



Exostosin 1 is expressed in human odontoblasts



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A B S T R A C T

Objective: Dental pulp is soft connective tissue maintaining the vitality of the tooth, while odontoblasts form the dentin. Our earlier DNA microarray analysis revealed expression of putative tumour suppressor exostosin 1 (EXT-1) in odontoblasts. EXT-1 is essential for heparan sulphate synthesis, which may play a role in the dentin mineralization. Since the absence of the functional EXT-1 causes bone tumours, expression in odontoblasts is interesting. Our aim was to analyse further the EXT-1 expression in human tooth.

Designs: DNA microarray and PCR techniques were used to study the EXT-1 expression in mature native human odontoblasts and pulp tissue as well as in newly-differentiated cultured odontoblast-like cells. Immunohistochemistry was performed to study EXT-1 protein in mature human teeth, teeth with incomplete root and developing teeth.

Results: Markedly higher EXT-1 was observed in mature odontoblasts than in pulp at mRNA level with DNA microarray and PCR techniques. Immunohistochemistry of mature tooth revealed EXT-1 both in odontoblasts and the predentin but not in the dentin. EXT-1 was also observed in the odontoblasts of incomplete root, but the localization of the staining was different. In developing foetal tooth, staining was detected in ameloblasts and the basal lamina.

Conclusions: The detection of EXT-1 in both mature and newly-differentiated cells indicates a role in the odontoblast function, and EXT-1 staining in the predentin indicates a function in the dentin formation. Detection of EXT-1 in developing teeth indicates a role in tooth development.

1. Introduction

Exostosin 1 (EXT-1) is a transmembrane glycoprotein, localized in the endoplasmic reticulum and Golgi apparatus, and together with EXT-2 synthesizes the cell surface heparan sulphate (McCormick et al., 1998; McCormick, Duncan, & Tufaro, 1999). EXT-1 is a putative tumour suppressor gene, and the absence of the functional protein causes hereditary multiple exostoses (Ahn et al., 1995; Cook et al., 1993; Trebicz-Geffen et al., 2008). Torus palatinus and torus mandibularis are common types of exostoses found in the oral region.

Dentin is the hard tissue of tooth, and in many respects similar to bone. Odontoblasts are non-dividing terminally differentiated cells whose primary function is dentin formation. Odontoblasts form a single-cell layer along the junction of dentin and pulp. Odontoblasts secrete and mineralize the secondary dentin throughout life to compensate for the natural wear of the enamel and form reactionary dentin

after irritation, e.g. caries. Dental pulp is soft connective tissue that contains blood vessels and nerves and maintains the vitality of the dentin. (Nanci, 2008)

We have previously compared the gene expression profiles of mature native human odontoblasts and pulp tissue with DNA microarray and in that study observed the expression of several genes less-known function in odontoblasts, but not in pulp (Pääkkönen, Vuoristo, Salo, & Tjäderhane, 2008). Among these was a putative tumour suppressor, EXT-1, whose expression in odontoblasts is of interest due to the terminally differentiated stage of the cells. EXT-1 is particularly interesting because mutation in EXT-1 gene causes bone tumours (Trebicz-Geffen et al., 2008; Wuys & Van Hul, 2000). Thus, it is possible that EXT-1 plays a role in odontoblast differentiation. Heparan sulphate has previously been detected in predentin and may play a role in the dentin mineralization (Embery, Hall, Waddington, Septier, & Goldberg, 2001). This makes EXT-1, which is an enzyme

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needed for heparan sulphate synthesis (McCormick et al., 1998; McCormick et al., 1999), an interesting protein in the respect of odontoblast function in dentin formation. The aim of this study was to further study EXT-1 expression in the dentin-pulp complex of mature and developing human tooth.

2. Materials and methods

2.1. Tissue samples

Intact third molars (n = 29) were extracted from healthy adult patients at the Oulu Health Care Centre Dental Specialty Care Unit as part of the normal dental treatment. The foetal teeth tissue was extracted from fetuses aborted legally at 14 weeks of gestation. The study was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District.

2.2. Native odontoblast and pulp samples and total RNA isolation

The collection of mature native odontoblasts and pulp tissue separately for the experiments has been described previously in detail (Tjäderhane, Salo, Larjava, Larmas, & Overall, 1998). Briefly, the teeth were cut 5 mm apically from the cement-enamel junction, and the pulp tissue was carefully removed with forceps. The pulp samples were frozen immediately in liquid nitrogen and pulverized for RNA extraction. The odontoblasts were scraped with dental excavator from the walls of the pulp chamber. Total RNA was isolated using Trizol reagent and protocol (GIBCO-BRL, Gaithersburg, MD, USA) pooling pulp tissues of 2–3 teeth or odontoblasts of 3–4 teeth during the RNA isolation to achieve optimal amount for the Trizol method as has been previously described (Palosaari et al., 2000). RNA was further purified Qiagen Rneasy Mini Kit (Hilden, Germany) according to the manufacturer's instructions and A260/A280 ratio of 1.8 was considered acceptable for analysis. To decrease the effect of genetic variation and environmental factors into results, two individual pools (n = 12 and n = 17) collected from patients similar in respect to age and sex were formed for both pulp and odontoblasts.

2.3. Cultured odontoblast-like cells

Method for culturing pulp-derived odontoblast-like cells, representing newly-differentiated odontoblasts, has been previously described in detail by Couble et al. (2000). Dental pulp cells were obtained from sound human third molar germs that were extracted for orthodontic reasons from patients aged 14–16 years. Informed consent was obtained from the patients in accordance with French legal requirements (Article 672-1, Public Health Code). Apical ends of pulp tissues were removed to prevent periodontal cell contamination and the explants (about 2 mm³) were grown in Eagle's basal medium (Invitrogen, Grand Island, NY, USA) supplemented with ascorbic acid, antibiotics, foetal calf serum. 10 mM sodium β-glycerophosphate was added to the medium to differentiate the cells into odontoblast-like cells. After two to three weeks of culture, the cells cultured with sodium β-glycerophosphate differentiated into odontoblasts exhibiting typical features at the morphological and functional levels as described earlier (Couble et al., 2000). Total RNA was extracted from cultured odontoblast-like cells, coming from 3 different patients, using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.4. DNA microarray

Data of DNA microarray analysis for mature native odontoblasts was performed earlier (Pääkkönen et al., 2008), and stored at Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/projects/geo/>) under number GSE8694 was used.

Affymetrix HGU133plus 2.0 array was used for analysis of mature native pulp tissue and cultured odontoblast-like cells. Experimental procedures for GeneChip (Affymetrix, Santa Clara, CA, USA) were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual. Microarray analysis was performed once.

Probe sets representing EXT-1 and associated presence/absence calls as well as *p*-values were extracted from the data. dChip-software (<http://www.hsph.harvard.edu/cli/complab/dchip/>) was used to compare EXT-1 expression levels in mature odontoblasts and pulp tissue.

2.5. Real-time quantitative PCR

The two odontoblast and pulp tissue RNA pools mentioned above were used for reverse transcriptase reaction using AMV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) reactions for EXT-1 were performed to samples with Stratagene Mx3005P using Dynamo Flash SYBR Green qPCR Kit and protocol (Finnzymes, Espoo, Finland). The primers used for EXT-1 were GCA AAG ACT GGC AAA AGC ACA A and GAA TGG CAA CTC CCA TCC ATT G, for β-actin AAC TGG GAC ATG GAG AAA A and AGA GGC GTA CAG GGA TAG CAC A and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) CAC CAT CTT CCA GGA GCG AG and GAC TCC ACG TAC TCA GC. EXT-1 expression levels in each sample were quantified by comparative Ct method (2^{-ΔΔCT}) with β-actin and GAPDH as control gene. One of the pulp samples was selected as the reference with value 1. The analyses were performed in triplicates for each sample. Averages and SD-values were calculated for each sample.

2.6. Immunohistochemical analysis

For the immunohistochemical stainings, 6-μm thick formalin fixed and demineralized paraffin sections of four intact fully formed human third molars, two third molars with incomplete root formation and foetal developing tooth were obtained. Endogenous peroxidase activity was inhibited by incubation with 0.3% H₂O₂ in methanol for 3 h. The sections were then pepsinated for 1 h in 37 °C, followed by incubation in 5% normal goat serum in 2% BSA/PBS solution to prevent any nonspecific binding. Anti-EXT-1 antibody (1:500), generated against mouse EXT-1 (Trebicz-Geffen et al., 2008) was used for overnight incubation at +4 °C. For negative controls, the primary antibody was replaced with preimmune serum from the same rabbit. The sections were treated with biotinylated anti-rabbit IgG (DAKO A/S, Glostrup, Denmark), incubated with VECTASTAIN R Elite ABC-reagent (Vector Laboratories, Burlingame, CA, USA), stained with AEC (DAKO, Carpinteria, CA, USA), according to the manufacturer's instructions and counterstained with Mayer's hematoxylin.

3. Results

3.1. DNA microarray

Three probe sets detecting EXT-1 were included on the microarray used. Analysis of DNA microarray data obtained from native odontoblasts (the GEO database GSE8694) and pulp tissue revealed EXT-1 expression in native odontoblasts (all three probes positive) and at lower level in pulp tissue where only one of the probe sets gave positive signal. In the probe set giving positive signal in pulp tissue 2-fold decrease in comparison with native odontoblasts was observed. EXT-1 gene expression was also observed in newly-differentiated cultured odontoblast-like cells with two of the probe sets giving positive signal.

3.2. Quantitative PCR

Quantitative PCR was performed for EXT-1 using two individual mature odontoblast and pulp samples. Markedly higher EXT-1 expres-

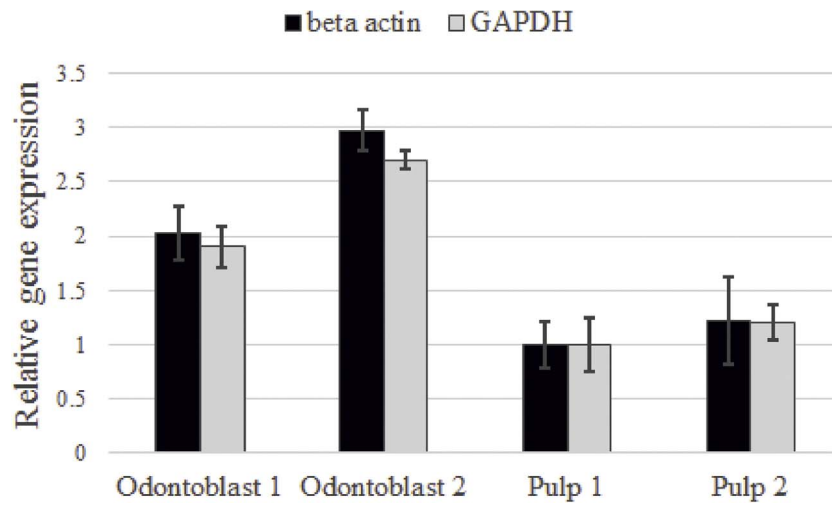


Fig 1. Markedly higher EXT-1 expression was observed in odontoblasts than in pulp tissue by qPCR analysis. qPCR analysis was performed to two individual odontoblast (Odontoblasts 1 and 2) and two pulp samples (Pulp 1 and 2) in triplicates. β -actin and GAPDH were used as control genes and the expression level in Pulp 1 sample was selected as the reference with value 1. Bars represent means and SD-values of relative gene expression.

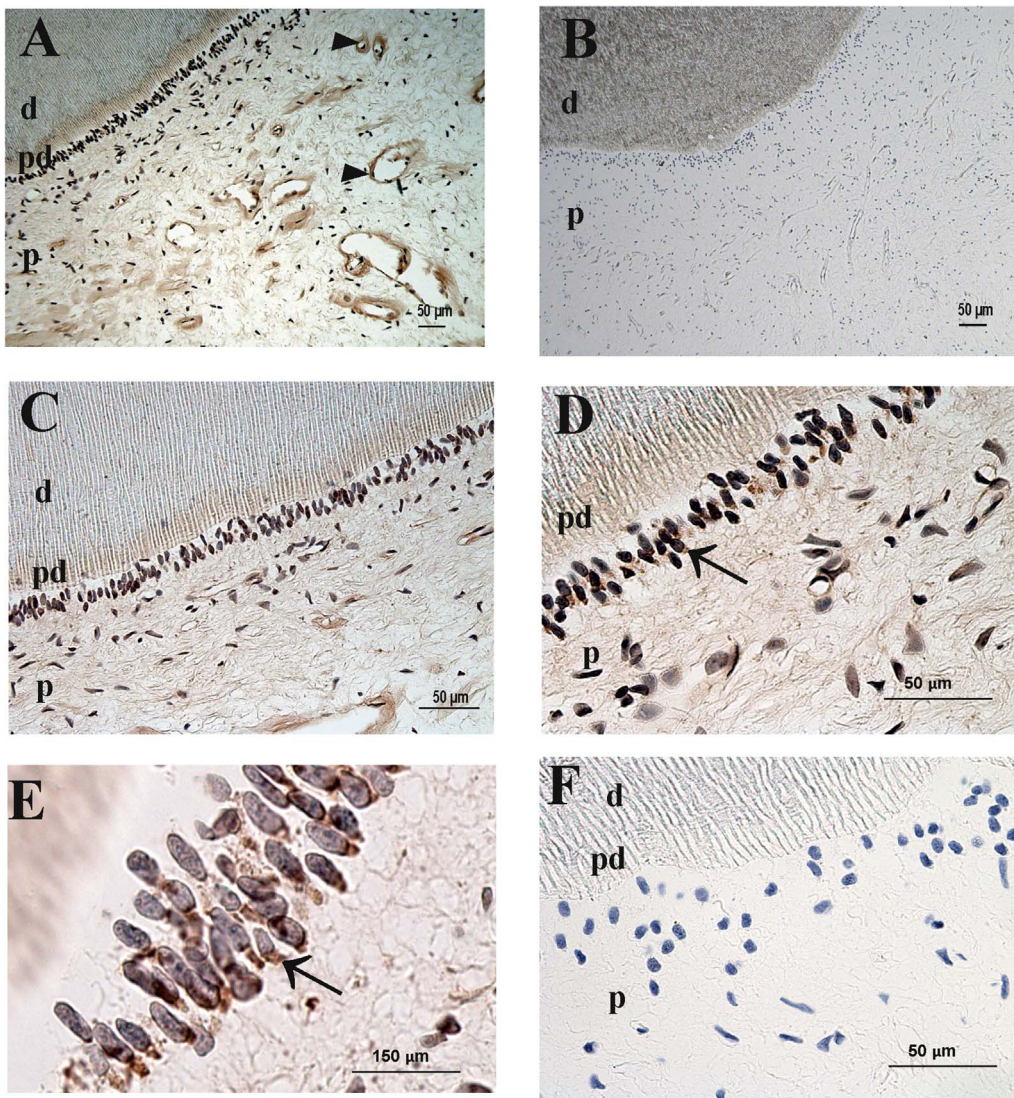


Fig. 2. Immunohistochemical analysis of EXT-1 expression in mature human tooth. EXT-1 staining was observed in the predentin but not in the mineralized dentin (d) (A, C). Staining was observed in the odontoblast layer, adjacent to the nucleus of each odontoblast (D, E, arrow) but not in the pulp (p), except in vascular endothelium (arrowhead) (A, C). No staining was observed in the negative controls (B, F).

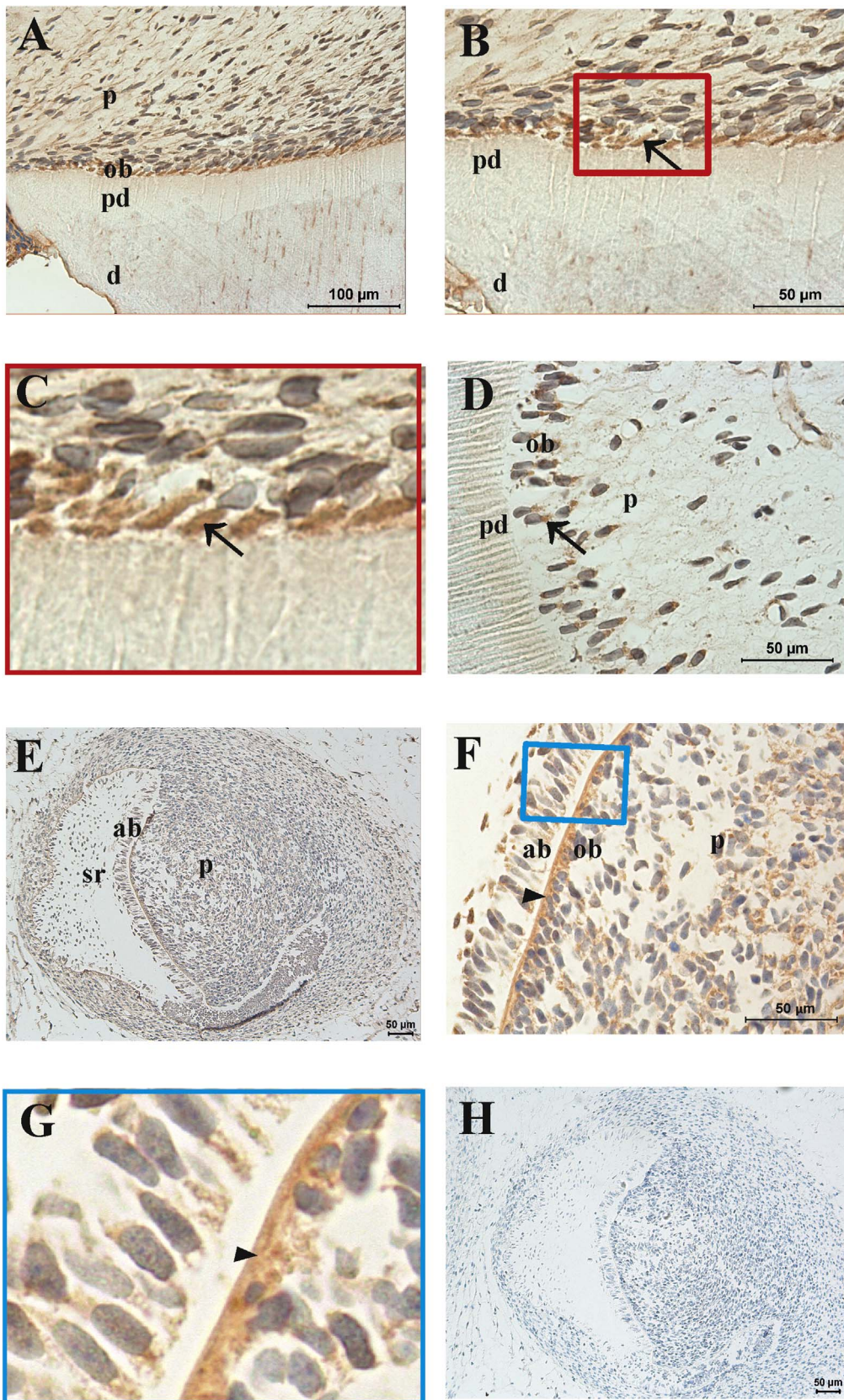


Fig. 3. EXT-1 staining in developing human tooth. In tooth with incomplete root development, EXT-1 staining was seen in the odontoblast layer (ob) but not in the predentin (pd) (A–C). The staining at the root area was localized on different side of the odontoblast nuclei (A–C, arrows) than in the crown area (D). In bell-stage developing tooth (E–G), EXT-1 was observed in ameloblasts (ab) and mantle dentin (arrowhead) as well as in the pulp. No staining was observed in the negative controls (H). C is magnification of B (red square) and G is magnification of F (blue square).

sion was detected in odontoblasts than in the pulp tissue (Fig. 1).

3.3. Immunohistochemical analysis of EXT-1 in mature and developing

Intense EXT-1 staining was observed in the odontoblast layer and the predentin of the mature third molars both at the crown and root areas, but not in the dentin (Fig. 2A, C). In the pulp tissue staining was detected only in vascular endothelium (Fig. 2A). Each odontoblast had an area of intense staining, likely corresponding to the Golgi apparatus, on the pulpal side to the nucleus (Fig. 2D, E). No staining was observed in the control sections (Fig. 2B, F).

In third molars with incomplete root development, clear EXT-1 staining was observed in the odontoblast layer localized on dentinal side of nuclei at the root area (Fig. 3A–C). At the crown area of the same teeth, the staining pattern was similar to the crown area of fully developed teeth (Fig. 3D). In bell-stage developing tooth, EXT-1 was observed in ameloblasts and mantle dentin (Fig. 3E–G) as well as in the developing dental pulp (3F). No staining was visible in the negative controls (Fig. 3H).

4. Discussion

In this study, we analysed the expression of EXT-1, a putative tumour suppressor, observed earlier in odontoblasts but not in dental pulp (Pääkkönen et al., 2008). Odontoblasts are, by definition, terminally differentiated non-dividing cells. Thus, the expression of the genes controlling cell proliferation is of interest, and might reveal factors regulating the odontoblast post-mitotic status. The information gained might, in turn, help to find treatment methods enabling the induction of the tertiary dentin formation under carious lesion.

mRNA analyses revealed markedly higher EXT-1 expression in mature odontoblasts than in pulp. EXT-1 has been shown to enhance heparan sulphate, a glycosaminoglycan, biosynthesis (McCormick et al., 1998). Heparan sulphate chains are associated with many pericellular and extracellular proteins and are involved in several biological processes, including inflammation and tumour metastasis (Bernfield et al., 1999; Lortat-Jacob, 2009). Pericellular heparan sulphate molecules are essential for the activation of FGF receptors and ligands in addition to chemokines (Rapraeger, Krufka, & Olwin, 1991). Heparan sulphate has been observed in predentin and may be important to dentin mineralization (Embery et al., 2001). EXT-1 is related to endochondral ossification of bones through heparan sulphate biosynthesis (Kozziel, Kunath, Kelly, & Vortkamp, 2004), and therefore it is probable that it also plays a role in the odontoblast function.

Immunohistochemical analysis of mature teeth revealed positive EXT-1 labelling in the predentin and in the odontoblasts on the pulpal side of the nucleus, probably in the Golgi apparatus, where the protein has been localized earlier (McCormick et al., 1999). In the developing root, the staining in odontoblasts was seen on the dentinal side of the nuclei. This difference in the localization of EXT-1 staining likely reflects the different localization of the Golgi apparatus in the secretory and mature odontoblasts (Couve, 1986). The staining in the predentin but not in the mineralized dentin indicates that EXT-1 plays a role in the dentin formation. In bell-stage developing tooth, EXT-1 staining was observed in ameloblasts and in the mantle dentin between odontoblasts and ameloblasts, indicating that EXT-1 also plays a role in the enamel formation. At the bell-stage the staining was also observed in the developing pulp tissue indicating formation of heparan sulfates in the developing pulp tissue. EXT-1 mRNA has been previously found in mouse tooth primordia (Stickens, Brown, & Evans, 2000), and our results confirm the expression also in developing human teeth.

The expression of a putative tumour suppressor gene in mature and in newly differentiated odontoblasts, both in the odontoblasts of developing root and cultured odontoblast-like cells, also indicates a

possible function in maintaining the odontoblast differentiation stage. EXT-1 may prove to be a potential target gene for treatment, e.g. the ability to regulate the EXT-1 production to optimize the quality of the tertiary dentin would lead to better treatment outcomes.

Conflict of interest

The authors declare no conflicts of interest.

Ethical approval

The study was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District.

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