Developmental changes in primary cilia in the mouse tooth germ and oral cavity

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ABSTRACT

The primary cilium, a sensory apparatus, functions as both a chemical and mechanical sensor to receive environmental stimuli. The present study focused on the primary cilia in the epithelial-mesenchymal interaction during tooth development. We examined the localization and direction of projection of primary cilia in the tooth germ and oral cavity of mice by immunohistochemical observation. Adenylyl cyclase 3 (ACIII)-immunolabeled cilia were visible in the inner/outer enamel epithelium of molars at the fetal stage and then conspicuously developed in the odontoblast layer postnatally. The primary cilia in ameloblasts and odontoblasts—shown by the double staining of acetylated tubulin and γ -tubulin—were regularly arranged from postnatal Day12, projecting apart from each other. The periodontal ligament possessed ACIII-positive cilia, which gathered on both sides of the dentin/cement and alveolar bone in postnatal days. In the oral cavity, numerous long primary cilia immunoreactive for ACIII were condensed at subepithelial stromal cells in the oral processes in fetuses, while postnatally a small number of short cilia were dispersed throughout the stroma of the oral cavity. These findings suggest that the primary cilia showing stage- and region-specific morphology are involved in the epithelial-mesenchymal interaction during tooth development via mechano- and/or chemoreception for growth factors.

The primary cilium is a single, immotile cilium extending from the centriole surrounded by the Golgi apparatus in most cell types of mammals (22). It is now believed that the primary cilium has specialized functions as a fundamental structure for mechanical and chemical sensing by individual cells, transferring the signals to the nucleus and cell organellae to induce adequate cellular responses (9). As mechanoreceptors, primary cilia in the collecting tubules of the kidney detect and respond to fluid flow in the

lumen and induce a Ca^{2+} -response in cells when the ciliary axoneme is bent by fluid movement (5). Recent studies indicated a possible need to regulate the length of primary cilia in response to extracellular environmental changes. For instance, kidney cells can adjust the length of cilia in reaction to fluid stimuli; a deficiency in the regulation of the length and shape causes some disorders, termed ciliary diseases (14, 21). Moreover, the primary cilium functions as a chemoreceptor which perceives the extracellular environment via membrane-bound receptors and ion channels expressed on cilia (23). The primary cilium is also known to be associated with the coordination of various signaling pathways to control cell proliferation and differentiation via hedgehog (Hh), Wnt, and sonic hedgehog (Shh) signaling pathways (6, 10).

The structures and functions of primary cilia have

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been described for various kinds of cells. Primary cilia in the tooth germ have been investigated using animal models or tissue cultures. Here, tooth development involves a typical epithelial-mesenchymal interaction, and accumulating evidence indicates that the primary cilia may take a leading part in such interaction (7). Electron microscopic observation of the centriole and associated Golgi apparatus in the differentiating ameloblasts of the rat showed that the movement and separation of ciliated centrioles affected the morphological and functional differentiation of ameloblasts (16). As for the primary cilia of odontoblasts, investigations using human and mouse dental pulps have reported that this organelle influences the terminal differentiation of odontoblasts and may be functionally connected with tooth pain transmission (19). An *in vitro* study using human periodontal ligament stem cells considered primary cilia as a critical signaling center required for the Shh pathway activation related to odontogenesis (8). Although many studies have reported on the features and functions of primary cilia in the tooth germ, few reports have dealt with the developmental changes in primary cilia in the tooth germ from the bell stage to the root completion stage.

The present study analyzed the development and morphological characteristics of the primary cilia in the mouse tooth germ including odontoblasts, ameloblasts, and related cells during tooth development. Additionally, we examined for the first time the localization of primary cilia in the oral cavity in fetal and postnatal mice. This study realized the immunohistochemical detection of cilia using three different markers: type III adenylyl cyclase (ACIII), which converts ATP to cyclic AMP and is associated with ciliary length control (1, 11); acetylated tubulin to label totally cilial axoneme (13); and γ -tubulin which is localized at the bases of cilia.

MATERIALS AND METHODS

Animals and tissue sampling. Pregnant ddY mice were supplied by Japan SLC (Shizuoka, Japan). Heads of E16.5 embryos, neonates of postnatal day-0, 1, 2, 3, 6, 12, 19, and adult mice were used in the present study. The mice were sacrificed by an intraperitoneal injection of an overdose of pentobarbital sodium (Schering Plough Animal Health, the Netherlands). E16.5 embryos and Day0–12 neonates were fixed with 10% formalin. Day19 neonates and adult mice were perfused with a physiological saline through the heart, followed by 4% paraformaldehyde plus 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The

heads were removed, immersed in the same fixative for 24 h, and decalcified with 5% EDTA for 1–3 weeks at 4°C. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

Immunohistochemistry. The decalcified tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections, about 16 μ m in thickness, were mounted on poly-L-lysine-coated glass slides. After pretreatment with 0.3% Triton-X 100-containing PBS (pH 7.2) for 1 h, the sections were preincubated with a normal donkey serum. For single immunofluorescence staining, the sections were incubated with one of the following antibodies: rabbit anti-ACIII (1 : 1,400; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-acetylated tubulin (1 : 1,400; Sigma-Aldrich, St. Louis, MO). The antigen-antibody reactions were visualized by incubation with Cy3-labeled anti-rabbit or mouse IgG (Jackson ImmunoResearch, West Grove, PA). The nuclei were stained with SYTOX green (SyTO 13; Invitrogen, Carlsbad, CA). For double immunofluorescence staining, sections were incubated with a mixture of rabbit anti-acetylated tubulin (T6793, Sigma) and mouse anti- γ tubulin antibody (T3320, Sigma). The sites of the antigen-antibody reactions were detected by incubation with a combination of Alexa Flour 488-labeled anti-rabbit IgG (Invitrogen) and Cy3-labeled anti-mouse IgG (Invitrogen). The stained sections were observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan).

Silver-intensified immunogold method for electron microscopy. The formaldehyde-fixed and decalcified tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT compound, and quickly frozen in liquid nitrogen. Frozen sections of 16 μ m in thickness were mounted on poly-L-lysine-coated glass slides, incubated with rabbit anti-ACIII antibody overnight, and subsequently reacted with goat anti-rabbit IgG covalently linked with 1-nm gold particles (1 : 200 in dilution; Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmicated, dehydrated, and directly embedded in Epon (Nissin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

RESULTS

Primary cilia in tooth germ

We first investigated the localization of primary cilia in the tooth germ of fetuses (E16.5) and neonates (Day0–3, 6, 12, and 19) and in the molars of adult mice, using two antibodies against ACIII and acetylated tubulin.

Molars at the fetal stage displayed numerous short cilia immunoreactive for ACIII and acetylated tubulin in the inner/outer enamel epithelium but fewer cilia in the stellate reticulum (Fig. 1A). A broad distribution of similar primary cilia was found in the mesenchyme, including the dental papilla. In the fetal enamel organ, ACIII-immunolabeled primary cilia were concentrated in the enamel knots which regulate the patterning of the tooth crown including the cusp formation and release many signaling molecules such as bone morphogenetic protein (BMP)-2/4, Shh, and Wnt as well as fibroblast growth factor (FGF)-4 (Fig. 1B).

At Day0, ACIII-positive primary cilia were detectable in differentiated ameloblasts of molars (Fig. 1C), but thereafter rapidly decreased in number and stainability, almost disappearing by Day3 (Fig. 1D). In contrast, numerous thick ACIII-immunoreactive cilia were visible in odontoblasts and dental pulp cells at these stages; the cilia were longer in the odontoblast layer than those in the dental pulp. On subsequent days (Days 6, 12, and 19), the ACIII antibody intensely stained primary cilia in odontoblasts, but not in ameloblasts (Fig. 1E). The length of cilia in odontoblasts tended to be longer according to the differentiation of the odontoblasts. The lack of ACIII-immunolabeled cilia in ameloblasts did not mean the disappearance of cilia themselves, however. Immunostaining for acetylated tubulin detected many primary cilia in the ameloblast layer as well as the odontoblast layer on postnatal days, though their lengths were shorter than those in odontoblasts (Fig. 1F).

Next, we tried to decide the direction of primary cilia in molar ameloblasts and odontoblasts by double staining of acetylated tubulin and γ -tubulin, the latter of which is localized at the base of the cilia. The direction of the cilia was irregular from E16.5 to Day6. At later stages, cilia in both ameloblasts and odontoblasts largely aligned along the axis of the enamel/dentin. The regular arrangement of cilia in odontoblasts first appeared near the root apex and then extended along the entire length of the odontoblast layer by Day12 (Fig. 2). In a major population of odontoblasts, the γ -tubulin antibody marked the base of primary cilia projecting toward the dental

pulp with a high polarity (Fig. 2B, C). The direction of primary cilia in odontoblasts was confirmed under an electron microscope by the silver-intensified immunogold method (Fig. 3). Odontoblasts possessed an ACIII-immunolabeled long cilium extending toward the dental pulp, though they appeared to be embedded in the cytoplasm of odontoblasts. In contrast, the cilia of ameloblasts were largely directed toward the outer enamel epithelium (Fig. 2A). Thus, cilia of ameloblasts and odontoblasts extended in opposite directions (Fig. 2D).

Primary cilia in the periodontal ligament

The dental follicle which gives rise to the periodontium—including the periodontal ligament—possessed primary cilia immunoreactive for ACIII from the fetal stage to the postnatal stages (Fig. 4A). According to the development, the primary cilia were concentrated in periodontal ligament cells between the dentin/cement and the alveolar bone. In the adult, ACIII-immunolabeled primary cilia arranged themselves in a line on both sides of the teeth and alveolar bone; in some regions, they tended to gather on the tooth side. However, they were scarce in the central zone of the periodontal ligament (Fig. 4B). Even when stained for acetylated tubulin, a small number of primary cilia in the adult were scattered in the central zone of the periodontal ligament. Double staining with the anti- γ -tubulin antibody showed the irregular direction of primary cilia in the fetal dental follicle, as well as in the neonatal and adult periodontal ligament (Fig. 4C).

Primary cilia in the oral cavity

In considering the relationship of primary cilia in regard to the development of the maxillofacial region, the present study paid attention to the distribution of primary cilia in the processes of the oral cavity. Primary cilia immunoreactive for ACIII existed more numerous in the midline palatine process and the maxillary/mandibular processes than in other flat regions in the fetal period (E16.5) (Fig. 5A, B). Thick and long cilia were condensed at the subepithelial stroma in each process, in contrast to short cilia in the basal layer of the epithelium and the deeper stroma. In postnatal development, cilia in the subepithelial stromal cells became shorter at the processes of the oral cavity.

Immunostaining for acetylated tubulin detected primary cilia in each process from the fetal stage to Day3; the cilia did not display any condensed distribution in the processes, but rather displayed a similar density as for other flat regions. From Day6, tubulin-

Fig. 1 Immunohistochemistry of primary cilia in the tooth germ of mice using antibodies against ACIII (A–E) and acetylated tubulin (F). Primary cilia and nuclei are labeled red and green, respectively. In a fetus (E16.5), ACIII-immunoreactive cilia are visible in the inner (*ie*) and outer (*oe*) enamel epithelia (A); cilia gather in the enamel knots (*ek* in B). On postnatal days (Day0 and Day3), the ACIII antibody stains numerous primary cilia in odontoblasts (*od* in C, D). The primary cilia in ameloblasts (*am*) are shorter than those in odontoblasts (C), and disappear by Day3 (D). At Day12, ACIII-immunoreactive cilia exist only in odontoblasts (E) but immunostaining for acetylated tubulin detects primary cilia in ameloblasts as well as odontoblasts (F). *dp*: dental pulp Bars: 40 μ m (A, B), 20 μ m (C–F)

immunoreactive primary cilia in the oral processes decreased in number and almost disappeared by Day19. The direction of cilia shown by double stain-

ing with anti- γ -tubulin antibody in all processes was irregular from the fetal stage to the postnatal stage.

Fig. 2 Double staining of acetylated tubulin (green) and γ -tubulin (red) in molars at Day12 (A, B) and Day19 (C). Primary cilia of ameloblasts are largely directed toward the outer enamel epithelium (A). In odontoblasts, the primary cilia tend to extend toward the dental pulp (B, C), though some of them extend in different directions (arrows in A and B). A schematic drawing for the direction of primary cilia is shown in Fig. 2D. Bars: 10 μ m

DISCUSSION

The primary cilia play critical roles associated with the epithelium-mesenchyme interaction in various tissues, including the tooth germ (2, 7, 17). Accumulating evidence shows that many growth factors/receptors and cross-talk via these factors between adjacent tissues precisely regulate tooth development. The primary cilia are responsible for both Shh and Wnt signalings in several developmental processes because the Shh receptor Ptch1 and its activator Smo are localized on the cilia. The same signalings are also important for odontogenesis (10, 15). In the present study, we revealed the distribution of primary cilia and their morphological characteristics in the tooth germ and developing oral cavity.

Primary cilia in tooth germ

ACIII is a reliable marker of functional primary cilia in immunohistochemical analyses (1). Numerous

Fig. 3 Electron microscopy of the primary cilium in odontoblasts. Gold particles showing the existence of ACIII label a primary cilium (arrow is base) in an odontoblast. Bar: 1 μ m

Fig. 4 Primary cilia in the dental follicle and periodontal ligament. At Day12, ACIII-immunoreactive cilia are dispersed in the dental follicle (A). ACIII-positive primary cilia gather along the surface of dentin and bone in the adult, but are rarely seen in the middle zone of the periodontal ligament (B). Double immunostaining shows an irregular direction for the primary cilia in the adult periodontal ligament (C). *df*: dental follicle, *od*: odontoblasts, *pl*: periodontal ligament Bars: 40 μ m

Fig. 5 ACIII-immunoreactive primary cilia in the oral cavity at E16.5. Many cilia gather in the subepithelial stroma of the midline palatine process (*mpp* in A). A similar subepithelial condensation of the ACIII-immunoreactive cilia can be seen in the maxilla/mandibular processes (*mxp* and *mdp* in B). Bars: 100 μ m

ACIII-immunoreactive primary cilia were seen distributed in the enamel organ of fetal molars except for the stellate reticulum. It is worth noting that the ACIII-expressing cilia accumulated more in the enamel knot compared to other regions of the enamel organ. This structure participates in the differentiation of the dental papilla and odontoblasts by expressing many signaling molecules such as FGF, BMP, Shh,

and Wnt families (20). In particular, it is known that the primary cilia are associated with Shh and Wnt signaling during tooth development (7). Therefore, our data further support the possibility that the primary cilia are involved in the epithelium-mesenchymal interaction in the enamel knot.

Around Day3, the predominant occurrence of ACIII-positive primary cilia shifted from the inner/

outer enamel epithelia to the odontoblast layer. Immunostaining for acetylated tubulin was able to detect many cilia in both ameloblasts and odontoblasts until the weaning period (Day19). If the expression of ACIII is a sign of receptor function, this finding indicates the progressive involvement of primary cilia in odontogenesis using the ACIII-cAMP signaling pathway. In terms of length of primary cilia in odontoblasts, ACIII-immunoreactive primary cilia in the postnatal stages tended to be longer than in the fetal stage. It has been reported that the ACIII-cAMP signaling pathway may control the length of cilia (10) and that there exists a need to adjust the length of cilia in response to environmental stimuli; otherwise, a lack of the adequate length change can cause some ciliary diseases (12, 18).

Primary cilia of molar odontoblasts were irregularly arranged in the early neonatal stages, while around Day19 they were largely directed toward the dental pulp along the axis of the enamel/dentin, in agreement with a previous study (19). The direction of primary cilia in molar ameloblasts was almost opposite to that of cilia in odontoblasts. In the process of the enamel/dentin formation, odontoblasts move toward the dental papilla and ameloblasts move apart from odontoblasts to form the enamel and dentin. There is a possibility that the direction of primary cilia in odontoblasts and ameloblasts affects their movement. The regular arrangement may be effective for functioning as a mechanosensor for cellular orientation (19). This finding suggests the possibility that certain receptor molecules are expressed in primary cilia of odontoblasts to receive growth factors necessary for differentiation.

Primary cilia in the periodontal ligament and oral cavity

The periodontal ligament is the fibrous connective tissue that joins the cementum with the alveolar bone and also serves as a shock absorber against mechanical forces. Primary cilia in fibroblasts of connective tissues have been reported to associate with mechanoreception (3), but information on the periodontal ligament is limited. Although the present study showed that ACIII-immunoreactive cilia were numerous on both sides of the teeth and alveolar bone, they tended to gather on the cementum side, which received more intense occlusal force than the side of the alveolar bone. Therefore, it is likely that primary cilia function as mechanoreceptors to contribute to maintenance of the periodontium. Although the direction of primary cilia shown by double immunostaining was irregular, the above is reasonable

based on the fact that the periodontal ligament receives forces from every direction.

No previous studies have dealt with primary cilia of the developing oral cavity. During early development, ACIII-expressing long primary cilia were condensed at each process of oral cavity, but they obviously decreased according to the structural accomplishment. The subepithelial stromal cells in the processes of fetal oral cavity possessed longer ACIII-positive cilia than those in the epithelia. Therefore, it is possible that these primary cilia in the processes express receptors to receive growth factors during development, possibly functioning as a chemical sensor. It is worth noting that PDGF receptors are densely expressed in the subepithelial stroma (4), though we failed to detect any receptor on the primary cilia.

In conclusion, the tooth germ and oral tissues during development possess numerous primary cilia expressing ACIII. The morphological findings on primary cilia suggest that the primary cilia in oral cavity play a critical role in the epithelial-mesenchymal interaction.

REFERENCES

1. Bishop GA, Berbari NF, Lewis J and Mykityn K (2007) Type III adenylyl cyclase localizes to primary cilia throughout the adult mouse brain. *J Comp Neurol* **505**, 562–571.
2. DeRouen MC and Oro AE (2009) The primary cilium: a small yet mighty organelle. *J Invest Dermatol* **129**, 264–265.
3. Donnelly E, Williams R and Farnum C (2008) The primary cilium of connective tissue cells: imaging by multiphoton microscopy. *Anat Rec (Hoboken)* **291**, 1062–1073.
4. Hisamoto M, Goto M, Muto M, Nio-Kobayashi J, Iwanaga T and Yokoyama A (2015) A systematic analysis for localization of predominant growth factors and their receptors involved in murine tooth germ differentiation using *in situ* hybridization technique. *Biomed Res (Tokyo)* **36**, 205–217.
5. Iwanaga T, Miki T and Takahashi-Iwanaga H (2011) Restricted expression of somatostatin receptor 3 to primary cilia in the pancreatic islets and adenohypophysis of mice. *Biomed Res (Tokyo)* **32**, 73–81.
6. Kero D, Novakovic J, Vukojevic K, Petricevic J, Kalibovic Govorko D, Biocina-Lukenda D and Saraga-Babic M (2014) Expression of Ki-67, Oct-4, γ -tubulin and α -tubulin in human tooth development. *Arch Oral Biol* **59**, 1119–1129.
7. Liu B, Chen S, Cheng D, Jing W and Helms JA (2014) Primary cilia integrate hedgehog and Wnt signaling during tooth development. *J Dent Res* **93**, 475–482.
8. Martínez C, Smith PC, Rodriguez JP and Palma V (2011) Sonic hedgehog stimulates proliferation of human periodontal ligament stem cells. *J Dent Res* **90**, 483–488.
9. Michaud EJ and Yoder BK (2006) The primary cilium in cell signaling and cancer. *Cancer Res* **66**, 6463–64637.
10. Ohazama A, Haycraft CJ, Seppala M, Blackburn J, Ghafoor S, Cobourne M, Martinelli DC, Fan CM, Peterkova R, Lesot H, Yoder BK and Sharpe PT (2009) Primary cilia regulate Shh activity in the control of molar tooth number. *Development* **136**, 897–903.

11. Ou Y, Ruan Y, Cheng M, Moser JJ, Rattner JB and van der Hoorn FA (2009) Adenylate cyclase regulates elongation of mammalian primary cilia. *Exp Cell Res* **315**, 2802–2817.
12. Pazour GJ, Dickert BL, Vucica Y, Seeley ES, Rosenbaum JL, Witman GB and Cole DG (2000) Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene *tg737*, are required for assembly of cilia and flagella. *J Cell Biol* **151**, 709–718.
13. Piperno G and Fuller MT (1985) Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J Cell Biol* **101**, 2085–2094.
14. Resnick A and Hopfer U (2007) Force-response considerations in ciliary mechanosensation. *Biophys J* **93**, 1380–1390.
15. Rohatgi R, Milenkovic L and Scott MP (2007) Patched1 regulates hedgehog signaling at the primary cilium. *Science* **317**, 372–376.
16. Sasano Y (1986) Dynamic behavior of ciliated centrioles in rat incisor ameloblasts during cell differentiation. *Arch Histol Jpn* **49**, 437–448.
17. Satir P (2008) Primary cilia: integral to development and disease. *Dev Dyn* **237**, 1953–1954.
18. Smith LA, Bukanov NO, Husson H, Russo RJ, Barry TC, Taylor AL, Beier DR and Ibraghimov-Beskrovnaya O (2006) Development of polycystic kidney disease in juvenile cystic kidney mice: insights into pathogenesis, ciliary abnormalities, and common features with human disease. *J Am Soc Nephrol* **17**, 2821–231.
19. Thivichon-Prince B, Couble ML, Giamarchi A, Delmas P, Franco B, Romio L, Struys T, Lambrichts I, Ressenkoff D, Magloire H and Bleicher F (2009) Primary cilia of odontoblasts: possible role in molar morphogenesis. *J Dent Res* **88**, 910–915.
20. Vaahtokari A, Aberg T, Jernvall J, Keränen S and Thesleff I (1996) The enamel knot as a signaling center in the developing mouse tooth. *Mech Dev* **54**, 39–43.
21. Verghese E, Weidenfeld R, Bertram JF, Ricardo SD and Deane JA (2008) Renal cilia display length alterations following tubular injury and are present early in epithelial repair. *Nephrol Dial Transplant* **23**, 834–841.
22. Wheatley DN, Wang AM and Strugnell GE (1996) Expression of primary cilia in mammalian cells. *Cell Biol Int* **20**, 73–81.
23. Whitfield JF (2004) The neuronal primary cilium—an extra-synaptic signaling device. *Cell Signal* **16**, 763–767.