Bone 51 (2012) 407–417

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

The intraflagellar transport protein IFT80 is required for cilia formation and osteogenesis

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ARTICLE INFO

Article history:
Received 24 March 2012
Revised 20 June 2012
Accepted 23 June 2012
Available online 4 July 2012

Edited by: J. Aubin

Keywords:
Intraflagellar transport
Cilia
Osteoblast differentiation
Skeleton development
Hedgehog signaling

ABSTRACT

Intraflagellar transport (IFT) proteins are essential for the assembly and maintenance of cilia, which play important roles in development and homeostasis. IFT80 is a newly defined IFT protein. Partial mutation of IFT80 in humans causes diseases such as Jeune asphyxiating thoracic dystrophy (JATD) and short rib polydactyly (SRP) type III with abnormal skeletal development. However, the role and mechanism of IFT80 in osteogenesis is unknown. Here, we first detected IFT80 expression pattern and found that IFT80 was highly expressed in mouse long bone, skull, and during osteoblast differentiation. By using lentivirus-mediated RNA interference (RNAi) technology to silence IFT80 in murine mesenchymal progenitor cell line-C3H10T1/2 and bone marrow derived stromal cells, we found that silencing IFT80 led to either shortening or loss of cilia and the decrease of Arl13b expression — a small GTPase that is localized in cilia. Additionally, silencing IFT80 blocked the expression of osteoblast markers and significantly inhibited ALP activity and cell mineralization. We further found that IFT80 silencing inhibited the expression of Gli2, a critical transcriptional factor in the hedgehog signaling pathway. Overexpression of Gli2 rescued the deficiency of osteoblast differentiation from IFT80-silenced cells, and dramatically promoted osteoblast differentiation. Moreover, introduction of Smo agonist (SAG) promotes osteoblast differentiation, which was partially inhibited by IFT80 silencing. Thus, these results suggested that IFT80 plays an important role in osteogenesis through regulating Hedgehog/Gli signal pathways.

Introduction

Primary cilia are microtubule-based organelles localized on the surface of almost all vertebrate cells including osteoblasts and osteocytes. These organelles are extended and maintained by the transport of particles along the axoneme mediated by intraflagellar transport (IFT) bidirectional machinery. IFT proteins are organized into two complexes. Complex A containing IFT144, 140, 139, and 122 proteins mediates retrograde transport of cargoes from the tip to the base of the cilia, while complex B containing IFT172, 88, 81, 80, 74/72, 57/55, 52, 46, 27, and 20 mediates anterograde transport of specific cargoes from the base to the tip. The movement of IFT proteins is carried out by two different microtubule-based motors: the anterograde (towards the cilia tip) motor is kinesin-II, which is composed of Kif3a and Kif3b motor subunits; the retrograde (towards the cell body) motor is cytoplasmic dynein-Ib. IFT complexes carry axonemal subunits to the site of assembly at the tip of the axoneme and are necessary for axonemal growth [1]. In recent years, the importance of IFT proteins for the development and function of the skeleton has been demonstrated due to the findings of skeletal abnormalities in human cilia-associated disorders [2–6] and in IFT-related mouse knockout studies [7–13]. Increasing studies have shown that primary cilia/IFT regulate embryonic bone development [8,14–18] and mechanically regulate bone formation in adults [19,20]. However, the function and mechanism of IFT/cilia proteins in osteoblast differentiation and function are still largely undefined.

Most recently, Xiao et al. [21] reported that targeted deletion of PKD1 (polycystin-1) in osteoblasts results in osteopenia phenotype, and impaired osteoblastic differentiation. While Kif3a deficiency reverses the skeletal abnormalities in Pkd1 deficient mice by restoring the balance between osteogenesis and adipogenesis [18]. Qiu et al. [17] demonstrated that osteoblast specific deletion of *Kif3a* causes increased cell proliferation, impaired osteoblastic differentiation, and enhanced adipogenesis in vitro. They further found that conditionally deleted *Kif3a* in osteoblasts results in the reduction or shorten of primary cilia and develops osteopenia *in vivo* and suggested that Kif3a regulates osteoblastic differentiation and function through multiple pathways including hedgehog, intracellular calcium and Wnt signaling. These findings highlighted important roles of IFT and cilia related proteins in osteoblast differentiation and bone development.

Abbreviations: IFT, intraflagellar transport; BBS, Bardet–Biedl syndrome; JATD, Jeune asphyxiating thoracic dystrophy; SRPIII, short rib polydactyly type III; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; RUNX2, Runt-related transcription factor 2; OCN, osteocalcin; BSP, Bone sialoprotein.

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doi:10.1016/j.bone.2012.06.021

A number of studies have shown that the skeletal phenotypes observed in a variety of IFT and ciliary component knockout lines can be attributed to abnormal hedgehog signaling (Hh) [8,12,22]. Hh signaling is one of the major signaling pathways that regulate osteogenesis and embryonic bone development and post-embryonic bone homeostasis [23,24]. In vertebrates, the Hh family consists of three members: Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh (Dhh) [24]. Hh protein binding to the transporter-like receptor Patched (Ptch) releases Ptch inhibition of Smoothened (Smo) allowing the transduction of the Hh signal to the primary cilium. This in turn activates Gli transcription factors that mediate the transcription of Hh target genes in cells [25–27]. Without a cilium, hedgehog signaling is abrogated, leading to a variety of skeletal malformations as well as embryonic lethality. For example, deletion of IFT88 in limb mesenchyme resulted in shortening of the bone in the limbs due to alterations in Ihh signaling and endochondral bone formation [8]. Conditional deletion of IFT88 or Kif3α in chondrocyte lineage by using Col2α1-cre lead to abnormal hedgehog signaling topography and apparent growth plate dysfunction [22,28], which are similar to conditional deletion of Ihh in postnatal cartilage ($Ihh^{flox/flox}$, Col2a-CreER) [29].

IFT80 is a newly identified IFT protein, which encodes a 777-residue protein that contains seven WD40 domains and is a component of the IFT complex B [30]. WD40 domains are short motifs of approximately 40 amino acids that form circular beta propeller structures. During intraflagellar transport, this complex helps carry materials from the base to the tip of cilia. Partial mutations of *IFT80* in humans cause Jeune asphyxiating thoracic dystrophy (JATD) and short rib polydactyly type III (SRPIII). Both diseases have severe bone abnormalities including shortening of the long bones and constriction of the thoracic cage [31–33]. SRP type III is a more severe disorder with a range of extra skeletal malformations, including cleft lip or palate, cystic renal disease, gastrointestinal, urogenital, brain and/or cardiac malformations. These two diseases often lead to death prenatally or in infancy due to respiratory insufficiency. However, currently, it is still unclear if the abnormal bone phenotype result from the effect of IFT80 mutation on osteogenesis or indirect effect of mutation of *IFT80* in human tissues. Therefore, in this study, to identify the role and mechanism of IFT80 in osteoblast differentiation, we first identified the gene expression pattern of this newly discovered protein in various mouse tissues, including skull and bone among others, and confirmed IFT80 is predominantly expressed in bone as well as during osteoblast differentiation. We further determined the effect of IFT80 on osteoblast differentiation and activation and on the Hh/Gli signaling transduction pathway. Our results demonstrated that the IFT80 gene plays an essential role in osteoblast differentiation and likely is involved in Hh/Gli signal pathway.

Materials and methods

Cell lines and cell culture

HEK293T human embryonic kidney cell line, C3H10T1/2 murine mesenchymal progenitor cell line and RAW264.7 murine monocyte/macrophage cell line were obtained from American Type Culture Collection (ATCC). For preparation of mouse BMMs and BMSCs, animal procedures were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of University at Buffalo (UB). Both femora and tibias were removed and soft tissues were detached from the bone of 6-week-old C57BL/J wild type mice. The metaphysis from both ends was resected and BMMs were collected by flushing the diaphysis with PBS. BMSCs were expanded as described previously [34]. For osteoblast differentiation, C3H10T1/2 cells and BMSCs were respectively seeded at a density of 5×10^4 cells/cm^2 and maintained in complete growth media for 24 hours (α-modified Eagle's medium with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C, then the cells were induced with osteogenic medium (OS media), i.e. the growth medium with 10^{-8} M dexamethasone, 50 μg/ml ascorbic acid and 10 mM β-glycerol-phosphate [35] for the indicated times based on different experiments. For osteoclast differentiation, RAW264.7 cells and BMMs were respectively induced with 10 ng/ml RANKL and 20 ng/ml M-CSF for 0, 24 and 96 hours [36].

IFT80 shRNA lentivirus packaging, tittering and cell infection

To identify the function of IFT80 gene in osteoblast differentiation and osteoblastic signaling pathways, we used lentivirus-mediated mouse pGIPZ-IFT80 shRNA plasmids and control pGIPZ-scrambled shRNA (Open Biosystems, RMM4532-NM 026641) to package IFT80 recombinant lentivirus according to the manufacturer's instructions. Briefly, five individual vector pGIPZ-IFT80 shRNAs were respectively co-transfected with the packaging plasmids, pCMV-Dr8.2 and pCMV-VSVG (Addgene) [37] into HEK293T cells (ATCC) using calcium phosphate co-precipitation method. The medium was replaced with fresh complete growth media after co-transfection for 8 hours. After 48–72 hours, the lentiviral supernatant was harvested and the titer was determined by infecting HEK293T cells with serial dilutions of concentrated lentivirus in the presence of 4 μg/ml polybrene (Sigma). For infection of target cells, the viral supernatant was added to C3H10T1/2 cells or BMSCs. Following incubation for 24 hours, the virus-containing media was removed and replaced with fresh growth medium. At 48 hours following the transfection, the cells were analyzed by RT-PCR, western blot and immunostaining to test silent efficiency of the IFT80 gene. For osteoblast differentiation, after the infection of the cells for 24 hours, the virus-containing media was replaced with OS media for the indicated times based on the different experiments.

Production of recombinant retrovirus

Retroviral vector pBMN-Gli2 was constructed by inserting a full-length 6.18 kb Gli2 cDNA (access no. NM_027770) into the EcoRI and Not I site of pBMN-I-GFP vector (pBMN) (Addgene), and packaging was performed as the protocol from Dr. Garry Nolan Laboratory, Stanford University. Briefly, retrovirus vectors pBMN-I-GFP (control vector) and pBMN-Gli2 were separately transfected into the phoenix-ecotropic packaging cell line by CaCl2 precipitation method [38]. Following the transfection, the cells were placed in a 32 °C humidified incubator for 48 hours. The media containing infectious virus was harvested and filtered through a 0.45 mm filter for tittering assay. The retrovirus carrying Gli2 cDNA was then used to infect 70–80% subconfluent proliferating C3H10T1/2 cells with the presence of 8 μg/ml Polybrene to enhance the efficiency of infection. The Gli2 protein expression was confirmed by performing immunostaining and western blot [36].

RT-PCR and quantitative real time RT-PCR analyses

All animal procedures were approved by IACUC of UB. Total RNA was extracted from 14-day-old C57BL/6J mouse tissues including the lung, spleen, kidney, muscle, liver, heart, brain and calvaria (free of periosteum), OS media-BMSCs and C3H10T1/2 cells, or RANKL/M-CSF-induced BMMs and RAW264.7 cells using TRIzol reagent (Invitrogen). cDNA was synthesized from 1 μg total RNA using the SuperScript III reverse transcriptase kit (Invitrogen) in a final volume of 20 μl. Primers were designed with the IDT SCI primer design tool (Integrated DNA Technologies, San Diego, California). RT-PCR experiments were performed with a Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, CA), and real-time PCR experiments were performed with an ABI prism 7500 (Applied Biosystems, Grand Island, NY) in triplicate. Sequence and product lengths for each primer pair were: BMP2, forward primer 5′ GTTTGG CCTGAAGCAGAGAC 3′ , reverse