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PERSPECTIVES

Hedgehog Signaling in Postnatal Bone

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Abstract

While it has been known for some time that hedgehog signaling plays a critical role in regulating skeletal development, its role in skeletal homeostasis is only beginning to be understood. New data from several groups using mouse models suggest this pathway continues to be important in controlling growth plate maturation and bone homeostasis in adult mice. Loss of hedgehog signaling in postnatal chondrocytes, either genetically or pharmacologically, leads to loss of epiphyseal growth plates with premature fusion and shortened stature. Hedgehog signaling is also important for regulating the delicate balance between osteoblast and osteoclast function in adult bone, and increased hedgehog signaling can either increase or decrease bone mass depending on the strength and spatial-temporal pattern of activation. While initial observations suggest that targeting the hedgehog pathway may be of therapeutic interest, care must be taken in choosing the most effective strategy. *IBMS BoneKEy*. 2008 July;5(7):243-252.

Introduction

Hedgehogs (Hhs) are a family of secreted proteins that regulate myriad fundamental processes during both embryonic development and adult homeostasis. Hh was first described in the pioneering and Nobel prize-winning work of Christiane Nüsslein-Volhard and Eric Weischaus as a gene important for regulating segment polarity in *Drosophila melanogaster* (1). While there is only one Hh in the fly, there are three hedgehogs in vertebrates – desert hedgehog (Dhh), sonic hedgehog (Shh) and indian hedgehog (Ihh). Vertebrate Hhs have distinct and overlapping expression patterns and regulate the formation and maintenance of multiple tissues, including bone. Recently, several groups have sought to understand the role of Hh signaling in postnatal skeletal growth and homeostasis and their work will be reviewed in this *Perspective*.

The Hedgehog Signaling Pathway

The Hh signaling pathway has been most thoroughly studied in *Drosophila* and is largely conserved, evolutionarily, from flies to vertebrates (Fig. 1). Hh is an extracellular morphogen that is subject to multiple post-translational modifications prior to the secretion of a mature, active protein. Through an autocatalytic proteolytic cleavage event, the C-terminal domain is removed and a cholesterol moiety is attached (2), followed by the addition of a palmitate molecule to the N-terminus (3). Hh-producing cells express Dispatched (Disp), a 12-transmembrane domain-containing protein that regulates Hh release. Upon secretion, Hh ligands are thought to aggregate, associate with lipoprotein particles and form gradients whose diffusion distance and signaling potency are controlled by these post-translational modifications. Hh gradients may further be regulated by heparin sulfate proteoglycans (4).

Hh signals are received and transduced by two receptors: Patched (Ptch) and Smoothened (Smo). Ptch is a 12-transmembrane domain-containing protein that binds Hh and, with the aid of several co-receptors, senses extracellular Hh levels. In the absence of Hh ligand, Ptch represses Smo function through a poorly defined mechanism (Fig. 1). Hh binding alleviates

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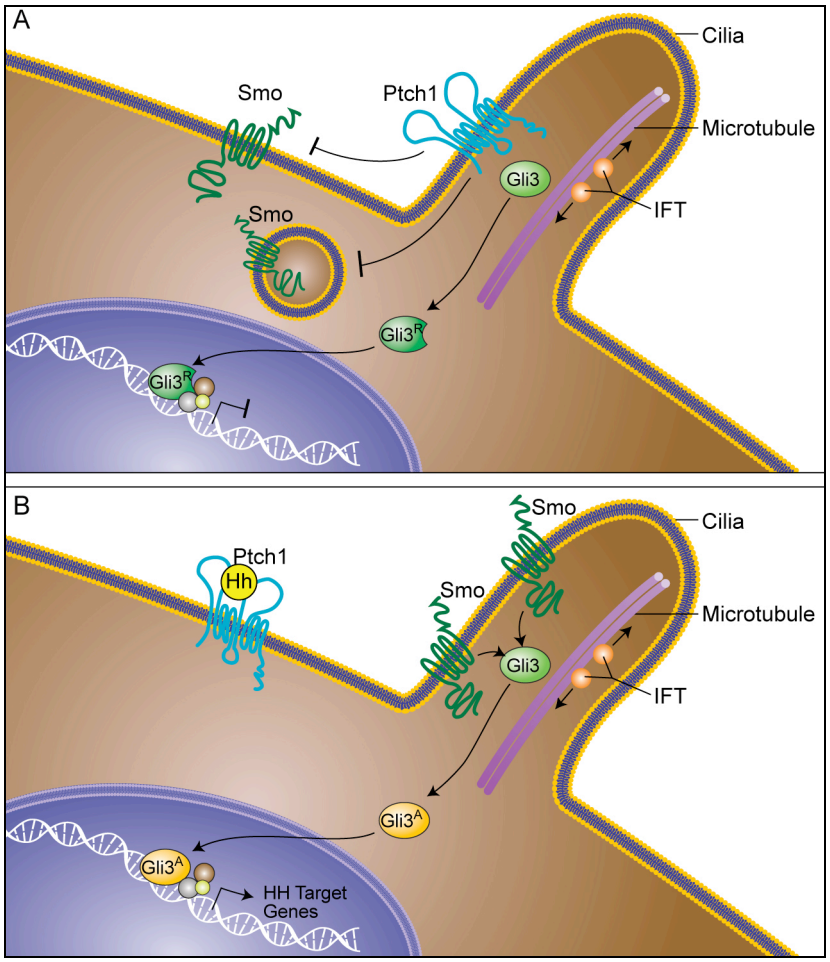

Fig. 1. The hedgehog signaling pathway. A) In the absence of hedgehog (Hh), Ptch1 inhibits Smo localization to cilia and function. Gli3 is processed to the repressor ($Gli3^R$) form that traffics to the nucleus and inhibits transcription. B) Binding of Hh to Ptch1 removes inhibition of Smo, allowing Smo to localize to the primary cilia and to stimulate conversion of Gli3 to the activator form ($Gli3^A$). $Gli3^A$ migrates to the nucleus and stimulates transcription of Hh-responsive genes. [Intraflagellar transport (IFT)]

this repression, allowing Smo to be stabilized on the cell membrane and to signal to cubitus interruptus (Ci), a zinc-finger transcription factor. This signal is propagated by a complex of cytoplasmic proteins that includes Costal 2 (Cos2), Fused (Fu) and Suppressor of Fused (Sufu), and is further regulated by a number of signal transduction molecules including protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). A major output of Hh signaling is the control of Ci processing between activator and repressor forms. The ratio of these forms is critical for transducing the correct information from the Hh gradient and generating the appropriate output. There are three mammalian homologues of Ci: Gli1, Gli2 and Gli3. In the absence of Hh signal, Gli3, whose processing most closely resembles that of Ci, is converted to an N-terminal repressor form that inhibits transcription (Fig. 1). Following Hh induction, Gli3 is processed to a full-length activator form that accumulates in the nucleus and stimulates gene expression. Gli1, which is transcriptionally induced by Hh, can activate and amplify Hh responses, but is not required for Hh signaling *in vivo* (5). Gli2 mainly acts as a transcriptional activator of

Hh signaling. A unique feature of vertebrate Hh signaling is the requirement of primary cilia, microtubule-based organelles that protrude from most mammalian cells. Gli2 and Gli3 are present in cilia under steady state conditions, while Smo traffics to (and Ptch1 leaves from) the cilia following Hh stimulation (6;7) (Fig. 1). Importantly, mice with mutations in a number of intraflagellar transport (IFT) proteins, components required for the assembly and maintenance of cilia, have phenotypes similar to those caused by loss of either *Gli2*/*Gli3* or *Shh*/*Ihh* function (8-13).

The role of Hh in bone development has been reviewed elsewhere (14) and will be covered only briefly here to place current findings into context. Ihh, the major Hh regulator in the developing skeleton, is first expressed in mouse limb cartilage condensates at E11.5 and later becomes restricted to the prehypertrophic chondrocytes in the growth plate (15). Ihh regulates bone growth by directly regulating chondrocyte proliferation and osteoblast differentiation (16;17). IHH also controls chondrocyte maturation indirectly by positively regulating expression of parathyroid hormone-related protein (PTHrP) (15;18). In *Ihh* null mice the growth plate fails to develop, PTHrP is not expressed and osteoblasts fail to differentiate (15). Postnatally, IHH immunoreactivity has been demonstrated in human growth plate chondrocytes and in rodent growth plate chondrocytes and osteoblasts (19-23). PTCH1, the endogenous receptor for IHH, is expressed on chondrocytes, osteoblasts and perichondrial/periosteal cells (15).

The Hedgehog Pathway in Human Genetics

While mutations leading to inappropriate Hh activation are often associated with tumor formation (24), the importance of this pathway in regulating the skeleton is underscored by the number of human syndromes caused by mutations in pathway components.

Depending on their position, mutations in IHH can cause either brachydactyly type A-1 (25) or acrocapitofemoral dysplasia (26). Heterozygous missense mutations between amino acids 95-154 of *IHH* lead to autosomal dominant brachydactylyl type A-1, a mild skeletal dysplasia confined mostly to shortened middle phalanges (25;27;28). Acrocapitofemoral dysplasia is an autosomal recessive disorder characterized by cone-shaped epiphyses in the hands and hips and premature epimetaphyseal fusion leading to early growth arrest and shortened limbs and stature. Mutations associated with acrocapitofemoral dysplasia are also in the amino terminus of IHH, but outside the region where brachydactyly type A1 mutations cluster (26).

EXT1 and *EXT2* encode glycosyltransferases required for heparin sulfate proteoglycan formation. Mutations in these genes lead to hereditary multiple exostoses, a skeletal disorder characterized by the presence of boney protuberances (called exostoses or osteochondromas) arising in the epiphyseal growth plates (29). One function of these genes may be to constrain the range of the Hh gradient as a mouse hypomorphic allele of *Ext1* leads to an expanded area of Hh signaling and delays chondrocyte hypertrophy (30). It is important to note that heparin sulfate proteoglycans regulate the function of multiple pathways, and skeletal malformations caused by mutations in *EXT1* and *EXT2* are molecularly complex and difficult to interpret.

Mutations in *PTCH1* cause autosomal dominant Gorlin syndrome, also known as nevoid basal cell carcinoma syndrome, due to haploinsufficiency and ectopic activation of the Hh pathway (31). Aside from developing numerous basal cell carcinomas, Gorlin syndrome patients present with numerous skeletal anomalies, including an enlarged head with frontal bossing, hypertelorism, increased stature, calcification of the falces, spine and rib anomalies, polydactyly and cystic bone lesions (31).

The importance and complexity of the Hh pathway is further illustrated by the discoveries that mutations in *GLI3* can result in three overlapping, but distinct, disorders: Pallister-Hall syndrome (PHS) (32), Greig cephalopolysyndactyly syndrome (GCPS) (33) and postaxial polydactyly type A (PAP-A) (34). Skeletal manifestations of PHS include polydactyly, syndactyly and several visceral abnormalities and are thought to be due to constitutive expression of the repressor form of GLI3. GCPS is characterized by polydactyly, syndactyly and craniofacial abnormalities including macrocephaly and hypertelorism and is thought to be caused by loss of function of GLI3 leading to haploinsufficiency. PAP-A phenotypes are mostly restricted to postaxial polydactyly.

The role of Hh signaling in regulating skeletal growth has also been highlighted in recent genome-wide studies of stature. Two groups looking at single-nucleotide polymorphisms (SNPs) associated with human height identified SNPs linked with three Hh pathway genes (*PTCH1*, *IHH* and hedgehog interacting protein (*HIP*)) (35;36). Hh was the mostly highly represented pathway in these studies, comprising >10% of all associated SNPs. These findings are compelling and consistent with recent findings in mice demonstrating a role for the Hh pathway in maintaining the growth plate (see below).

The Role of Hedgehog Signaling in Bone Homeostasis

Similar to the *Ihh* knockout mouse phenotype and to human mutations in *IHH* leading to acrocapitofemoral dysplasia, inhibition of IHH activity later in life through either genetic removal or pharmacologic blockade leads to premature loss of the growth plate and shortened stature. Using a tamoxifen-inducible cre/*lox* system, Maeda *et al*. removed Ihh from chondrocytes perinatally (37). Loss of *Ihh* in neonates led to a decrease in *Pthrp* expression, premature terminal differentiation of chondrocytes and abnormal mineralization of the growth plate and joints. The growth plate was lost from mutant mice within a week of *Ihh* removal, leading to dramatically shorter long bones. Kimura *et al*. saw similar effects using a recently developed small molecule hedgehog antagonist (HhAntag) (38). Administration of HhAntag to P10 mice caused a rapid global inhibition of Hh signaling, leading to a permanent disruption to the developing bone within 2 days. The growth plate was the most severely affected bone region as the columnar organization of the proliferating chondrocytes was disrupted, proliferation was decreased, chondrocytes entered premature hypertrophy and the growth plate fused. Following birth, IHH continues to regulate chondrocyte independently of PTHrP. Our group has reported that increased Hh signaling promotes chondrocyte hypertrophy around the second ossification center, whereas reduced Hh signaling decreases chondrocyte hypertrophy in this region (40).

All groups mention disruption of the articular surfaces, observations with significant implications for human osteoarthritis that should be studied more closely. Also, these experiments strongly suggest that IHH plays a critical role in maintaining the growth plate, an important observation given that Hh antagonists are currently being developed for therapeutic treatment of human diseases, including several pediatric tumors (39).

Similar to the *Ihh* null mouse phenotype (15), perinatal removal of chondrocyte-derived Ihh also leads to reduced osteoblast differentiation and function. Markers of both early and mature osteoblasts are reduced and a loss of trabeculation is seen, leading to decreased bone mineral density (BMD) in the metaphyseal region (37). Ihh may regulate osteoblast differentiation through the Wnt/β-catenin pathway, as this pathway is known to be critical for osteoblast formation and is downregulated in Ihh mutant mice (37;41-43). Consistent with this observation, inhibition of Hh signaling with HhAntag also negatively affects osteoblast proliferation and function, and bone formation is inhibited (38). Following 4 days of HhAntag administration, the bone collar is noticeably reduced and the number of osteoblasts is reduced by 50% (38).

Similarly, Ohba *et al*. administered cyclopamine, a Smo antagonist, to 8-week-old mice for one month and witnessed a mild decrease in BMD, with decreased trabeculation, decreased bone deposition and fewer osteoblasts (44). Interestingly, a significant increase of bone mass was observed following withdraw of HhAntag treatment (38).

Two groups recently chose to study the effects of activating the Hh pathway on bone homeostasis (44;45). As Ptch1 represses Smo function, removing it leads to ectopic Smo, and hence Hh, activation (46). Our group performed both gain- and loss-of-function experiments in bone using transgenic mouse lines to remove floxed alleles of either *Ptch1* or *Smo* from mature osteoblasts (45;47). Mice in which *Ptch1* was removed from mature osteoblasts (referred to hereafter as $Ptch1^{MOb}$) had some skeletal features reminiscent of Gorlin syndrome, including frontal bossing, large calvaria and hypertelorism. Microcomputed tomography analysis of $Ptch1^{MOb}$ mice further revealed a dramatic decrease in both cortical and trabecular bone mass. Interestingly, Ohba *et al*. looked at mice globally heterozygous for *Ptch1* (referred to hereafter as *Ptch1*(+/–)) and saw the opposite: increased bone mass. Both groups reported increased osteoblast differentiation *in vitro*, increased numbers of osteoblasts *in vivo* and accelerated bone deposition. The major difference between the groups' observations was the degree of osteoclast formation. The long bones of *Ptch1*(+/–) mice contained ~50% more osteoclasts than controls while those of $Ptch1^{MOb}$ mice contained a >3-fold increase. Both groups demonstrate, through co-culture experiments, that loss of Ptch1 specifically in osteoblasts increases both the expression of receptor activator of NF-κB (RANKL), through PTHrP, and osteoclastogenesis (44;45). *In vitro*, as *in vivo*, osteoblasts from *Ptch1*(+/–) mice confirm a more modest induction of osteoclast formation than those from $Ptch1^{MOb}$ mice (2-fold vs. 8-fold). Thus, while bone deposition was increased in both mutant mice, this was offset in $Ptch1^{MOb}$ due to accelerated bone turnover leading to osteopenia.

Loss-of-function mutant mice in which *Smo* was removed utilizing the same mature osteoblast-specific Cre (referred to hereafter as Smo^{MOb}) showed a complimentary phenotype. Smo^{MOb} mice had increased trabecular bone mineral densities and cortical thickness, diminished *Pthrp* and *Rankl* expression and fewer osteoclasts. Further experiments suggest that Hh signaling also controls *Pthrp* expression in osteoblasts, similar to its role during cartilage development. PTHrP signaling in osteoblasts mediates the effect of Hh signaling in promoting *Rankl* expression by activating protein kinase A (PKA) and its target transcription factor target cAMP responsive element-binding protein (CREB).

Summary

The Hh signaling pathway plays important roles in regulating both skeletal development and homeostasis and is a key modulator of crosstalk between mesenchymal stem cells, osteoblasts and osteoclasts (Fig. 2). The Hh pathway is more active in early osteoblasts than in mature osteoblasts and activating it in mature osteoblasts further stimulates osteoclastogenesis (45). *Rankl* expression is increased in the bones of old vs. young mice and the cultured osteoblasts from older mice are more osteoclastogenic than those from young mice (45), suggesting that Hh signaling might become re-activated in osteoblasts from older mice. In young mice the growth and differentiation activities of osteoblasts exceed that of osteoclasts and thus bone mass is increased, while in older mice this balance is reversed, resulting in net bone loss. While both *Ptch1*(+/–) and $Ptch1^{MOb}$ mice showed increased osteoblast and osteoclast formation, the skeletal outcome was markedly different: increased bone formation vs. increased bone resorption. These data suggest that partial vs. full activation of the Hh pathway may have formative vs. resorptive influences, respectively, and/or extra-osteoblastic Hh signaling may function to further enhance bone deposition or inhibit osteoclast formation. Thus, the cell type in which Hh is activated and/or the degree to which it is activated is likely to be critical for determining bone quality. It is interesting

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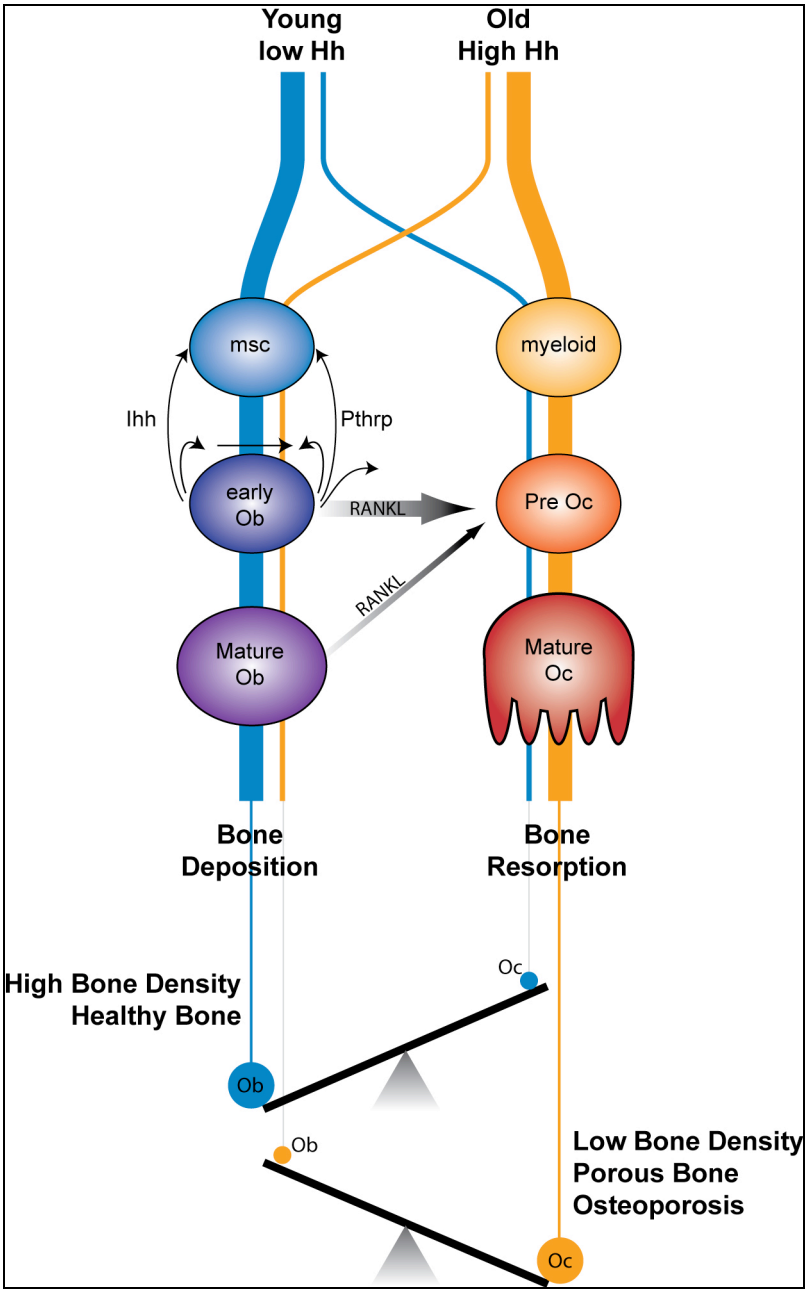

Fig. 2: Model for hedgehog signaling in postnatal bone. In young mice or mice with mild activation of Hedgehog (Hh) signaling (in blue), bone formation is favored. Hh signaling is greater in early osteoblasts than in mature osteoblasts/osteocytes. Hh stimulates both osteoblast differentiation from mesenchymal precursors and osteoclast formation from myeloid precursors (via Pthrp and Rankl). In young animals or instances of mild Hh activation, the osteoblast cascade prevails and bone formation is dominant. In old mice or mice with high activation of Hh signaling (in yellow), bone resorption is favored. Osteoblasts from either old mice or mice with highly elevated Hh activation are more osteoclastogenic than those of younger or control animals, respectively. Under these conditions the osteoclast cascade is dominant and increased bone resorption occurs.

that PTHrP, which is regulated by IHH, similarly has anabolic or catabolic effects on bone depending on dosing regimen (48) and suggests that localized regulation of PTHrP and IHH may have a dramatic impact on bone health. Pharmacologic inhibition of Hh

in the young is not recommended as it inhibits bone growth and is teratogenic. In older individuals moderate augmention of Hh signaling, or perhaps intermittent cycles of Hh activation and inhibition, may tip the balance towards increasing osteoblast function and improve bone quality.

While we are beginning to understand the role of Hh signaling in bone homeostasis, a number of important questions still remain to be answered. Given the number of physiological differences between rodents and humans, do the lessons we are learning from mouse models hold true for humans? Is Hh signaling dysregulated in osteoporosis and/or osteoarthritis? Finally, what might be the best strategy for manipulating Hh signaling to improve bone mass in aged humans?

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