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Original Article

The temporospatial relationship between mouse dental pulp stem cells and tooth innervation

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KEYWORDS
Dental pulp stem cells;
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Nerve of pulp

Abstract *Background/purpose:* Dental pulp stem cells (DPSCs) exhibit versatile differentiation capabilities, including neural differentiation, prompting the hypothesis that they may be implicated in the neurodevelopment of teeth. This study aimed to explore the temporospatial dynamics between DPSCs and tooth innervation, employing immunofluorescence staining and fluorescent dye injections to investigate the distribution of DPSCs, neural stem cells (NSCs), nerve growth cones, and sensory nerves in developing mouse tooth germs at various stages.
Materials and methods: Immunofluorescence staining targeting CD146, Nestin, and GAP-43, along with the injection of AM1-43 fluorescent dye, were utilized to observe the distribution of DPSCs, NSCs, nerve growth cones, and sensory nerves in mouse tooth germs at different developmental stages.
Results: Positive CD146 immunostaining was observed in microvascular endothelial cells and pericytes within and around the tooth germ. The percentage of CD146-positive cells remained consistent between 4-day-old and 8-day-old second molar tooth germs. Conversely, Nestin expression in odontoblasts and their processes decreased in 8-day-old tooth germs compared to 4-day-old ones. Positive immunostaining for GAP-43 and AM1-43 fluorescence revealed the entry of nerve growth cones and sensory nerves into the pulp in 8-day-old tooth germs, while these elements were confined to the dental follicle in 4-day-old germs. No co-

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localization of CD146-positive DPSCs with nerve growth cones and sensory nerves was observed.
Conclusion: DPSCs and NSCs were present in dental pulp tissue before nerves penetrated the pulp. The decline in NSCs after nerve entry suggests a potential role for DPSCs and NSCs in attracting neural growth and/or differentiation within the pulp.
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Introduction

Tooth formation is a continuous process, and it is characterized by a series of stages known as the bud, cap, and bell stages. When several increments of dentin are produced, the differentiated ameloblasts deposit some enamel matrix.[1] The dental pulp is termed when the dental papilla becomes surrounded by dentin during dentinogenesis.[2] Not until dentinogenesis begins, do the nerve fibers enter the dental papilla.[3,4] The initial innervation of the developing teeth is concerned with the sensory innervation of the future periodontal ligament and pulp.[4] However, the temporospatial relationship between dental pulp stem cells (DPSCs) and the innervation of teeth is not clear.

Similar to mesenchymal stem cells, which are able to differentiate into neurons,[4–6] DPSCs have also been demonstrated good results in vitro neural differentiation[7,8] and participated in the development of nervous system in experimental animal studies.[9] DPSCs not only possess mesenchymal stem cell ability, but also express mesenchymal stem cell markers, such as CD146.[10] CD146 is a cell-surface molecule belonging to the immunoglobulin superfamily and expressed in human endothelial cells and pericytes.[11,12] CD146-positive endothelial cells and/or pericytes have the ability for neural differentiation and peripheral nervous system development.[12,13] Moreover, CD146 is a functional cell surface marker which can be used to evaluate the potency of the mesenchymal stem cells from dental pulp, including neural potency and has been reported as one of the DPSC markers.[14,15] Therefore, CD146 was chosen as the marker for localizing DPSCs in this study.

Neural stem cells (NSCs) have the capabilities of self-renewal and differentiation into different neural cells types, including neurons, astrocytes, oligodendrocytes and glia.[10,16] Nestin is a protein marker of NSCs and is also expressed in hair follicular stem cells[16,17] and their directly differentiated progeny[16] and stem cells in peripheral nervous system.[18,19] So Nestin was used as the marker for NSCs in this study.

The styryl dye FM1-43 and its fixable analog AM1- 43 have been utilized for the discovery of sensory cells and nerves.[20] AM1-43 is suitable for the examination of hard tissues[21] and as an excellent marker of sensory nerves for studying dental innervation.[20] AM1-43 fluorescent dye was used in this study to examine the distribution of sensory nerves in fixed demineralized tooth germ sections.

The nerve growth cone is a distinctive structure located at the tip of a growing axon or branch. Growth-associated protein-43 (GAP-43) is a neuron growth associated protein, participating in neuronal pathfinding and branching during development and regeneration, and thus has been used as a marker for nerve growth cones.[22,23] The staining of GAP-43 allows the growth cone to be recognizable at the tip of the axon.[23,24] GAP-43 is transported to the growth cone during axonal outgrowth during development and express in the peripheral nervous system.[25,26] To observe the nerve growth front in the developing tooth germs, GAP-43 was chosen as the marker for nerve growth cones.

Data related to the relationship between DPSCs and neural differentiation to date is all derived from cell culture experiments. No immunohistochemical studies using developing tooth germ sections to observe the temporospatial relationship between DPSCs and nerves have been reported. The aim of this study was to investigate and observe the temporospatial relationship between DPSCs, NSCs and the innervations of teeth. The expressions of CD146, Nestin, AM1-43 and GAP-43 were assessed by immunofluorescence in mouse developing tooth germ sections of different stages. It was hypothesized that the neurodevelopment of teeth might be related to DPSCs in the dental pulp. It could be inferred that DPSCs and growing nerves were present in chronological order. Furthermore, the number of NSCs diminished after nerve entering the pulp tissue and thus might play a role in regulate nerve growth or neural differentiation in the pulp tissue.

Materials and methods

Sample selection

Equal numbers of Institute of Cancer Research (ICR) mice aged 3-day-old and 7-day-old were divided into control and experimental groups, each consisting of two mice. The experimental mice were injected subcutaneously at the back skin with AM1-43 fluorescent nerve terminal probe (Biotium, Hayward, CA, USA; 2 μg/g body weight) dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/mL. Control mice were injected with the same amount of PBS alone. One day after the injection, animals were anesthetized by inhalation of carbon dioxide and sacrificed. The following data were acquired from mandibular second molars of 4-day-old and 8-day-old ICR mice. The mandibles were dissected and fixed with 4% paraformaldehyde in PBS at 4 °C for 9–48 h. The maxillae and mandibles from 4-day-old ICR mice were processed without demineralization and 8-day-old ICR mice were processed after demineralization. The tissues were washed

with PBS, infused with paraffin, and cut into 5 μm paraffin sections using a microtome (Leica RM2125 RTS, Wetzlar, Germany). Approval for animal use was obtained from the National Taiwan University College of Public Health Institutional Animal Care and Use Committee (IACUC).

Immunofluorescence staining

Slides were first immersed in the Trilogy™ (Merck KGaA, Darmstadt, Germany) for 10 min to remove paraffin and another 5 min for antigen retrieval. Sections were then incubated in 10% normal goat serum (ab7481, Abcam Inc., Cambridge, UK) for 30 min. The incubation of the primary antibody was performed with Nestin (ab6142, Abcam, 1:20—1:200) or CD146 (ab75769, Abcam, 1:250) or GAP-43 (ab75810, Abcam, 1:500) at 4 °C overnight. Negative controls were not incubated with the primary antibody. The slides were washed three times in PBS for 5 min each and then incubated with secondary antibodies (ab150115, Abcam, 1:200, ab150083, Abcam, 1:200) at room temperature for 2 h followed with wash steps. Sections were incubated with DAPI (62,248, Sigma, Burlington, MA, USA, 1:1000) for 30 min and rinsed off. Sections were mounted with prolonged gold anti-fade reagent (P36934, Thermo Fisher, Waltham, MA, USA) and coverslipped. Fluorescence images of confocal microscopy were acquired by Zeiss LSM 510 Meta and analyzed by LSM Image Browser software (Carl ZEISS, Jena, Germany). The positive immunostaining area was marked and calculated by Image J (NIH, USA).

Statistical analysis

The relationship between the positive immunostaining signals in control and experimental groups was evaluated with t-test. All analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA). A *P*-value <0.05 was considered to be significant.

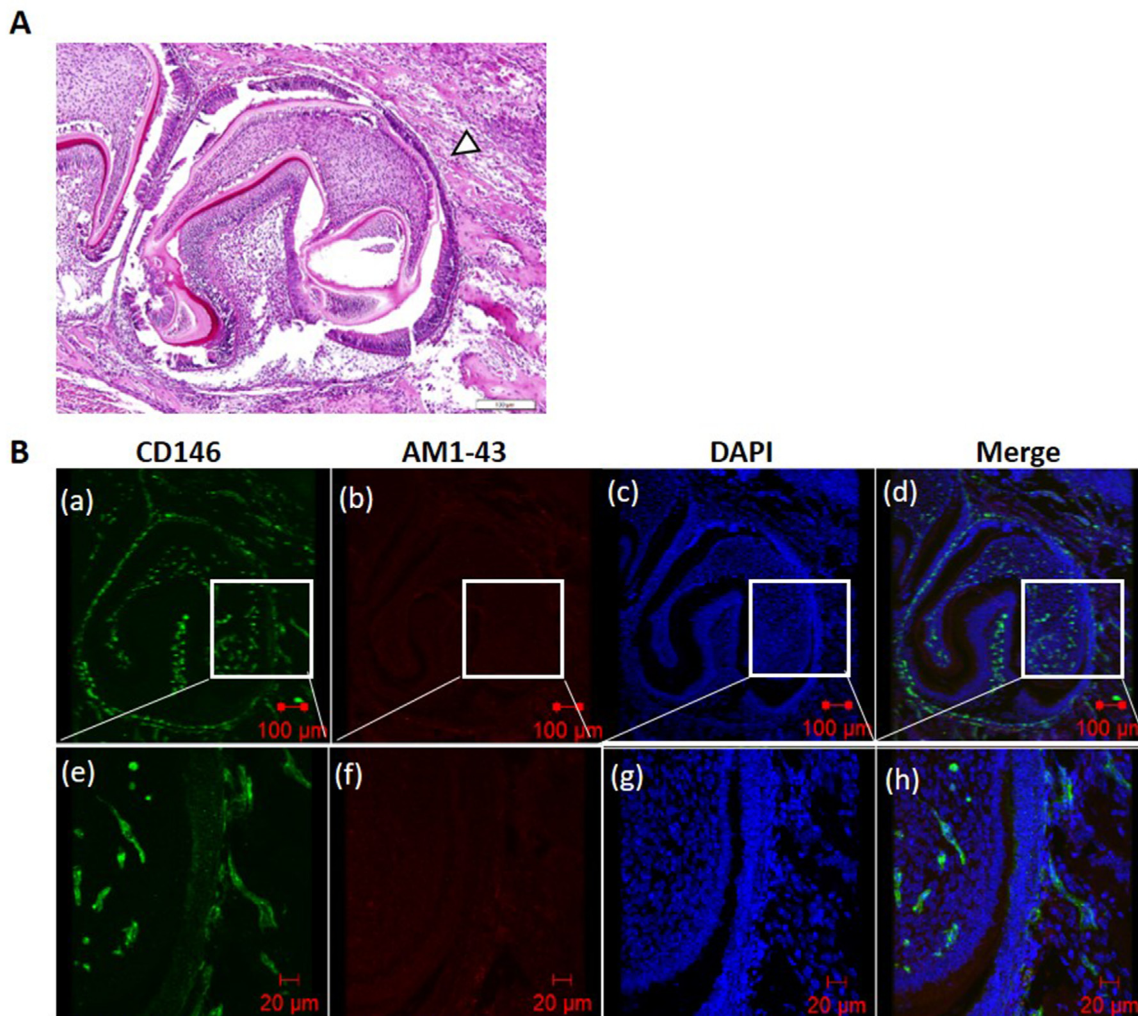

Figure 1 Immunofluorescence staining of 4-day-old tooth germ from ICR mouse mandibular second molar. A. H&E section highlighting the main regions used for the zone of the 4-day-old tooth germ for this study. Scale bar = 100 μm. Arrowhead: cervical portion of the 4-day-old tooth germ. B. Low-magnification and high-magnification confocal images of the mandibular molar showed the CD146 (green), AM1-43 (red), and DAPI (blue) labeled cells. Scale bars = 100 μm (a—d), 20 μm (e—h). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Results

We first used CD146 immunofluorescence to locate DPSCs in this study. In the 4-day-old tooth germ (Fig. 1A), the positive immunostaining for CD146 was in microvascular endothelial cells both inside and outside the tooth germ. The positive immunostaining area in this 4-day-old tooth germ accounted for 4.7% of the entire tooth germ (Fig. 1B). The positive immunostaining for CD146 in the 8-day-old tooth germ (Fig. 2A) displayed similar distribution as in 4-day-old tooth germ. The CD146 positive area in the 8-day-old tooth germ accounted for 4.1% of the entire tooth germ (Fig. 2B), which was not statistically significant.

Sensory nerve fibers and neurons can be labeled by AM1-43 fluorescent nerve terminal probe, and thus it was used to observe the innervation in the developing tooth germ. In the 4-day-old tooth germ (Figs. 1A–3A and 5A), the nerve fibers labeled with AM1-43 were outside the tooth germ and did not enter the pulp (Fig. 1B; Fig. 3B; Fig. 5B). In contrast, in the 8-day-old tooth germ (Figs. 2A–4A and 6A), there were nerve fibers labeled with AM1-43 inside and outside the tooth germ, which means that the nerve already entered the pulp (Fig. 2B; 4B; 6B). The distributions of sensory nerves and DPSCs were different. The CD146 positive DPSCs were present in the pulp in advance of sensory nerve fibers and neurons.

NSCs can be labeled by Nestin. In the 4-day-old tooth germ (Fig. 3A), immune-reactivity for Nestin appeared in the odontoblasts and its processes; the positive staining area in the tooth germ of this stage accounted for 5.8% of the entire tooth germ (Fig. 3B). Different from the 4-day-old tooth germ, the area of Nestin positive staining area in the 8-day-old tooth germ (Fig. 4A) only accounted for 0.8% of the entire tooth germ (Fig. 4B), which was statistically significant ($P < 0.05$). The location of the sensory nerve labeled by AM1-43 (Figs. 3B and 4B) was not co-localized with the Nestin positive areas.

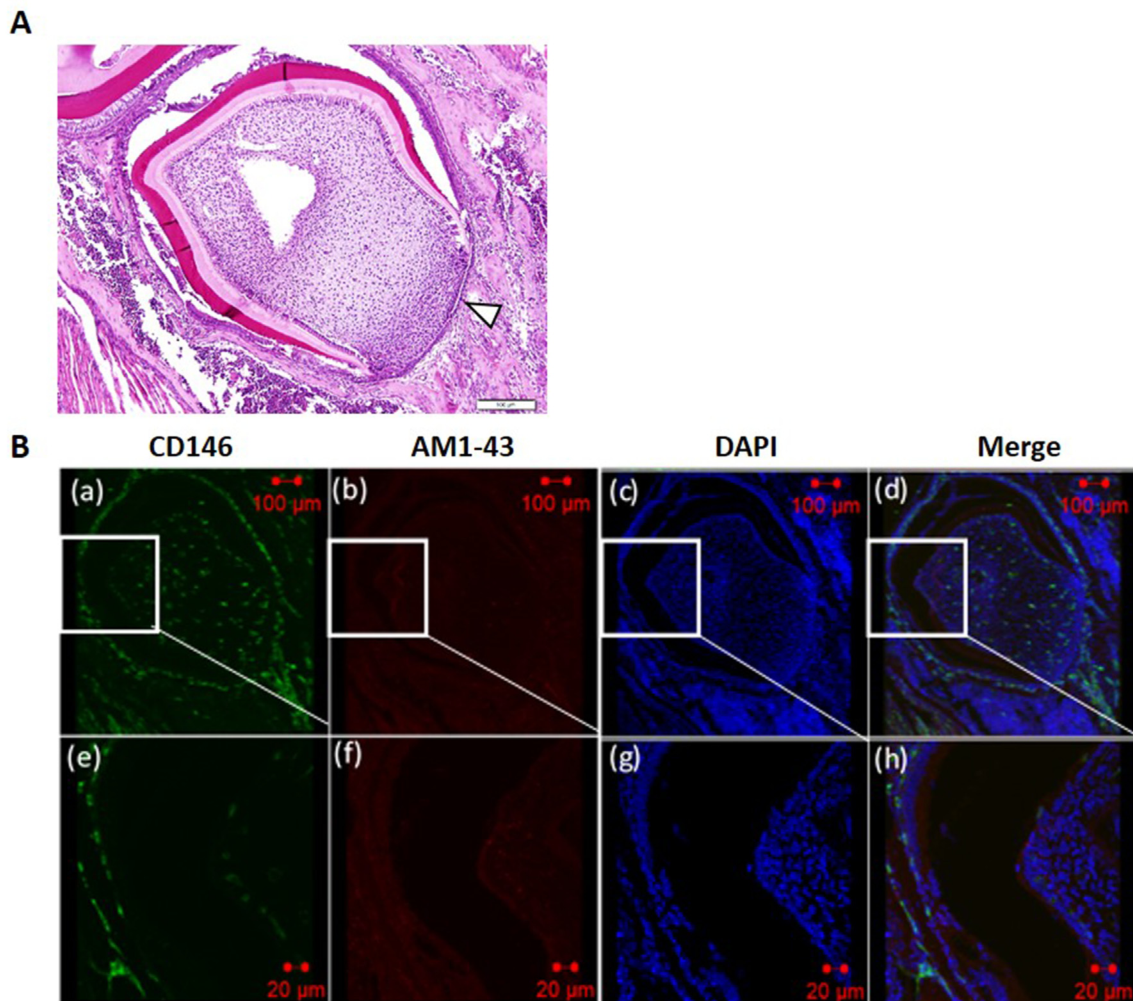

Figure 2 Immunofluorescence staining of 8-day-old tooth germ from ICR mouse mandibular second molar. A. H&E section highlighting the main regions used for the zone of the 8-day-old tooth germ for this study. Scale bar = 100 μm. Arrowhead: cervical portion of the 8-day-old tooth germ. B. Low-magnification and high-magnification confocal images of the mandibular molar showed the CD146 (green), AM1-43 (red), and DAPI (blue) labeled cells. Scale bars = 100 μm (a–d), 20 μm (e–h). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

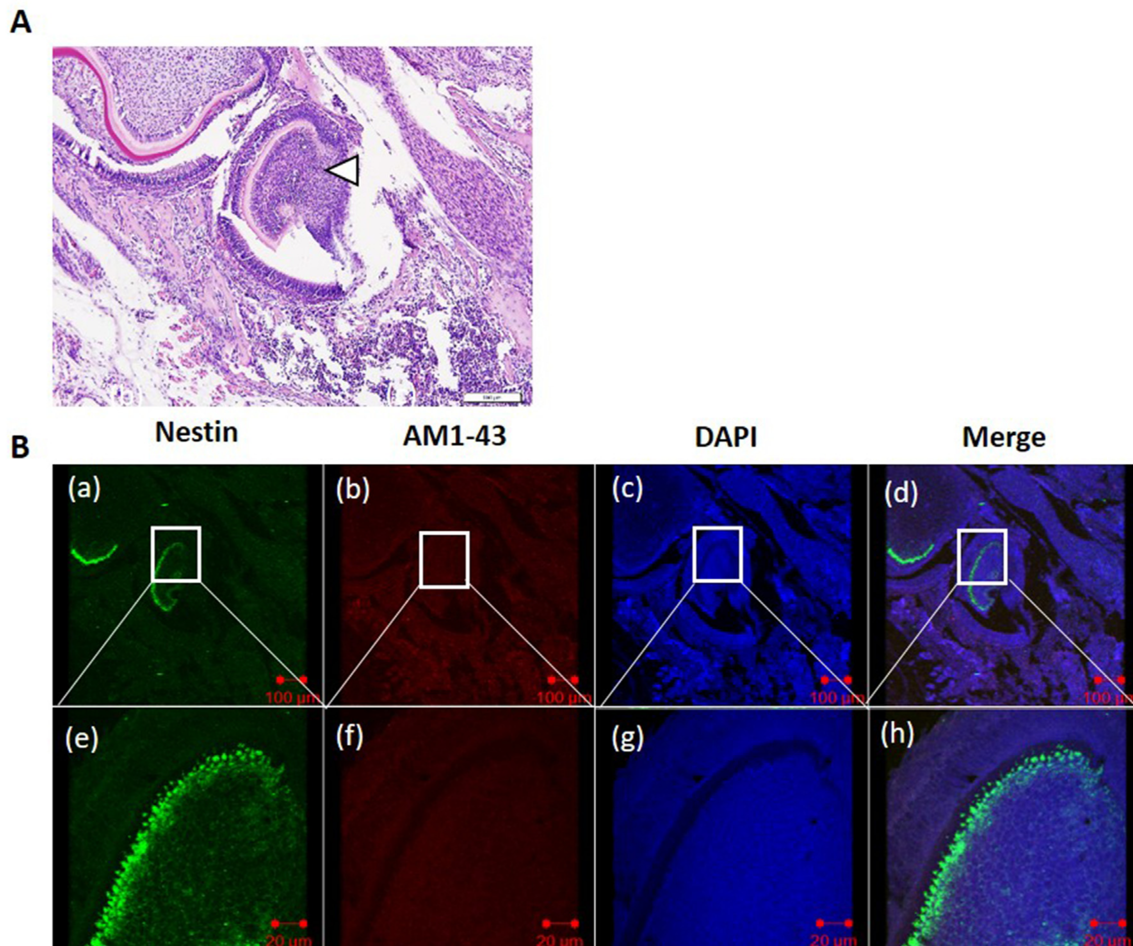

Figure 3 Immunofluorescence staining of 4-day-old tooth germ from ICR mouse mandibular second molar. A. H&E section highlighting the main regions used for the zone of the 4-day-old tooth germ for this study. Scale bar = 100 μm. Arrowhead: cervical portion of the 4-day-old tooth germ. B. Low-magnification and high-magnification confocal images of the mandibular molar showed the Nestin (green), AM1-43 (red), and DAPI (blue) labeled cells. Scale bars = 100 μm (a—d), 20 μm (e—h). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The nerve growth cone is a distinctive structure located at the tip of a growing axon or branch. GAP-43 is a neuron-specific protein related to axon growth and regeneration and has been used as a marker for nerve growth cones. Immunofluorescence labeling showed GAP-43 signals were around the dental follicle in the 4-day-old tooth germ (Fig. 5A). Consistent with the labeling results of AM1-43 (Fig. 5B), GAP-43 positive signals were noted around the dental follicle, in the cervical portion of and inside the tooth germ in the 8-day-old tooth germ (Fig. 6B).

Discussion

DPSCs are multipotent stem cells located in the dental pulp of teeth.[27,28] They possess the ability to differentiate into various cell types, including odontoblasts and neural cells.[27] Teeth are densely innervated structures, crucial for sensation and physiological functions. DPSCs are of interest due to their potential role in guiding nerve innervation, possibly through their neurogenic potential, paracrine signaling, and microenvironment interactions. Investigating this relationship has implications for tooth regeneration, pain perception, and broader applications in regenerative medicine.

To date, all data concerning the relationship between DPSCs and neural differentiation have originated from cell culture experiments. Surprisingly, there have been no immunohistochemical studies utilizing developing tooth germ sections to investigate the temporospatial relationship between DPSCs and nerves. The primary objective of this study was to explore and observe the temporospatial association between DPSCs, NSCs, and tooth innervation. We employed immunofluorescence to evaluate the expressions of CD146, Nestin, AM1-43, and GAP-43 in mouse developing tooth germ sections at various stages.

Human natal DPSCs express CD146 and neurogenic marker Nestin, resembling mesenchymal stem cells (MSCs).[27,28] While CD146 expression is observed in DPSCs both before and after differentiation, Nestin expression significantly increases after differentiation and noticeably increased Nestin is expressed in DPSCs after neurogenic induction.[29—31] These

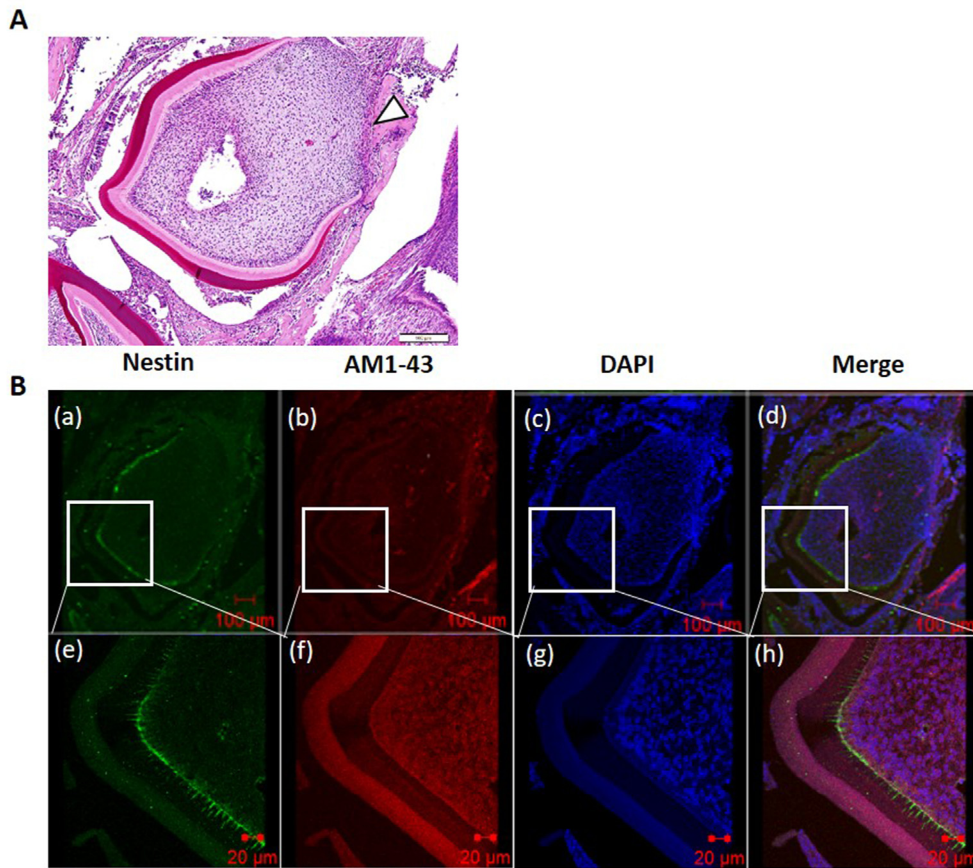

Figure 4 Immunofluorescence staining of 8-day-old tooth germ from ICR mouse mandibular second molar. A. H&E section highlighting the main regions used for the zone of the 8-day-old tooth germ for this study. Scale bar = 100 μm. Arrowhead: cervical portion of the 8-day-old tooth germ. B. Low-magnification and high-magnification confocal images of the mandibular molar showed the Nestin (green), AM1-43 (red), and DAPI (blue) labeled cells. Scale bars = 100 μm (a—d), 20 μm (e—h). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

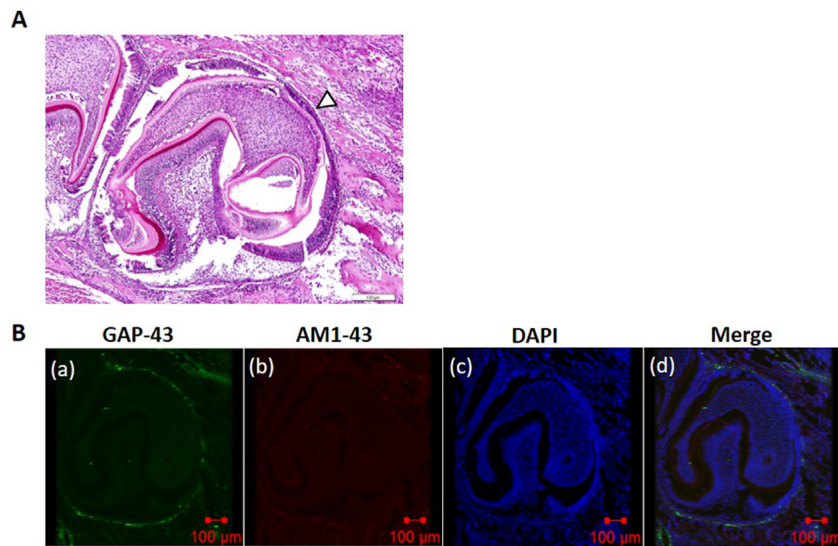

Figure 5 Immunofluorescence staining of 4-day-old tooth germ from ICR mouse mandibular second molar. A. H&E section highlighting the main regions used for the zone of the 4-day-old tooth germ for this study. Scale bar = 100 μm. Arrowhead: cervical portion of the 4-day-old tooth germ. B. Confocal image of the mandibular molar showed the GAP-43 (green), AM1-43 (red), and DAPI (blue) labeled cells. Scale bars = 100 μm (a—d). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

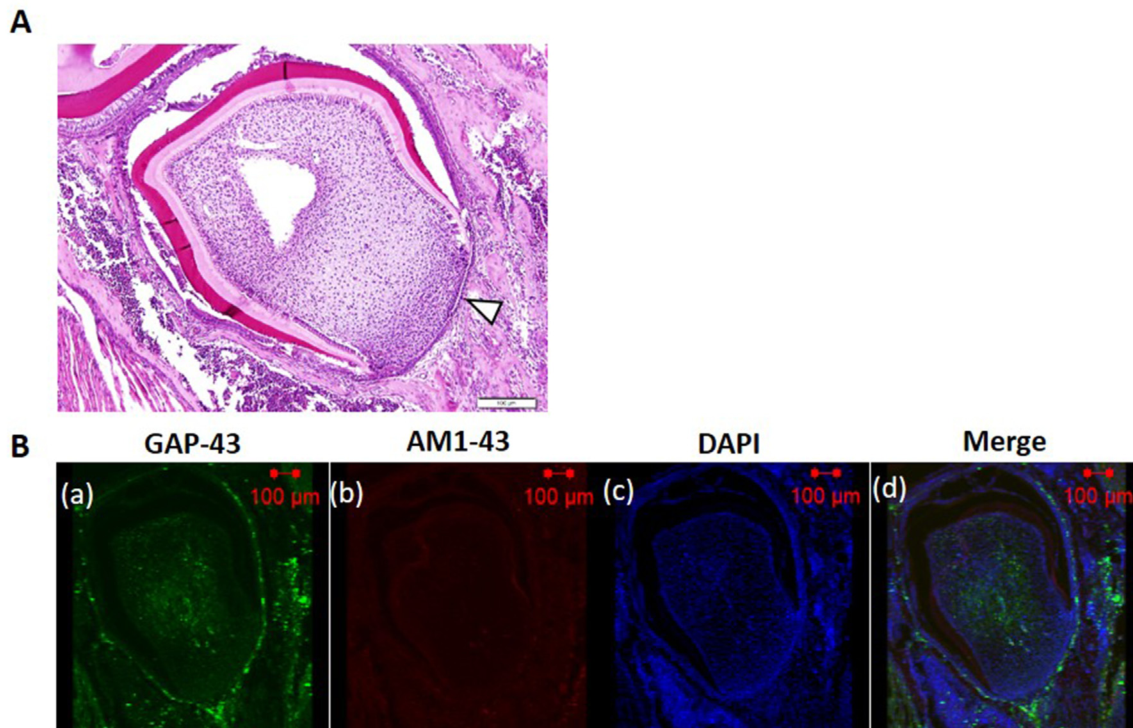

Figure 6 Immunofluorescence staining of 8-day-old tooth germ from ICR mouse mandibular second molar. A. H&E section highlighting the main regions used for the zone of the 8-day-old tooth germ for this study. Scale bar = 100 μm. Arrowhead: cervical portion of the 8-day-old tooth germ. B. Confocal image of the mandibular molar showed the GAP-43 (green), AM1-43 (red), and DAPI (blue) labeled cells. Scale bars = 100 μm (a–d). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

findings imply a potential connection between innervation and neural stem cells. Thus, we used CD146 as the marker for DPSCs and Nestin as the marker for NSCs in our study. Although there are some other DPSC markers, such as CD29, CD105, CD146 and Stro-1, these markers are good for cell sorting but not suitable for immunofluorescence studies in sections.[32–34] Our methods for nerve immunostaining aligned with previous studies.[21] Our study's findings contribute to understanding the DPSCs' role in tooth development and nerve innervation in the real situation.

Our hypothesis centered on the potential correlation between DPSCs in dental pulp and the neurodevelopment of teeth. We posited that DPSCs and developing nerves might coexist in a chronological sequence. Our study observed the distribution changes of DPSCs, NSCs, sensory nerves and nerve growth cones in the mandibular second molar germs of mice at different developmental stages. Our study showed that sensory nerves and nerve growth cones were initially located outside the mandibular second molar germs of 4-day-old mice in the bell stage. However, they later entered the pulp in 8-day-old tooth germs, which were in the late bell stage. Interestingly, the entry of nerves into the pulp in 8-day-old tooth germs coincided with a significant decrease in positive immunostaining for NSCs compared to 4-day-old tooth germs, with a ratio of 1:7. Conversely, the percentages of positive immunostaining for DPSCs both inside and outside the tooth germ remained similar between 4-day-old and 8-day-old tooth germs. Furthermore, we observed a reduction in the number of NSCs following the entry of nerves into the pulp tissue, suggesting a potential role for NSCs in regulating nerve growth or neural differentiation within the pulp tissue.

Our study reveals a dynamic relationship between sensory nerves, nerve growth cones, and dental stem cells during tooth development. Sensory nerves and growth cones initially exist outside the tooth germ and subsequently enter the pulp during later stages. Interestingly, this transition coincides with a marked decrease in positive immunostaining for NSCs but no significant change in DPSCs. These findings suggest a potential role for DPSCs in supporting nerve innervation during tooth development, warranting further exploration in understanding tooth regeneration and sensory function.

In conclusion, from the fourth to the eighth day, the nerve of the molar tooth germ was observed slowly entering into the pulp from the outside of the tooth germs. Neural stem cells had always appeared in odontoblasts and their processes, and stem cells had always appeared in the pulp.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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