMPs are members of a highly conserved gene family and can substitute for each other in many assays (Kawabata et al., 1998; Monsoro-Burq et al., 1996), it is not likely that a close relative of BMP2, like BMP7, acts as the proposed secondary signal. Further studies will be necessary to test if other growth factors, regulated by Ihh, can function to propagate the Ihh signal.

Because the experiments by Zou et al. showing *Pthlh* upregulation by an activated BMP receptor were carried out in chicken embryos (Zou et al., 1997), it was important to confirm that the results of this study did not reflect a species-specific difference between chick and mouse bone development. Therefore the culture experiments were repeated using limb explants of stage HH 32 chick embryos. Similar to the mouse experiments no evidences were found for BMP signaling acting downstream of Ihh in regulating *Pthlh* expression and hypertrophic differentiation. After overexpression of constitutively activated *Bmpr-Ia* the infected chicken limbs were analyzed at comparable stages to our study. However, the virus was injected at stage HH14 into the presumptive limb field, leading to the activation of BMP signaling before the onset of hypertrophic differentiation (Zou et al., 1997). The limb culture

system allowed activating BMP signaling specifically at later stages of chondrocyte development, after the establishment of the different regions of the growth plate is completed. Therefore the effect seen in the studies by Zou et al. might detect a different role for BMP signals at earlier stages of skeletal development (Zou et al., 1997). Continuous activation of BMP signaling might keep all chondrocytes in a proliferating state thereby preventing the initiation of the hypertrophic differentiation process.

4.2.2 BMP signaling regulates several aspects of chondrocyte development

Several Ihh-independent functions of BMP signaling were identified in the present study. One of these is regulating the process of terminal hypertrophic differentiation. Blocking of BMP signaling by Noggin resulted in an increased number of *Spp1*-expressing terminal hypertrophic cells. In contrast, cyclopamine treatment, which leads to an advanced onset of hypertrophic differentiation, did not increase *Spp1* expression. Double treatment of limbs with Noggin and cyclopamine or with Noggin and Pthlh resulted in accelerated terminal hypertrophic differentiation. Thus double treatment experiments support the idea that BMP and Ihh/Pthlh signaling control different aspects of hypertrophic differentiation: the Ihh/Pthlh system regulates the onset of hypertrophic differentiation whereas BMP signaling controls the pace of the differentiation process itself. Similar experiments treating cultures of mouse metatarsals with BMP2 have shown an enlargement of the hypertrophic region (De Luca et al., 2001). One possible explanation might be that BMP2 slows down the differentiation process in addition to increasing the number of cells undergoing hypertrophic differentiation.

The present and previous studies have shown that Ihh and BMP signaling regulate chondrocyte proliferation (De Luca et al., 2001; Karp et al., 2000; Long et al., 2001; St-Jacques et al., 1999). Treatment of *ColII/Ihh* mice with Noggin or co-treatment of wild type limbs with BMP2 and cyclopamine revealed that the two signals are necessary in concert for proper chondrocyte proliferation. Blocking either BMP or Ihh pathway results in inhibition or severe reduction of chondrocyte proliferation, demonstrating that the two signaling systems act in parallel.

A third function of BMP signaling identified in this study is the regulation of *Ihh* expression. After treatment with only BMP2 a slight enlargement of the *Ihh* expression domain was observed. Correspondingly, treatment with Noggin resulted in reduced *Ihh* expression. Double treatment with cyclopamine and BMP2 led to a greater increase in *Ihh* expression than did treatment with cyclopamine alone, whereas double treatment with cyclopamine and Noggin reduced the cyclopamine-induced upregulation of *Ihh*. These experiments

demonstrated that BMP signaling regulates *Ihh* expression. In this respect it is interesting to note that in the presence of cyclopamine BMP2 induces *Ihh* expression closer to the periarticular region as treatment with cyclopamine alone. One possible explanation is that BMPs upregulate the expression of *Ihh* in absence of the Ihh/Pthlh pathway. This could furthermore mean that Pthlh not only blocks chondrocyte differentiation but simultaneously determines the competence of chondrocytes to react to BMP signals with the upregulation of *Ihh* expression. Whether the induction of *Ihh* expression is a direct consequence of BMPs regulating *Ihh* expression on a transcription level or if it is a secondary consequence of BMPs regulating chondrocyte differentiation remains to be addressed in future experiments.

An interesting finding is that BMP signaling integrates chondrocyte proliferation and differentiation during bone development. As the distance between the joint region and the *Ihh*-expressing cells is determined by the Ihh/Pthlh system, one could expect that upregulation of chondrocyte proliferation would not increase this distance. However, treatment of explants with BMP2 protein resulted in an enlargement of both, the zone of proliferating and the region of hypertrophic chondrocytes. The negative effect on the onset of hypertrophic differentiation seems to be mediated by the upregulation of *Ihh* expression by BMP signaling. Furthermore, as discussed above, BMP signals delay the hypertrophic differentiation process itself. By acting at several stages of chondrocyte differentiation BMP signaling seems to regulate the size of a skeletal element without disturbing the differentiation process relative to developmental age. A slight increase of BMP signaling would thus result in slightly larger bones, which would however develop at an unaltered pace. Similarly, a slight decrease in BMP signaling would lead to shortened bones, also without a change in the rate of development. Such an effect can be seen in several of the *Bmp* mutant mice like the short ear mutant *(Bmp5)* (King et al., 1994) or mice carrying a targeted deletion of *Bmp6* or *Bmp7* genes (Dudley et al., 1995; Luo et al., 1995; Solloway et al., 1998). They display mild skeletal phenotypes including shorter bones without severe disturbance of the overall differentiation process.

4.2.3 The *Bmp* gene family

Several *Bmp* genes are expressed in specific regions of the developing cartilage elements and might thus be important for different aspects of chondrocyte differentiation during normal development. *Bmp7* is expressed in the proliferating chondrocytes distal to *Ihh* (Haaijman et al., 2000; Solloway et al., 1998) and may be responsible for regulating chondrocyte proliferation and *Ihh* expression. Other *Bmps*, including *Bmp2*, *Bmp3*, *Bmp4* and *Bmp7*,

are expressed in the perichondrium/periosteum (Daluiski et al., 2001; Haaijman et al., 2000 and Fig.14C,D; Pathi et al., 1999; Zou et al., 1997), and may signal back to adjacent chondrocytes to regulate chondrocyte proliferation, chondrocyte differentiation or *Ihh* expression. *Bmp6* is expressed in the prehypertrophic and hypertrophic chondrocytes (Solloway et al., 1998; Vortkamp et al., 1996) and is a good candidate to regulate the process of hypertrophic differentiation.

Analysis of mutations in single *Bmp* genes has not given significant insight into their role during skeletal differentiation. Targeted disruption of either *Bmp2* or *Bmp4* leads to early embryonic lethality (Dunn et al., 1997; Winnier et al., 1995; Zhang and Bradley, 1996). Mutations in *Bmp5*, *Bmp6* or *Bmp7* display only mild skeletal phenotypes indicating a highly redundant role of *Bmp* genes in regulating bone development (Dudley et al., 1995; King et al., 1994; Kingsley et al., 1992; Luo et al., 1995; Solloway et al., 1998). Due to the proposed redundancy of BMP function, it is likely that for some of the processes addressed in this study the overall level of BMP signaling is more critical than the signal from a single factor. In addition, the receptors of BMPs can add to the redundant functions of BMP signals, since several BMPs can signal not only through the BMP receptors but also through the close related activin receptors (Kawabata et al., 1998; Massague, 2000). The double knockout mice of *Bmp7* and *Bmpr-1b* support this idea, as they show more severe skeletal phenotype than each of the single mutants does (Yi et al., 2000). BMPs signal through the heterodimers of type I and type II of BMP or activin receptors, which in turn activate members of the Smad family of transcription factors, Smad1, Smad5 and Smad8. Upon activation these Smad proteins can form heterodimers with either Smad4 or Smad6 to direct the transcription of target genes (Kawabata et al., 1998; Massague, 2000; von Bubnoff and Cho, 2001). To fully understand the role of *Bmp* genes during bone development it will be necessary to analyze how BMP signals are interpreted by BMP receptors and Smad pathways.

4.2.4 Interaction of BMP signaling with the Ihh/Pthlh pathway

Based on the results of the present and of previous studies, the following model for the regulation of chondrogenesis by the Ihh/Pthlh and BMP signaling pathways is suggested (Fig. 30). Ihh produced by prehypertrophic chondrocytes induces the expression of several *Bmp* genes in the perichondrium and in the proliferating chondrocytes. Ihh, furthermore, induces the expression of *Pthlh* in the periarticular region. Pthlh, in turn, negatively regulates the onset of hypertrophic differentiation. The range of Pthlh activity determines the distance

from the joint region at which chondrocytes initiate the hypertrophic differentiation program and thus the size of the domain of chondrocytes that are competent to proliferate. Ihh and BMP signaling together regulate the level of chondrocyte proliferation thereby pushing some cells out of the Pthlh signaling range. These cells are then released from the block of hypertrophic differentiation and activate the expression of *Ihh*, which, as discussed above, might be directly or indirectly regulated by BMP signaling. Since Ihh signaling regulates the expression of both, *Bmp* genes and *Pthlh*, it tightly controls its own activation. Additionally, BMP signaling negatively regulates the differentiation of terminal hypertrophic chondrocytes. This delay in terminal hypertrophic differentiation might be essential for chondrocytes to acquire the enlarged size of hypertrophic cells and to undergo the accompanying changes in gene expression and matrix composition. By regulating chondrocyte proliferation, *Ihh* expression and the pace of hypertrophic differentiation, BMP signaling might thus keep the overall pace of cartilage development in phase.

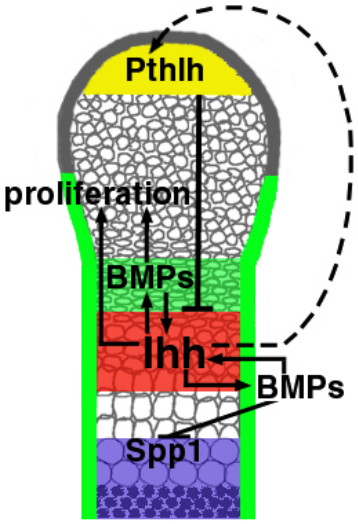

Fig. 30 Interaction of BMP and Ihh signaling

During endochondral ossification *Ihh* is expressed in the differentiating chondrocytes (red). Ihh induces the expression of *Pthlh* in periarticular chondrocytes (yellow) independent of BMP signaling. The range of Pthlh signaling keeps chondrocytes in a proliferating state and determines the distance from the joint region at which chondrocytes can undergo hypertrophic differentiation. Ihh in addition regulates the expression of *Bmp* genes in the perichondrium/periosteum and in part of the proliferating chondrocytes (green). BMP and Ihh signaling together upregulate chondrocyte proliferation thereby pushing cells out of the range of Pthlh signaling. These chondrocytes are released from the block of differentiation and upregulate *Ihh* expression upon BMP signaling. BMP signaling furthermore negatively regulates the development of terminally differentiated hypertrophic chondrocytes (blue). By regulating chondrocyte proliferation, *Ihh* expression and terminal hypertrophic differentiation, BMP signaling integrates the different steps of endochondral ossification.

4.3 Interaction of FGF and Ihh/Pthlh signaling

4.3.1 FGF signaling regulates bone development

FGF signaling is another important pathway that has been shown to control the different steps of bone development by regulating cell proliferation and differentiation. In vertebrates the FGF family consists of at least 22 members which signal through four tyrosine kinase receptors, FGFR1, FGFR2, FGFR3 and FGFR4 (Ornitz and Itoh, 2001). In the skeleton the expression pattern of *Fgfr1*, *Fgfr2* and *Fgfr3* was found in the skull and the appendicular skeletal elements (Fig. 6 in this study) (Delezoide et al., 1998; Iseki et al., 1997). In the developing skeletal elements the expression for only three of *Fgf* genes, *Fgf8*, *Fgf17* and *Fgf18*, could be detected in the perichondrium (Liu et al., 2002; Xu et al., 1999).

In previous studies the roles of FGF receptors and FGF themselves were analyzed by gene targeting approach in mice. FGFR4 was not found to regulate skeletal development, since the *Fgfr4 -/-* mutant mice do not reveal any skeletal malformations (Weinstein et al., 1998). FGFR1 and FGFR2 were found to play important roles in early embryogenesis, since targeted deletions of either *Fgfr1* or *Fgfr2* result in neonatal lethality of mutant mice before the limb formation (Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994). Due to the early lethality their role during endochondral ossification could not be addressed in these mutant mice. In contrast, FGFR3 plays a specific role only during later embryogenesis. The targeted deletion of the *Fgfr3* gene results in deafness and overgrowth of skeleton. Morphologically the skeletal elements of *Fgfr3* mutant mice revealed increased zones of proliferating and hypertrophic chondrocytes (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1998). From all known *Fgf* knockout mice only *Fgf18 -/-* mutant mice and mice with limb specific targeted deletion of *Fgf8* display a skeletal phenotype (Liu et al., 2002; Moon and Capecchi, 2000). *Fgf18 -/-* mice reveal increased zones of proliferating and hypertrophic chondrocytes similar as in *Fgfr3 -/-* mice, thus implicating FGF18 as one of the ligands of FGFR3 (Liu et al., 2002). Targeted deletion of *Fgf8* results in early embryonic lethality before the initiation of limb formation, whereas limb specific deletion of *Fgf8* results in shortening of the skeletal elements in limbs with missing radius and thumb (Moon and Capecchi, 2000). The observed skeletal phenotype, however, can be explained by insufficient FGF8 signaling during early patterning events rather than by its direct effect in regulating cartilage development.

In the present study different functions of FGF signaling in regulating chondrocyte proliferation and differentiation and its interactions with the Ihh/Pthlh pathway and BMP signaling have been analyzed. First it was demonstrated that the treatment of limb explants

with FGF2 mimics the phenotype of activated FGFR3 signaling in mouse models, by reducing the domains of proliferating chondrocytes and of the *Ihh*- and *ColX*-expressing hypertrophic chondrocytes. In addition cells in the middle of FGF2 treated skeletal elements highly expressed *Spp1*, indicating an accelerated differentiation into the terminal hypertrophic state. Similarly the expression of *MMP13*, another marker for terminal hypertrophic chondrocytes and bone, was upregulated in these cells. To test that the terminal hypertrophic chondrocytes were not transformed into the osteoblasts the expression of *osteocalcin*, a specific marker for osteoblasts, was analyzed. *Osteocalcin* was upregulated in the periosteum flanking the region of terminal hypertrophic cells, but was not present in the middle of skeletal elements in the cells, expressing *Spp1*. Interestingly, untreated control limbs at E14.5 showed no *osteocalcin* expression supporting a role of FGF signaling in accelerating the differentiation process not only of chondrocytes but also of perichondral cells into primary osteoblasts of the periosteum. Overall, the results of the present study clearly demonstrated that upon activated FGF signaling the hypertrophic differentiation is accelerated in chondrocytes.

The upregulation of *Spp1* expression has not been described in FGFR3ach mice. This might indicate that the regulation of terminal hypertrophic differentiation is mediated by a different receptor. *Fgfr1* is strongly expressed in terminal hypertrophic chondrocytes (Fig. 6B) (Delezoide et al., 1998) and might thus be the main receptor to regulate this process.

4.3.2 FGF signaling accelerates hypertrophic differentiation

Analysis of mouse models for human achondroplasia with activated FGF signaling, as well as *Fgfr3* knockout mice, have shown that the expression of *Ihh* and *BMP4* is regulated by FGF signaling (Naski et al., 1998). However the interactions of FGF signaling with the Ihh/Pthlh and BMP signaling pathways were not clarified. To analyze their epistatic interactions with the Ihh/Pthlh system the distance between the *Ihh*-expressing chondrocytes and the joint region was carefully analyzed. This distance was reduced by FGF signals in limb cultures and in FGFR3ach mice. As this distance demarcates the onset of hypertrophic differentiation, the FGF induced effect can be interpreted as an advanced onset of chondrocyte differentiation. As Ihh also regulates the onset of hypertrophic differentiation it was interesting to analyze if the FGF induced effect is mediated by the Ihh/Pthlh signaling system. Co-treatment of limb cultures with Pthlh and FGF2 (this study) as well as Pthlh treatment of metatarsal bones of achondroplasia or FGFR3-/- mice (Chen et al., 2001) resulted in a delay of chondrocyte differentiation, thus placing FGF signals either upstream or

parallel to Pthlh. Furthermore treatment of limbs of *ColII/Ihh* mice with FGF2 has shown that FGF signaling could not accelerate the onset of hypertrophic differentiation in these mice. This result indicates that FGF signaling acts upstream of the Ihh in regulating the onset of hypertrophic differentiation. As activated FGF signaling results in a reduced expression of *Ihh* in limb culture and in transgenic mice, FGF signals seem to regulate the onset of hypertrophic differentiation by directly regulating *Ihh* expression. Interestingly, *Bmp7* expression, which is normally found in a small zone of proliferating chondrocytes, adjacent to *Ihh*-expressing cells, is shifted toward the joint region by activated FGF signaling. This result indicates that FGF signaling advances chondrocyte differentiation even in the pool of proliferating chondrocytes.

An important role of FGF signaling in regulating chondrocyte proliferation has been also identified in the present study. In contrast to regulating the onset of hypertrophic differentiation, FGF signaling regulates chondrocyte proliferation in parallel or downstream of Ihh. The negative role of FGF signaling in regulating chondrocyte proliferation seems to be mediated through the several receptors. Also the *Fgfr3* knockout mice reveal only mild overgrowth of skeletal elements (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1998).

A third function of FGF signaling is an accumulation of terminal hypertrophic cells similar to the Noggin treatment. It has already been discussed that Ihh signaling seems not to regulate terminal hypertrophic differentiation, since neither Pthlh, nor cyclopamine treatment could upregulate *Spp1* expression. Consequently, double treated limbs with FGF2 and Pthlh or FGF2 and cyclopamine showed an induced expression of *Spp1* similar to that in limbs treated with FGF2 only. Therefore, FGF signaling regulates terminal hypertrophic differentiation of chondrocytes independent of the Ihh/Pthlh system as has been shown for a block of BMP signaling by Noggin. Moreover, this result indicates a possible antagonistic relationship between FGF and BMP signaling in regulating terminal hypertrophic differentiation of chondrocytes.

Summarizing the results of the present study, the role of FGF signaling in regulating chondrocyte development might be interpreted in a different way. Instead of reducing hypertrophic differentiation of chondrocytes FGF signaling advances hypertrophic differentiation, both by inducing the onset and by accelerating the differentiation process itself. In concert with the reduced rate of chondrocyte proliferation, the accelerated differentiation process leads to the decreased domains of hypertrophic chondrocytes. Thus by controlling proliferation and differentiation of chondrocytes FGF signaling adapts the differentiation process to the proliferation rate.

4.3.3 FGF and BMP signaling act as antagonists during cartilage development

This study has demonstrated that BMP signals positively regulate chondrocyte proliferation, induce the expression of *Ihh* and delay terminal hypertrophic differentiation of chondrocytes. Block of BMP signaling by Noggin results in a similar phenotype, as does treatment with FGF2: reduced chondrocyte proliferation, decreased *Ihh* expression and induced terminal hypertrophic differentiation of chondrocytes. Therefore, BMP and FGF signals seem to regulate the same stages of chondrocyte development, however mediating opposite effects. Co-treatment experiments with BMP2 and FGF2 were used to analyze the epistatic relationship between the two signaling systems. All three parameters tested, proliferation, *Ihh* expression and terminal hypertrophic differentiation, showed a partial rescue of the BMP2- as well as of the FGF2-induced phenotype. Furthermore, by varying the concentrations of the two factors in co-treatment experiments it was found that the balance of their concentrations was critical for the resulting phenotype. As in an epistatic relationship the downstream signal should determine the phenotype, these results strongly indicate that FGF and BMP signals act in independent pathways having antagonistic effects on chondrocyte development. Similar antagonistic interactions have been found for FGF and BMP signals during the development of many organ systems as for example tooth formation, limb bud outgrowth, embryonic blood formation and neurogenesis (Merino et al., 1998; Neubuser et al., 1997; Wilson et al., 2001; Xu et al., 1999; Zuniga et al., 1999). However their relationship during the development of the skeletal elements has not been analyzed before.

As FGFs and BMPs signal through different types of kinase receptors and intracellular mediators, it will be important to investigate at which point both pathways merge. Both signaling pathways could for example regulate the same promoters. In such case the balance of downstream transcription factors could determine the activation level of the same specific promoters. Alternatively both signaling pathways could interact cytoplasmatically on the level of the signal transduction cascade. Intracellularly the BMP signals are mediated through transcription factors of the Smad family, such as Smad1, Smad5 and Smad8. Activated Smads interact with Smad4 to transport the BMP signal into the nucleus (Massague, 2000). At least two intracellular pathways, the extracellular signal-regulated kinase (Erk) pathway and the phosphatidilinositol-3-OH kinase (PI(3) kinase) pathway mediate FGF signals (Marshall, 1995; Umbhauer et al., 1995). Interestingly, Kretzschmar et al. have analyzed Erk mediated signaling of the Epidermal growth factor (EGF) and its interaction with BMP signals in cell cultures (Kretzschmar et al., 1997). They found that activation of the Erk pathway inhibits BMP induced phosphorylation of Smad1, thus preventing transport of the Smad1-Smad4 complex into the nucleus (Kretzschmar et al., 1997). More recent studies have

demonstrated similar interactions of BMP signals and the Erk kinase pathway during endoderm determination and osteoblast differentiation (Gallea et al., 2001; Goswami et al., 2001). It will be interesting to analyze if FGF and BMP signals interact in comparable ways regulating chondrocyte proliferation and differentiation. In the present study additionally was demonstrated that FGF and BMP signaling might regulate their expression via positive feed back relationship.

4.3.4 BMP signaling rescues defects in achondroplasia mice

Achondroplasia, the most common form of inherited human dwarfism, results from an activation of FGFR3 signaling (Bellus et al., 1995; 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. Deciphering the role of FGF signaling during bone differentiation and its interaction with other signaling systems is necessary to understand the molecular origin of the dwarfism phenotype and to ultimately find new ways for specific treatment. The results of this study indicate that at least under limb culture conditions, BMP signaling can improve the achondroplasia phenotype on the molecular level by increasing chondrocyte proliferation and delaying hypertrophic differentiation of chondrocytes.

Furthermore, the results of this study have demonstrated the importance of analyzing the exact integration of different signaling systems. 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 achondroplasia phenotype in bone. In contrast, BMP signaling seems to act at very similar stages of chondrocyte development, as does FGF signaling. Therefore manipulating the BMP signaling pathway might be a more promising approach to improve the achondroplasia phenotype. Further *in vitro* and *in vivo* studies are however necessary to understand the molecular mechanism of their interaction.

4.4 Conclusions

Based on the results of this study the following model of regulating chondrocyte development by Ihh/Pthlh, BMP and FGF signaling is proposed (Fig. 31). Ihh, which is expressed in the prehypertrophic chondrocytes, induces the expression of various *Bmps* in the flanking

perichondrium/periosteum and in the proliferating chondrocytes. Ihh and BMP signals act in parallel to induce chondrocyte proliferation, whereas FGF signaling inhibits chondrocyte proliferation independent 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 and thereby indirectly defines the pool of chondrocytes that are competent to proliferate. Chondrocytes which are released from the range of Pthlh signal change their competence and in response to BMP signals upregulate *Ihh* expression. In contrast FGF signals act as antagonist 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 FGF signaling accelerates the rate of terminal hypertrophic differentiation, whereas BMPs have been shown to hinder this process. By regulating proliferation, *Ihh* expression and the rate of terminal hypertrophic differentiation, these signaling pathways might adjust the process of hypertrophic differentiation in response to the proliferation rate and keep the overall skeletal development in phase.

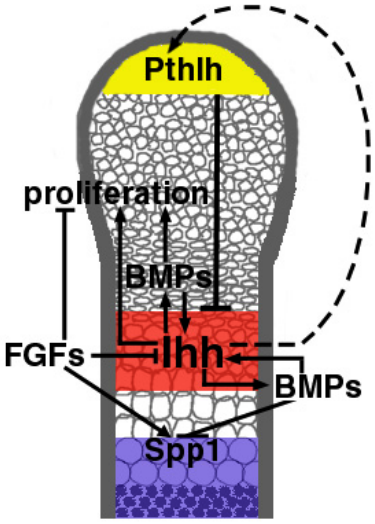

Fig. 31 Interaction of the FGF, the BMP and the Ihh/Pthlh signaling pathways

Ihh, expressed from the prehypertrophic chondrocytes (red), induces the expression of *Pthlh* in the periarticular chondrocytes (yellow). Pthlh in turn determines the distance from the joint region at which chondrocytes undergo hypertrophic differentiation and the pool of chondrocytes competent to proliferate. Ihh in addition induces the expression of different *Bmp* genes in the proliferating and hypertrophic chondrocytes. Both signals, Ihh and BMPs, positively regulate chondrocyte proliferation, whereas FGF signals, derived from the perichondrium and possibly the chondrocytes themselves, negatively regulate chondrocyte proliferation. Chondrocytes that are released from Pthlh signaling change their competence and react to BMP signals with the upregulation of *Ihh* expression. FGF signaling in contrast antagonizes the BMP induced expression of *Ihh*. By acting upstream of Ihh, FGF and BMP signals indirectly regulate the onset of hypertrophic differentiation. FGF signals in addition accelerate the differentiation of terminal hypertrophic chondrocytes (blue), expressing *Spp1*, whereas BMP signaling delays this process. By regulating the proliferation rate, the level of *Ihh* expression and the pace of terminal hypertrophic differentiation, the BMP and FGF signals integrate the onset of hypertrophic differentiation with the proliferation rate.