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Complexity of Odontoblast and Subodontoblast Cell Layers in Rat Incisor

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Research Article

ABSTRACT

Structural and functional interrelationships between odontoblast (OCL) and sub-odontoblast cell layers (SOCL) are relevant to tissue formation and regulation in health and disease, but are incompletelyunderstood. This study employed contemporary immunohistochemical techniques to gain important new insights on tissue complexity within the apical half of the rodent mandibular incisor.

Whole incisors were removed from freshly killedWistar rats, fixed in 4% paraformaldehyde,demineralized, and prepared for standard immunohistochemistry. Tissues were labelled with primary antibodies to vimentin, α actin, NaKATPase, alpha tubulin and COX 1, and secondary antibodies alexafluor 488 and 594, before examination by fluorescence microscopy.

Odontoblasts presented in stratified or pseudo-stratified arrangements, rather than simple columnar form. Additional stellate cells,α-actin immunoreactive, were observed predominantly in the distal half of the OCL.

Cells in the cell rich zone (crz) also showed complexity, with high cell density, and cells presenting with large nuclei and sparse cytoplasm. These cells showed intense NaKATPase immunoreactivity.

Some of these cells sent processes towards and into the OCL, whilst other processes remained within the crz. No processes directed downwards.

The OCL of the rat mandibular incisor presents a hitherto undescribed complexity, in terms of cellular arrangement and composition, the functional significance of which is not immediately apparent.

The observation that cellular processes from crz tothe OCL and lateral processes within crz suggest functional connectivityand cell to cell communication within the layer. The intense immunoreactivity to NaKATPase suggests functional specification and a high degree of tissue activity. Structural observations will require further investigations.

INTRODUCTION

The odontoblasts represent a specialized cell population with primary roles in the secretion and mineralization of dentine matrix. Further roles include regulation dentinal of dentine permeability Bishop [1], involvement of immuneresponses Horst et al.

[2], enzyme production Karim et al. [3], signalling and mechano-transduction Magloire et al. [4], through the generation of action potentials Allard et al. [5]. thermosensitivity Son et al. [6], and additional sensory roles Byers and Westenbroek [7], Okumura et al. [8]. Odontoblasts are usually described in columnar or pseudo stratified formation with one cytoplasmic process extending from each cell into the tubular structure of dentine Luukko et al. [9]. Their location facilitates their roles in regulating the pulp dentine barrier Bishop and preserving tissue integrity in response to noxious stimuli Arana-Chavez and Massa [10]. Interactions between the odontoblast cell layer (OCL) and sub-odontoblast cell layer (SOCL) are likely to be important in these roles.

The continuously growing rat incisor is a well-established research module, facilitating study of cellular life cycles Ohshima and Yoshida [11], from tissue formation through maturation to senescence and response to injury Harada and Ohshima [12], Harada et al. [13]. Tummers and Thesleff [14], Investigations involving the effects of therapeutic agents Murray et al. [15], bioactive molecules Sloan and Smith [16], responses to injury D'Souza et al. [17]. Surgical procedure and materials Murray et al. [18] have been described.

This study applied contemporary immunohistochemistry to gain new understanding of structural and functional complexity within the rat incisor OCL and SOCL, a deeper understanding of which may underpin therapeutic applications Smith et al. [19].

MATERIALS AND METHODS

Harvest and preparation of pulp tissues

Isolation and fixation of whole rodent pulps

Mandibular incisors (n=20) were extracted from freshly culled Wistar rats (300-600 gm weight) and split longitudinally after grooving with high-speed bur and water cooling. Pulp tissue was transferred with tweezers to fresh 4% paraformaldehyde solution at 4°C for 3-4 hours before 4 washing twice with PBS, and subsequent cryopreservation in graded sucrose solutions (10%, 20%, and 30%, 24 h each at 4°C).

Demineralized teeth: Ten incisors were extracted and sectioned horizontally into apical and incisal regions with adiamond disk under constant water cooling. These were fixed in 4% paraformaldehyde at 4˚C for 24 h Boushell et al. [20], before demineralization in 17% EDTA (pH 7.4 at 4°C for 4-6 weeks Cho et al. [21]. Changing the solution twice every week, washing in PBS for 10 minutes cryopreservation as previously. Samples were then immersed in optimal cutting temperature medium (OCT, Tissue Tek, Netherlands), snap frozen in isopentane and liquid nitrogen, and stored at -80°C. Sections of 8 µm thickness were prepared from frozen blocks with a cryotome (SHANDON Cryotome FSE, Thermo Fisher Scientific, USA) and mounted on slides allowed to bench dry. Slides were stored in a Coplin Jar and washed thrice TBS, TBS-T, and TBS for 5 minutes using a 3D rocking platform (Stewart Scientific, UK).

Staining sections

Primary antibodies **(Table 1)** were then applied, and slides stored in a humidified chamber at 4°C overnight.

Table 1. List of primary antibodies used in this study.

Antibody name	Type	Concentration	CAT No.	LOT No.	Supplier
Vimentin	Anti-mouse monoclonal	1:5000	MU074-UC	MU074-1013	Biogenex
NaKATPase	Anti-rabbit monoclonal	1:500	Ab76020	YH022206	Abcam
α-smooth muscle actin	Anti-rabbit monoclonal	1:100	Ab32575	GR32377-19	Abcam
Alpha tubulin	Anti-rabbit monoclonal	1:1000	GTX-102078	40478	GenTex
COX 1	Anti-goat polyclonal	1:100	Sc-1752	G1210	Santa Cruz

The following day, slides were transferred back to a Coplin Jar and washed thrice in TBS, TBS-T, and TBS for 20 minutes before first secondary antibodies **(Table 2)** were applied for 1 hr in a dark, humid environment. Slides were then washed again before applying second secondary antibodies **(Table 2)** for 1 hr in a dark, humid environment.

Table 2. List of secondary antibodies used in this study.

Antibody name	Type	Concentration	CAT No.	LOT No.	Supplier
Alexa Fluor	Anti-goat 488	1:500	A11055	1081957	Invitrogen
Alexa Fluor	Anti-goat 594	1:500	A11058	1003216	Invitrogen
Alexa Fluor	Anti-goat 488	1:500	A21206	737675	Invitrogen
Alexa Fluor	Anti-goat 594	1:500	A21207	997874	Invitrogen
Alexa Fluor	Anti-goat 488	1:500	A21202	1423052	Life Technologies

Slides then were washed and dried as previously before were covered with Vectashield hard setting mounting medium with DAPI (Vector laboratories Inc., Burlingame USA), one drop of PBS + glycerol and a glass cover slip, sealed at the periphery with nail varnish prior to examination under the fluorescence microscope (Olympus BX61) at 10 X, 20 X and 60 X magnification.

Examination of stained sections: Relevant images were captured with a microscope-mounted Olympus XM 10 monochrome camera and examined using Image J software (Java- based image processing program- National Institute of Health USA).

Calculations: the length and thickness measurements of different components of the dental pulp were made using Olympus microscope Cell F software.

RESULTS

Figure 1 shows low power images from the apical half of a de-mineralized specimen, the labial side presented in **Figure 1A** and the lingual side in **Figure 1B.** The section was stained with antibodies to the structural protein vimentin (vim: green) and NaKATPase (nak: red); with DAPI as a nuclear stain (dapi: blue). Both images provide an overview of the hard and soft-tissues including dentine (de), odontoblast cell layer (od) and odontoblast cellular processes within dentine (odp). Immediately beneath the odontoblast cell layer (OCL) is the cell rich zone (crz), with varying thickness and cell density. In both images A and B, vim-IR was observed in the distal regions of the odontoblasts and in their processes (odp), with the nuclei located in the proximal regions of the cells. These images appear to show the odontoblasts arranged in a stratified or pseudo stratified pattern, the cell bodies being globular rather than tall columnar inshape, and nuclei arranged in two or more lines. The interstitial cells in the crz and the pulpwere immuno-reactive for vimentin (vim-IR). NaKATPase IR was intense in the crzandof lower intensity in the OCL and bulk of the pulp. The sections also6 illustrate differences between labial and lingual sides of the pulp, with apparent differences in average OCL thickness (thicker labially ((72 µm) than lingually (66 µm)). The crz also thicker labially (53 µm) than lingually (37.5 µm).
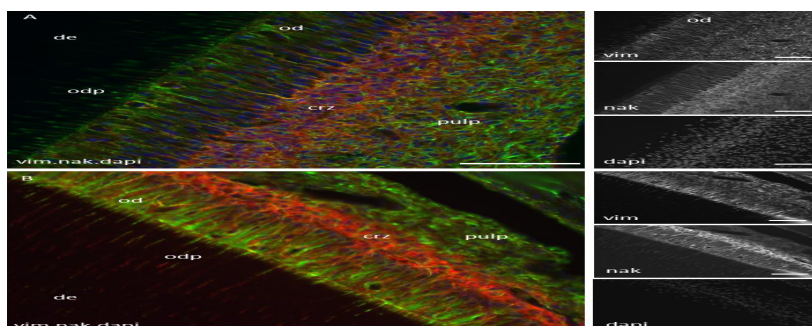

Figure 1. Overview of the cellular components and dentine of the apical half of demineralized rat incisor root: A (labial) and B (lingual) shows sections stained with antibodies to vimentin (vim: green), NaKATPase (nak: red) and nuclear stain (dapi: blue). In both A and B, the dentine (de) is clearly identified, containing several odontoblast processes (odp), and just beneath is the pulp which contains a layer of odontoblast cells (od), a cell rich zone (crz) and the bulk of the pulp (pulp). The right side panels show the individual images of Vim-IR, NaKATPase-IR and dapi. Calibration bars of 100 µm are shown in all images.

Figure 2 shows a relatively high power image from the apical half of a demineralized mandibular rat incisor. The section was stained with antibodies to vimentin (vim: green), α-smooth muscle actin (actin: red) and nuclear stain with dapi (dapi: blue). Both A and B focus on the od with the presence of actin-IR cells scattered within the OCL (arrow). These cells had reduced vim-IR compared with the surrounding od, and appeared to be found only in the distal half (close to dentine) of the OCL, with appearances suggestive of connectivity between the actin-IR cells.
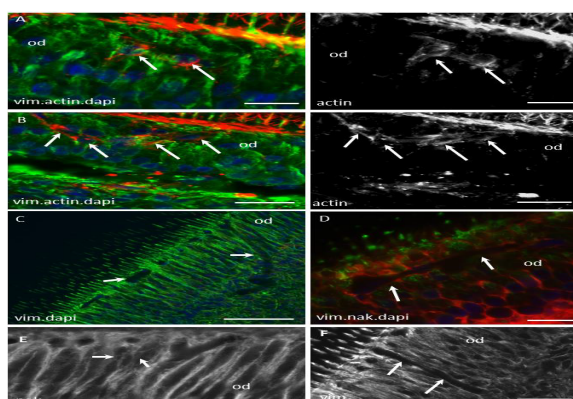

Figure 2. Demineralized sections from the mid part of the mandibular rat incisor showing odontoblast cell layer and related vascularity: Section A and B stained with antibodies to vimentin (vim: green), α-smooth muscle actin (actin: red) and nuclear stain (dapi: blue). Sections are high magnification images showing the odontoblast (od) cell layer, with the presence of other cells (arrowed) within the odontoblast cell layer, at the distal part of odontoblast cell layer close to the pulp/predentine junction. These cells show α-actin-IRbut were negative to vim as shown in the images to the right for individual stains. Section C stained with antibody to vimentin (vim: green) and dapi (dapi: blue) showing many blood vessels, with some entering the odontoblast cell layer from the pulp, others dividing and many near the mineralizing front. D shows a section stained with antibodies to vimentin (vim: green), NaKATPase (nak: red) and dapi in blue, showing a blood vessel that runs parallel to the odontoblast cell layer and another one parallel to dentine. Section E demineralised tissue with NaKATPase-IR showing dividing blood vessel. Section F is a demineralised tissue with vim-IR showing blood vessel crossing the entire odontoblast cell layer. Calibration bars are 50 µm in all images.

C, D, E and F were stained with antibodies to vimentin (vim: green) and NaKATPase (nak: red) and nuclear stain dapi (dapi: blue) and show again the globular shape of the odontoblasts and the wavy lines of nuclei. Vascular complexity was observed within the OCL and SOCL with many small blood vessels in different patterns, including relatively wide blood vessels in the crz narrowing as they enter the OCL and crossing its thickness before bifurcating at the mineralizing front (panel C), tubes close to the mineralizing front (panels C and D), a single blood vessel running parallel to the OCL before bifurcating to form Y shaped vessels (panel E) and a single blood vessel running parallel to the od and crossing the OCL to reach the mineralizing front (panel F).Most blood vessels within the OCL were located close to the mineralizing front.

Figure 3 was stained with antibodies to vimentin (vim: green), NaKATPase (nak: red) and nuclear stain dapi (dapi: blue) and shows at higher power a cell whose cell-body is located in the crz (arrow) with a cellular extension toward the OCL, which runs between the od, extending to about half of the thickness of the OCL. The globular shape of odontoblasts was again noted. B shows a non-demineralized section of pulp tissue after staining with antibodies to vimentin (vim: green) and nuclear stain dapi (dapi: blue) and provide further evidence for the presence of cellular processes extending from cells within the crz toward the OCL, some of which infiltrating the OCL and running between the od (arrows), while others sent short horizontal processes that remained within that zone (arrows with star*). There was no evidence of cellular processes extending from the cells of the crz downwards toward the bulk of the pulp.

Figure 4A shows a low magnification image of a demineralized specimen after staining with antibodies to vimentin (vim: green), α-smooth muscle actin (actin: red) and nuclear stain with dapi in blue. Both labial (top of each image) and lingual (bottom of each image) sides are visible, with higher immunoreactivity in the lingual side.
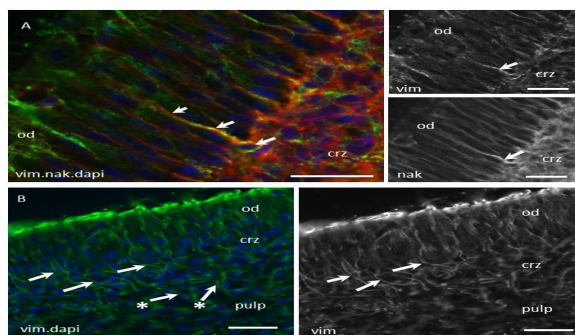

Figure 3. Sections of rat mandibular incisor showing the odontoblast and subodontoblast cell layers: A shows high magnification images from a demineralised section stained with antibodies to vimentin (vim: green), NaKATPase (nak: red) and dapi (dapi: blue). The arrows in the colour image refer to a cell body and its process that appear to pass from the cell rich zone (crz) to the odontoblast cell layer (od). The panels to the right are the individual images for vimentin and NaKATPase. B show section stained with antibodies to vimentin (vim: green) and dapi (dapi: blue). The odontoblast cell layer (od), cell rich zone (crz) and bulk of the pulp (pulp) can be identified. These images show that some subodontoblast cells in the cell rich zone send long cellular processes towards the odontoblast cell layer (arrow), some of which infiltrate the odontoblast cell layer and run between the odontoblasts. Some of the subodontoblast cells also send short horizontal processes that remain in the cell rich zone (arrows with star). No cellular processes were seen to extend from the cell rich zone towards the bulk of the pulp. Calibration bars of 50 μm in A and 100 μm in B.
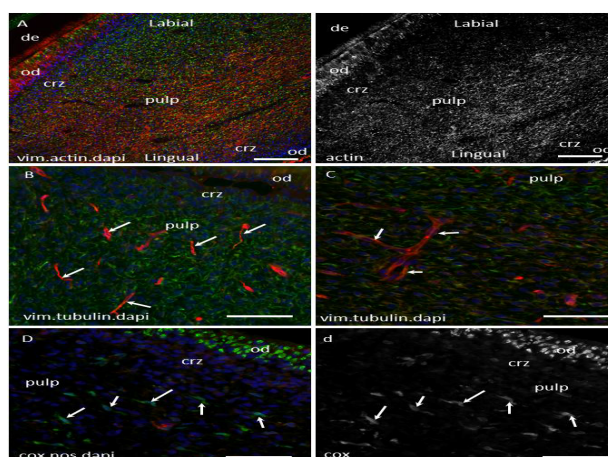

Figure 4. Sections of rat mandibular incisor showing an overview of the whole thickness of the pulp and cellular heterogeneity:A shows section stained with antibodies to vimentin (vim: green), α smooth muscle actin (actin: red) and nuclear stain with dapi (dapi: blue). In this section both labial and lingual compartments of odontoblast (od) and cell rich zone (crz) are clear with the labial side to the top and lingual to the bottom. This section may provide evidence of the distribution of α smooth muscle actin within the bulk of the pulp being more in the lingual side than on the labial side. This can clearly be distinguished with the black and white image to the right which shows α-actin IR. Section B and C stained with antibodies to vimentin (vim: green), tubulin (tub: red), and dapi as nuclear stain. B shows cells with immunoreactivity to tubulin in the pulp area. C shows cells with positive immunoreactivity to tubulin may be arranged in specific styles; sometimes it forms lobes or tubes and sometimes appears as group of cells with lumen (arrows). Section D stained with antibodies to Cyclooxygenase-1 (cox-1: green), NOS (nos: red) with dapi as nuclear stain (dapi: blue) showing cells within the sub-odontoblast area with positive immunoreactivity to cox-1 (arrows) as shown, (d) is the individual black and white image showing COX-1 IR cells. All COX-1 IR and tub-IR cells have very little or no immunoreactivity to vimentin. Calibration bars is 100 μm in A and 50 μm in B, C and D.

B, C and D show non-demineralised sections, stained with antibodies to vimentin (vim: green), tubulin (tub: red) in A, vimentin (vim: green), tubulin (tub: red) in B and C and cyclooxygenase-1 (cox1: green), nitric oxide synthase-1 (nos-1: red) in D with dapi as nuclear stain in blue in all figures. These sections show cellular heterogeneity within the bulk of the pulp. Panel B show cells with tubulin-IR scattered among the vimentin-IR cells appearing as single spindle like cells. Panel C shows tubulin-IR cells or aggregates of cells forming specific shapes and patterns like tubes, lobes or star shapes. Sometimes these aggregations of cells surround a lumen. Panel D shows cells with cyclooxygenase-1 immunoreactivity scattered throughout these cell layers. All cells in panels A, B and C showed less vimentin-IR than the surrounding cells.

Observations are summarized in the cartoon shown in **(Figure 5).**

DISCUSSION

Previous studies have reported that in the rat mandibular incisor and during the phase of active dentine deposition, the odontoblasts are tall and columnar, with nuclei at the proximal end of the cell body, the Golgi apparatus immediately above the nuclei, with the endoplasmic reticulum occupying nearly all of the distal part of the cell body Arana-Chavez and Massa.
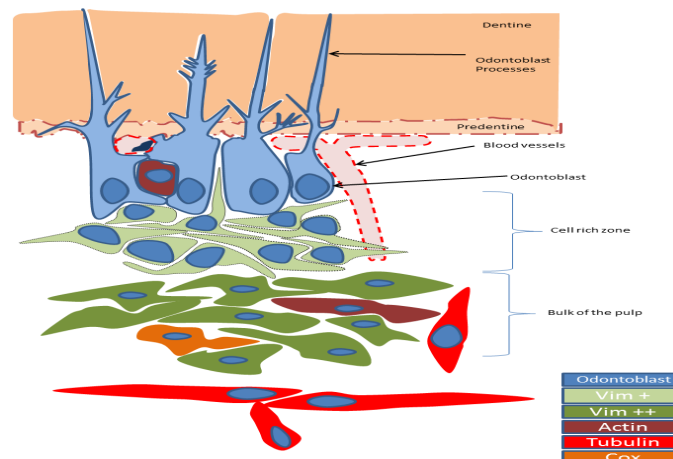

Figure 5. A cartoon illustrating structural complexity and cellular heterogeneity within the odontoblasts and subodontoblast cell layers of the apical half of rat mandibular incisor. The odontoblast cell layer is a complex layer presenting in a stratified or pseudo-stratified arrangement with the odontoblast cells appearing in globular shape. Note the presence of a different cell type with α-actin IR within the odontoblasts layer. There is avascular network of small arterioles located near the mineralizing front. Some cells in the cell rich zone appear to send long processes toward and into the odontoblast cell layer which suggests the possibility of functional cross talk between these two layers. Other cells in the crz send short horizontal processes with no evidence of processes directed towards the bulk of the pulp. The bulk of the pulp shows some degree of cellular heterogeneity.

The current study presents a different picture, with odontoblasts appearing globular in shape, wide proximally and narrower at the distal part of the cell body. In addition, few sections showed the odontoblasts in a single layer; rather, it appeared as a stratified or pseudo stratified arrangement two or more cells thick, with the nuclei appearing as wavy lines. Possible interpretations may be that not all odontoblast cell bodies are in close contact with dentine, raising questions whether some odontoblasts may serve supporting roles alongside active cells, or that there is some sort of succession, with cells taking turns to deposit dentine matrix while other cells are dormant. The OCL is generally regarded as odontoblast exclusive, with occasional antigen-presenting dendritic cell Tsuruga et al. [22], playing a regulatory role in odontoblast function and differentiation Ohshima et al. [23]. The presence of dendritic cells also highlights the potentially important interface that the odontoblasts occupy. Sections observed in the current study appear to confirm the presence of an additional and hitherto un-described cell population within the OCL. These cells were morphologically distinct from odontoblasts and were immunoreactive to α- smooth muscle actin and negative to vimentin (the opposite of odontoblasts). It is conceivable that these cells may be dendritic cells, contributing to a complex immunological defense mechanism with the class I Major Histocompatibility Complex (MHC)-presenting odontoblasts acting as antigen recognizing cells Farges et al. [24], Horst et al. [25], Keller et al. [26] and the class II MHC presenting dendritic cells as antigen presenting cells Yoshiba et al. [27]. and potentially attracted to the area by the odontoblasts Durand et al. [28] Holland and Botero [29].

Immediately below the odontoblasts is the crz, with its densely packed cells, which are believed to be capable of differentiation to other cell types, including odontoblasts when conditions dictate Couble et al. [30], Fitzgerald et al. [31], Luukko et al. [8]. Previous studies have suggested that if odontoblasts are irreversibly damaged or killed, they will be replaced by cells derived from the crz Fitzgerald et al. [31]. after an increase in its mitotic activity Murray et al. [32-34]. Cells in the crz exhibit a range of shapes, from polygonal to stellate with cellular processes, which facilitate cellular interactions and actively contribute to complex signalling pathways in the dental pulp Bongenhielm et al. [35]. Woodnutt et al. [36]. Yamaguchi et al. [37]. Findings from the current study confirm the presence of cellular processes that extend between cells of the crz, with no evidence of any processes directed downwards to the core of the pulp. By contrast, cellular processes were frequently seen to extend from the crz towards the odontoblast layer, extending approximately half the thickness of its pseudo-stratified structure. This finding may indicate previously unrecognized

cellular interactions between the crz and the odontoblast layer, with the possibility of sensory, defensive, mechano-sensitive Magloire et al. [4] or other regulatory functions.

Cells of the rat incisor crz werefound to have poorly developed endoplasmic reticulum and Golgi apparatus, suggesting that they are unlikely to be involved in protein synthesis and secretion. An abundance of well-developed mitochondria Gotjamanos [38], suggest that these cells have high metabolic rates Zhai et al. [39]. Cells of the transporting epithelia Silverthorn et al. [40]. share some histological and morphological features with the crz cells. These include the cellular projections (which increase the surface area) and well-developed numerous mitochondria. These features and the strong IR to NaKATPase suggest that these cells may be involved in active transport and reabsorption of fluids and ions from the pulp to the OCL. Data from the current study confirm previous studies on the differences in thickness of the OCL and crz labially and lingually in the apical half of the rat incisor Ohshima and Yoshida, with the OCL taller labially than lingually (70-80 µm vs 55-63 µm respectively), and the crz more prominent and thicker labially than lingually (50-60 µm vs 33-40 µm respectively). The core of the pulpis known to contain additional cell types, including macrophages, dendritic and immune cells Luukko et al. [8]. The current observation of cells with different immunoreactivity within the core of the pulp may be of considerable importance. Cells that were immunoreactive to cyclooxygenase-1 might suggest functional and signalling activity. Cells that were immunoreactive to tubulin appear in two distinct patterns, either as single cells scattered throughout the pulp, or as a specific pattern of aggregated cells, sometimes associated with a narrow empty lumen. This heterogeneity of cells may be associated with a variety of functional and vital roles within the pulp complex which remain unclear.

One additional finding from the current work is the expression of α-smooth muscle actin in the cells of the core of the pulp. This expression appears to be less on the labial and increasing in intensity

towards the lingual side. In conclusion, the findings of this study indicate that the OCL, crz and the core of the pulp are heterogeneous and more structurally complex than previous reports have suggested. The functional implications of such complexity are currently unclear, but it may highlight important and

complex cellular interactions that may involve odontoblast-odontoblast, odontoblast-odontoblasts processes and/or odontoblast-subodontoblast cell layers. These observations warrant further investigation in both animal and human tissues.

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REFERENCES

1. Bishop M. Extracellular fluid movement in the pulp; the pulp/dentin permeability barrier. Proc Finn Den Soc. Suomen Hammaslaakariseuran toimituksia. 1991;88:331-335.

2. Horst O, et al. TGF-β1 inhibits TLR-mediated odontoblast responses to oral bacteria. J Dent Res. 2009;88:333-338.

3. Karim A, et al. Immunohistochemical localization of procollagens II. Electron microscopic distribution of procollagen I antigenicity in the odontoblasts and predentin of rat incisor teeth by a direct method using peroxidase linked antibodies. J Histochem Cytochem. 1979;27:1070-1083.

4. Magloire H, et al. Odontoblast: a mechanosensory cell. J Exp Zool B Mol Dev Evol. 2009;312: 416-424.

5. Allard B, et al. Voltage-gated sodium channels confer excitability to human odontoblasts: possible role in tooth pain transmission. J Bio Chem. 2006;281:29002-29010.

6. Son AR, et al. Odontoblast TRP channels and thermo/mechanical transmission. J Dent Res. 2009;88:1014-1019.

7. Byers MR, and Westenbroek RE. Odontoblasts in developing, mature and ageing rat teeth have multiple phenotypes that variably express all nine voltage-gated sodium channels. Arch Oral Biol. 2011;56:1199-1220.

8. Okumura R, et al. Odontoblast as a sensory receptor cells? The expression ot TRPV1 (VP1) channels. Arch Hisol Cytol. 2005;68:251-257.

9. Luukko A, et al. Structure and function of the dental pulp in : KM Hargreaves and S. Cohen, Edition, Pathways of the pulp, Mosby Elsevier, St. Louis, Missouri, USA. 2011.

10. Arana-Chavez VE and Massa LF. Odontoblasts: the cells forming and maintaining dentine. Int J Biochem Cell Biol. 2004;36:1367-1373.

11. Ohshima H and Yoshida S. The relationship between odontoblasts and pulp capillaries in the process of enamel- and cementum-related dentin formation in rat incisors. Cell Tissue Res. 1992;268:51-63.

12. Harada H and Ohshima H. New perspectives on tooth development and the dental stem cell niche. Arch Hisol Cytol. 2004;67:1-11.

13. Harada H, et al. FGF10 maintains stem cell compartment in developing mouse incisors. Development 2002;129:1533-1541.

14. Tummers M and Thesleff I. Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species Development. 2003;130:1049-1057.

15. Murray P, et al. Comparison of pulp responses following restoration of exposed and non-exposed cavities. J Dent. 2002;30:213-222.

16. Sloan AJ and Smith AJ. Stimulation of dentine-pulp complex of rat incisor teeth by transforming growth factor-B isoforms 1-3 in vitro. Arch Oral Biol. 1999;44:149-156.

17. D'Souza RN, et al. Characterization of Cellular Responses Involved in Reparative Dentinogenesis in Rat Molars. J Dent Res. 1995;74:702-709.

18. Murray PE, et al. Analysis of incisor pulp cell populations in Wistar rats of different ages. Arch Oral Biol. 2002b;47:709-715.

19. Smith AJ, et al. Dental regeneration and materials: a partnership. Clin Oral Invest. 2008;12:103-108.

20. Boushell LW, et al. Immunohistochemical localization of matrixmetalloproteinase-2 in human coronal dentin. Arch Oral Biol. 2008;53:109-116.

21. Cho A, et al. A method for rapid demineralization of teeth and bones. Open Dent J. 2010;4:223-229.

22. Tsuruga E, et al. Appearance and distribution of dendritic cells and macrophages in dental pulp during early postnatal morphogenesis of mouse mandibular first molars. Histochem Cell Biol. 1999;112:193-204.

23. Ohshima H, et al. The distribution and ultrastructure of class II MHC-positive cells in human dental pulp. Cell Tissue Res. 1999;295:151-158.

24. Farges, et al. Cytokine production by human odontoblast-like cells upon Toll-like receptor-2 engagement. Immunobiology. 2011;216:513-517.

25. Horst OV, et al. Caries induced cytokine network in the odontoblast layer of human teeth. BMC immunol. 2011;12:9.

26. Keller JF, et al. Toll-like receptor 2 activation by lipoteichoic acid induces differential production of pro-inflammatory cytokines in human odontoblasts, dental pulp fibroblasts and immature dendritic cells. Immunobiology. 2010;215:53-59.

27. Yoshiba K, et al. Class II antigen-presenting dendritic cell and nerve fiber responses to cavities, caries, or caries treatment in human teeth. J Dent Res. 2003;82:422-427.

28. Durand SH, et al. Lipoteichoic acid increases TLR and functional chemokine expression while reducing dentin formation in in vitro differentiated human odontoblasts. J Immunol. 2006;176:2880-2887.

29. Holland GR and Botero TM. Pulp biology: 30 years of progress. Endodontic Topics. 2014;31:19-35.

30. Couble ML, et al. Odontoblast differentiation of human dental pulp cells in explant cultures. Calcified Tissue Int. 2000;66:129-138.

31. Fitzgerald M, et al. Autoradiographic analysis of odontoblast replacement following pulp exposure in primate teeth. Arch Oral Biol. 1990;35:707-715.

32. Murray P, et al. Comparison of pulp responses following restoration of exposed and non-exposed cavities. J Dent. 2002a;30:213-222.

33. Murray P, et al. Human odontoblast cell numbers after dental injury. J Dent. 2000a;28:277-285.

34. Murray PE, et al. Tooth slice organ culture for cytotoxicity assessment of dental materials. Biomaterials. 2000b;21:1711-1721.

35. Bongenhielm U, et al. Effects of neuropeptides on growth of cultivated rat molar pulp fibroblasts. Regul peptides. 1995;60:91-98.

36. Woodnutt D, et al. Neurotrophin receptors and nerve growth factor are differentially expressed in adjacent nonneuronal cells of normal and injured tooth pulp. Cell Tissue Res. 2000;299:225-236.

37. Yamaguchi M, et al. Neuropeptides stimulate production of interleukin-1β, interleukin-6, and tumor necrosis factor-α in human dental pulp cells. Inflamm Res. 2004;53:199-204.

38. Gotjamanos T. The odontoblastic and subodontoblastic cell layers of the rat incisor pulp. Aust Dent J. 1969;14:302-311.

39. Zhai XY, et al. Digital three dimensional reconstruction and ultrastructure of the mouse proximal tubule. J Am Soc Nephrol. 2003;14:611-619.

40. Silverthorn DU, et al. Human physiology: An integrated approach. Fourth edition. Pearson/Benjamin Cummings, San Francisco, USA. 2007.