Odontoblast differentiation is regulated by an interplay between primary cilia and the canonical Wnt pathway

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ABSTRACT

Primary cilium is a protruding cellular organelle that has various physiological functions, especially in sensory reception. While an avalanche of reports on primary cilia have been published, the function of primary cilia in dental cells remains to be investigated. In this study, we focused on the function of primary cilia in dentin-producing odontoblasts. Odontoblasts, like most other cell types, possess primary cilia, which disappear upon the knockdown of intraflagellar transport-88. In cilia-depleted cells, the expression of dentin sialoprotein, an odontoblastic marker, was elevated, while the deposition of minerals was slowed. This was recapitulated by the activation of canonical Wnt pathway, also decreased the ratio of ciliated cells. In dental pulp cells, as they differentiated into odontoblasts, the ratio of ciliated cells was increased, whereas the canonical Wnt signaling activity was repressed. Our results collectively underscore the roles of primary cilia in regulating odontoblastic differentiation through canonical Wnt signaling. This study implies the existence of a feedback loop between primary cilia and the canonical Wnt pathway.

1. Introduction

Primary cilia are small sensory organelles that are regarded as crucial for the development of various tissues [1]. Primary cilia are assembled from plasma-membrane-tethered basal bodies, which act as a basis for ubiquitous the extension of their microtubule (MT)-based axonemes from the cell surface, encapsulated by a membranous sheath. Axonemes take on a 9 + 0 MT configuration, which serves as a track for intraflagellar trafficking. Intraflagellar transport (IFT) protein was discovered by Kozminski et al. [2] and further characterized by Cole et al. [3]. This machinery involves large protein assemblies composed of complexes A and B; the former participate in retrograde trafficking while the latter perform anterograde transport in cilia. Each component of IFT has been investigated extensively [4], and some components are responsible for human ciliopathies [5-7]. IFT88, also known as polaris, is one of the principal components of the IFT-B complex and is indispensable for ciliogenesis [5].

Various signaling pathways are associated with primary cilia, such as the canonical [8, 9] and non-canonical Wnt pathways [8, 10, 11], the hedgehog (Hh) signaling pathway [12, 13], and the platelet-derived growth factor receptor α pathway [14]. Technically, primary cilia inhibit canonical Wnt signaling by restricting β-catenin nuclear entry [8, 9]. Activation of the canonical Wnt signaling pathway rescues β-catenin from degradation by a complex with glycogen synthase kinase 3 beta (GSK3 β), leading, in turn, to β-catenin-mediated gene transcription [11].

Several studies have revealed the presence of primary cilia in odontoblasts [15-18], which produce dentin, as well as the oral environment sensors built into teeth [19]. Odontoblasts line the outermost surface of dental pulp [19], and therefore show a palisading array on a longitudinal section. Moreover, primary cilia are aligned vertically on the dentin walls, with

their tips oriented toward the pulp core [18]. However, very little is known about the physiological functions of odontoblastic cilia. In this study, we employed the KN-3 cell line, which has been regarded as an authentic model for inducing odontoblasts from iPS cells [20], to analyze the functions of primary cilia in odontogenesis. The deletion of primary cilia through *Ift88* knockdown increased the activity of canonical Wnt signaling, which facilitated the formation of dentin. In addition, the activation of the canonical Wnt pathway also decreased the ratio of ciliated cells. To our knowledge, this is the first report to show that primary cilia regulate odontoblastic differentiation through Wnt signaling pathways, forming a feedback system with the canonical Wnt signaling.

2. Materials and Methods

2. 1. Antibodies and reagents

For immunocytochemistry, we employed monoclonal anti-acetylated (Ac)-α tubulin (Tu) (6-11B-1; Sigma–Aldrich, St. Louis, MO, USA), and rabbit polyclonal anti-γTu (Abcam, Cambridge, Cambridgeshire, UK) antibodies. For immunoblotting, we employed anti-intraflagellar transport (IFT)-88 protein (Proteintech Group, Inc. Chicago, IL, USA), anti-GAPDH (Millipore, Billerica, MA, USA), anti-Cyclin D1 (St John's Laboratory Ltd., University Way, London, UK), anti-Cyclin E2 (Bioss Antibodies Inc., Woburn, MA, USA), anti-dentin sialoprotein (DSP; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-β-catenin (BD Biosciences, Franklin Lakes, NJ, USA) antibodies. As secondary antibodies for immunocytochemistry, Alexa Fluor 568 goat anti-rabbit IgG, and Alexa Fluor 488 goat anti-mouse IgG were purchased from Life Technologies (Carlsbad, CA, USA). Secondary antibodies for immunoblotting, horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG, were purchased from Cell Signaling Technology. Recombinant WNT3A was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). SB216763 was purchased from

Sigma-Aldrich.

2.2. Cells

A rat clonal dental pulp cell line with odontoblastic properties (KN-3) [21] was cultured in α-minimum essential medium (αMEM) containing 10% fetal bovine serum (FBS). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. To analyze the primary cilia, the cells were seeded and cultured for 72 h and then serum starved in serum-free αMEM for 24 h. Rat gingival cells and dental pulp cells (DPCs) were isolated from the gingival epithelium and dental pulp in the upper molar germs of four-day-old rats [22, 23]. The media, in which the gingival cells and DPCs were cultured were used as condition media. To monitor the odontogenic differentiation, DPCs were collected every 3 days at confluency. For agonist assays, 100 ng/mL of recombinant WNT3A was added to the cultures. For inhibition assays, 2 μM of SB216763 was added to the cultures.

2.3. Animals and preparation of tissue.

The crania of Wister rats (embryonic day 16; post-natal days 0, 3, 7, and 14) were harvested and immersed in 4% paraformaldehyde (w/v) at 4 °C overnight. Tissues were then embedded in OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in liquid nitrogen. Serial vertical frozen sections (5 μm thick) were cut on a cryostat at −20°C and mounted on silane-coated slides.

2.4. Staining

Cells were cultured on a Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Fisher Scientific Inc., Waltham, MA, USA), and fixed in chilled 100% methanol for 15 minutes at -20 °C. The frozen section was fixed in 4% paraformaldehyde (w/v) for 20 minutes. For

hematoxylin and eosin staining, Mayer's Hematoxylin (MUTO PURE CHEMICALS CO., LTD., Bunkyou-ku, Tokyo, Japan) and 0.1% Eosin Y Ethanol Solution (Wako Pure Chemical Industries, Ltd., Osaka, Osaka, Japan) were used. For immunocytochemistry, primary anti-Ac-αTu (1:2000) and anti-γTu (1:1000) antibodies were used for detecting primary cilia and basal bodies, respectively. As secondary antibodies, Alexa-Fluor-labeled immunoglobulins were used at a ratio of 1:400.

2.5. Electron Microscopy

For scanning electron microscopy of the KN-3 cells, cell monolayers grown on coverslips were fixed in 2% (w/v) paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer with a pH of 7.4 for 1 h at room temperature. For the transmission electron microscopy of the KN-3 cells, cells on coverslips were fixed in 2% (w/v) paraformaldehyde, 2.5% glutaraldehyde, and 2% (w/v) tannic acid in 0.1 M cacodylate buffer with a pH of 7.4 for 1 h at room temperature. The remainder of the procedure was performed as previously described [24].

2.6. Plasmid construction

Lentiviral expression vector encoding shRNA for *Ift88* (sh-*Ift88*) was constructed by inserting double-stranded DNA fragments comprising the following sense-loop-antisense DNA sequences: 5'- GCA GGA AGA CTG AAA GTG AAT CTC GAG ATT CAC TTT CAG TCT TCC TGC -3'. For the lentiviral expression of a fluorescence ubiquitination-based cell-cycle indicator [Fucci; AmCyan1-hGeminin (1-110)], the coding region of the pRetroX-SG2M-Cyan Vector (#631462, Clontech Laboratories, Mountain View, CA, USA) was amplified with KOD Plus Neo using Fwd (5'-TATGGCCCTGTCCAACAAGTTCATCG-3') and Rev (5'-GTTACAGCGCCTTTCTCCGTTTTTCTG-3') primers and then ligated to the Eco47III site of CSII-CMV-MCS-IRES2-Bsd (provided by Dr. Hiroyuki Miyoshi (RIKEN BioResource

Center) along with the packaging plasmids pCAG-HIVgp and pCMV-VSV-G-RSV-Rev). W7/pGEM T-easy was kindly provided by Dr. Takafumi Noma [25]. Lentiviral pLKO.1 (Dharmacon Inc., Lafayette, CO, USA) was used as the parental vector.

2.7. Lentivirus preparation

The 293T packaging cells in Dulbecco's modified Eagle medium (DMEM) were seeded at 7×10^6 cells per 10 cm tissue-culture plate. The cells were cultured for 12 hours at 37 °C in a humidified atmosphere with 5% CO_2. Subsequently, the lentiviral expression vector and packaging vectors were transfected into the cells using polyethylenimine “Max” (Polysciences, Inc., Warrington, PA, USA). After 16 hours, the medium was removed from the plate, and fresh medium was added. After 48 hours, the medium containing the lentivirus was harvested and concentrated using the Lenti-X Concentrator (Clontech Laboratories).

2.8. Lentivirus infection

KN-3 cells were seeded at 1×10^5 cells per well of six-well culture plates in αMEM. The cells were cultured overnight at 37 °C in a humidified atmosphere with 5% CO_2. Subsequently, lentivirus in a medium containing 8 μg/mL hexadimethrine bromide (polybrene) was added to the well. After overnight cultivation, the medium containing lentiviral particles was removed from the well and fresh medium without polybrene was added. To select the cells expressing sh-*Ift88* and Fucci after 48 hours, 1 μg/mL puromycin and 0.25 μg/mL blasticidin were added to the wells, respectively.

2.9. RT-PCR and real-time RT-PCR

Cells were collected, and total RNA was extracted by following the manufacturer's instructions (RNeasy kit, (Qiagen, Hilden, Germany)). Total RNA (1 μg) was reverse-transcribed with

RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific Inc.) at 42 °C for 60 minutes, according to the manufacturer's protocol. RT-PCR was performed by using LaboPass™ G-Taq (Hokkaido System Science, Hokkaido, Japan) in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Real-time RT-PCR was performed by using the Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) in a StepOne Plus™ Real-Time PCR System (Applied Biosystems). Reactions were performed in a 10 μL reaction mixture containing 1 μL of cDNA, 0.5 μL of each primer (10 μM), and 5 μL of SYBR qPCR Mix. The primer sets for each target are listed in Table 1. The absence of non-specific PCR products was checked with melting-curve and electrophoresis analyses. The relative copy numbers were computed based on data obtained through a serial dilution of a representative sample for each target gene.

2.10. Immunoblot analysis

Cells were lysed in the RIPA buffer. The lysate, which was diluted in 1× SDS sample buffer with 2-mercaptoethanol, was boiled for 3 minutes and was then subjected to SDS–PAGE on 10% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes with a blotting apparatus. The membranes were then incubated for 1 h in a blocking buffer (5% dry non-fat milk in PBS) and subsequently incubated overnight with anti-IFT88 (1:1000), anti-GAPDH (1:1000), anti-cyclin D (1:500), anti-cyclin E (1:500), anti-DSP (1:200), and anti-β-catenin (1:1000) antibodies in the blocking buffer. Next, the membrane was washed five times in PBS and then incubated for 2 h with HRP-conjugated anti-mouse (1:3000) or anti-rabbit (1:3000) IgG in the blocking buffer. After extensive washes with PBS, immunoreactive proteins were detected by using an Immobilon Western (Millipore).

2.11. Cell-cycle analysis

The expression of Fucci-SG2M was analyzed by using an ArrayScan VTI HCS Reader (Thermo Fisher Scientific Inc).

2.12. Alkaline phosphatase (ALP) activity assay

Cells were collected in 200 μL RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, and 0.1% SDS). The ALP activity in the cleared lysates was measured with an Alkaline Phosphatase Detection Kit (Sigma-Aldrich) according to the manufacturer's protocol. ALP activities in DPCs and KN-3 cells were normalized to the total protein content and the DNA content, respectively. The DNA content was also quantified using 15 μL of the same lysate by adding 15 μL of 0.02 N NaOH and 60 μL of 0.1 g/mL 3,5-diaminobenzoic acid. After incubation in the dark for 45 min at 65 °C and then mixing with 2 N HCl, the samples were spectrophotometrically analyzed at wavelengths of 414 and 492 nm.

2.13. Alizarin red S staining

As shown in Fig. 2D, KN-3 cells were cultured in a medium conditioned by primary rat gingival (control) or pulp (for inducing mineralization) cells. Cells cultured in 96-well plates were washed with phosphate-buffered saline and then fixed in 4% paraformaldehyde (w/v) for 15 min at room temperature. The cells were then washed with water and stained with 1% Alizarin red S for 5 min at room temperature. After five washes with water, images of the stained cells were captured and digitally processed.

2.14. Statistics

The data are presented as means ± standard deviations; the statistical significance of the

differences in mean values was assessed by performing Student's unpaired *t*-tests. Differences among the mean values were considered significant at a *P*-value < 0.05. All the experiments were repeated at least twice, and similar results were obtained.

3. Results

3.1. Inhibition of primary cilia formation in KN-3 cells

To identify the primary cilia in odontoblasts, we analyzed KN-3 cells through immunocytochemistry (Fig. 1A), scanning electron microscopy (Fig. 1B), and transmission electron microscopy, which revealed the 9 + 0 axonemal configuration (Fig. 1C). The axonemes and basal bodies of primary cilia were stained with antibodies against acetylated α-tubulin (Ac-αTu) and γ-tubulin (γTu), respectively. These results suggest that the identity of the ciliary projections in KN-3 was that of genuine primary cilia (Fig. 1A–C).

Furthermore, to test whether primary cilia are indispensable for the differentiation of odontoblasts, we treated KN-3 cells with a lentiviral expression vector for shRNA for Intraflagellar transport (Ift)-88 (sh-*Ift88*) to inhibit ciliogenesis (Fig. 1D–F). The treatment of the cells with shRNA reduced the expression of *Ift88* to approximately 30% of that in the control (Fig. 1D), and resulted in a substantial decrease in the IFT88 protein level (Fig. 1E). Under this condition, there was a significant decrease in the ratio of ciliated cells (Fig. 1F). These results suggest that IFT88 plays a crucial role in the formation of primary cilia in KN-3 cells (Fig. 1D–F).

3.2. Primary cilia regulate the differentiation of odontoblasts

No differences were observed in the cell-cycle between IFT-88 knocked-down cells and control cells under conditions, in which primary cilia were present (suppl. Fig. 1). Next, we focused on the roles of primary cilia in the differentiation of odontoblasts. Interestingly, non-

ciliated KN-3 cells increased the expression level of dentin sialoprotein (DSP) (Fig. 2B), which was further witnessed with the increased expression of dentin sialophosphoprotein (Dspp) mRNA (Fig. 2A). Since *DSPP* encodes two major noncollagenous dentin matrix proteins, DSP and dentin phosphoprotien (DPP), *Dspp* and DSP were used as molecular markers for the maturation of odontoblasts [26].

In contrast, alkaline phosphatase (ALP) activity was significantly suppressed in non-ciliated cells (Fig. 2C). ALP induces the mineralization of odontoblasts [27] and is used as an odontoblastic terminal differentiation maker. Furthermore, non-ciliated KN-3 cells showed a slowed mineralization in the presence of a conditioned medium from either gingival cells or dental pulp cells (DPCs) [23] (Fig. 2D); this result is consistent with the decreased ALP expression (Fig. 2C).

Collectively, the loss of primary cilia has dual effects on odontoblasts: induction of dentin matrix and suppression of extracellular matrix maturation (hard tissue formation) by suppressing the activity of ALP.

3.3. Elongation of primary cilia during tooth development.

In order to further clarify the role of primary cilia in odontoblastic differentiation, we analyzed the developmental changes in primary cilia in terms of tooth morphogenesis from embryonic day (E) 16 to post-natal day (P) 14 (Fig. 3). Odontogenesis starts at the dental lamina, a thickening of the epithelium in the upper and lower jaw. Individual teeth develop through the bud, cap, bell, and crown stages of the tooth epithelium. At the crown stage, dental papilla cells differentiate into odontoblasts [28]. The odontoblasts produce dentin, which form the crown of the tooth. After completing tooth crown formation, root formation follows [29]. At the bell stage at E16, the primary cilia in the dental papilla were short (Fig. 3). When odontoblasts emerged in the bell stage at P0 and the dentin commenced

calcification in a crown stage at P3, the primary cilia in odontoblasts were short and were directed in a random manner (arrowhead in Fig.3). When enamel calcification commenced in the crown stage at P7, the odontoblastic primary cilia were elongated and aligned vertically on the dentin walls, with their tips oriented toward the pulp core (Fig. 3). This trend was sustained at P14, when the morphogenesis of the tooth root was finalized. These results collectively suggest that the primary cilia in odontoblasts are associated with the differentiation of odontoblasts.

3.4. Primary cilia inhibit canonical Wnt signaling in odontoblasts

Next, we investigated the molecular mechanisms responsible for regulating odontoblastic differentiation. Since recent studies have suggested the involvement of the canonical Wnt signaling pathways in the ciliogenesis of fibroblasts [9], we evaluated the role of primary cilia on the canonical Wnt pathways in KN-3 cells (Fig. 4A, B). In order to find the role of primary cilia in the canonical Wnt pathway, the levels of β-catenin (Fig. 4A) and *Axin2* mRNA (Fig. 4B) were quantified. Both gene products are representative markers of the canonical Wnt pathway [30]. As the two parameters were increased in cilium-less KN-3 cells (Fig 4A, B), we concluded that the primary cilia dampened the canonical Wnt pathway. On the other hand, as primary cilia are known to be involved in the hedgehog (Hh) pathway [12, 13, 31], which determines molar tooth differentiation [31], we further examined the activity of the Hh signaling pathway (Fig. 4C). We did not find any changes in the expression of the

Hh signaling pathway in cilium-less KN-3 cells, indicating that the Hh pathway dose not involve the primary cilia of KN-3 cells.

3.5. Canonical Wnt signal ligand and activator recapitulate the loss of the cilium phenotype

In order to reconfirm the roles of primary cilia in the canonical Wnt pathway, we employed either recombinant WNT3a or SB216763 (an inhibitor of glycogen synthase kinase 3 β that protects β-catenin from degradation) as an activator of the canonical Wnt pathway. Treatment of KN-3 cells with WNT3a or SB216763 caused the accumulation of b-catenin (Fig. 5A) and the expression of *Axin2* mRNA (Fig. 5B), both of which shared the trends observed in *Ift88* shRNA treated cells. Collectively, these results demonstrated that we could modulate the canonical Wnt pathway by using either recombinant protein or pharmacological reagents to reproduce the results obtained with cilia-defective KN-3 cells.

It is worth noting that, as shown in Fig. 5A and C, the expression of IFT88 was significantly decreased by WNT3A and SB216763. Therefore, these results suggest that ciliogenesis, per se, is also affected by the canonical Wnt pathway.

3.6. Wnt/β-catenin signaling controls the differentiation of odontoblasts

Next, we investigated whether the activation of the canonical Wnt pathway contributes to the regulation of odontoblastic differentiation. As shown in Fig. 6A and B, both *DSPP* mRNA and DSP protein levels were significantly increased by WNT3A or SB216763. On the contrary, the activity of ALP was suppressed by WNT3A and SB216763 (Fig. 6C). These results suggest that activation of the canonical Wnt signaling pathway recapitulates the phenomena observed in cilia-defective cells (compare Fig. 2A–C with Fig. 6).

3.7. WNT/β-catenin signaling inhibits ciliogenesis in odontoblasts

Taking into consideration the fact that the levels of IFT88 protein and mRNA were significantly decreased by WNT3A and SB216763 (Fig. 5A, C), IFT88 is indispensable for ciliogenesis [5]. With this in mind, we investigated the effect of the canonical Wnt pathway on ciliogenesis. Administration of WNT3a and SB216763 reduced the ratio of cells with cilia (Fig. 7). All in all (Fig. 5A, C and 7), the canonical Wnt pathway inhibited ciliogenesis, possibly through suppression of *Ift88*.

3.8. Primary cultured odontoblasts reproduced the role of canonical Wnt pathways in odontoblastic differentiation and ciliogenesis

In order to clarify the relationship between ciliogenesis and odontoblastic differentiation, we then compared the time course of ciliogenesis and odontoblastic marker expression in the primary cultured DPCs [23]. To exclude contamination by ameloblasts in DPC cultures, the expression of *amelogenin* (*Amel*), a specific molecular marker of ameloblasts, was checked through RT-PCR (Fig. 8A). The level of *Dspp* mRNA reached a peak at 12 D.I.V. (days in vitro) (Fig. 8B), indicating the differentiation of DPCs into odontoblasts. ALP activity began to increase at 9 D.I.V. (Fig. 8C), followed by mineralization at around 15 D.I.V; the latter was detected through Alizarin red S staining (Fig. 8D). Accompanied by increased expression of *Ift88* mRNA (Fig. 8E), the ratio of ciliated cells started to increase at around 12 D.I.V. (Fig. 8F). Primary cilia were observed in the differentiated odontoblasts (Fig. 8G). In these cells, the DSP protein level reached a peak at 15 D.I.V., three days after the maximal expression of *Dspp* mRNA (Fig. 8B and H). Moreover, the IFT88 protein and mRNA levels peaked at 15 D.I.V. (Fig. 8E and H). The difference between the bands detected in Fig. 2B and Fig. 8H may be ascribed to the difference in the cellular background (suppl. Fig. 2). Specifically, the cells used in Fig. 2B and Fig.8H were KN-3 cells and DPCs, respectively.

During this stage, β-catenin was significantly decreased with the differentiation (Fig. 8H). Real-time RT-PCR analysis showed decreased expression of *cMyc* [32], Wnt-1-induced secreted protein gene (*Wisp*) 1, *Wisp2*, and *Wisp3* [33] at 15 D.I.V. (Fig. 8I). This coincides well with the amount of β-catenin (Fig. 7H).

4. Discussion

In the present study, we showed detailed molecular lines of evidence that primary cilia modulate the differentiation of odontoblasts. IFT88 is a critical cilia-associated protein required for proper ciliary structure and function. Therefore, the disruption of IFT88 brings about shortening of the ciliary axoneme [7]. We observed the same phenotype in odontoblasts (Fig. 1F).

Moreover, *Ift88* knockdown in cultured odontoblasts promoted DSP production (Fig. 2A, B) and inhibited ALP activity (Fig. 2C). Furthermore, in primary cultured DPCs, the changes in ALP activity were consistent with the changes in the ratio of ciliated cells (Fig. 8C, F and Fig. 9A), while the changes in DSP protein levels were consistent with the changes in IFT88 protein levels (Fig. 8H and Fig. 9A). Finally, the primary cilia in odontoblasts should play certain roles in odontoblastic differentiation because the extension of the primary cilia was observed along with odontoblastic differentiation in vivo (Fig. 3). These in vitro and in vivo results collectively suggest that ALP activity is controlled by IFT88 through primary cilia, while DSP production is directly or indirectly regulated by IFT88 (Fig. 9B).

In *Ift88* knocked-down odontoblasts, the ratio of ciliated cells decreased, whereas canonical Wnt pathways were activated (Fig. 4A, B). Therefore, primary cilia seemed to switch off the canonical Wnt signaling. This is consistent with the results of previous studies [9]. However, the role of the Hh pathway in odontogenesis is trivial (Fig. 4C) compared with

that involved in the determination of the molar tooth number [34]. Therefore, signaling through primary cilia may play different roles depending on the differentiation stages.

The finding that canonical Wnt signaling increased the *Dspp* expression level (Fig. 6A) was consistent with the results of a previous study [35]. Moreover, in DPCs, WNT1 inhibits ALP activity and the formation of mineralized nodules [36], which is consistent with our results (Fig. 6C). However, in osteoblastic MC3T3-E1 cells, the Wnt/low-density lipoprotein-receptor-related protein 5 pathway suppressed the ALP activity [37]. The mechanism of ALP activity regulated by the canonical Wnt signaling pathway may be different between osteoblasts and odontoblasts, though they are usually categorized into hard-tissue-forming cells.

From another perspective, the levels of IFT88 mRNA and protein were significantly decreased by recombinant WNT3A protein and SB216763 (Fig. 4A, C). In these cells, the cilia-positive ratio was as the same as in those treated with sh-*Ift88* (compare Fig. 1F with Fig. 7). The fact that the ratio of ciliated cells was increased towards the maturation of cultured DPCs coincided very well with the reduced β-catenin in the course of differentiation (Fig. 8 and Fig. 9A). However, in chondrocytes, GSK3β is inhibited by lithium chloride, which, in turn, activate the canonical Wnt pathway [38] and elongates the primary cilia [39]. The mechanism of ciliogenesis through the canonical Wnt signaling pathway may be different between chondrocytes and odontoblasts, though they are usually categorized as hard-tissue-forming cells. Taking these results into consideration, the canonical Wnt pathway inversely regulates ciliogenesis (Fig. 9B).

During the differentiation of odontoblasts from DPCs, which are undifferentiated mesenchymal cells, the canonical Wnt signaling system is turned on to decrease IFT88, resulting in the inhibition of ciliogenesis (Fig. 9A). Subsequently, the canonical Wnt pathway is inhibited by a yet-unidentified trigger that increases the expression of IFT88, and then facilitates ciliogenesis (Fig. 9B). Prior to mineral deposition, DSP, which buttresses the extracellular matrix (ECM)

of dentin, is produced (Fig. 9B). Interestingly, considering the expression time courses of these genes along with differentiation (Fig. 9A), primary cilia may not regulate the DSP production proper (Fig. 9B). Either the inhibition of the canonical Wnt signaling pathway or an increased level of IFT88 downregulates the expression of DSP protein (Fig. 9B). The elucidation of the detailed mechanism that controls DSP production through the canonical Wnt signaling pathway or IFT88 is a future research task. On other hand, cilia enhance the ALP activity, which in turn facilitates the mineralization of ECM in the maturation of odontoblasts (Fig. 9B).

Therefore, the primary cilia in odontoblasts are indispensable for inducing physiological odontogenesis. This is further strengthened by a previous study showing that genetic deletion of Kif3a in dental mesenchyme results in an arrest of odontogenesis [40]. Considering the fact that KIF3a is indispensable for ciliogenesis [41], cilia must play a pivotal role in the regulation of odontogenesis. Regarding other components required for ciliogenesis, DPC-specific *Ift140* knockout mice showed thinner dentin and slowed dentin formation compared to the wild type [42]. One of the latest studies showed that, in DPC-specific *Ift80* knockout mice, molar root development was impaired and incisor eruption was delayed through reduced DPSC proliferation and differentiation, as well as disrupted odontoblast polarization [43]. Moreover, in some patients with ciliopathy, some phenotypic changes have been recorded in the teeth [44]. These studies, including our own, suggest that primary cilia are required for proper tooth development.

In conclusion, we clarified one of the pivotal roles of IFT88 in odontoblasts, which are essential for ciliogenesis and controls odontogenesis, especially via the canonical Wnt pathway, by modulating the ALP activity and DSP production. Furthermore, a feedback loop between primary cilia and the canonical Wnt pathway, which negatively regulates ciliogenesis, was discovered. Signals other than the canonical Wnt signal may inhibit the canonical Wnt signal, thus promoting ciliogenesis. That is to say, the inhibition of the canonical Wnt signal may lead

to the promotion of ciliogenesis. We believe that this is one of the most important directions for understanding the mechanisms of odontogenesis regulation through primary cilia.

Declaration of competing interest

All authors state that they have no conflicts of interest.

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Figure Legends

Fig. 1. *Ift88* knockdown changes the gene expression profiles of odontoblasts. (A–C) Primary cilia in KN-3 cells. (A) KN-3 cells were stained with anti-acetylated α-tubulin (Ac-αTu) and anti-γ-tubulin (γTu) antibodies, as well as DAPI. Scale bars: 10 μm. (B) KN-3 cells were analyzed through scanning electron microscopy. Scale bars: 10 μm (low-power field) and 1 μm (high-power field). (C) KN-3 cells were analyzed through transmission electron microscopy. Abbreviation: BB, basal body. Scale bars: 500 nm (low-power field) and 100 nm (high-power field). (D, E) Expression levels of *Ift88* mRNA and IFT88 protein in *Ift88* knockdown odontoblastic KN-3 cells. (D) Expression levels of *Ift88* mRNA. The expression of *Ift88* was efficiently knocked down. The values represent the means ± SD. **P* < 0.05. (E) Representative immunoblot analysis of IFT88. Positions of molecular weight markers are shown on the right side of the images. Substantial and specific reductions in protein levels were observed. (F) The ratio of ciliated cells among *Ift88* knockdown KN-3 cells. The values represent the means ± SD. **P* < 0.05. There was a decrease in the ratio of ciliated cells among sh-*Ift88*-expressing KN cells. Abbreviations: NC, negative controls with the secondary antibody only; BB, basal body; EV, empty vector.

Fig. 2. Odontoblastic differentiation in *Ift88* knockdown odontoblasts. (A-C) KN-3 cells were cultured in αMEM containing 10% FBS for 72 h and then starved in serum-free αMEM for 24 h. (A) Real-time RT-PCR analysis for *Dspp* mRNA. Increases in *DSPP* mRNA through *Ift88* knockdown in KN-3 cells. The values are presented as the means ± SD. **P* < 0.05. (B) Representative immunoblot analysis of DSP. The DSP protein level was increased through *Ift88* knockdown in KN-3 cells. Positions of molecular weight markers (55, 40, and 35 kDa) are shown on the right side of the images. (C) Terminal differentiation of odontoblasts was monitored by assaying ALP activity. A decrease in ALP activity through *Ift88* knockdown

was observed in KN-3 cells. The signals were normalized to the DNA content in the samples. The values are presented as the means ± SD. *$P < 0.05$. (D) KN-3 cells were cultured in medium conditioned by primary rat gingival or dental pulp cells (DPCs). Mineralization was then detected through Alizarin red S staining. Mineralization was delayed by *Ift88* knockdown in KN-3 cells. Abbreviations: EV, empty vector.

Fig. 3. Change in the length of primary cilia during tooth development. The primary cilia in odontoblasts were identified from the bell (embryonic day (E) 16) to root development (post-natal day (P) 14) stage with anti-Ac-αTu and anti-γTu antibodies. Scale bars: 100 μm (HE staining) and 10 μm (immunocytochemistry and NC). Abbreviations: IEE, inner enamel epithelium. NC, negative control without a primary antibody.

Fig. 4. Primary cilia affect the activity of the canonical Wnt signaling pathway. (A) Immunoblot analysis of the retrovirally transduced cells. β-catenin accumulation was increased in sh-*Ift88*-expressing KN-3 cells. Molecular weight markers (100, 75, and 37 kDa) are shown on the right side of the images. (B) Expression levels of *Axin2* mRNA. The expression of *Axin2* was significantly increased. The values represent the means ± SD. *$P < 0.05$. (C) Real-time RT-PCR analysis of the mRNA expression levels of components of the hedgehog (Hh) signaling pathway in KN-3 cells. The mRNA levels of all components of the Hh signaling pathway did not change in sh-*Ift88*-expressing KN-3 cells. The values are presented as the means ± SD. *$P < 0.05$. Abbreviations: EV, empty vector.

Fig. 5. Activation of canonical Wnt signaling pathways through recombinant WNT3a or SB216763 (glycogen synthase kinase 3 beta inhibitor) in KN-3 cells. (A) Immunoblot analysis of the cells treated with the recombinant protein or inhibitors. β-catenin accumulation was

increased through treatment with WNT3a or SB216763. Moreover, IFT88 protein levels were decreased through activation of the canonical Wnt signaling pathway. Positions of molecular weights (75 and 37 kDa) are shown on the right side of the images. (B, C) Real-time RT-PCR analysis of *Axin2* and *Ift88* mRNA in KN-3 cells treated with the recombinant protein or inhibitors. (B) The expression level of *Axin2* mRNA was increased through treatment with WNT3a or SB216763. The values are presented as the means ± SD. *$P < 0.05$. (C) The expression level of *Ift88* mRNA was decreased through treatment with WNT3a or SB216763. The values are presented as the means ± SD. *$P < 0.05$.

Fig. 6. Activation of the canonical Wnt signaling pathway suppresses ciliogenesis. (A, B) *DSPP* mRNA and DSP protein levels decreased through activation of the canonical Wnt signaling pathway in odontoblasts. (A) Real-time RT-PCR analysis of *Dspp* mRNA. The values are presented as the means ± SD. *$P < 0.05$. (B) Immunoblot analysis. Molecular weight markers (55, 40, and 35 kDa) are shown on the right side of the images. (C) Terminal differentiation of odontoblasts was monitored by assaying ALP activity. The ALP activity was decreased through treatment with both WNT3a or SB216763. The signals were normalized to the DNA content in the samples. The values are presented as the means ± SD. *$P < 0.05$.

Fig. 7. Activation of the canonical Wnt signaling pathway suppressed ciliogenesis. The ratio of ciliated cells was decreased through treatment with Wnt3a or SB216763. The values are presented as the mean values ± SD. *$P < 0.05$.

Fig. 8. Changes in the ratio of ciliated cells in differentiating dental pulp cells (DPCs). (A) The gene expression profiles of differentiating DPCs were analyzed through RT-PCR. Amelogenin (Amel) mRNA was not detected at any point in time. Molecular weight markers

are shown on the right side of the images. (B) The gene expression profiles of differentiating DPCs were analyzed through real-time RT-PCR. The level of target mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA. *Dspp* mRNA expression increased at 12 D.I.V. The values are presented as the means ± SD. *$P < 0.05$. (C) Terminal differentiation of odontoblasts was monitored by assaying ALP activity. The signals were normalized to the total protein content in the samples. The values are presented as the means ± SD. *$P < 0.05$. (D) Mineralization was detected after 15 D.I.V. through Alizarin red S staining. (E) The gene expression profiles of differentiating DPCs were analyzed through real-time RT-PCR. The level of target mRNA was normalized to that of *Gapdh* mRNA. *Ift88* mRNA expression increased at 15 D.I.V. (F) The ratio of differentiating DPCs with primary cilia. The values are presented as the means ± SD. *$P < 0.05$. (G) Primary cilia were detected through immunocytochemistry using anti-Ac-αTu and anti-γTu antibodies. Scale bars: 10 μm. NC, negative control without a primary antibody. (H) Immunoblot analysis of DPCs. DSP and IFT88 levels peaked at 15 D.I.V. On the other hand, β-catenin accumulation decreased with the progression of odontoblastic differentiation. Molecular weight markers are shown on the right side of the images. (I) The expression levels of genes regulated by the canonical Wnt signaling pathway were analyzed through real-time RT-PCR. The level of the target mRNA was normalized to that of *Gapdh* mRNA. Expression of *cMyc*, Wnt-1-induced secreted protein (Wisp) *1*, *Wisp2*, and *Wisp3* decreased after 15 D.I.V. Abbreviations: NC, negative control with the secondary antibody only.

Fig. 9. Schematic representation of the role of primary cilia in odontoblastic differentiation. (A) Expression of molecular marker is summarized along with differentiation of odontoblasts. (B) Molecular pathway underlying the maturation of odontoblast. Inhibition of the canonical Wnt signaling pathway increases the IFT88 levels, leading to ciliogenesis and

increased ALP activity. On the other hand, *DSPP* mRNA and DSP protein levels decrease through inhibition of the canonical Wnt signaling pathway and activation of the IFT88 pathway. The expression of *DSPP* mRNA and DSP protein appears to have nothing to do with primary cilia.

Supplemental Fig. 1. The effect of *Ift88* knockdown on the cell-cycle in odontoblasts. (A) The expression levels of cell-cycle-related genes were analyzed through real-time RT-PCR. The level of target mRNA was normalized to that of *Gapdh* mRNA. The mRNA level of both genes did not change through sh-*Ift88*-expresson in KN-3 cells. (B) Representative immunoblot analysis of Cyclin D and E. Positions of molecular weight markers are shown to the right of the images. The protein levels of both Cyclin D and E did not change through sh-*Ift88* expression in KN-3 cells. (C) The expression of Fucci-S/G2/M was monitored by ArrayScan VTHI HCS Reader. The expression level of Fucci-S/G2/M did not change by sh-*Ift88*-expression in KN-3 cells. The values are presented as the means ± SD. Abbreviations: EV, empty vector.

Supplemental Fig. 2. The original data of the DSP immunoblot. The original picture of the immunoblot analysis shown in Fig. 2B**.** (A) and Fig. 8H. Molecular weight markers are indicated on the right side.