IFT80 is required for stem cell proliferation, differentiation, and odontoblast polarization during tooth development

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Abstract
Primary cilia and intraflagellar transport (IFT) proteins control a wide variety of processes during tissue development and homeostasis. However, their role in regulation of stem cell properties during tooth development remains elusive. Here, we revealed that dental pulp stem cells (DPSCs) express IFT80, which is required for maintaining DPSC properties. Mice with deletion of IFT80 in odontoblast lineage show impaired molar root development and delayed incisor eruption through reduced DPSC proliferation and differentiation, and disrupted odontoblast polarization. Impaired odontoblast differentiation resulted from disrupted hedgehog (Hh) signaling pathways. Decreased DPSC proliferation is associated with impaired fibroblast growth factor 2 (FGF2) signaling caused by loss of IFT80, leading to the disruption of FGF2-FGFR1-Pi3K-AKT signaling in IFT80-deficient DPSCs. The results provide the first evidence that IFT80 controls tooth development through influencing cell proliferation, differentiation, and polarization, and Hh and FGF/AKT signaling pathways, demonstrating that IFT proteins are likely to be the new therapeutic targets for tooth and other tissue repair and regeneration.

Introduction
Tooth development, like many other organs' formation, requires multi-lineage cells controlled by different signaling pathways. During tooth formation, a subpopulation of mesenchymal cells differentiates into odontoblasts, which are the columnar polarized cells located at the outer edges of dental pulp, expressing dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP), and producing dentin. Ameloblasts are derived from dental epithelium and secrete enamel matrix, which is the hardest and highly mineralized tissue. Dental pulp is the soft tissue inside the tooth, and their function is to support dentin formation and regeneration¹,².

Primary cilia are highly conserved microtubule-based solitary organelles that are present on almost all vertebrate cells. Formation and function of primary cilia require intraflagellar transport (IFT) proteins and other ciliary proteins³,⁴. Mutation of those proteins usually causes cilia defects, leading to a wide range of diseases called ciliopathies. These disorders target multiple tissues, of which bone and tooth are most common tissues⁵–⁷. The arising evidence demonstrates that primary cilia play important roles in tooth development⁸,⁹. For example, Wnt1-Cre; Kif3a⁻/⁻ mice (deletion of ciliary motor-Kif3a) in dental mesenchyme) show impaired incisor and molar development⁹,¹⁰. Abnormal molar development was also found in Evc⁻/⁻ and IFT88orpk mice⁷. Hypomorphic alleles of IFT88 (Tg737orpk) showed increased Hh signaling and formation of supernumerary molar in the diastema of both the lower and upper jaws¹²,¹³. Previously, we reported that IFT80, one of the IFT complex B proteins, promotes osteogenesis¹⁴ and deletion of IFT80 in osteoblast precursor cells leads to decreased bone mass...
with impaired osteoblast differentiation through regulating Hh signaling pathway. However, their role in regulation of stem cell properties and odontogenesis during tooth development remains elusive.

Fibroblast growth factors (FGFs) are growth factors that are important for bone and tooth development and regeneration. There are 22 FGF proteins that interact with four highly conserved transmembrane tyrosine kinase receptors (FGFR1–4). Among FGFs, FGF2 is highly expressed in odontoblast lineage cells. Several studies supported that FGF2 promotes the pulp cell proliferation, self-renewal, migration, and early differentiation. However, the relationship between FGF signaling and primary cilia/IFT proteins has not been established.

In this study, we generated IFT80 conditional knockout mouse by breeding IFT80-floxed mice with Osterix (OSX)-Cre transgenic mice and examined their phenotypic and molecular changes in tooth development. We found for the first time that IFT80 governs tooth development through influencing DPSC proliferation, differentiation, and odontoblast polarization by regulating Hh and FGF/AKT signaling pathways, demonstrating that IFT proteins are likely new therapeutic targets for tooth and other tissue repair and regeneration.

Results
Conditional deletion of IFT80 impaired incisor development

OSX is a transcription factor during osteoblast differentiation from stem cells and OSX+ cells are essential for bone development. Recent studies demonstrate that OSX is also expressed in pulp cells during differentiation of odontoblasts. Therefore, we generated OSX;IFT80<sup>fl/ff</sup> mice to study the function of IFT80 in tooth development. We observed that incisors were completely absent in 15-day-old OSX;IFT80<sup>fl/ff</sup> mice, and severely underdeveloped and maloccluded in 1-month-old and 3-month-old OSX;IFT80<sup>fl/ff</sup> mice (Fig. 1a). The average incisor eruption age was around postnatal day 7 in OSX;IFT80<sup>+/+</sup> mice, whereas it was delayed to postnatal day 14 for lower incisors and postnatal day 21 for upper incisors in OSX;IFT80<sup>fl/ff</sup> mice. Mandibular incisors were isolated from their sockets for morphological analysis. OSX;IFT80<sup>fl/ff</sup> incisors were obviously shorter but more curved at all examined time points (Fig. 1b). The mean length of lower
incisors in **OSX;IFT80^ff** mice was only 0.61-fold of that in **OSX;IFT80^+/+** mice at 1 month old (Fig. 1b). Examination of skulls by micro computed tomography showed the malocclusion and defects in both mandibular and maxillary incisors in **OSX;IFT80^ff** mice (Fig. 1c). These data suggest that IFT80 is critical for incisor development. Notably, **OSX;IFT80^ff** mice also showed markedly decreased bone mass in craniofacial bones as well as alveolar bones (Fig. 1c).

Conditional deletion of IFT80 impaired pulp cell proliferation in the cervical loop

Histological analysis revealed that the cervical loops and odontoblast layers were smaller in **OSX;IFT80^ff** mice compared with those in **OSX;IFT80^+/+** mice (Fig. 2a, A1–A4 and B1–B4, B1–B4), suggesting that the proliferation might be compromised in this area. Therefore, we performed Ki67 staining to detect cell proliferation. As we expected, the results showed that proliferating cells were...
significantly reduced in the cervical loop and the dental pulp in OSX;IFT80+/− mice compared to OSX;IFT80+/+ control mice (Fig. 2c). Together, these data implied that IFT80 is required for the odontoblast lineage cell proliferation and incisor growth.

**Conditional deletion of IFT80 caused shorter molar root, less mineralized dentin, and disrupted odontoblast differentiation**

We next examined molar development and found that molars were normally erupted in both OSX;IFT80+/− and OSX;IFT80+/+ mice. The crowns of molars were well-formed but the roots were shorter in OSX;IFT80+/− mice compared with those in OSX;IFT80+/+ mice (Fig. 3a and Fig. S1A and S1B). Quantitative analysis of the root and crown length of first molars in mandible showed that the roots from OSX;IFT80+/− mice were significantly shorter than those from OSX;IFT80+/+ mice (Fig. 3b), whereas crown length was similar in both groups. Thus, the crown-to-root ratio was significantly increased in OSX;IFT80+/− mice (Fig. 3b).

To further uncover the role of IFT80 in dentin formation and mineralization, we performed backscattered electron analysis. OSX;IFT80+/− mice had well-organized
and patterned dentinal tubules. In contrast, the dentinal tubules in OSX;IFT80f/f mice were disorganized with less mineralization (Fig. 3c). Odontoblasts are responsible for the production of calcified dentin matrix; therefore, these data suggested that IFT80 is involved in odontoblast differentiation and function. We further observed odontoblast morphology in the early stage of root formation. At postnatal day 14, odontoblasts were elongated and highly polarized in the proximal portion of the root in OSX;IFT80+/+ mice (Fig. 3d), and the nuclei were polarized in the pulp side of the odontoblasts. These cells were attached to each other with their terminal webs (Fig. 3d, cyan arrows). However, in OSX;IFT80f/f mice, odontoblasts were disorganized along the proximal portion of the root. Some of the odontoblasts displayed a reversed cell orientation with the nuclei close to the dentin side of the odontoblasts (Fig. 3d). In addition to the morphology changes, we also found that the expression of odontoblast marker genes (DMP1 and DSPP) in dental pulp was strikingly reduced in OSX;IFT80f/f mice by quantitative PCR (qPCR) (Fig. 3e), confirming that IFT80 deletion impaired odontoblast differentiation.

Deletion of IFT80 disrupted cilia formation in odontoblasts and dental pulp cells

To gain insight into the mechanism that IFT80 deficiency caused defective dental pulp cell differentiation and tooth development, we first examined cilia formation (Fig. 4a). In molar section, cilia were found on almost every ameloblast and the cilia length was similar between OSX;IFT80+/+ mice and OSX;IFT80f/f mice (Fig. 4a, b, c). However, in OSX;IFT80f/f odontoblasts, cilia formation was severely disrupted. The ciliated cell population reduced to 20% in OSX;IFT80f/f mice compared with 80% in OSX;IFT80+/+ mice (Fig. 4b). Moreover, the cilia length of ciliated odontoblasts was significantly shorter in OSX;IFT80f/f mice than that in OSX;IFT80+/+ mice (Fig. 4c). Additionally, the ciliated cell population but not cilia length were also reduced in dental pulp cells in OSX;IFT80f/f mice (Fig. 4b, c).

Deletion of IFT80 disrupted cilia formation and Hh signaling in DPSCs

To further examine the role of IFT80 in dental pulp cell proliferation and differentiation in vitro, we isolated primary DPSCs from the dental pulp of mouse incisor. DPSCs isolated from IFT80f/f mice were infected with adenovirus-expressing Cre recombinase to delete IFT80 (named as IFT80f/fΔ). Adenovirus expressing green fluorescent protein (GFP)-infected IFT80f/fΔ DPSCs were used as control (still marked as IFT80f/fΔ). Western blot and qPCR confirmed that Cre adenovirus transduction significantly reduced IFT80 expression in DPSCs (Figs. 5a, b).

We compared cilia formation between IFT80f/f and IFT80f/fΔ DPSCs by immunostaining. Consistent to the results from in vivo tooth slide staining (Fig. 5a, b), the results showed that the ciliated population in IFT80f/fΔ DPSCs was reduced to <30% and the average cilia length was decreased to 1.56 μm compared with 80% and 3.72 μm in IFT80f/f DPSCs, respectively (Fig. 5c, d). These results demonstrated that IFT80 is required for cilia formation in both dental pulp cells and odontoblasts. Primary cilia are essential for Hh signaling transduction, and our previous study has demonstrated that IFT80 is required for Hh signaling transduction in osteoblast differentiation15; therefore, we tested Hh signaling activation in IFT80f/f and IFT80f/fΔ DPSCs. As expected, sonic Hh (Shh) activated Gli promoter activity as well as Gli1 and Ptc1 expression in IFT80f/fΔ DPSCs (Fig. 5e, f), indicating Hh pathway activation. However, these responses were impaired in IFT80f/fΔ DPSCs (Fig. 5e, f), confirming disrupted Hh signaling transduction in IFT80f/fΔ DPSCs.

Deletion of IFT80 impaired DPSC proliferation through defect of FGF signaling rather than Hh signaling

Since our in vivo results suggested that OSX;IFT80f/f mice has less proliferating cells in dental pulp (Fig. 2c), we then compared the proliferation rate between IFT80f/f and IFT80f/fΔ DPSCs by MTS assay and Ki67 staining. The results showed that IFT80 deletion inhibits DPSC proliferation (Fig. 6a, b). To further test if disrupted Hh signaling in IFT80f/fΔ DPSCs contributes to the DPSC proliferative defect, Hh agonist purmorphomine was used to stimulate DPSCs in both IFT80f/f and IFT80f/fΔ groups. The results showed that activation of Hh signaling by purmorphomine did not affect DPSC proliferation in both groups (Fig. 6c, d), suggesting that the pathway(s) other than Hh signaling existed to regulate DPSC proliferation and were affected by loss of IFT80.

FGF signaling regulates the proliferation of many types of cells including dental pulp cells15,26. We found that FGF2 dramatically promotes proliferation of IFT80f/fΔ DPSCs, but failed to increase the proliferation of IFT80f/fΔ DPSCs (Fig. 6e, f), indicating that deletion of IFT80 impaired FGF2 signaling transduction, which caused defective DPSC proliferation.

Deletion of IFT80 inhibited DPSC proliferation via downregulation of FGF-Pi3K-AKT signaling

Phosphorylated FGFR activates phosphatidylinositol 3-kinase (PI3K) and AKT pathway, which converts FGF signaling to the proliferative effect. Consistently, we found that, in response to FGFR stimulation, AKT phosphorylation increased in IFT80f/fΔ DPSCs, which was inhibited by PD173074 (FGFR inhibitor) or API-2 (AKT inhibitor) treatment (Fig. 7a, b). In IFT80f/fΔ DPSCs, AKT expression was comparable to IFT80f/f; however, AKT
phosphorylation level induced by FGF2 in IFT80\textsuperscript{d/d} DPSCs was much lower than that of IFT80\textsuperscript{f/f} DPSCs (Fig. 7a, b), confirming that FGF2-FGFR signaling was blocked in IFT80\textsuperscript{d/d} DPSCs. We further found that PD173074, LY294002 (PI3K inhibitor), API-2, or rapamycin (mammalian target of rapamycin (mTOR) inhibitor) inhibited the proliferation of FGF2-induced IFT80\textsuperscript{f/f} DPSC but not IFT80\textsuperscript{d/d} DPSCs (Fig. 7c). These data implied that IFT80 is essential for DPSC proliferation via regulating FGF2-FGFR1-PI3K-AKT-mTOR signaling pathway.

**Discussion**

Although primary cilia have been observed in DPSCs and odontoblasts more than a decade ago\textsuperscript{27}, the function of primary cilia and IFT proteins in DPSCs has not been well addressed. Our study reveals for the first time that IFT80 is required for tooth development by regulating DPSC proliferation, differentiation, and odontoblast polarization. Deletion of IFT80 in DPSCs impairs FGF2-FGFR1-PI3K-AKT signaling that inhibits DPSC proliferation. Moreover, IFT80 deletion impairs cilia formation and Hh signaling transduction, which eventually inhibits DPSC differentiation and tooth development.

Primary cilium projects from the cell surface of most mammalian cells and coordinates of various signaling pathways during development and homeostasis. Although the exact roles of primary cilia in tooth development remain elusive, the emerging evidence indicates that primary cilia in both dental epithelium and mesenchyme are pivotal for early odontogenesis and later tooth development\textsuperscript{28,29}. A most recent study showed that IFT140, one of the IFT protein that formed IFT complex A, is highly expressed in odontoblasts and knockout of IFT40 leads to delayed and defective dentin formation due to poor odontogenic differentiation, abnormal primary cilia, and decreased Hh signaling\textsuperscript{30}. In this study, we used an IFT80 conditional knockout mouse model, which is one of the components of IFT complex B, and found the similar dental phenotypes—reduced odontoblast proliferation and shorter root formation. Deletion of IFT140 or IFT80 all causes defects in cilia formation, suggesting that primary cilia, instead of individual IFT protein, play an essential role in the odontoblast proliferation and
differentiation. However, inconsistently, hypomorphic alleles of IFT88 (Tg737orpk) showed increased Hh signaling and formation of supernumerary molars in the diastema of both the lower and upper jaws, indicating that IFT protein likely also regulates cilia-independent pathway during tooth development.

Hisamoto et al.31 studied the pattern of cilia in tooth germ and oral cavity and found that the cilia in odontoblasts first appears near the root apex and then extends along the odontoblast layer31. This might explain why we observed severe root formation defects in IFT80 knockout mice. Their study also indicated the stage-specific and region-specific cilia formation and cilia length regulation. A reasonable assumption is that the cell differentiation status determines the cilia length, which closely related to their sensory function29,31. In IFT80 knockout mice, we also observed the cilia length change in both pulp cells and odontoblasts (Fig. 4). Whether cilia length is directly associated with differentiation status needs further study. Our data here suggest that in addition to odontoblast differentiation and pulp cell proliferation (Fig. 2), odontoblast polarization (Fig. 3) requires functional cilia.

Primary cilium serves as a signaling hub and a variety of receptors and signaling proteins locate in cilia3,32. For example, the key components of Hh signaling including Patched1 (Ptc1), Smoothened, and Gli transcription factors are present in primary cilia33,34. It is well known that Hh signaling transduction requires IFT proteins and other ciliary protein35. In our previous study, we reported that IFT80 is essential for Hh signaling in osteoblasts, chondrocytes, and bone marrow derived mesenchymal stem cells14,15,36,37. Consistently, in this study, we found that IFT80 deletion in DPSCs disrupts cilia formation and Hh signaling transduction (Figs. 4 and 5). In addition to Hh signaling, emerging studies have suggested that IFT proteins and primary cilia mediate platelet-derived growth factor signaling38, Notch39, insulin-like growth factor40,41, and epidermal growth factor32 signaling pathways. Besides these critical pathways, cilia formation is also regulated by other signaling pathways like FGF signaling. Mutation of FGFR1, a receptor of FGF signaling, in zebrafish shortens cilia42. Whether IFT proteins regulate FGF signaling was unknown. In our study, we found that loss of IFT80 in DPSCs dramatically blocked FGF2-induced DPSC proliferation (Fig. 6), indicating that IFT80-mediated primary cilium formation is important for FGF2 transduction. It is still not clear how primary cilia loss effects FGF2 signaling. One possible explanation is that the receptor for FGF2 is normally located in primary cilia and loss of cilia leads to the dysregulation of FGFR.
Another possible explanation is that primary cilia indirectly effect FGF2 signaling by targeting the downstream signaling, such as PI3K-AKT.

FGF2 signaling has been recognized as an important signaling that regulates both cell proliferation and cell differentiation; however, its functions are complicated and stage-dependent. Our work showed that IFT protein/primary cilia regulates the FGF2 signaling, which indicated a new regulatory mechanism of FGF2 signaling during odontogenesis and tooth development. Further study regarding how IFT and cilia regulate stem cell self-renewal and differentiation is going on. Collectively, this study demonstrated for the first time that IFT80 is a critical regulator of DPSC proliferation and tooth development. IFT80 maintains cilia formation and stimulates DPSC proliferation. Thus, we revealed a novel role and mechanism of IFT80 in the regulation of DPSC and tooth development, and provided new insights for bone and tooth regenerative therapeutic design and therapy.

Materials and methods

Mice

All experiments performed on mice were approved by the University at Buffalo Institutional Animal Care and Use Committee. The generation of IFT80lox/lox mice model (two LoxP sites flanking exon 6 of IFT80) was previously described. OSX-Cre transgenic mice (also known as OSX1-GFP::Cre (Jackson #006361)) were crossed with IFT80+/− mice to generate OSX-Cre;IFT80lox/lox mice, which were crossed with IFT80lox/lox mice to obtain OSX-Cre;IFT80lox/lox mice (named as OSX:IFT80lox/lox). OSX-Cre mice were used as control mice (named as OSX:IFT80lox/lox).

Reagents

Recombinant mouse Shh N-terminus (1 μg/mL, R&D Systems, Minneapolis, MN, USA) or purmorphomine (2 μM, Tocris Bioscience, 4551) was used to activate Hh signaling. FGF2 (10 ng/mL, R&D Systems, Minneapolis, MN, USA) was used to activate FGF signaling. PD173074 (1 μM, Tocris, 3044) was used to inhibit FGFR. LY294002 (1 μM, Tocris, 3044) was used to inhibit FGFR. LY294002 (1 μM, Tocris, 3044) was used to inhibit FGFR.
(15 μM, Sigma, L9908), API-2 (1 μM, Tocris, 2151), rapamycin (0.5 μM, Selleckchem, S1039) were employed to inhibit PI3K, AKT, and mTOR, respectively.

**Backscattered and resin-casted scanning electron microscopy**

Extracted and cleaned molars were fixed and cut longitudinally. Acid etching was performed with 37% phosphoric acid for 5 s and followed by washing with 5.25% sodium hypochlorite for 5 min on the dentin surface as described before. The specimens were dehydrated in a graded ethanol series (30%, 50%, 70%, 85%, and 100% for 15 min) and washed with hexamethyldisilizane. The samples were air dried overnight and then coated with carbon. A Hitachi SU70 scanning electron microscope (Hitachi Instruments, Schaumburg, IL, USA) was used to perform the analyses.

**Histology**

Mouse mandibles were excised, fixed with 10% natural-buffered formalin (VWR International, West Chester, PA, USA), and decalcified in 10% EDTA (ethylenediaminetetraacetic acid, Thermo Fisher Scientific) for 2 weeks at 4°C. Paraffin-embedded samples were sectioned and stained with hematoxylin and eosin.

**DPSC isolation, culture, and differentiation**

The incisors were isolated from the mandibles that were dissected from 6-week-old IFT80f/f mice. Whole dental pulp was gently collected from the interior of the incisor and exposed to enzymatic digestion with collagenase type I (3 mg/mL) and dispase (4 mg/mL) for 1 h at 37°C with shaking. The digested tissues were homogenized by repetitive pipetting and the released cells were centrifuged at 200 × g for 10 min. The cells were cultured in α-modified Eagle’s medium (α-MEM, Life Technologies) containing 10% fetal bovine serum (FBS, Life Technologies), 2 mM l-glutamine (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). DPSCs could adhere to the plastic dish for 24 h and then the medium was changed to remove floating debris. Culture medium was replaced every 3 days until the cells reach 80% confluence. Then, the cells were detached by 0.25% Trypsin-EDTA (Life Technologies) and subcultivated at a ratio of 1:2.5–4.

DPSCs from IFT80f/f mice were infected with adenovirus that overexpress either Cre (Ad-CMV-Cre, #1405, Vector Biolabs) or GFP (Ad-GFP, #1060, Vector Biolabs). Ad-CMV-Cre infection causes IFT80 deletion in IFT80f/f DPSCs, which were then marked as IFT80f/f. Ad-GFP were used as an infection control and Ad-GFP-treated IFT80f/f DPSCs were still marked as IFT80f/f.

**Western blot**

Cells were harvested and homogenized with radioimmunoprecipitation assay buffer. Proteins were denatured in sodium dodecyl sulfate (SDS) buffer and separated with SDS-polyacrylamide gel electrophoresis...
gels. Proteins were transferred to polyvinylidene difluoride membranes and then blocked with 5% skimmed milk (OXOID). Membranes were incubated with primary antibody overnight at 4 °C, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1:10,000, Novex, Carlsbad, CA, USA) at room temperature for 1 h. Visualization was performed with WesternBright ECL HRP (Bio-Rad). β-Actin (1:500, Santa Cruz) was used as the internal control.

The same procedure was used to determine the IFT80 (1:400, PAB15842, Abnova), ACT (1:300, sc-8312, Santa Cruz), and p-AKT (1:300, sc-7985-r, Santa Cruz).

Quantitative PCR
Total RNA was extracted from cultured DPSCs with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then synthesized to complementary DNA (cDNA) with total RNA by RNA to cDNA EcoDry Premix Kit (Clontech, Palo Alto, CA, USA). qPCR was performed with ABI PRISM 7500 Real-Time PCR Machine (Invitrogen, Carlsbad, CA, USA) and SYBR Green PCR Master Mix (Invitrogen). Sequence and product length for each primer pair were listed in Supplementary Table S1. Gene expression was normalized to the housekeeping gene GAPDH and calculated according to the 2−ΔΔCT method. All reactions were run in triplicate.

Immunocytochemistry and immunofluorescence
Deparaffinized sections or fixed cells were permeabilized, blocked, and incubated with primary anti-Ki67 antibody (1:50 for slides, 1:200 for cells, ab16667) overnight at 4 °C, and then incubated with Alexa Fluor 568-conjugated anti-rabbit IgG (1:100 for slides, 1:200 for cells, ab16667) overnight at 4 °C, and then incubated with Alexa Fluor 568-conjugated anti-rabbit IgG (1:100, Invitrogen) antibody or for 1 h at room temperature. DAPI (6-diamidino-2-phenylindole, Sigma) was used as a counterstain for nuclei.

The same staining procedure was used for acetylated α-tubulin (1:500, T6793, Sigma). For primary cilia staining of DPSCs, the cells were serum starved for 48 h to induce ciliogenesis.

MTS assay
The MTS assay was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega).

Reporter assay
To test Gli promoter activity, reporter assay was performed with Gli-responsive luciferase reporter construct (8×Gli-Luc) (gift from Dr. Fernandez-Zapico). DPSCs (1×10⁶) were transfected with 3 µg 8×Gli-Luc and 0.6 µg pRL-TK Renilla luciferase (Promega, internal control) with Fugene HD transfection reagent. Cells were induced with OS medium for 3 days and then stimulated with 1 µg/mL of recombinant mouse Shh N-terminus (R&D Systems, Minneapolis, MN, USA) for 8 h. Cells were harvested and colored by the Dual-luciferase Assay Kit (Promega, Madison, WI, USA) and the relative luciferase activity was measured with the Veritas microplate luminometer (Turner Biosystem, Sunnyvale, CA, USA). The experiment was conducted in triplicate.

Statistical analysis
All data are presented as mean ± SEM (n = 3 or more as indicated in figure legends). Comparisons between two groups were performed by Student’s t test and comparisons among grouped samples were analyzed by two-way analysis of variance followed by Tukey’s multiple comparison. P < 0.05 was considered to be statistically significant. The program GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) was used for these analyses.

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Conflict of interest
The authors declare that they have no conflict of interest.

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