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REVIEW

The Hedgehog signalling pathway in bone formation

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The Hedgehog (Hh) signalling pathway plays many important roles in development, homeostasis and tumorigenesis. The critical function of Hh signalling in bone formation has been identified in the past two decades. Here, we review the evolutionarily conserved Hh signalling mechanisms with an emphasis on the functions of the Hh signalling pathway in bone development, homeostasis and diseases. In the early stages of embryonic limb development, Sonic Hedgehog (Shh) acts as a major morphogen in patterning the limb buds. Indian Hedgehog (Ihh) has an essential function in endochondral ossification and induces osteoblast differentiation in the perichondrium. Hh signalling is also involved intramembrane ossification. Interactions between Hh and Wnt signalling regulate cartilage development, endochondral bone formation and synovial joint formation. Hh also plays an important role in bone homeostasis, and reducing Hh signalling protects against age-related bone loss. Disruption of Hh signalling regulation leads to multiple bone diseases, such as progressive osseous heteroplasia. Therefore, understanding the signalling mechanisms and functions of Hh signalling in bone development, homeostasis and diseases will provide important insights into bone disease prevention, diagnoses and therapeutics.

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HEDGEHOG SIGNAL TRANSDUCTION

The Hedgehog (Hh) signalling pathway is evolutionarily conserved and plays critical roles in development and homeostasis. Disruption of Hh signalling leads to tumour formation and other diseases.[1–5] The Hh gene was first identified in *Drosophila melanogaster* and was named according to the phenotypes of the *Drosophila* mutant embryo, which displayed disorganised bristles that resembled Hh spines.[6]

Hh protein maturation

The Hh protein undergoes several steps of processing, including proteolytic cleavage, glycosylation and lipid modification. Newly synthesised Hh protein is first translocated to the endoplasmic reticulum (ER) and is autoproteolytically cleaved into C-terminal Hh (Hh-C) and N-terminal Hh (Hh-N). During this process, Hh-N is dually modified by the addition of palmitate and cholesterol to the N- and C-termini, respectively.[7–8] Although Hh-C is critical for catalysing the autoproteolytic cleavage, it is rapidly degraded thereafter in the proteasome.[9] The dually lipid-modified Hh-N is secreted and associates with the lipid bilayer of the plasma membrane. With the assistance of Dispatched (Disp), a transmembrane transporter-like protein, Hh protein is released from Hh-producing cells and exerts its effect up to a distance of 300 μm in the vertebrate limb bud.[10–12]

Hh receptor complex and regulation of the Hh pathway in Drosophila

Patched (Ptc) is a transmembrane protein and was the first identified Hh-binding protein that inhibits Hh signalling in the absence of Hh protein binding.[13–15] Ptc suppresses the activity of the seven transmembrane protein Smoothened (Smo) by triggering its degradation and/or intracellular vesicle trafficking[16–18] (Figure 1a). The intracellular Hh signalling components include an important complex composed of Costal 2 (Cos2), Fused (Fu), Suppressor of Fused (SuFu) and Cubitus interruptus (Ci). When the Hh ligand binds to Ptc, the inhibition of Smo by Ptc is relieved.[19–21] Smo is then stabilised on the plasma membrane and activated. Phosphorylation of Smo by casein kinase 1 (CK1), casein kinase 2 (CK2), G protein-coupled receptor (Gpcr) Kinase 2 (Gprk2) and protein kinase A (PKA) plays a critical role in Smo activation. The cytoplasmic tail of Smo can recruit Cos2, a kinesin-like protein. Cos2 is critical because it associates with Ci, the transcriptional effector of Hh signalling; regulates Ci's processing; and anchors Ci in the cytoplasm.[22–24] In the absence of Hh, Ci is phosphorylated by PKA, CK1 and glycogen synthase kinase-3β (Gsk3β) in the Cos2 complex, partially degraded by a Slimb (Slmb)-regulated ubiquitination pathway and the proteasome to be converted into its repressor form CiR (Figure 1a). However, in the presence of Hh ligand, Cos2 is recruited to Smo, released from Ci and phosphorylated by Fu. In this case, Ci is activated and not cleaved. The full-length Ci

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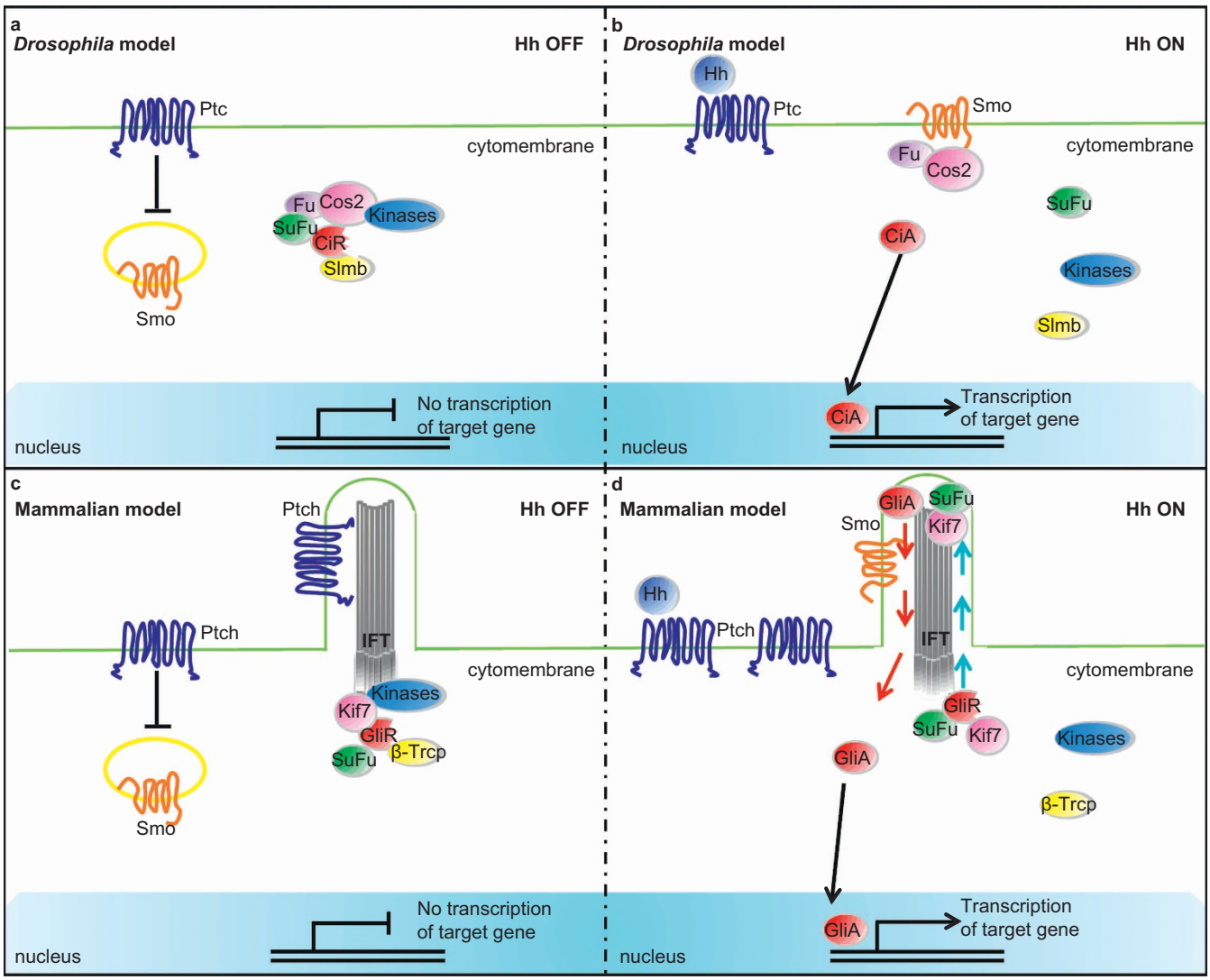

Figure 1 The Hedgehog signalling pathway in *Drosophila* and vertebrates. (**a**) In *Drosophila*, Ptc inhibits Smo activity by suppressing the membrane stabilisation of Smo in the absence of Hh ligand. The Cos2, Ci, Fu and Sufu complex recruits kinases, such as PKA, CK1, Gsk3β, and promotes the cleavage of full-length Ci to become its repressor form (CiR) in a Slmb-dependent manner. Hh signalling transduction is blocked. (**b**) In *Drosophila*, Smo inhibition by Ptc is removed in the presence of Hh ligand. Smo is relocated to the plasma membrane and activated by several kinases, such as CK1, CK2, Gprk2 and PKA. The Fu-Cos2 complex is recruited to Smo and releases Ci. The released Ci is not cleaved and remains in its active form (CiA). CiA translocates into the nucleus and activates Hh downstream gene expression. (**c**) In vertebrates, Ptch1 is located in the cilium, whereas Smo is kept outside of cilium in the absence of Hh ligands. Gli is phosphorylated by kinases, such as PKA, CK1 and Gsk3β, which promote the processing of the repressor form (GliR) in a β-Trcp-dependent manner. Hh signalling is blocked. (**d**) In vertebrates, when Hh ligands bind to Ptch1, Smo inhibition is relieved. Ptch1 exits from the cilium, whereas Smo is translocated to cilium. The repressor form of the Gli (GliR), Sufu and Kif7 complex travels from the base of the cilium to the top via intraflagellar transport (IFT). Kif7 blocks the function of Sufu at the top of the cilium. Gli is not processed and is maintained its active form (GliA). Activated Gli travels from the top of the cilium to the cytoplasm via IFT and translocates to the nucleus to transcript target genes thereby activating Hh signalling.

then translocates into the nucleus and activates the transcription of Hh target genes[25–26] (Figure 1b).

Hh pathway in mammals

In mammals, the Hh signalling pathway is mostly conserved. However, this pathway requires more components and, most importantly, the mammalian Hh signal transduction requires a distinct cell organelle, the cilium.[27–29] Approximately 800 cilium proteins have been found in mammals.[30–31] The relationship between cilium and Hh signalling is best understood among cilium-transduced signalling pathways.[29,32] The Hh homologous proteins in mammals are Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh).[33–35] In the absence of Hh ligands, protein Ptc homologues 1 and 2 (Ptch1 and Ptch2), the mammalian homologues of Ptc, are enriched on and around cilium.[36] Smo, the mammalian counterpart of *Drosophila* Smo, is kept outside of the cilium and inactive (Figure 1c). When Hh ligands bind Ptch1, Ptch1 exits the cilium and Smo inhibition is relieved and accumulates in the primary cilium.[36–38] In addition to Ptch1, in the presence of Hh protein, Ptch2 can form a receptor complex by oncogenes (CDO), brother and CDO (BOC) and growth arrest specific (GAS) on the cell surface, which is critical in Hh signal trans-

duction and gradient establishment.[39] Suppressor of fused homologue (Sufu) and kinesin family member 7 (Kif7), the mammalian homologues of *Drosophila* SuFu and Cos2,[40] are both located in the primary cilium and act as dynamic regulators of Hh signal transduction.[41–46] Kif7 plays a dual role, as it does in *Drosophila*. Glioma-associated oncogene family members (Gli1/2/3) are the mammalian homologues of Ci. In the absence of Hh protein, Kif7 and PKA convert Gli3, and to a lesser extent Gli2, to their repressor forms via proteolytic processing at the base of the cilium and Hh signal transduction is blocked[43–44] (Figure 1c). In the presence of Hh ligands, Smo is relocated to the cilium and is phosphorylated, which abolishes PKA function and allows for the movement of Kif7 and the Gli2/3-SuFu complex from the base of the cilium to the top.[47–48] In this process, Kif7 plays a role in facilitating protein trafficking and disassociating binding between Gli and Sufu,[48–49] which leads to Gli2/3 activation as active forms that relocate to the nucleus to activate the expression of Hh target genes, such as *Ptch1*, *Gli1* and *Hhip1*.[50–51] Ptch1 itself is a transcriptional target of Hh signalling (Figure 1d); therefore, it forms a negative feedback system in Hh signalling.[40] Interestingly, Stk36, the mammalian homologue of Fused, becomes a component that is required in ciliogenesis rather than a key regulator of Hh signalling in *Drosophila*.[52] In some situations, Hh signalling is not transduced through Gli, which is referred to as non-canonical Hh signalling.[39,53–54] However, more studies are necessary to understand how the cilium controls specific roles of each component in Hh signalling and trafficking in the cilium.

HH SIGNALLING AND BONE DEVELOPMENT

There are two processes of bone development in vertebrates: intramembranous ossification in most craniofacial bones and endochondral ossification in other parts of the skeletal system. During endochondral ossification, mesenchymal progenitor cells condense and differentiate into chondrocytes first. These chondrocytes go through a tightly regulated developmental programme of proliferation, prehypertrophy, hypertrophy and apoptosis and are eventually replaced by osteoblasts in the ossification centre.[55] Perichondral cells, the cell sheath surrounding chondrocytes, differentiate into osteoblasts and migrate to the ossification centre together with blood vessels to form the trabecular bones. In contrast, during intramembranous ossification, condensed mesenchymal progenitor cells differentiate into osteoblasts and form bone directly.

In general, Shh acts at early stages of development to regulate patterning and growth.[56] Ihh acts later in the process of endochondral bone formation in limb development.[57]

Hh and limb patterning

During early limb development, *Shh* is expressed in the posterior margin of the limb bud called the zone of polarising activity (ZPA).[58] The limb bud is the primordium of the future limb. Shh acts as an important morphogen that patterns the anteroposterior axis of the future limb.[59] Ectopic *Shh* expression in the anterior limb bud leads to mirror image digit duplication.[60] Gli3 is expressed in an anterior to posterior gradient[61] and can be downregulated by Hand2, which is expressed in the posterior region, where Shh signalling is high.[61] Hh mutations can lead to either abnormal digit number or changes in digit identity.[62–64] Towers *et al.* recently reported that Shh expression in the chick limb bud is regulated by an intrinsic cell cycle clock.[65] According to that model, the periodic expression of Shh regulated by cell cycle progression can be reset, whereas anterior-posterior position cannot be changed.[65]

Hh and endochondral ossification

Ihh is expressed in the prehypertrophic chondrocytes adjacent to the proliferation zone. Parathyroid hormone-related peptide (PTHrP), which resembles parathyroid hormone (PTH), is expressed by periarticular cells during endochondral ossification.[66] Ihh and PThrP form a feedback loop to regulate growth plate and long bone development.[18,67] Ihh stimulates PTHrP expression in periarticular chondrocytes.[57,68–69] PTHrP diffuses into the growth plate region to promote the proliferation of chondrocytes. Chondrocytes exit the cell cycle and undergo hypertrophy when PTHrP expression drops below a critical level.[70] In the absence of Ihh, the expression of PTHrP is reduced,[67] leading to an accelerated hypertrophy of chondrocytes.[69,71–72] Loss of endochondral ossification due to abolished osteoblast differentiation is also observed in the absence of Ihh signalling.[67,73] Bone morphogenetic proteins (BMPs), fibroblastic growth factors (FGFs) and mechanical loading may have effects on this feedback system.[57]

Jemtland *et al.* demonstrated that Ihh is also expressed in osteoblasts postnatally in rats and mice.[74–75] Gli2 and Gli3 are essential for mouse skeletal development, whereas Gli1 is not critical in this process.[2,76–77] Gli1 acts synergistically with Gli2 and Gli3 in osteogenesis.[78] Removing Gli3 rescues the Ihh null mice phenotype in chondrocyte hypertrophy,[58,79–80] whereas removing Gli3 and activating Gli2 at the same time restore Runx 2 expression in the absence of Ihh.[80–81] Therefore, the function of Ihh is mainly to suppress the Gli3 repressor function in regulating chondrocyte hypertrophy during cartilage development, whereas osteoblast differentiation requires Hh signalling to activate Gli2 activator activity. Our lab has shown that Wnt/β-catenin signalling is required downstream of Hh signalling in regulating osteoblast differentiation during endochondral bone development by establishing double mutant mice.[82] In this model, Hh signalling is activated and Wnt/β-catenin is inactivated by generating a chondrocyte-specific deletion of Ptch1 and β-catenin in mouse. By examining the expression of the Ihh signalling target genes *Hip* and *Gli* and the Ihh downstream gene *Pthrp*, strong activation was found in *Ptch1* single mutant and *Ptch1*, β-catenin double mutant mice. This study demonstrates that β-catenin is not required in Hh signalling. Bone formation was blocked in β-catenin single mutants and *Ptch1*, β-catenin double mutant mice, which indicates that Wnt/β-catenin is required for bone formation and acts downstream of Hh signalling.[64,82] Recent studies have also demonstrated that Ihh induces collagen type X (Col10α1) expression through the direct regulation of the Col10 α1 promoter via Gli1 or Gli2 or indirect interaction with the Runx2/smad pathway.[83] The Bmp pathway also interacts with the Hh pathway during endochondral ossification. It has been reported that BMP signalling acts downstream of the Hh pathway and regulates osteoblast cell differentiation from perichondrial cells.[84]

Hh and intramembranous ossification

In intramembranous or dermal bone formation, Hh signalling is also required. $Ihh^{-/-}$ mice are observed to have smaller calvaria with reduced expanse, thickness and mineralization and widened sutures.[67,85–86] Lenton *et al.* further studied the mechanism underlying this phenotype. They found that *Ihh*, which is expressed at the osteogenic edge of growing cranial bones, promotes bone formation by regulating osteogenic differentiation rather than proliferation. Loss of *Ihh* leads to a reduction of *BMP2/4*, suggesting that *BMP2/4* is downstream of *Ihh* in the intramembrane ossification process.[86]

Rice *et al.* and Jenkins *et al.* found that deletion of Gli3 or RAB23, the repressors of Hh signalling, results in an increased ossification of the calvarial bone, causing craniosynostosis.[87–88] In zebrafish, Huycke *et al.* demonstrated that in the craniofacial bone opercle (OP), a dermal bone model, Hh signalling mediates early morphogenesis in intramembranous bone formation and that Ihh is expressed in active osteoblasts along the growing OP in the second phase of morphogenesis.[89] In addition, Shh expression is found in cranial bones.[90] Taken together, these findings show that Hh serves as a positive regulator in intramembranous ossification.

Hh and joint formation

During synovial joint formation, ectopic Hh signalling in the cartilage leads to joint fusion. Overexpression of *Shh* in the cartilage caused joint fusion.[91] Mak *et al.* further demonstrated that Ihh and Wnt signalling interact with each other in regulating synovial joint formation in developing cartilage by upregulating Ihh signalling and inactivating Wnt signalling in a mouse model simultaneously.[82,91–92] Ihh in the joint must be kept at a low level to prevent joint fusion.

HH SIGNALLING AND BONE HOMEOSTASIS

Bone remodelling is a lifelong process that regulates bone mass and quality. During this process, osteoblasts of mesenchymal stem cell origin are responsible for bone formation, whereas osteoclasts derived from monocytes are responsible for bone resorption. These two cell types maintain the balance of bone formation and resorption during bone homeostasis through a coupling and feedback mechanism. Osteoblasts secret the receptor activator of NFkB ligand (RANKL) and Osteoprotegerin (OPG). The former binds to the receptor activator of NFkB (RANK) on monocytes to stimulate osteoclast differentiation in the presence of monocyte colony stimulating factor (M-CSF). The latter is a decoy receptor of Rankl and blocks osteoclast induction by competing with Rankl to bind Rank.

Ihh is expressed in growth plate chondrocytes in postnatal humans and rodents as well as osteoblasts in postnatal human and mice.[68,75,93] The growth plate is composed of chondrocytes undergoing constant mitosis at the end of each long bone and elongates the long bones by pushing the old chondrocytes into the middle shaft. The chondrocytes in growth plates exhibit increased apoptosis under the control of oestrogen levels in puberty.[94] Increased bone mass is observed in Ptch1-deficient mice and patients. An *in vitro* study showed that Ptch1-deficient osteoblast precursor cells differentiate into osteoblasts at an accelerated rate as a result of an enhanced response to runt-related transcription factor 2 (Runx2) and reducing the generation of Gli3 repressor.[95] Consistent with this result, Gli1-haploinsufficient mice exhibit reduced bone mass with impaired osteoblast differentiation and increased osteoclastogenesis.[96] Furthermore, Kingston *et al.* found that Hh signalling plays an important role in mature osteoblasts. Activated Hh signalling in mature osteoblasts in adult mice leads to fragile long bones with significantly reduced bone density. The authors demonstrated that the reduced bone mass is due to enhanced bone resorption by osteoclasts. They further showed, at the cellular and molecular levels that increased Hh signalling in mature osteoblasts promoted RANKL expression by upregulating PTHrP expression. PTHrP then acts through PKA and its target transcription factor CREB to regulate Rankl expression. Thus, Hh signalling indirectly induces osteoclast maturation and promotes bone resorption.[97] Another *in vitro* study showed that Shh upregulates Osx expression in osteoblast cell lines,[98] increases osteoblast production and indirectly upregulates osteoclast activity, resulting in more bone resorption and less bone strength.[97,99] Furthermore, Ihh and Ptch1 are upregulated during the initial stage of fracture repair[100–102] and Shh is activated in osteoblasts at the remodelling site of fractures to regulate osteoblast proliferation, differentiation and osteoclast formation as well as vascularization.[103–105] Tissue engineering experiments using implanted Ihh/MSCs/scaffold complexes showed increased bone repair ability.[106] The latest study by Benjamin's group showed that $Gli1^+$ cells are located in the perivascular region and act as mesenchymal stem cells to contribute to organ fibrosis, especially after kidney, lung, liver and heart injury.[107] Zhao *et al.* demonstrated that $Gli1^+$ cells in mouse incisors expressed MSCs surface markers and contributed to dentin tubules after tooth injury.[108] However, whether stem cells mediate the effects of Hh signalling in bone repair remains unknown. Taken together, these findings shown that the Hh signalling pathway plays a critical role in bone homeostasis.

HH SIGNALLING AND BONE DISEASE

Hh signalling is a key factor in regulating bone development, homeostasis and repair. Abnormalities of the Hh pathway result in various bone diseases. Gao *et al.* showed that mutations in *Ihh* resulting human brachydactyly type A1 (BDA1), which is characterised by shortened or missing middle phalanges.[109–110] One of the mutations was knocked into the mouse Ihh gene to establish the DBA1 mouse model. It was found that a BDA1 mutation (E95K) affects the range and capacity of Ihh signalling via its interaction with Hh co-receptors, such as Ptch1 and Hip1.[111] A *GLI3* mutation is reported to cause Grieg cephalopolysyndactyly, Pallister–Hall syndrome or postaxial polydactyly type 3, which are characterised by various bone anomalies, such as syndactyly, polydactyly, abnormality of limbs or skull or hip dislocations.[112–114] VACTERL Syndrome, which involves vertebral defects and limb abnormalities, is also related to *Gli2* or *Gli3* mutations.[115] *Shh* mutations are observed in patients with Smith–Lemli–Opitz syndrome (SLOS), which is characterised by syndactyly and polydactyly in bone abnormalities[116] *PTCH1* mutations cause Gorlin syndrome, which is also known as nevoid basal cell carcinoma syndrome, in which bone abnormalities include polydactyly, rib anomalies, ectopic ossification, spina bifida and others.[117–119] Genome-wide association studies (GWAS) have shown that Hh signalling is an important regular of human height.[120] GWAS have also revealed Shh as an important regulating gene for polydactyly.[121] Jean *et al.* found that Hh signalling is upregulated in patients with progressive osseous heteroplasia (POH).[92] POH was previously found to be caused by a null mutation of *GNAS*, which encodes $G\alpha_s$.[122–125] $G\alpha_s$ transduces signals from G protein-coupled receptors (GPCRs). The main symptom of POH is progressive ankylosis and growth retardation caused by ectopic ossification from mesenchymal progenitor cells.[122–123] In *Prrx-1-cre*, $Gnas^{fl/-}$, *Prrx-1-cre* and $Gnas^{fl/fl}$ mice, Jean *et al.* studied the underlying mechanism of POH. The authors demonstrated that Hh signalling activation is sufficient and necessary to cause heterotopic ossification and that $G\alpha_s$ inhibits Hh signalling through cAMP and PKA.[92] In normal soft tissues, Hh signalling must be rigorously suppressed by $G\alpha_s$ to prevent bone formation. Xuelian He *et al.* also indicated that $G\alpha_s$ inhibits Hh signalling to prevent medulloblastoma,[126] which shows that understanding the mechanisms underlying bone diseases has a broad impact in other fields such as brain tumour formation. Tiet and Alman identified that disruption of the Ihh-PTHrP feedback loop and upregulating Hh signalling results in cartilaginous neoplasms such as enchondromas and osteochondromas during childhood.[127] In addition, Hh signalling has been reported to play a role in promoting osteoblast differentiation[128–132] and

proliferation[89] and to inhibit adipocyte differentiation,[133] which implies that Hh signalling can regulate bone density and might become a target for the treatment of patients with osteoporosis. Given the multiple important roles of Hh signalling in bone development and homeostasis, it is not surprising that disruption of Hh signalling causes many bone diseases.

SUMMARY

The Hh signalling pathway is critical for embryonic bone development as well as bone remodelling throughout postnatal life. Disruption of Hh signalling causes severe bone diseases. Enhancing Hh signalling in bone fracture patients may improve the bone repair process. Applying Hh inhibitors may have a promising effect in treating POH. In addition, due to the genetic relationship between Hh and Wnt/β-catenin signalling, maintaining the appropriate level of Hh and Wnt/β-catenin signalling is critical in bone formation. Extreme expression of Hh and Wnt/β-catenin signalling results in either insufficient bone formation in skeleton-like osteoporosis or ectopic ossification in soft tissues. Furthermore, given that Hh is downregulated in postnatal bones,[134] it may be associated with age-related bone diseases. Therefore, a better understanding of the functional mechanisms of Hh signalling in bone might have an important clinical impact.

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