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The Sonic Hedgehog–Patched–Gli Signaling Pathway Maintains Dental Epithelial and Pulp Stem/Progenitor Cells and Regulates the Function of Odontoblasts

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This study aimed to elucidate the role of the Sonic hedgehog (Shh)–Patched (Ptch)–Gli signaling pathway in maintaining dental epithelial and pulp stem/progenitor cells and regulating the function of odontoblasts. Doxycycline (dox)-inducible histone 2B (H2B)–green fluorescent protein (GFP) transgenic mice ingested dox at prenatal embryonic days 14.5 or 15.5 and their offspring were collected from postnatal day 1 (P1) to week 3 (P3W). Immunohistochemistry for Gli1, Ptch1, and Ptch2 and *in situ* hybridization for *Shh* and *Ptch1* were conducted. Mandibular incisors of postnatal day 2 H2B-GFP transgenic and wild-type mice were cultivated in a nutrient medium with Shh antibody for 4 days and subsequently processed for immunohistochemistry for Sox2. In molars, dense H2B-GFP-label-retaining cells (H2B-GFP-LRCs) were densely distributed throughout the dental pulp during P1 to postnatal week 2 (P2W) and decreased in number by postnatal P3W, whereas the number of dense H2B-GFP-LRCs in the subodontoblastic layer increased in number at P2W. Gli1+ and Pthc1+ cells were distributed throughout the enamel organ and dental pulp, including the odontoblast and subodontoblastic layers. *Shh* mRNA was expressed in the inner enamel epithelium and shifted into odontoblasts after dentin deposition. *Ptch1* mRNA was expressed in the inner enamel epithelium and cuspal pulpal tissue on P1 and decreased in intensity from postnatal week 1 to P3W. In incisors, the apical bud contained H2B-GFP-LRCs, Gli1+ cells, and Ptch1+ cells. The addition of Shh antibody to explants induced a decrease in the number of Sox2+ cells due to the increase in apoptotic cells in the apical bud. Thus, the Shh–Ptch–Gli signaling pathway plays a role in maintaining quiescent adult stem cells and regulating the function of odontoblasts.

Keywords: adult stem cells, dental pulp, gli protein, hedgehog proteins, mice, patched-1 receptor, odontogenesis, tooth germ

INTRODUCTION

Committed multipotent stem cells, or adult stem cells, exist in renewal tissues such as the epidermis, intestinal crypt, and bone marrow and are crucial for physiological tissue renewal and regeneration after injury (1). Current stem cell biology suggests that quiescent (removed from the cell cycle) and active (present in the cell cycle) subpopulations of stem cells may coexist within renewal tissues (2). Epithelial stem cells (ESCs) in the epidermis and mesenchymal stem cells (MSCs) in the dermis play crucial roles in the process of skin regeneration following injury (3). In contrast, dental epithelial tissue, or enamel, never regenerates after tooth injury, and exogenous injury to the dentin induces scar formation, referred to as tertiary dentin *via* pulpal irritation (4). A weak injury activates preexisting odontoblasts, resulting in the production of reactionary dentin, whereas a heavy injury elicits degeneration of injured odontoblasts followed by replacement with new odontoblast-like cells that contribute to reparative dentin (4). Dental pulp stem/progenitor cells play a crucial role in the differentiation of odontoblast-like cells in the process of pulpal healing (5). It has been difficult to identify the location of these cells in the mature pulp using the 5-bromo-2′-deoxyuridine (BrdU) pulse-chase method, because the pulp tissue rarely proliferates after tooth eruption. The prenatal BrdU labeling method, in which BrdU is injected into the pregnant mother to label proliferative cells in the prenatal stages, enables us to identify long-term label-retaining cells (LRCs) in the perivascular niche (5, 6). These LRCs are considered to be quiescent stem cells that actively proliferate following exogenous stimuli (5, 7, 8) and play an important role in pulpal homeostasis and the reparative process. These cells maintain their regenerative capability throughout life and are capable of producing odontoblast-like cells to repair injured dentin. Dental MSCs share characteristics similar to bone marrow-derived MSCs in terms of proliferative capacity, cell markers, and differentiation capability into plural lineages, such as osteoblasts, chondrocytes, and adipocytes (9). Sonic hedgehog (Shh) signaling plays crucial roles in the development of numerous organs, and Gli1+ cells are co-localized with LRCs during molar tooth development (10, 11). Even BrdU labeling in the stem cells becomes sparse if these LRCs continuously provide transit-amplifying cells (5, 7, 8). In contrast, long-term LRCs labeled by BrdU are regarded as quiescent stem cells (10). However, the localization of stem/progenitor cells in the dental pulp, the differences in their differentiation capacity in different cell populations, and the mechanisms maintaining their niches are not fully understood.

Rodent incisors peculiarly evolved as continuously growing teeth. The homeostasis of this tooth type is maintained by the balance between continuous cell proliferation at the apical end and attrition of the incisal edge (12, 13). The adult stem cells that provide transit-amplifying cells exist in the apical end of rodent incisors (14), and the epithelial bulge, including the stem cell niche, shows the human head-like appearance referred to as an apical bud or cervical loop (12, 13). Sox2+ apical bud cells provide cells of all lineages in the enamel organ (15). The LRCs in the apical bud that have been identified by prenatal

TABLE 1 | Number of animals for histological, immunohistochemical, and statistical analyses of chronological changes in GFP, Gli1-, Ptch1-, and Shh-positive cells.

		P1	P1W	P2W	P3W	Total
Histological analysis		6	6	6	7	25
GFP	Incisor				(2)	(2)
	Molar	(3)	(3)		(3)	(9)
Gli1	Incisor				(2)	(2)
	Molar	(3)	(3)	(3)	(3)	(12)
Ptch1	Incisor				(2)	(2)
	Molar	(1)	(2)	(2)	(1)	(6)
Ptch1 (in situ)	Incisor	(1)	(1)	(1)	(1)	(4)
	Molar	(1)	(1)	(1)	(1)	(4)
Ptch2	Incisor				(1)	(1)
Shh (in situ)	Incisor	(1)	(1)	(1)	(1)	(4)
	Molar	(1)	(1)	(1)	(1)	(4)
Total		6	6	6	7	25

P1, postnatal day 1; P1W, postnatal week 1; P2W, postnatal week 2; P3W, postnatal week 3.

TABLE 2 | Number of incisor explants for histological, immunohistochemical, and statistical analyses of Ki67-, Sox2-, TUNEL, and GFP-positive cells.

		Control	5E1	Total
B6 mice		9	10	19
	Ki67	(8)	(7)	(15)
	Sox2	(7)	(8)	(15)
	TUNEL	(3)	(3)	(6)
TetOP-H2B-GFP mice		5	5	10
	Sox2	(3)	(4)	(7)
	GFP	(3)	(3)	(6)
Total		14	15	29

5E1, Shh antibody; H2B, histone 2B.

BrdU labeling are regarded as quiescent stem cells (10) similar to bulge cells of the hair follicle, cycling position 4 LRCs of the intestinal crypt, and endosteal hematopoietic stem cells of the bone marrow (2). Recent studies have demonstrated that Shh signaling in neurovascular bundles regulates the maintenance of MSCs in mouse incisors (16) and that the Bmp–Shh signaling network regulates the fate of ESCs *via* the maintenance of stem cell niche in the developing molars (17). Canonical hedgehog (Hh) signaling is activated by the binding of Hh ligands to Patched (Ptch), a transmembrane receptor. Gli1 and Gli2 are known as mediators of Hh signaling, regulating the expression of their target genes (18). In the continuously growing incisors, the Hh signaling cascade regulates both Sox2+ stem cells (17) and ameloblast differentiation (19). Hh ligands bind Ptch1 or Ptch2 to release Smoothened (Smo), which activates the transcription of downstream targets such as Gli1 and Gli2 (20–22). Dense BrdU-LRCs, Gli1+ cells, and Ptch1+ cells were actually co-localized in the outer enamel epithelium of the apical bud, the mesenchyme beneath the bud, and the perivascular niche in the central pulp in
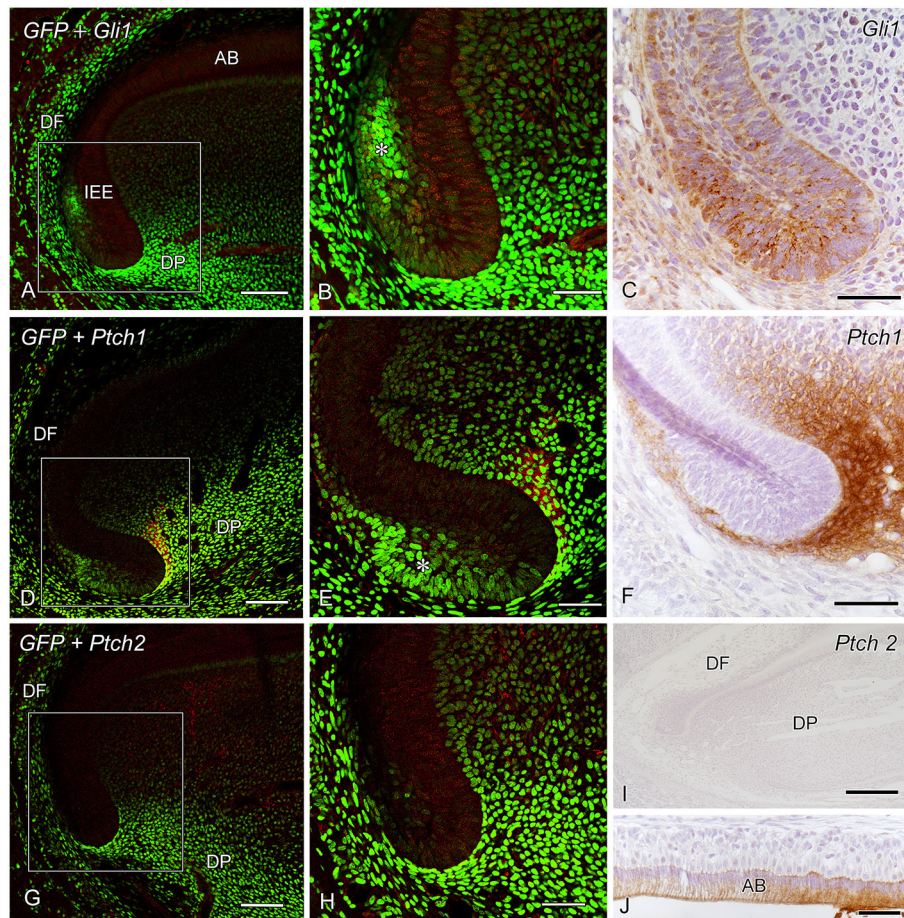
FIGURE 1 | Green fluorescent protein (GFP) expression and Gli1 immunoreactivity (IR) **(A,B)**, Gli1 IR **(C)**, GFP expression and Patched1 (Ptch1) IR **(D,E)**, Ptch1 IR **(F)**, GFP expression and Ptch2 IR **(G,H)**, and Ptch2 IR **(I,J)** in the apical end of incisors at postnatal week 3 (P3W) (*DF* dental follicle, *DP* dental papilla). **(A,B,D,E,G,H)** GFP+ cells are observed throughout the dental follicle (*DF*) and the apical dental papilla (*DP*) in addition to the outer enamel epithelium of the apical bud (*). **(B,E,H)** Higher magnification of the boxed areas in **(A,D,G)**. **(A–C)** Gli1+ cells intensely localize in the inner enamel epithelium (*IEE*) and ameloblast layer (*AB*) in addition to the apical bud (*). **(D–F)** Ptch1+ cells are intensely localized in the apical dental papilla beneath the apical bud (*). **(G–I)** Intense Ptch2 expression is observed in the maturation ameloblasts (AB). Scale bars: 250 μm **(I)**, 100 μm **(A,D,G)**, and 50 μm **(B,C,E,F,H,J)**.

developing teeth (10). It is known that Shh signaling regulates the production of ameloblast progenitors of mouse incisors (19) and that Shh–Patch–Gli1 signaling cascade plays a crucial role in the maintenance of quiescent stem cells in the apical mesenchyme (16). However, the direct role of Shh signaling in the maintenance of quiescent stem cells in the apical bud is not fully understood.

Adult stem cells in the dental pulp are considered quiescent stem cells and contribute to pulpal healing following tooth injury by proliferating actively under pathological conditions (5, 7, 8). We succeeded in labeling the putative stem cells in the developing teeth by the prenatal BrdU labeling method in Wistar rats and Crlj:CD1 (ICR) mice (5, 6). However, the BrdU labeling method possesses several inherent problems; first, non-proliferating cells at the timing of BrdU injection are never labeled, because BrdU is incorporated into the nucleus of proliferating cells in the cell cycle. Second, the labeled cells must be fixed for visualization of the BrdU deposition. Third, the toxic effects of

BrdU on host cells or animals cannot be excluded (23). Finally, transit-amplifying cells such as odontoblast-lineage cells are also densely labeled with BrdU, because the timing of BrdU injection overlaps with their final cell division (5–7, 24, 25). To overcome this issue, we used doxycycline (dox)-inducible histone 2B–green fluorescent protein (H2B–GFP) transgene (26, 27). The ingestion of dox by the pregnant mother at prenatal day 14–15 labels all cells in the pups that express GFP and decreases the intensity of GFP with the number of cell divisions. This method demonstrated the existence of periodontal H2B-GFP-LRCs in the mature teeth, whereas the BrdU labeling method failed to label the periodontal BrdU-LRCs (28). This study aimed to elucidate the role of the Shh–Ptch–Gli signaling pathway in maintaining dental ESCs and MSCs and in regulating the function of odontoblasts in the incisors and molars using dox-inducible H2B-GFP transgenic mice, immunohistochemistry, and *in situ* hybridization.
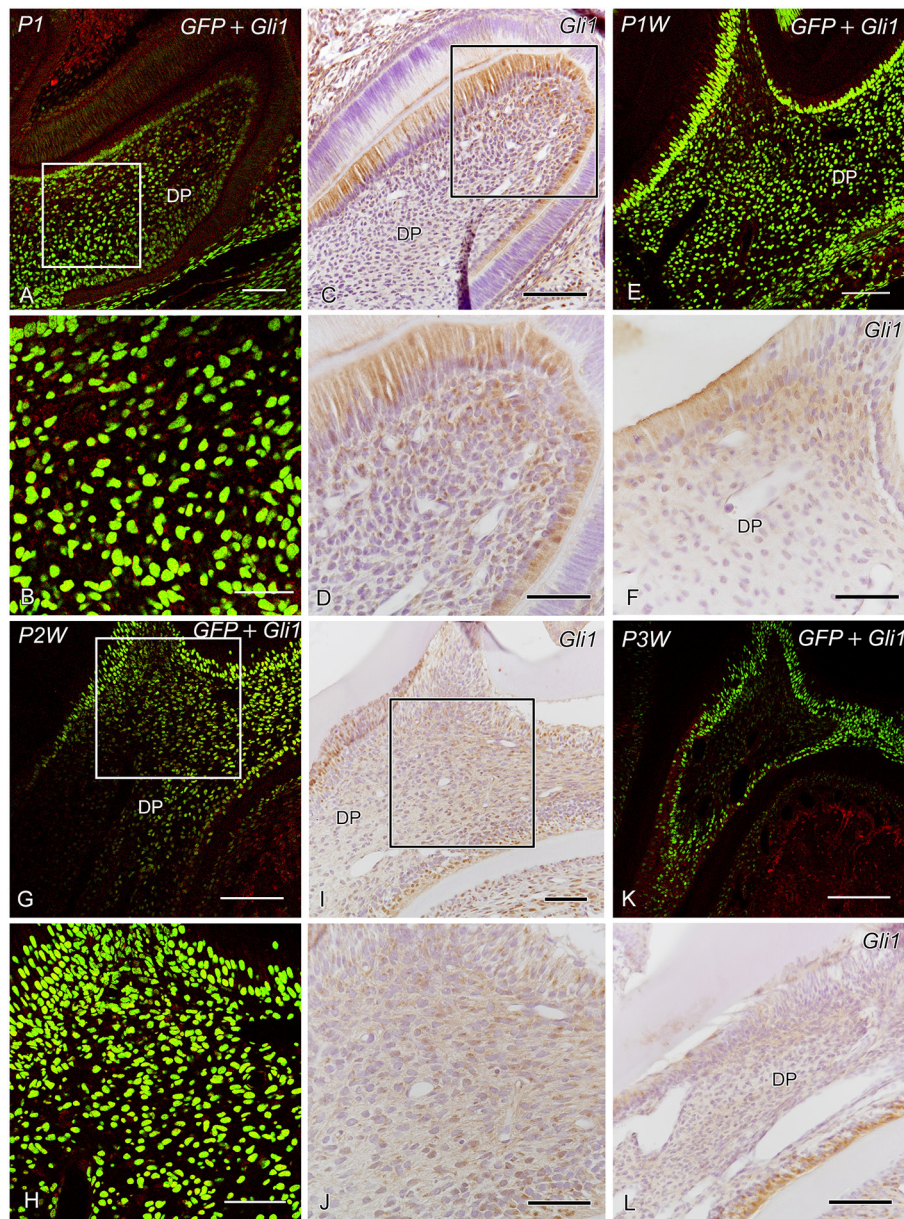
FIGURE 2 | GFP expression and Gli1 IR **(A,B,E,G,H,K)** and Gli1 IR **(C,D,F,I,J,L)** in the developing molars at postnatal day 1 (P1) **(A–D)**, P1W **(E,F)**, P2W **(G–J)**, and P3W **(K,L)** (*DP* dental pulp). **(A,B,E,G,H,K)** Dense GFP$^+$ cells are observed throughout the dental pulp during P1–P2W and decrease in number at P2W, whereas the number of dense GFP$^+$ cells in the subodontoblastic layer increases in number at P2W. In contrast, granular GFP$^+$ cells increase in number at P3W in the dental pulp. **(B,D,H,J)** Higher magnification of the boxed areas in panels **(A,C,G,I)**. **(A–L)** Gli1$^+$ cells are distributed throughout the enamel organ and dental pulp, including the odontoblast and subodontoblastic layers. Scale bars: 100 μm **(A,C,E,G,I,K,L)** and 50 μm **(B,D,F,H,J)**.

MATERIALS AND METHODS

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. All 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 (Permit Number: #27 Niigata Univ. Res.282-4). WT C57BL/6J (B6) mice were obtained from 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, ME,
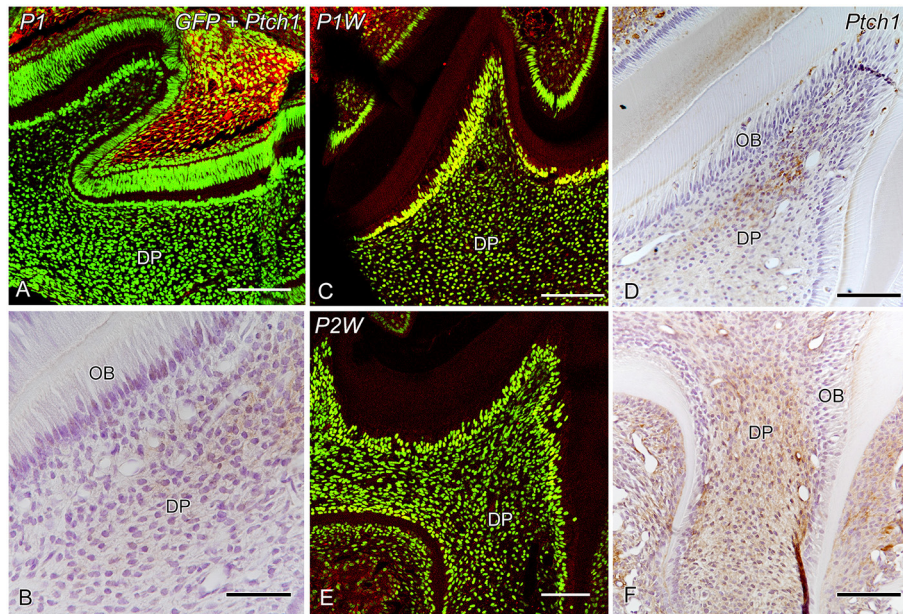
FIGURE 3 | GFP expression and Ptch1 IR **(A,C,E)** and Ptch1 IR **(B,D,F)** in the developing molars at P1 **(A,B)**, P1W **(C,D)**, and P2W **(E,F)** (*DP* dental pulp, *OB* odontoblasts). **(A–F)** Intense Ptch1$^+$ cells are located in the enamel organ and dental pulp. **(B)** Higher magnification of the boxed area in **(A)**. Scale bars: 100 μm **(A,C,D,E,F)** and 50 μm **(B)**.

USA) (26). For GFP transgene expression, dox (Sigma D9891, 2 mg/mL, supplemented with sucrose at 50 mg/mL) was ingested *via* a pregnant mother to label cells of the pups at embryonic day (E) 14.5 or E15.5.

Histological Procedure

Postnatal mice at day 1 (P1), week 1 (P1W), week 2 (P2W), and week 3 (P3W) were used for *in vivo* experiments (**Table 1**). The animals were transcardially perfused with physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia *via* an intraperitoneal injection of chloral hydrate (maximum dose of 350 mg/kg). The heads, including incisors and molars, were removed *en bloc* and immersed in the same fixative for an additional 12 h. Following decalcification at 4°C in a 10% ethylenediamine tetraacetic acid (EDTA)-2Na solution, the samples were processed for embedding in paraffin. The paraffin-embedded teeth were cut sagittally into 5 μm sections. The paraffin sections were mounted on Matsunami adhesive silane-coated glass slides (Matsunami Glass, Osaka, Japan).

Mouse Incisor Cultures

Mouse incisor organ culture was performed as previously described (29). Specifically, incisors were dissected from the lower jaws of B6 or TetOP-H2B-GFP mice at P2 (**Table 2**) and put onto membrane filters (0.1 μm pore size, OMNIPORE™, Millipore, Bedford, MA, USA) in a Trowell system and then cultured for 4 days at 37°C in Dulbecco's Modified Eagle's Medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum, 100 μg/mL ascorbic acid (Seikagaku Kogyo,

Tokyo, Japan), and 100 U/mL penicillin–streptomycin (Gibco BRL). The incisor germs of the experimental group were added with Shh antibody 5E1 (26 μg/mL, the Developmental Studies Hybridoma Bank, Iowa City, IA, USA). For histological analysis of incisor explants, serial sections were made of whole explants in the mesial–distal direction.

Immunohistochemical Analysis

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). Immunohistochemistry for Ki67, Sox2, Gli1, Ptch1, Ptch2, and Shh was conducted as previously reported (6). A rabbit anti-Ptch2 polyclonal antibody diluted to 1:100 (Novus Biologicals; catalog number: NB200-119) was used for Ptch2 immunohistochemisty. Immunohistochemical negative controls were included lacking primary antibodies, showing no specific immunoreaction. The sections were examined with a confocal laser microscope (FV300; Olympus, Tokyo, Japan) or a light microscope.

In situ Hybridization

In situ hybridization was performed as described previously (30). Digoxigenin-labeled probes for *Shh* (31) and *Ptch1* (32) were prepared according to the manufacturer's protocol (Roche). Sense probes transcribed as negative controls did not show any significant staining.
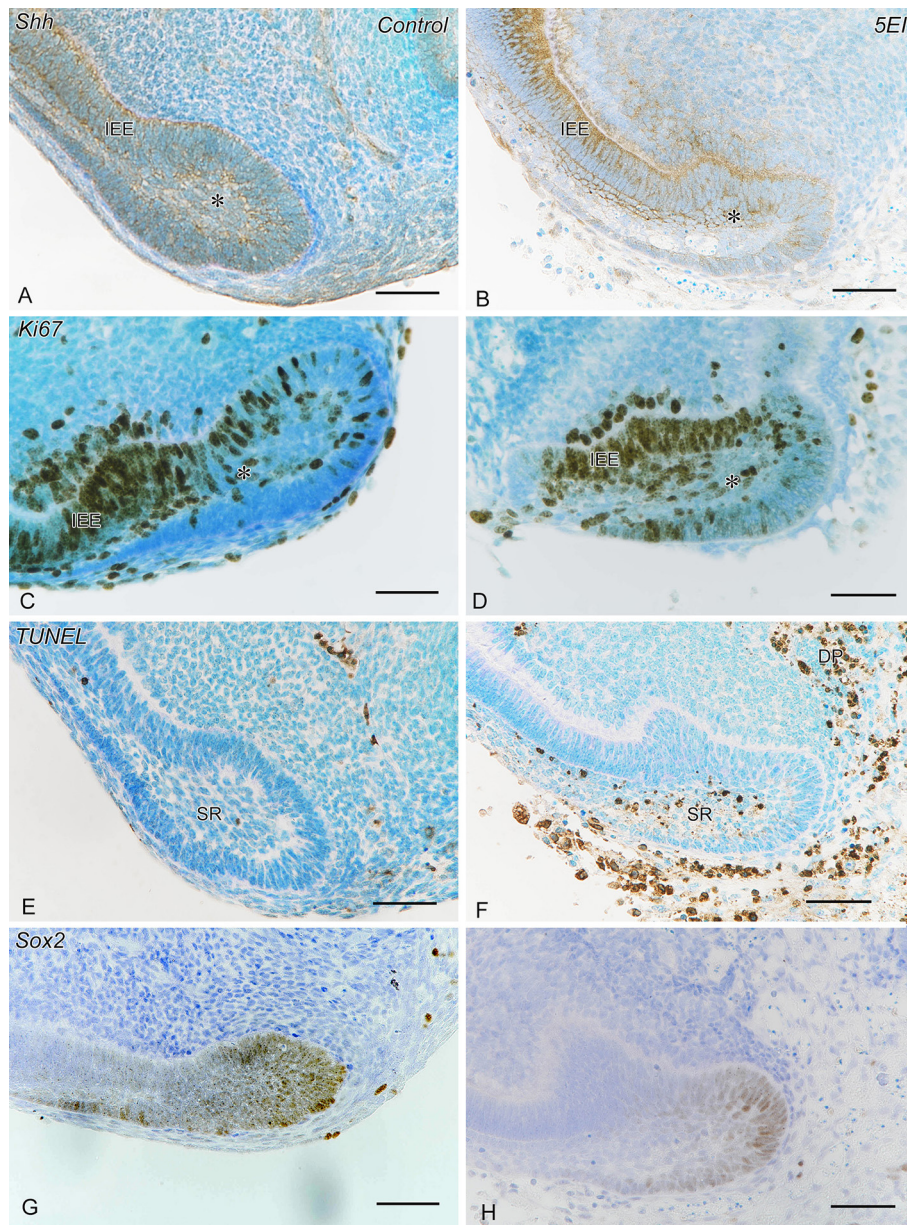
FIGURE 4 | Shh IR **(A,B)**, Ki67 IR **(C,D)**, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) **(E,F)**, and Sox2 IR **(G,H)** in control **(A,C,E,G)** and experimental (Shh antibody: 5E1) explants **(B,D,F,H)**. **(A–D)** Shh expression and Ki67$^+$ cells are observed in the apical bud (*) and the inner enamel epithelium (*IEE*) in both the control and 5E1 groups. **(E,F)** TUNEL$^+$ cells of stellate reticulum (*SR*) and dental papilla (*DP*) increase in number in the 5E1 group compared with those in the control. **(G,H)** Sox2$^+$ cells decrease in number in the 5E1 group compared with those in the control. Scale bars: 50 μm.

Statistical Analysis of GFP, Gli1, Ki67, TUNEL, and Sox2 Labeling

The number of dense and granular GFP$^+$ and Gli1$^+$ cells in the dental pulp and apical bud [a grid of (0.24–3.5 × 10^4 μm^2) was selected] were counted in developing teeth (**Table 1**). The number of Ki67$^+$, Sox2$^+$, TUNEL$^+$, and GFP$^+$ cells [a grid of (8.7–9.1 × 10^4 μm^2) was selected] were counted in the incisor explants (**Table 2**). Furthermore, the number of cells/unit area at different time points in the developing teeth was compared using one-way analysis of variance after the confirmation of data normality and homogeneity of variance, followed by Bonferroni's test for multiple comparisons. The proportion of cells was compared between control and experimental groups using Student's *t*-test by statistical software (SPSS 20.0J for Windows; SPSS Japan, Tokyo, Japan). The samples showing non-normal distribution were compared using Kruskal–Wallis or Mann–Whitney tests.
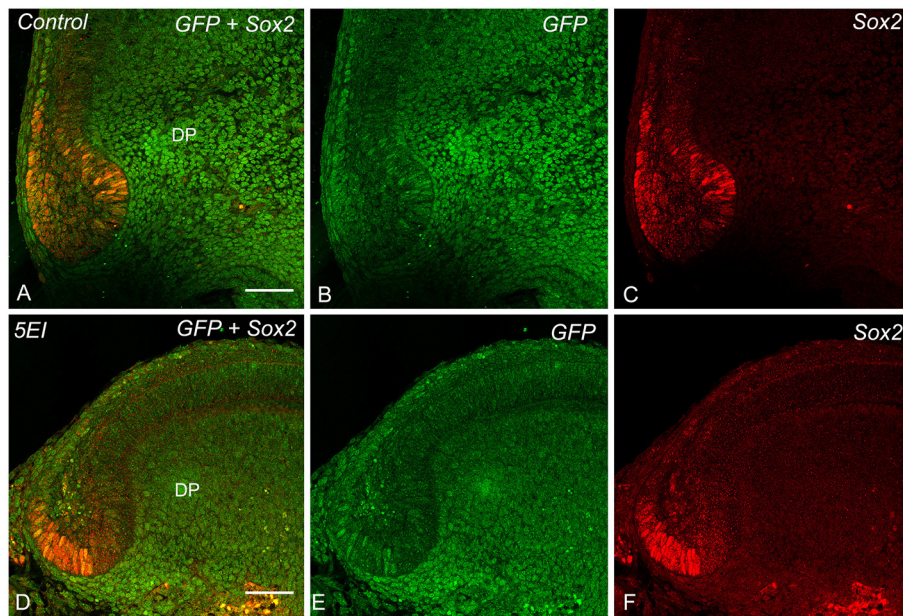
FIGURE 5 | GFP expression and Sox2 IR **(A,D)**, GFP expression **(B,E)**, and Sox2 IR **(C,F)** in control **(A–C)** and 5E1 explants **(D–F)**. Sox2$^+$ cells decrease in number in the 5E1 group in the TetOP-H2B-GFP explants compared with those in the control. Scale bars: 50 μm.

RESULTS

GFP Expression and Gli1, Ptch1, and Ptch2 Immunoreactivity (IR) in the Apical End of Incisors

GFP$^+$ cells were distributed through the dental follicle and apical dental papilla in addition to the outer enamel epithelium of the apical bud (**Figures 1A,B,D,E,G,H**). Gli1$^+$ cells were localized in the inner enamel epithelium and ameloblast layer in addition to the apical bud (**Figures 1A–C**). Ptch1$^+$ cells were intensely localized in the apical dental papilla beneath the apical bud (**Figures 1D–F**). Intense Ptch2 expression was observed in the maturation ameloblasts (**Figures 1G–J**).

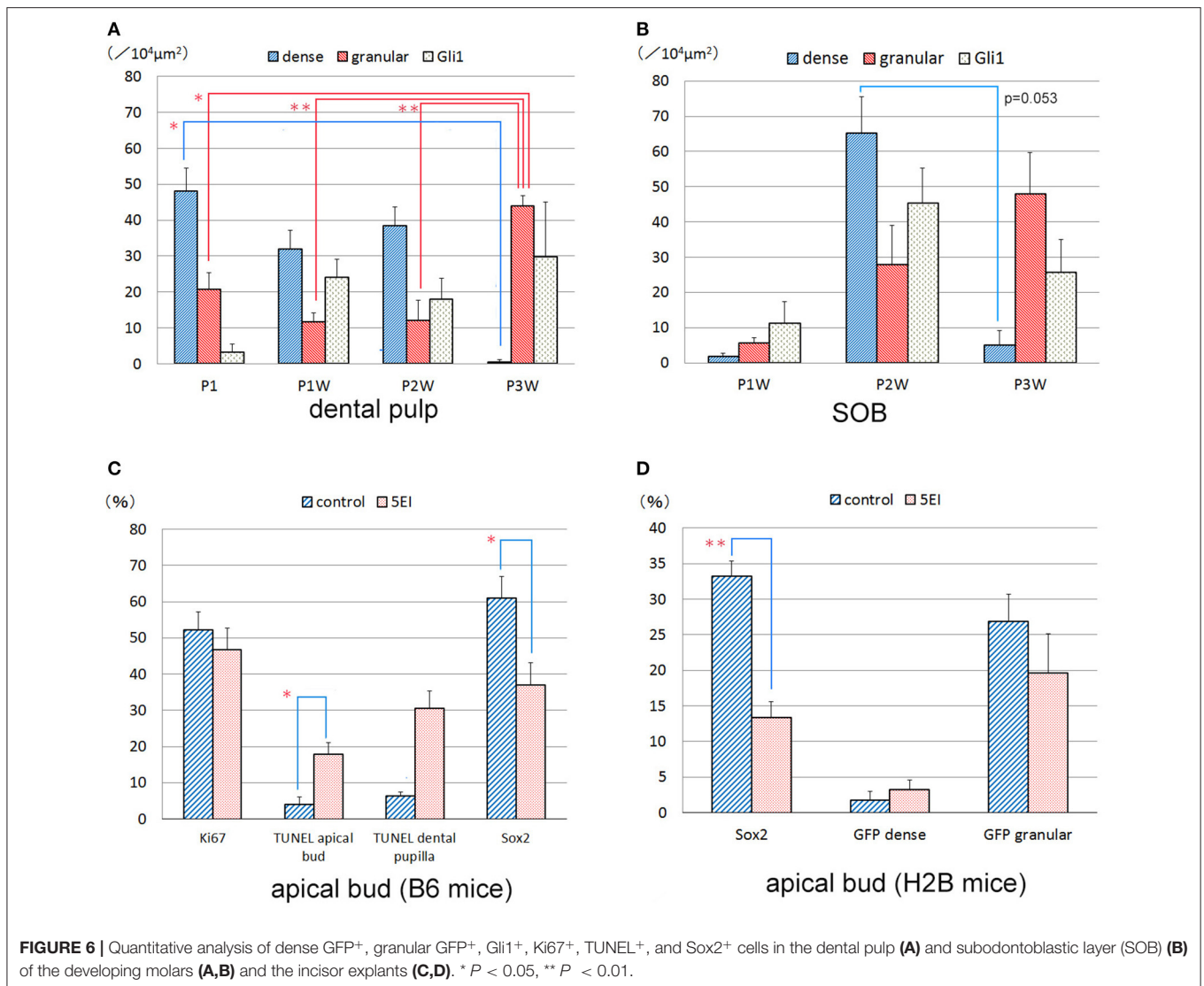## GFP Expression and Gli1 and Path1 IR in the Developing Molars

Dense GFP$^+$ cells were distributed throughout the dental pulp during P1–P2W and decreased in number after P2W, whereas the number of dense GFP$^+$ cells in the subodontoblastic layer increased in number at P2W (**Figures 2A,C,E–G,I,K,L**, **6A,B**). In contrast, granular GFP$^+$ cells increased in number at P3W in the dental pulp. Gli1$^+$ cells were distributed throughout the enamel organ and the dental pulp, including the odontoblast and subodontoblastic layers (**Figures 2**, **6A,B**). Intense Ptch1$^+$ cells were located in the enamel organ, odontoblast layer, and dental pulp (**Figure 3**).

GFP Expression, Shh, Ki67, and Sox2 IR and TUNEL Assay in the Apical End of Incisor Explants

Shh expression and Ki67$^+$ cells were observed in the apical bud and inner enamel epithelium in both the control and 5E1 groups (**Figures 4A–D**) in B6 mice. TUNEL$^+$ cells of the stellate reticulum increased in number in the 5E1 group compared with those in the control group, whereas Sox2$^+$ cells decreased in number in the 5E1 group (**Figures 4E–H**, **6C**). TUNEL$^+$ dental papilla cells increased in number in the 5E1 group, although there was no significant difference between the control and 5E1 groups (**Figures 4E,F**, **6C**). A significant decrease in the number of Sox2$^+$ cells in the 5E1 group was also observed in TetOP-H2B-GFP mice (**Figures 5**, **6D**).

Shh and Ptch1 mRNA Expression Levels in the Apical End of Incisors and in Developing Molars

In the apical end of the incisors, Shh was expressed in the inner enamel epithelium (**Figure 7A**), whereas Ptch1 expression was observed in the epithelial and mesenchymal tissues (**Figure 7B**). Intense Shh was exclusively expressed in the inner enamel epithelium at P1 (**Figure 7C**), and its expression decreased in intensity in the ameloblasts in the developing molars during P1W–P3W (**Figures 7E,G**). In contrast, the odontoblasts and other pulp cells began to express Shh during P1W–P3W. Intense Ptch1 expression was observed in the inner enamel epithelium and dental pulp, including odontoblasts at P1 (**Figure 7D**) and decreased in intensity during P1W–P3W (**Figures 7F,H**).

FIGURE 6 | Quantitative analysis of dense GFP+, granular GFP+, Gli1+, Ki67+, TUNEL+, and Sox2+ cells in the dental pulp **(A)** and subodontoblastic layer (SOB) **(B)** of the developing molars **(A,B)** and the incisor explants **(C,D)**. * $P < 0.05$, ** $P < 0.01$.

DISCUSSION

The present analysis of LRCs in the apical end of incisors of TetOP-H2B-GFP mice demonstrated that dense H2B-GFP-LRCs were distributed through the dental follicle and apical dental papilla in addition to the outer enamel epithelium of the apical bud. These findings are similar to the distribution pattern of BrdU-LRCs in ICR mice with prenatal BrdU labeling, except for the LRCs observed in the dental follicle (10). Prenatal BrdU labeling by three intraperitoneal injections of BrdU (once per day from E15 to 17) also failed to detect BrdU-labeled LRCs in the periodontal ligament (PDL) of the developing molars (28). These findings indicate that dense H2B-GFP-LRCs, having not proliferated during E15–17, remain in the mature PDL. These dense LRCs were primarily distributed in the tooth-related zone. Thus, the static stem/progenitor cell population in the PDL are distinct from these cells in the apical bud and dental pulp, undergoing cell division during E15–17. The coincidence

of the distribution patterns of H2B-GFP-LRCs and Gli1-IR in the dental follicle of incisors is similar to the findings in the PDL in molars (28). This study demonstrates that *Shh* is expressed in the inner enamel epithelium, whereas *Ptch1* expression is observed in the epithelial and mesenchymal tissues. Thus, this study using B6 mice confirmed that the Shh-Ptch1-Gli1 signaling cascade plays a crucial role in the maintenance of quiescent stem cells that respond to Shh signals from the inner enamel epithelium *via* Ptch1 localized in the outer enamel epithelium and stellate reticulum, as previously demonstrated in ICR mice (10).

Dense GFP+ cells were distributed throughout the dental pulp during P1–P2W and decreased in number at P3W, whereas the number of dense H2B-GFP-LRCs in the subodontoblastic layer increased in number at P2W in this study. Gli1+ and Ptch1+ cells were also distributed throughout the dental pulp. These findings were similar to the distribution pattern of BrdU-LRCs in ICR mice with prenatal BrdU labeling, except for the LRCs in the subodontoblastic layer (6, 10, 33). H2B-GFP-LRCs
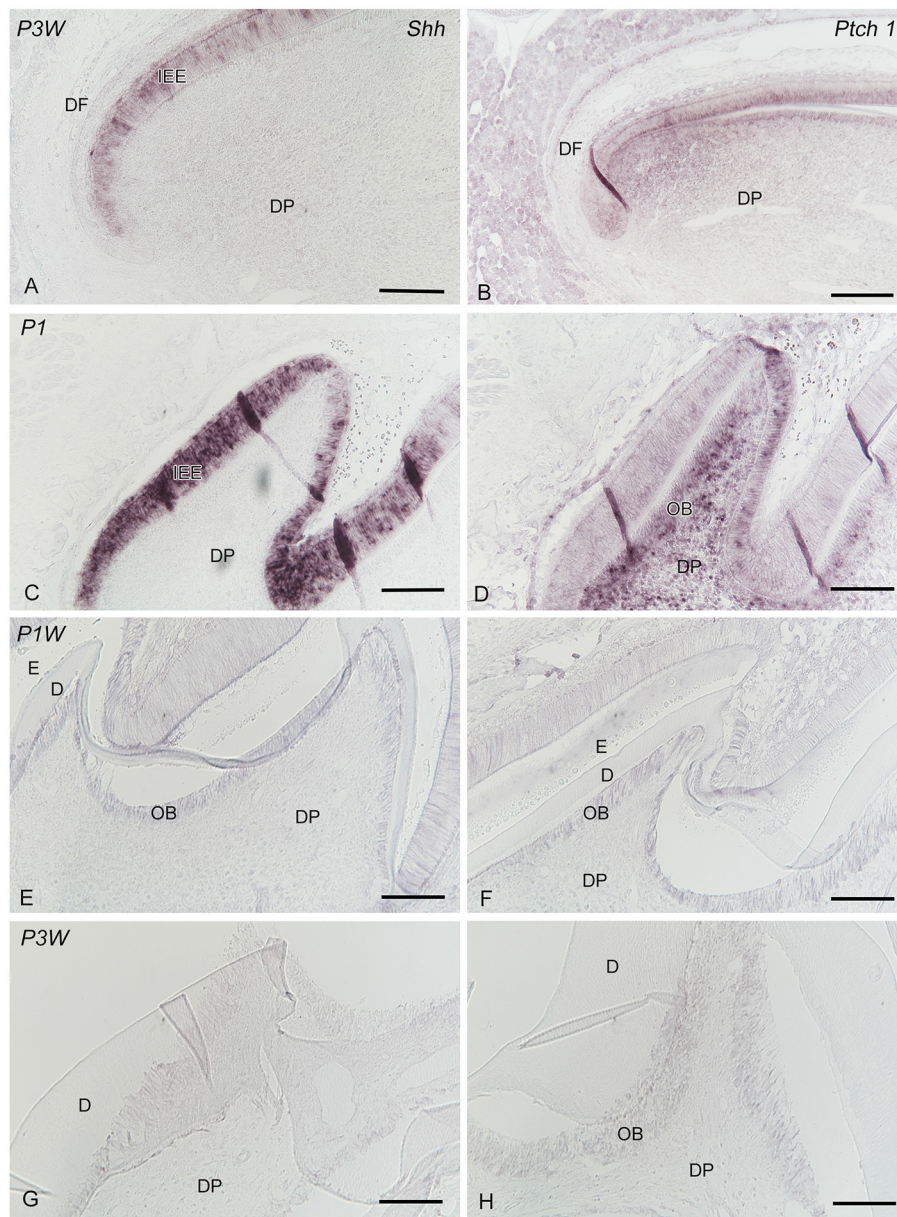
FIGURE 7 | *In situ* hybridization of *Shh* **(A,C,E,G)** and *Ptch1* **(B,D,F,H)** in the incisors **(A,B)** and developing molars **(C–H)** at P1 **(C,D)**, P1W **(E,F)**, and P3W **(G,H)** (*D* dentin, *DP* dental papilla or papilla, *DF* dental follicle, *E* enamel or enamel space, *IEE* inner enamel epithelium, *OB* odontoblasts). **(A)** *Shh* is intensely expressed in the inner enamel epithelium. **(B)** The epithelial and mesenchymal tissues demonstrate *Ptch1* expression. **(C)** *Shh* is exclusively expressed in the inner enamel epithelium. **(E,G)** *Shh* expression in the ameloblasts decreases in intensity, whereas the odontoblasts and the other pulp cells begin to express *Shh* during P1W-P3W. **(D)** *Ptch1* expression is observed in the inner enamel epithelium and the dental pulp, including odontoblasts **(F,H)** *Ptch1* expression decreases in intensity during P1W–P3W. Scale bars: 100 μm.

in the subodontoblastic layer also express insulin-like growth factor (IGF)-binding protein 5 (IGFBP5)/*Igfbp5*, which plays a crucial role in regulating the survival and apoptosis of dental pulp stem/progenitor cells during both tooth development and pulpal healing following tooth injury (33). The expression pattern of Ptch1 was similar to that of Gli1, and this also overlapped with H2B-GFP-LRCs. These findings suggest that the quiescent dental pulp stem/progenitor cells are regulated by Shh signaling.

This study demonstrated that inhibition of Shh signaling with anti-Shh antibodies (5E1) induced an increase in apoptotic cells and a decrease in Sox2+ cells in the apical bud. These findings are similar to the recent report that inhibition of Hh signaling in the incisor stem cell niche with cyclopamine decreased the number of Sox2+ stem cells as a direct consequence of increased cell death (34). Two types of Hh, Shh and desert hedgehog, react with Ptch1 and Ptch2 independently in the vicinity of the apical

bud, and subsequently multiple modes of Hh signaling regulate the maintenance of stem cells and their differentiation *via* the complex relationship between Hh ligands and their receptors. Interestingly, Shh expression and cell proliferation in the inner enamel epithelium seemed normal, and apoptotic cells appeared mainly in the stellate reticulum in this study. Furthermore, inhibition of Shh signaling did not influence the number of H2B-GFP-LRCs and Sox2+ cells in the outer enamel epithelium. These findings suggest that Shh signaling plays crucial roles in the transition between stem and transit-amplifying cells, resulting in a decreased number of Sox2+ cells in the inner enamel epithelium. Although there was no significant difference between the control and 5E1 groups, apoptotic cells increased in number in the apical mesenchyme of incisors, suggesting that inhibition of Shh signaling also affects the stem cell niche of the apical mesenchyme.

CONCLUSION

This study demonstrated that dense H2B-GFP-LRCs were distributed throughout the dental pulp during P1–P2W and decreased in number at P3W, whereas the number of dense H2B-GFP-LRCs in the subodontoblastic layer increased in number at P2W in the developing molars. Gli1+ and Ptch1+ cells were distributed throughout the dental pulp, whereas *Shh* mRNA was expressed in the inner enamel epithelium and shifted into odontoblasts after dentin deposition. *Ptch1* mRNA was expressed in the inner enamel epithelium and cuspal pulpal tissue on P1 and decreased in intensity from P1W to P3W. The apical bud contained H2B-GFP-LRCs, Gli1+ cells, and Ptch1+ cells in continuously growing incisors. The addition of 5E1 to the explants *in vitro* experiments induced a decrease in the number of Sox2+ cells due to increased cell death in the apical bud. Thus, the Shh–Ptch–Gli signaling pathway plays a role in maintaining quiescent adult stem cells and regulating the function of odontoblasts. There may be some discrepancies between proteins and mRNAs regarding the expression patterns of the Shh–Ptch–Gli signaling pathway compared with the previous studies (16, 17, 34–37). Whether this is attributed to the specificity of antibodies or the reduced translation of mRNA in the restricted cell types remains unsolved. Furthermore, the functional significance of dense H2B-GFP-LRCs in the subodontoblastic layer and the PDL remains to be clarified under

pathological conditions following exogenous injury. Recent studies demonstrate that the subodontoblastic cell population may differ from odontoblasts in their origin (38) and that odontoblasts forming the primary dentin are different from odontoblast-like cells responsible for reparative dentinogenesis in their phenotype (39). Further experimental studies using TetOP-H2B-GFP and nestin-enhanced GFP transgenic mice (38) are needed to clarify the responses of subodontoblastic cells to tooth injuries in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee and the President of Niigata University.

AUTHOR CONTRIBUTIONS

FUNDING

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