Original Article

Regulation of IGF-I by IGFBP3 and IGFBP5 during odontoblast differentiation in mice

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ABSTRACT

Objectives: Although intracellular signaling pathways of insulin-like growth factor I (IGF-I) related to the proliferation of dental pulp cells have been investigated, the switching mechanism from cell proliferation to differentiation during odontogenesis remains elusive. This study aimed to elucidate the role of IGF binding protein (IGFBP) 3 and 5 in regulation of IGF-I during odontoblast differentiation in mouse incisors.
Methods: The detailed expression patterns of IGF-I, IGF-I receptor (IGF-IR), IGFBP3, and IGFBP5 together with that of an odontoblast differentiation marker, nestin, were examined by immunohistochemistry and/or in situ hybridization using paraffinized sections of TetOP-H2B-GFP mouse incisors at postnatal 4 weeks.
Results: Undifferentiated dental papilla cells and preodontoblasts (preOB) showed intense IGF-I- and IGF-IRα-positive reactions, and the expression was observed in differentiated odontoblasts, such as immature odontoblasts (iOB) and mature odontoblasts (mOB). IGFBP3/Igfbp3 was transiently expressed in preOB and early iOB, and the intensity of expression gradually reduced with the progression of odontoblast differentiation. In contrast, immunohistochemical analysis for IGFBP5 identified a positive reaction in the undifferentiated dental papilla cells and differentiated odontoblasts, and the expression of Igfbp5 was reduced in the differentiated odontoblasts.
Conclusion: The present study demonstrated the expression patterns of IGF-I, IGF-IR, IGFBP3, and IGFBP5 during odontoblast differentiation in mouse incisors. These results suggested that IGFBP3 regulates the transition from the proliferative to differentiation stage by inhibiting the action of IGF-I on the proliferation of dental papilla cells, and that IGFBP5 plays an important role in the maintenance of the differentiated odontoblasts during tooth development.

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1. Introduction

Elucidation of the mechanism of odontoblast differentiation has been one of the most important issues in dental pulp biology. The process of odontoblast differentiation has been classified into preodontoblast (preOB) and odontoblast stages based on the morphology and secretory activities [1]. Odontoblasts have been further divided into the following subgroups: immature (iOB), mature (mOB), and resting odontoblasts [1—3]. During odontogenesis, the odontoblast lineage cells undergo cell proliferation followed by cell differentiation, resulting in dentin formation. In dental mesenchyme, undifferentiated cells with high proliferative activity are referred to as dental papilla cells. The polarized cells, without proliferative activity, that begin to acquire their differentiation markers, such as nestin and Dsp, are named preodontoblasts. The cells beginning to produce predentin are characterized by immature features, including minute projections on their distal ends, and are engaged in the production of mantle dentin, where no capillaries are present in the odontoblast layer. These immature cells are referred to as iOB (immature odontoblast). Subsequently, they increase in height and develop a thick cellular process during active dentin formation, resulting in the formation of a

pseudostratified layer; fenestrated capillaries are located close to the predentin. Based on their mature morphology and capacity for dentin deposition and sufficient nutritional supply, these odontoblasts were termed as mOB (mature odontoblast). Regarding the mechanism of differentiation, numerous reports have demonstrated that the differentiation of odontoblasts from the neural crest-derived dental papilla cells is induced by several growth factors, such as the transforming growth factor (TGF) family [4], insulin-like growth factors (IGFs) [5], and fibroblast growth factors (FGFs) [6,7]. However, since the results of these previous studies were mainly based on *in vitro* culture analyses, it seemed to be difficult to evaluate the precise effects of various growth factors on cell proliferation, differentiation, and secretion. Particularly, the regulatory mechanism of transition from cell proliferation to differentiation during odontogenesis remains to be elucidated.

IGF-I plays important roles in tooth development, including pulpal cell proliferation and root development [8,9]. IGF-I initiates signaling pathways by binding to its receptor (IGF-I receptor: IGF-IR), and is involved in numerous biological events, such as cell proliferation, migration, and inhibition of apoptosis [10]. Recently, several reports have demonstrated that IGF-I activates both mitogen-activated protein kinase and Akt/mTOR pathways, resulting in active proliferation of dental pulp cells [5,11]. In addition, another study has revealed that ephrinB1 also acts downstream of Akt/mTOR pathway [12]. In addition to *in vitro* studies, analysis by *in situ* hybridization has shown that *Igf-I* is expressed in dental pulp cells, including odontoblasts in rat incisors [13]. In contrast, IGF-II can also bind to IGF-IR and stimulate cell proliferation in dental pulp, although its effect on cell proliferation is less prominent than that of IGF-I [14]. Thus, intracellular events through the interaction between IGF-I or IGF-II and IGF-IR have been investigated in dental pulp biology. To date, however, functional significance and interaction of other members associated with IGF-I such as IGF binding proteins (IGFBPs) and insulin with IGF-I, remains to be elucidated.

IGFBPs are a family of proteins that bind with high affinity to IGFs and regulate their availability to IGF-IR [15]. Binding of IGF to IGFBPs inhibits access of IGF to IGF-IR, resulting in the inhibition of cell proliferation, differentiation, survival, and other IGF-stimulated signaling pathways. By early 1990s, all six members of the IGFBP family had been cloned and several key structural and sequence similarities were identified. Among them, IGFBP3 is the most abundant circulating IGFBP and prolongs the circulating half-life of IGFs [16]. The functional roles of IGFBP3 involve the transport of IGFs in plasma, the control and regulation of the efflux from the vascular space and their clearance, and the modulation of the interaction of IGFs with their receptor. As with IGFBP3, IGFBP5 also inhibits the action of IGFs under most circumstances, although it has a modest binding preference for IGF-II over IGF-I [17]. Furthermore, several IGFBPs, including IGFBP3 and IGFBP5, have also been reported to exhibit IGF-independent activities, including the regulation of cell proliferation, migration, survival, and apoptosis [18—20]. Since IGFBPs exhibit a variety of functions regulating essential cellular processes, such as cell proliferation, migration, survival, and differentiation, thorough the IGF-dependent and -independent activities, it is important to understand the spatiotemporal regulation of IGFs and IGFBPs in a given tissue. In the field of dentistry, several studies have demonstrated that IGFBPs are expressed in periodontal ligament cells *in vivo* and IGFBP2 and IGFBP3 can regulate the action of IGF-I on human dental pulp cells *in vitro* [21,22]. In addition, our unpublished data suggested that IGFBP5 plays a pivotal role in regulating the survival and apoptosis of putative dental pulp stem cells during both tooth development and pulpal healing following tooth injury. However,

the mechanism of regulation of IGF-I by IGFBPs during odontoblast differentiation remains to be elucidated.

It is well-known that rodent incisors are continuously growing teeth and epithelial and mesenchymal stem cell compartments are eternally maintained in their apical end [23,24], indicating that we have to distinguish dental pulp stem/progenitor cells from other cell populations. Histone 2B (H2B)-green fluorescent protein (GFP) mice have been used for identifying the label-retaining cells (LRCs) [25]. In TetOP-H2B-GFP mice, the H2B-GFP expression is doxycycline (dox)-inducible and is gradually decreased according to the number of cell divisions during the chasing periods. Previous studies using these mice have demonstrated that epithelial [26] and mesenchymal [27] stem cells in the continuously growing incisors of mice have been shown to be H2B-GFP-LRCs. Thus, this study aimed to elucidate the role of IGFBP3 and IGFBP5 in regulating the action of IGF-I during odontoblast differentiation in TetOP-H2B-GFP mouse incisors.

2. Materials and methods

2.1. Mice

All animal experiments complied with the guidelines of the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of the Environment, and the Science Council of Japan and were carried out in accordance with the Act on Welfare and Management of Animals. TetOP-H2B-GFP mice [B6; 129S4-Gt(ROSA)26-Sor?tm1(rtTA*M2) Jae?Colla1?tm7(tetO-HIST1H2BJ/GFP)Jae?/J] were purchased from Jackson Laboratories [25]. For green fluorescent protein transgene expression, doxycycline (2 mg/mL, supplemented with 50 mg/mL sucrose) was added to drinking water at embryonic day 16.5. TetOP-H2B-GFP mice were sacrificed at postnatal (P) 4 weeks (n = 3) and perfused with physiological saline transcardially, followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) under deep anesthesia via an intraperitoneal injection of chloral hydrate (350 mg/kg). Following decalcification in a 10% EDTA-2Na solution for 3 weeks at 4 °C, the specimens were dehydrated through serially diluted concentrations of ethanol and embedded in paraffin, and 4 μm thick sagittal sections were cut. The paraffin sections were mounted on Matsunami adhesive silane (MAS)-coated glass (Matsunami Glass Ind., Osaka, Japan) slides and stained with hematoxylin and eosin.

2.2. Immunohistochemical analysis

Immunohistochemistry was conducted as described in our previous report [28], with a mouse anti-nestin monoclonal antibody (diluted to 1:500) (catalog number MAB353, Merck Millipore, Billerica, MA, USA), a rabbit anti-GFP polyclonal antibody (diluted to 1:1000) (catalog number 598, Medical & Biological Laboratories Co., Nagoya, Japan), a rabbit anti-IGFBP3 polyclonal antibody (diluted to 1:200) (catalog number ab217205, Abcam, Cambridge, UK), a goat anti-IGFBP5 polyclonal antibody (diluted to 1:50) (catalog number AF578, R & D Systems Inc., Minneapolis, MN, USA), a rabbit anti-IGF-I polyclonal antibody (diluted to 1:200) (catalog number sc-9013, Santa Cruz, CA, USA), a rabbit anti-IGF-IRα polyclonal antibody (diluted to 1:200) (catalog number sc-712, Santa Cruz, CA, USA), and a rat anti-Ki67 monoclonal antibody (diluted to 1:100) (catalog number M7249, Dako Japan, Tokyo, Japan).

2.3. In situ hybridization

Section *in situ* hybridization was performed as previously described [29]. Digoxigenin-labeled probes for *Igfbp3* and *Igfbp5*

were purchased from GenoStaff (Tokyo, Japan). Following the fixation, the specimens were decalcified with Morse solution (10% sodium citrate and 22.5% formic acid) for 24 h, dehydrated through serially diluted concentrations of ethanol and xylene, and embedded in paraffin. Subsequently, 5-μm-thick paraffinized sections were mounted on MAS-coated glass slides, deparaffinized, dehydrated, and predigested with proteinase K. The sections were then acetylated with 0.25% acetic anhydride in triethanolamine for 10 min and incubated overnight at 70 °C with hybridization buffer containing a digoxigenin-labeled probe for *Igfbp3* or *Igfbp5*. After hybridization, the slides were washed using serially diluted concentrations of sodium citrate—sodium chloride solution and treated by two consecutive incubations with blocking reagent (Roche, Diagnostics Corp, Indianapolis, IN, USA) and anti-digoxigenin antibody (Roche). The sections were stained with 4-nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

3. Results

3.1. Odontoblast differentiation in mouse incisor

On the apical side close to the apical bud region, numerous Ki67-positive dental papilla cells were identified and lacked H2B-GFP labels (Fig. 1a, b, e, f). H2B label-retaining cells (H2B LRCs) were located in the center of pulp tissue, associated with neurovascular bundles, in addition to differentiated odontoblasts and subodontoblastic cells beneath them (Fig. 1a, c, d). In odontogenic epithelium, H2B LRCs were localized in the outer enamel epithelium in the apical bud (Fig. 1b). During odontogenesis, Ki67-positive dental papilla cells gradually became polarized and Ki67-negative preOB began to express nestin, which is type VI intermediate filament and a known differentiation marker for odontoblasts (Fig. 1g, i, j). Nestin-positive and Ki67-negative iOB possessed an H2B label

and initiated the secretion of dentin matrix (Fig. 1d, h, j, k). From this differentiation stage, odontoblasts continuously showed a nestin-positive reaction (Fig. 1k and l).

3.2. Expression patterns of IGF-I and its receptor in mouse incisors

Undifferentiated dental papilla cells and preOB, in particular the apical end of their cytoplasm, showed intense IGF-I- and IGF-IRα-positive reactions (Fig. 2a, b, e, f). The expression of these proteins was maintained in differentiated odontoblasts, such as iOB and mOB (Fig. 2 c, d, g, h).

3.3. Expression patterns of IGFBP3 and IGFBP5 in mouse incisors

IGFBP3/*Igfbp3* was transiently expressed in preOB and early iOB, and the expression was gradually reduced with progression of odontoblast differentiation (Fig. 3a—g). In addition, dental epithelium, including apical bud region, expressed *Igfbp3*, although immunohistochemistry for IGFBP3 could not detect any positive reaction in enamel epithelium (Fig. 3a—h). In contrast, immunohistochemical analysis for IGFBP5 identified the positive reaction in undifferentiated dental papilla cells and differentiated odontoblasts, although the expression of *Igfbp5* was reduced in differentiated odontoblasts (Fig. 3i-o). Dental epithelium, including apical bud region, also expressed IGFBP5/*Igfbp5* (Fig. 3i, j, p).

4. Discussion

The current study confirmed the expression patterns of IGF-I and its receptor during odontoblast differentiation in mouse incisors. The results of this study were consistent with that of a previous study using *in situ* hybridization analysis [13]. IGF-I and IGF-IR were intensely expressed in undifferentiated dental papilla
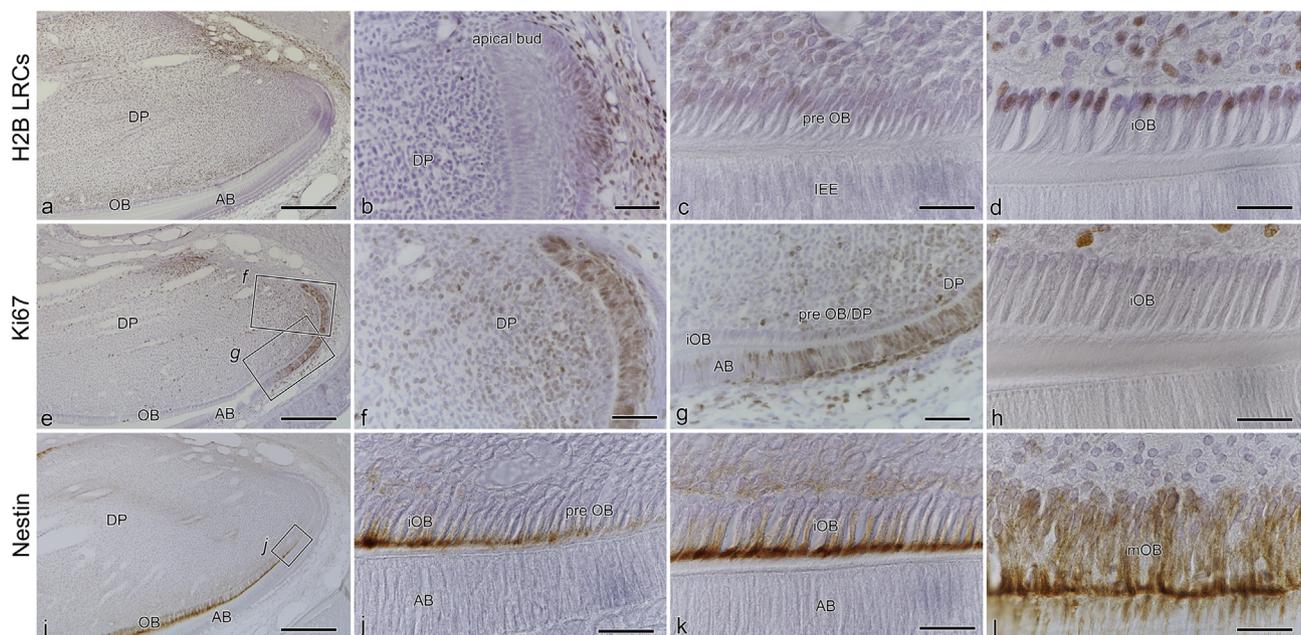

Fig. 1. GFP- (a—d), Ki67- (e—h), and nestin-immunoreactivities (i—l) in mouse incisor at postnatal 4 weeks. Doxycycline was administered at embryonic day 16.5 for the induction of H2B-GFP transgene expression. f and g represent magnifications of the boxed areas in e. Similarly, j represents a higher magnification of the boxed area in i. (b, c, e, and f) In the apical end close to the apical bud region, numerous Ki67-positive dental papilla cells were identified and lack H2B-GFP labels. In odontogenic epithelium, H2B label-retaining cells (H2B LRCs) are localized in the outer enamel epithelium in the apical bud. (a and d) H2B LRCs are located in the center of pulp tissue, associated with neurovascular bundles, in addition to differentiated odontoblasts and subodontoblastic cells beneath them. (g, i, and j) During odontogenesis, Ki67-positive dental papilla cells gradually became polarized and Ki67-negative preOB began to express nestin. (d, h, and k) Nestin-positive and Ki67-negative iOB possess an H2B label and initiate the secretion of dentin matrix. (k and l) From this differentiation stage, odontoblasts continuously showed nestin-positive reaction. AB, ameloblasts; D, dentin; DP, dental pulp; iOB, immature odontoblasts; OB, odontoblasts; preOB, preodontoblasts. Bars, 250 μm (a, e, and i), 50 μm (b, f, and g), 25 μm (c, d, h, and j-l).
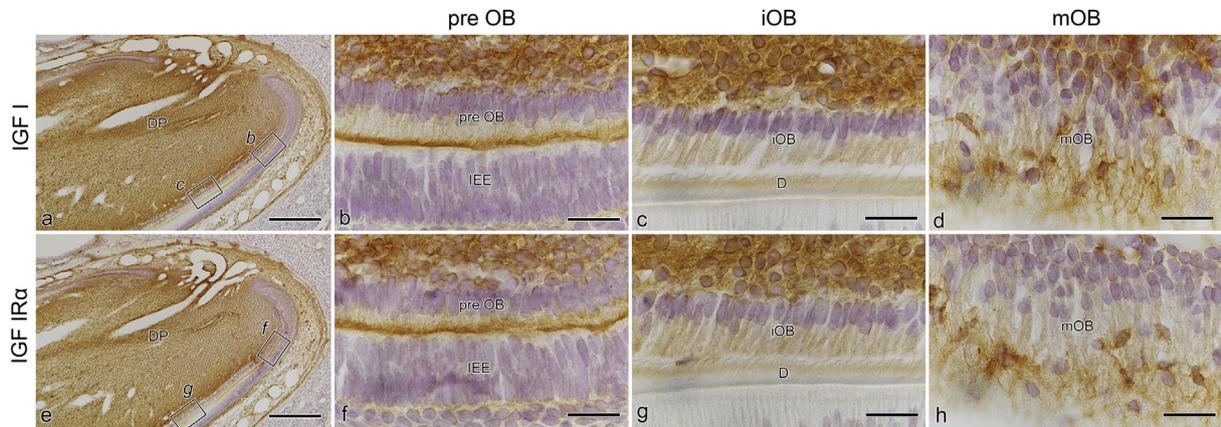
Fig. 2. IGF-I- (a−d) and IGF-IRα-immunoreactivities (e−h) in mouse incisor at postnatal 4 weeks b and c represent magnifications of the boxed areas in a. Similarly, f and g represent magnifications of the boxed areas in e. (a, b, e, and f) Undifferentiated dental papilla cells and preOB, in particular, the apical end of their cytoplasm, show intense IGF-I- and IGF-IRα-positive reactions. (c, d, g, and h) The expression of these proteins is maintained in differentiated odontoblasts, such as iOB and mOB. D, dentin; DP, dental pulp; IEE, inner enamel epithelium; iOB, immature odontoblasts; preOB, preodontoblasts; mOB, mature odontoblasts. Bars, 250 μm (a and e), 25 μm (b-d, f-h).

cells, suggesting that IGF-I stimulates proliferation of these cells. Indeed, these undifferentiated cells showed no H2B label and Ki67-positive reaction, which indicates high proliferative capacity of these cells. This notion was supported by the evidence that exogenous IGF-I enhanced the proliferation of human dental pulp stem cells [5]. In addition to dental papilla cells, the expressions of IGF-I and IGF-IR were widely observed in dental pulp of mouse incisors in the current study. In general, the multifunction of IGF-I has been reported, including cell proliferation, migration, and inhibition of apoptosis [10]. Thus, it is reasonable to suppose that the function of
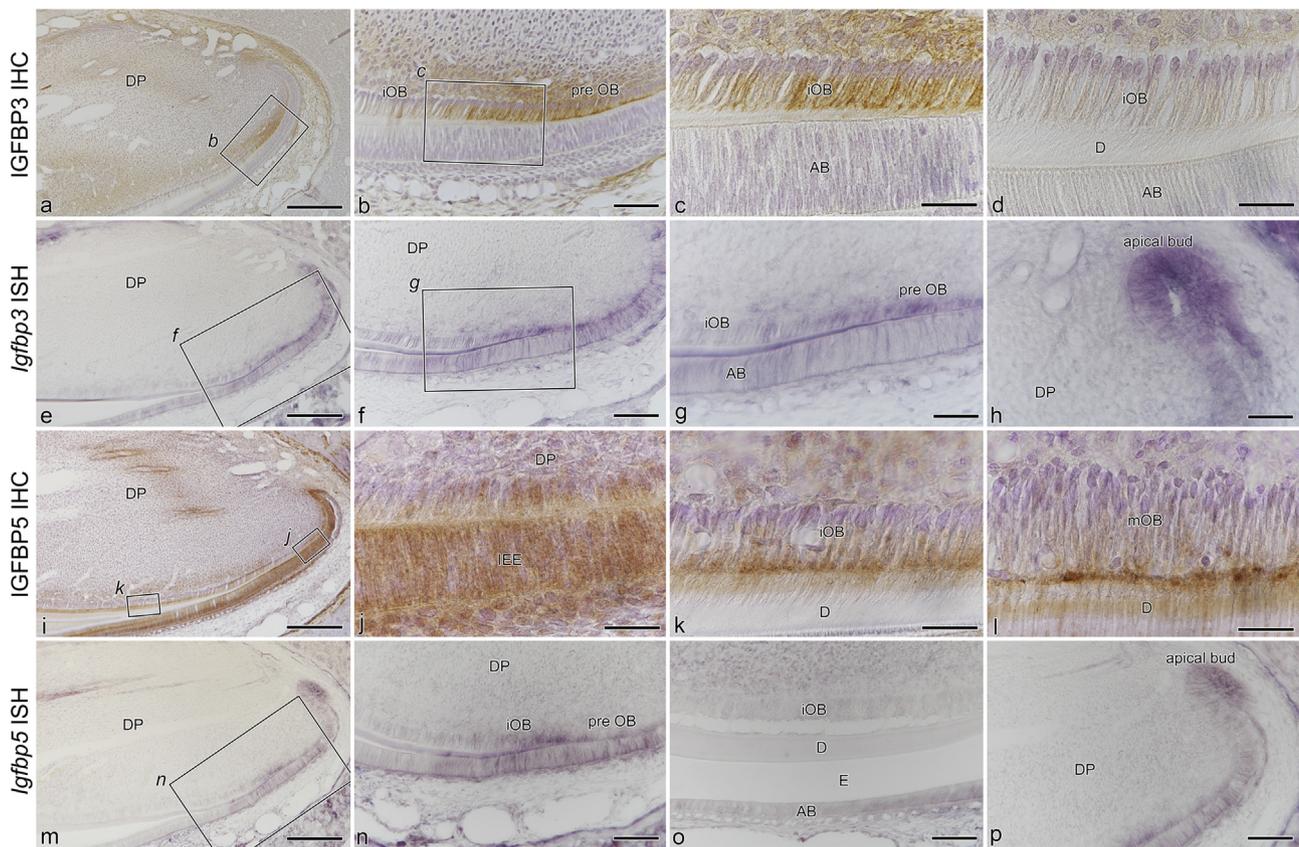

Fig. 3. IGFBP3- (a−d) and IGFBP5-immunoreactivities (i−l) and _in situ_ hybridization for _Igfbp3_ (e−h) and _Igfbp5_ (m−p) in mouse incisor at postnatal 4 weeks b, c, f, g, and n represent a higher magnification of the boxed area in a, b, e, f, and m, respectively. Similarly, j and k represent a higher magnification of the boxed areas in i. (a−h) IGFBP3/_Igfbp3_ was transiently expressed in preOB and early iOB, and the expression was gradually reduced with the progression of odontoblast differentiation. Dental epithelium, including apical bud region, expresses _Igfbp3_. (i−p) Immunohistochemical analysis for IGFBP5 identified positive reaction in undifferentiated dental papilla cells and differentiated odontoblasts, although the expression of _Igfbp5_ reduced in differentiated odontoblasts. Dental epithelium, including the apical bud region, also expressed IGFBP5/_Igfbp5_. AB, ameloblasts; D, dentin; DP, dental pulp; E, enamel space; iOB, immature odontoblasts; preOB, preodontoblasts; mOB, mature odontoblasts. Bars, 250 μm (a, e, i and m), 100 μm (f and n-p), 50 μm (b, g, and h), 25 μm (c, d, j-l).

IGF-I varies depending on the differentiation stages or types of cells. Since several molecules are related to the regulation of IGF-I, comprehensive study including the analysis of interaction with these IGF-related members is required to understand the spatio-temporal specific function of IGF-I in the dental pulp.

Regarding the roles of IGFBPs in odontoblast differentiation, the analyses by immunohistochemistry and *in situ* hybridization revealed the expression patterns of IGFBP3 and IGFBP5 in the current study. Intriguingly, IGFBP3 was transiently expressed in preOB and early iOB, whereas the expression of IGFBP5 was continuously observed in odontoblast lineage cells. Immunohistochemical analyses for nestin and Ki67 revealed that the stage showing the expression of IGFBP3 was consistent with the transition phase from cell proliferation to differentiation. This finding indicated that IGFBP3 inhibits the action of IGF-I on proliferation of dental papilla cells, resulting in the progression of the differentiation of odontoblast lineage cells. In contrast, the expression pattern of IGFBP5 coincided with that of IGF-I in odontoblast lineage cells. since IGF-I was also expressed in differentiated odontoblasts in addition to undifferentiated cells, IGF-I might act as a regulator for cell survival or maintenance under the circumstances [10]. Therefore, it was speculated that IGFBP5 regulates the action of IGF-I on cell survival and maintenance of differentiated odontoblasts constitutively. Although there was a discrepancy between immunohistochemistry and *in situ* hybridization with respect to the expression pattern of IGFBP5 in the current study, this difference may be attributed to the recognition of accumulated proteins in intracellular and extracellular areas during immunohistochemical analysis. To understand the regulatory mechanism of IGFBPs during odontoblast differentiation, further investigation is needed to elucidate the interaction of other IGFBPs with IGF-I.

Separate from the dental mesenchyme, the expressions of IGFBP3 and IGFBP5 were recognizable in the odontogenic epithelium in this study. The analysis by *in situ* hybridization demonstrated that apical bud and inner enamel epithelium expressed *Igfbp3* and *Igfbp5*. Although inner enamel epithelium did not show IGF-I positive reaction, it was surmised that IGFBPs, derived from dental epithelium, exhibited an effect on the regulation of odontoblast differentiation. Previous studies have revealed that IGF-I is expressed in the apical bud region and secretory ameloblasts and regulates the secretion of enamel matrix [30,31]. With respect to the role of IGFBPs in the apical bud region, it is generally accepted that epithelial stem cells, providing the enamel forming cells, are localized there. Thus, it is reasonable to assume that IGFBPs play an important role in the maintenance of epithelial stem cells in the apical bud region. Future studies should include detailed investigations of the interactions between dental epithelium and mesenchyme regulating the activity of IGF-I.

5. Conclusion

The present study demonstrated the expression patterns of IGF-I, IGF-IR, IGFBP3, and IGFBP5. These results suggested that IGFBP3 regulates the transition from proliferative stage to differentiation stage by inhibiting the action of IGF-I on proliferation of dental papilla cells, and IGFBP5 plays an important role in the maintenance of differentiated odontoblasts during tooth development.

Ethical statement

All of the animal experiments were conducted in compliance with a protocol that was reviewed by the Institutional Animal Care and Use Committee and approved by the President of Niigata University (SA00092).

CRediT authorship contribution statement

Chisato Aizawa: Data curation, Formal analysis, Investigation, Writing - original draft. **Kotaro Saito:** Data curation, Formal analysis, Funding acquisition, Investigation, Writing - original draft. **Hayato Ohshima:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing - original draft.

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Conflicts of interest

The authors declare no conflicts of interest with respect to the authorship and/or publication of this article.

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