

Bone growth: Coordinating chondrocyte differentiation

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Two signalling molecules – Indian hedgehog and parathyroid hormone-related peptide – have been found to function in a negative feedback loop that is crucial for the coordinated regulation of the rate and extent of endochondral bone growth.

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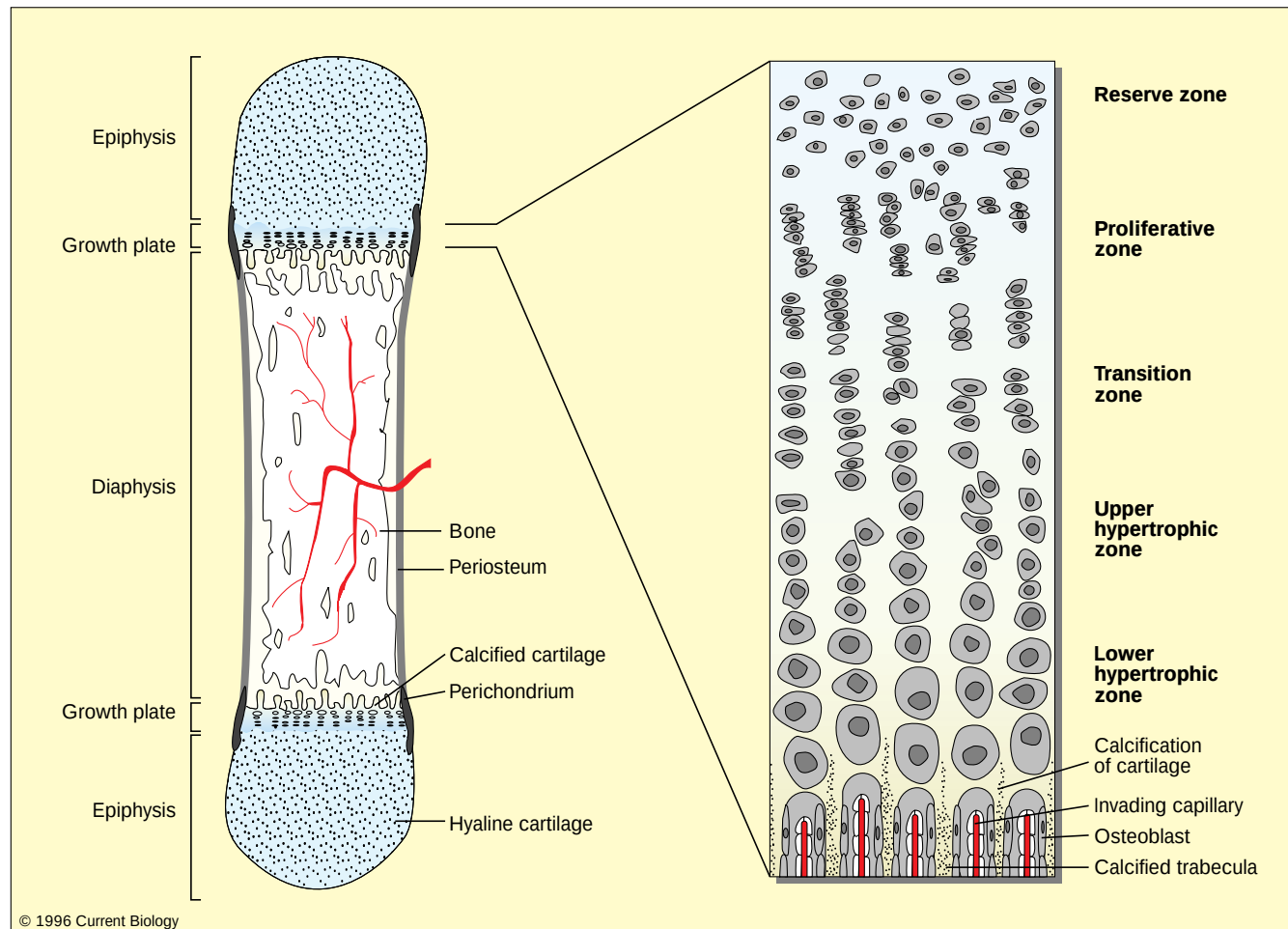
An important aspect of bone growth in higher vertebrates is endochondral ossification, the complex, multistep process whereby the cartilaginous template of the axial and appendicular skeleton is replaced by bone [1]. Early in development, this process is initiated when chondrocytes in the centre of the cartilaginous template are stimulated to proliferate and then to proceed through stages of maturation and hypertrophy. In the region of cellular hypertrophy, the surrounding matrix and vascular tissue undergo calcification. The hypertrophic chondrocytes give way to invading osteoblasts and the calcified cartilage is subsequently replaced by bone and bone marrow. This process radiates outwards with the formation of the growth plates at the ends of the long bones that separate the distal, cartilaginous epiphyses from the medial, bony diaphyses (Fig. 1). Chondrocytes within the growth plates are organized in columns, and the width of each growth plate is maintained by cellular proliferation on the epiphyseal margin, balanced by cellular loss on the diaphyseal margin. The initiation of endochondral ossification and the highly ordered progression of the chondrocytes through the growth plate must be under strict spatial and temporal control, given that they are important determinants of both the rate and extent of long-bone growth.

Our understanding of the molecular mechanisms regulating endochondral ossification has come primarily from studies of chondrocyte behaviour *in vitro* following the addition of exogenous factors and of human and murine models of skeletal dysplasia caused by naturally occurring or engineered mutations in specific genes. Two recent papers, by Lanske *et al.* [2] and Vortkamp *et al.* [3], have enhanced our understanding considerably by providing the first convincing evidence that two signalling molecules — Indian hedgehog (Ihh), synthesized by chondrocytes in the growth plate, and parathyroid hormone-related peptide (PTHrP), acting *via* receptors expressed on chondrocytes — regulate chondrocyte maturation and hypertrophy in a coordinated way.

PTHrP is a paracrine factor that is synthesized in a number of different tissues. It is structurally homologous to PTH and both bind to a common receptor. Although many functions for PTHrP have been proposed, the most direct information has come from studies of mice homozygous for a targeted mutation of the PTHrP gene [4]. These PTHrP mutant mice were relatively normal early in development, but died at birth. The mice at birth had a distinct phenotype, characterized by a domed skull, short snout and mandible, protruding tongue and disproportionately short limbs, whereas their nonskeletal organs and tissues appeared normal. On examination of the skeleton of the PTHrP mutant mice, abnormalities were present specifically in bones formed by endochondral ossification, which appeared to have undergone premature ossification.

Microscopic examination of the growth plates of bones in the PTHrP mutant mice revealed a marked reduction in the height of the zones of resting and proliferating chondrocytes, as the result of a decreased number of cell divisions, and also the chondrocytes were arranged irregularly. PTHrP thus appears to be needed for normal proliferation of chondrocytes, and the premature bone formation was presumably a consequence of the reduced proliferation and premature hypertrophy of chondrocytes within the growth plate. These findings have been corroborated by a more recent study [5] in which transgenic mice overexpressing PTHrP were found to have a form of short-limbed dwarfism, where the rate of endochondral ossification is significantly delayed as a result of persistent chondrocyte proliferation and spatially and temporally abnormal chondrocyte hypertrophy.

The transition zone between chondrocyte proliferation and hypertrophy that is affected in the PTHrP mutant mice is precisely the region of the growth plate where the PTH/PTHrP receptor is expressed, making this receptor a likely mediator of PTHrP function within the growth plate. This hypothesis has been tested by the generation of mice homozygous for a targeted deletion of the PTH/PTHrP receptor gene [2]. The PTH/PTHrP receptor mutant mice were markedly similar to the PTHrP mutant mice, both in terms of phenotype (domed skull, short snout and mandible, protruding tongue and disproportionately short limbs) and endochondral bone development (irregular and shorter columns of proliferative chondrocytes and accelerated mineralization). The similar consequences of the absence of PTHrP or the PTH/PTHrP receptor clearly suggests that this receptor mediates the effect of PTHrP on chondrocytes.

Figure 1

On the left is a diagrammatic representation of a long bone during embryonic development. The ossification centre that originated within the cartilage model of the bone has migrated outward toward the ends

of the bone and growth plates have formed. On the right, the growth plate is expanded to show the organization and differentiation of the chondrocytes.

Ihh had also been included in the repertoire of factors that might regulate chondrocyte proliferation and hypertrophy, by virtue of its expression by predominantly prehypertrophic chondrocytes in the vertebrate growth plate, and its membership of the Hedgehog family of conserved, secreted proteins that are known to provide key signals in embryonic patterning in many organisms [6,7]. In *Drosophila* development, the segment polarity gene *Hedgehog* (*Hh*) regulates embryonic segmentation and anterior posterior patterning of the imaginal discs. In higher vertebrates, *Ihh* is one of at least three Hedgehog family genes, including *Sonic hedgehog* (*Shh*) and *Desert hedgehog* (*Dhh*).

Hedgehog proteins undergo autoproteolysis generating the amino and carboxyl domains. The amino domain is involved in signalling and is highly conserved between species and between members of the Hedgehog family. In the chick, the amino domain of Ihh shares 93 % sequence

identity with that of *Shh*, and this domain can elicit the same biological responses as *Shh* in the developing chick limb bud [3]. Furthermore, the different Hedgehog proteins appear to function *via* the same signal transduction pathway. Two key genes are involved in the *Drosophila* Hedgehog transduction pathway: *patched* (*ptc*), which encodes a transmembrane protein, and *cubitus interruptus* (*ci*), which encodes a putative transcription factor. Their vertebrate homologues *Patched* (*Ptc*) and *Gli* are expressed adjacent to sites of *Shh*, *Ihh* and *Dhh* expression, and are activated in tissues responding to *Shh* and *Dhh* [8,9].

In order to discover more about the function of *Ihh*, and how its role differs from that of *Shh*, the expression pattern of *Ihh* was examined in the developing chick embryo [3]. Expression of *Ihh* was detected at two major sites in the embryo: in the endoderm of the developing midgut and lung, and in the cartilage of developing long

bones in the limbs. No expression of *Ihh* was observed early in limb development, when *Shh* is expressed, suggesting that *Ihh* acts later in limb development, during the formation of the skeletal elements.

The expression pattern of *Ihh* in the developing cartilage elements of the chick wing was examined by *in situ* hybridization and compared to the expression patterns of two collagen types — *Col-IX*, expressed by all chondrocytes, and *Col-X*, specific to hypertrophic chondrocytes (reviewed in [10]) — as well as *bone morphogenetic protein-6* (*Bmp-6*), which is expressed by prehypertrophic and hypertrophic chondrocytes [8]. At the stage of chondrocytes proliferation, *Col-IX* expression was detected in all cells, whereas *Ihh* and *Bmp-6* expression was restricted to the middle of the area of *Col-IX* expression and there was no expression of *Col-X*. During the subsequent formation of the growth plates, the *Ihh* expression domain split into two regions, each distal to and slightly overlapping the region of *Col-X* expression.

Ihh was therefore expressed in the region of transition between proliferating and hypertrophic chondrocytes, and this pattern was maintained during bone growth. To establish whether *Ihh* has a role in the transition from proliferation to hypertrophy, *Ihh* was misexpressed during cartilage formation in the chick wing using a replication-competent retroviral vector [3]. Infected cartilage elements were found to be broader and shorter than their uninfected counterparts and lacked hypertrophic cartilage. The developing bones persisted as continuous cartilaginous cores and there were no normal growth plates, no detectable expression of *Col-X* or *Bmp-6*, and bone appeared to be forming from the periosteum.

Overexpression of *Ihh* thus appears to block the differentiation pathway from proliferating to hypertrophic chondrocytes, and the absence of expression of *Bmp-6* in infected cartilage elements suggests that misexpressed *Ihh* might act before overt hypertrophic differentiation. Indeed, the misexpressed *Ihh* was found to block transcription of the endogenous *Ihh* gene, suggesting that the differentiation of proliferating chondrocytes was blocked by *Ihh* before the transition to an *Ihh*-expressing cell type [3]. Interestingly, both *Ptc* and *Gli* were found to be strongly induced in the perichondrium and not in the cartilage core after *Ihh* misexpression, implying that the negative-feedback effect of *Ihh* on cartilage was indirect and mediated by the perichondrium. Furthermore, PTHrP was found to be strongly upregulated following *Ihh* misexpression in the periarticular regions of infected cartilage elements [3].

The phenotypic consequences of *Ihh* misexpression are thus opposite in effect to what was observed for the PTHrP [4] and PTH/PTHrP receptor mutant mice [2], but are similar to those observed for the mice with the targeted

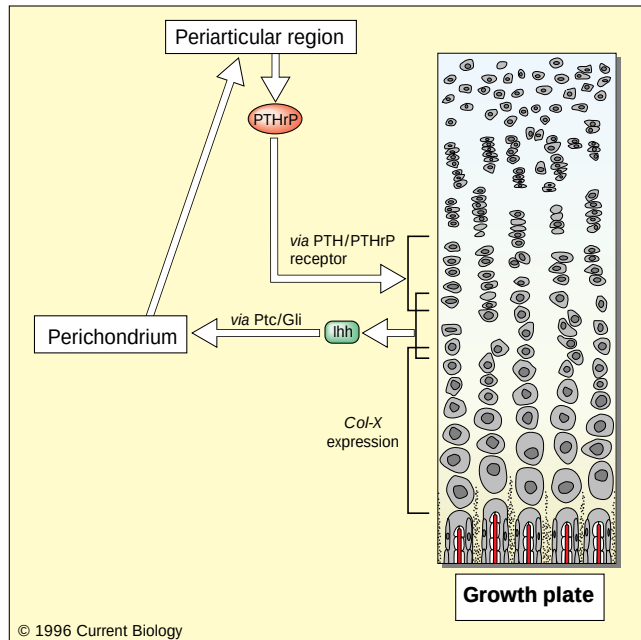
overexpression of PTHrP [5]. In addition, the *Ihh* signal appears to be mediated by the perichondrium and act on chondrocytes within the growth plate that express the PTH/PTHrP receptor and are distal to the chondrocytes expressing *Ihh*. Furthermore, the increased expression of PTHrP following *Ihh* misexpression places PTHrP downstream of *Ihh* in the regulation of cartilage differentiation. This circumstantial evidence suggested that PTHrP and the PTH/PTHrP receptor might be involved in the *Ihh* negative feedback loop.

Experiments that have provided strong support for this hypothesis were made possible through further investigations of the PTHrP and PTH/PTHrP receptor mutant mice, which have proved to be ideal experimental models. The level and pattern of *Ihh* expression were found to be unchanged in PTHrP mutant mice, demonstrating that *Ihh* is not reciprocally regulated by PTHrP. To determine whether PTHrP mediates the effect of *Ihh*, hind-limb explants from control or PTHrP mutant mice were incubated with control medium or medium containing recombinant *Shh* protein — which had been shown to be interchangeable with *Ihh* — or PTHrP [3]. Both exogenously added PTHrP and *Shh* were able to repress hypertrophic differentiation of the control wild-type mice, whereas the premature hypertrophy of the PTHrP mutant mice could be repressed by the addition of exogenous PTHrP, but not *Shh*. Thus, PTHrP is required for the response to *Shh/Ihh*.

That the PTH/PTHrP receptor mediates the action of PTHrP in the feedback loop with *Ihh* was verified by treating hind-limb explants from mutant or wild-type mice with PTHrP and *Shh* [2]. Treatment of explants from PTH/PTHrP receptor mutant mice with PTHrP or *Shh* did not mitigate the mutant phenotype, whereas the same treatment of wild-type explants led to expansion of the less differentiated chondrocyte population and suppression of differentiation into hypertrophic *Col-X*-expressing chondrocytes. The pattern of *Ihh* expression in the PTH/PTHrP mutant mice was unchanged, compared to wild-type mice, except that the chondrocytes expressing *Ihh* were nearer to the articular end of the bone in mutant embryos. From these results it was concluded that chondrocytes expressing the PTH/PTHrP receptor are the physiological target of the *Ihh*/PTHrP pathway.

On the basis of all this evidence, a model has been proposed [2,3] for the role of *Ihh* and PTHrP in the regulation of endochondral ossification (Fig. 2). In the model, *Ihh* is produced by cells that are committed to hypertrophy. *Ihh* then signals *via* *Ptc* and *Gli* in the perichondrium to increase the synthesis of perichondrial PTHrP, which acts *via* its receptor on cells in the growth plate, prior to their conversion to *Ihh*-expressing cells. Activation of the PTH/PTHrP receptor maintains the proliferative nature

Figure 2



A model for the regulation of chondrocyte differentiation [2,3]. *Ihh* is produced by chondrocytes within the growth plate that are committed to hypertrophy. *Ihh* then acts on cells in the perichondrium, signalling via a pathway that includes *Ptc* and *Gli*. The response to *Ihh* in the perichondrium results either directly or indirectly in the activation of *PTHrP* expression in the periarticular region. *PTHrP* then acts on the *PTH/PTHrP* receptor on cells in the growth plate; activation of the receptor maintains the proliferative nature of the chondrocytes.

of the chondrocytes. The level of *Ihh* expression is suggested to regulate the number of cells leaving the proliferative state. As cells progress to the hypertrophic state, they stop expressing *Ihh*, thereby attenuating the negative-feedback loop and allowing the cells to proceed to hypertrophy. This model provides a firm foundation for further studies aimed at understanding the many interacting processes required to balance the growth and ossification of developing bones.

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