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Function and regulation of primary cilia and intraflagellar transport proteins in the skeleton

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Abstract

Primary cilia are microtubule-based organelles that project from the cell surface to enable transduction of various developmental signaling pathways. The process of intraflagellar transport (IFT) is crucial for the building and maintenance of primary cilia. Ciliary dysfunction has been found in a range of disorders called ciliopathies, some of which display severe skeletal dysplasias. In recent years, interest has grown in uncovering the function of primary cilia/IFT proteins in bone development, mechanotransduction, and cellular regulation. We summarize recent advances in understanding the function of cilia and IFT proteins in the regulation of cell differentiation in osteoblasts, osteocytes, chondrocytes, and mesenchymal stem cells (MSCs). We also discuss the mechanosensory function of cilia and IFT proteins in bone cells, cilia orientation, and other functions of cilia in chondrocytes.

Keywords

primary cilia; IFT proteins; osteoblasts; chondrocytes; osteocytes; MSCs; bone; cilium

Introduction

Primary cilia are small microtubule-based (MT) organelles that project from the cell surface. They function as antennae to sense a diverse range of extracellular signals, such as growth factors and hormones, through various surface receptors that are localized on the ciliary membrane, and transmit signals into the nucleus. Cilia are thus important for embryonic and postnatal development of many vital organs.^{1–3} It is now widely believed that cilia play important roles in proliferation, differentiation, and functions of many types of cells.^{4–8} Defects of cilia and/or cilia-related proteins are found in wide range of human diseases,

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Conflicts of interest

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including polycystic kidneys, hydrocephalus, and vision and hearing loss. Ciliary defects also result in severe skeletal abnormalities, suggesting the indispensable role of cilia in bone development.^{3, 9–15}

The healthy vertebrate skeleton is composed of cartilage, bone, and other connective tissues that are all derived from the embryonic mesenchyme.^{16–18} Skeletal formation and homeostasis are strictly controlled by systemic and local regulators such as WNT, Hedgehog (HH),¹⁹ Notch,²⁰ bone morphogenetic protein 2 (BMP2),²¹ and thyroid hormones.^{22, 23} More than 300 genetic mutations are found in the abnormal development and growth of the skeleton. These genetic mutations often lead to growth failure and bony deformities that severely affect quality of life and general health, and sometimes lead to premature death.^{16, 17, 24, 25, 26} Recently, the role of the cilia has become a topic of intense interest in the field of skeletal development.²⁷

The first evidence of primary cilia in the skeleton is the discovery of cilia on chondrocytes about 40 years ago.²⁸ Since then, a growing number of publications have reported the essential roles of cilia in almost every aspect of chondrocyte biology, including endocytosis, osmotic response, and apoptosis.^{29–31} Some studies revealed that cilia on cartilage display specific orientations that can be altered in chondrocyte-related diseases.^{32–38} Other studies have shown that cilia-related proteins play important roles in chondrocyte differentiation by regulating the HH signaling pathway.^{33, 39–42} Further studies suggested that a matrix–cilium–Golgi continuum exists in chondrocyte to transduce biomechanical signals from the extracellular matrix (ECM) to the cell.^{32, 43, 44} Cilia are also present in primary osteoblasts and osteocytes, pre-osteoblasts (MC3T3-E1 line), and osteocyte-like cells (MLO-Y4 line).^{45–48} Recently, the fine ultrastructure and spatial orientation of the osteocyte centrosome were observed by transmission electron microscopy (TEM) in tibial cortical bone.⁴⁹ TEM revealed that the cilia on osteocytes are mainly oriented perpendicular to the long axis of tibia bone.⁴⁹ Additionally, cilia and cilia-related proteins are largely involved in osteoblast differentiation and mechanical stimulation–induced osteogenesis. Loss of these proteins or cilia leads to bone defects and impaired osteoblast differentiation and function.^{50–55} Recent studies reported that primary cilia are also present on mesenchymal stem cells (MSCs), the common progenitor of osteoblasts and chondrocytes.⁵⁶ Cilia on MSCs are believed to direct differentiation and transduce mechanical signaling.^{56, 57} Unlike the majority of vertebrate cells, hematopoietic lineage cells lack primary cilia.⁵⁸ Although osteoclasts are derived from hematopoietic stem cells,^{45, 59} whether primary cilia are absent in osteoclasts remains unknown. However, some studies have shown that cilia on osteoblasts/osteocytes indirectly regulate osteoclast formation by altering the ratio of osteoprotegerin (OPG) to receptor activator of nuclear factor kappa B ligand (RANKL) in the bone microenvironment.⁶⁰

In this review, we discuss current understanding of the structure of cilia and cilia-related proteins. We further summarize recent studies of cilia and cilia-related proteins in the regulation of osteogenesis, chondrogenesis, and mechanotransduction of osteoblasts, chondrocytes, and MSCs. We also include other functions of cilia in chondrocytes, including endocytosis, osmotic responses, and apoptosis.

Cilia structure and cilia-related proteins

While primary cilia were first described one century ago, cilia received little research attention until the associations between intraflagellar transport (IFT) and human polycystic kidney disease (PKD), and later between IFT and HH signaling pathways, were discovered.^{61–64} Scientists are realizing more and more that primary cilia, which are present on the surface of the majority of cells in vertebrates, have profound influences on tissue development and homeostasis.^{65–69}

Cilia are specialized cellular compartments or organelles. When the cells are in G₀ stage, a primary cilium is generated from basal body, which is an organelle formed from a centriole.^{70, 71} The basal body provides a nine-fold symmetric template to build the nine-fold symmetry axonemal structure (the axoneme, nine peripheral MT doublets).⁷² The basal body also regulates protein entry into the cilium and the position of the cilium, which is correlated with cell polarity.^{71, 73} The axoneme serves as the backbone of the cilium (Fig. 1). Although the mechanisms relating to the formation and regulation of cilia are unclear, studies suggest that cilia formation and function universally rely on IFT, a specialized transport process mediated by a large multiprotein complex.^{74–76} IFT drives macromolecules from the base to the tip of the cilium (anterograde transport) and from the tip of the cilium back to the cell body (retrograde transport).⁷⁷ The large multiprotein complex is composed of the IFT complex, the BBSome, IFT motors, and associated cargo (Fig. 1). Heterotrimeric kinesin-II and cytoplasmic dynein-2 are IFT motors that carry out anterograde and retrograde transport, respectively.^{78, 79} In vertebrates, two kinesin-II complexes have been found, namely kinesin-2 family protein A (KIF3A)/KIF3B/kinesin-associated protein (KAP3) and KIF3A/KIF3C/KAP3.⁸⁰ Dynein-2 consists of a heavy chain (DYNC2H1), an intermediate chain (D2IC), a light intermediate chain (D2LIC), and a light chain (LC).^{79, 81, 82} The IFT complex, which serves as an adaptor between cargoes and IFT motors, can be divided into two complexes, complex A (IFT-A) and complex B (IFT-B).⁸³ IFT-A contains six proteins (IFT144, IFT140, IFT139, IFT122, IFT121, and IFT43)^{83–85} and IFT-B has fourteen proteins (IFT20, IFT22, IFT25, IFT27, IFT46, IFT52, IFT54, IFT57, IFT70, IFT74/IFT72, IFT80, IFT81, IFT88, and IFT172).^{3, 83, 85, 86} IFT81 and IFT74/72 form a tetrameric complex and interact with IFT88, IFT52, IFT46, and IFT27 to form the core of complex B.⁸⁷ IFT-B is involved in anterograde transport motored by kinesin-II, while IFT-A is involved with retrograde transport motored by dynein-2.^{84, 88} The multi-protein complex BBSome is formed by seven highly conserved Bardet-Biedl syndrome (BBS) proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, and BBS9); mutations of BBS proteins account for the syndrome. The BBSome is a component of the ciliary basal body and is indispensable for ciliogenesis and transportation within cilia.^{89–91} The BBSome assembles the complex of IFT-A and IFT-B and is required for the IFT complex assembling at the ciliary base, IFT transport, and IFT recycling along the cilia.^{92–94} The BBSome also functions as a scaffold to tether the IFT complex and cargo into a single unit before and during IFT.⁹⁵

More than 600 proteins are estimated to reside in the cilium. These multi-function proteins are synthesized in the cytosol and transported to cilia by IFT.⁷³ Some of the proteins are membrane receptors, such as polycystin-1 (PC-1) and polycystin-2 (PC-2, also known as

TRPP2) and platelet derived growth factor receptor (PDGFR),⁹⁶ others are signaling components such as Smoothened (SMO) and suppressor of fused (SUFU), and transcription factors such as glioma (GLI).^{97, 98} The latter proteins (SMO, GLI, and SUFU) are core components of the HH signaling pathway, which is among the best-studied cilia-related signaling pathways.⁹⁷ The mammalian HH family contains sonic hedgehog (SHH), desert hedgehog (DHH), and Indian hedgehog (IHH);⁹⁹ both IHH and SHH are important signaling proteins for skeleton development.^{100–103}

Cilia have been suggested as the HH transduction machine.⁹⁷ When HH is absent, HH receptor patched-1 (PTCH1) is located at the base of primary cilia and inhibits SMO function. Meanwhile, SUFU and GLI interact with each other within cilia and allow GLI to be processed into its repressor form. The repressor GLI exits the cilia to inhibit the expression of related gene. However, when HH is present, it binds to PTCH1 and triggers PTCH1 to move out of cilia and release the inhibition to SMO, allowing SMO translocation into cilia.^{104, 105} Active SMO translocates into cilia and activates GLI (GLI activator) by relieving the inhibition of SUFU. The GLI activator is transported to the nucleus to turn on the expression of related genes (Fig. 2).^{106–108} Ellis-van Creveld syndrome protein homolog (EVC) is located at the basal body and the base of the axoneme.¹⁰⁹ Recently, Dorn *et al.* suggested that the SMO-EVC2 complex is formed at the EVC zone, which is immediately adjacent to the transition zone. This complex is required for SMO function and HH signaling transduction (Fig. 2).^{110, 111} Thus, cilia and cilia-related proteins such as EVC are essential for HH signaling transduction.^{98, 108}

Cilia-mediated regulation of the canonical WNT pathway has also been studied recently (see Ref. 112). Studies showed that cilia limit the nuclear entry of β -catenin by sequestering it within the ciliary compartment, thereby dampening the canonical WNT signaling transduction.^{113, 114} Loss of cilia potentiates canonical WNT responses.¹¹³ The interplay between non-canonical WNT/planar cell polarity (PCP) signaling and cilia has also been studied (See Refs. 115 and 116). Despite these findings, how cilia regulate WNT signaling during bone development and remodeling is still not clear.^{117–119}

PDGF signaling is another pathway that directs bone formation, especially the osteogenic differentiation of MSCs.^{120, 121} PDGFR α is located in cilium, and PDGF signaling through the PDGFR α requires intact cilia (see Refs. 112 and 122). Ligand-dependent activation of the PDGFR α homodimer and the downstream activation of AKT and MEK1/2–ERK1/2 occur within the cilium and/or at the basal body.¹²³ Loss of cilia elevates the level of PDGFR α , but fails to transduce PDGF signaling.¹²³

In the following sections, we will summarize how cilia or cilia-related proteins regulate the differentiation and function of bone cells and associated signaling pathways to influence skeletal development and homeostasis.

Cilia and IFT proteins in bone cell differentiation

Both chondrocytes and osteoblasts are derived from MSCs.^{17, 18} Appropriate commitment of MSCs to chondrocytes and osteoblasts is important for skeletal development and remodeling. Moreover, chondrogenic and osteogenic processes also determine bone health

and function. The regulation of primary cilia and IFT proteins in bone cell differentiation is not well understood; however, it has been discovered that cilia and cilia-related proteins are required for MSC commitment and differentiation of osteoblasts and chondrocytes.

Cilia/IFT proteins in osteogenesis

Osteoblasts are specialized fibroblasts that develop from mesenchymal precursors. They secrete and mineralize the bone matrix and are responsible for bone formation. Inhibition of osteoblast differentiation or function usually leads to defective intramembranous and endochondral ossification.^{124–126} Currently, it is largely unknown how primary cilia/IFT proteins regulate osteoblast differentiation and function. Several recent studies have begun to uncover the role of cilia and cilia-related proteins in osteoblast differentiation. In the following sub-sections, we summarize the studies regarding IFT proteins (IFT88 and IFT80), IFT motors protein (KIF3A), and IFT-related proteins (EVC, ANKH, and polycystin) in osteogenesis.

IFT88 (also known as Tg737 or polaris)

IFT88 is a core component of the IFT-B complex. Using Cre recombinase-mediated deletion of a target gene in mature osteoblasts from E18.5,¹²⁷ Haycraft *et al.*¹²⁸ deleted the IFT88 gene (*Ift88*) in osteoblasts and osteocytes in transgenic mice expressing Cre recombinase under the control of the osteocalcin promoter (Oc-Cre). They found that while the osteoblast number and bone density decreased in 9-month-old *Ift88*-deleted mice on a mixed genetic background, on an inbred C57BL/6J genetic background *Ift88*-deletion resulted in increased bone volume/total volume ratio (BV/TV) and trabecular thickness at one month of age. It is not clear whether this difference results from different genetic background or age. Moreover, whether cilia formation was disrupted and what kind of signaling pathways were altered in the *Ift88*-deleted mice were not discussed. Further studies are necessary to uncover the postnatal complex role of IFT88/cilia in bone formation and remodeling.

IFT80

IFT80, the IFT-B complex protein, is highly expressed in osteoblast progenitor cells, osteoblasts, and bone.⁵⁰ Mutation of *Ift80* has been found in human Jeune asphyxiating thoracic dystrophy (JATD) and short rib polydactyly type III (SRP type III). Both diseases have severe bone abnormalities, including short limbs and a narrow thorax.^{129, 130}

The function of IFT80 in osteoblast differentiation has recently been studied.⁵⁰ Silencing *Ift80* in the murine mesenchymal progenitor cell line-C3H10T1/2 and bone marrow derived stromal cells (BMSCs) by lentivirus-mediated RNA interference (RNAi) blocked cilia formation and osteoblast differentiation.⁵⁰ The level of GLI2 protein was largely reduced in *Ift80*-silenced cells. Overexpression of GLI2 protein or treatment with Smoothed agonist (SAG, *N*-methyl-*N'*-(3-pyridinylbenzyl)-*N'*-(3-chlorobenzo[b]thiophene-2-carbonyl)-1,4-diaminocyclohexane) rescued deficient osteoblast differentiation in *Ift80*-silenced cells, suggesting that IFT80 is important for osteoblast differentiation by regulating the HH signaling pathway.⁵⁰ Rix and colleagues generated *Ift80*^{gt/gt} (gene-trap line) mice and found

them to be hypomorphic rather than true null mutants.¹³¹ While 98% of *Ift80*^{gt/gt} mice died during embryonic development, the 2% of the mice surviving to postnatal stages have a disease resembling JATD and SRP type III, with defects in the long bones, constriction of the rib cage, and polydactyly.¹³¹ In addition, HH signaling is disrupted in *Ift80*^{gt/gt} mouse embryonic fibroblasts (MEFs).¹³¹ However, how IFT80 regulates osteoblast differentiation and function and related pathways *in vivo* has not been determined because of the embryonic lethality. Currently, our laboratory has generated a conditional knockout mouse model of *Ift80* (*Ift80*^{flox/flox}) and deleted *Ift80* in the osteoblast lineage with Osterix-Cre (which activates Cre in osteoblast precursor cells¹³²) on an inbred C57BL/6J genetic background. *Ift80*; Osterix-cre mice display an osteopenia phenotype with impaired osteoblast differentiation (unpublished data).

KIF3A

In mammals, KIF3A is a subunit of the kinesin-II motor protein. Global deletion of KIF3A causes embryonic lethality with cilia disruption, randomized establishment of left-right asymmetry and skeletal abnormalities.¹³³ Qiu *et al.* generated and compared Oc-Cre;*Kif3a*^{flox/+} (conditionally deletion of *Kif3a* in mature osteoblasts in one allele), *Kif3a*^{flox/null} mice (global deletion of *Kif3a* in one allele by using CMV-Cre mice) and *Kif3a*^{Oc-cKO} (conditionally deleted *Kif3a* in mature osteoblasts using Oc-Cre, which is limited to mature osteoblasts) mice.¹³⁴ Interestingly, they found that haploinsufficiency of KIF3A in Oc-Cre;*Kif3a*^{flox/+} and *Kif3a*^{flox/null} heterozygous mice had no bone phenotype, however, *Kif3a*^{Oc-cKO} mice, with 75% reduction of *Kif3a* transcripts in bone and osteoblasts, showed osteopenia due to impaired osteoblast function.¹³⁴ They further revealed that *Kif3a*^{Oc-cKO} mice impaired intracellular calcium responses to fluid flow shear stress with attenuated signal transduction in the WNT and HH signaling pathways.¹³⁴ Additionally, deletion of *Kif3a* in osteoblasts inhibits osteogenesis but enhances cell proliferation and adipogenesis.¹³⁴ In contrast, *Kif3a*^{flox/null} mice have normal osteogenesis but show impaired adipogenesis with decreased peroxisome proliferator activated receptor-gamma (PPAR γ) expression and increased GLI2 expression.¹³⁵ Compared to these models, the model generated by the deletion of *Kif3a* in osteoblast progenitor cells using Col1(I) 2.3-Cre (2.3 kb fragment of the α 1(I)-collagen promoter¹³⁶) does not cause obvious abnormalities in skeletal size and patterning in mice (described below).¹³⁷ Thus, these findings suggest that KIF3A likely has a gene dosage-dependent effect on bone development and postnatal bone homeostasis, and that variable Cre activity and efficiency in conditional knockout mice likely cause a large variety of bone phenotypes.

EVC

Ellis-van Creveld syndrome (EVC) is characterized by restricted growth, narrow rib cage, supernumerary fingers/toes, and orofacial abnormalities.^{138, 139} Mutation of the genes *EVC1* and *EVC2* were reported in EVC.^{140, 141} Reduced expression of *Evc2* mRNA in mouse C3H10T1/2 cells by small interfering RNA (siRNA) inhibited SHH-induced *Gli* expression and osteogenic differentiation.¹¹⁰ Dorn *et al.* suggested that a SMO-EVC complex formed in the EVC zone is important for HH signal transduction (Fig. 2). In EVC syndrome, mutant *Evc2* is not present in cilia. Although mutant *Evc2* is present in cilia in Weyers Acrofacial

Dysostosis, it is not confined to the EVC zone. Additionally, both diseases exhibit impaired HH signal transduction.¹¹⁰ Homozygous *Evc*-deficient mice (*Evc*^{-/-} mice) display short limbs and ribs and small dysplastic incisors and molars, phenotypes that are similar to EVC patients.^{109, 142} While EVC is localized in primary cilia of osteoblasts, deletion of *Evc* does not lead to cilia loss. *Evc*^{-/-} mice display defective bone collar development with impaired osteoblast differentiation. Osteoblasts from *Evc*^{-/-} mice had significantly decreased expression of *Ptch1*, *Gli1* and *Hhip* (Hedgehog interacting protein) after a stimulation with purmorphamine (SMO agonist, which activates the HH signaling pathway).¹⁴² These findings suggest the important role of EVC in osteoblast differentiation.¹⁴² Considering that the global deletion of *Evc* likely alters chondrocyte differentiation in *Evc*^{-/-} mice (see below), conditional deletion of *Evc* in the osteoblast lineage may be employed to uncover the precise role of EVC in osteoblast differentiation and function.

ANKH (or ANK)

ANKH protein is a putative transmembrane transport protein with eight predicted transmembrane domains.¹⁴³ ANKH localizes to the cilia and basal body.^{105, 143} In humans, mutations of *ANKH* are found in craniometaphyseal dysplasia (CMD), chondrocalcinosis, and calcium-pyrophosphate dihydrate deposition disease (CPPD).^{143, 144} The hypoactive mutation of *ANKH* leads to CMD, which is characterized by thicker cranium and abnormalities at the ends of the long bones in the limbs. The hyperactive mutation of *ANKH* causes CPPD, which includes deposition of calcium pyrophosphate dihydrate in and around joints.¹⁴³ It is believed now that ANKH functions at the plasma membrane as an exporter of pyrophosphate (PPi) and controls tissue calcification through regulating PPi levels.^{144, 145} Mutation of the gene in the mouse (*Ank*) leads to arthritis accompanied by ectopic calcification, joint destruction, and formation of bony outgrowths.¹⁴⁵ However, how ANKH functions in bone cells is largely unknown. It is also unclear whether the ciliary localization of ANKH would affect its function. Most recently, Carr *et al.* confirmed the endogenous expression of ANKH in the osteoblast cell line MC3T3-E1 and its ciliary and basal body localization.¹⁴³ They proposed that this localization may allow ANKH to locally modify inorganic PPi and calcium activities, and to take part in signaling events initiated from the cilia.¹⁴³ The precise role and mechanism of ANKH need to be further determined.

Polycystin

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited disorder that commonly results in renal failure in humans.¹⁴⁶ More than 85% of ADPKD patients have mutations in *PKD1* and/or *PKD2*.¹⁴⁷ *PKD1* encodes PC-1, which functions as a G protein coupled receptor (GPCR).¹⁴⁷ *PKD2* encodes PC-2 that is a receptor-activated calcium channel.¹⁴⁸ PC-1 interacts with PC-2 to form a heterodimers to co-localize in the primary cilia through interactions between the C-terminus of PC-2 and KIF3A (Fig. 1).^{148–150} *Pkd1* and *Pkd2* are expressed in mouse-derived MC3T3-E1 osteoblasts and MLO-Y4 osteocytes.¹⁵⁰ Lu *et al.* first found that loss of *Pkd1* in *Pkd1*^{null} mice results in abnormal skeletal phenotypes including spina bifida occulta and osteochondrodysplasia, in addition to renal and pancreatic cystic disease.⁵³ Furthermore, Xiao *et al.* used a *Pkd1*^{m1Bei} mouse model, which has an inactivating point mutation in the *Pkd1* gene caused by *N*-ethyl-*N*-

nitrosourea (ENU) mutagenesis, to study the function of *Pkd1* in bone formation. They found that homozygous *Pkd1*^{m1Bei/m1Bei} mice are embryonically lethal with a mineralized defect in both calvaria and long bones.¹⁵¹ Heterozygous mice (*Pkd1*^{m1Bei/+}) survive but have low mineral apposition rate (MAR) and bone mineral density (BMD). Serum levels of osteocalcin, OPG, RANKL, and tartrate-resistant acid phosphatase (TRAP) and the ratio of OPG/RANKL are reduced in *Pkd1*^{m1Bei/+} mice, suggesting that defects occur in both osteoblasts and osteoclasts.¹⁵¹ *Pkd1* has a gene dose-dependent increase in the expression of runt-related transcription factor-2 (RUNX2); overexpression of constitutively-active PC-1 C-terminal constructs results in increased expression of RUNX2 and osteoblast differentiation markers, demonstrating that PC-1 is required for osteogenesis.¹⁵¹ To elucidate the direct role of PC-1 in osteoblast function and skeletal homeostasis, Xiao *et al.* further specifically inactivated *Pkd1* in mature osteoblasts by crossing Oc-Cre mice with *Pkd1*^{flox/m1Bei} mice to generate Oc-Cre;*Pkd1*^{flox/m1Bei} mice.¹⁵² Similar to the results from the deletion of *Kif3a* (*Kif3a*^{Oc-cKO}), deletion of *Pkd1* in osteoblasts also resulted in a gene dose-dependent reduction in osteoblast differentiation and BMD by inhibition of phosphatidylinositol 3-kinase (PI3K)–AKT–GSK3β–β-catenin signaling pathways in the Oc-Cre;*Pkd1*^{flox/m1Bei} mice.¹⁵²

Besides the above studies, Qiu *et al.* generated heterozygous *Pkd1*^{+/-} mice by crossing floxed *Pkd1* mice¹⁵³ (*Pkd1*^{flox/flox}) with CMV-Cre transgenic mice (Cre recombinase expressed in all tissue including germ cells).¹³⁵ The *Pkd1*^{+/-} mice had a similar osteopenia phenotype as the *Pkd1*^{m1Bei/+} mice, including decreased BMD, trabecular bone volume, and cortical thickness. Moreover, they found that the loss of *Pkd1* results in impaired osteogenesis and enhanced adipogenesis accompanied by decreased *Runx2* expression, increased *Pparg* expression, and impaired HH signaling. Interestingly, by generating double mutant mice of *Pkd1* and *Kif3a*, the authors found that loss of *Kif3a* can rescue the defective osteogenesis and adipogenesis in *Pkd1*^{+/-} mice and restore the expression of *Runx2*, *Pparg*, and *Gli2*. These findings suggest that *Pkd1* and *Kif3a* counterbalance osteogenesis and adipogenesis through GLI2 regulation of RUNX2 and PPARγ.¹³⁵

Recently, Qiu *et al.* explored the *in vitro* role of PC-1 in the human osteoblastic MG-63 cell line using lentivirus-mediated short hairpin RNA (shRNA) to stably silence PKD1 mRNA.⁵² Stable knockdown of PKD1 mRNA in MG-63 cells increased cell proliferation and enhanced adipogenesis, but impaired osteoblastic differentiation, suggesting a role for *PKD1* in regulating human osteoblast commitment and function. *PKD1* deficiency in MG-63 cells increases cilia length that can be blocked by the protein kinase A (PKA) inhibitor H89.⁵² Additionally, silencing of *PKD1* impairs intracellular calcium and upregulates cyclic adenosine monophosphate (cAMP)/PKA signaling. These results indicate that the cAMP/PKA signaling is involved in cilia formation and osteogenesis.⁵²

Cilia in chondrogenesis

Chondrocytes arise from MSCs and are responsible for cartilage formation and function.¹⁵⁴ Chondrogenesis is a complex of multiple developmental programs, including chondrocyte proliferation, maturation, hypertrophy, and terminal differentiation.¹⁵⁵ A number of pathways, such as fibroblast growth factor (FGF), BMP, HH, and WNT pathways, are

highly involved in the regulation of chondrogenesis.¹⁵⁶ Mice with the cartilage-specific deletion of IFT/cilia display abnormal chondrocyte differentiation and cartilage morphology,³³ which is consistent with the conclusion that IFT/cilia are indispensable in chondrogenesis. We will discuss the details regarding the role of cilia in chondrogenesis in the following sections.

IFT46

IFT46 is the core protein of the IFT-B complex and is necessary for IFT-B stability.^{88, 157} Located in the primary cilia of chondrocytes, endogenous IFT46 is preferentially expressed in early hypertrophic chondrocytes in the growth plate.⁴¹ Gouttenoire *et al.* first identified IFT46 as a BMP-2-sensitive protein in mouse chondrocytes.⁴¹ Knockdown of *Ift46* mRNA in cultured chondrocytes using siRNA oligonucleotides stimulates expression of *Msx1*, *Fgfr1* (fibroblast growth factor receptor-1), *Col12a1* (type XII collagen), *Bgn* (biglycan), *Mmp10* (matrix metalloproteinase 10), and *Hsp47*, but does not affect *Col2a1*, *Col10a1*, *BMP* and *Smads* expression.⁴¹ Currently, there is no genetic *in vivo* evidence showing whether deletion of *Ift46* affects cilia formation or function in humans, or how IFT46 is involved in chondrocyte differentiation.

IFT80

IFT80 is highly expressed in the growth plate and during BMSCs chondrogenic differentiation.³⁹ Silencing *Ift80* mRNA in BMSCs disrupted cilia formation and chondrogenic differentiation, with decreased HH signaling but increased WNT signaling.³⁹ Overexpression of *GLI2* was shown to rescue chondrogenesis in IFT80-deficient cells.³⁹ This suggests that IFT80/cilia in BMSCs regulates chondrogenic differentiation through HH and WNT signaling pathways.

IFT88

Tg737^{orp} (ORPK) mice carry a hypomorphic mutation in *Ift88* and display defects in both appositional and endochondral growth and shorten or absent cilia.³⁴ *Tg737^{orp}* mice had smaller growth plates and disorganized articular cartilage. In addition, *Tg737^{orp}* mutant chondrocytes fail to fully undergo hypertrophy with a marked reduction in type X collagen sequestration.³⁴ Compared to *Tg737^{orp}* mice, the mice with specific deletion of *Ift88* in committed chondrocytes (*Ift88*;Col2 α -Cre) display postnatal dwarfism with premature loss of the growth plates.³³ *Ift88*;Col2 α -Cre mice have thicker articular cartilage with increased cell density resulting from reduced apoptosis in cartilage.⁴⁰ Irianto *et al.* studied the intrinsic mechanical properties of articular cartilage matrix in *Ift88*;Col2 α -Cre mice. Their results showed that the loss of cilia in the chondrocyte lineage significantly reduced the mechanical properties of cartilage, particularly in the deeper zones.¹⁵⁸ Chang *et al.* further found that the expression of PTCH1, GLI1, and secreted frizzled-related protein 5 (*Sfrp5*, an extracellular antagonist of WNT signaling pathway) was lower, while WNT/ β -catenin signaling activities were higher in the growth plate of the *Ift88*;Col2 α -Cre mice than in wild-type mice.¹⁵⁹ It was suggested that IFT88 regulates SFRP5 expression in chondrocytes through HH signaling; SFRP5 could subsequently regulate WNT signaling. Thus, WNT signaling is regulated indirectly through HH signaling.⁴⁰ Ochiai *et al.* found that *Ift88*;Col2 α -cre mice

have deformed post-natal cranial bases and disorganized synchondrosis growth plates accompanied by reduced endochondral bone and excessive intramembranous ossification along the perichondrium.¹⁶⁰ Heparan sulfate proteoglycans (HS-PGs), which are involved in restricting HH distribution, were downregulated in synchondrosis. Decreased HS-PGs led to the elevated HH signaling, which contributed to the excessive intramembranous ossification.¹⁶⁰ How the deletion of *Ift88* leads to the HS-PGs loss and defective HH signaling is still an unknown.

DYNC2H

DYNC2H1 is the heavy chain of the cytoplasmic dynein-2 motor of retrograde transportation in cilia.¹⁶¹ DYNC2H1 mutation in humans is associated with JATD and SRP.^{42, 161, 162} Merrill *et al.* reported that chondrocytes from patients with DYNC2H1 mutation displayed shortened cilia with bulbous distal ends.⁴² Moreover, the chondrocytes from DYNC2H1 mutant individuals showed abnormal cytoskeletal microtubule architecture.⁴² Whether the abnormalities affect chondrocyte differentiation and cartilage development is unknown.

Bardet-Biedl syndrome proteins

BBS is a heterogeneous autosomal recessive disorder characterized by retinal dystrophy, diabetes, polydactyly, renal dysfunction, learning difficulties, and hypogonadism.¹⁶³ Kaushik *et al.* studied the cartilage of *Bbs1*^{M390R/M390R} (a knockin strain, in which the *Bbs1* gene has been deleted from the genome and replaced with a mutated copy of the gene), *Bbs2*^{-/-} (deletion of *Bbs2*) and *Mkks*^{-/-} (deletion of *Bbs6*) mutant mice,⁸⁹ and found that compared to the wild-type mice, three *Bbs* mutant mice all displayed early signs of osteoarthritis (OA) with significantly lower articular joint thickness, lower proteoglycan content saturation and abnormal cell distribution and fibrillations at the joint surface.⁸⁹ This suggests that BBS functions in preserving the integrity of the joint surface by sustaining ECM production in the articular cartilage. They further found significantly fewer ciliated cells in cultures of mutant chondrocytes, but not in sections from mutant mice (both load-bearing joints and non-load-bearing xiphoid processes). Kaushik *et al.* explained that this difference may result from their limited immunofluorescence microscopy technique.⁸⁹ Zhang *et al.* studied HH signaling in BBS and showed that the MEFs with *Bbs* mutations (derived from *Bbs1*^{M390R/M390R} knockin, *Bbs2*^{-/-}, *Mkks*^{-/-}, and *Bbs7*^{-/-} mice) exhibited a decreased SHH response with accumulation of PTCH1 in cilia.¹⁶⁴ How BBS proteins are involved in the cilia and chondrocyte formation, and maintain the normal articular cartilage needs to be further studied.

EVC

EVC is localized at the base of cilia in chondrocytes.¹⁰⁹ Deletion of *Evc1* in mice (*Evc1*^{-/-}) results in the EVC-like phenotype (described above), with advanced chondrocyte maturation in the growth plate and delayed bone collar formation.¹⁰⁹ Loss of EVC1 does not affect cilia formation or GLI3 processing in chondrocytes. *Ihh* expression is normal in the growth plate of *Evc*^{-/-} mice, but IHH transduction is blocked, as evidenced by decreased *Gli1* and *Ptch1* expression.¹⁰⁹ Additional studies from Pacheco *et al.* revealed that EVC1 is essential for

intrasphenoidal synchondrosis development and that loss of EVC causes cranial base defects in mice.¹⁴² They also found that EVC1 is involved in chondrocyte proliferation and hypertrophy, and suggested that EVC1 takes part in both parathyroid hormone related peptide (PTHrP)-dependent and -independent IHH signaling pathways.¹⁴² Most recently, Caparrós-Martín *et al.* pointed out that EVC1 and EVC2 function as a complex. They found that mutation of either or both genes (*Evc1*^{-/-}, *Evc2*^{-/-}, or *Evc1*^{-/-}*Evc2*^{-/-}) cause a similar phenotype.¹¹¹ They further found SMO co-precipitates with the EVC1/EVC2 complex. *Evc* mutations reduce the recruitment of GLI3 to the cilia tip and impairs SUFU/GLI3 dissociation.¹¹¹ The EVC1/EVC2 complex also promotes HH signaling in the absence of SUFU, as silencing *Evc* mRNA in *Sufu*^{-/-} cells reduces HH signaling activity.¹¹¹ These data reveal a role for EVC1/EVC2 in HH-regulated normal endochondral bone formation.

KIF3A

Deletion of *Kif3a* in chondrocytes (*Kif3a*;Col2α1-Cre) leads to cilia loss in the growth plate, accompanied by disorganization of the growth plate and alterations in chondrocyte orientation.³³ *Kif3a*;Col2α1-Cre mice also displayed post-natal dwarfism, resulting from premature loss of the growth plate.³³ These are due to reduced cell proliferation and accelerated hypertrophic differentiation.³³ Furthermore, Koyama *et al.* observed cranial base growth retardation and dysmorphogenesis in *Kif3a*; Col2α1 mice.¹⁶⁵ Interestingly, the synchondrosis growth plate phenotype and HH activity and distribution in *Kif3a*;Col2α1-Cre mice were very similar to those observed in *Ift88*;Col2α1-Cre mice.^{160, 165} However, the phenotype in the *Ihh*;Col2α1-Cre mice does not fully resemble that of the *Kif3a*;Col2α1-Cre mice,¹⁶⁵ suggesting other signaling pathways are likely to be involved in cilia-mediated cranial base development and synchondrosis. Besides the growth plate, Kinumatsu *et al.* recently studied the role of KIF3A/cilia in temporomandibular joint (TMJ) development.¹⁶⁶ They found that *Kif3a*;Col2α1-cre mice had narrow and flat condyles with an irregular bony surface often fused with the articular disc. Alteration of IHH signaling topography was associated with ectopic bone formation in condyles, implicating KIF3A/cilia in coordination of HH in condylar growth.¹⁶⁶

Cilia/IFT proteins in MSC differentiation

MSCs are multipotent stem cells and have the potential to differentiate to mesenchymal tissues including bone and cartilage.^{167, 168} Primary cilia are shown to act as chemosensors and mechanosensors in many cell types and more than 90% of the human MSCs have primary cilia.⁵⁶ However, how cilia regulate MSC differentiation and function is largely unknown. Recently, Tummala *et al.* studied the function of cilia in osteogenic, adipogenic, and chondrogenic differentiation of human MSCs using *Ift88* mRNA-specific siRNA to disrupt primary cilia.⁵⁶ They found that the loss of cilia in MSCs significantly decreases the basal expression of three lineage specific transcription factors, PPARγ, SOX9, and RUNX2. When chemically induced to osteogenic and adipogenic differentiation, MSCs without cilia lost adhesion and were released from the slides.⁵⁶ These results suggest that cilia are required for the initiation of differentiation, as well as for maintaining the phenotype of differentiated cells.

Most recently, McMurray *et al.* studied the effect and relationship of cilia length and WNT signaling on human MSC differentiation.¹¹⁴ They found that surface topography increases primary cilia length through ROCK-dependent actin modulations. Disruption of the cilia using *Ift88* mRNA-specific siRNA enhanced MSCs response to WNT signaling; however, the topographical microenvironment can down-regulate this response WNT. Thus, these finding demonstrate that topography regulates MSCs WNT signaling through modulating primary cilia structure and function.¹¹⁴

In addition to the *in vitro* studies, some research groups have studied the *in vivo* role of cilia in the mesenchyme using *Prx1*-Cre transgenic mice to delete the target gene in early limb bud mesenchyme and a subset of craniofacial mesenchyme.¹⁶⁹ Interestingly, the deletion of *Ift172* with *Prx1*-Cre (*Ift172*^{f/t}; *Prx1*-Cre, one allele is the germline deletion and the other is specifically deleted in the developing limb bud mesenchyme) causes eight digits on each forelimb and a single extra digit on each hindlimb.¹⁷⁰ The deletion of *Ift88* by using *Prx1*-Cre (*Ift88*; *Prx1*-Cre) leads to polydactyly with the loss of anteroposterior digit patterning and shortening of the proximodistal axis.¹⁷¹ These abnormalities were associated with aberrant SHH pathway activity.¹⁷¹ Deletion of *Kif3a* with *Prx1*-Cre results in forelimb polydactyly and short limb bones with deregulated HH signaling.¹⁷² KIF3A was shown to be necessary to process fulllength GLI3 into the repressor form.¹⁷² However, kinesin-II is also suggested to play a role in the transportation of late endosomes, lysosomes and endoplasmic reticulum (ER)-to-Golgi anterograde traffic of vesicles in mammalian cells.¹⁷² These unique functions beyond cilia transportation may contribute to HH signal transduction and normal bone formation in *Kif3a*; *Prx1*-Cre mice. Although the deletion of either *Ift88* or *Kif3a* leads to cilia disruption and similar phenotypes, whether all phenotypes are secondary to the cilia loss is still not known.

Mechanosensory function of cilia in bone cells

Bone is a dynamic tissue and bone homeostasis is regulated by different signaling pathways including chemical and mechanical signaling. Mechanotransduction, which converts mechanical forces or loads into anabolic cell signals, is an important pathway in bone maintenance.^{151, 173} Mechanotransduction includes mechanosensing, signal transduction, and effector-cell responses.¹⁷⁴ Mechanosensing is conducted by mechanosensors in bone cells, including mechanosensitive calcium channels, integrins, cell-cell adhesion elements, cytoskeleton constituents, and membrane structures such as cilia.¹⁷⁴ A variety of mechanical stimuli have been found to influence bone cells *in vitro*, such as fluid flow, compressive strain, substrate stretch, gravity force, vibration, magnetic bead twisting, atomic force, and shockwaves.^{174, 175} *In vivo*, bone is a mechanosensitive organ; and how bone cells sense and response the mechanical stimuli, such as interstitial fluid flow, greatly affect bone homeostasis,^{47, 176} yet the molecular basis of mechanotransduction in bone cells has not been fully elucidated.

The sensory function of primary cilia has been greatly studied in kidney cells (see Ref. 177). For example, primary cilia mediate PC-2-dependent Ca²⁺ entry and intracellular Ca²⁺ release in response to fluid flow in kidney cells.^{178–180} Recently, several studies (discussed

below) have provided strong evidence that primary cilia also play a mechanosensory function in bone cells.

Mechanosensory function of cilia and IFT proteins in osteoblasts and osteocytes

Mechanical forces induce important effects during osteogenesis.^{174, 181} When potential mechanosensors are disrupted, the activities of the components in mechanotransduction pathways are also inhibited, preventing mechanically-induced osteogenesis.

The osteocyte has long been considered a major player in mechanotransduction in bone.^{182, 183} Since cilia in the kidney transduce the signals from fluid flow,^{184–186} Whitfield *et al.* proposed that cilia in osteocytes also act as mechanical sensors in bone. This hypothesis is supported by the following data. Cilia in osteoblasts recoiled upon cessation of flow, similar to the activity of cilia in kidney cells.⁶⁰ In MLO-A5 murine osteoblasts, cilia became shorter after exposure to periods of oscillatory fluid flow (OFF, short periods of mechanical stimulation, which are more like the natural loading).¹⁸⁷ Damage or removal of primary cilia by chloral hydrate (mitotic inhibitor, which disrupts ciliary microtubules) inhibited OFF-induced prostaglandin E₂ (PGE₂) release and mineral deposition, indicating that cilia are important mediators of OFF-induced mineral deposition in osteoblasts.¹⁸⁷ Emerging evidence has shown that cilia are specialized calcium signaling compartments in response to mechanical stimuli regulated by transient receptor potential (TRP) channels and/or polycystin calcium channels.^{181, 188, 189} Very recently, voltage-gated L-type calcium channel CaV1.2 has been found to localize to primary cilia in renal epithelia.¹⁹⁰ Knockdown of CaV1.2 mRNA in zebrafish results in classic primary cilia defects, such as renal cyst formation, hydrocephalus, and left-right asymmetry defects.¹⁹¹ How the mechanical forces transduce into calcium signaling through cilia and cilia-related proteins to regulate bone cells is discussed in the context of polycystin, KIF3A, and IFT88 in osteoblasts/osteocytes.

Polycystin

In renal epithelial cells, the polycystin complex (PC-1/PC-2) mediates Ca²⁺-dependent mechanosensitive signaling in response to urine flow and cilia bending.^{147, 192} These studies suggest that PC-1/PC-2 might also function as a mechanosensor or fluid sensor in bone cells. Later studies found that basal intracellular calcium levels decreased in *Pkd1*-deleted osteoblasts and that overexpression of *Pkd1* C-terminal constructs can rescue this defect and stimulate the *Runx2* P1 promoter via an intracellular calcium-dependent mechanism.⁵¹ Knockdown of PKD1 mRNA in the human osteoblastic MG-63 cell line by shRNA leads to attenuated cytosolic calcium signaling in response to pulsatile laminar fluid flow.⁵² Knockdown of *Pkd1* mRNA in mouse MC3T3-E1 cells results in the loss of response to external mechanical stimuli through intracellular calcium and AKT/ β -catenin pathway.¹⁹³ These data demonstrate that polycystin is required for flow-induced Ca²⁺ response in osteoblasts. To study the function of PC-1 in bone mechanotransduction, Xiao *et al.* generated *Pkd1* conditional knockout mice (*Pkd1*^{Dmp1-cKO}) by crossing *Dmp1*-cre (deletion in odontoblasts and osteocytes¹⁹⁴) with *Pkd1*^{flox/m1Bei} mice.¹⁹⁵ They found that *Pkd1*^{Dmp1-cKO} mice display reduced bone formation and expression of *Ptgs2* (COX2), *Jun*, *Wnt10b*, *Axin2*, and *Runx2* in response to axial compression in the forelimbs. Fluid shear

stress upregulates cytosolic calcium; this is abrogated in osteoblasts from *Pkd1*^{null/null} and *Pkd1*^{m1Bei/m1Bei} mice. These data suggest that PC-1 is a bone mechanosensor.¹⁹⁵

KIF3A

Kif3a;Colα1(I) 2.3-Cre mouse (2.3 kb fragment of the α1(I)-collagen promoter¹³⁶) is a good model to study the role of KIF3A/cilia in regulating mechanosensing in osteoblasts without the variables resulting from developmental defects.¹³⁷ To investigate the response of *Kif3a*;Colα1(I) 2.3-Cre mutants to mechanical ulnar loading, Temiyasathit *et al.* have exposed these mutants and littermate controls to a cyclic ulnar loading regimen of one minute per day for three consecutive days and measured the rate of new bone formation. The results showed that the rate of new bone formation is significantly reduced in the *Kif3a*;Colα1(I) 2.3-Cre group than in the control group, suggesting that KIF3A/cilia are critical to regulate mechanotransduction-mediated bone remodeling.¹³⁷ In addition, Leucht *et al.* also studied the role of cilia in mechanically sensitive bone repair using the *Kif3a*;Colα1(I) 2.3-Cre mouse model by applying “a motion device, which generated a spatial distribution of strain around an intra-osseous implant positioned in the mouse tibia”.⁵⁴ Peri-implant cells in the *Kif3a*;Colα1(I) 2.3-Cre mice failed to proliferate, deposit and then orient collagen fibers along the strain field in response to the mechanical stimulus. Those results suggested that cilia/KIF3A are indispensable for pre-osteoblasts to sense strain-related mechanical stimuli at a healing bone–implant interface.⁵⁴

IFT88

Osteoblasts and osteocytes can respond to dynamic fluid flow with characteristic increases in upregulating the expression of osteopontin (OPN), cyclooxygenase 2 (COX2) and the release of cytokine prostaglandin E₂ (PGE₂). Malone *et al.*⁶⁰ studied the role of cilia/IFT88 in mouse-derived osteoblasts/osteocytes *in vitro* in response to fluid flow. They found that silencing *Ift88* mRNA significantly inhibits the fluid flow induced the expression of *Opn* and *Ptgs2* as well as PGE₂ release, and blocks osteogenic and bone resorptive responses by increasing the OPG/RANKL ratio in MC3T3-E1 cells.⁶⁰ However, silencing *Ift88* mRNA does not affect Ca²⁺ flux and stretch-activated ion channels, which differs from the kidney and liver.⁶⁰ Further studies suggested that adenylyl cyclase 6 (AC6), an isoform of adenylyl cyclase that converts ATP to cyclic AMP (cAMP), and cAMP are involved in cilia/IFT88-mediated mechanotransduction in bone cells.¹⁹⁶ AC6 is preferentially localized in cilia of osteocytes and osteoblasts. When murine MLO-Y4 cells are exposed to flow, AC6 and Gd³⁺-sensitive channels decrease cAMP production, which leads to the expression of COX2. The decrease in cAMP was abolished in osteocytes treated with *Ift88*-specific siRNA.¹⁹⁶ More recently, Lee *et al.* investigate the *in vivo* function of AC6.¹⁹⁷ They found that although global knockout of the AC6 gene (*Adcy6*) in mice did not cause significant bone defects, new bone formation decreased in response to ulnar loading over three consecutive days. Also flow-induced COX2 expression decreased in primary bone cells isolated from *Adcy6* knockout mice compared to controls, implicating the role of cilia and AC6 in osteocyte mechanotransduction.¹⁹⁷ To determine the pathway coupled with the osteocytes and osteoblast progenitors during mechanical loading, Hoey *et al.* collected conditioned medium from osteocytes (MLOY4 cell line) stimulated by fluid flow and used it

to induce differentiation of MSCs (C3H10T1/2 cell line). When compared to conditioned medium from osteocytes cultured under static conditions, mechanically stimulated–conditioned medium promoted osteogenic differentiation by increasing the expression of OPN and COX2. Moreover, this promotion is specific between osteocytes and MSCs and not present between osteoblasts (MC3T3 cell line) and MSCs or between osteocytes and osteoblasts.⁵⁵ More interestingly, this coupling signaling between osteocytes and MSCs relies on the presence of cilia/IFT88, as the stimulation was abolished if primary cilia formation in osteocytes was inhibited (via siRNA targeting of *Ift88* mRNA) before mechanical stimulation. This suggested that primary cilia/IFT88 in osteocytes are essential for flow-mediated paracrine signaling in the bone.⁵⁵

Mechanosensory function of cilia in chondrocytes

Poole *et al.* demonstrated that the MTs of the chondrocyte ciliary axoneme are extremely stable, which makes the cilia capable of acting as a mechanosensor.^{44, 198} Jensen *et al.* analyzed the ultrastructure of cilia in chick embryo sternal chondrocytes.⁴⁴ Both tomography and TEM revealed the close association between the collagen fibers/condensed proteoglycans and the axoneme. Further studies suggested that the contact point on the membrane of the axoneme may be known receptors for cartilage matrix components such as $\beta 1$ integrin, CD44 or annexin V.^{44, 199, 200} The follow-up experiment indicated the expression of integrin and NG2 (a transmembrane proteoglycan) on chondrocyte primary cilia, whereas annexin V and CD44 were not detected.²⁰¹ It has been suggested that the matrix-cilium-Golgi continuum existed in chondrocytes to potentially regulate the cellular secretion of ECM components.^{32, 43, 44} Thus, primary cilia may function as a mechanosensor that transduces the ECM biomechanics to the cell body while facilitates the secretion of ECM (Fig. 3).

McGlashan *et al.* used a 3-dimensional (3D) agarose culture model to study the chondrocyte cilia in response to a compressive strain. The results showed that cyclic compression decreased the incidence and length of cilia on chondrocytes.²⁰² Compression-induced reduction in cilia length is reversible, suggesting these decreases may be through a feedback mechanism that reduces an excessively strong signaling from the mechanical stimulation.²⁰²

Wann *et al.* studied the downstream signaling after chondrocyte cilia are bent or pulled from loading.²⁰³ They generated conditionally immortalized wild-type and *Ift88*^{orp} mutant murine chondrocytes (cilia loss) and applied compressive strains in their 3D chondrocyte cultures system. Both wild-type and *Ift88*^{orp} chondrocytes had mechanically induced ATP release, suggesting the cilia do not act as the initial mechanoreceptors. However, they identified a novel role of the cilia in cartilage mechanotransduction called *cilia-mediated control of ATP reception* because *Ift88*^{orp} mutant cells failed to generate Ca^{2+} response when the expression of purine receptors was normal. In contrast, in wild-type chondrocytes, compression induced ATP release that triggered Ca^{2+} signaling, which then promoted aggrecan and sulfated glycosaminoglycan secretion. O'Connor *et al.* further demonstrated that transient receptor potential vanilloid 4 (TRPV4), a Ca^{2+} -permeable osmotically-sensitive cation channel, mediated the metabolic response of chondrocytes to dynamic loading.²⁰⁴ Inhibition of TRPV4 blocked the loading induced matrix accumulation and

mechanical properties, which may help to explain the joint arthropathy and osteoarthritis caused by TRPV4 function loss.²⁰⁴ This study suggests that cilia-TRPV-Ca²⁺ signaling is indispensable in the transduction of mechanical signals to support cartilage extracellular matrix synthesis and joint health.

Thompson *et al.* investigated the relationship between mechanical loading, HH signaling and cilia disassembly in articular chondrocytes. They found that mechanical loading (10% cyclic tensile strain) activates primary cilia-mediated HH signaling and the expression of ADAMTS-5 (a disintegrin and metalloproteinase with thrombospondin motifs 5, a catabolic enzyme leading to cartilage degeneration) in adult bovine articular chondrocytes.²⁰⁵ However, the chondrocyte cell line generated from the Tg737 (also known as ORPK, FVB/N strain) mouse, which lack cilia, fail to response the same loading, suggesting cilia are necessary for the pathway activation. More importantly, high strain (20% cyclic tensile strain) caused HDAC6-mediated cilia disassembly, which also blocked the response to loading. This provides an important feedback mechanism which controlling ADAMTS-5 expression in articular chondrocytes.²⁰⁵

Mechanosensory function of cilia in MSCs

Although numerous studies have focused on the role of mechanical stimulation in osteocytes,^{184, 206} increasing studies suggested that MSCs can also sense the mechanical stimulation and differentiate into osteoblasts to help the bone adapt to the physical environment (see Refs. 181 and 207). Accumulated evidence has shown that direct mechanical stimulation enhance osteogenic differentiation and ECM formation in MSCs.^{207–210} Various mechanosensors have been proposed in MSCs, including cytoskeleton, focal adhesions, primary cilia, membrane channels and gap junctions, which activate multiple pathways to mediate osteogenic lineage commitment.¹⁸¹ Hoey *et al.* first studied the mechanosensory role of cilia in MSCs by applying OFF to human MSCs (hMSCs).⁵⁷ OFF up-regulated the osteogenic gene *BMP2* expression and cell proliferation. However, knockdown of *Ift88* mRNA by siRNA in hMSCs significantly increased proliferation but failed to increase *Ptgs2* or *Bmp2* expression in response to mechanical stimulation, demonstrating that while cilia promote osteogenic differentiation they limit cell proliferation in response to OFF in hMSCs.⁵⁷

Cilia orientation of chondrocytes and osteocytes

Chondrocytes are highly organized into columns parallel to the long axis of the bone and form the so-called postnatal growth plate.²¹¹ The growth plate can be divided transversely into germinal, proliferative, and hypertrophic zones, each with different stages and shapes of chondrocytes.^{212, 213} The proliferation of chondrocytes is perpendicular to this axis. However, to maintain columnar organization, the daughter cell moves (rotates) around the mother cell after division to merge into the stacked columnar.^{37, 211} This is termed *chondrocyte rotation* (Fig. 4); however, little is known about how it is regulated.

Aszodi *et al.* visualized cilia in the native ECM by using whole-mount immunohistochemical and multiphoton microscopy techniques.²¹⁴ Cilia project either from the epiphyseal or the methaphyseal side of the chondrocytes in all zones of growth plate,

which making the most of cilia are similarly aligned relative to the longitudinal axis of the bone.^{214–216} Song *et al.* also found that KIF3A/primary cilia mediate chondrocyte rotation. They found that specific deletion of *Kif3a* in chondrocytes (*Kif3a*;Col2 α 1-Cre) in mice leads to the disruption of cell shape and columnar orientation in the growth plates, but does not affect cell polarity and axis of cell division.³³ Further study showed that the defects in chondrocyte rotation are accompanied by the alterations in the localization of activated focal adhesion kinase (FAK) and the disruption of the actin cytoskeleton.³³ Planar cell polarity (PCP) signaling regulates cell polarity, migration, and orientation. It is possible that PCP-like signaling is altered after KIF3A/cilia disruption in the growth plates, leading to the defects in chondrocyte rotation.³³ Highly expressed on chondrocytes, β 1 integrins mediate adhesion to cartilage matrix proteins.²¹⁵ Conditional deletion of β 1 integrins in chondrocytes also leads to the disruption of chondrocyte rotation, suggesting that communication with the ECM is involved in chondrocyte rotation.^{33, 215} As mentioned above, integrins and NG2 are localized in chondrocyte cilia.²⁰¹ Thus, deletion of KIF3A/cilia may disrupt the communication of chondrocytes with the ECM, and thus interfere with rotation.

More recently, Ascenzi *et al.* suggested that the localization and orientation of cilia in the chondrocytes are also involved in chondrocyte rotation after division.³⁷ They found that loss of cilia clusters at 10° with respect to the anterior-posterior direction in chondrocytes occurred in *Smad1*- and *Smad5*-specific double knockout mice (*Smad1/5*;Col2 α 1-Cre), leading to the disruption of chondrocytes rotation and a disorganized growth plate.³⁷ Deletion of *Smad1/5* disrupt IHH signaling in the growth plate.²¹⁷ However, how SMAD1/5 or IHH regulates cilia orientation remains unknown.

Farnum *et al.* studied cilia orientation in articular surfaces and found it to be more definable in load-bearing than in non-load-bearing areas.²¹⁸ Cilia project to the subchondral bone in load-bearing regions of the superficial zone; in the radiate zone, cilia either face the articular surface or the subchondral bone.²¹⁸

Recently, Uzbekov *et al.* found that primary cilia display the orientation on osteocytes in rat tibial cortical bone by using a TEM technique.⁴⁹ They found that cilia are mainly oriented perpendicular to the long axis of tibia bone. Interestingly, mother and daughter centrioles change their original mutual orientation during the osteocyte differentiation process.⁴⁹

Although currently the studies regarding the orientation of cilia in osteocytes is limited, it is worth mentioning that the orientation of cilia in osteocytes is mainly perpendicular to the long axis of weight-bearing bone,⁴⁹ while cilia orientation in chondrocytes is parallel the long axis. So far there is a lack of evidence to draw a conclusion or general picture about whether there is a difference in cilia orientation between chondrocytes and osteocytes or how cilia orientation is regulated. Further studies are needed to understand the connection between the function of osteocytes and the orientation of cilia.

Other functions of cilia and IFT proteins in chondrocytes

Endocytosis

Endocytosis is a process by which cells internalize small molecules, macromolecules, and particles and target them to specific sealed organelles within the cytoplasm.²¹⁹ Clathrin-dependent endocytosis is responsible for the internalization of a variety of ligand-receptor complexes.²²⁰ In chondrocytes, endocytosis is important for matrix turnover (see Ref. 221). In the internalization of matrix macromolecule hyaluronan, CD44 (the receptor for hyaluronan) on the surface of chondrocytes is important for the assembly and maintenance of the cartilage matrix.^{222, 223}

The ciliary pocket is a membrane domain in which the cilium is rooted.²²⁴ The actin-based cytoskeleton and clathrin-coated pits or vesicles were discovered along the ciliary pocket membrane and ciliary base, suggesting that active membrane trafficking happens in the vicinity of the cilia (Fig. 3).^{224, 225} Rich *et al.* proposed that the ciliary pocket is a site for endocytosis.²⁹ They observed that primary cilia of femoral cartilage chondrocyte are deeply invaginated in a ciliary pocket close to the nucleus, rather than projecting from the cell surface. Clathrin-coated vesicles, endosomal proteins, and CD44 co-localize with the ciliary pocket in murine femoral chondrocytes, suggesting the chondrocyte ciliary pocket contains the sites for endocytosis.²⁹ Whether loss of cilia will lose the ciliary pocket and, thereafter, abolish chondrocyte endocytosis needs further study.

Osmotic responses

In situ chondrocytes are osmotically sensitive. Chondrocyte changes its cell size and matrix synthesis or undergoes death in response to changes in extracellular osmolarity.^{226–229} The extracellular osmolarity in cartilage is higher than the surrounding tissues. The main reason is that proteoglycans in the extracellular matrix carry fixed negative charge and attract a high concentration of cations.²²⁸ Matrix degradation in certain situations, for example cartilage wounding or degenerative diseases such as OA, would reduce the extracellular osmolarity.^{230–232} Rich *et al.* reported that *ex vivo* live murine femoral chondrocytes have shorten cilia in response to a one-minute hypo- or hyper-osmotic challenge.²⁹ This length change could result from cilia retraction or cilia disruption. However, osmotic change does not alert the primary cilia incidence.²⁹ Interestingly, osteoarthritic cartilage is exposed to a hypo-osmotic environment but exhibits longer cilia. In OA, damage of the collagen network leads to swelling, thus decreasing the interstitial osmotic pressure.²⁹ Further studies are needed to elucidate the relationship between osmotic and cilia length and the mechanism behind these phenomena.

TRPV4 is expressed in cilia, as well as in other parts of the chondrocyte cell membrane. TRPV4 can be activated by hypo-osmotic cell swelling, heat stress, phorbol esters (like 4 α -phorbol 12,13-didecanoate [4 α PDD]) and arachidonic acid and its metabolites.²³³ Exposure to 4 α PDD increases intracellular Ca²⁺ in chondrocytes, which can be blocked by the selective TRPV4 antagonist GSK205.³⁰ Phan *et al.* studied the relationship between the cilia and TRPV4 in porcine articular chondrocytes using chloral hydrate to inhibit cilia formation.³⁰ They found that the loss of cilia in the chondrocytes results in almost full loss

of Ca^{2+} signaling in response to 4aPDD or hypo-osmotic stress, suggesting that intact cilia structure is necessary for TRPV4 to function as an osmotically sensitive ion channel.³⁰

Apoptosis

Andrea *et al.* reported that a portion of hypertrophic cells with well-preserved morphology were absent of cilia.³⁵ On the basis of the evidence that most of these chondrocyte undergo apoptosis, it has been proposed that cilia were disassembled in the early stages of apoptosis.³⁵ But it remains largely unknown about the mechanisms that the cilia regulate the apoptosis or how apoptosis regulates cilia disassembly. It has been reported in the human proximal tubular HK-2 epithelial cells, shorter cilia (knockdown of Kif3a or Ift88 mRNAs) were more sensitive to cisplatin-induced apoptosis.³¹ Whether this relationship exists in chondrocytes needs further studies.

Summary and future directions

Since scientists have realized that primary cilia are not a vestigial organelle, cilia biology has drawn intense and expanded interest. It has been known that primary cilia are involved in the development and function of many organs, including the bone. But how primary cilia regulate cellular events is just beginning to be understood. HH signaling, which is essential for bone development, is largely considered to rely on cilia structure and function. However, defects in HH signaling cannot account for all of the phenotypes observed in IFT/cilia mutant mice. Therefore whether and how other pathways, such as FGF, WNT, and PDGF signaling, are regulated by cilia in bone cells are not known. It is also possible that additional pathways are involved in skeletal development through regulating primary cilia.

Great progress has been made in understanding how IFT and cilia-related proteins such as BBS and EVC are involved in cilia assembly. Moreover, very little is known about how individual IFT proteins function within the ciliary complex. Mutations in ciliary proteins result in diverse clinical symptoms with a wide variety of skeletal phenotypes, which suggests that different ciliary proteins likely have unique functions. Thus, the further studies are important for understanding the unique function and transcriptional regulation of different ciliary proteins. Over the past few years, many studies have elucidated ciliary assembly and disassembly. It is now clear that cilia are highly dynamic structures, which are assembled when cells are in G_0 stage.⁷¹ Interestingly, several components, such as specific genes and inflammatory cytokines, were found to facilitate cilia formation and to elongate cilia.^{234–236} However, whether the elongation of cilia affects cilia function and mechanotransduction remains unknown. Future studies will hopefully uncover new aspects of the dynamic nature and regulatory mechanism by integrating the subcellular structure of the cilia with their environment signaling.

Although evidence has emerged that cilia function in bone patterning and development, their precise role and mechanism in mechanotransduction in bone cells remains unclear. Much effort should be put into elucidating the roles of different ciliary proteins in mechanotransduction and the signaling pathways beyond calcium signaling downstream of mechanotransduction. Notably, cilia orientation differs in loadbearing and nonload-bearing regions, suggesting that there is an important relationship between the position of cilium and

its mechanotransduction function. Ultrastructural studies *in situ* under different conditions, such as fluid flow to uncover this relationship, might prove to be useful for learning more about this.

Another interesting question is whether osteoclasts have cilia structure and IFT protein expression during their differentiation stages. Recent studies have suggested that IFT proteins may have their unique functions independent of cilia.^{237, 238} In non-ciliated cells, such as in T lymphocytes, IFT proteins and the kinesin-II motors are present and participate in exocytosis, which is important for T cell activation.²³⁸ Both osteoclasts and T lymphocytes are derived from hematopoietic stem cells; further studies are needed to determine whether cilia/IFT proteins exist in osteoclast lineage cells, and whether IFT has a specific function in osteoclasts to direct osteoclast differentiation and function.

Although significant effort is being focused on understanding fundamental concepts of cilia function, many questions remain, including in the area of bone development. With a better understanding of the roles of cilia in bone development and diseases, therapeutics may be developed to target cilia to treat patients with bone disorders derived from cilia abnormalities. For example, based on the known mechanical sensor role of cilia in the kidney, recent studies suggest a crucial role of cilia in bone adaptation in response to the extracellular mechanical environment. Therapeutics that target cilia or cilia related proteins may mimic the anabolic effect of physical loading, and therefore may be used in the treatment of osteoporosis.

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Fig. 1.

Schema of cilia structure, IFT complexes, and BBSome. Basal bodies are modified centrioles, which provide a template to build the axoneme, the backbone of cilia. IFT occurs in two directions. *Anterograde transport* is from the base to the tip of the cilium and *retrograde transport* is from the tip back to the cell body. Anterograde transport is operated by a kinesin-II motor and the IFT-B complex. Kinesin-II complexes are composed of either KIF3A/KIF3B/KAP3 or KIF3A/KIF3C/KAP3 in vertebrates. IFT-B complex is composed of 14 IFT proteins (IFT20, IFT22, IFT25, IFT27, IFT46, IFT52, IFT54, IFT57, IFT70, IFT74/IFT72, IFT80, IFT81, IFT88 and IFT172). IFT81 and IFT74/72 form a tetrameric complex and interact with IFT88, IFT52, IFT46, and IFT27 to form the core of complex B. Retrograde transport is regulated by Dynein-2 and IFT-A complex. Dynein-2 is composed of the heavy chains D2IC, D2LIC, and LC. IFT-A contains six proteins (IFT144, IFT140, IFT139, IFT122, IFT121, and IFT43). The BBSome, composed of seven highly conserved BBS proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9), assembles IFT-A and IFT-B together to facilitate IFT complex and cargo transport. Polycystin-1 (PC-1), a G protein couple receptor (GPCR), and PC-2, a receptor-activated calcium channel, form a heterodimer that is located in cilia. EVC-1 and EVC-2 are located at the basal body and at the base of the axoneme.

Fig. 2.

The primary cilium is a HH signal transduction machine. When HH is absent, the HH receptor PTCH1 is located at the base of the cilium and inhibits SMO function. SUFU processes GLI in the cilia into its repressor form (GLIR), which inhibits related gene expression. However, when HH is present, it binds to PTCH1 and triggers PTCH1 to move out of cilia, which releases the inhibition of SMO and allows SMO to translocate into the cilium. Active SMO goes into cilia and forms a complex with EVC2 at the EVC zone and activates GLI (GLIA) by relieving the inhibition by SUFU. GLIA then turns on related gene expression. Adapted from Refs. 108 and 110.

Fig. 3.

Cilia in chondrocytes with ciliary pocket and matrix–cilium–golgi continuum. Ciliary pocket is a membrane domain, in which the cilium is rooted. It is also an endocytic membrane domain with actin-based cytoskeleton and clathrin-coated pits. CD44, the receptor for hyaluronan, was found in the clathrin-coated pits with the ciliary pocket. The collagen fibers are closely associated with the axoneme. The contact points on the membrane of the axoneme are receptors for cartilage matrix components such as integrin and NG2. The matrix–cilium–Golgi continuum exists in chondrocytes to potentially regulate the cellular secretion of ECM components. Adapted from Ref. 224.

Fig. 4.

Chondrocytes rotation and cilia orientation in the articular and growth plate cartilage. The cilia in cartilage have a clear orientation. In articular cartilage, cilia in the superficial articular cartilage project away from the articulating surface.³⁶ In the growth plate, chondrocytes in the proliferative and hypertrophic zones are formed as stacked columns that are parallel to the longitudinal axis of the bone. Cilia in these areas are found parallel to this axis as well.³⁵ However, the proliferation of chondrocytes is perpendicular to this axis and their daughter cells are displayed laterally to each other. To maintain columnar organization, the daughter cells rotate after division to merge into the stacked columnar. The cilia are believed to have an important role in the regulation of chondrocyte rotation.^{33, 35, 37} Adapted from Refs. 35 and 239.