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# Microtubule-associated Protein 1b, a Neuronal Marker Involved in Odontoblast Differentiation

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## Abstract

**Introduction:** Map-1B belongs to the family of proteins that govern the dynamic state and organization of microtubules within cells. MAP-1B is a microtubule-associated protein highly expressed during the development of the nervous system. Its expression, regulated by the fragile X mental retardation protein (FMRP), is essential to stabilize microtubules during the elongation of dendrites and neurites. Other microtubules-associated molecules such as tau or MAP2 seem to act similarly. The aim of this work was to identify the MAP-1B expression in *in vitro* and *in vivo* human odontoblasts during development and carious processes. The expression of MAP2 and tau was also studied. **Materials and Methods:** In cultured cells, MAP-1B expression was analyzed by real-time polymerase chain reaction, flow cytometry, and Western blot. Its distribution was visualized by *in situ* hybridization and immunocytochemistry both *in vitro* and *in vivo*. The expression of FMRP, MAP2, and tau was identified by real-time polymerase chain reaction and immunocytochemistry. **Results:** MAP-1B is specifically expressed in odontoblasts from adult third molars as well as incisor germs from human embryos. In adult carious teeth, it is also expressed in newly differentiated dentin-forming cells. *In vitro*, MAP-1B expression is related to the differentiation state of odontoblasts. MAP-1B clearly underlines the cellular architecture of cell bodies and processes of differentiated cells. FMRP, MAP2, and tau are also detected *in vivo*. **Conclusion:** On the basis of these data, MAP-1B could be considered as a new protein involved in the terminal differentiation of odontoblasts. (*J Endod* 2009;35:992–996)

## Key Words

Fragile X mental retardation protein, human tooth pulp, MAP-1B, MAP2, odontoblasts, tau

Odontoblasts are post-mitotic neural crest-derived cells aligned in a single layer on the edge of the dental pulp. Their terminal differentiation implies withdrawal from the cell cycle, cytotologic polarization, formation of a cellular process, and transcriptional and translational modifications allowing the synthesis of dentin matrix (1, 2). The analysis of the established complementary DNA subtractive library specific for human odontoblasts led us to identify the expression of various neural proteins (3) including the microtubule-associated protein 1B (MAP-1B).

MAP-1B belongs to the family of proteins that govern the dynamic state and organization of microtubules within cells by binding with  $\beta$ -tubulin (constitutive proteins of microtubules). It is the first MAP to be expressed during the development of the nervous system and particularly in neurons that are actively extending axons (4–10). Its expression controlled by the fragile X mental retardation protein (FMRP) gradually decreases but remains detectable at the adult stage (11). MAP-1B is still expressed in the regions of the central and peripheral nervous system where neurogenesis and/or axogenesis can occur throughout adult life. Added to this, MAP2 and tau (two microtubule-associated proteins) seem to play a synergistic role in neurite outgrowth (7). The expression of MAP-1B is also detected in nonneuronal cells as in the liver, kidney, and testis (12, 13) in which its function remains to be elucidated.

In the present study, we first identified the gene expression and localization of MAP-1B in human cultured odontoblasts compared with pulp cells. Then using *in situ* hybridization and immunocytochemistry, we provided evidence for the expression and distribution of MAP-1B in differentiated odontoblasts during human odontogenesis, in mature teeth, and under carious lesions.

## Materials and Methods

### Preparation of Tissues

Five sound unerupted human third molars were collected from 14- to 18-year-old patients with informed consent from their parents in accordance with the French Public Health Code and according to a protocol approved by the local ethics committee. Pulpes were fixed in 4% paraformaldehyde phosphate-buffered saline solution and routinely treated for freezing embedding. Serial sections were collected on 3-aminopropyltriethoxysilane-coated slices and air dried.

Two teeth obtained from 20-week-old fetuses were collected from medical induction in the Department of Anatomy and Pathology (Timone Hospital, Marseille, France) according to the French ethical law and the agreement of the local ethics committee (14). Cryostat sections were treated for immunohistochemistry as described previously. Three carious erupted human molars were collected with informed consent as described earlier. Teeth

were fixed in 10% neutral-buffered formalin, demineralized, and embedded as described (15).

## Cell Culture

Odontoblasts were obtained by culture of human dental pulps as previously described (16). Briefly, explants were grown in Eagle's basal medium (Invitrogen Life Technologies, Grand Island, NY) supplemented with 50 µg/mL ascorbic acid, 100 IU/mL penicillin and 50 µg/mL streptomycin (Roche Diagnostic, Mannheim Germany), and 10% fetal calf serum (Biowest, Nuaille, France). Cells were maintained in culture at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 4 to 6 weeks. In these conditions, cultured cells exhibited a fibroblastic morphology. When 10 mmol/L β-glycerophosphate was added to the medium, cells differentiated into odontoblast cells.

## Reverse-Transcription Polymerase Chain Reaction

Total RNA from cultured pulp cells and cultured odontoblasts were extracted with the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany); 100 ng of total RNA was reverse transcribed and amplified by polymerase chain reaction (PCR) with the Titan One Tube RT-PCR System (Roche Diagnostics). Primers were as follows: MAP-1B: forward, 5'-CCTCA GATGTGGGTGGCTATTAA-3'; reverse, 5'-TGTCTTG GGGTGCCTGTATTCA-3'; tau: forward, 5'-TCATTAGGCAACATCCATCATA-3'; reverse, 5'-CACCTCGT CAGCTAGCGT-3'; and MAP2: forward, 5'-TCAG AGCCAATC GCAGAG-3'; reverse, 5'-TGTGTGTC TGTGTGATCCGATTTT-3'. Thirty cycles of PCR amplification were performed with an annealing temperature of 56°C. PCR products were analyzed on 2% agarose gel. The specificity of the PCR amplification product was confirmed by enzymatic restriction.

## Real-time PCR

It was performed in a Light Cycler instrument (Roche) with the Fast Start DNA Master SYBR Green I kit according to the manufacturer's specifications as described (17). The primers used were as follows: MAP-1B: forward primer: 5'-CCGGAGACAGTACCTTCGGAG-3', reverse primer: 5'-CCGACGACCACCAGCAAGTAG-3' and cyclophilin A: forward primer: 5'-ATGGCACTGGTGGCAAGTCC-3', reverse primer: 5'-TTGCCATTC CTGG ACCCAAA-. MAP-1B messenger RNA levels were normalized to the cyclophilin A housekeeping gene with the RelQuant software (Roche).

## Statistical Analysis

Results were expressed as mean values ± standard deviation obtained from multiple cultures of human odontoblasts (three independent experiments). Statistical analysis was determined with a Student t test ( $p < 0.05$ ).

## In Situ Hybridization

An asymmetric linear amplification with the 593-bp complementary DNA probe obtained by reverse-transcription (RT) PCR was used. Sections were then counterstained with Masson's Hemalun, air dried, and observed in dark-field illumination.

## Flow Cytometry

Cells obtained after trypsin/EDTA treatment of cultures were stained for MAP-1B (clone AA6; Sigma Aldrich, St Louis, MO) or isotype-matched mouse immunoglobulin (Ig) G2a (clone UPC 10, Sigma Aldrich) using the "fix and perm" cell permeabilization kit (Invitrogen) according to the manufacturer's instructions. Staining was revealed by goat antimouse F(ab')<sub>2</sub> IgG-FITC (Invitrogen).

## Immunohistochemistry

Sections and cultures were incubated with anti-MAP-1B monoclonal mouse antibody (Clone AA6, 1:100, Sigma Aldrich), anticlass II b tubulin monoclonal mouse antibody (7B9 clone 1:500; generous gift from A. Frankfurter, University of Virginia, Charlottesville, VA), anti-FMRP monoclonal mouse antibody (1:100; Chemicon International Inc, Temecula, CA), anti-MAP2 monoclonal mouse antibody (1:100, Sigma Aldrich), or antitau polyclonal antibody (1:50, Sigma Aldrich). Staining was revealed with Alexa Fluor 594 goat antimouse IgG (1:1500 Molecular Probes, Eugene, OR, USA). Labeling on paraffin wax sections was processed as described (18). Negative controls were conducted with appropriate isotype-matched mouse IgG.

## Western Blotting

Proteins from odontoblast cell cultures were extracted in Laemmli's buffer, separated on a 5% sodium dodecyl sulphate polyacrylamide gel, and transferred to nitrocellulose membrane. Bound anti-MAP-1B monoclonal antibody (1:500 in PBS-2% bovine serum albumin) was detected with horseradish-peroxidase-conjugated goat antimouse IgG (dilution 1:10,000; DAKO, Glostrup, Denmark). The negative control was done with appropriate isotype-matched mouse IgG. Blots were developed using ECL (Millipore Corporation, Billerica, MA).

## Results

### Expression and Localization of MAP-1B in Human Odontoblasts *In Vivo*

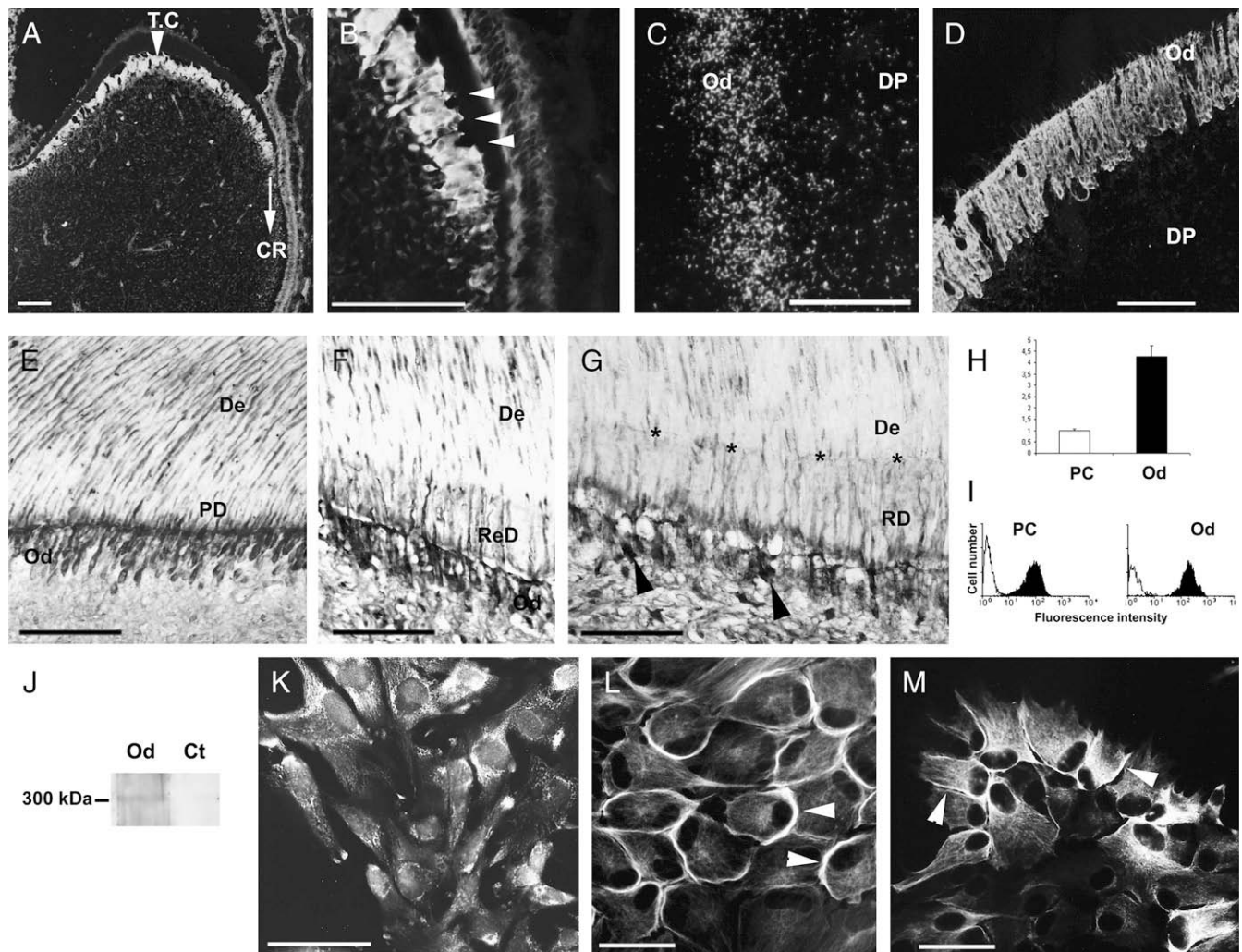
In embryonic incisor tooth germs, the labeling was restricted to the odontoblast layer particularly in the polarized cells with a decreasing gradient from the tip of the cusp to the cervical region (Fig. 1A and B). RT-PCR performed from whole pulps and cultured odontoblasts displayed a 593-bp PCR product cleaved in three fragments by Alu I. The PCR product accurately represented the MAP-1B sequence (data not shown) and was used as a probe for *in situ* hybridization. With the antisense probe, a signal restricted to the odontoblast layer was detected in contrast to the rest of the tissue (Fig. 1C). No specific labeling was revealed with the sense probe. In human mature dental pulps, MAP-1B was detected in the odontoblast layer, particularly in the crown region underlying the cell bodies and processes (Fig. 1D).

### Expression of MAP-1B in Carious Teeth

The contralateral area to the carious tissue exhibited a normal morphology of dentin, Predentin, and odontoblasts (Fig. 1E). MAP-1B protein was identified in the odontoblastic layer as revealed by the strong immunostaining observed in this area (Fig. 1E). In the peripheral zone to the carious lesion corresponding to the reactionary dentin, the cell number was reduced in the odontoblastic layer, and the dentinal tubules appeared to become more irregular in shape. A MAP-1B immunostaining was identified in the odontoblasts (Fig. 1F). In the central zone of the carious lesion corresponding to the reparative dentin, newly differentiated odontoblast-like cells were also positive (Fig. 1G).

### Expression of MAP-1B in Cultured Cells

Real-time PCR showed a significant increase of MAP-1B gene expression in odontoblast compared with pulp cells (4.27-fold;  $p = 0.004$ ) (Fig. 1H). Flow cytometry confirmed the higher MAP-1B level detected by real-time PCR in odontoblasts compared with pulp cells (Fig. 1I). In extracts of odontoblast cell cultures,



**Figure 1.** MAP-1B expression in human odontoblasts and in pathologic teeth. (A and B) Immunohistochemical staining of MAP-1B in embryonic incisor tooth germ at the bell stage development (20-week-old embryos). A positive immunostaining gradient was observed in polarized odontoblasts from the tip of the cusp (TC) (arrowhead in B) to the cervical region (CR). (C) The expression of MAP-1B gene in mature tooth pulp identified by in situ hybridization. (D) Immunohistochemical staining of MAP-1B in the mature teeth. A-D: bars = 100  $\mu$ m. Od: odontoblast layer, DP: dental pulp cells. (E, F, and G) Immunohistochemical localization of MAP-1B in teeth under carious process. (E) Immunostaining of MAP-1B in the odontoblast layer (Od) laterally to the carious tissues. (F) Immunostaining of MAP-1B in the periphery of the carious lesion. (G) Immunostaining of MAP-1B in the central zone under the carious lesion corresponding to the reparative dentin (RD). Newly differentiated odontoblast-like cells expressed MAP-1B (arrowheads). The asterisks represent the calcitropic line, which is the limit between sound dentin and reparative dentin. Od = odontoblasts; De = dentin; PD = predentin; ReD = reactionary dentin; RD = reparative dentin. E-G: bars = 50  $\mu$ m. (H) Real-time PCR analysis of MAP-1B gene expression in cultured odontoblasts (Od) or in pulp cells (PC). (I) Flow cytometry analysis revealed that MAP-1B was more intensely expressed in odontoblasts (Od) than in pulp cells (PC). Results are representative of two independent experiments. (J) Western blotting of MAP-1B expression, identified as a 300-kD molecule in contrast to control (Ct). (K) Immunofluorescence labelling of MAP-1B in cultured pulp cells. (L) At the onset of odontoblast polarization, MAP-1B labelling underlined the plasma membrane particularly around the cell bodies (arrowheads). (M) In fully polarized cultured odontoblasts, the staining was observed at the base of the cell processes (arrowheads). K-M: bars = 50  $\mu$ m.

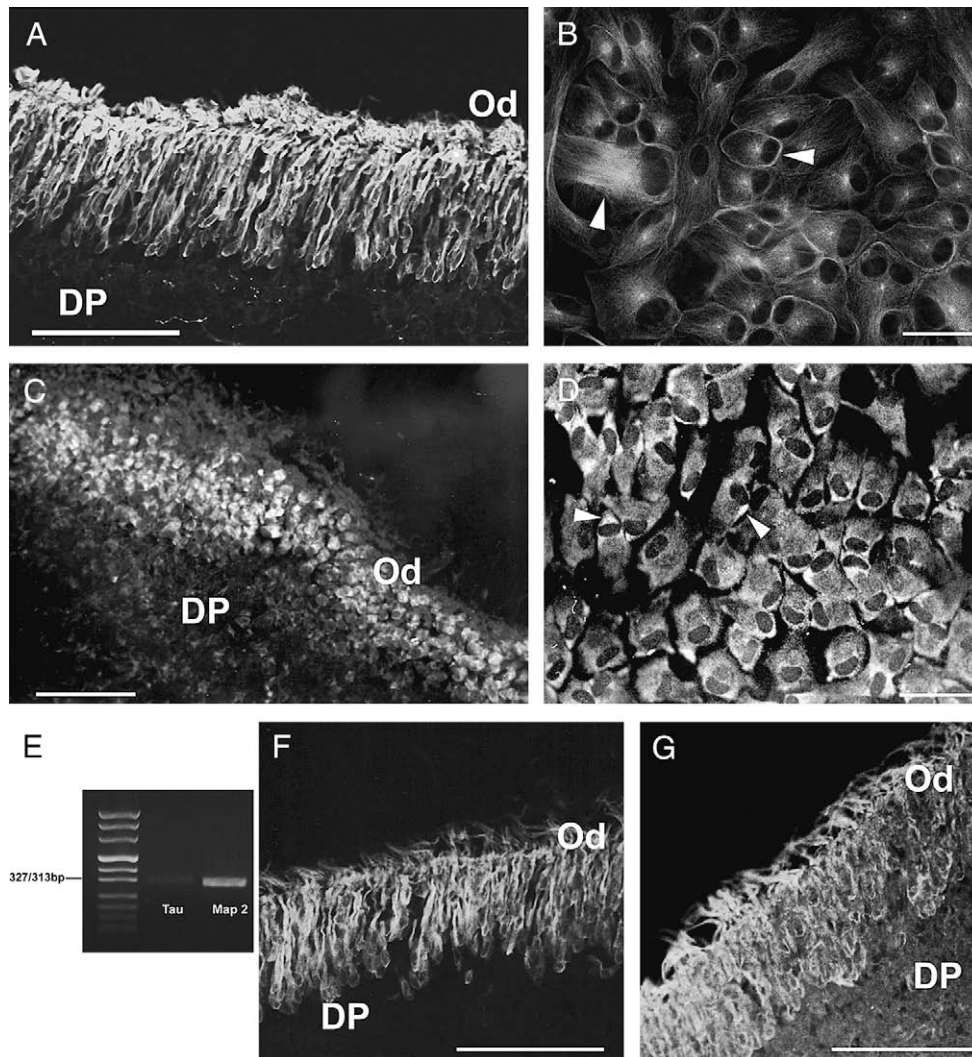
MAP-1B was identified as a unique band of 300 kd by Western blot (reducing conditions). No immunoreactive band was observed in the control when the primary antibody was replaced with nonimmune mouse IgG (Fig. 1J). Intracellular MAP-1B staining could be related to odontoblasts gradient of polarization. In cultured pulp cells, labeling appeared as diffuse patches at the periphery of the cells or concentrated around the nucleus area (Fig. 1K). At the onset of the process of cell polarization, when the cells acquired a polygonal shape with an excentric position of the nucleus, the labeling underlines the plasma membrane particularly around the cell bodies (Fig. 1L). At a later stage, when cultured odontoblasts are fully polarized with an elongated process, the staining was also observed along the membrane of the cell processes (Fig. 1M).

### Expression of $\beta$ -Tubulin

$\beta$ -Tubulin intensely underlined the cell membrane of human odontoblasts compared with pulp cells. Nerve fibers were also positive (Fig. 2A). In cultured cells, the staining was also present in the plasma membrane and developing cell processes.  $\beta$ -Tubulin was evidenced in the perinuclear area revealing the odontoblast centrosome (Fig. 2B).

### Expression of FMRP

In vivo, the FMRP protein was positively identified in human dental pulp cells and in odontoblasts as intense dots (Fig. 2C). In cultured odontoblasts, FMRP underlined the cell bodies and the base of the cell processes (Fig. 2D).



**Figure 2.** (A) The expression of  $\beta$ -tubulin in human odontoblasts of human third molars. Bar = 100  $\mu$ m. (B) In cultured odontoblasts  $\beta$ -tubulin underlined the plasma membrane and the developing processes (*arrowheads*). Bar = 50  $\mu$ m. (C) In vivo expression of FMRP in odontoblasts. Bar = 100  $\mu$ m. (D) In cultured odontoblasts, a positive immunostaining underlined the plasma membrane of the cell bodies and the base of cell processes (*arrowheads*). Bar = 50  $\mu$ m. (E and F) The expression of tau and Map2 in odontoblasts. (E) The expression of MAP2 and tau messenger RNAs by RT-PCR analysis in cultured odontoblasts. (F) The expression of MAP2 in the odontoblast layer of the human third molar pulp. (G) In vivo expression of tau in odontoblasts. F and G: bars = 100  $\mu$ m. Od = odontoblasts; DP = dental pulp.

### Expression of Tau and MAP2

Tau and MAP2 (respectively 327- and 313-bp PCR products) were expressed in *in vitro* differentiated odontoblasts as revealed by RT-PCR (Fig. 2E). In the odontoblast layer of human third molars, an intense membrane staining was observed with anti-MAP2 and anti-tau antibodies (Fig. 2F and G).

### Discussion

We presented evidence that MAP-1B is expressed by cultured odontoblasts originating from human dental pulp cells and previously well characterized (3, 16, 19, 20). We showed also that MAP-1B transcripts are specifically expressed by human odontoblasts *in vivo*. This particular pattern could be related to the gradient of odontoblast differentiation (eg, cell processes elongation). Indeed, cell polarization is accompanied by the formation of a cell process containing numerous

longitudinally arranged microtubules (21). A close correlation could exist between the expression of MAP-1B and the formation of odontoblast process as described in nonneuronal cells bearing cell processes or cilia (13, 22). This hypothesis is strengthened by our results showing that  $\beta$ -tubulin detected in odontoblasts was mainly concentrated in cell extensions. This process may involve FMRP known to regulate MAP-1B translation and microtubules stability (11, 23). FMRP was suspected to play a role in tooth development (24), and its intracellular localization similar to axons (23) suggests that the same control could occur in odontoblasts although a coincidental coexpression could not be excluded. Thus, MAP-1B might facilitate the formation of cell processes and could be involved in the odontoblast polarization by inducing microtubules stability and promoting microtubules assembly. This hypothesis was checked by the histologic analysis of an MAP-1B mutant mouse line (generous gift of Professor D'Avila) (25). No defect in the tooth morphogenesis or odontoblast polarization could be observed (data not shown). This could be caused by a functional redundancy

of MAP-1B and other molecules, such as tau or MAP2, acting similarly (5, 26, 27) even if the residual persistent MAP-1B expression in this hypomorphic mouse line could not be excluded.

Finally, the presence of MAP-1B in reactionary (overexpression of dentin matrix by original odontoblasts) and reparative dentin (a scar tissue elaborated by newly differentiated odontoblast-like cells) suggests that it may also be involved in the transport of molecules essential for the synthesis of dentin in response to carious lesions (28).

In conclusion, we show for the first time that MAP-1B is specifically expressed by fully differentiated odontoblasts in vitro, during odontogenesis, and in reparative processes under carious lesions. Interestingly, our findings are consistent with the distribution of nestin (an intermediate filament expressed in neural stem cells and early development of the central nervous system) in human teeth (29, 30). In this regard, MAP-1B might be considered as a new marker of functioning odontoblasts enhancing the neuronal aspect of these cells.

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