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Indian Hedgehog: Its Roles and Regulation in Endochondral Bone Development

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Abstract Normal endochondral bone development requires the coordination of chondrocyte proliferation and differentiation. Indian hedgehog (Ihh) is a morphogen produced by chondrocytes in the early stage of terminal differentiation and plays several key roles in this process. Ihh regulates growth of adjacent proliferative chondrocytes and can also regulate the rate of differentiation of chondrocytes indirectly through its stimulation of parathyroid hormone-related protein (PTHrP). In this review, we focus on recent studies that have identified new functions of Ihh and how Ihh itself is being regulated. J. Cell. Biochem. 96: 1163–1173, 2005. © 2005 Wiley-Liss, Inc.

Key words: Indian hedgehog; endochondral bone development; parathyroid hormone-related peptide; Runx2; TGFβ; BMP

Skeletal tissues are essential components of our bodies, supporting our weight and providing points of attachment for skeletal muscle groups. All bones develop from mesenchymal cells, but depending on their function, location, and shape, they can develop through one of two different processes. Intramembranous ossification is the process by which flat bones such as the facial bones and cranium develop. It involves direct differentiation of mesenchymal progenitor cells into bone-forming osteoblasts. During this process, new bone matrix is synthesized and mineralized by osteoblasts. Long bones of the limbs as well as the ribs develop by the alternative process known as endochondral ossification. This process involves the formation of a cartilage primodrium and growth plate, where chondrocytes initially undergo proliferation and a series of differentiation steps secreting a cartilage template that is eventually replaced by bone [Olsen et al., 2000].

During endochondral bone development, committed mesenchymal pre-chondrogenic cells undergo condensation, and secrete a number of matrix proteins that form the cartilaginous template. Cells in the center of the condensate will differentiate into mature chondrocytes, while cells on the periphery become the perichondrium, forming the boundary of the cartilage. Mature chondrocytes at the center of the cartilage actively undergo proliferation, forming columns of proliferative cells, whereas those at the epiphyseal ends divide at a much slower rate, becoming the reserve chondrocytes. Chondrocytes express specific arrays of gene products as they differentiate into post-mitotic hypertrophic cells. The appearance of these gene products demarcates the various stages of chondrocyte development as shown in Figure 1. Mature chondrocytes express collagen type II and Sox9, and as they reach the pre-hypertrophic stage, they express PTH/PTHrP receptors (PPR) and Indian hedgehog (Ihh). When they become hypertrophic chondrocytes, the cells express type X collagen and vascular endothelial growth factor (VEGF) [Olsen et al., 2000].

Elongation of bones during growth of the organism results from chondrocyte proliferation whereas the differentiation of chondrocytes into the hypertrophic state is required for the secretion of specific factors and matrix proteins that allow vascular invasion and matrix

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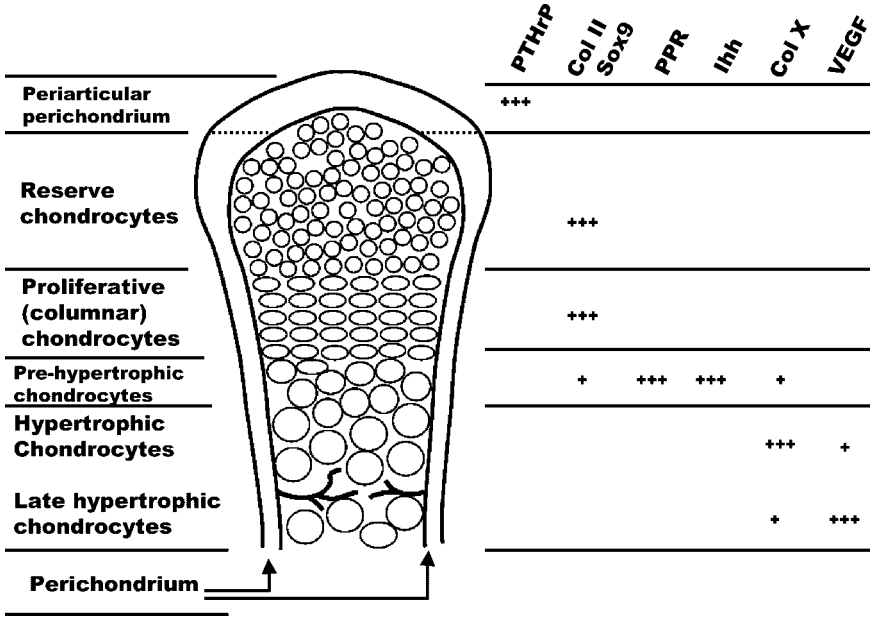

Fig. 1. Endochondral ossification occurs at growth plates, where chondrocytes proliferate and undergo a series of differentiation steps. At each stage, they express different arrays of marker genes. Reserve and proliferative chondrocytes express high levels of collagen II (Col II) and Sox 9. As they become pre-hypertrophic chondrocytes, they transiently express PTH/PTHrP receptor (PPR) as well as Indian hedgehog (Ihh), and as they become hypertrophic chondrocytes, they progressively express collagen X (Col X) and vascular endothelial growth factor (VEGF). Parathyroid hormone-related peptide (PTHrP) is exclusively expressed by the cells in the periarticular perichondrium.

calcification. Hypertrophic chondrocytes finally undergo apoptosis as the cartilaginous template is replaced by true bone matrix secreted by osteoblasts to form trabecular bone. Coordination of the growth and differentiation of chondrocytes within the growth plate are synchronized with those of the surrounding perichondrium that directly differentiates into osteoblasts (perichondrial bone formation), forming initially the periosteum and later the cortical bone. Perichondrial development, chondrocyte proliferation as well as differentiation all affect bone development, and they must be carefully coordinated by multiple local and systemic factors [Olsen et al., 2000]. This review focuses on the role of one of these factors, Indian hedgehog, in skeletal development.

Hedgehog (Hh) proteins are secreted morphogens that are essential for multiple developmental processes in both invertebrates and vertebrates. Hh is synthesized as a 45 kDa precursor, but is cleaved to an active 19 kDa N-terminal fragment, which is subsequently modified by attachment of cholesterol and palmitic acid. Secreted active Hh fragments can regulate cellular activities of neighboring and distant cells. In *Drosophila*, where Hh was first discovered and is better understood, the long-range effects of Hh are facilitated by Hh–cholesterol interactions with heparan sulfate proteoglycans (HSPG) in the surrounding intracellular matrix (see below). Hh-target cells express two components of the Hh signaling system on the cell surface: Patched (Ptc), a 12-transmembrane protein, and Smoothened (Smo), a 7-transmembrane protein. In the absence of Hh, Ptc represses the activity of Smo, which allows proteolytic processing of a downstream zinc-finger transcription factor, Cubitus intereptus (Ci) at its C-terminal end forming a transcriptional repressor. When Hh binds to Ptc it relieves Ptc repression of Smo and activated Smo stabilizes intact Ci, which then acts as a transcription activator, and hence stimulates transcription of target genes [reviewed in: Ingham and McMahon, 2001].

Hedgehog proteins are conserved in vertebrates but there are multiple proteins and receptors and their roles are less well defined. There are three vertebrate Hh proteins: Desert hedgehog (Dhh), Sonic hedgehog (Shh), and Indian hedgehog (Ihh). All of them have unique sets of functions in regulation of different

developmental processes. Dhh has the closest sequence similarity to the *Drosophila* Hh, and is essential for the development of peripheral nerves and spermatogenesis. Shh is involved in establishing lateral asymmetry, the anterior–posterior limb axis, and development of the central nervous system. Ihh is a master regulator of endochondral bone development [McMahon et al., 2003].

Hedgehog signaling is also more complex in vertebrates. There are two mammalian Ptc homologues both bind Hh proteins with similar affinity and both can interact with mammalian Smo. Ptc1 is widely expressed throughout the mouse embryo and serves as the extracellular receptor for multiple Hh proteins, and is itself upregulated by Hh signaling. Ptc2 on the other hand, is more discreetly expressed with high levels in the skin and spermatocytes where it is thought to act as the receptor for Dhh co-expressed in the testis [Carpenter et al., 1998]. There are also three Ci homologues in vertebrates, known as Gli1, 2, and 3. While Gli1 functions as an activator, Gli2 and 3 can function as either transcriptional activators or inhibitors depending on the cellular context. Gli1 is upregulated while Gli3 is downregulated by Hh [Ruiz i Altaba et al., 2002].

Ablation of either *Shh* or *Ihh* genes in transgenic mice causes different forms of skeletal defects, indicating that both are essential for skeletal development but they play different roles in this process. Shh-deficient transgenic mice have abnormalities of early skeletal development with severe growth retardation, lack of vertebrae, and distal limb structures [Chiang et al., 1996]. This suggests a role for Shh in regulation of skeletal patterning. Ihh-deficient transgenic mice have short limbs but they are considerably more developed than those of Shh-deficient animals. The absence of mineralized bone structures in Ihh-deficient animal indicates a role for Ihh in the coordination of multiple cellular events during endochondral bone development including chondrocyte proliferation and differentiation as well as osteoblast differentiation [St.-Jacques et al., 1999]. Mutations in the Ihh gene have been linked to two inherited skeletal developmental defects: brachydactyly type A-1 and acrocapitofemoral dysplasia, clearly implicating Ihh as a key regulator of skeletal development in humans [Gao et al., 2001; Hellemans et al., 2003]. In this review, we will discuss the multiple roles that Ihh plays in endochondral bone development, with special focus on recent studies that have extended our understanding of how Ihh signaling interacts with other signaling pathways, how Ihh itself is being regulated, as well as identification new functions of Ihh.

FUNCTIONS OF IHH IN ENDOCHONDRAL BONE DEVELOPMENT (Fig. 2)

Ihh and PTHrP Form a Feedback Loop That Regulates Chondrocyte Differentiation at Different Stages

Seminal work by Vortkamp et al. [1996] first demonstrated that Ihh and parathyroid hormone related peptide (PTHrP) participate in a feedback loop, which coordinates chondrocyte proliferation and differentiation in fetal developing bones (Fig. 2). As shown in Figure 1, Ihh is expressed and secreted by pre-hypertrophic chondrocytes preceding and overlapping with

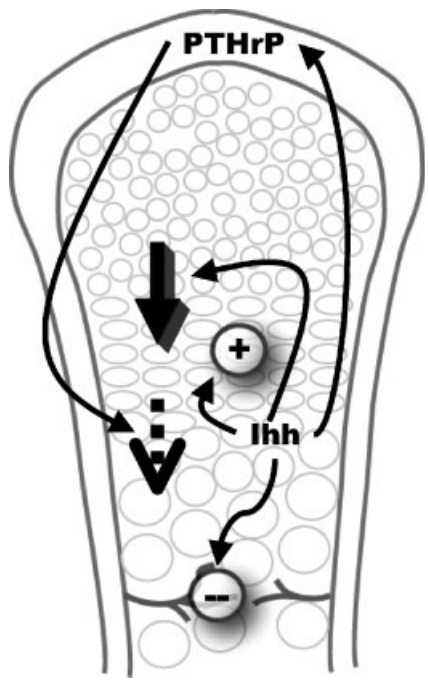

Fig. 2. Ihh is a master regulator of endochondral bone development. It regulates and coordinates multiple cellular events during this process. By upregulating PTHrP, Ihh slows down the differentiation rate of proliferative chondrocytes into hypertrophic chondrocytes (•••▶). Ihh directly stimulates chondrocyte proliferation (+), and promotes differentiation of reserve chondrocytes into proliferative chondrocytes (➡). It also adversely affects endothelial cell development during vascular invasion, which is likely linked to its effect on perichondrial differentiation (–).

expression of PPR. Ihh, either directly or indirectly induces PTHrP production from the periarticular perichondrium. PTHrP is able to diffuse to the PPR expressed by proliferative and pre-hypertrophic chondrocytes. Activation of PPR in these cells delays their rate of differentiation into hypertrophic chondrocytes thus shutting off the supply of Ihh by keeping chondrocyte in the proliferative state. This feedback loop between Ihh and PTHrP is clearly important for regulation of normal endochondral bone development as disruption of any component of the system results in abnormal limb development [St.-Jacques et al., 1999]. Since these initial studies, numerous functional studies have further defined the roles of PTHrP and Ihh and their regulation.

To directly study the roles of Ihh, transgenic mice deficient in Ihh ($Ihh^{-/-}$) were generated [St.-Jacques et al., 1999]. Almost all of these mice died at birth due to respiratory failure as a result of restrictive underdevelopment of the rib cage. Consistent with earlier studies, PTHrP was not detected in the periarticular regions of cartilaginous structures in $Ihh^{-/-}$ mice, and chondrocyte differentiation was affected. Chondrocyte hypertrophic differentiation was initially delayed and later occurred at abnormal locations in $Ihh^{-/-}$ mice close to the epiphyseal ends of the bones, instead of at the center. Studies performed by Karp et al. [2000] further compared the phenotypes of $Ihh^{-/-}$ mice and $Ihh^{-/-}PTHrP^{-/-}$ double knock out mice. The two groups of mice had very similar skeletal defects and a constitutively active PPR was able to correct the defects in chondrocyte hypertrophy in $Ihh^{-/-}$ mice. These data provided strong evidence to support the idea that Ihh regulates chondrocyte hypertrophy indirectly by stimulating PTHrP.

Recent studies performed by Kobayashi et al. [2002] indicated that PTHrP and Ihh are also involved in regulating chondrocyte differentiation during an earlier stage. They used the Cre-loxP system to repress expression of PPR in collagen type II-expressing chondrocytes. As expected they found that the decreased PTHrP signaling upregulated Ihh expression and signaling in the growth plate, however, to their surprise, they also found that the periarticular reserve chondrocyte region was smaller, but their proliferation rate was increased. Since there is normally no PPR expressed in reserve chondrocytes, and at the same time, Ptc1 expression was increased in that region in these mice, the authors concluded that the increased Ihh expression and signaling had accelerated the differentiation of reserve chondrocytes to the faster-dividing columnar chondrocytes. Taken together, these studies demonstrate a pivotal role for Ihh and PTHrP in coordinating chondrocyte differentiation at multiple stages during endochondral bone development.

Ihh Directly Promotes Chondrocyte Proliferation

In addition to the effect of Ihh on chondrocyte differentiation, the study using $Ihh^{-/-}$ mice also found the limbs of these mice were shorter than wild type mice, due to a decrease in chondrocyte proliferation [St.-Jacques et al., 1999]. Based on the observation that both Ptc1 and Gli were expressed in proliferative chondrocytes adjacent to the pre-hypertrophic cells, the authors proposed a direct role for Ihh in regulation of chondrocyte proliferation. This hypothesis has gained further support from the study comparing phenotypes of $Ihh^{-/-}PTHrP^{-/-}$ double knock out mice with $PTHrP^{-/-}$ mice [Karp et al., 2000]. $PTHrP^{-/-}$ mice had slightly shorter limbs than wild type mice and the proportion of cells undergoing division in the proliferative chondrocyte zone was also smaller. However, double knock out mice had even shorter limbs than those of the $PTHrP^{-/-}$ mice, supporting the idea that Ihh regulates chondrocyte proliferation independent of PTHrP. Furthermore, overexpression of a constitutively active PPR was unable to correct the growth defects of the $Ihh^{-/-}$ mice. The slightly decreased chondrocyte proliferation rate in $PTHrP^{-/-}$ mice suggests that PTHrP may also have the ability to promote proliferation or this may only be a secondary effect of PTHrP delaying chondrocyte hypertrophic differentiation.

The mechanism by which Ihh promotes chondrocyte proliferation has been linked to cell-cycle regulators. In mice with chondrocyte-specific knock out of either Ihh or Smo there is a decrease in chondrocyte proliferation that is accompanied by reduced expression of cyclin D1 [Long et al., 2001]. Cyclin D1 promotes cell-cycle progression through G_1/S phase transition and since cyclin D1 is expressed at low levels in slowly dividing reserve chondrocytes, but at high levels in rapidly dividing columnar chondrocytes [Yang et al., 2003] it was suggested that cyclin D1 mediates Ihh-dependent

proliferative effects. This is supported by studies in *Drosophila* where Hh signaling directly induces cyclin D transcription through Ci [Duman-Scheel et al., 2002]. Similarly, Shh induces cyclin D expression in order to sustain cell cycle progression in mammalian neuronal precursor cells [Kenney and Rowitch, 2000]. It will be interesting to determine if Ihh can also increase cyclin D transcription in chondrocytes through Gli.

Ihh Regulates Perichondrial Development and Angiogenesis

Recent studies exploring new functions of Ihh in endochondral bone development identified Ihh as a regulator of perichondrial differentiation and development. Initially it was observed that Ihh has a direct role in promoting osteoblast differentiation in the perichondrium, as there was no cortical bone development in $Ihh^{-/-}$ mice [St.-Jacques et al., 1999; Long et al., 2004]. It was subsequently found that $Ihh^{-/-}$ mice had a thinner perichondrium, suggesting that Ihh may regulate cell differentiation in the perichondrium, and therefore cortical bone defects may result from the lack of proper development of the perichondium [Colnot et al., 2005].

The effect of Ihh on early perichondrial development also affects vascular invasion, which occurs at a later stage of endochondral bone development. In $Ihh^{-/-}$ mice, endothelial cells had the ability to invade the partially mineralized cartilaginous matrix, but they were only briefly sustained [Colnot et al., 2005]. The same phenomenon was observed when bone rudiments isolated from these mice were transplanted under the renal capsule of wild type mice. Endothelial cells from the knock out mice initially invaded the rudiments but again disappeared shortly after and were replaced by endothelial cells originated from the wild type mice. This study suggested that it was the lack of Ihh itself, rather than the cartilage environment that caused the disappearance endothelial cells in $Ihh^{-/-}$ animals. Ihh, Ptc1, and Gli1 are all expressed in the perichondrium during early chondrogenic development, and endothelial cells normally develop from cells adjacent to that area [Colnot et al., 2005]. It can be concluded that Ihh signaling during the early developmental stage affects subsequent endothelial cell development and retention in the cartilage matrix.

REGULATION OF IHH SIGNALING IN THE GROWTH PLATE

Heparan sulfate proteoglycans (HSPG) are cell surface or secreted extracellular molecules that regulate the activities and distribution of various signaling molecules, including Hh proteins. HSPG consist of a core protein to which heparan sulfate glycosaminoglycan chains are attached. Based on the core protein structures, they are divided into different families including glypicans, syndecans, and the secreted perlecans. They can facilitate long-range diffusion of signaling proteins, present them to target cells, or restrict the activity range of these molecules [Lin, 2004].

Ihh proteins, despite their lipid modifications, have been demonstrated to migrate over a long distance [Gritli-Linde et al., 2001]. Studies in *Drosophila* provided evidence that HSPG may be involved in the long-range Hh diffusion. *Drosophila* Tout-velu (*Ttv*) mutants have abnormal Hh signaling [Bellaiche et al., 1998]. *Ttv* is related to the human exostasin (*EXT*) gene family of glycosyltransferases that elongate the heparan sulfate (HS) chains in HSPG, and hence are essential for the synthesis and function of HSPG. Ttv facilitates lipid-modified Hh protein movement, and Hh-secreting cells can only activate neighboring cells in the *Ttv* mutant [Bellaiche et al., 1998], indicating that HSPG facilitates the long-range Hh diffusion in *Drosophila*.

The role of HSPG in Ihh migration and signaling in mammalian species appears to be more complex than in *Drosophila*. Mutations in *EXT1* and *2* are associated with hereditary multiple exostoses in humans [Cook et al., 1993]. Affected individuals have reduced skeletal size and multiple chondrosarcomas as well as osteosarcomas, indicating that EXTs are involved in bone development. Transgenic mice with reduced levels of Ext1 have been generated [Koziel et al., 2004]. As expected, these mice had low levels of HSPG, however, they had elevated, rather than diminished, PTHrP expression in the periarticular perichondrium and delays in hypertrophic chondrocyte differentiation with larger zones of proliferative chondrocytes. Similar results were also obtained independently using $Ext1^{+/-}$ heterozygous mice [Hilton et al., 2005]. Since these phenotypes are similar to those of mice over-expressing *Shh* in their growth plates, it was concluded that HSPG

actually restricts Ihh migration in embryonic endochondral bone formation.

Seemingly contrasting results have been shown in studies of another HSPG, syndecan 3. This protein is expressed specifically by proliferative chondrocytes, raising the possibility that it may have a role in Ihh effects on this group of cells. Indeed, overexpression of syndecan 3 in embryonic chicken chondrocytes caused increased chondrocyte proliferation, and slowed down chondrocyte hypertrophy, all of which resulted from increased Ihh signaling [Shimo et al., 2004]. Recent studies performed in mouse embryos also found syndecan 3 expressed exclusively by proliferative chondrocytes in growth plates [Pacifici et al., 2005]. Syndecan 3 may not regulate the range of Ihh, but rather help present Ihh molecules specifically to proliferative chondrocytes, mediating the Ihh proliferative effect. To explain the difference in the effects of Ext1 and syndecan 3, it has been suggested that syndecan linkage to both HS and chrondroitin sulfate (CS) are less restrictive to Hh movement than other HSPG that are linked to HS chains exclusively [Gritli-Linde et al., 2001]. These studies in vertebrates point to the importance of HSPGs in fine-tuning of Hh activities with different HSPGs restraining the range of Ihh or facilitating its proliferative effect.

IHH REGULATION OF PTHRP: DIRECT AND INDIRECT REGULATION (Fig. 3)

While studies in $Ihh^{-/-}$ animals have identified Ihh as the major regulator of PTHrP synthesis in the developing limb, it is not clear how this is mediated (Fig. 3). In normal animals, most PTHrP synthesis is localized to cells in the periarticular perichondrium [Vortkamp et al., 1996], however, Ptc, the receptor for Ihh is detected at highest levels in perichondrial cells adjacent to the site of Ihh production. It is noteworthy that Ihh was recently proposed to be able to directly induce PTHrP expression without any secondary messenger from the perichondrium if the range of Ihh signaling was increased. In transgenic mice with reduced expression of Ext1 and hence an extended Ihh signaling range, weak Ptc expression was detected at the periarticular perichondrium where PTHrP is expressed [Koziel et al., 2004]. These findings suggest that when Ihh is present in high enough concentrations, perhaps during the early stages of endochondral development, it is able to directly regulate the expression of PTHrP, however, in the growing limb, the source of Ihh may become sufficiently distant from PTHrP-producing cells that additional relays of Ihh signaling are required. For this reason it has been suggested that Ihh may regulate PTHrP production indirectly and several of the proposed mediators are discussed below.

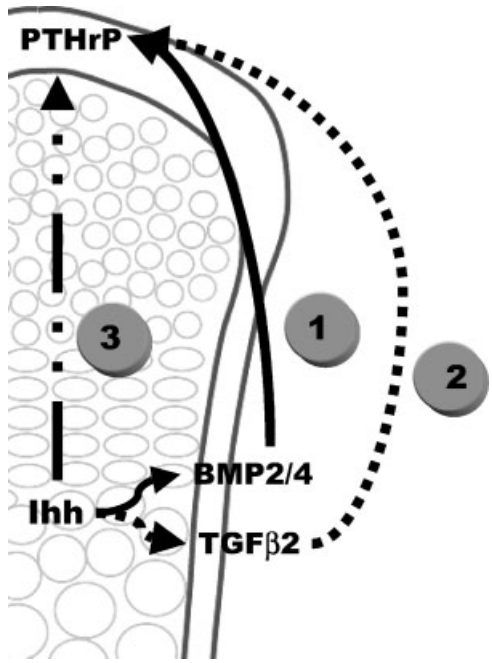

Fig. 3. Three proposed mechanisms by which Ihh upregulates PTHrP expression. (**1**) Ihh stimulates BMP2/4 expression in the perichondrium which in turn upregulates PTHrP. (**2**) Ihh stimulates TGFβ2 expression in the perichondrium which upregulates PTHrP. (**3**) Ihh directly stimulates PTHrP production at the periarticular perichondrium.

Bone Morphogenic Proteins as Mediators of Ihh Regulation

The transforming growth factor-β (TGFβ) superfamily includes TGFβs and bone morphogenic proteins (BMPs). Several of these proteins are key regulators of endochondral bone development [Moses and Serra, 1996]. Early studies demonstrated that BMPs when introduced at intramuscular sites in adult mice induce ectopic endochondral bone development [Wozney et al., 1988]. Studies of the roles of this family of proteins in endochondral bone development have been complicated by the large number of BMP proteins that are differentially expressed and regulated in the developing skeleton

[Vortkamp et al., 1996; Pathi et al., 1999]. TGFβ and BMPs bind to serine/threonine kinase receptors. The type II receptors bind the extracellular ligands causing them to heterodimerizes with type I receptors. Receptor heterodimerization results in cross-phosphorylation, which allows binding of downstream Smad proteins, and subsequently activation or inhibition of target genes [Moses and Serra, 1996]. Several groups have been investigating the roles of different BMP proteins in regulation of chondrocyte proliferation and differentiation.

Within the developing limbs, BMP2, 4, and 7 are expressed in the perichondrium, while BMP6 is found in pre-hypertrophic and hypertrophic chondrocytes [Vortkamp et al., 1996; Pathi et al., 1999]. Zou et al. [1997] first reported that overexpressing a constitutively active mutant of BMP type IA receptor induced PTHrP expression in the periarticular perichondrium. Based on these data and studies in many different species showing that BMPs are one of the major targets of Hh signaling [Methot and Basler, 1999], it was proposed that BMPs expressed in the perichondrium can act downstream of Ihh. Indeed, ectopic Ihh expression in chicken embryos induced BMPs 2 and 4 in the perichondrium [Pathi et al., 1999], and BMP activation of BMP receptors in the periarticular perichondrium can increase PTHrP expression. However, recent work has refuted the idea that BMP2 or 4 can mediate Ihh regulation of PTHrP. Using organ cultures of embryonic limb explants, Minina et al. [2001] showed that Ihh-dependent delay of hypertrophic differentiation was not overcome by Noggin protein, which has the ability to bind to BMP2 and 4, and block their signaling. Similarly, BMP2 was not able to rescue the accelerated hypertrophic differentiation resulting from cyclopamine treatment, which blocks Ihh signaling. BMP2 was also not able to induce PTHrP expression in cyclopamine-treated limb explants. These results argue against the hypothesis that BMPs act as mediators of Ihh up-regulation of PTHrP in the perichondrium.

To make the situation more complicated, several lines of evidence have shown that BMPs can also directly stimulate Ihh expression in pre-hypertrophic chondrocytes [Minina et al., 2002]. Embryonic chicken chondrocytes expressing constitutively active BMP type I receptors had increased Ihh expression, which was not blocked by PTHrP, implying that BMPs stimulation of Ihh is independent of the effect of BMPs on chondrocyte hypertrophy. Similarly, BMP2-treated embryonic limb explants also had increased Ihh expression, which was blocked by Noggin [Brunet et al., 1998].

All of these studies implicate a connection between Ihh and BMPs in the growth plate, and it is clear that both are able to induce each other's expression under some conditions. However, further work remains to clarify their interactions under normal conditions.

TGFβs as Mediators of Ihh Regulation

There is growing evidence that TGFβ proteins may act as relays between Ihh and PTHrP in the developing limb. The three TGFβ subfamily members: TGFβ1, 2, and 3 are all expressed in the perichondrium, pre-hypertrophic, and hypertrophic chondrocytes. TGFβ signaling is similar to that of BMPs, although TGFβs bind to different sets of serine/threonine kinase receptors and activate different sets of intracellular Smads [Moses and Serra, 1996].

TGFβs can regulate chondrocyte differentiation by upregulation of PTHrP [Serra et al., 1999; Pateder et al., 2001]. Using embryonic metatarsal rudiment organ cultures, TGFβ1 was shown to decrease the area of hypertrophic chondrocytes in the growth plate, accompanied by increased PTHrP expression and this effect was ablated in rudiments isolated from $PTHrP^{-/-}$ transgenic mice [Serra et al., 1999]. More recently, Alvarez et al. [2002] showed that TGFβ2 expressed in the perichondrium acts as a signal relay between Ihh and PTHrP. Again using embryonic rudiment organ cultures, Shh (as a functional analog of Ihh) induced TGFβ2 and 3 expression in the perichondrium, while TGFβ1 levels were not changed. As expected, Shh treatment caused hypertrophy delay in intact rudiments, but it had no effect on perichondrium-free rudiments. Moreover, Shh was not able to induce PTHrP expression and subsequently was not able to slow down the rate of chondrocyte differentiation in rudiments isolated from $TGF\beta2^{-/-}$embryos. These data demonstrated the importance of TGFβ2 expressed in the perichondrium in mediating Ihh regulation of PTHrP and hence chondroycte hypertrophic differentiation. However, as noted by the authors the phenotype of the $TGF\beta2^{-/-}$ mice is less severe than that of $Ihh^{-/-}$ or $PTHrP^{-/-}$ mice [St.-Jacques et al., 1999] and

therefore, TGFβ2 is likely only one of the mediators of Ihh expression of PTHrP.

REGULATION OF IHH EXPRESSION (Fig. 4)

Even though Ihh is one of the most important factors coordinating chondrocyte proliferation and differentiation surprisingly little is known about the mechanisms by which Ihh is regulated (Fig. 4). Factors that have been shown to either enhance or inhibit Ihh expression are outlined below along with what is known about the mechanisms that may mediate their effects.

Stimulators of Ihh Expression

As noted in the previous section, BMPs can regulate Ihh expression in pre-hypertrophic chondrocytes [Grimsrud et al., 2001]. While the mechanism of BMP regulation of Ihh is not known, evidence in both primary chondrocytes as well as chondrocytic cell lines suggest that both Smad proteins and the transcription factor Runx2 could be involved.

A recent study using chromatin immunoprecipitation (ChIP)-based cloning methods has shown that the 5′ flanking region of the *Ihh* gene is able to bind to Smad 4, one of the downstream messengers of BMP signaling [Seki and Hata, 2004]. Multiple putative BMP-responsive elements were identified within that region of the *Ihh* promoter. Furthermore, the same study demonstrated that BMP7 increased Ihh mRNA levels, suggesting that at least in the cell system used in this study, BMP signaling can directly upregulate Ihh mRNA, probably via Smad 4.

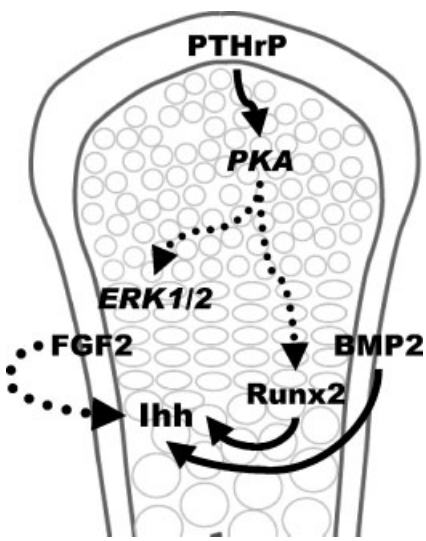

Fig. 4. Ihh is regulated by multiple local factors in the growth plate. BMP2 has been shown to increase Ihh expression, while FGF2 has been shown to decrease it. PTHrP activated protein kinase A (PKA) pathway can decrease Ihh expression by inhibiting the activity of extracellular signal-regulated kinases (ERK1/2). Alternatively, this can also be mediated by PTHrP downregulation of Runx2. (➡) positive regulation; (•••▶) negative regulation.

Runx2 (or cbfa-1) belongs to the runt transcription factor family. It was initially identified as the positive regulator of osteoblast differentiation. Transgenic mice deficient of *Runx2* ($Runx2^{-/-}$) lacked mineralized bone matrix and chondrocytes were unable to undergo hypertrophic differentiation, as indicated by the absence of Ihh and collagen type X, suggesting that Runx2 was required for both chondrocyte and osteoblast differentiation [Kim et al., 1999]. A recent study focused on regulation of Ihh demonstrated that Runx2 can directly induce Ihh transcription [Yoshida et al., 2004]. They showed that $Runx2^{-/-}$ mice had dramatically reduced Ihh expression that could be restored by overexpression of Runx2. The same authors identified seven putative Runx2 binding elements within the 5′ flanking region of *Ihh* and showed that three of these binding elements are important for Ihh transcriptional activation by Runx2. At least one study in chondrocytes has shown that BMP2 upregulated Runx2 mRNA [Takazawa et al., 2000] and many studies in both chondrocytes and osteoblasts have demonstrated cooperative regulation of gene expression by Runx2 and Smads [Leboy et al., 2001]. Therefore, BMP regulation of Ihh expression could be mediated by a combination of Smad and Runx2 transcription factors.

Retinoic acid (RA) has also been reported to increase Ihh levels in chondrocytes [Yoshida et al., 2001]. Yoshida et al. first demonstrated in primary chicken chondrocytes that RA increased Ihh transcription in a process that required de novo protein synthesis. A retinoic acid response element was found in the 5′ flanking region of *Ihh*, indicating that Ihh transcription can be stimulated by RA.

The extracellular matrix surrounding cells in the growth plate can also act as a source of stimulation for Ihh. Using the CFK2 chondrocytic cell line, we have investigated the roles of ERK1/2 and p38 MAP kinases in regulation of Ihh expression [Lai et al., 2005]. We showed that both ERK1/2 and p38 MAP kinases are positive regulators of Ihh expression and mediate the stimulation of Ihh by β1-integrins in

these cells that are being stimulated by matrix proteins secreted by the cells.

Inhibitors of Ihh Expression

Achondroplasia is the most common cause of dwarfism in humans, and it is caused by gain-of-function mutations in the fibroblast growth factor-receptor3 (FGFR3). FGFR3 is expressed in both proliferative and hypertrophic chondrocytes. FGFs bind to and activate this receptor and cause growth arrest in chondrocytes, leading to short limbs. Early studies also suggested that FGFs inhibit chondrocyte differentiation [Iwata et al., 2000], although this hypothesis has been challenged by more recent studies. Minina et al. [2002] using an organ culture from embryonic limb explants, has demonstrated that FGF2 reduced the rate of chondrocyte proliferation, as well as promoting chondrocyte hypertrophic differentiation as indicated by the shorter distance between the joint and hypertrophic chondrocytes. However, the effect of FGFs in chondrocyte hypertrophy was not observed in Ihh-overexpressing transgenic mice. In addition, they showed that FGF2 reduced Ihh expression in pre-hypertrophic chondrocytes, suggesting that FGF2 by suppressing Ihh expression promotes chondrocyte hypertrophic differentiation. This conclusion was supported by other studies showing that transgenic mice expressing FGFR3 with a gain-of-function mutation had decreased Ihh and PPR expression [Chen et al., 2001]. Taken together, these studies suggest that FGF signaling inhibits Ihh expression in order to suppress chondrocyte proliferation and promote chondrocyte hypertrophy. The mechanism by which FGF inhibits Ihh has not been established.

PTHrP stimulation of its receptors in pre-hypertrophic chondrocytes can inhibit Ihh expression. Yoshida et al. [2001] first demonstrated this in primary chicken chondrocytes showing that PTH directly down-regulated Ihh mRNA levels, independent of its effect on differentiation. Ihh regulation was mimicked by a cAMP analog and was not blocked by either cycloheximide or actinomycin D, suggesting that PTH directly inhibits Ihh gene transcription, possibly via the cAMP/PKA pathway. There are several mechanisms by which stimulation of PKA may mediate Ihh inhibition. A putative cAMP response element was identified in the 5′ flanking region of *Ihh* that could mediate PPR regulation of Ihh transcription, however this has not yet been tested. Alternatively, Runx2 may be the focus of PTHrP inhibition of Ihh. Runx2 levels can be negatively regulated by PTHrP through activation of the cAMP/PKA pathway, resulting in decreased Runx2 expression in primary embryonic chick chondrocytes [Li et al., 2004]. Since Runx2 has been shown to stimulate Ihh transcription the PKA-stimulated loss of Runx2 would be expected to decrease Ihh transcription. Our lab has shown that PTH inhibited Ihh in CFK2 cells by inhibition of ERK1/2 MAP kinase [Lai et al., 2005].

PROSPECTS

Numerous studies have clearly demonstrated the crucial role of Ihh in endochondral bone development, but surprisingly few studies have investigated the regulation of Ihh itself. We are just starting to identify some of the key components mediating Ihh regulation, and more experiments are required to elucidate the complete pathways. Of particular interest is the transcriptional regulation of *Ihh*. There has only been one report studying the promoter region of *Ihh*, which suggests an essential role of Runx2 for *Ihh* transcription. As noted above the expression of Runx2 can be stimulated by factors such as BMPs or inhibited by PTHrP. Runx2 phosphorylation can also regulate its transcriptional activity [Xiao et al., 2002] and additional factors also interact with Runx2 to regulate its transcriptional activity [Vega et al., 2004]. It is tempting to speculate that Runx2 may serve as a convergent point of multiple pathways orchestrating the transcription of *Ihh*, however, we have yet to determine if any of the factors regulating Runx2 play a role in regulation of Ihh in the growth plate.

Similarly, we are only beginning to appreciate the roles of Ihh in regulating and coordinating different aspects of endochondral bone development. With new functions still being identified, we know very little of how Ihh regulates these processes, especially at the molecular levels. The mechanisms of even well established functions such as upregulation of PTHrP remain controversial. We still do not fully understand how Ihh directly promotes chondrocyte proliferation or how Ihh affects perichondrial development and vascular invasion. These are only some of the more important issues that we need to investigate.

In conclusion, it is worth mentioning that our discussion has focused only on Ihh regulation of endochondral ossification during bone development, but similar processes particularly chondrocyte hypertrophic differentiation, occur at early stages of cartilage degeneration during osteoarthritis as well as during bone regeneration after fracture. Moreover, the role of Ihh in skeletal development is clearly not restricted to chondrocytes, it also plays a major role in regulating osteoblast differentiation. Our continued understanding of Ihh regulation, signaling and functions will have broader implication in both bone development and pathophysiology.

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