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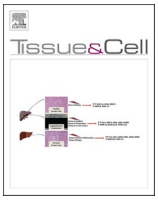

S100A4 is expressed in human odontoblasts and odontoblast-like cells

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ABSTRACT

Introduction: The calcium-binding protein S100A4 has been found in fibroblasts in many tissues. It has been well studied in the regulating process of inflammation and tumor metastasis. However, as a calcium regulating protein, its expression in the dental pulp has not yet been elucidated. The aim of this study was to investigate the in situ expression of S100A4 in dental pulp tissue.

Methods: Five intact and three carious human teeth were collected and sectioned. Hematoxylin and eosin (HE) staining was used to locate areas reflecting the characteristics of the tooth. Based on this initial evaluation, the slides adjacent to these areas were immunohistochemically stained with S100A4 and dentin sialophosphoprotein (DSPP) antibodies.

Results: In both the crown and root, S100A4 staining was observed in the odontoblast layer and in odontoblast-like cells, but not in other pulp cells. In contrast, DSPP was expressed in most cells of the dental pulp.

Conclusion: S100A4 is expressed in the odontoblast layer and in odontoblast-like cells in mature human pulp tissue. This protein can be used as a marker to differentiate these two kinds of cells from pulp cells.

1. Introduction

S100 calcium-binding proteins (termed S100 proteins) are low-molecular-weight proteins harboring an EF-hand-type calcium-binding domain (Gonzalez et al., 2020). The S100 protein family comprises 25 proteins, which are expressed only in vertebrates (Pietzsch, 2011). S100 proteins exhibit a cell-specific expression pattern and are involved in regulating processes, such as cell proliferation and differentiation, apoptosis, and inflammation (Donato et al., 2013). Of all the S100 family members, S100A4 is the most extensively studied. It has been found expressed in tumor and stromal cells, myeloid cells, adipocytes, fibroblasts, immunocytes, and vascular cells (Zhang et al., 2011; Yan et al., 2009; Kalluri and Neilson, 2003). Therefore, it has many names, including metastasin-1 (MTS-1), PEL-98, 18A2, 42A, P9KA, CAPL, calvasculin, and fibroblast specific protein-1 (FSP-1) (Gonzalez et al., 2020).

In oral tissue, S100A4 has been found to be expressed in periodontal ligament cells, and the expression of S100A4 correlates with the pathogenesis of periodontal diseases (Zhou et al., 2015) and periapical granulomas (Tamura et al., 2021), mainly because of its regulating function in inflammation. Expression of S100A4 has also been positively correlated with clinical grading and lymph node metastasis of malignant maxillofacial tumors (Hu et al., 2015). Most of the previous studies have focused on the two fields mentioned above: inflammation regulation and tumor metastasis. However, one of the main functions of S100A4 is to regulate calcium metabolism (Gonzalez et al., 2020). The odontoblasts and odontoblast-like cells in the dental pulp tissue have the capacity to mineralize, which is related to calcium metabolism. However, the expression of S100A4 in the dental pulp has not yet been elucidated (Widbiller et al., 2019). The aim of the present study was to investigate the in-situ expression of S100A4 in intact and carious mature human teeth. Our findings may provide a reference for further studies of human tooth development.

2. Materials and methods

2.1. Chemicals and reagents

Deionized water was produced from a Milli-pore system (Milli-poreSigma, MA, USA). All unspecified solvents and reagents were

Abbreviations: DSPP, Dentin sialophosphoprotein; OPN, Osteopontin; OCN, Osteocalcin; RUNX2, Runt-related transcription factor 2; MSX2, Msh homeobox 2; DLX5, Distal-less homeobox 5; ALP, Alkaline phosphatase; OSX, Osterix.

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Table 1
Descriptive statistics of the teeth.

Donor Number	Donor Gender	Doner Age	Dental Notation *	Tooth Number	Intact or Caries	Pulpal Condition
1	F	34	20	1	Intact	Normal
2	F	24	32	2	Caries	Normal
3	M	60	30	3	Caries	Irreversible pulpitis
4	F	36	1	4	Carries	Normal
5	F	23	1	5	Intact	Normal
6	F	29	16	6	Intact	Normal
7	F	14	5	7	Intact	Normal
			28	8	Intact	Normal

* Dental notation: universal numbering system.

obtained from Sigma Aldrich (MO, USA). The antibodies and conjugates used in immunohistochemical staining were purchased from Abcam (Berlin, Germany): anti-S100A4 rabbit polyclonal IgG (ab41532); anti-DSPP rabbit polyclonal IgG (ab216892); biotinylated goat anti-rabbit IgG (ab6720); horseradish peroxidase (HRP)-conjugated streptavidin (ab7403) and DAB substrate kit (ab64238). The antigen retrieval buffer was 1 mM ethylenediamine tetraacetic acid (EDTA) adjusted to pH 8.0 with NaOH. The tris-buffered saline (TBS) buffer was 0.05 M TBS adjusted to pH 7.4 with concentrated hydrochloric acid (HCL). The slides were washed with TBS plus 0.025 % Triton X-100.

2.2. Tooth collection and section

The materials for the present study consisted of eight mature human teeth. Five were intact teeth extracted for orthodontic reasons, and three were carious teeth. The teeth were collected according to a protocol approved by the Ethical Committee of Peking University with the donors' informed consent. Descriptive statistics of the teeth are included in Table 1.

Immediately after the teeth were extracted, the following approaches were undertaken to facilitate proper fixation of the pulp tissues and correct orientation of the specimens in the paraffin block. A groove parallel to the long axis of the tooth was cut with a bur equipped with an air–water spray to expose the pulp chamber. The teeth were immersed

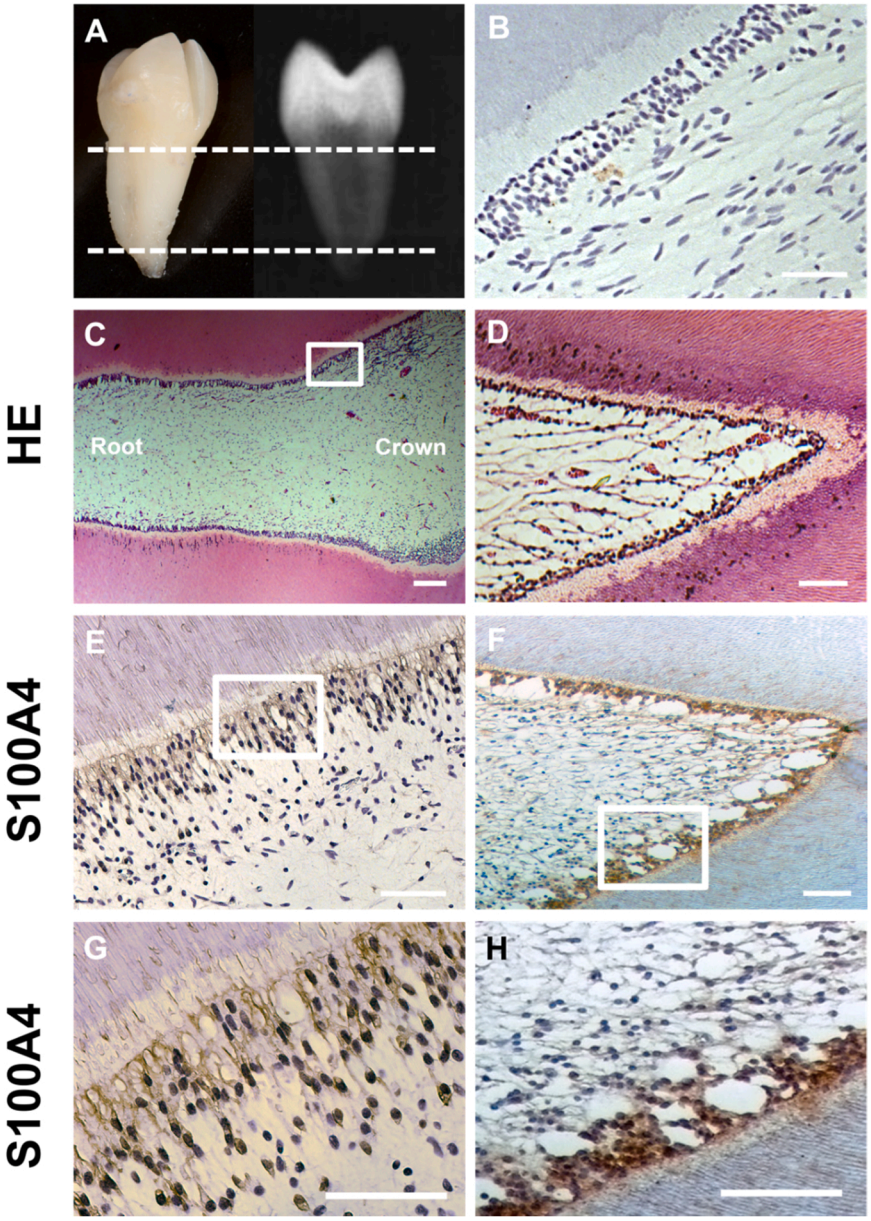

Fig. 1. Intact tooth (bar = 50 μm). (A) Photograph of intact tooth 5 (upper right first premolar) in a 14-year-old female taken after pulpal fixation and radiograph of the tooth after extraction. (B) Negative control. (C) HE stained section taken at the level of the upper line in (A). (D) HE stained section taken at the level of the lower line in (A). (E) Immunohistochemical analysis of S100A4 expression in the rectangular area of (C). Staining was observed in the odontoblast layer but not in the pulp. (F) Immunohistochemical analysis of S100A4 expression in the adjacent area of (D). (G, H) High power view of the rectangular area in (E, F). S100A4 staining was observed in cell bodies and processes.

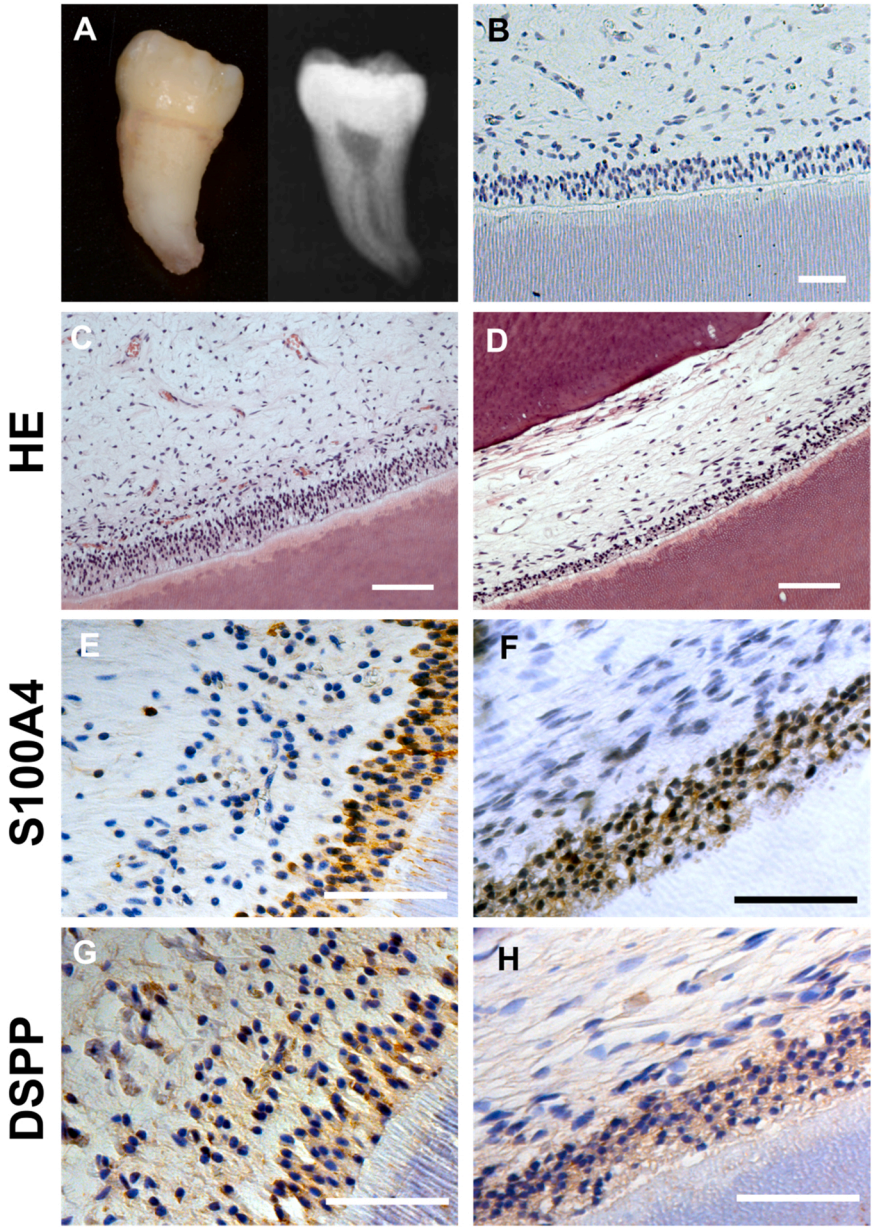

Fig. 2. Intact tooth (bar = 50 μm). (A) Photograph of intact tooth 16 (upper left third molar) in a 29-year-old female and radiograph of the tooth after extraction. (B) Negative control. (C) HE stained section of the crown area. (D) HE stained section of the apical area. (E, F) Immunohistochemical analysis of S100A4 expression in the crown and apical area. S100A4 was expressed in the odontoblast layer but not in the pulp. (G, H) Immunohistochemical analysis of DSPP expression in the crown and apical area. Staining was observed in both the odontoblast layer and the pulp.

overnight in a 10 % neutral buffered formalin solution at 4 ℃.

The teeth were demineralized using 10 wt. % EDTA buffered to pH 7.2. The demineralization buffer was renewed every other day. The end point of demineralization was tested by adding 1 mL of 10 % dipotassium oxalate to 1 mL of the demineralization buffer. If there was no precipitate, the demineralization procedure was complete. All specimens were subsequently washed in running tap water overnight, dehydrated in ascending grades of ethanol, cleared in xylene, infiltrated and embedded in paraffin (melting point 56 °C) according to standard tissue processing procedures. With the microtome set at 6 μm, longitudinal serial sections were taken of the whole tooth, until the pulp tissue was exhausted.

2.3. Hematoxylin and eosin staining

Every eighth section was deparaffinized and rehydrated according to standard tissue processing procedures and then stained with hematoxylin and eosin (HE) for screening purposes. These sections were used to locate the areas that best reflected the characteristics of the tooth. Based on this initial evaluation, the slides adjacent to this area were immunohistochemically stained (the other six slides were used for another study).

2.4. Immunohistochemical staining

The chosen slides were deparaffinized and rehydrated according to standard tissue processing procedures. Antigen retrieval proceeded in the antigen retrieval buffer with heating at 98 ℃ for 20 min using a scientific microwave oven. After cooling and washing, the slides were incubated with 0.3 % H_2O_2 in TBS for 3 h to inhibit endogenous peroxidase activity. The sections were blocked in 10 % normal serum with 1 % bovine serum albumin (BSA) in TBS for 15 min to prevent any nonspecific binding, and the area around the sections was wiped with tissue paper. Primary antibody (diluted 1:200) in TBS with 1 % BSA was applied and the sections were incubated overnight at 4 °C. For negative controls, the primary antibody was replaced with pre-immune serum from the same rabbit. Biotinylated secondary antibody (goat anti-rabbit IgG) was applied to the slides which were then incubated for 10–15 min

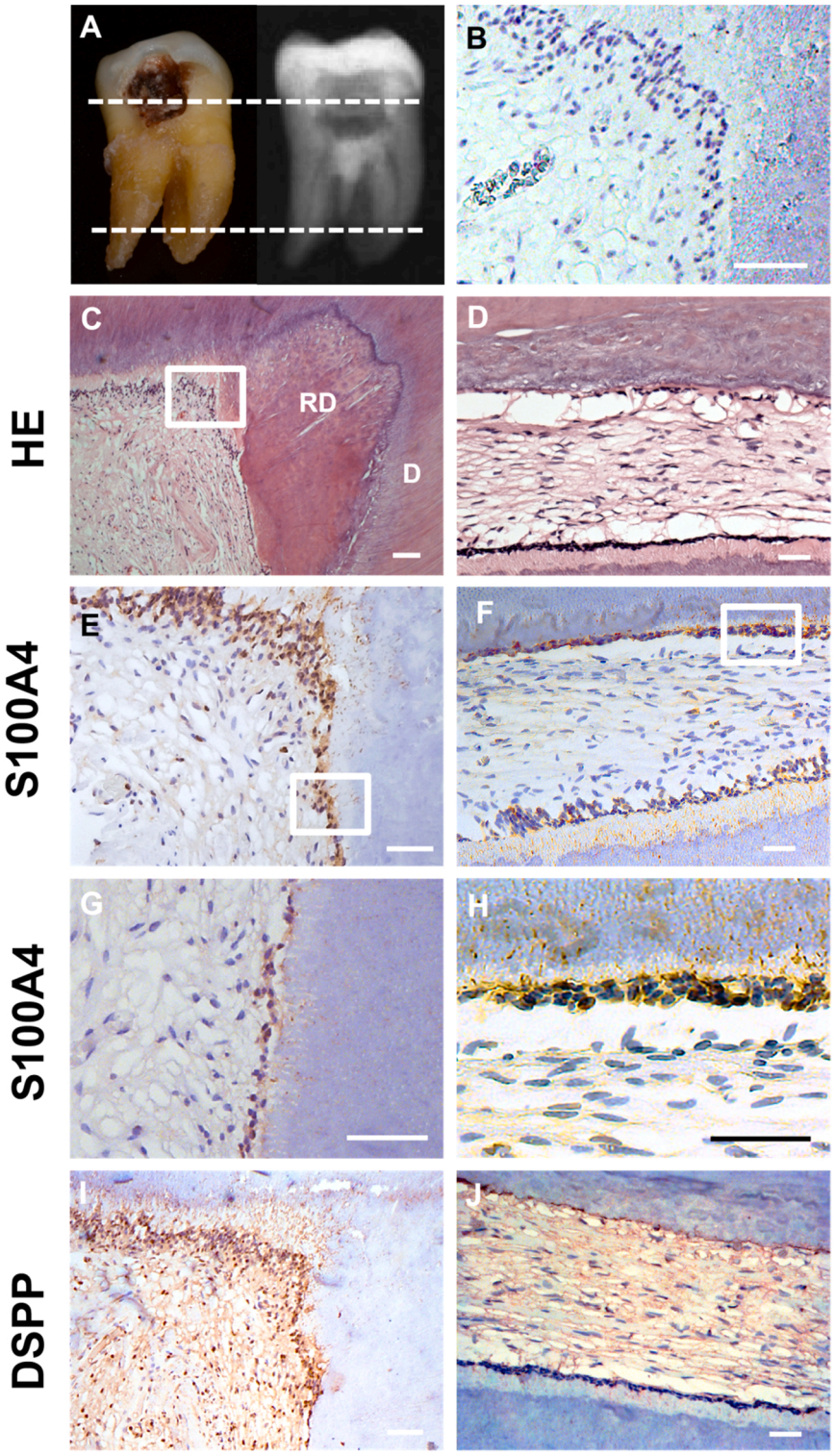

Fig. 3. Carious tooth (bar = 50 μm). (A) Photograph of carious tooth 30 (lower right first molar) in a 60-year-old male and radiograph of the tooth after extraction. (B) Negative control. (C) HE stained section taken at the level of the upper line in (A). RD: reparative dentin, D: normal dentin. (D) HE stained section taken at the level of the lower line in (A). (E) Immunohistochemical analysis of S100A4 expression in the rectangular area of (C). Staining was observed in the odontoblast layer and odontoblast-like cells but not in the pulp. (F) Immunohistochemical analysis of S100A4 expression in the adjacent area of (D). (G) High power view of the rectangular area in (E). S100A4 staining was observed in cell bodies of the odontoblast-like cells. (H) High power view of the rectangular area in (F). S100A4 staining was observed in cell bodies of the odontoblast layer in the apical area. (I, J) Immunohistochemical analysis of DSPP expression in the adjacent area of (C, D). Staining was observed in both the odontoblast layer and the pulp.

at room temperature. HRP-conjugated streptavidin was applied for 10–15 min at room temperature. Then the sections were developed with DAB for 5–10 min and counterstained with hematoxylin for 20 s

3. Results

Fig. 1 shows a representative image of S100A4 expression in an intact human tooth. Intense S100A4 staining was observed in the odontoblast layer in both the crown and root areas. Each odontoblast had homogeneous staining in the cell body and process. No staining of S100A4 was observed in other dental pulp cells. All five intact teeth exhibited the same results. When compared with DSPP staining, S100A4 staining showed tissue specificity. As shown in Fig. 2, DSPP staining was observed in almost all the pulp tissue, but S100A4 staining was only observed in the odontoblasts. Fig. 3 is a representative image of S100A4 expression in a carious human tooth. After exposure to severe challenges, reparative dentin, which lacks typical dentinal tubules, forms in the carious tooth. The cells beneath the reparative dentin do not exhibit

the typical elongated polarized shape of odontoblasts. These cells are known as odontoblast-like cells. Intense S100A4 staining can also be observed in the odontoblast-like cells. All three carious teeth exhibited the same results.

4. Discussion

To analyze the biological properties of the cells in the dental pulp in detail, a pure and homogeneous population of cultivable cells is required. However, a straightforward, reproducible procedure for the direct isolation and cultivation of primary odontoblasts derived from human teeth has not been easy to achieve. Most of the previous studies used odontoblast-like cells differentiated from human dental pulp cells or dental pulp stem cells (Sharpe, 2016). One reason for this is the lack of specific molecules to identify cells presumed to be of odontoblastic lineage (Gallorini et al., 2021). A comparative gene expression analysis between the odontoblast layer and other pulp tissue showed that most genes (OPN, OCN, RUNX2, MSX2, DLX5, ALP, OSX and DSPP) were similarly expressed without significant differences between the two tissues (Widbiller et al., 2019). DSPP, a widely accepted odontoblast marker, is also expressed similarly in both tissues, possibly because most of the crown construction is finished after primary dentinogenesis and the odontoblasts remain in an inactive state. Thus, DSPP seems to be primarily expressed in odontoblasts or odontoblast-like cells during differentiation or progressive mineralization (Quispe-Salcedo et al., 2012; Widbiller et al., 2018). Likewise, further mineralization-associated genes (ALP, OPC and OCN) are not upregulated in mature odontoblasts because they are not in a state of active mineralization (Widbiller et al., 2019).

Unexpectedly, but extremely significantly, S100A4 was specifically expressed in odontoblasts and odontoblast-like cells when compared with pulp cells. In a previous study, cells obtained from the dentin–pulp interface of human teeth were collected and cultivated. The S100A4 gene expression of these cells was roughly 10–100 fold higher than that of pulp cells (Gallorini et al., 2021). These findings are consistent with our results. Furthermore, our study demonstrated in situ expression of S100A4, proving that the cells from the dentin–pulp interface were odontoblasts.

According to previous studies, S100A4 is also expressed in the peripheral and central nervous system in Schwann cells and in astrocytes with neuroprotective functions (Dmytriyeva et al., 2012; Sandelin et al., 2004). It is notable that Schwann cells and a relevant portion of odontoblasts originate from one single glia stem cell type in the neural crest (Kaukua et al., 2014). Thus, odontoblasts probably express S100A4 as an embryonic odontoblast stem cell marker more strongly and more specifically than pulp stem cells.

In addition, the present study found that odontoblast-like cells express S100A4. Opinions on the origin of these cells remain controversial. Traditionally, it was thought that pulp-derived stem/progenitor cells differentiated into odontoblast-like cells in the presence of severe challenges and produced a mineralized dentin matrix (reparative dentin) (Smith et al., 2001). However, a recent study identified that reparative dentin deposition occurred in the radicular pulp of teeth affected by deep caries (Ricucci et al., 2017). These areas were remote from the site of infection in the coronal dentin, casting doubt on the mechanism by which stem/progenitor cells received differentiating signals. The researchers then proposed that odontoblast-like cells might be a kind of fibroblast (Ricucci et al., 2018). Our present study demonstrated that odontoblast-like cells express S100A4, which is present in the primary odontoblasts but not in the fibroblasts in the pulp tissue. This finding is consistent with the traditional opinion, and provides more evidence for the origin of odontoblast-like cells.

Although the current study, along with the previous study (Gallorini et al., 2021), demonstrated the differential expression of S100A4 in mature teeth, there are inevitable limitations in these in vitro studies. The prominent function of S100A4 has been described as a link between metastasis and inflammation, and as a mediator of pro-inflammatory pathways (Ambartsumian et al., 2019). It has been repeatedly reported that mineralized nodule formation and differentiation is negatively correlated with S100A4 expression (Duarte et al., 2003). However, the odontoblasts or odontoblast-like cells retained a moderate capacity to mineralize while highly expressing S100A4. The function of S100A4 in odontoblasts and odontoblast-like cells should be investigated in a developmental animal model in further studies.

5. Conclusion

S100A4 is expressed in odontoblasts and odontoblast-like cells in mature human pulp tissue. The differential expression of S100A4 makes it a potential marker for differentiating cells in dental pulp and investigating the origin and function of odontoblast-like cells.

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CRediT authorship contribution statement

Xue Cai: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Lu Zhang**: Resources, Methodology, Validation. **Xiaoyan Wang**: Supervision, Project administration, Visualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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