Hedgehog Signaling in Chondrocytes

9

Kay Grobe

Abstract

The skeleton develops from a densely packed, avascular mesenchyme, called the skeletal blastema. Chondrogenesis from this mesenchyme requires a balance between negative and positive maturational factors during initial chondrocyte proliferation and differentiation, as well as during postnatal chondrocyte development and homeostasis. Accurate regulation of this developmental program is crucial for the ultimate size of skeletal elements, as premature or delayed maturation often results in their severe shortening. One essential group of regulators of chondrogenesis comprises members of the Hedgehog (Hh) morphogen family. Hh's act as long-range morphogens during chondrocyte development and endochondral ossification. Mutations in Hh effectors, receptors, and co-receptors, as well as in ciliary proteins that act as modulators of Hh reception, result in skeletal and craniofacial deformities. In addition to their essential roles in chondrogenesis, both Sonic Hh and Indian Hh family members serve as crucial regulators of endochondral ossification, a process in which calcified hypertrophic cartilage is resorbed and replaced by bone. Finally, dysregulated Hh signaling contributes to cartilage and bone pathologies in the adult. This chapter summarizes the current understanding of Hh production and signaling in chondrocytes in development and disease.

K. Grobe, Ph.D.
Medical Faculty, Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, Waldeyerstr. 15, 48149, Münster, Germany
e-mail: kgrobe@uni-muenster.de

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9.1 Hedgehog Functions in Chondrocyte Biology

Two distinct processes form vertebrate skeletons during development: intramembranous and endochondral ossification. Intramembranous bones are directly formed by specialized, terminally differentiated mesenchymal stem cells called osteoblasts. Osteoblasts synthesize densely cross-linked collagen and specialized proteins, including osteocalcin and osteopontin, to form the basis of the cranial vault, some facial bones, and parts of the mandible and clavicle. Endochondral ossification that forms the rest of the skeleton during development, in contrast, relies on the replacement of a cartilaginous template with bone (Yoshida et al. 2004). As a first step in this process, the cartilaginous template (or cartilage anlage) generated from mesenchymal progenitors expresses collagens I, III, and V as a result of mesenchymal cell condensation and chondroprogenitor cell differentiation, called chondrogenesis (Goldring et al. 2006). Fibroblast growth factor 8 and Sonic hedgehog (Shh) (Kmita et al. 2005) are two essential modulators of cell proliferation within this cartilage template (Hall and Miyake 2000), and bone morphogenetic protein (BMP; most BMPs are transforming growth factor beta family members) signaling contributes to the formation of precartilaginous condensations and subsequent chondrocyte differentiation (Yoon et al. 2005). The BMP antagonist, Noggin, further permits precartilage cell differentiation into chondrocytes (Yoon and Lyons 2004; Pizette and Niswander 2000). This process is marked by the expression of cartilage-specific collagens II, IX, and XI. The proliferation of these cells requires Indian hedgehog (Ihh) signaling in parallel with BMP function or BMP signaling acting as a modulator of Ihh function (Minina et al. 2001; Vortkamp 2001). Finally, cells undergo terminal differentiation, or chondrocyte hypertrophy, and apoptosis in the intervening interzone. During chondrocyte hypertrophy, there is a notable increase in cell size, up to 20-fold of its initial resting size. The hypertrophic zone is further characterized by the expression of collagen X and alkaline phosphatase and the subsequent calcification of the matrix (St-Jacques et al. 1999). This process involves matrix remodeling by matrix metalloprotease (MMP)-9, MMP-13, and MMP-14 and vascularization mediated by vascular endothelial growth factor (VEGF) activity. In this process, the hypertrophic cartilage is finally replaced by bone, except for resting chondrocytes embedded in a dense extracellular matrix (ECM) lacking blood vessels, nerves, or lymphatics at the ends of (opposing) bones (called articular cartilage).

A similar sequence of chondrocyte proliferation and differentiation occurs in the postnatal growth plate, leading to rapid growth of the skeleton (Onyekwelu et al. 2009). At birth, the articular cartilage of many joints in humans and mice is still indistinguishable from the epiphyseal growth plate. Soon after birth, however, a secondary ossification center appears within the epiphyseal cartilage, dividing it into the future metaphyseal growth plate proximally and the articular surface distally (for further details, see Chap. 4). Ihh that is still produced in the metaphyseal growth plate directly and indirectly induces parathyroid hormone-related peptide (PTHrP) expression in periarticular resting zone chondrocytes (Karaplis et al. 1994; Lanske et al. 1996; Vortkamp et al. 1996; Chung et al. 2001; Kronenberg 2006) (Fig. 9.1). PTHrP, in turn, induces continued proliferation and inhibits the

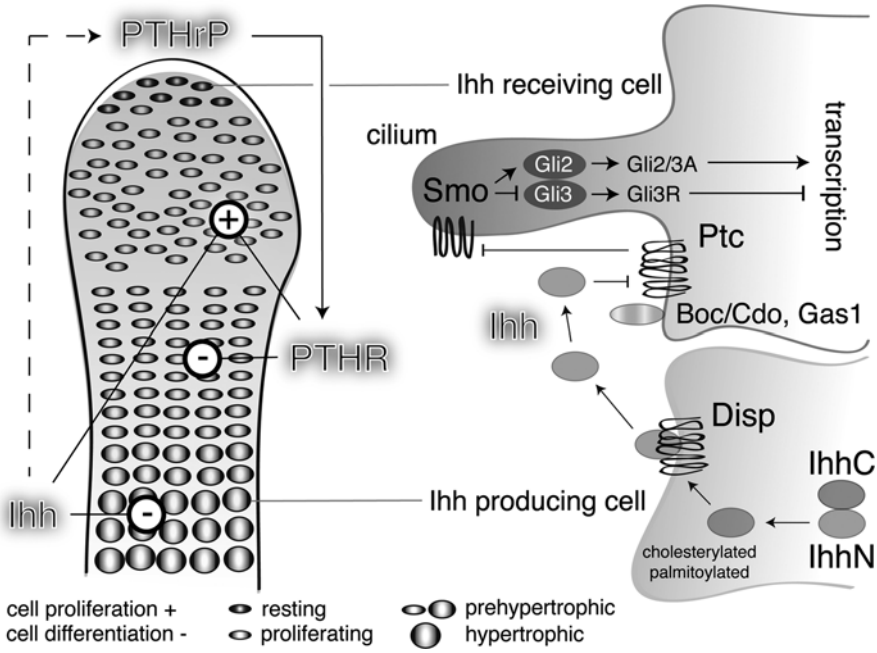

Fig. 9.1 Overview of bone development and Hh signaling. *Left*: Ihh and PTHrP/PTHR regulate chondrocyte proliferation and differentiation during endochondral bone formation. PTHrP is synthesized by resting chondrocytes and perichondrial cells. Secreted PTHrP diffuses toward the prehypertrophic zone, where it binds to and activates its receptor, PTHR. PTHR activity maintains chondrocyte proliferation (+) and delays chondrocyte differentiation (−) into prehypertrophic and hypertrophic chondrocytes. After chondrocytes stop proliferating at the transition from a proliferating into a hypertrophic phenotype, they start to synthesize Ihh, which indirectly increases the synthesis of PTHrP. Ihh and PTHrP thus participate in a negative feedback loop that regulates the proliferation rate of growth plate chondrocytes. Besides increasing PTHrP synthesis, Ihh also stimulates the proliferation of chondrocytes and directly inhibits their terminal differentiation. *PTHrP* parathyroid hormone-related protein, *Ihh* Indian Hedgehog, *PTHR* parathyroid hormone receptor. *Right*: The Ihh ligand in producing cells undergoes a series of autoprocessing/lipidation reactions that result in its secretion in multimeric, dual-lipidated form and its firm tethering to the cell surface. All Hh family members are then released from the cell surface via the activity of the 12-span transmembrane protein Dispatched (*Disp*). Ihh binding to the receptor Patched (*Ptc*) on the receiving cell releases the 7-pass transmembrane protein Smo from constitutive inhibition, allowing for Smoothened (*Smo*) translocation to the primary cilium. This activates glioblastoma (Gli)2/Gli3 transcription factors (Gli2/3A) and inhibits the generation of Gli3 repressors (Gli3R). In the presence of Hh, the co-receptors Boc, Cdo, and Gas1 assist in the release of Smo from Ptc inhibition and thereby contribute to Hh pathway activity

progression to maturation in proliferating and prehypertrophic chondrocytes. This process, in turn, maintains the length of chondrocyte columns and thus the architecture of the epiphyseal growth plate. In addition, Ihh acts independently of PTHrP on the periarticular chondrocytes and regulates the differentiation of columnar chondrocytes within the proliferative zone (Kobayashi et al. 2005). In postnatal joints,

PTHrP and Ihh remain expressed in a zone-specific manner (Onyekwelu et al. 2009), potentially regulating mineralization by chondrocytes at the osteochondral interface of the immature joint (Jiang et al. 2008). Here, Ihh expression is particularly strong in chondrocytes at the articular surface, indicating a role in resisting chondrocyte hypertrophy, mineralization, and/or ossification.

Local chondrocytes in the postnatal skeleton are also influenced through endocrine hormones (particularly thyroid hormone and estrogen). Ihh signaling is further known to induce BMP-4, a mitogenic factor, in a PTHrP-independent manner. Notably, this induction depends on mechanical stimulation (Wu et al. 2001). Cyclic mechanical stress induces Ihh expression in chondrocytes; gadolinium, an inhibitor of stretch-activated channels, inhibits Ihh induction. This suggests that the Ihh gene is mechanoresponsive because of the involvement of primary cilia in Hh signaling (discussed in more detail below). Finally, functional interactions between Ihh and the Wingless/Wnt pathways regulate cartilage growth plate control and joint segmentation (Spater et al. 2006). Loss of activity of Wnt9a (a secreted signaling molecule) transiently downregulates Ihh expression and reduces Ihh signaling activity in prehypertrophic chondrocytes; in vivo chromatin immunoprecipitation revealed a direct interaction between the β-catenin/lymphoid enhancer-binding factor 1 (constituting part of the Wnt receptor/signal transduction complex) and the Ihh promoter. Another report demonstrated that Ihh, Wnt5b, and Wnt11 control chondrogenesis in parallel pathways (Church et al. 2002) and that Ihh can cause parallel inhibition of Lrp (Wnt co-receptor) and Sfrp (Wnt antagonist) in chondrocytes (Choi et al. 2012). The conclusion that Wnt5a signaling in the prehypertrophic zone of the cartilage growth plate may be increased, however, is not supported because of the unchanged Wnt5a levels in Ihh mutant mice in vivo (St-Jacques et al. 1999; Long et al. 2001).

9.2 Hedgehog Morphogen Production and Reception

Vertebrates produce three structurally and functionally closely related Hh's (Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh). Of these, the function of Shh has been best characterized (reviewed by McMahon et al. 2003), including its role in the development of the head process and in the development of limbs: limb budding, anterior/posterior patterning of the limb skeleton, and specification of vertebrate digit identities (Capdevila and Johnson 2000). Shh expressed in the forebrain also mediates the development of the mid- and upper face, the frontonasal process, and the maxillary processes (Byrnes et al. 2009). Dysregulation of the Shh pathway therefore results in a wide and complex array of skeletal and craniofacial defects, including syndactyly, holoprosencephaly, hypotelorism, cleft palate, and cyclopia (Belloni et al. 1996; Chiang et al. 1996). Desert Hh is expressed in peripheral nerves and in male gonads (Bitgood and McMahon 1995), suggesting a functional role restricted to these tissues. The third vertebrate Hh family member is Ihh. Both Ihh and Shh functions have been studied in cartilage and bone patterning throughout the axial, appendicular, and facial skeletons (Hammerschmidt et al. 1997; Capdevila

and Johnson 2000; Chai and Maxson 2006), as well as in calvarial ossification and suture morphogenesis (Pan et al. 2013). Ihh is mainly produced by post-mitotic prehypertrophic chondrocytes adjacent to the proliferative zone that express the parathyroid hormone (PTH)/PTHrP receptor (PTHR) and stimulate the proliferation of chondrocytes at the growth plate and later in development. Ihh further regulates chondrocyte hypertrophic and osteoblast differentiation, either directly or via PTHrP (Nakamura et al. 1997; Mak et al. 2008; Vortkamp et al. 1996) (Fig. 9.1). In the latter system, a negative feedback loop between Ihh and PTHrP regulates the rate of chondrocyte differentiation: Ihh produced by prehypertrophic chondrocytes induces PRTrP expression, which prevents further differentiation of chondrocytes expressing PTHR. Ihh knockout mice show appositional chondrocyte differentiation and loss of PTHrP and either die during mid-gestation because of yolk sac defects or die at birth because of rib cage deformities and respiratory failure (Byrd et al. 2002). Chondrocyte-specific (*Col2α1Cre;Ihhd/Ihhd*) mice also die at birth, showing delayed chondrocyte hypertrophy, reduced calvarial bone size and ossification, abnormal mineralization of axial and appendicular bones, and widened cranial sutures (Razzaque et al. 2005). These findings demonstrate that chondrocyte-derived Ihh not only is responsible for the regulation of the endochondral skeleton by regulating both chondrocyte proliferation and differentiation, but it is also essential for osteoblast differentiation. Ihh expression in chondrocytes depends on the runt-related transcription factors (Runx)2 and Runx3 (Yoshida et al. 2004). Runx2−/− mice die after birth and completely lack bone formation due to absence of osteoblast differentiation and delayed chondrocyte maturation (Komori et al. 1997; Otto et al. 1997), and Runx3−/− mice show mildly reduced chondrocyte maturation. Runx2/3 compound mutant mice completely lack Ihh expression (Yoshida et al. 2004).

All Hh homologs undergo the same three-step conserved maturation pathway in producing cells (Fig. 9.1). Production of the active Hh protein begins with autocleavage of a HhNC precursor protein into a N-terminal (HhN) signaling domain and the HhC autoprocessing domain. This cleavage reaction is linked to the covalent attachment of a cholesterol moiety to the carbonyl of the C-terminal HhN glycine residue (Porter et al. 1996a, b; Cohen 2003). In a second step, Hh acyltransferase attaches a palmitoyl group to the NH_2-terminal cysteine of the Hh signaling domain (Pepinsky et al. 1998). The dually lipidated molecule constitutes the active morphogen (Jacob and Lum 2007; Taylor et al. 2001). Upon secretion to the cell surface, lipidated Hh's multimerize prior to their release (Dierker et al. 2009a) and transport to cells expressing the Hh receptor Patched (Ptc) (Panakova et al. 2005; Zeng et al. 2001). The paradoxical situation is that a membrane-tethered molecule serves as a long-range morphogen; this requires specific mechanisms for its release and transport. The 12-pass transmembrane protein Dispatched (Disp) is essential for the release of lipid-modified Hh's (Burke et al. 1999; Caspary et al. 2002; Kawakami et al. 2002). Disp is therefore critical for full signaling within the chondrocyte target field in developing bones and consequently for the establishment of a normal skeletal growth plate (Tsiairis and McMahon 2008). The exact mechanism of Disp-dependent release of lipidated Hh, however, is not yet resolved. Other suggested players in Hh transport include Hh micelle formation by unknown mechanisms

(Zeng et al. 2001), Hh transport together with lipoprotein particles (Panakova et al. 2005; Eugster et al. 2007), Hh transport on filopodia (called cytonemes) (Bischoff et al. 2013; Roy et al. 2011), Hh association with the soluble glycoprotein Scube2 (Creanga et al. 2012; Hollway et al. 2006; Johnson et al. 2012; Kawakami et al. 2005; Tukachinsky et al. 2012; Woods and Talbot 2005), or simple diffusion of solubilized Hh after its proteolytic processing from the cell surface (called shedding) (Dierker et al. 2009b; Ohlig et al. 2011; Ohlig et al. 2012). Notably, cytonemes have not been reported on chondrocytes, making this transport mechanism unlikely. Moreover, the very dense extracellular matrix (ECM) of the developing skeleton makes most of these suggested mechanisms—in particular Hh transport on filopodia and transport via large lipoprotein particles or exosomes—hard to envision. It has been firmly established, however, that Hh long-range function depends on the expression of heparan sulfate proteoglycans (HSPGs). Again, the underlying mechanism of HSPG-mediated Hh transport is not clearly defined, but it is assumed that these versatile molecules somehow aid Hh transport by "facilitated diffusion" or Hh stabilization against degradation (Lin 2004; Muller et al. 2013).

In contrast to the components that act to release Hh, Hh signaling components in receiving cells have been studied in more detail (Cohen 2003; Robbins et al. 2012; Ingham and McMahon 2001). Hh proteins induce signaling on receiving cells by direct binding to the Hh receptor Ptc, a 12-pass transmembrane protein (Fuse et al. 1999). The amount of Hh available for Ptc binding is regulated by other Hh-binding proteins, such as Hh-interacting protein (Chuang and McMahon 1999) and growth arrest-specific protein 1 (Gas1) (Evangelista et al. 2006; Lee et al. 2001). Furthermore, the Interference Hh protein family (Ihog in *Drosophila* and CDO and BOC in humans) (Wilson and Chuang 2006; Kavran et al. 2010) and HSPGs (Bornemann et al. 2004; Beckett et al. 2008) modulate Hh signaling. Hh binding to Ptc (together with Hh binding to Boc/Cdo and Gas1 (Allen et al. 2011)) induces internalization of the receptor/ligand complex and relieves Ptc-mediated catalytic inhibition of the seven-pass transmembrane protein Smoothened (Smo) (Taipale et al. 2002). Active Smo then transduces the Hh signal to the cytoplasm, resulting in processing and activation of the glioblastoma (Gli) family of transcription factors (Gli1–Gli3) (Hatsell and Frost 2007). Gli1, in contrast to Gli2 and Gli3, lacks an amino-terminal repressor domain and thus represents a constitutive activator of the Hh pathway (Hatsell and Frost 2007; Hynes et al. 1997; Karlstrom et al. 2003). Yet, in mouse development, Gli1 is not essential since Gli1−/− mutants survive from birth to adulthood with a normal phenotype (Park et al. 2000). In contrast, Gli2 and Gli3 are required for mouse development and carry an N-terminal repressor domain in addition to the C-terminal activator domain and thus can act as both activators and repressors (Sasaki et al. 1999; Ruiz i Altaba 1999). Their bifunctionality is determined by the presence of Hh signaling: the absolute concentration of Hh ligands specifically induces defined Gli transcription factor activation, resulting in Hh concentration-dependent activation of target genes (Ogden et al. 2004; Harfe et al. 2004). In the absence of Hh signaling, Gli3 is complexed with suppressor of fused (SuFu), which leads to Gli3 phosphorylation by several kinases. This targets Gli3 for proteolytic processing into the truncated repressor form (Gli3R) that locates

to the nucleus and inhibits transcription of target genes (Persson et al. 2002). Upon Smo activation in the presence of Hh signaling, however, SuFu is sequestered away from Gli3, proteolytic processing is inhibited, and full-length Gli3 (Gli3A) induces target gene transcription. Gli2 can likewise be converted into a repressor by proteolytic processing in the absence of Hh signaling and is activated by high levels of Hh. However, Gli2 C-terminal processing is less effective than that of Gli3. Therefore, Gli2 mostly remains transcriptionally active even at low levels of Hh signaling in vivo (Fuccillo et al. 2006).

Gli-regulated Hh-dependent target genes include Wnts, BMP, and the Ptc receptor itself. Importantly, upregulation of Ptc in response to Hh signaling constitutes a negative feedback loop by increasing the relative amount of free Ptc on the cell surface, which in turn inhibits Smo activity and signaling. In addition, Ptc directly reduces Hh levels in the ECM by ligand internalization upon binding (Jeong and McMahon 2005). In chondrocytes, another direct consequence of Ihh signaling is the Wnt5A-dependent, yet PTHrP-independent, degradation of Nkx3.2 proteins that are normally expressed in chondrocyte precursor cells and in early-stage chondrocytes (Choi et al. 2012). In these cells, Nkx3.2 proteins enhance chondrocyte differentiation and survival while inhibiting chondrocyte hypertrophy and apoptosis.

9.3 Primary Cilia in Hedgehog Perception

Primary cilia are involved in the regulation of Hh signal transduction, although the precise mechanisms are not fully elucidated (Tran et al. 2008). A primary cilium consists of a singular, immotile organelle, which is present on most cells, including chondrocytes, during interphase (Scherft and Daems 1967). Currently, it is thought that primary cilia provide an environment that facilitates interactions between different Hh pathway components (Ruat et al. 2012), such as Ptc, Smo, and Gli proteins that require ciliary transport in order to activate Hh-dependent gene expression (Keady et al. 2012). Upon Hh binding to Ptc and following Smo stimulation, Smo is translocated to the cilium and subsequently interacts with Gli's, leading to their activation. Gli's then move down the cilium to enter the nucleus and transduce the Hh signal (Huangfu and Anderson 2005, 2006).

For these reasons, the targeted inactivation of intraflagellar transport (IFT) proteins, such as components of the kinesin-like protein motor complex and retrograde dynein motors, has been found to affect Hh signal transduction (Ruat et al. 2012). Conditional inactivation of the Kif3a subunit of the kinesin-2 intraflagellar transport motor in mesenchymal skeletal progenitor cells, for example, resulted in severe patterning defects in the craniofacial area, the formation of a split sternum, and the development of polydactyly, deformities reminiscent of those described in mice with deregulated Hh signaling (Koyama et al. 2007).

In Kif3a-deficient mesenchymal tissues, both the repressor function of Gli3 and the activation of the Shh transcriptional targets Ptc and Gli1 are compromised (Kolpakova-Hart et al. 2007). This is consistent with the finding that Gli signaling

depends on Kif3a function (Haycraft et al. 2005; Huangfu and Anderson 2005). Kif7, which plays a role in the turnover of Sufu and the exclusion of Sufu-Gli complexes from the primary cilium, regulates the activity of Gli transcription factors through both Sufu-dependent and Sufu-independent mechanisms (Hsu et al. 2011). Mutations in the IFT protein DynC2H1 cause short-rib polydactyly syndrome, a lethal autosomal recessive condition that features cerebral and skeletal abnormalities, including appendicular malformations (Dagoneau et al. 2009; El Hokayem et al. 2012; Merrill et al. 2009). Finally, partial mutation of intraflagellar transport 80 (IFT 80) in humans causes Jeune asphyxiating thoracic dystrophy and short-rib polydactyly syndrome. IFT80 is mainly expressed in growth plate chondrocytes, and IFT80 knockdown impairs chondrocyte cilia formation and chondrogenic differentiation in mouse bone marrow-derived stromal cells by downregulating Hh signaling (Wang et al. 2013). In addition to merely acting as a location for Hh signaling regulation, the primary cilium also plays a role in mechanosensitive Hh signaling in adult articular chondrocytes (Thompson et al. 2014). Mechanical strain promotes Ihh expression and Hh pathway activation; cilia disassembly due to high-magnitude strain prevents this process. However, in comparisons of Ihh- and Kif3a-deficient mice, chondrogenesis differs significantly, indicating that Ihh actions may not solely depend on molecular association of Hh reception components with cilia (Koyama et al. 2007; Kolpakova-Hart et al. 2007).

9.4 Hedgehog Functions in Chondrocyte Pathobiology

Osteoarthritis (OA) is linked to the irreversible degeneration of articular cartilage in adult joints, often due to initial injury. In this disease, articular cartilage chondrocytes undergo phenotypic and gene expression changes that resemble their end-stage differentiation in the growth plate during skeletal development, suggesting that Ihh and the Ihh/PTHrP axis continue to play a role in OA. Indeed, Ihh expression is upregulated in human OA cartilage, and this upregulation correlates with OA progression and changes in chondrocyte morphology. Consistent with this observation, transgenic mice with induced Ihh expression exhibit increased chondrocyte hypertrophy and cartilage damage resembling human OA. In these mice, higher levels of Hh signaling in chondrocytes caused a more severe osteoarthritic phenotype (Lin et al. 2009). Two other genetic studies in mice confirmed this finding, showing that conditional deletion of Ihh in chondrocytes attenuates OA progression (Zhou et al. 2014a, b). Only mild OA changes were observed in Ihh-deficient mice, while control mice displayed significantly more cartilage damage. OA markers such as collagen X and MMP-13 were decreased in Ihh-deficient mice, and the activity of cathepsins and MMPs in knee joints of animals with deletion of Ihh was decreased. Consistent with this finding, PTHrP inhibits mineralization in articular cartilage that is associated with OA (Terkeltaub et al. 1998), and histone deacetylase four was suggested to have chondroprotective properties by inhibiting the Ihh transcription factor Runx2 (Cao et al. 2014). Therefore, the PTHrP/Ihh axis continues to participate in the maintenance of articular cartilage, and dysregulation of this system likely contributes to the pathogenesis of OA.

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