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Activation of α SMA expressing perivascular cells during reactionary dentinogenesis

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Abstract

Aim—To examine the contribution of perivascular cells expressing α SMA to reactionary dentinogenesis.

Methodology—An inducible, Cre-loxP *in vivo* fate-mapping approach was used to examine the contribution of the descendants of cells expressing the α SMA-CreERT2 transgene to reactionary dentinogenesis in mice molars. Reactionary dentinogenesis was induced by experimental mild injury to dentine without pulp exposure. Student *t*-test was used to determine statistical significance at $*p = 0.05$.

Results—The lineage tracing experiments revealed that mild injury to dentine first led to activation of α SMA-tdTomato⁺ cells in the entire pulp chamber. The percentage of areas occupied by α SMA-tdTomato⁺ in injured ($7.5 \pm 0.7\%$) teeth were significantly higher than in teeth without injury ($2 \pm 0.5\%$). After their activation α SMA-tdTomato⁺ cells migrated towards the site of injury, gave rise to pulp cells and a few odontoblasts that became integrated into the existing odontoblast layer expressing Col2.3-GFP and *Dspp*.

Conclusion—Mild insult to dentine activated perivascular α SMA-tdTomato⁺ cells giving rise to pulp cells as well as a few odontoblasts that were integrated into the pre-existing odontoblast layer.

Keywords

Odontoblasts; Perivascular cells; Pulp biology; Reactionary dentine; α SMA

INTRODUCTION

Odontoblasts are exclusively dentine-producing cells. These highly specialized tall columnar cells are located at the periphery of the dental pulp and differentiate from neural crest-derived dental papilla cells at the late bell stage of tooth development (Kawashima *et al.* 2016). The differentiation of the dental papilla into odontoblasts is dependent on signals and growth factors derived from the inner dental epithelium (Balic & Thesleff 2015).

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Conflict of Interest statement

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Available evidence suggests the similar signaling pathways including the local release of signaling molecules from the damaged/demineralized dentine are involved in the initiation of reparative and reactionary dentinogenesis (Smith *et al.* 1995, 2016, Couve *et al.* 2013, Bleicher 2014, Kawashima & Okiji 2016,). It is well established that in reparative dentinogenesis, these signaling molecules regulate the generation of odontoblasts-like cells from residents MSCs. On the other hand, in reactionary dentinogenesis, it is thought that increase in the synthetic and secretory activity of odontoblasts results from interactions between these signaling molecules with pre-existing odontoblasts without the involvement of resident MSCs (Smith *et al.* 1995, 2016).

After differentiation, odontoblasts secrete unmineralized predentine, a type I collagen-rich matrix considered to be similar to the osteoid in bone (Bleicher 2014). Predentine mineralizes at the mineralization front to form dentine. The mineralization of dentine is initiated and controlled by deposition of hydroxyapatite crystals and non-collagenous proteins (NCP) secreted by the odontoblasts (Qin *et al.* 2007). Dentine phosphoprotein (DPP) and dentine sialoprotein (DSP) are specific cleavage products of a single gene named dentine sialophosphoprotein (*DSPP*) with roles in dentine formation and mineralization (Prasad *et al.* 2010). Expression of *DSPP* and DSP has been used as a marker to distinguish differentiated odontoblasts from undifferentiated progenitors and osteoblasts.

Dentine secreted by odontoblasts until the completion of root formation is defined as primary dentine. Following primary dentinogenesis, after the complete eruption of the tooth, odontoblasts remain functional and secrete secondary dentine (Kawashima & Okiji 2016). Secondary dentine is secreted throughout life at a much slower rate than primary dentine and results in a decrease in the size of the pulp chamber. Primary and secondary dentine secreted by odontoblasts are characterized by tightly packed dentinal tubules that span the entire thickness of the dentine (Kawashima & Okiji 2016). The odontoblasts cell bodies, located at the dentine-pulp interface are connected at their apical end by numerous junctional complexes and form a physiological barrier that protects the dental pulp from exogenous noxious stimuli (Kawashima & Okiji 2016, Sole-Magdalena *et al.* 2018).

The dentine-pulp complex has a regenerative potential that leads to the formation of tertiary dentine (Sloan *et al.* 2007, Simon *et al.* 2011). Strong noxious stimuli that lead to the destruction of existing odontoblasts is followed by formation of reparative dentine secreted by a new generation of odontoblast-like cells derived from resident Mesenchymal stem cells (MSCs) in dental pulp tissue (Smith *et al.* 2012). Studies have identified several populations of MSCs in continuously growing mice incisors (Feng *et al.* 2011, Kaukua *et al.* 2014, Zhao *et al.* 2014). α SMA-expressing perivascular cells have been identified as one of the MSC population in mice molars (Vidovic *et al.* 2017). These studies together have shown that injury to odontoblasts and dental pulp results in the activation of these resident MSCs in dental pulp tissue, their migration to the site of injury and their differentiation into odontoblast-like cells that generate osteodentine and/or reparative dentine, that creates a bridge to protect the exposed pulp. On the other hand, in response to mild stimuli and absence of damage to odontoblasts and pulp, pre-existing live odontoblasts upregulate their secretory activity and secrete reactionary dentine matrix (Smith *et al.* 1995). Therefore,

odontoblasts are a unique population of cells that survive for the life of a tooth and mediate primary, secondary and tertiary dentinogenesis.

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Recent studies have shown direct contacts between odontoblasts in the periphery of the pulp and nerves ending and other cells in dental pulp (Khatibi Shahidi *et al.* 2015, Kawashima & Okiji 2016, Sole-Magdalena *et al.* 2018). These new findings suggest a possible interaction between pre-existing odontoblasts and MSCs in dental pulp tissue and involvement of MSCs in reactionary dentinogenesis secreted by pre-existing odontoblasts. To gain insight into this possibility, in the present study the fate and progenies of α SMA-tdTomato⁺ cells in dental pulp during reparative dentinogenesis was examined.

MATERIALS & METHODS

Animal models:

Animal protocols were approved by the Institutional Animal Care Committee at University of Connecticut Health Center. α SMACreERT2/Ai9, and Col2.3-GFP mice used in this study have been described previously (Kalajzic *et al.* 2008, Grcevic *et al.* 2012, Vidovic *et al.* 2017). For *in vivo* lineage tracing experiments α SMACreERT2/Ai9 cross between α SMACreERT2 with Ai9 Cre reporter mice, (Jackson Labs, Bar Harbor, ME, USA) and α SMACreERT2/Ai9,Col2.3-GFP (a cross between α SMACreERT2/Ai9 with Col2.3-GFP mice (Vidovic *et al.* 2017) were used. In lineage tracing studies, four-week-old mice were injected intraperitoneally (i.p.) with corn oil (vehicle, VH) and tamoxifen (TM, 75 μ g/g body weight) twice at 24 h intervals. The intact/uninjured and injured teeth from VH-injected α SMACreERT2 mice were examined as controls for spontaneous Cre activation in pulp tissue *in vivo*.

Experimental dentine Injury:

Two days after the second TM injection, mice were anesthetized with an intraperitoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Experimental dentine injuries were prepared on the mesial pit on the occlusal surface of maxillary first molars using a 0.3mm round tungsten carbide bur. Cavities were sealed with light-cured composite resin associated with an adhesive system as described previously (Vidovic *et al.* 2017). Animals were sacrificed with CO₂ at various time points (2, 7, 14 days, 4 and 6 weeks) and processed for multiple analyses.

Histological analysis:

Following sacrifice, maxilla was dissected, fixed in 4% paraformaldehyde for two days at 4°C and decalcified in 14% EDTA for seven days. Decalcified tissues were placed in 30% sucrose solution overnight and embedded in cryomatrix (Thermo Shandon, Pittsburgh, PA, USA). Seven-micrometre sections were obtained using the Leica cryostat and mounted using a CryoJane tape transfer system (Instrumedics, St Louis, MO, USA). Sections were examined and imaged using a Zeiss microscope and filter cubes optimized for DAPI, GFP and Td-Tomato variants. Adjacent sections were processed for haematoxylin/eosin and analyzed by light microscopy as described previously (Vidovic *et al.* 2017).

For analysis of the fate of α SMA-tdTomato⁺ and Col2.3⁺ cells, all sections through the site of injury (at least ten sections per tooth) and sections of control uninjured teeth (12–20 sections per tooth) were evaluated. In these experiments, the areas of red-labeled cells in the pulp chambers were calculated as a ratio to total area using ImageJ software (National Institutes of Health). Results are presented as the mean $\pm$ S.E of labeled areas/mm² in 3–5 animals per time point.

Immunohistochemical analysis:

Cryosections were incubated in blocking reagent for 20 min at 20°C. After washing with PBS, the sections were incubated with the anti-mouse CD31 primary antibody (1:200, BD Biosciences, San Jose, CA, USA) at 4°C overnight. Sections were washed and exposed to donkey anti-goat IgG labeled with Alexa Fluor 488 (1:500, Thermo Fisher Scientific, Waltham, MA, USA) for one h at 20°C.

Fluorochrome labeling and evaluation of growth in reactionary dentine after experimental injury:

The dynamic process of mineral deposition was examined by IP injection of fluorescent dyes dissolved in 2% NaHCO₃ (pH=7.4). The first injection of demeclocycline (DC, 60 mg/kg) was two weeks after dentine injury. Two weeks later calcein green (CG, 6 mg/kg) Fluorochrome label (Sigma Aldrich, St. Louis, MO, USA) was administered. One day after the injection of last dye, animals were sacrificed and processed for histology.

RNAscope in situ hybridization:

In situ hybridization was conducted using RNAscope BROWN kit for *Dspp* according to the manufacturer's instructions (Advanced Cell Diagnostics, Hayward, CA, USA).

Statistical analysis of data.

Results represent mean $\pm$ SEM of at least three independent experiments. Statistical analysis was performed by GraphPad Prism 6 software (La Jolla, CA, USA) using one-way ANOVA analysis with the Bonferroni's multiple comparison post-test or paired two-tailed Student *t*-test. Statistical significance was determined at **p* < 0.05.

RESULTS

Injury to dentine and induction of reactionary dentine:

To gain a better understanding of the possible involvement of α SMA-tdTomato⁺ cells in reparative dentinogenesis injuries limited to dentine were created on the maxillary first molars in 4-week-old transgenic mice. The lack of damage to the pulp and the odontoblast layer was confirmed using Col2.3-GFP transgenic animals that revealed the presence of an intact 2.3-GFP⁺ layer of odontoblasts after a limited injury to the dentine (Figure 1A) and histological examination (Figure 1C). Histological analysis of molars four weeks after injury to dentine and Fluorochrome mineral labeling (Figure 2A) revealed two distinct and closely associated labeled lines in dentine indicating deposition of a small amount of new reparative dentine after injury (Figure 2B).

Activation of α SMA-tdTomato⁺ during reactionary dentinogenesis.

The effects of mild injury to the dentine leading to reactionary dentinogenesis on α SMA-tdTomato⁺ cells were examined using Cre-mediated genetic lineage tracing with α SMA-CreERT2 transgenic mice (Grcevic *et al.* 2012, Vidovic *et al.* 2017). In these animals, CreERT2 recombinase is targeted by the regulatory elements of α SMA. The administration of TM at specific time points (pulse) activates CreERT2 in cells expressing the transgene. Crossing these mice with Cre-dependent Ai9 reporter mice (Rosa26-tdTomato) enabled the visualization of α SMA⁺ cells and their progenies expressing the tdTomato fluorescent protein (referred to as α SMA-tdTomato⁺ cells) over an extended period of time.

In these lineage tracing experiments animals were first injected with TM (twice, 24h intervals), followed by an experimental injury to the dentine (Figure 3A). The fate of α SMA-tdTomato⁺ cells was monitored at 2–14 days after injury (Figure 3A).

The specificity of Cre activation in pulp tissue *in vivo* was confirmed by examining the distribution and fate of α SMA-tdTomato⁺ cells in injured and uninjured maxillary molars in VH-injected animals (Figure 1B) that revealed the absence of α SMA-tdTomato⁺ cells.

In TM-injected animals with intact (without injury) maxillary molars, a few α SMA-tdTomato⁺ cells were detected in the dental pulp and bone marrow of the alveolar bones 2–14 days after injury (Figure 3B). Immunohistochemical staining with CD31 antibody that identifies endothelial cells revealed that in these animals, α SMA-tdTomato⁺ cells were in close association with CD31⁺ cells indicating their perivascular location (Figure 4B and 4C).

In TM-injected animals with injury to maxillary molars, there were significant activation and increases in the number α SMA-tdTomato⁺ cells throughout the pulp (chamber and root) as early as two days after injury as compared to molars without injury (Figure 3C as compared to Figure 3B). Immunohistochemical analysis revealed mobilization of the α SMA-tdTomato⁺ in injured teeth (Figure 4B and 4C). Unlike in intact teeth, in teeth with mild injury, the majority of α SMA-tdTomato⁺ cells were not in close association with CD31⁺ cells (Figure 4B d-f and 4C d-f) had migration to the site of dentine injury. The percentage of areas occupied by α SMA-tdTomato⁺ in injured ($7.5 \pm 0.7\%$) teeth were significantly higher than

in teeth without injury ($2 \pm 0.5\%$). These observations indicated that damage to dentine resulted in the activation and mobilization of α SMA-tdTomato⁺ cells.

Progenies of α SMA-tdTomato⁺-derived cells during reactionary dentinogenesis.

To further examine the progenies of α SMA-tdTomato⁺-derived cells, we crossed α SMACreERT2/Ai9 mice with Col2.3-GFP transgenic mice shown to be expressed by both odontoblasts and osteoblasts (Balic *et al.* 2010). Experimental mild injury to dentine was performed on 4-week old α SMACreERT2/Ai9; Col2.3-GFP mice after TM injections (Figure 5A). The fate of cells was examined 4 and six weeks after injury.

Histological examination of teeth 4 and six weeks after injury to dentine revealed α SMA-tdTomato⁺ cells (Figure 5Ba, 5Cb, 5Cg) in the existing odontoblast layer expressing Col2.3-GFP (Figure 5Bb, 5Cc, 5Ch) and *Dspp* (Figure 5Be, 5Bf). Furthermore, unlike in teeth without injury, in teeth with injury varying number of cells co-expressing both α SMA-tdTomato and Col2.3-GFP (Figure 5Bc, Bd, Cd, Ce, Ci, Cj) were detected. The lack of remodeling in the odontoblast layer indicated that double labeled cells in odontoblast layer were new odontoblasts derived from α SMA-tdTomato⁺ cells.

In addition to their small contribution to newly formed odontoblasts, α SMA-tdTomato⁺ cells were also detected in the pulp at the site of the injury. Closer examination of α SMA-tdTomato⁺ cells in the pulp of the injured teeth showed that in contrast to uninjured teeth, most of these cells were not associated with endothelial cells and were elongated and fibroblastic-like cells (Figure 5). These observations provided evidence for the contribution of α SMA-tdTomato⁺ cells to cells other than perivascular cells in dental pulp during reparative dentinogenesis.

DISCUSSION

The observations revealed that despite the lack of apparent damage to the odontoblasts and dental pulp, injury to dentine leading to reactionary dentinogenesis, also resulted in activation of perivascular MSCs expressing α SMA-tdTomato in dental pulp tissue. The lineage tracing experiments revealed the contribution of α SMA-tdTomato to odontoblasts and fibroblast-like cells at the site of injury. However, it is possible that activation and recruitment of α SMA-tdTomato⁺ cells to the site of injury in this study was related to their contribution to new odontoblast replacing those that were lost by apoptosis. Although the histological examination revealed a lack of apparent damage to odontoblasts, it remains possible that experimental cavity preparation in this study led to apoptosis in some odontoblasts and underlying pulp cells.

The contribution of α SMA-tdTomato⁺ to new odontoblasts during reactionary dentinogenesis and in the absence of apparent damage to odontoblasts and pulp tissue in this study is comparable to the previously reported contribution of pericyte-derived cells to the odontoblast-like cells in “restorative dentin,” a mineralizing tissue formed at the tips of incisors in response to continuous wear and absence of damage to the pulp (Pang *et al.* 2016)

The signaling pathways leading to activation of α SMA-tdTomato⁺ during reactionary dentinogenesis although unknown most likely involves the newly discovered direct contacts between odontoblasts, fibroblasts, and nerves in dental pulps (Ichikawa *et al.* 2012, Khatibi Shahidi *et al.* 2015, Shibukawa *et al.* 2015). The diffusion of signaling factors through the dentinal tubules may initiate that activation of α SMA-tdTomato⁺, (Murray *et al.* 2002, Bleicher 2014, Kawashima & Okiji 2016) and/or by transmission of sensory stimuli by odontoblasts

The information from this study provides evidence for various mechanisms and signaling pathways that mild insult to dentin can activate responses in pulp tissue. This study provides evidence that mild injuries to dentin leading to reactionary dentinogenesis also activate perivascular cells expression α SMA-tdTomato shown to be one of the MSC population in the dental pulp. The results also identified contributions of α SMA-tdTomato⁺ cells to new fibroblast-like and other pulp cells as well as a few new odontoblasts that become integrated into the odontoblast layer.

Conclusions

Mild insult to dentine activated perivascular α SMA-tdTomato⁺ cells giving rise to pulp cells as well as a few odontoblasts that were integrated into the pre-excising odontoblast layer.

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Figure 1.

Reparative dentinogenesis and Specificity of Cre activation A. Bright field (a) and epifluorescence image (b) of section of a first maxillary molar from Col2.3-GFP transgenic animal immediately after dentine injury. Note the presence of intact Col2.3-GFP⁺ cells underlying dentine at the site of injury indicating the lack of pulp injury. B. Bright field (a and c) and epifluorescence (b and d) images of sections through maxillary molars without injury (a, b) and with injury (c, d) from VH-injected αSMACreERT2/Ai9 transgenic animals isolated four weeks post injection and post injury. Sections were processed for staining with DAPI (blue). Note the lack of αSMA-tdTomato⁺ cells in dental pulp and alveolar bone(ab) in these images. In all images, dental pulp is denoted by dashed lines, the site of injury by an asterisk and alveolar bone (ab). Scale bars = 100μm.

Figure 2.

Deposition of new mineralized tissue during reactionary dentinogenesis. A. Scheme of vital dye, fluorochrome labeling in 4 weeks old mice after dentine injury. The first vital dye of democlocycline (DC, yellow) was injected two weeks after dentine injury and followed by injection of calcein green (CG, green) after additional two weeks. Animals were sacrificed 24 hours after the second injection. b. Representative bright field and epifluorescence images of sections through a maxillary molar without (a-c) and with (d-f) dentine injury. Higher magnifications of boxed areas in b and e are shown in c and f respectively. Note the two separate lines of fluorochrome labels (indicated by an arrow in f) in the mineralized tissue below the site of dentine injury (indicated by *). Also, note the presence of both fluorochrome labels in underlying alveolar bone (ab) in images from intact (c) and injured molars (f) undergoing physiological bone remodeling. Note the lack of fluorochrome labels in the dentine of the intact molar (f). In all images, dental pulp is denoted by dashed lines, the site of injury by an asterisk and alveolar bone (ab). Scale bars = 100µm.

Figure 3.

The response of α SMA-tdTomato⁺ cells to dentine injury. **A** Scheme of lineage tracing experiment in α SMACreERT2/Ai9 transgenic mice during reactionary dentinogenesis. Four-week-old animals were injected with TM twice at a 24-hour interval. Dentine injury was performed 48 hours after the second injection. Labeled cells were chased at indicated time points after injury. **B** Representative bright field and epifluorescence scans of sagittal sections through intact maxillary first molars without injury from TM-injected α SMACreERT2/Ai9 transgenic mice at 2, 7 and 14 days post TM injection. **c, f** and **i** are higher magnifications of boxed area shown in **b, e** and **h** respectively. Note that in teeth without injury few α SMA-tdTomato⁺ cells are detected in dental pulp tissue. Also note that lack of significant changes in numbers and locations of these cells between days 2–14 (**b, e, h**). α SMA-tdTomato⁺ cells are not detected close to the dentine layer (**c, f, i**). α SMA-tdTomato⁺ cells are detected in bone marrow of the alveolar bone (**ab**). **C**. Representative bright field and epifluorescence scans of sagittal sections through maxillary first molars with dentine (injury indicated by *) at various time points. **c, f**, and **i** are higher magnifications of boxed areas in **b, e** and **h** respectively. Note the activation of α SMA-tdTomato⁺ in dental pulp at the site of injury and distant areas in molars with dentine injury as early as two days

(b). Note the expansion of α SMA-tdTomato⁺ cells between days 2–14 after injury (b, e, h). Also note the accumulation of α SMA-tdTomato⁺ cells under the site of injury and close to the dentine layer at all time points (c, f, i). α SMA-tdTomato⁺ cells are detected in bone marrow of the alveolar bone (ab). In all images, dental pulp is denoted by dashed lines, the site of injury by an asterisk and alveolar bone (ab). Scale bars = 100 μ m.

Figure 4.

Changes in the localization of α SMA-tdTomato⁺ cells in response to dentine injury. A. Scheme of lineage tracing experiment in α SMACreERT2/Ai9 transgenic mice during reactionary dentinogenesis. Four-week-old animals were injected with TM twice at a 24-hour interval. Dentine injury was performed 48 h after the second injection and cells were chased at 14 days after injury. B & C Representative bright field and epifluorescence images of sections through maxillary molars of α SMACreERT2/Ai9 animals 14 days after TM injection without dentine injury (Ba-c and Ca-c) and with dentine injury (Figure Bd-f and Cd-f). Sections were processed for staining with CD31 antibody (green). c and f in both panels B and C are higher magnifications of boxed areas in b and e respectively. Note that in the intact molar most of α SMA-tdTomato⁺ cells (red) are in proximity to CD31⁺ endothelial cells (green) (indicated by arrowheads) whereas, in the molar with injury, α SMA-tdTomato⁺ cells are not in proximity with CD31⁺ endothelial cells and have migrated towards the site of injury. In all images, dental pulp is denoted by dashed lines and the site of injury by an asterisk. Scale bars = 100 μ m. Dashed lines outline the pulp. Scale bars=100 μ m.

Figure 5.

Contributions of α SMA-tdTomato⁺ cells to dental pulp and odontoblasts during the regeneration process. **A** Scheme of lineage tracing experiment in α SMACreERT2/Ai9 transgenic mice during reactionary dentinogenesis. Four-week-old animals were injected with TM twice at a 24-hour interval. Dentine injury was performed 48 h after the second injection and animals were sacrificed 4 and six weeks after injury. **B** Representative epifluorescence images of sections through a maxillary molar from α SMAAi9/Col2.3-GFP animal six weeks after dentine injury. Images from an adjacent section processed for RNAscope in situ hybridization for *Dspp* are shown in **e** and Higher magnifications boxed areas in **c** and **f** are shown in **d** and **e** respectively. Note α SMA-tdTomato⁺ cells (indicated by arrow heads in **a**) in dentine layer expressing 2.3-GFP⁺ (**b**) and *Dspp*⁺ (**e**, **f**) odontoblasts (**b**). Also, numerous cells co-expressing α SMA-tdTomato and Col2.3-GFP (yellow, indicated by arrow heads in **c** and **d**) are detected in the layer of odontoblasts (indicated by

arrow heads) at the site of injury C Representative bright field and epifluorescence images of sections through two maxillary molars from α SMAAi9/Col2.3-GFP animal four weeks after dentine injury. Note α SMA-tdTomato⁺ cells (indicated by arrow heads in b and g) in dentine layer expressing 2.3-GFP⁺ (c, h) Also note numerous cells co-expressing α SMA-tdTomato and Col2.3-GFP (yellow, indicated by arrow heads in (d and i) are detected in the layer of odontoblasts (indicated by arrow heads) at the site of injury. In all images, dental pulp is denoted by dashed lines and the site of injury by an asterisk and alveolar bone (ab). Scale bars = 100 μ m.