Original Article

The putative role of insulin-like growth factor (IGF)-binding protein 5 independent of IGF in the maintenance of pulpal homeostasis in mice

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ABSTRACT

Although insulin-like growth factor binding protein 5 (IGFBP5) may play a crucial role in activating the functions of periodontal and bone marrow stem cells, the factors responsible for regulating the maintenance of dental pulp stem cells (DPSCs) remain to be clarified. This study aimed to elucidate the role of IGFBP5 in maintaining pulpal homeostasis during tooth development and pulpal healing after tooth injury in doxycycline-inducible TetOP-histone 2B (H2B)-green fluorescent protein (GFP) transgenic mice (GFP expression was induced at E14.5 or E15.5) by using TUNEL assay, RT-PCR, *in situ* hybridization for *Igfbp5*, and immunohistochemistry for IGFBP5, Nestin, and GFP. To observe the pulpal response to exogenous stimuli, the roots of the maxillary first molars were resected, and the coronal portion was autografted into the sublingual region. Intense IGFBP5/*Igfbp5* expression was observed in cells from the center of the pulp tissue and the subodontoblastic layer in developing teeth during postnatal Week 4. Intense H2B-GFP-expressing label-retaining cells (LRCs) were localized in the subodontoblastic layer in addition to the center of the pulp tissue, suggesting that slowly dividing cell populations reside in these areas. During postoperative days 3—7, the LRCs were maintained in the dental pulp, showed an IGFBP5-positve reaction in their nuclei, and lacked a TUNEL-positive reaction. *In situ* hybridization and RT-PCR analyses confirmed the expression of *Igfbp5* in the dental pulp. These findings suggest that IGFBP5 play a pivotal role in regulating the survival and apoptosis of DPSCs during both tooth development and pulpal healing following tooth injury.

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1. Introduction

It is well-known that dental pulp contains adult stem cells, referred to as dental pulp stem cells (DPSCs), which are responsible for pulpal healing after tooth injury. To gain a better understanding of the biological characteristics of DPSCs, the ability to precisely identify these cells *in vivo* has been one of the most important issues in dental pulp biology. Our recent studies utilizing three and five intraperitoneal injections of BrdU into pregnant ICR mice and Wister rats (prenatal BrdU labeling), respectively, demonstrated that the incorporation of BrdU into the nucleus during cell division allows putative adult stem/progenitor cells to be labeled as dense label-retaining cells (LRCs) [12,13]. Dense LRCs expressing surface markers for mesenchymal stem cells, including STRO-1 and CD146, are enriched in the perivascular niche in the center of the dental pulp of postnatal animals, suggesting that dental pulp stem/progenitor cells can be identified as dense LRCs in the mature tissue. However, this prenatal BrdU labeling method has some intrinsic limitations for the identification of DPSCs. One major problem of using BrdU to label DPSCs is that non-dividing quiescent stem cells cannot be labeled because BrdU incorporation requires cell division. Furthermore, functional assays with viable LRCs isolated based on the intensity of BrdU labeling is impossible, since the detection of BrdU-positive cells requires the cell fixation. Finally, differentiated odontoblasts are also densely labeled, as the timing

of BrdU administration overlaps with the proliferation and differentiation of odontoblast-lineage cells. To circumvent these problems, histone 2B (H2B)-green fluorescent protein (GFP) mice have been used for identifying the LRCs [7]. In this transgenic mouse, the H2B-GFP expression is doxycycline (dox)-inducible and is gradually diluted according to the number of cell divisions during the chasing periods. To date, several studies using H2B-GFP mice have demonstrated that LRCs can be identified in the specialized niches of many organs such as hematopoietic, neural, and epithelial stem cells in the skin, small intestine, and prostate gland [7,8,11,31,34]. In the field of tooth biology, epithelial [5] and mesenchymal [35] stem cells in the continuously growing incisors of mice have been shown to be H2B-GFP-LRCs, although the use of transgenic mice to identify DPSCs in teeth with limited growth, such as mouse molars, remains to be performed. Thus, H2B-GFP mice could provide new insight regarding the localization and dynamics of DPSCs during both tooth development and pulpal healing following tooth injury.

Tooth replantation/transplantation is a common procedure in dentistry for conservative treatment, and it induces at least two types of healing patterns in the dental pulp cavity in some animal models: tertiary dentin and bone tissue formation [4,10,14,21,23,28,30,32]. Our previous studies have shown that the pulpal healing pattern is affected by whether or not dense LRCs are maintained in the pulp cavity. If dense LRCs remain in the pulp chamber following tooth replantation/transplantation, these cells actively proliferate and differentiate into odontoblast-like cells, resulting in induction of tertiary dentin formation. Interestingly, dense LRCs remained in the center of the dental pulp for a long time after autogenic tooth replantation [26], whereas these cells were not maintained there in the case of allogenic tooth transplantation [16]. The tooth replantation/transplantation procedure may not be suitable for observing odontoblast differentiation, since reparative dentinogenesis does not always occur in the dental pulp. We established an experimental model for tooth crown transplantation into the sublingual region [20,24]. In this model, the deposition of dentin matrix is observed at the pulp-dentin border, while the bone tissue is separated from the dentin matrix in the pulp cavity. In any case, the new experimental model, where LRCs are maintained more effectively and tertiary dentin always occur, had been needed to analyze the effect of allografts on the survival of LRCs.

We have improved the experimental procedure for tooth crown transplantation into the sublingual region, where the roots of the extracted molars are removed and the pulp floor is conserved [26]. Using this improved transplantation model to constantly induce tertiary dentin in the pulp cavity, we compared the fates of dense LRCs between autografts and allografts. In the autogenic transplants, the dense LRCs remained for a long time in the niche associated with the perivascular environment in the center of the pulp. In contrast, dense LRCs disappeared from this niche by postoperative Week 4 in the allografts. The loss of dense LRCs, which occurs in cases without immunological rejection, is thought to be due to the extensive apoptosis taking place in these cells, except for in the newly differentiated odontoblast-like cells. These findings suggest that the host and recipient interactions in the allografts disturb the maintenance of putative stem/progenitor cells, resulting in the loss of these cell types. Microarray analysis using autogenic and allogenic models has provided that Insulin-like growth factor-binding proteins (IGFBPs) are a candidate of the factors responsible for regulating DPSC maintenance without apoptosis following tooth injury. The clarification of these factors is one of the most important issues to be addressed in the future to refine clinical treatment using DPSCs.

IGFBPs are a family of proteins that bind with high affinity to insulin-like growth factors (IGFs), regulating their ability to interact with a signaling receptor, type I IGF receptor (IGF-IR) [3]. Binding of

IGF to IGFBPs restricts IGF's access to the IGF-IR, resulting in the inhibition of cell proliferation, differentiation, survival, and other IGF-stimulated signaling events. In addition to the IGF-dependent activity of IGFBPs, IGF-independent IGFBP functions also has been reported to be important for the regulation of cell proliferation [2,33]. IGFBP5, the most conserved member of the IGFBP family in all vertebrates, has been shown to regulate cell proliferation, migration, survival, and apoptosis [6,29]. Furthermore, our recent study using continuous growing incisors demonstrated 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 [1]. However, there are no available data on the expression pattern and functional significance of the IGF-independent role of IGFBP5 in the maintenance of DPSCs. Thus, this study aimed to investigate the role of IGFBP5 in the maintenance of DPSCs during tooth development and pulpal healing following autogenic tooth crown transplantation into the sublingual region using a doxycycline-inducible TetOP-H2B-GFP transgenic mouse model.

2. Methods

2.1. Mice

All animal experiments complied with the guidelines by 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. All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and performed according to the recommendations or under the conditions proposed by the committee (SA00091). WT C57BL/6J [B6] and Crlj:CD1(ICR) mice were obtained from the Charles River Laboratories of Japan (Yokohama, Japan). TetOP-H2B-GFP mice [B6; 129S4-Gt(ROSA)26Sor<tm1(rtTA*M2) Jae>-Colla1<tm7(tetO-HIST1H2BJ/GFP)Jae>/J] were purchased from the Jackson Laboratory (Bar Harbor, Maine, US) [7]. For GFP transgene expression, doxycycline (Sigma D9891, 2 mg/ml, supplemented with sucrose at 50 mg/ml) was administered to a mother via drinking water in order to label cells pups at E14.5 or E15.5. Three-week-old labeled birthed animals were used for the autogenic tooth transplantation into the sublingual region.

2.2. Autogenic tooth crown transplantation into the sublingual region

The upper-right first molar was extracted with a pair of modified dental forceps under anesthesia by an intraperitoneal injection of chloral hydrate (the maximum dose of 350 mg/kg), and the roots were resected with a surgical knife. The resected samples without the roots were immediately transplanted into the sublingual region after cutting the ventral side of the tongue of the same animals, and the section was sutured with a nylon suture. The upper-left first molar of the same animal was used as a control.

2.3. Histological procedure

Mice at postnatal Days 1 (n = 3), Week 1 (n = 3), and Week 4 (n = 3) were used for histological analyses, including immunohistochemistry and *in situ* hybridization. The materials for transplantation were collected in groups of 3–6 animals at intervals of 1, 3, and 7 days after the operations mentioned above (n = 15). At each stage, the animals were transcardially perfused with physiological saline followed by 4% paraformaldehyde in a 0.1 M

phosphate buffer (pH 7.4) under deep anesthesia by an intraperitoneal injection of chloral hydrate (350 mg/kg). The maxillae or the tongues, including the transplanted teeth, were removed *en bloc* and immersed in the same fixative for an additional 24 h. Following decalcification in a 10% EDTA-2Na solution for 2 weeks at 4 °C, the specimens were dehydrated through an ethanol series and then embedded in paraffin. Next, 4-μm-thick sagittal sections of the transplants, including the surrounding tissue, 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 (H&E). For the immunofluorescence microscopy, 50 μm frozen sections were cut sagittally with a freezing microtome and mounted on glass slides. The sections were examined under a confocal laser-scanning microscope (FV300, Olympus, Tokyo, Japan).

2.4. Immunohistochemical analysis

Immunohistochemistry was conducted essentially according to our previous report [25], with a mouse anti-Nestin monoclonal antibody diluted 1:500 (catalog no. MAB353, Merck Millipore, Billerica, MA, USA), a rabbit anti-IGFBP3 polyclonal antibody diluted 1:200 (catalog no. ab217205, Abcam, Cambridge, UK), a goat anti-IGFBP5 polyclonal antibody diluted 1:50 (catalog no. AF578, R & D Systems Inc., Minneapolis, MN, USA), a rabbit anti-IGF-I polyclonal antibody diluted 1 : 200 (catalog no. sc-9013, Santa Cruz, CA, USA), and a rabbit anti-GFP polyclonal antibody diluted 1:1000 (catalog no. 598, Medical & Biological Laboratories Co., Nagoya, Japan). Apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (catalog no. S7100, Merck Millipore). For double staining, Nestin-immunohistochemistry was conducted first, followed by GFP-immunohistochemistry. GFP-immunohistochemistry was performed as described above, except that the sections were stained with 4-nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche, Diagnostics Corp, Indianapolis, IN, USA) for color development.

2.5. In situ hybridization

Section *in situ* hybridization was performed as previously described [18]. Digoxigenin-labeled probes for *Igfbp5* were purchased from GenoStaff (Tokyo, Japan). Following fixation, the specimens were decalcified with Morse's solution (10% sodium citrate and 22.5% formic acid) for 24 h, dehydrated through an ethanol series and xylene, and embedded in paraffin. Subsequently, 5-μm-thick paraffin 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 the incubated overnight at 70 °C in hybridization buffer containing a digoxigenin-labeled probe for *Igfbp5*. After hybridization, the slides were washed in a series of sodium citrate—sodium chloride solutions and treated with two consecutive incubations with blocking reagent (Roche) and anti-digoxigenin antibody (Roche). The sections were stained with 4-nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

2.6. Reverse transcriptase-polymerase chain reaction

The dental pulp was dissected from the intact or transplanted first molars of mice for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by oligo-dT-primed cDNA synthesis from 2 mg of RNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). One microliter of cDNA was used for each 25 mL conventional PCR with the TaKaRa Ex Taq DNA Polymerase (Takara Bio Inc., Shiga, Japan) and 5 pmol each of the forward and reverse primers. The primer sequences are as follows: β-actin (5′-TGGAATCCTGTGGCATCCATGAAA-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′), IGFBP5 (5′-ACTGTGACCG-CAAAGGATTC-3′ and 5′-TTGTCCACACACCAGCAGAT-3′), IGFBP3 (5′-CACATCCCAAACTGTGACAA-3′ and 5′-CCATACTTGTCCACACACCA-3′), and IGF-I (5′-GTCGTCTTCACACCTCTTCTACCT -3′ and 5′-TAAAGCCCCTCGGTCCACACAC-3′). The PCR cycles consisted of an initial denaturation step at 94 °C for 4 min, followed by 35 amplification cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min.

2.7. Statistical analysis

The proportions of GFP-positive cells in the odontoblast and subodontoblastic layer and the center of the dental pulp (the 3 different locations, the mesial, medial, and distal sides of the dental pulp, were selected and the means were calculated) in the crown region (a 150×25 μm^2 grid was selected) were calculated separately. In the root region, the values for the odontoblast layer and the center of dental pulp (a 150×25 μm^2 grid was selected) were calculated separately. Data were obtained from samples of 3 teeth 4 weeks postnatal for the H2B-GFP labeling analysis (doxycycline was added to the drinking water at E15.5 for GFP transgene expression). The proportions of GFP- or TUNEL-positive cells in the pulp chamber of the transplants (a 150×25 μm^2 grid was selected) were calculated separately. Data for the H2B-GFP labeling and apoptosis analyses were obtained from samples of 12 teeth 1 [n = 3], 3 [n = 3], and 7 days after the operations [n = 3] in the transplanted teeth and in the control teeth [n = 3]. The proportions of IGFBP5- or TUNEL-positive cells to H2B-GFP-LRCs in the center of the dental pulp of the transplants (a 100×100 μm^2 grid was selected) were calculated separately. Data were obtained from the samples of 16 teeth for the IGFBP5 immunohistochemistry and apoptosis analysis: 3 [n = 3], and 7 days after the operations [n = 6] in the transplanted teeth for IGFBP5 immunohistochemistry, and 3 [n = 3], and 7 days after the operations [n = 4] in the transplanted teeth for the apoptosis analysis. All of the data are presented as the means of each group. Furthermore, the proportions of cells in the pulp chamber for the different time points after the operations (1—7 days) were compared using one-way analysis of variance (ANOVA) for multiple comparisons, adjusted by Bonferroni's test, or using Student's *t*-test with statistical software (SPSS Statistics 24 for Windows; SPSS Japan, Tokyo, Japan).

3. Results

3.1. The expression pattern of IGFBP5/Igfbp5 during tooth development

During tooth development, IGFBP5-positive reaction was observed in the nuclei of differentiated odontoblasts, and these cells expressed weak *Igfbp5* on postnatal Day 1 (Fig. 1a and b). Furthermore, IGFBP5-positive cells were scattered throughout the dental papilla. On postnatal Week1, cells at the pulp horn intensely expressed IGFBP5/*Igfbp5* (Fig. 1e, f, i and j). Some enamel epithelial cells and surrounding bone cells also expressed IGFBP5/*Igfbp5* (Fig. 1e and f). On postnatal Week 4, cells in the center of the dental
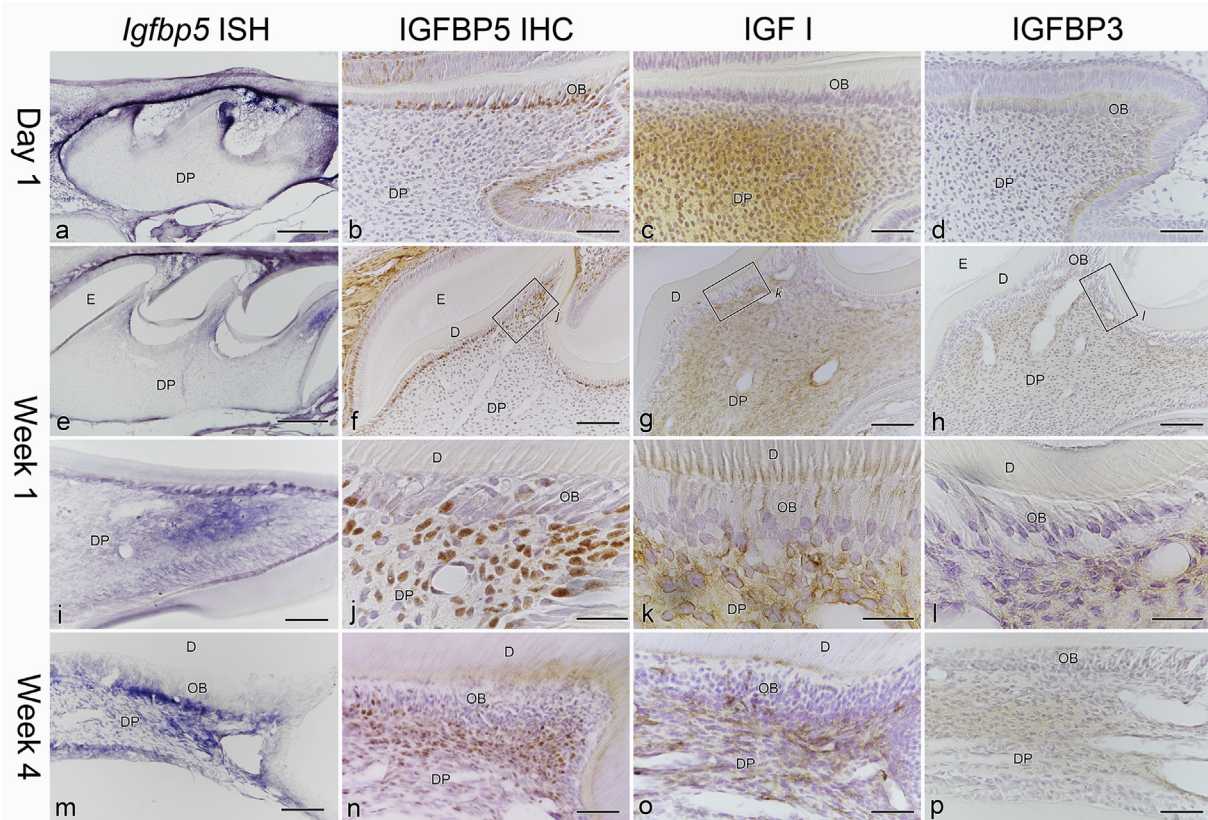
Fig. 1. *In situ* hybridization of *Igfbp5* (**a, e, i, m**) and the IGFBP5- (**b, f, j, n**), IGF-I- (**c, g, k, o**), and IGFBP3-immunoreactivities (**d, h, l, p**) in developing mouse upper first molars from postnatal 1 day to 4 weeks. **j, k,** and **l** are a higher magnification of the boxed area in (**f**), (**g**), and (**h**), respectively. (**a, b**) On postnatal Day 1, an IGFBP5-positve reaction is observed in the nuclei of differentiated odontoblasts, and these cells express weak *Igfbp5*. IGFBP5-positive cells are scattered throughout the dental papilla. (**e, f, i, j**) On postnatal Week1, cells at the pulp horn intensely express IGFBP5/*Igfbp5*. Some enamel epithelial cells and surrounding bone cells also express IGFBP5. (**m,n**) On postnatal Week 4, cells in the center of dental pulp and subodontoblastic layer show intense IGFBP5-positive reaction. (**c, d, g, h, k, l**) The dental pulp broadly express intense IGF-I and faint IGFBP3, although differentiated odontoblasts lack these positive reactions. An IGFBP3-positive reaction is not localized in the nuclei of the pulpal cells, but rather in the extracellular matrix. (*D* dentin, *DP* dental pulp, *E* enamel, *OB* odontoblasts). *Bars* 250 μm (**a, e**), 100 μm (**f-h, m**), 50 μm (**b-d, i, n-p**), 25 μm (**j-l**).

pulp and in the subodontoblastic layer showed intense IGFBP5/*Igfbp5* expression (Fig. 1m and n). On the other hand, the dental pulp broadly expressed intense IGF-I and faint IGFBP3, although the differentiated odontoblasts lacked these positive reactions. IGFBP3-positive reaction was not localized in the nuclei of the pulpal cells, but rather in the extracellular matrix (Fig. 1c, d, g, h, k, l, o and p).

3.2. The localization of H2B-GFP-LRCs

Doxycycline was administered to a mother via drinking water to label pups at E14.5 or E15.5 to induce GFP transgene expression. In postnatal Week 4, H2B-GFP-LRCs were located in the odontoblast and subodontoblastic layer and in the center of the pulp chamber in association with CD31-positive capillary vessels in the crown region, although immunohistochemistry for GFP using 3, 3′-diaminobenzidine (DAB) for the color development failed to detect intense H2B-GFP expression in Nestin-positive odontoblasts (Fig. 2a—c, f, h and i). Intriguingly, there was a significantly high percentage of intense LRCs in the subodontoblastic layer compared to the odontoblast layer and the center of pulp tissue in the crown region (Fig. 2a, b and j). On the other hand, intense and diluted LRCs were distributed in the odontoblast layer in the root region and in the pulp floor (Fig. 2d, e and j). In the root region, the percentage of intense LRCs was significantly high in the odontoblast layer compared to that in the crown region (Fig. 2b, d and j). In addition, the LRCs in the center of the dental pulp and subodontoblastic layer both showed an IGFBP5-positive reaction (Fig. 2g).

3.3. Autograft — root resected transplantation into the sublingual region —

Doxycycline was added to the drinking water at E15.5 to induce GFP transgene expression and 3-week-old animals were used for the operation. On Day 3, the number of TUNEL-positive cells in the odontoblast and subodontoblastic layer were significantly increased, although the center of the dental pulp did not show a TUNEL-positive reaction (Fig. 3o). Intense and diluted H2B-GFP-LRCs remained in the center of the pulp chamber and 76.2% of these LRCs also showed an IGFBP5-positive reaction (Fig. 3a, b, m and n). In contrast, only 5.3% of the LRCs in the center of the pulp chamber showed a TUNEL-positive reaction (Fig. 3c, d and n). On Day 7, the number of TUNEL-positive cells in the dental pulp was significantly decreased (Fig. 3o and h). The number of H2B-GFP-expressing LRCs was increased compared to the control group and were distributed throughout the dental pulp (Fig. 3k and m). In addition, 76.8% of these LRCs coexpressed IGFBP5 (Fig. 3e, f and n), and some of them were committed into newly differentiated odontoblast-like cells (Fig. 3k). On postoperative Days 3—7, IGF-I, IGFBP3, and IGFBP5 expression was observed in the dental pulp (Fig. 3j, l and p).

4. Discussion

The present study clearly shows that IGFBP5-positve reaction is observed in the nuclei of dental papilla/pulp cells and that the
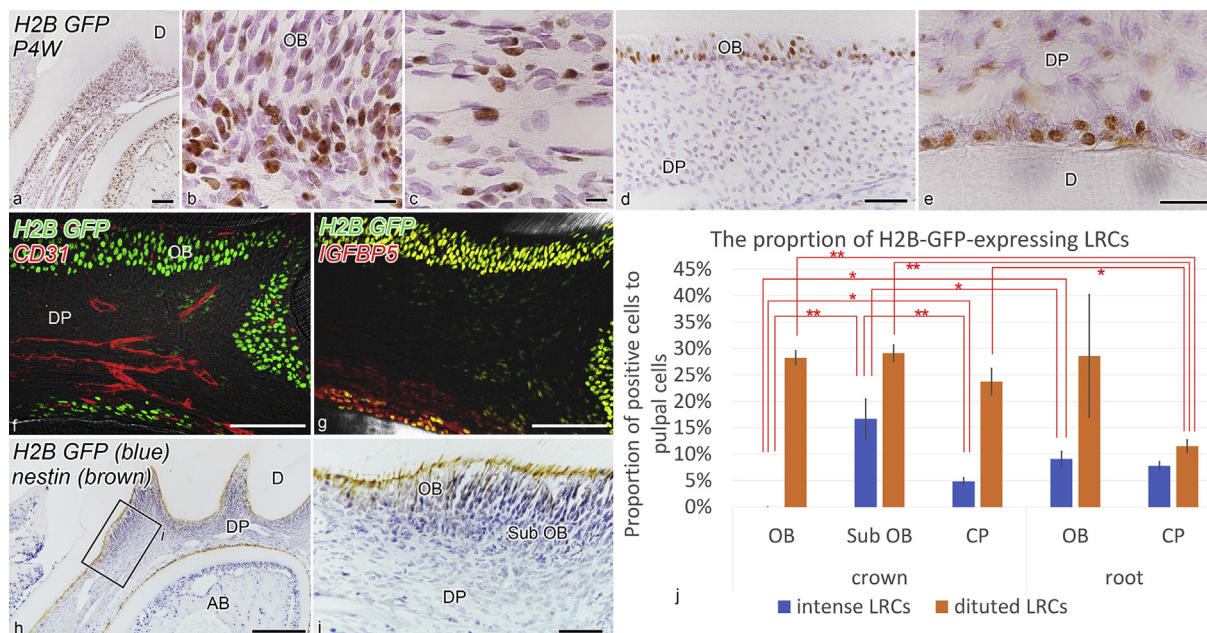
Fig. 2. GFP-immunoreactivity (**a-e**), double immunofluorescence staining for H2B-GFP (green) and CD31 (red) (**f**) or IGFBP5 (red) (**g**), double staining for Nestin- (brown) and GFP-immunoreactivity (blue) (**h, i**) of the mouse upper first molars in the crown (**b, c, f, g, i**) and root region (**d**) and on the pulp floor (**e**) on postnatal 4 weeks, and quantitative analysis of the proportions of H2B-GFP-positive cells to pulpal cells (**j**) (*P values are <0.05, **P values are <0.01). **i** is a higher magnification of the boxed area in (**h**). Doxycycline was administered to a mother via drinking water to label pups at E14.5 or E15.5 to induce GFP transgene expression. (**a-c, f, h, i**) In postnatal Week 4, H2B-GFP-LRCs are located in the odontoblasts and the subodontoblastic layer and in the center of the pulp chamber, where they are associated with CD31-positive capillary vessels in the crown region. Intense LRCs are enriched in the subodontoblastic layer and in the center of dental pulp. (**d, e**) Intense and diluted LRCs are distributed in the odontoblast layer in the root region and on the pulp floor. (**g**) The LRCs in the center of the dental pulp and subodontoblastic layer show a concomitant IGFBP5-positive reaction. (**j**) There are a significantly higher percentage of intense LRCs in the subodontoblastic layer compared to in the odontoblast layer and the center of the pulp tissue in the crown region. In the root region, the percentage of intense LRCs is significantly higher in the odontoblastic layer compared to in the crown region. (*AB* alveolar bone, *D* dentin, *DP* dental pulp, *OB* odontoblasts, *Sub OB* subodontoblastic cells, *CP* the center of the dental pulp). *Bars* 250 μm (**h**), 100 μm (**a, f, g**), 50 μm (**d, i**), 25 μm (**e**), 10 μm (**b, c**).

number of positive cells increases in the cells in the center of the dental pulp and subodontoblastic layer, where stem/progenitor cells capable of differentiating into odontoblasts are present [13,27], during the progression of tooth development. Intriguingly, the expression pattern of IGFBP5/*Igfbp5* in mature pulp tissue is consistent with the localization of the stem/progenitor cells in the dental pulp. Although IGF-I is broadly expressed in the dental papilla/pulp, the finding showing IGFBP5 expression in the nuclei of pulpal cells there suggests that IGFBP5 plays an IGF-independent role in the regulation of stem/progenitor cell survival and apoptosis. Indeed, another study revealed that periodontal ligament stem cells (PDLSCs) express IGFBP5 [15]. Since IGFBP3 expression was observed in the extracellular matrix, other IGFBPs including IGFBP3, might regulate IGF-I activity in the dental papilla/pulp. Further investigation is needed to clarify the detailed IGFBP5 signaling pathway in the regulation of cell survival and apoptosis in DPSCs.

In the present study, we identified a slowly dividing population of dental pulp cells *in vivo* using TetOP-H2B-GFP mice. In our previous studies, prenatal BrdU labeling was used as a technique to detect DPSCs *in vivo*. In this BrdU labeling method, non-dividing, truly quiescent stem cells cannot be labeled due to the requirement for cell division, although some dense LRCs possess stem cell properties, such as high cell proliferating activity and the capacity to differentiate into odontoblast-like cells following tooth injury. The use of TetOP-H2B-GFP mice for the identification of DPSCs overcame the limitation of the conventional BrdU labeling method. H2B-GFP expression was induced at E14.5 or E15.5 and then allowed to become diluted in proliferating cells until postnatal Week 3 or 4. In postnatal Week 4, intense H2B-GFP-LRCs were located in the subodontoblastic layer in addition to the center of the

pulp chamber, where they were associated with CD31-positive capillary vessels in the crown region. The findings in this study regarding the localization of LRCs are nearly consistent with the results of our previous studies using the BrdU pulse chase approach, except for the observation of an enrichment of intense H2B-GFP-LRCs in the subodontoblastic layer. These findings indicate that cells destined to become progenitor cells in the subodontoblastic layer reside in the dental papilla tissue during early tooth morphogenesis and remain in a quiescent state throughout the pulpal developmental process. It is thought that subodontoblastic progenitor cells act as spares for odontoblasts, which are susceptible to suffering from exogenous stimuli, such as attrition, abrasion, dental caries, and tooth drilling [19]. Furthermore, our recent study showing differential expression of *Nestin*-GFP in the coronal and root region of *Nestin-EGFP* transgenic mice [22] suggested that the tissue constitution of the dental pulp is different between the coronal and root areas, i.e., the subodontoblastic layer resides only in the crown region [19]. Indeed, the LRCs were not distributed beneath the odontoblast layer in the root region in the present study. Thus, it is reasonable to suppose that a slowly dividing cell population is present in the subodontoblastic layer to make advanced preparations for an exogenous trauma. In contrast, immunohistochemistry for GFP using DAB for color development failed to detect intense H2B-GFP expression in Nestin-positive odontoblasts. The different intensity of the odontoblasts between bright- and dark-field microscopy may be attributed to a technical limitation in the tissue preparation method, although the exact cause remains to be elucidated. Indeed, our unpublished data have shown discrepancies in the H2B-GFP intensity between the bright- and dark-field microscopy during early tooth development. In future work, detailed investigations are
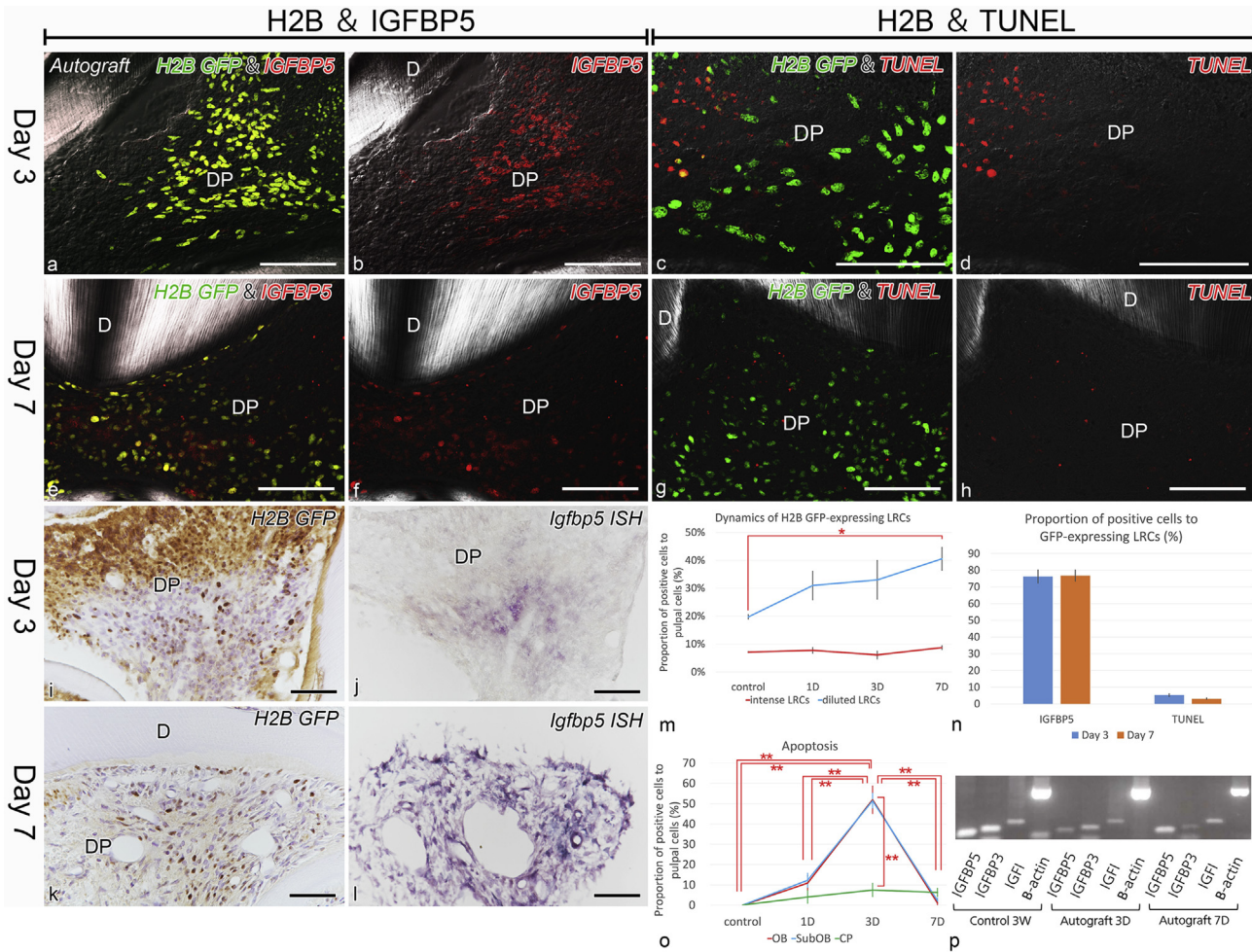
Fig. 3. Double immunofluorescence staining for H2B-GFP- (green) and IGFBP5 (red) (**a, b, e, f**) or TUNEL assays (red) (**c, d, g, h**), GFP-immunoreactivity (**i, k**), and *in situ* hybridization against *Igfbp5* (**j, l**) in the autografted teeth at 3 (**a-d, i, j**) and 7 (**e-h, k, l**) days after operation. Quantitative analyses of the proportions of H2B-GFP- (**m**) or TUNEL-positive cells (**o**) to pulpal cells and IGFBP5- or TUNEL-positive cells to H2B-GFP-LRCs (**n**). The result of the RT-PCR analysis of the β-actin, IGFBP3, IGFBP5, and IGF-I gene expression levels in the dental pulp cells obtained from control and autografted teeth at 3 and 7 days after operation (**p**) (*P values are <0.05, **P values are <0.01). Doxycycline was administered to a mother via drinking water to label pups at E15.5 to induce GFP transgene expression and 3-week-old animals were used for the operation. (**o**) On Day 3, the number of TUNEL-positive cells in the odontoblasts and in the subodontoblastic layer are significantly increased, although apoptotic activity is not upregulated in the center of the dental pulp. (**a, b, m, n**) Intense and faint H2B-GFP-LRCs remain in the center of the pulp chamber, and 76.2% of these LRCs show concomitant IGFBP5-positive reactions. (**c, d, n**) In contrast, only 5.3% of the LRCs in the center of the pulp chamber show a TUNEL-positive reaction. (**o, h**) On Day 7, the number of TUNEL-positive cells in the dental pulp are significantly decreased. (**e, f, n**) H2B-GFP-expressing LRCs are distributed throughout the dental pulp, and 76.8% of these LRCs show IGFBP5 expression. (**k**) Some, but not all, LRCs are committed into newly-differentiated odontoblast-like cells. (**j, l, p**) On postoperative Days 3–7, the expression levels of IGF-I, IGFBP3, and IGFBP5 in the dental pulp are confirmed by the results of the *in situ* hybridization and RT-PCR analysis. (*D* dentin, *DP* dental pulp, *OB* odontoblast, *Sub OB* subodontoblastic cells, *CP* the center of the dental pulp). *Bars* 100 µm (**a-h**), 50 µm (**i-l**).

needed to elucidate the relationship between the intensity of the H2B-GFP and odontoblast differentiation during tooth development. Furthermore, our recent study reported that H2B-GFP-LRCs can be detected in periodontal ligament, although the prenatal BrdU labeling method failed to detect LRCs in this tissue [17]. Thus, the H2B-GFP transgenic mouse model is advantageous as the quiescent stem cells can be completely labeled independent of the cell division timing, resulting in more precise *in vivo* visualization of the DPSCs. In the future, functional assays with H2B-GFP-LRCs isolated on the basis of their labeling intensity will be performed to clarify the differentiation capacity of these labeled cells.

Regarding the relationship between the LRC dynamics and the IGFBP5 expression pattern following tooth injury, we demonstrated in the present study that surviving H2B-GFP-LRCs expressed intense IGFBP5. The experimental mouse model for tooth crown transplantation into the sublingual region, in which the roots were resected, is a heavy injury model where existing odontoblasts

degenerate and, subsequently, the dental pulp stem/progenitor cells actively proliferate and differentiate into odontoblast-like cells [26]. In the present study, extensive apoptosis was observed in odontoblasts and subodontoblastic cells on postoperative Day 3. In contrast, the center of the pulp tissue did not show especially high apoptotic activity, and both the intense and diluted H2B-GFP-LRCs were maintained in that tissue throughout the experimental period. On Day 7, decreased apoptotic activity was detected in the dental pulp, followed by reorganization of the pulp tissue. H2B-GFP-LRCs were committed in newly differentiated odontoblast-like cells, suggesting that the LRCs possess the capacity to differentiate into odontoblast-like cells. Intriguingly, intense LRCs in the dental pulp were maintained, while the number of diluted LRCs was significantly increased compared to the control group. Since our previous study reported that active cell proliferation occur in the dental pulp following the operation [26], it is thought that the dilution of the GFP expression due to active cell proliferation

occurred in the dental pulp. Based on the findings that the number of intense H2B-GFP-LRCs is maintained throughout the experimental period, it is possible that progenitor cells in the subodontoblastic layer migrate toward the center of the pulp tissue and contribute to providing new pulpal cells, in addition to the DPSCs, in the center of the dental pulp. On the other hand, the dental pulp expressed *Igfbp5* during postoperative Days 3—7, and almost all H2B-GFP-LRCs expressed IGFBP5 and lacked apoptotic activity. Interestingly, IGFBP5 was localized in the nuclei of the LRCs. Taken together, these findings demonstrate that an IGF-independent action of IGFBP5 might play a key role in the regulation of cell survival and apoptosis in dental pulp stem/progenitor cells following tooth injury. Recently, it has been reported that IGFBP5 administration activates the functions of PDLSCs and bone marrow stem cells *in vitro* [9] and periodontal tissue regeneration *in vivo* [15,9]. For further detailed investigation, loss-of-function analysis will be required to clarify the functional significance of IGFBP5 in the inhibition of apoptosis in DPSCs following tooth injury.

5. Conclusion

Based on the use of H2B-GFP mice, the results of the present study clearly show that a slowly dividing cell population resides in the dental pulp and that IGFBP5 may play an important role in the activation of cell survival and in the inhibition of apoptosis in DPSCs during both tooth development and in the process of pulpal healing following tooth injury. However, detailed analyses regarding the differentiation capacity of the H2B-GFP-LRCs and the functional significance of IGFBP5 in the maintenance of these cells remains to be performed.

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