Expression patterns of nestin and dentin sialoprotein during dentinogenesis in mice

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ABSTRACT

Differentiated odontoblasts could not be identified by one unique phenotypic marker, but the combination of expression of dentin phosphoprotein (Dpp), dentin sialoprotein (Dsp), dentin matrix protein 1 (Dmp1), and nestin may be valuable for the assessment of these cells. However, the findings using these proteins remain controversial. This study aimed to compare two odontoblast differentiation markers: nestin and Dsp in the process of dentinogenesis in mice. We performed immunohistochemistry and/or *in situ* hybridization technique for nestin and Dsp using 3-week-old incisors as well as postnatal 1-day- to 8-week-old molars. Preodontoblasts began to express nestin and Dsp proteins and *Dsp* mRNA, which increased in their intensity according to the progress of odontoblast differentiation in both incisors and developing molars. Nestin was consistently expressed in the differentiated odontoblasts even after the completion of dentin matrix deposition. The expression of *Dsp* mRNA coincided with the odontoblast secretory activity for dentin matrix deposition. In contrast, other pulpal cells, predentin matrix and dentinal tubules also showed a positive reaction for Dsp protein in addition to differentiated odontoblasts. In conclusion, nestin is valuable as a differentiation marker for odontoblasts, whereas *Dsp* mRNA is a functional marker for their secretory activity.

Dentin is a hard connective tissue that forms the bulk of the tooth, and it is a bone-like matrix characterized by multiple closely packed dentinal tubules that traverse its entire thickness and contain the cytoplasmic processes of odontoblasts, which are responsible for the formation and maintenance of the dentin. The cell bodies of the odontoblasts are aligned along the inner aspect of the dentin, beneath a layer of predentin, where they also form the peripheral boundary of the dental pulp (35). The

odontoblasts are terminally differentiated ectomesenchymal cells that synthesize several collagenous and non-collagenous proteins (NCPs) (45). The morphologically discernible differentiation of the odontoblast begins with the dental papilla cells adjacent to the inner enamel epithelium (7). The dental papilla cells adjoining the acellular zone rapidly enlarge and elongate to become preodontoblasts first and then odontoblasts as their cytoplasm increases in volume to contain increasing amounts of protein-synthesizing organelles. The morphology of odontoblasts reflects their functional activity and ranges from an active synthetic phase to a quiescent phase (35). However, the preferred use of terminology regarding the classification of odontoblasts is controversial, because several terms for differentiated odontoblasts have been used by different researchers, *i.e.*, secretory, transitional, and aged odontoblasts (12), young and old odontoblasts (51), or

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immature and mature odontoblasts (39).

Rodent incisors are continuously growing teeth, for which all stages of odontogenesis—including amelogenesis and dentinogenesis—can be surveyed if the sections of the tooth are prepared from the apical end to the incisal edge (39, 48). Recent molecular biological studies have demonstrated the existence of a niche for self-renewing adult stem cells in these rodent incisors, which is referred to as an “apical bud” (21–23, 40, 56). Thus, continuously growing incisors are useful materials for analyzing the developmental process of dentinogenesis, although the incisal edge shows the unique feature that an inflammatory reaction is induced by the attrition of the incisal tip (39). In contrast, rodent molars are teeth with limited growth similar to human teeth. The rate of dentin deposition increases in the early stage of coronal dentin formation and subsequently almost ceases, suggesting the transition from the active to quiescent stages of secretory odontoblasts (57). Hence, both rodent incisor and molar teeth should be compared for the exact understanding of dentinogenesis.

The selection of odontoblast differentiation markers is important for the proper assessment of the differentiation process of odontoblasts. Among these markers, NCPs have been widely studied, especially

the small integrin binding ligand, N-linked glycoprotein (SIBLING) family, which is composed of dentin sialophosphoprotein (Dspp), dentin matrix protein 1 (Dmp1), osteopontin (Opn), bone sialoprotein (Bsp), and matrix extracellular phosphoglycoprotein (Mepe) (16). Dspp is proteolytically cleaved into dentin sialoprotein (Dsp) and dentin phosphoprotein (Dpp) (49, 55). However, differentiated odontoblasts could not be identified by a unique phenotypic marker; a combination of markers such as Dsp, Dpp, Dmp1, and nestin has been applied to identify these cells (18). Dspp and/or its cleaved products Dsp and Dpp are believed to be tooth-specific (11), although they are also expressed in preameloblasts (6, 8, 10, 15, 43, 44). Recent studies have shown the expression of Dspp in bone (41, 42), cementum (3), and some non-mineralized tissues (2, 37, 38), while its expression in non-dental tissues is lower than that in dentin. Dmp1 is an acidic phosphoprotein predominantly expressed in dentin, bone, and cementum (17, 30). A lower level of expression for these proteins has also been found in non-mineralized tissues such as the brain, kidney, pancreas, and salivary gland (53). Among them, the intermediate filament nestin is considered as a marker for differentiated odontoblasts in developing teeth and newly-differentiated odontoblast-like cells ap-

Table 1 Comparison of nestin and dentin sialoprotein (Dsp) immunoreactivity and Dsp mRNA expression during dentinogenesis in a 3-week-old incisor

	apical bud	dental papilla	preodontoblasts	odontoblasts	
				immature	mature
nestin	+/-	-	+	++	+++
Dsp	+	-	+/-	++	+++
Dsp mRNA	-	-	+/-	++	+++

–: negative, +/-: very weak, +: weakly positive, ++: medium positive, +++: intensely positive

Fig. 1 Expression of nestin (**a, d, g, j, m, p**) and Dsp proteins (**b, e, h, k, n, q**), and Dsp mRNA (**c, f, i, l, o, r**) in a 3 week-old incisor. Figs. **d, g, j, m**, and **p** are higher magnified views of the boxed areas in **a**. Figs. **e, h, k, n**, and **q** are higher magnified views of the boxed areas in **b**. Figs. **f, i, l, o**, and **r** are higher magnified views of the boxed areas in **c**. Nestin and Dsp proteins are intensely expressed in odontoblasts, although other pulpal cells also show weak positive reactions (**a, b**). The expression of Dsp mRNA coincides with the odontoblast secretory activity for dentin matrix deposition (**c**). Faint positive reactions for nestin and Dsp are observed in the dental papilla and follicle around the apical bud, respectively (**d, e**), whereas there is no Dsp mRNA in this area (**f**). Nestin is expressed in preodontoblasts and immature odontoblasts, and the apical end of their cytoplasm begins to show intense immunoreactions according to the progress of odontoblast differentiation (**j, m**). Finally, mature odontoblasts show intense immunoreactions in their entire cytoplasm including their cellular processes (**p**). Stellate reticulum also shows faint immunoreactions for nestin (**g**). Intense Dsp immunoreactions are localized in the Golgi area of immature and mature odontoblasts as well as other pulpal cells (**k, n, q**). Dentinal tubules and predentin matrix also show positive reactions for Dsp (**q**) in addition to preameloblasts, ameloblasts, and stellate reticulum (**h, k, n**). Preodontoblasts begin to express Dsp mRNA and increase in their intensity according to the progress of odontoblast differentiation (**i, l, o**), and finally mature odontoblasts intensely express Dsp mRNA in their cytoplasm (**r**). Arrows: enamel matrix, arrowheads: odontoblastic capillaries, AB: ameloblasts, B: bone, D: dentin, DP: dental pulp, E: enamel, IEE: inner enamel epithelium, iOB: immature odontoblasts, mOB: mature odontoblasts, pAB: preameloblasts, PD: predentin, pOB: preodontoblasts, SR: stellate reticulum Bars 500 μ m (**a–c**), 50 μ m (**d–f**), 25 μ m (**g–r**)

Fig. 1

pearing following tooth injuries such as cavity preparation and tooth replantation/transplantation (1, 24, 26, 28, 36, 46, 54). Regarding immunohistochemistry for Dmp1, we failed to obtain a specific immunoreaction in the differentiated odontoblasts in our preliminary experiments (data not shown). Thus, this study aimed to compare two odontoblast differentiation markers: nestin and Dsp in the process of dentinogenesis in mice, using immunohistochemistry and/or *in situ* hybridization technique for nestin and Dsp in 3-week-old incisors as well as postnatal 1-day- to 8-week-old molars.

MATERIALS AND METHODS

Tissue preparation. 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. Materials were collected in groups of 2 to 5 ICR mice at intervals of 1 (n = 3), 3 (n = 2), 5 (n = 2), 7 days (n = 3) and 3 (n = 5), 5 (n = 3) and 8 weeks (n = 2) after birth. At each stage, the animals were perfused with physiological saline transcardially followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) under deep anesthesia by an intraperitoneal injection of chloral hydrate (the maximum dose of 350 mg/kg). The maxillae were removed *en bloc* and immersed in the same fixative for an additional 12 h at 4°C. Following decalcification in Morse's solution (10% sodium citrate and 22.5% formic acid) (47) for 1–4 days at 4°C, the specimens were dehydrated through ethanol series and embedded in paraffin, and sagittal sections of maxillae were cut at 4 µm. The paraffin sections were mounted on Matsunami adhesive silane (MAS)-coated glass slides (Matsunami Glass Ind., Osaka, Japan) and stained with H&E.

Immunohistochemical analysis. For the immuno-per-

oxidase procedure, the sections were processed for the EnVision method (DAKO Japan, Tokyo, Japan) using a mouse anti-nestin monoclonal antibody diluted 1 : 100 (Millipore, Temecula, CA, USA) and the avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA, USA) method using goat anti-Dsp polyclonal antibody diluted 1 : 50 or 1 : 500 (Santa Cruz Biotechnology, Inc., CA, USA), since the previous reports used the different dilutions of anti-Dsp antibody and we had to confirm the suitable concentration. The sections were counter-stained with hematoxylin. Immunohistochemical controls were performed by replacing the primary antibodies with PBS. These immunostained sections contained no specific immunoreaction.

For double immunofluorescent staining for nestin and Dsp, frozen sections (40 µm in thickness) from 3-week-old incisors were treated by three consecutive incubations with a mouse anti-nestin monoclonal antibody diluted 1 : 50 (Millipore), biotinylated anti-mouse IgG diluted 1 : 100 (Vector), and FITC-conjugated streptavidin diluted 1 : 250 (Vector). After washing with PBS and blocking with Avidin/Biotin Blocking kit (Vector), they were then consecutively incubated with goat anti-Dsp polyclonal antibody diluted 1 : 25, biotinylated anti-goat IgG (Vector), and Texas red-conjugated streptavidin diluted 1 : 100 (Vector). The sections were examined with a confocal laser scanning microscope (FV300; Olympus, Tokyo, Japan).

In situ hybridization. Tissue section *in situ* hybridization was conducted as previously described (34). A digoxigenin-labeled probe for *Dsp* mRNA (43) was prepared according to the manufacturer's protocol. Following the fixation, the specimens were decalcified with Morse's solution (10% sodium citrate and 22.5% formic acid) for 24 h (47), dehydrated through ethanol series and xylene, and embedded in

Fig. 2 Expression of both nestin and Dsp (a, d, g, j, m), Dsp (b, e, h, k, n), and nestin (c, f, i, l, o) in a 3 week-old incisor. Second and third rows are differently sliced views in the same section as well as fourth and fifth rows. The first row is the position including preodontoblasts, immature odontoblasts, and inner enamel epithelium. The second row is the position including immature odontoblasts and preameloblasts. The fourth row is the position including mature odontoblasts. The first row is near and the fourth and fifth rows are far from the apical bud, whereas the second and third rows are the positions between them. Nestin and Dsp are expressed in preodontoblasts and immature odontoblasts (a–f), and the apical end of their cytoplasm begins to show intense immunoreactions for nestin according to the progress of odontoblast differentiation (g, i). Finally, mature odontoblasts contain intense immunoreactions for nestin in their entire cytoplasm including their cellular processes (j, m, l, o). Stellate reticulum and stratum intermedium also show faint immunoreactions for nestin and Dsp (a–f). Intense Dsp immunoreactions are localized in the Golgi area of immature and mature odontoblasts (d, e, j, k). Predentin matrix and dentinal tubules also show positive reactions for Dsp (g, h, m, n) in addition to preameloblasts (d, e). D: dentin, DP: dental pulp, IEE: inner enamel epithelium, iOB: immature odontoblasts, mOB: mature odontoblasts, pAB: preameloblasts, PD: predentin, pOB: preodontoblasts, SI: stratum intermedium, SR: stellate reticulum Bar 100 µm.

Fig. 2

paraffin. Five- μ m-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 incubated overnight at 70°C with hybridization buffer containing a digoxigenin-labeled probe for *Dsp* mRNA. After hybridization, the slides were washed in a series 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).

RESULTS

Incisor at Week 3 (Table 1)

Nestin and *Dsp* were intensely expressed in odontoblasts, although other pulpal cells also showed weak positive reactions (Fig. 1a, b). The expression of *Dsp* mRNA coincided with the odontoblast secretory activity for dentin matrix deposition (Fig. 1c). Faint positive reactions of nestin and *Dsp* were observed in the dental papilla and follicle around an apical bud, respectively (Fig. 1d, e). Nestin was expressed in preodontoblasts and immature odontoblasts, and the apical end of their cytoplasm began to show intense immunoreactions according to the progress of odontoblast differentiation (Fig. 1j, m). Finally, ma-

ture odontoblasts contained intense immunoreaction in their entire cytoplasm including their cellular processes (Fig. 1p). Stellate reticulum in the enamel organ also showed faint immunoreaction for nestin (Fig. 1g). Intense *Dsp* immunoreactions were localized in the Golgi area of immature and mature odontoblasts as well as other pulpal cells (Fig. 1k, n, q). Dentinal tubules and predentin matrix also showed positive reactions for *Dsp* (Fig. 1q) in addition to stellate reticulum (Fig. 1h, k). Preodontoblasts began to express *Dsp* mRNA and increased their intensity according to the progress of odontoblast differentiation (Fig. 1l, o), and finally mature odontoblasts showed intense expression of *Dsp* mRNA in their cytoplasm (Fig. 1r).

Double immunofluorescent staining in incisor at Week 3

Nestin and *Dsp* were expressed in preodontoblasts and immature odontoblasts (Fig. 2a–f), and the apical end of their cytoplasm began to show intense immunoreactions for nestin according to the progress of odontoblast differentiation (Fig. 2a, c, d, f). Finally, mature odontoblasts contained intense immunoreactions for nestin in their entire cytoplasm including their cellular processes (Fig. 2j, m, l, o). Stellate reticulum and stratum intermedium also showed faint immunoreactions for nestin and *Dsp* (Fig. 2a–f). Intense *Dsp* immunoreactions were localized in the Golgi area of immature and mature

Table 2 Comparison of nestin and *Dsp* immunoreactivity and *Dsp* mRNA expression during dentinogenesis in developing molars

	Day 1			Week 1			Week 3			Week 5			Week 8		
	DP		OB	DP	OB		DP	OB		DP	OB		DP	OB	
		C	R		C	R		C	R		C	R		C	R
nestin	–	+	N/A	–	++	+	+/-	+++	++	–	++	++	–	++	++
<i>Dsp</i>	+	+	N/A	+	++	+	++	+++	++	+++	+++	+++	+++	+++	+++
<i>Dsp</i> mRNA	+/-	++	N/A	+/-	+++	++	+/-	+++	++	+/-	+	+	–	+/-	–

–: negative, +/-: very weak, +: weakly positive, ++: medium positive, +++: intensely positive, N/A: not available

C: coronal dentin, DP: dental pulp, OB: odontoblasts, R: root dentin

Fig. 3 Expression of nestin (a, d, g, j, m) and *Dsp* proteins (b, e, h, k, n) and *Dsp* mRNA (c, f, i, l, o) in a 1 day-old molar. Figs. d, e and f are higher magnified views of cervical areas of Figs. a, b, and c, respectively. Figs. g, j, and m are higher magnified views of the boxed areas in a. Figs. h, k, and n are higher magnified views of the boxed areas in b. Figs. i, l, and o are higher magnified views of the boxed areas in c. Odontoblasts consistently express nestin and *Dsp* proteins and *Dsp* mRNA (a–c). Other pulpal cells show positive reaction for *Dsp* protein as well as bone cells (b, e, h, k, n). Intense expression of *Dsp* mRNA is observed in the part of the enamel organ (c). Preodontoblasts begin to express nestin and *Dsp* proteins and *Dsp* mRNA (d–i), and increase their intensity according to the progress of odontoblasts differentiation (j–o). Preameloblasts also show immunoreactions for *Dsp* protein and *Dsp* mRNA (k, l), and positive reaction for only *Dsp* protein is observed in the dentinal tubules (n). Arrows: enamel matrix, AB: ameloblasts, B: bone, D: dentin, DP: dental pulp, EO: enamel organ, IEE: inner enamel epithelium, iOB: immature odontoblasts, pAB: preameloblasts, PD: predentin, pOB: preodontoblasts Bars 500 μ m (a–c), 50 μ m (d–f), 25 μ m (g–o).

Fig. 3

odontoblasts (Fig. 2d, e, j, k). Dentinal tubules and predentin matrix also showed positive reactions for Dsp (Fig. 2g, h, m, n) in addition to preameloblasts (Fig. 2d, e).

Molar at Day 1 (Table 2)

Odontoblasts consistently expressed nestin and Dsp proteins and *Dsp* mRNA (Fig. 3a–c). Other pulpal cells showed positive reaction for Dsp protein as well as bone cells (Fig. 3b, e, h, k, n). Intense expression of *Dsp* mRNA was observed in the part of the enamel organ (Fig. 3c). Preodontoblasts began to express nestin and Dsp proteins and *Dsp* mRNA (Fig. 3d–i), which increased their intensity according to the progress of odontoblast differentiation (Fig. 3j–o). Preameloblasts also showed immunoreaction for Dsp protein and expressed *Dsp* mRNA (Fig. 3k, l), and positive reaction for only Dsp protein was observed in the dentinal tubules (Fig. 3n).

Molar at Week 1 (Table 2)

Odontoblasts consistently expressed nestin and Dsp proteins and *Dsp* mRNA (Fig. 4a–c). Other pulpal cells showed a positive reaction for Dsp protein as well as bone cells (Fig. 4b, e, h, k). Dentinal tubules and predentin matrix in the coronal dentin showed intense expression of Dsp protein (Fig. 4k). Immature and mature odontoblasts expressed intense nestin and Dsp proteins and *Dsp* mRNA in their cytoplasm (Fig. 4g–l).

Molar at Week 3 (Table 2)

Root formation progressed at this stage when odontoblasts continued to express nestin and Dsp proteins and *Dsp* mRNA (data not shown). Nestin immunoreactivity in the odontoblast cell processes in the predentin of coronal dentin was more intense than that in the root and pulp floor dentin, and this tendency continued until Week 5. Other pulpal cells and dentinal tubules showed intense positive reaction for Dsp protein (data not shown).

Molar at Weeks 5 and 8 (Table 2)

We adopted two different concentrations (1 : 50 and 1 : 500) for Dsp immunohistochemistry. The Dsp immunoreaction increased in intensity according to the progress of dentinogenesis and the use of different dilution of antibody was recommended to get the suitable stainability in dentin-pulp complex at each stage. Odontoblasts consistently expressed nestin and Dsp proteins during Weeks 5–8 (Fig. 5a–f), although the expression of *Dsp* mRNA in the odontoblasts became weak in intensity at Week 5 (data not shown) and almost disappeared except for some odontoblasts beneath the enamel-free area (9) and in the pulpal floor at Week 8 (Fig. 5g, h, i, k). Increased immunoreaction for Dsp protein was recognized in the coronal dentinal tubules and dental pulp according to aging (Fig. 5c, d), and intense expression of *Dsp* mRNA appeared in some cementoblasts at Week 8 (Figs. 5j, l).

DISCUSSION

Dsp

The present study clearly demonstrated that the expression of *Dsp* mRNA coincided with the odontoblast secretory activity for dentin matrix deposition in both 3-week-old incisors and developing molars, judging from the previous data: the rate of dentin deposition in the medial coronal portion almost ceases after 60 days in rats (57) and the progress of dentinogenesis in mice is faster than that in rats (26, 27). In contrast, other pulpal cells, predentin matrix and dentinal tubules showed positive reactions only for Dsp protein and increased in their intensity according to the progress of tooth development. Previous *in situ* hybridization experiments showed that *Dsp* and *Dspp* mRNA were expressed only by odontoblasts and not by other cell types except for preameloblasts in the mouse incisor and molar at Day 11 (43) or developing rat incisors and molars during embryonic day 20 to postnatal day 20 (4, 19) and developing mouse molars during Day 1 to Week 8 (5, 58). These observations are consistent with the

Fig. 4 Expression of nestin (a, d, g, j) and Dsp proteins (b, e, h, k) and *Dsp* mRNA (c, f, i, l) in a 1 week-old molar. Figs. d, e, and f are higher magnified views of cervical areas of Figs. a, b, and c, respectively. Figs. g and j are higher magnified views of the boxed areas in a. Figs. h and k are higher magnified views of the boxed areas in b. Figs. i and l are higher magnified views of the boxed areas in c. Odontoblasts consistently express nestin and Dsp proteins, and *Dsp* mRNA (a–c). Other pulpal cells show positive reaction for Dsp protein as well as bone cells (b, e, h, k). Dentinal tubules in the coronal dentin show intense expression of Dsp protein (k). Immature and mature odontoblasts express intense nestin and Dsp proteins and *Dsp* mRNA in their cytoplasm (d–l). Arrowheads: odontoblastic capillaries, AB: ameloblasts, B: bone, D: dentin, DP: dental pulp, E: enamel, EO: enamel organ, iOB: immature odontoblasts, mOB: mature odontoblasts, pAB: preameloblasts, PD: predentin Bars 250 µm (a–c), 50 µm (d–f), 25 µm (g–l)

Fig. 4

Fig. 5 Expression of nestin (**a, b**) and Dsp proteins (**c, d, e, f**) and Dsp mRNA (**g–l**) in an 8 week-old molar. Figs. **b, d, f**, and **h** are higher magnified views of the boxed areas in **a, c, e**, and **g**, respectively. Odontoblasts consistently express nestin protein (**a**) and Dsp protein which is demonstrated at two different antibody concentrations, 1 : 50 (**c**) and 1 : 500 (**e**). The Dsp immunoreaction increases in intensity according to the progress of dentinogenesis and the use of different dilution of antibody produces the suitable stainability in dentin-pulp complex at each stage. The expression of Dsp mRNA almost disappears (**g**) except for some odontoblasts beneath the enamel-free area (EFA) (**i**) and in the pulpal floor (**k**). Increased immunoreactions for Dsp protein are recognized in the coronal dentinal tubules and dental pulp (**c, e**), and intense expression of Dsp mRNA appears in some cementoblasts (**j, l**). B: bone, CB: cementoblasts, D: dentin, DP: dental pulp, JE: junction epithelium, OB: odontoblasts, PDL: periodontal ligament, rOB: resting odontoblasts Bars 250 µm (**a, c, e, g**), 50 µm (**b, d, f, h, i, k, l**)

results from immunohistochemical experiments demonstrating the highly specific manner of expression of Dsp protein in developing rat molars during Days 0–17 (4, 10, 13) and developing mouse incisors or molars during Day 1 to 13.5 months (5, 20, 58). However, our immunostaining for Dsp gradual-

ly increased in intensity in predentin matrix, dentinal tubules and odontoblasts, and appeared in many cells of the dental papilla or pulp. The discrepancy between the expressions of Dsp mRNA and Dsp protein could be explained from three possible perspectives. First, the antibodies may detect epitopes

Table 3 Summary of nestin and Dsp immunoreactivity and Dsp mRNA expression in each cell type during dentinogenesis in developing molars

	dental papilla	preodontoblasts	immature odontoblasts	mature odontoblasts	resting odontoblasts
nestin	+/-	+	++	++	++
Dsp	+/-	+	++	+++	+++
Dsp mRNA	-	+	++	++	+/-

-, negative, +/-: very weak, +: weakly positive, ++: medium positive, +++: intensely positive

for other proteins similar to Dsp that are synthesized by pulpal cells (43). Second, pulpal cells may take up Dsp protein into their cytoplasm that has been secreted by differentiating and differentiated odontoblasts. Lastly, it is probable that *Dsp* mRNA is expressed at undetectable levels in the pulpal cells, and subsequently Dsp protein gradually accumulates in their cytoplasm according to the progress of dentinogenesis. The last idea is supported by the findings of a recent study that demonstrated that DSPP is also expressed in lower levels in bone and non-mineralized tissue (58).

Regarding the regenerative process after tooth injuries such as cavity preparation and pulpal capping following pulp exposure, newly differentiated or surviving odontoblast-like cells express Dsp protein in addition to the immunoreaction in the tertiary dentin (14, 25), suggesting that this protein is a reliable marker for regenerated odontoblasts and tertiary dentin. However, immunohistochemistry for Dsp is unable to determine the up-regulation of Dsp production in the afflicted odontoblasts, because Dsp protein has already been accumulated in their cytoplasm. Analysis for *Dsp* mRNA is necessary for precise distinction between the accumulation of protein and up-regulation of its production. The present study demonstrated that some odontoblasts beneath the enamel-free area expressed intense *Dsp* mRNA at Week 8. Tertiary dentin is formed under this area according to aging (31), because this area lacking the enamel covering is directly exposed to the oral environment. Thus, the continuous irritation via the enamel-free area could induce the up-regulation of Dsp synthesis in the odontoblasts beneath this area. The intense expression of *Dsp* mRNA in the odontoblasts adjacent to the pulpal floor at Week 8 suggests that their secretory activity correlates with the continuous deposition of secondary dentin in this area.

Nestin

The present immunohistochemistry for nestin confirmed that nestin is valuable as a differentiation

marker for odontoblasts (26). Furthermore, our double immunohistochemistry for nestin and Dsp proteins clearly demonstrated that the timing of their expressions in the odontoblast-lineage cells was synchronized: preodontoblasts began to express both proteins and increased their intensity according to the progress of odontoblast differentiation, and finally mature odontoblasts showed intense expression of both proteins in their cytoplasm. However, the immunoreaction for Dsp was accumulated in the other pulpal cells, whereas nestin was exclusively expressed in the differentiated odontoblasts in the mature dental pulp. Thus, nestin is a more reliable marker for differentiated odontoblasts compared with Dsp protein. Although the expression patterns of *nestin* mRNA in the dental pulp of developing and aged teeth remain to be elucidated at the present, it is assumed that *nestin* mRNA is consistent with nestin protein judging from the cytoskeletal characteristics of intermediate filament nestin and the facts demonstrated by the previous study focusing on the pancreatic cancer (29). Furthermore, our preliminary study has confirmed the usefulness of nestin as a marker for differentiated odontoblast-like cells even in the environment of *in vitro* culture (author's unpublished data). Further study is needed to clarify the exact nature of nestin as a marker for differentiated odontoblasts.

Classification of odontoblast-lineage cells (Table 3)

Based on the present results, we reconsider the terminology for classification of odontoblast-lineage cells. Preodontoblasts began to express nestin and Dsp proteins and *Dsp* mRNA, which increased their expression intensities according to the progress of odontoblast differentiation. Since the matrix-producing cells lack their proliferative activity and acquire a differentiation marker such as heat-shock protein 25 during odontogenesis in rats (32, 33), proliferating cells are defined as "dental papilla cells" and the polarized cells beginning to acquire their differentiation markers such as nestin and Dsp are named "preodontoblasts". Matrix-producing cells increasing

the expression of their nestin and *Dsp* are referred to as odontoblasts, and these cells are further divided into subgroups. In our previous study using continuously growing rat incisor teeth, we have divided these cells into three types: immature, mature, and post-odontoblasts, according to their morphology, the rate of dentin deposition, and their nutritional supply (the location and ultrastructure of the odontoblastic capillaries) (39). 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 "immature odontoblasts". Subsequently, they increase in height and develop a thick cellular process during active dentin formation resulting in the pseudostratified layer and fenestrated capillaries are always located close to the predentin. Based on their mature morphology and capacity for dentin deposition and sufficient nutritional supply, these odontoblasts can be described as "mature odontoblasts". The intense expression of *Dsp* mRNA in mature odontoblasts in this study was precisely correlated with their functional activity. In contrast, the term "post-odontoblasts" is not suitable for the general terminology used in the process of dentinogenesis, because their appearance is a peculiar status due to an inflammatory reaction caused by the attrition of the incisal tip (39, 50, 52). The expression of *Dsp* mRNA in the odontoblasts became weak in intensity at Week 5 and almost disappeared at Week 8 in this study, suggesting the existence of odontoblasts that have vacated an active synthetic phase and committed to a quiescent phase. The term "resting odontoblasts" seems suitable for the definition of these cells (13, 14).

In conclusion, nestin is valuable as a differentiation marker for odontoblasts, whereas *Dsp* mRNA is a functional marker for their secretory activity. Regarding the classification of odontoblasts, immature, mature, and resting odontoblasts are universal terminology in teeth with limited growth including human teeth from morphological and functional perspectives. Further study should continue to provide the information on the additional molecular markers that starts to be expressed in mature odontoblasts (or ceases to be expressed in immature odontoblasts) to clarify the precise distinction among different types of odontoblasts.

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