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Differential expression between "DSP-only" and DSP-PP$_{523}$ transcripts in rat molar teeth

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Abstract

Objective—To compare the expression patterns of two multiple transcripts derived from DSP-PP gene during tooth development. One is *DSP-only* transcript (i.e. does not encode PP) and the other is *DSP-PP$_{523}$* transcript, a main *DSP-PP transcript*.

Design—Unique antisense and sense riboprobes were generated from DSP-only and DSPPP$_{523}$ cDNAs for *in situ* studies to examine *DSP-only* and *DSP-PP$_{523}$* transcript expression in developing molars. Paraffin-embedded sections (5–7 μ m) from embryonic 20 day, postnatal 2, 3 and 6 days were deparaffined and hydrated. Tissues were prehybridized, then hybridized with DSP-only and DSP-PP$_{523}$ anti-sense (AS) or sense (S) Digoxigenin labeled-riboprobes overnight, and washed. Anti-Digoxigenin antibodies conjugated to alkaline phosphatase were used to detect the presence of bound riboprobes by color reaction with NBT/BCIP. Stro-1 antibody was used for immunohistochemical analysis of Stro-1 protein expression in rat molars.

Results—We found that unlike the *DSP-PP$_{523}$* transcript, the *DSP-only* transcript does not express in the entire polarized mature odontoblasts but is expressed in the areas subjacent to the mature odontoblast layer. In addition, DSP-only transcript is expressed in the dental pulp. Interestingly, Stro-1 protein, a stem cell marker, was also identified in the areas subjacentto odontoblasts and in dental pulp.

Conclusion—Differential expression of *DSP-only* and *DSP-PP$_{523}$* transcripts suggest that these two kinds of transcripts may play different roles during dentinogenesis. *DSP-PP$_{523}$* transcript is expressed in mature odontoblasts, which actively participates in dentin formation. *DSP-only* transcript might have a different function.

Keywords

DSP-only transcript; DSP-PP$_{523}$ transcript; Differential expression; a cell layer subjacent to odontoblast layer

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1. Introduction

Odontoblast cells are responsible for dentin formation. As they mature, odontoblasts actively synthesize extracellular matrix (e.g., collagen type I and noncollagenous proteins such as dentin sialoprotein (DSP) and phosphophoryn (PP)), which participates in dentin mineralization. DSP and PP proteins are derived from a dentin sialoprotein-phosphophoryn gene (DSP-PP) (also termed dentin sialophosphoprotein, DSPP) (MacDougall et al., 1997; Ritchie & Wang, 1997). Multiple *DSPP* transcripts (i.e., *DSP-PP*$_{523}$, *DSP-PP*$_{240}$, *DSP-PP*$_{171}$ and *DSP-only*) were identified in rat dental pulp (Ritchie, Hou, Veis, & Butler, 1994; Ritchie & Wang, 1996, 2000; Ritchie et al., 2001). Likely different transcripts might have different roles during tooth development and dentin formation.

The major *DSP-PP* transcript, *DSP-PP*$_{523}$, is first expressed in preodontoblasts, and then extensively expressed in mature columnar odontoblasts (D'Souza et al., 1997; Ritchie et al., 1997; Ritchie, Shigeyama, Somerman, & Butler, 1996). *DSP-PP*$_{523}$transcript generates a DSP-PP$_{523}$ precursor protein, which undergoes cleavage to produce DSP$_{430}$ and PP$_{523}$ proteins (Godovikova & Ritchie, 2007; Ritchie, Yee, Tang, Dong, & Fuller, 2012). PP proteins have long been known to be responsible for dentin mineralization (Linde & Lussi, 1989; Linde, Lussi, & Crenshaw, 1989). More recent studies have shown that DSPP knockout mice developed defective dentin and enlarged chambers similar to the phenotype of dentinogenesis imperfecta III patients (Sreenath et al., 2003).

The cDNA corresponding to the "*DSP-only*" transcript codes for 324 amino acids (i.e., the 17 amino acid leader sequence along with the 307 amino acid sequence of the secreted protein). No PP sequence is present in the transcript (Ritchie & Li, 2001). 286 of the amino acids encoded by this cDNA are identical to the published rat DSP$_{430}$ sequence. However, the subsequent 21 amino acids are unique to this DSP-only cDNA. The presence of *DSP-only* transcript was validated by RNA protection assay (Ritchie & Li, 2001). *DSP-only* transcripts were also reported in a porcine tooth model (Yamakoshi et al., 2003; Yamamoto, Oida, & Yamakoshi, 2015), To date, there is no information about rat *DSP-only* transcript *in situ* expression during tooth development. In this paper we sought to compare the expression of this *DSP-only* transcript with that of the main *DSP-PP*$_{523}$ transcript during tooth development in mice in order to examine whether these two transcripts share the same expression patterns.

2. Materials and methods

2.1. Generation of unique riboprobes from DSP-only and DSP-PP$_{523}$ cDNAs for in situ studies

Rat DRP-PP$_{523}$ cDNA was inserted into PGEM7Z(+). The DSP-PP$_{523}$ cDNA is comprised of 17 amino acid Leader sequence (designated as L;), DSP$_{430}$ (), PP$_{523}$ () coding sequences and 3′ noncoding sequence (Ritchie et al., 2001). ().DSP-only cDNA (nucleotide positions 1–909) was also inserted into pGEM7Z(+) which is comprised of Leader sequence (17 amino acids; L), DSP$_{307}$ and a short 3′noncoding sequence (see Fig. 1A) (Ritchie & Li, 2001). The relative sequence homology of DSP-only and DSP-PP$_{523}$ cDNAs is illustrated in Fig. 1B.

Using restriction enzyme XbaI and SP6 RNA polymerase, the DSP-PP_{523} anti-sense (AS) digoxingenin riboprobe (Roche Diagnostics GmbH, Mannheim Germany) was generated. Using restriction enzyme BamHI and T7 RNA polymerase, the DSP-PP_{523} sense (S) digoxingenin riboprobe was generated (see Fig. 1A). With *BglI* restriction enzyme (cut at nucleotide position 825) and SP6 RNA polymerase, DSP-only AS riboprobe (i.e., 322 nucleotides; specific for the *DSP-only* transcript) was generated and with XbaI and T7 RNA polymerase, DSP-only S digoxigenin riboprobe was generated (Fig. 1A and B).

2.2. Tissue fixation and paraffin embedding for in situ studies

All rats were of the Sprague-Dawley strain. All animal experiments were carried out in accordance with NIH Publications No. 8023, revised 1978. The jaws of embryonic 20d, postnatal 2, 3 and 6 day-old rats were excised and fixed in 4% paraformaldehyde in PBS. The tissues were extensively washed with PBS, dehydrated, cleared with xylene and embedded in paraffin.

2.3. In situ hybridization

Following the manufacturer's *in situ* hybridization procedures, paraffin-embedded sections (5–7 μ m) were hydrated, and then fixed with 4% buffered paraformaldehyde. Slides were washed in PBS, then treated with proteinase K (Ambion, Austin, Tex., USA; 2.5 μ g/ml in 10 mM Tris.HCl, pH 8.0, 50 mM EDTA) for 24 min at 37 °C, after which they were immersed in 0.2% glycine in PBS. Tissues were fixed again, then acetylated and washed in PBS. Tissues were prehybridized, hybridized with DSP-only and DSP-PP_{523} AS or S Digoxigenin labeled-riboprobes (100× dilution) overnight, and washed. Anti-Digoxigenin antibodies conjugated to alkaline phosphatase (Sigma, USA) were diluted 2000 fold to detect the presence of bound riboprobes. Samples were washed and treated with 2 mM levimisole in TBS to inhibit endogenous alkaline phosphatase activity.NBT/BCIP in 2 mM levimisole and 1× NTM were used for color development. The samples were then countered stained with Eosin and dehydrated in ethanol and xylene, followed by mounting with permount (Fisher scientific, USA) for photographing.

2.4. H & E staining

Paraffin-embedded sections (5–7 μ m) were heated at 60 °C for 30 min, and treated 2× (10 min each) with xylene. Slides were then hydrated by rinsing 2× with 100% ethanol (2 min each), followed by 95% ethanol, 80% ethanol, and finally with distilled water. Slides were then stained with hematoxylin first for 10 min, and followed by a 20 min deionized water rinse. The samples were then stained with eosin for 4 min, and subsequently rinsed in 80% ethanol, 95% ethanol twice and 100% ethanol twice, and finally xylene treatment twice (5 min each). The tissue sections were then preserved with permount (Fisher Scientific, USA).

2.5. IHC staining with stro-1 antibody

Tissue sections were deparaffinized and rehydrated through xylene and graded ethanols. Slides were rinsed in water and then in phosphate buffered saline (PBS). The Vectastain Universal Kit (Vector Laboratories, Burlingame, Calif., USA) was used following the instructions supplied by the manufacturer. Briefly, sections from postnatal 6 day rat molars

were incubated for 30 min in diluted blocking serum, and then incubated overnight at 4 °C with the mouse primary IgM antibody to human Stro-1 (Hybridoma Bank, Iowa City, Iowa). After rinsing in PBS for 5 min, sections were treated with prediluted biotinylated pan-specific universal secondary antibody (Goat anti-mouse IgM) for 30 min and then incubated with the streptavidin/peroxidase complex for 30 min. The immunostaining reaction was next developed with a diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Laboratories, Burlingame, Calif., USA) for 5 min.

3. Results

3.1. DSP-PP$_{523}$ and DSP-only transcripts were not expressed in molar teeth of embryonic 20 day rat

Using unique DSP-PP$_{523}$ and DSP-only riboprobes, neither transcript was detected in the rat molars (Fig. 2).

3.2. DSP-PP$_{523}$ transcripts were expressed in the odontoblast layer in postnatal 2 day and 3 day molars

Using a unique DSP-PP$_{523}$ riboprobe, a strong *DSP-PP$_{523}$* mRNA signal was detected by *in situ* hybridization in the odontoblast layer of postnatal 2 day molars (data not shown). *DSP-PP$_{523}$* mRNA was also detected in the odontoblast layers of post-natal 3 day molars which covered the entire odontoblast cells (Fig. 3A). Enlarged white left box (from Fig. 3A) shows that *DSP-PP$_{523}$* expression in the polarized/columnar/mature odontoblasts (Fig. 3B). Higher magnification of white right box (from Fig. 3A) shows that *DSP-PP$_{523}$* transcript is expressed in the entire mature odontoblasts and also expressed in the preameloblasts (Fig. 3C). DSP-PP$_{523}$ mRNA was also detected in the preodontoblasts (as indicated by a big arrow in Fig. 3C). No signal was detected using sense DSP-PP$_{523}$ riboprobe (Fig. 3G).

3.3. DSP-only transcripts were not expressed in the polarized and mature odontoblasts, but were expressed in the areas subjacent to the base of the odontoblasts in postnatal 2 day and 3 day molar

In situ hybridization with a unique DSP-only AS riboprobe shows that the *DSP-only* transcript is expressed at the base of the odontoblasts layer in 3 day molar. However, the *DSP-only* transcript was not detected in the entire columnar mature odontoblasts (Fig. 3D). Higher magnification of left box (from Fig. 3D) shows clearly that the *DSP-only* transcript is expressed in the areas subjacent to the base of odontoblast layer (Fig. 3E). Higher magnification of right box (from Fig. 3D) shows that *DSP-only* transcripts are also expressed at the base of odontoblast layer. The *DSP-only* positive signal was detected in the unpolarized odontoblasts (as indicat4ed by a big arrow in Fig. 3F) and at the base of polarized odontoblasts (Fig. 3F). No signal was detected using sense DSP-only riboprobe (Fig. 3H). Similar findings were observed for *DSP-only* expression for postnatal 2 day molars. *DSP-only* transcripts were expressed in cells subjacent to the base of odontoblast layer (data not shown). Thus, DSP-only positive areas appear to be localized beneath odontoblasts adjacent the predentin layer in developing teeth.

3.4. DSP-only transcripts were expressed in the dental pulp cells in 3 day molars

Using a unique anti-sense *DSP-only* transcript, *DSP-only* mRNA was also detected in the dental pulp (Fig. 3D–F). These DSP-only positive cells are clustered in the dental pulp (see small arrows in Fig. 3E and F).

3.5. Stro-1 protein expression near the base of odontoblast layer and in the dental pulp in postnatal 6 day molars

Stro-1 has been used as a stem cell marker (Lin, Lin, Tsai, Lin, & Chen, 2011; Miura et al., 2003). Isolated Stro-1 positive dental pulp cells were able to differentiate into odontoblasts. We used Stro-1 antibody to examine the Stro-1 expression in rat molars. Fig. 4A showed H & E staining of postnatal 6 day rat molars. Fig. 4B showed *DSP-PP*$_{523}$ transcript expression in odontoblasts and preameloblasts. Fig. 4C showed Stro-1 expression in the dental pulp of postnatal 6 day rat molar and near the base of odontoblast layer. As indicated by arrow in Fig. 4C, Stro-1 positive cells are located near blood vessels. Hallow arrows in Fig. 4C represent the Stro-1 positive signals near the base of odontoblasts. Fig. 5A shows H & E staining of postnatal 6 day rat molar. Stro-1 positive staining was also located at the base of the odontoblasts and not in the entire odontoblasts (Fig. 5B). Fig. 5C showed the Stro-1 staining sample was further counterstained with Hematoxylin staining. It is clear that Stro-1 positive staining is identified at the base of the odontoblasts (see arrows in Fig. 5C) and in the dental pulp (see arrow heads in Fig. 5C). When the secondary antibody (mouse primary IgM antibody to human Stro-1) was applied to the similar tissues, no signal was detected (see Figs. 4 D and 5 D).

4. Discussion

To better understand the role of the DSP-PP gene in tooth development and mineralization, researchers have followed *DSP-PP* mRNA expression at various developmental time periods. For example, using a DSP riboprobe (i.e., coding for the N-terminal portion of DSPP protein), Ritchie et al. reported *DSPP* transcript expression in polarized, secretary and mature odontoblasts of rat and mouse (Ritchie et al., 1997). D'souza et al. reported *DSPP* transcript expression in mouse odontoblasts in E18.5 d and new born mouse molars (D'Souza et al., 1997). Bleicher, Couble, Farges, Couble, and Maglorie (1999) reported the sequential expression of matrix protein genes in developing rat teeth. They subdivided the odontoblast layer into preodontoblasts, polarized odontoblasts, secretory odontoblasts and mature odontoblasts. And matrix proteins such as Col type I, osteocalcin and DSPP were expressed in the entire polarized odontoblasts in a sequential manner: first Col type I, second osteocalcin and third DSPP in rat incisor at E18.5d. Furthermore, as mineralization proceeds, Col type I and DSPP expression increase and osteocalcin expression decreases. All of these studies revealed that *DSPP* mRNA expression and other major matrix (Col type I and osteocalcin) expression occurs in the entire odontoblasts.

In this paper, we have compared *DSP-only* transcript expression with *DSP-PP*$_{523}$ transcripts. *DSP-PP*$_{523}$ transcripts (a major *DSP-PP* transcript in rat tooth) are expressed in the entire polarized/secretory/mature odontoblasts (Fig. 3A–C). *DSP-PP*$_{523}$ mRNA generates DSP-PP$_{523}$ precursor protein, which undergoes cleavage to produce DSP$_{430}$ and PP$_{523}$ proteins

(Godovikova & Ritchie, 2007). It is well accepted that PP functions as a nucleator to initiate mineral deposition (Linde & Lussi, 1989; Linde et al., 1989). Actually the DSPP KO mice exhibited thin dentin, hypomineralization, increased predentin width and enlarged pulp chamber (Guo et al., 2014; Sreenath et al., 2003), with a high frequency of pulp exposure (Guo et al., 2014). These characteristics are consistent with those of patients with type III dentinogenesis imperfecta (Sreenath et al., 2003).

In contrast to *DSP-PP*$_{523}$ expression, our present studies demonstrated that *DSP-only* transcripts are not expressed in the entire odontoblasts (Fig. 3D–F). The major extracellular matrix secreted by odontoblasts were reported to express in the entire odontoblasts. The fully synthesizing odontoblasts contain many active endoplasmin reticulums and golgi apparati. The absence of *DSP-only* transcripts in entire body of odontoblasts suggests that DSP-only proteins are not likely involved in active dentin synthesis and dentin mineralization. Thus differential expression of *DSP-PP*$_{523}$ and *DSP-only* transcripts suggest these two kinds of transcripts may play different roles during dentinogenesis.

DSP-only transcripts were also reported in a porcine tooth model (Yamakoshi et al., 2003; Yamamoto et al., 2015). Porcine *DSP-only* transcript were expressed in both odontoblasts and dental pulp but at a much lower amount compared to those of DSP-PP expression. Our *DSP-only* transcript expression was reported to occupy 40% of the total 5 day molar dental pulp *DSP-related RNA* (Ritchie & Li, 2001). Our data agree with those of Yamamoto's data (Yamamoto et al., 2015) that *DSP-only* transcripts are expressed in dental pulp.

It is worth noting that *DSP-only* transcripts are expressed at the base of both the unpolarized and polarized odontoblasts (Fig. 3E–F). This observation post a question whether both *DSP-only* and *DSP-PP*$_{523}$ transcripts are expressed in the polarized mature odontoblasts. Alternatively, *DSP-only transcripts* likely could be present in another group of cells, which are different from polarized/mature odontoblast cells.

Because *DSP only* transcripts were expressed in the areas near the base of the odontoblast layer and in the dental pulp, we asked whether these *DSP-only* transcript positive cells represented a group of cells that are less differentiated than odontoblasts. Stro-1 is considered a marker for dental pulp mesenchymal stem cells, and Stro-1 positive cells isolated by cell sorter were able to differentiate into odontoblasts (Lin et al.,2011; Miura et al., 2003). Thus we tested for the presence of the Stro-1 marker.

Previously Yoshiba et al. reported that Stro-1 protein expression was in cells around vascular and neural structures in the dental pulp (Yoshiba et al., 2012). Stro-1 protein expression was found in the human dental pulp tissues (Bottcher, Scarparo, Batista Jr, Fossati, & Grecca, 2013; Martens et al., 2012). We found Stro-1 expression in dental pulp cells and in the cells near the vascular structures (Fig. 4C). In addition, we found the Stro-1 expression in the area near the base of odontoblast layer (Figs. 4 C, 5 B and C). Stro-1 is present in mesenchymal stem cells. It is unlikely that Stro-1 expressed in the part of columnar/mature odontoblasts.

Stro-1 positive staining cells that were also located subjacent to odontoblasts similar to those of *DSP-only* transcript positive cells. We speculate that the Stro-1 positive staining at the base of the entire odontoblast layer (including both preodontoblasts and columnar/

mature odontoblasts) represents another group of less differentiated cells. Likely the newly identified DSP-only positive cells in the dental pulp are less differentiated cells, which might migrate to the odontoblast layer located subjacent to the predentin. These DSP-only positive cells might potentially differentiate into mature odontoblast later. Future work is needed to pursue the role of these *DSP-only* expressing cells during tooth development and tooth regeneration.

5. Conclusions

This is the first report to address the differential expression pattern during dentinogenesis of one (i.e., *DSP-only* transcript) of the multiple transcripts (i.e., $DSP\text{-}PP_{523}$, $DSP\text{-}PP_{240}$ and $DSP\text{-}PP_{170}$ and *DSP-only* transcripts) derived from the rat DSPP gene. $DSP\text{-}PP_{523}$ is the major DSPP derived transcript, which generates DSP and PP proteins. $DSP\text{-}PP_{523}$ mRNA was mainly expressed in the entire odontoblasts (i.e., polarized/secretory/mature odontoblasts). *DSP-only* transcripts were not expressed in entire polarized/secretory/mature odontoblasts but were present at the base of the odontoblasts and expressed in the dental pulp. Stro-1 positive cells were also located at the base of the odontoblasts further support this interpretation. These *DSP-only* transcript positive cells likely might represent a less differentiated odontoblast lineage cells.

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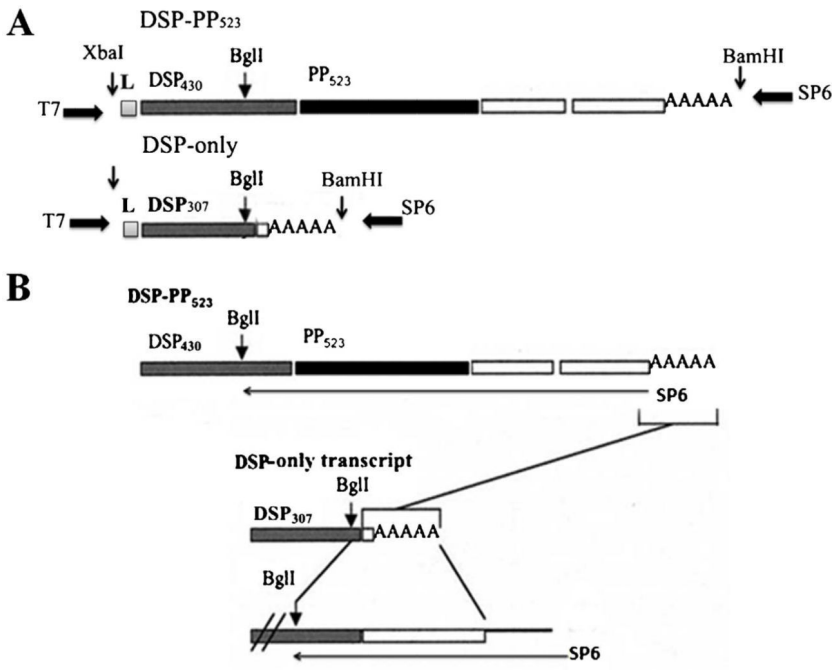

Fig. 1.

Diagram of rat *DSP-PP*$_{523}$ and *DSP-only* transcripts and riboprobe generation. A. The DSP-PP$_{523}$ cDNA is comprised of a 17 amino acid Leader sequence (designated as L), DSP$_{430}$, PP$_{523}$ coding sequences and 3′ noncoding sequence (□). DSP-only cDNA is comprised of a Leader sequence (17 amino acids; L), DSP$_{307}$ and a short 3′noncoding sequence (□). B. Sequence homology comparison between DSP-PP$_{523}$ and DSP-only cDNAs. 286 amino acids are identical to the published rat DSP$_{430}$ sequence from DSP-PP$_{523}$ cDNA. The BglI site (nucleotide position 825) is indicated by an arrow (↓) in both DSP-PP523 and DSP-only cDNA. The DSPonly sequence shares sequence homology (□) with the 3′ noncoding sequence of DSP-PP$_{523}$ (as shown by connected brackets).

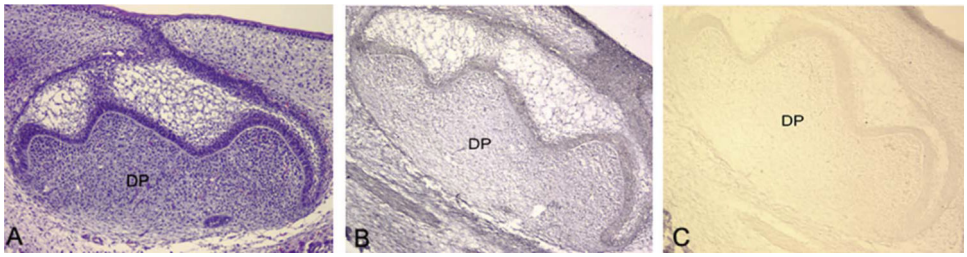

Fig. 2.

No *DSP-PP523* and *DSP-only* transcript expression in molar teeth of 20-day-old rat embryo. A: Hematoxylin & Eosin staining of embryonic 20 day molar. B: DSPPP523-AS riboprobe detected no DSP-PP523 transcript expression in 20 day rat embryonic molar. C:DSP-only unique AS riboprobe detected no DSP-only transcript expression in rat embryonic 20 day molar.

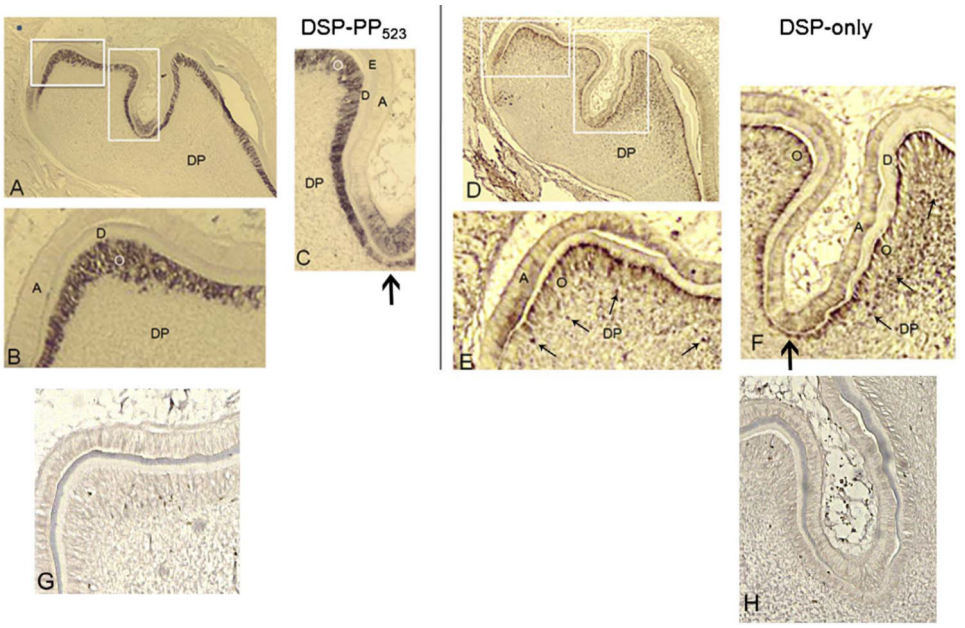

Fig. 3.

DSP-PP$_{523}$ and *DSP-only* transcript expression pattern in molar teeth of postnatal 3 day molars. Panels A-C represent *DSP-PP*$_{523}$ transcript expression in molar tooth. A. *In situ* hybridization with DSP-PP$_{523}$ AS riboprobe. 100× magnification. B. Enlarged white left box (from Fig. 3A). C. Higher magnification of white right box (from Fig. 3A). A big arrow represents the area of preodontoblasts. Panels D-F represent *DSP-only* transcript expression in molar tooth. D. *In situ* hybridization with DSPonly AS riboprobe shows that the *DSP-only* transcript is expressed at the base of the odontoblasts and in the dental pulp. E. Higher magnification of left box (from Fig. 3D). F. Higher magnification of right box (from Fig. 3D). Small arrows represent DSP-only mRNA expression in dental pulp in Fig. 3E and F. A big arrow represents the area of preodontoblasts (Fig. 3F). G. *In situ* hybridization with DSP-PP$_{523}$ S riboprobe: no signal. H. *In situ* hybridization with DSP-only S riboprobe: no signal.

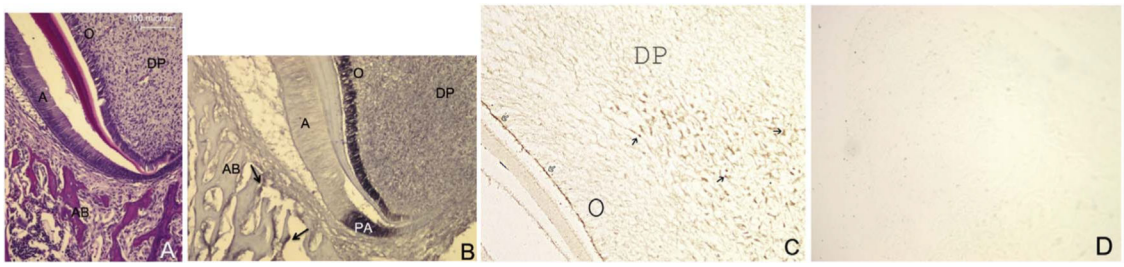

Fig. 4.

DSP-PP$_{523}$ transcript and Stro-1 protein expression in postnatal 6 day molar tooth in rat. A. H & E staining of 6 day molar tooth. B. *DSP-PP*$_{523}$ mRNA expression in preameloblasts and odontoblasts as well as in osteoblasts (indicated by arrows). C. Strol-1 protein expression in day 6 molar. Arrows (→) represent Stro-1 positive cells in dental pulp. Harrow arrows (⇨) represent Stro-1 positive staining at the base areas of odontoblasts. AB: alveolar bones. A: ameloblasts. DP: dental pulp. O: odontoblasts. PA: preameloblasts. D. Only the secondary antibody (mouse primary IgM antibodies to human Stro-1) was applied to the tissues as a negative control.

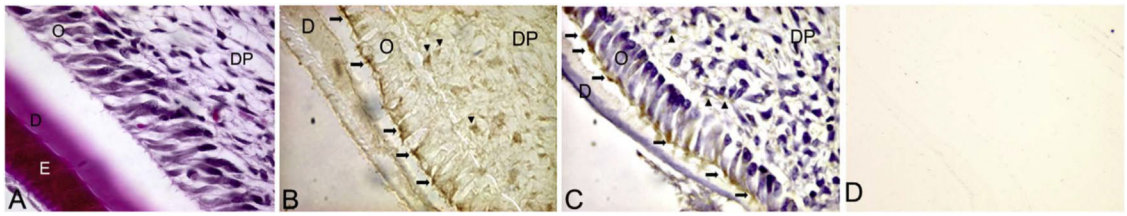

Fig. 5.

Stro-1 staining of postnatal 6 day rat molar. A. H & E staining of 6 day first molar. B. Stro-1 antibodies were used for immunohistochemical analysis. Stro-1 was detected in the base of the odontoblasts (as indicated by arrows) and in the dental pulp (represented by arrow heads). White color dash line represents the border between odontoblast layer and dental pulp. C. Stro-1 and Hematoxylin staining of 6 day first molar. Stro-1 and Hematoxylin stained sections clearly show the odontoblasts and the Stro-1 positive staining is located at the base of the odontoblasts. 1000× magnification. DP: dental pulp. O: odontoblasts. D: dentin. E: enamel. D. Only the secondary antibody (mouse primary IgM antibodies to human Stro-1) was applied to the tissues as a negative control.