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Expression of *Fibroblast growth factor receptor1*, *-2c*, and *-3c* transcripts in mouse molars after tooth eruption

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The title of the paper

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Tsuyoshi Kano, Tsuyoshi Morita and Otto Baba. The first draft of the manuscript was written by Tsuyoshi Kano and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Abstract

A previous study suggested that fibroblast growth factor (FGF) signaling plays an important role in dentin formation during tooth development. In this study, to examine dentin formation after tooth eruption involving secondary and tertiary dentin, we analyzed the expression patterns and expressing cells of *Fgfr1*, *-2c*, and *-3c* in mouse maxillary first molars ($\underline{M_1}$). Since it is difficult to recover the mRNAs from mineralized tissues, we tested methods for extraction after fixation and decalcification of teeth. We successfully obtained consistent results with quantitative real-time PCR (qPCR) using *β-acti*n transcripts for validation. qPCR for *Dentin sialo phosphoprotein* (*Dspp*), *Fgfr1*, *-2c*, and *-3*c transcripts was performed on mice at ages of 2-20 weeks. The results showed that the highest expression levels of *Dspp* and *Fgfr2c* occurred at 2 weeks old followed by lower expression levels after 4 weeks old. However, the expression levels of *Fgfr1* and *Fgfr3c* were constant throughout the experimental period. By *in situ* hybridization, *Dspp*, *Fgfr1*, and *Fgfr3c* transcripts were detected in odontoblasts at ages of 2 and 4 weeks. Also, *Dspp* and *Fgfr1* transcripts were detected in odontoblasts facing reactionary dentin at 8 weeks old. These results

suggest that FGF-FGFR signaling might be involved in the regulation of odontoblasts even after tooth eruption, including secondary and tertiary dentin formation. Moreover, our modified method for extracting mRNA from mineralized tissues after fixation and decalcification successfully produced consistent results.

Key words Dentin formation · FGF receptor · Odontoblasts · Secondary dentin · Tooth eruption

Mini-Abstract

By utilizing our modified RNA extraction method, we determined the expression of FGFRs after tooth eruption, which suggested the commitment of FGF signaling to the homeostasis of odontoblasts.

Introduction

Dentin is unique in its characteristics. Odontoblasts forms primary, secondary, and tertiary (reactionary) dentin during their lifetimes, and the morphological, biochemical, and physiological properties of these respective matrices are all different. Though odontoblasts after the eruption of the teeth seem to be rather quiescent, they occasionally maintain the homeostasis of the dentinal tubule and dental pulp, for example by forming tertiary dentin (Smith et al. 1995; Goldberg et al. 2011; Charadram et al. 2012; Couve et al. 2014). Clarifying the factors regulating odontoblasts after tooth eruption might therefore provide insights into pulp biology and further endodontic treatment. Primary dentin formation is initiated by the interaction between enamel epithelium and dental mesenchymal cells, followed by dentin formation to make up the crown and root dentin. It is important to note that dentin continues to form slowly even after primary dentin formation. This slowly formed dentin is called secondary dentin (Nanci 2018). After tooth eruption, odontoblasts in dental pulp always receive extrinsic stimuli through the dentinal tubules and/or dentin matrix, and some stimuli might trigger secondary dentin formation or tertiary dentin formation (Smith et al. 1995; Goldberg et al. 2011; Charadram et al. 2012; Couve et al. 2014).

Fibroblast growth factor receptors (FGFRs) are expressed in the developing tooth (Li et al. 2014; Du et al. 2018). At early cap stage, *Fgfr1c* transcripts are detected in the cells located at dental papilla and dental follicles, while *Fgfr2c* transcripts are detected in dental follicles and outer dental epithelium at the buccal side. At early bell stage, *Fgfr1c* transcripts are detected in cells of inner dental epithelium and dental papilla, and *Fgfr2c* transcripts are detected in cells of dental follicles (Kettunen et al. 1998). *Fgfr3c* transcripts are detected in dental papilla after odontoblasts and ameloblasts differentiation (Kettunen et al. 1998). These results suggest that the expression patterns of FGFRs are variable depending on the timing of tooth development. FGF ligands expressed in the enamel organ activate mesenchymal cells through FGFRs. Contrarily, FGF ligands expressed in mesenchymal cells activate epithelial cells through FGFRs (Kettunen et al. 1998; Porntaveetus et al. 2011). These reports suggest the involvement of FGF-FGFR signaling during primary dentin formation.

Our previous study determined the expression of transcripts for *Fgf18* in rat molars by RT-PCR and *in situ* PCR (Baba et al. 2015). The results clearly showed that the expression of *Fgf18* in odontoblasts was

upregulated during root formation rather than crown formation. Interestingly, the expressions of *Fgfr2c* and *Fgfr3* transcripts were detected in both crown and root dentin formation, in odontoblasts and some dental pulp cells (Baba et al. 2015). These results also suggest the involvement of FGF-FGFR signaling for the regulation of odontoblasts for dentin formation. Currently, few studies reported on FGF signaling and related factors after crown formation. In the present study, we examined the expression of FGFR by odontoblasts after tooth eruption for the first time. We also describe a new protocol for RNA extraction from mouse molars after tooth eruption for genetic analysis.

Materials and Methods

Tissue preparation for quantitative real-time PCR (qPCR) and histological sections

All animal experiments were approved by and followed the guidelines of the Animal Welfare Committee of Tokushima University (No.T-29-8). Tissue preparation was performed as described previously (Baba et al. 2015). Briefly, ICR the mice (CLEA Japan Inc., Tokyo, Japan) at ages of 2, 4, 8, 12, 16, and 20 weeks were perfused via the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and dissected maxillae were further fixed in the same fixative for 1 day at 4°C, followed by decalcification in 8% ethylenediaminetetraacetic acid (EDTA, pH 7.4) at 4°C for 1 to 3 weeks. The samples decalcified for 1 week were used in qPCR, and those decalcified for 2 to 3 weeks were made into paraffin sections for histological observations and *in situ* hybridization.

Quantitative real-time PCR (qPCR)

The decalcified maxillary first molars ($\underline{M_1}$) from each age group were homogenized with proteinase K solution (5.0 μg/ml) for 70 min at 45°C, and an additional homogenization process at 70°C for 20 min was performed to remove bound mono-methylol groups to the bases in the RNA strands.

Then, total RNAs of $\underline{M_1}$ in each age group were extracted using TRI REAGENT® (Molecular Research Center, Inc., USA). To generate cDNAs of specific target gene transcripts, reverse transcription was performed with reverse primers using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). qPCR was performed using MyGo Mini orange (IT-IS Life Science Ltd. Ireland) with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific Inc., USA). The PCR conditions were as follows: initial denaturation of 120 s at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. All gene expressions were quantified with *β-actin* as an internal control. Relative gene expressions were calculated using the ΔΔCt method. All primer sequences were designed using Primer3Plus (https://primer3plus.com). Each primer sequence is listed in Table 1.

Statistical analyses were performed as described previously (Morita et al. 2016). Data analyses were performed with one-way analysis of variance for multi-group comparisons, and the significance was determined using Tukey's comparison test using SPSS Statistics software (version 24, IBM Inc., Chicago, USA). A P-value of < 0.05 was considered statistically significant. All data were expressed as the means ± SD. Three different samples were analyzed for each group.

Histological preparation of the maxillary first molars (M_1)

Histological preparation was performed as described previously (Baba et al. 2015). Briefly, the fixed and decalcified specimens were embedded in paraffin and cut into bucco-palatal sections (4 μm) at the M_1 level. Some of the sections were stained with hematoxylin and eosin (HE) for histological observation.

Digoxigenin (DIG)-labeled probes to detect *Dspp*, *Fgfr1, -2c, and -3c* transcripts

DIG-labeled probes were generated as previously described (Baba et al. 2015). Briefly, a cDNA pool was generated from the incisors and molars of mice at the age of postnatal day (P) 7 using an oligo-dT primer (Transcriptor First Strand cDNA Synthesis Kit: Roche). The DNA fragments corresponding to *Dspp*, *Fgfr1*, *-2c,* and *-3c* transcripts possessing T3 and T7 promoter sequences were generated by RT-PCR. The DIG-labeled single-stranded antisense and sense probes were generated by utilizing a DIG RNA Labeling Kit (Roche). All primer sequences were designed using Primer3Plus (https://primer3plus.com) and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast). The respective primer sequences are presented in Table 1.

In situ hybridization (ISH)

ISH was performed as previously described (Baba et al. 2015). Briefly, deparaffinized sections were treated with proteinase K (10 μg/ml, Sigma) in Tris buffer (pH 8.0) at 37°C for 10 min, followed by a 0.2 M HCl treatment at room temperature for 10 min. Then, those sections were dipped into 0.1 M triethanolamine containing 0.5% acetic anhydride for 15 min as an acetylation process. The hybridization was performed using either antisense or sense probes for 20 h at 50°C. After several washing steps, expression loci were immunohistochemically detected using a DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer's instruction. Counter-staining was performed with methyl green solution.

Results

I) Evaluation of the method for extracting total RNA from fixed and decalcified samples

To validate our method for extracting RNA, we performed the following two experiments by employing 48 mice at age of 2 weeks old.

i) RNA was extracted from the mandibular first molars (M_1) at the age of 2 weeks and treated with the following conditions: (a) unfixed and undecalcified, (b) fixed and undecalcified, (c) fixed and decalcified for 1 week, (d) fixed and decalcified for 4 weeks. qPCR for *β-actin* was performed on the cDNA generated from RNA extracted by each condition, and cycle quantification (Cq) values were evaluated. As shown in Fig. 1-A, the Cq value for the group (a) was significantly lower than the others, while no significant difference was detected among (b), (c), and (d). In fact, extracted RNA was decreased after fixation; however, RNA could be extracted constantly from each sample even after decalcification.

ii) qPCR for *β-actin* was performed on the cDNA generated from RNA extracted from the M_1 at 2-20 weeks old, fixed, and decalcified for 1 week. No significant differences were detected among the Cq values of any age group (Fig. 1-B).

II) Expression of *Dspp* and *Fgfr1, -2c, and -3c* transcripts in maxillary first molars (M_1) at 2-20 weeks old

qPCR for *Dspp* and *Fgfr*s transcripts was performed on the cDNA generated from total RNA extracted from the M_1, at 2-20 weeks old. Totally 126 mice were employed for this experiment. The expression level of *Dspp* transcripts in M_1 was highest at 2 weeks old, while the expression level was less than 50% at 4 weeks old and 30% at 8 weeks old. The expression levels of *Dspp* transcripts were constant after 8 weeks (Fig. 2-A). The expressions of *Fgfr1 and Fgfr3c* transcripts were constantly detected during the experimental period (Fig. 2-B, 2-D). The expression level of *Fgfr2c* transcripts was also the highest in M_1 at 2 weeks old, while the expression was less than 30% of that level at later stages, though it was constantly detected (Fig. 2-C).

III) *In situ* hybridization (ISH) for *Dspp* and *Fgfr1, -2c, and -3c* transcripts in maxillary first molars (M_1) at 2, 4, and 8 weeks old

Histological analyses, HE staining and ISH, were performed by employing 12 mice.

2 weeks old

In 2-week-old mice, M_1 is just about to erupt. Dentin matrix was actively formed by odontoblasts, and the exact thickness of the predentin layer was observed (Fig. 3-a). Reflecting this activity, the expression of transcripts for *Dspp* was intensely detected in odontoblasts facing the predentin at the crown and root areas (Fig. 3-b, g). Transcripts for *Fgfr1* and *Fgfr3c* were weakly detected in odontoblasts (Fig. 3-c, e, h, j). Transcripts for *Fgfr2c* were not detected in the dental pulp area (Fig. 3-d, i).

4 weeks old

In 4-week-old mice, M_1 has already erupted and root dentin has become thicker (Fig. 4-a). The expression of *Dspp* transcripts was less intensely detected than that at 2 weeks old in odontoblasts facing the predentin (Fig. 4-b, g). Transcripts for *Fgfr1* and *Fgfr3c* were weakly detected in odontoblasts, especially at the pulp horn sites (Fig. 4-c, e, h, j). Transcripts for *Fgfr2c* were not detected in the dental pulp area (Fig. 4-d, i).

8 weeks old

In 8-week-old mice, the dental pulp space has become smaller (Fig. 5-a, f), and tertiary or reactionary dentin matrix was observed especially in the coronal dentin area (Fig. 6-a, c). The expression of *Dspp* and/or *Fgfr1* transcripts was detected in the limited number of odontoblasts those facing the reactionary dentin (Fig. 6-b, d). Transcripts for *Fgfr2c* and *Fgfr3c* were not detected in the dental pulp area (Fig. 5-d, e, i, j).

Positive reactions were not detected using sense probes for each transcript during the whole experimental period.

Discussion

Impact of fixation and decalcification on quantitative real-time PCR (qPCR) results

In the present study, we attempted to elucidate the dynamics of odontoblasts after tooth eruption, including secondary and tertiary dentin formation. However, there are a couple of problems when examining odontoblasts and dental pulp after tooth eruption, especially when extracting RNA. Odontoblasts are housed in dental pulp, which is surrounded by (sometimes highly) mineralized dentin and enamel matrices, which makes it difficult to homogenize the tissues and extract RNA. Moreover, odontoblasts extend their processes into dentinal tubules, which will be a barrier to collect cells themselves, even after the pulp cavity is exposed (Oida et al. 1982). Both together may result in the imperfect salvage of RNA in odontoblasts and dental pulp.

In consideration of this, we employed a method for the extraction of RNA as follows. First, the animals were perfused with paraformaldehyde solution to preserve RNA in the tissue, followed by decalcification to soften the mineralized tissues. Finally, samples were homogenized with proteinase K to remove the surrounding protein. Gene amplification by PCR using RNA extracted from formalin-fixed tissues is known to reduce its efficiency (Werner et al. 2000; Von Ahlfen et al. 2007). This is due to the binding of mono-methylol groups to the bases in the RNA strands. This binding can be avoided by RNA extraction at higher temperatures, which improves the efficiency of PCR amplification (Masuda et al. 1999; Wimmer et al. 2018). The effect on RNA by decalcification has also been reported using various decalcification solutions. EDTA and formic acid solutions can lower the Cq values of qPCR compared to strong acid solutions (Singh et al. 2013).

In our experiments targeting the intrinsic control *β-actin*, the unfixed and undecalcified group (Group a) showed the lowest Cq values (number of PCR cycles), while the fixed and undecalcified group (Group b) and the fixed and decalcified for 1 week group (Group c) showed values about 2 cycles higher than Group a (Fig. 1-A). These results suggest that fixation affected the efficiency of RNA extraction. In fact, we could not detect transcripts in the extracts from fixed specimens without proteinase K treatment (preliminary data, not shown). In addition, the decalcification process using EDTA had a slight affect (Fig. 1-A). However, stable and constant Cq values for *β-actin* expressions from 2 to 20 weeks old were observed (Fig. 1-B). Therefore, in the present study we concluded that RNA extracted from fixed and decalcified samples can be evaluated by qPCR.

In light of the above reports and results, we developed the protocol as described in the Materials & Methods. The tissues were fixed and decalcified by EDTA followed by proteinase K treatment. Reverse transcription was performed using a reverse primer for target genes to generate shorter amplicons for steady transcription. By utilizing this protocol, we could determine the temporal changes in transcript expression

levels for *Dspp* and *Fgfrs*. In previous studies (Baba et al. 2004; Quispe-Salcedo et al. 2012), Dsp was detected in the dental pulp area by immunohistochemistry (IHC), while its transcripts could not be detected by ISH after the age of 8 weeks. Our present study clearly showed that the expression level of transcripts for *Dspp* was less than 30%, which explains the discrepancy between the results of IHC and ISH.

The discrepancy between the results by quantitative real-time PCR (qPCR) and *in situ* hybridization (ISH)

Although the expression of *Fgfr2c* transcripts was highly detected at 2 weeks old by qPCR (Fig. 2-C), the transcripts for *Fgfr2c* were not detected in the dental pulp at 2-8 weeks old by ISH (Fig. 3-d, i, Fig.4-d, i, Fig. 5-d, i). In this case, we speculate that the quantity of *Fgfr2c* transcripts at 2 weeks old might be still less than the detection level by ISH. Regarding the expression of *Fgfr3c* transcripts, the expressions were detected at 2 weeks old (Fig. 3-e, j), but not at 8 weeks old by ISH (Fig. 5-e, j). While, the expression levels of *Fgfr3c* were somewhat constant by qPCR (Fig. 2-D, No statistically significant differences were found between all stages). In this context, the expression levels of *Fgfr3c* transcripts might be around the detection limit by ISH, and small amounts of transcripts may result in the subtle expression pattern by ISH. However, the enough amounts of transcripts as like expression of *Dspp*, the expression patterns of transcripts by qPCR (Fig. 2-A) were well consistent with those by ISH (Fig. 3-b, g, Fig. 4-b, g, Fig. 5-b, g). So that, we believe that our method is still considerably reliable.

Expression of *Fgfr1* after tooth eruption

In mouse tooth development, transcripts for *Fgfr1c* were expressed in the mesenchymal cells. They are also detected in cells in the sub-odontoblastic layer at the bell stage (Kettunen et al. 1998, Rice et al. 2003), and are only detected in odontoblasts on P1 to P7 (Kettunen et al. 1998). Previous studies have reported many possible candidate ligands for *Fgfr1c* by utilizing BAF3 cells (Ornitz et al. 1996; Zhang et al. 2006). Among them, Fgfr1c is commonly used for Fgf2 and Fgf23 signaling, and Fgf23 signals through a complex of α-Klotho and Fgfr1c (Urakawa et al. 2006). In *Fgf23*-deficient mice, a disrupted odontoblast layer and ectopic mineralized matrix are observed in the pulp cavity (Chu et al. 2010). In addition, The Klotho protein directly binds to multiple Fgfrs (Kurosu et al. 2006). In *Klotho*-deficient mice, some odontoblast-like cells are embedded in the secondary dentin that show osteodentin-like features (Suzuki et al. 2008). In the present study, *Fgfr1* transcripts were continuously detected in mouse M_1. However, the expression level of *Fgfr1* transcripts by ISH was lower in odontoblasts of 4-week-old compared to 2-week-old mice. Therefore, we hypothesized that FGF signaling via FGFR1 is involved in the early stage of dentin formation (primary dentin) and is formed in cooperation with other receptors during secondary dentin formation.

Although *Dspp* and *Fgfr1* transcripts were not detectable in most of the odontoblasts at 8 weeks old, limited expressions were found in odontoblasts at the pulpal horn area, where the tertiary dentin was formed (Fig. 6-a, c). At the cusp tip of mouse molars, there is a region of dentin without an enamel cap,

which is called the enamel-free area (Yamamoto and Nawa 1995), and *Dspp* transcripts are expressed by odontoblasts in these regions (Quispe-Salcedo et al. 2012). In this context, the expression of *Fgfr1* transcripts might be involved in tertiary dentin formation in response to physiological stimuli.

Given the above, FGF signaling via FGFR1 might differentially regulate odontoblasts depending on their differentiation status and physiological condition. Moreover, signaling via FGFR1 might control secondary and/or tertiary dentin formation.

Expression of *Fgfr2c* after tooth eruption

Fgfr2c transcripts in mice by ISH are observed in the dental follicle and the stellate reticulum (Kettunen et al. 1998). In rats, *Fgfr2c* has been detected in odontoblasts even in crown and root areas, as well as in some dental pulp cells (Baba et al. 2015). Tissue-specific ablation of *Fgfr2* in the dental epithelium results in the hypoplasia of enamel and dentin in maxillary incisors (Lin et al. 2009). In the present study, the expression of *Fgfr2c* was downregulated after tooth eruption. Taken together, we speculate that FGFR2 plays a critical role in epithelial-mesenchymal interactions, but has a lesser role in dentin formation after tooth eruption, such as in secondary and tertiary dentin formation.

Expression of *Fgfr3c* after tooth eruption

A previous study reported that *Fgfr3c* is not detected in the dental papilla at the early bell stage (Rice et al. 2003) but is observed in the dental papilla cells of the crown formation stage (Kettunen et al. 1998). In the present study, *Fgfr3c* transcripts were also detected in dental papilla in the maxillary molars at P3 (data not shown). Our results using rats have shown that *Fgfr3* expression is detected in odontoblasts and a small number of pulp cells at P7 and P14 (Baba et al. 2015). *Fgf18* during rat root formation is detected in the coronal and root odontoblasts and some cells in the sub-odontoblastic layer (Baba et al. 2015). In addition, expanded layers for proliferation of chondrocytes and hypertrophic cells at epiphyseal areas of long bones were observed in both *Fgf18*-deficient and *Fgfr3*-deficient mice, suggesting that FGF18 signals through via FGFR3 to regulate the proliferation and differentiation of chondrocytes (Ohbayashi et al. 2002). Thus, we conclude that FGFR3 may be involved in the proliferation and differentiation of odontoblasts for dentin formation.

In conclusion, our newly modified protocol for extracting RNA from mineralized tissue enabled us to determine the expression pattern of transcripts for DSPP and FGFRs in erupted molars. Our results suggest the commitment of FGF signaling to the homeostasis of odontoblasts after tooth eruption, including secondary and tertiary dentin formation.

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Compliance with Ethical Standards

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

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Figure legends

Table 1 Primer sequences (5'→3')

Fig. 1 Effects of fixation and decalcification on qPCR. **A** The effects of fixation and decalcification on qPCR were evaluated utilizing the mean Cq values of qPCR using 2-week-old mandibular first molars. Total RNA was extracted from the molars; **a**, unfixed and undecalcified; **b**, fixed and undecalcified; **c**, fixed and decalcified for 1 week; **d**, fixed and decalcified for 4 weeks. Increased Cq levels were found when total RNA was extracted from the fixed and decalcified sample. **B** Mean Cq values of mandibular first molars at 2, 4, 8, 12, 16, and 20 weeks when total RNA was extracted from the fixed and decalcified (1 week) sample. No statistically significant differences were found between any groups in **B.** All data are presented as means ± SD (n = 3). Significance was determined using Tukey's comparison test. *$p < 0.05$

Fig. 2 The expressions of *Dspp* (**A**), *Fgfr1* (**B**), *-2c* (**C**), and *-3c* (**D**) in maxillary first molars. **A** The expression level of *Dspp* transcripts was highest at 2 weeks old, decreased at 4 weeks old, and was lower

at 8 weeks old. No significant changes were observed in the groups after 8 weeks. **B** The expression of *Fgfr1* transcripts was constantly detected from 2 to 20 weeks old. **C** The expression levels of *Fgfr2c* transcripts were the highest at 2 weeks old. Lower level expression was constantly observed without significant differences after 4 weeks old. **D** The expression of *Fgfr3c* transcripts was constantly detected from 2 to 20 weeks old. No statistically significant differences were found between any groups in **B** and **D**. All data are presented as means ± SD (n = 3). Significance was determined using Tukey's comparison test. *$p < 0.05$

Fig. 3 Bucco-palatal sections of mice maxillary first molars at 2 weeks old. HE staining (**a**) and ISH using antisense probes to detect transcripts for *Dspp* (**b**), *Fgfr1* (**c**), *Fgfr2c* (**d**), and *Fgfr3c* (**e**). High magnification images (**f-j**) of the respective boxed areas in (**a-e**) representing the crown areas. Transcripts for *Dspp* were detected in odontoblasts (**b**, **g**) and were not detected in cells adjacent to Hertwig's epithelial root sheath (**b**, arrowheads). Transcripts for *Fgfr1* (**c**, **h**) and *Fgfr3c* (**e**, **j**) were weakly expressed in odontoblasts, while transcripts for *Fgfr2c* were not detected (**d**, **i**). Bars 100 μm (**a–e**) and 50 μm (**f–j**). **D**, dentin; **P**, dental pulp; **Od**, odontoblast

Fig. 4 Bucco-palatal sections of mice maxillary first molars at 4 weeks old. HE staining (**a**) and ISH using antisense probes to detect transcripts for *Dspp* (**b**), *Fgfr1* (**c**), *Fgfr2c* (**d**), and *Fgfr3c* (**e**). High magnification images (**f-j**) of the respective boxed areas in (**a-e**), representing the crown areas. Transcripts for *Dspp* were detected in odontoblasts (**b**, **g**). However, the expression level was less than that at 2 weeks old. Transcripts for *Fgfr1* (**h**) and *Fgfr3c* (**j**) were weakly expressed in odontoblasts at the pulp horn area, while transcripts for *Fgfr2c* were not detected (**d**, **i**). Bars 100 μm (**a–e**) and 50 μm (**f–j**). **D**, dentin; **P**, dental pulp; **Od**, odontoblast

Fig. 5 Bucco-palatal sections of mice maxillary first molars at 8 weeks old. HE staining (**a**) and ISH using antisense probes to detect transcripts for *Dspp* (**b**), *Fgfr1* (**c**), *Fgfr2c* (**d**), and *Fgfr3c* (**e**). High magnification images (**f-j**) of the respective boxed areas in (**a-e**), representing the crown areas. Transcripts for *Dspp*, *Fgfr1*, *Fgfr2c*, and *Fgfr3c* were not detected. Bars 100 μm (**a–e**) and 50 μm (**f–j**). **D**, dentin; **P**, dental pulp; **Od**, odontoblast

Fig. 6 Bucco-palatal sections of mice maxillary first molars at 8 weeks old, showing tertiary dentin at the pulp horn area, representing the expressions of transcripts in tertiary (reactionary dentin) area. HE staining (**a**, **c**) and ISH using antisense probes to detect the transcripts for *Dspp* (**b**) and *Fgfr1* (**d**). Transcripts for *Dspp* and *Fgfr1* were detected in odontoblasts (**b**, **d**) adjacent to the reactionary dentin at the pulp horn area at 8 weeks old. Bars 50 μm (**a-d**). **D**, dentin; **P**, dental pulp; **Od**, odontoblast; **Rd**, reactionary dentin

Table 1

Primer sequences (5'→3')

Gene	real-time PCR	Size	*in situ* hybridization	Size	Gene Bank accession number
Dspp	F:5'-TAGCACCAACCATGAGGCTG-3'	60 bp	F:5'-GTGCCTCTTCTAACACATCCAGGAA-3'	319 bp	NM_010080
	R:5'-GGCGTCGTTTCATGTGTAGC-3'		R5'-CGATGCCTACCTGGCTCCTGA-3'		
Fgfr1	F:5'-CCAGAGCGATGTGTGGTCTT-3'	91 bp	F:5'-GAAGATCGCAGACTTTGGCTTA-3'	339 bp	NM_010206
	R:5'-AAGTTCCTCCACAGGCACAC-3'		R:5'-AACTGCTTGAACGTAGGTCTCTG-3'		
Fgfr2c	F:5'-TGCAGCTAGGACGGTAGACA-3'	99 bp	F:5'-GACGGTAGACAGTGAAACTTGG-3'	159 bp	NM_010207
	R:5'-TCGGAGCTATCTGTGTCGTC-3'		R:5'-TTCTCCATCTTCTCGGTGTTG-3'		
Fgfr3c	R:5'-GCTAACACCACCGACAAGG-3'	61 bp	F:5'-ATTCTAGGCAGTGACGTGGAG-3'	312 bp	NM_008010
	F:5'-CGTCCTCAAAGGTGACATTG-3'		R:5'-AGCCTCATCAGTTTCCATCAG-3'		
β-actin	F:5'-CGACAACGGCTCCGGCATGT-3'	87 bp			NM_007393
	R:5'-CTAGGGCGGCCCACGATGGA-3'				

Fig. 1

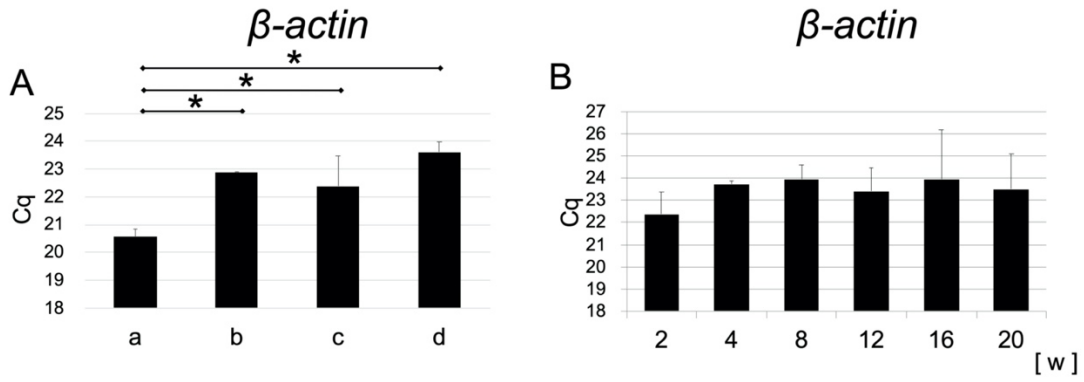

Fig. 2

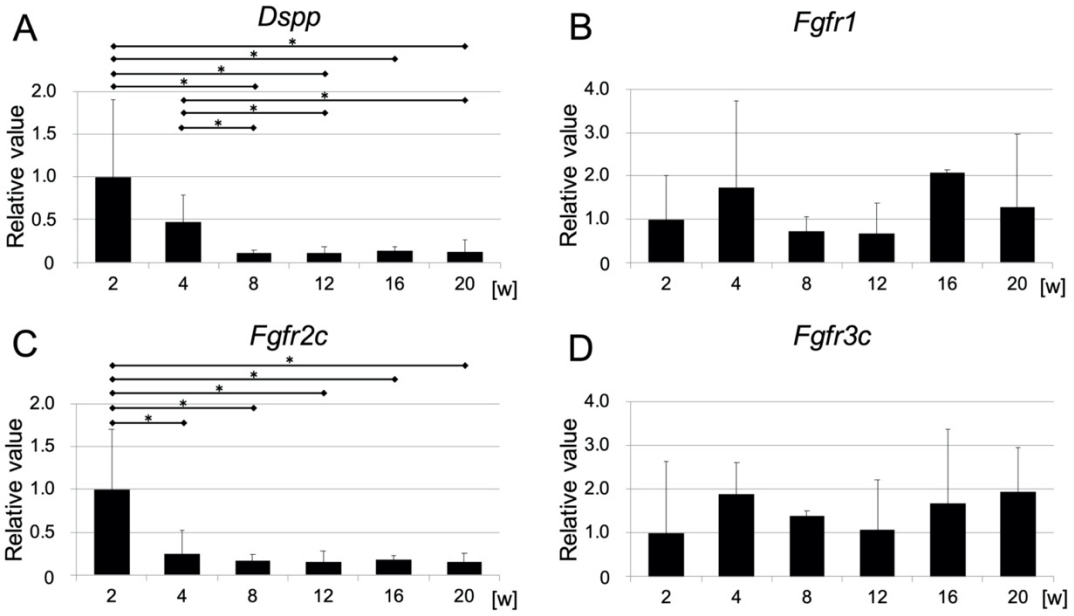
A
Dspp
Relative value
2.0
1.5
1.0
0.5
0
2
4
8
12
16
20
[w]
B
Fgfr1
Relative value
4.0
3.0
2.0
1.0
0
2
4
8
12
16
20
[w]
C
Fgfr2c
Relative value
2.0
1.5
1.0
0.5
0
2
4
8
12
16
20
[w]
D
Fgfr3c
Relative value
4.0
3.0
2.0
1.0
0
2
4
8
12
16
20
[w]

Fig. 3

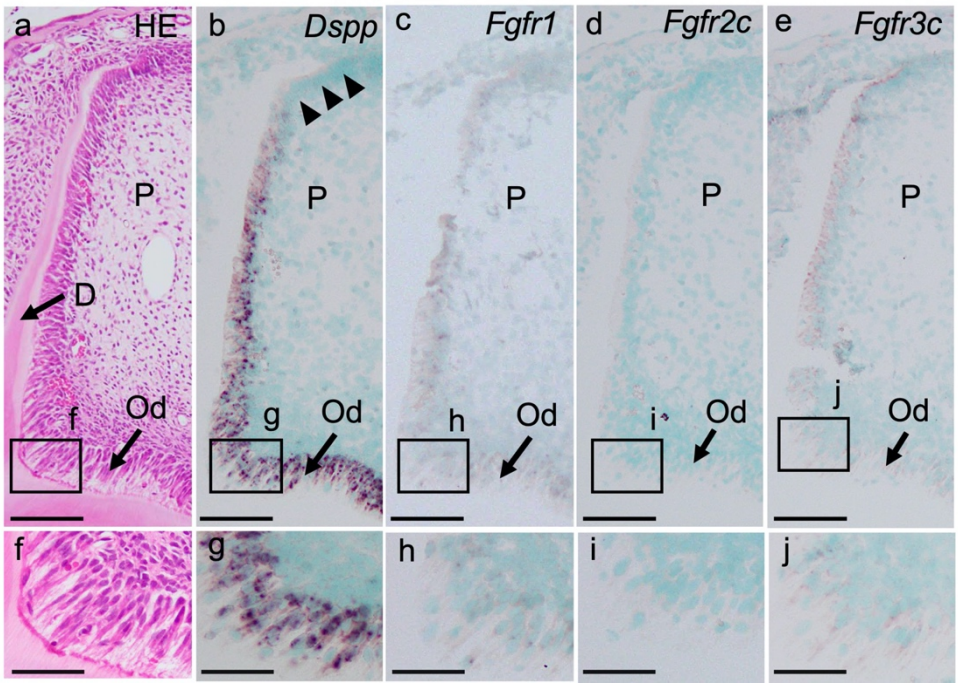
a
HE
P
D
f
Od
b
Dspp
P
g
Od
c
Fgfr1
P
h
Od
d
Fgfr2c
P
i
Od
e
Fgfr3c
P
j
Od
f
g
h
i
j

Fig. 4

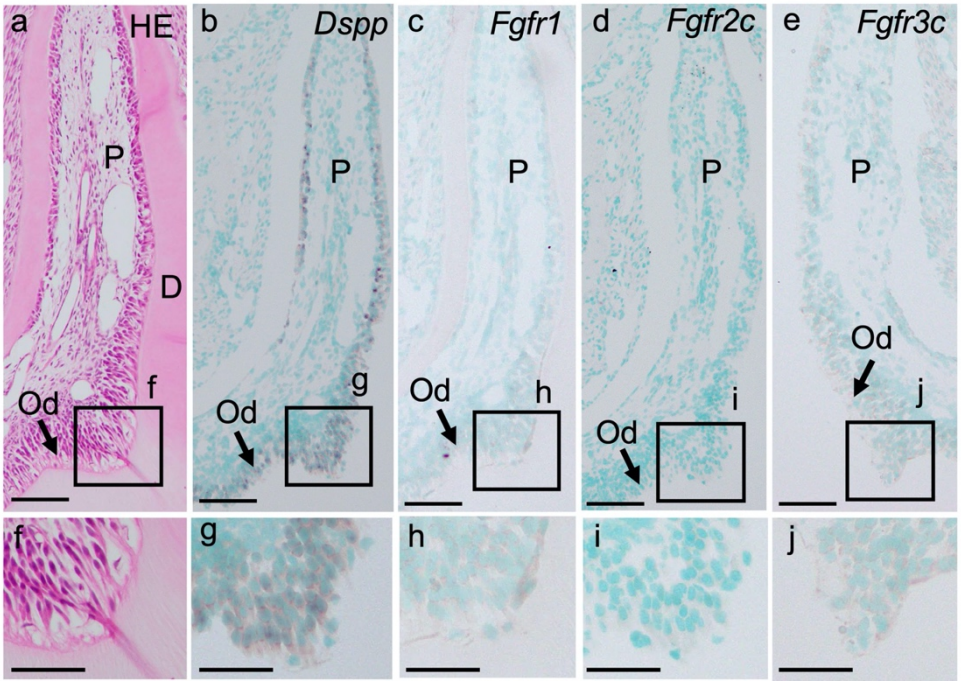
a HE
b Dspp
c Fgfr1
d Fgfr2c
e Fgfr3c
P
D
Od
f
g
h
i
j

Fig. 5

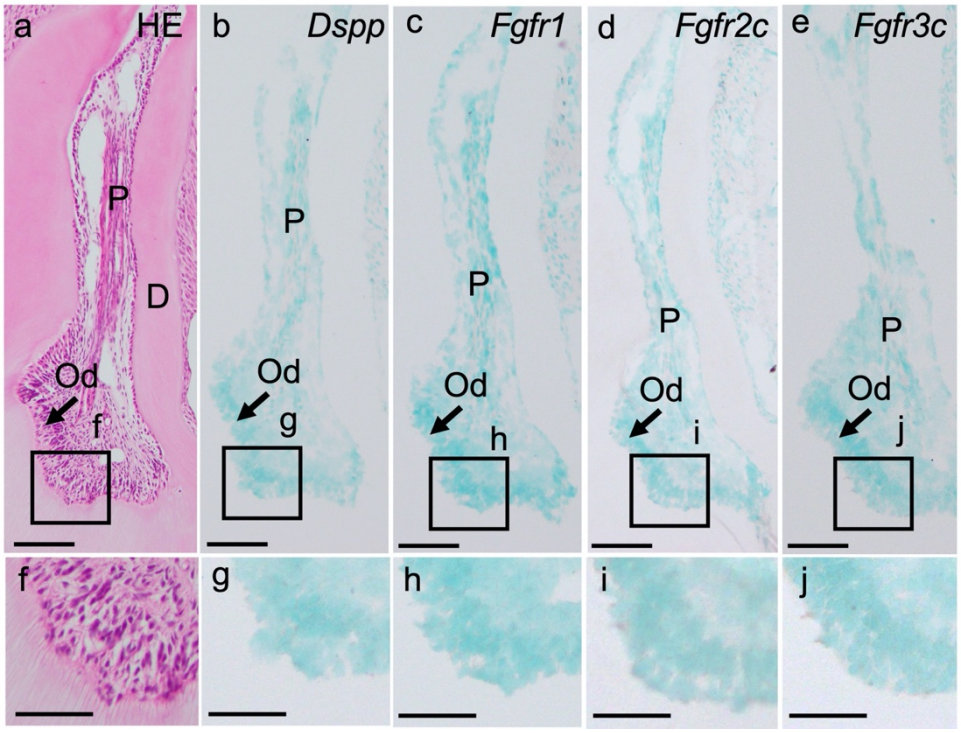
a HE
b Dspp
c Fgfr1
d Fgfr2c
e Fgfr3c
P
D
Od
f
g
h
i
j

Fig. 6