Dspp-independent Effects of Transgenic Trps1 Overexpression on Dentin Formation

Journal of Dental Research
2015, Vol. 94(8) 1128–1134
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for Dental Research 2015
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DOI: 10.1177/0022034515586709
jdr.sagepub.com

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Abstract

The Trps1 transcription factor is highly expressed in dental mesenchyme and preodontoblasts, while in mature, secretory odontoblasts, it is expressed at low levels. Previously, we have shown that high Trps1 levels in mature odontoblasts impair their function in vitro and in vivo. *Collal-Trps1* transgenic (Trps1-Tg) mice demonstrate defective dentin secretion and mineralization, which are associated with significantly decreased *Dspp* expression due to direct repression of the *Dspp* gene by Trps1. Here, by crossing Trps1-Tg and *Collal-Dspp* transgenic (Dspp-Tg) mice, we generated *Collal-Trps1;Collal-Dspp* double transgenic (double-Tg) mice in which *Dspp* was restored in odontoblasts overexpressing Trps1. Comparative micro-computed tomography analyses revealed partial correction of the dentin volume and no improvement of dentin mineralization in double transgenic mice in comparison with Trps1-Tg and wild-type (WT) mice. In addition, dentin of double-Tg mice has an irregular mineralization pattern characteristic for dentin in hypophosphatemic rickets. Consistent with this phenotype, decreased levels of Phex, Vdr, and Fam20c proteins are detected in both Trps1-Tg and double-Tg odontoblasts in comparison with WT and Dspp-Tg odontoblasts. This suggests that the Dspp-independent dentin mineralization defects in Trps1-Tg mice are a result of downregulation of a group of proteins critical for mineral deposition within the dentin matrix. In summary, by demonstrating that Trps1 functions as a repressor of later stages of dentinogenesis, we provide functional significance of the dynamic Trps1 expression pattern during dentinogenesis.

Keywords: dentinogenesis, gene expression, matrix biology, mineralized tissue/development, odontoblast(s), transcription factor(s)

Introduction

Dentin, a mineralized tissue that is the most abundant component of teeth, is produced by odontoblasts. Dentin formation requires the differentiation of odontoblasts from progenitor cells in dental papilla and the formation of a specialized extracellular matrix by these cells. Functional odontoblasts have a columnar shape and form a continuous single cell layer at the periphery of the dental pulp. Each odontoblast has a long cellular process that extends into the predentin and dentin. These processes contain an abundance of secretory vesicles, as the role of the mature odontoblast is to secrete the extracellular matrix and regulate its mineralization. The newly synthesized unmineralized matrix, termed predentin, separates odontoblasts from the mineralized matrix, called dentin (Linde and Goldberg 1993; Butler and Ritchie 1995; Ruch et al. 1995; Embery et al. 2001; Arana-Chavez and Massa 2004).

Like in bone, the organic dentin fraction is primarily composed of type I collagen. However, the molecular hallmark of dentin is an abundance of dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), which are products of a proteolytic processing of a single peptide encoded by the *DSPP* gene (Butler 1998; Yamakoshi 2009; Sun et al. 2010; Ravindran and George 2014). The importance of DSP and DPP for dentin development and mineralization has been underscored by the

identification of *DSPP* gene mutations in human hereditary dentin disorders: dentinogenesis imperfecta (DGI) and dentin dysplasia (MacDougall et al. 2006; Hu and Simmer 2007; Barron et al. 2008; Maciejewska and Chomik 2012). While mutations of *DSPP* cause isolated dentin defects, the presentation of DGI can also be a part of the osteogenesis imperfecta phenotype caused by mutations in one of the type I procollagen genes: *COL1A1* or *COL1A2*. In either case, the DGI phenotype is characterized by an abnormal dentin structure and severely impaired mineralization. Defective dentin is also observed in a genetically heterogeneous group of phosphate homeostasis disorders, such as hypophosphatemic rickets, suggesting the importance of systemic phosphate homeostasis for dentin formation. Hypophosphatemic teeth have enlarged pulp chambers, severely undermineralized dentin, and an irregular mineralization

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pattern (Souza et al. 2010; Opsahl Vital et al. 2012; Souza et al. 2013; Foster et al. 2014). Many of the genes associated with hypophosphatemia are highly expressed by odontoblasts, suggesting that they are involved in dentin mineralization acting locally and independently of systemic phosphate metabolism (Ruchon et al. 2000; Bai et al. 2002; Thompson et al. 2002; Boskey et al. 2009).

We have recently demonstrated severely disrupted dentin formation in *Colla1-Trps1* transgenic (*Trps1-Tg*) mice with sustained high expression of the transcription factor *Trps1* in secretory odontoblasts (Napierala et al. 2012). On the molecular level, marked downregulation of *Dspp* in odontoblasts of *Trps1-Tg* mice was detected. Although there is a significant overlap in dental phenotypes of *Trps1-Tg* and *Dspp*^{-/-} mice (Sreenath et al. 2003), the severity of dentin defects in *Trps1-Tg* mice suggests that other genes involved in dentinogenesis are also affected as a result of *Trps1* overexpression in odontoblasts. This is further supported by our recent in vitro studies demonstrating the decreased expression of *Phex* and *Vdr* in odontoblastic cell lines overexpressing *Trps1* (Kuzynski et al. 2014). The goal of this study was to provide a more detailed mechanism of defective dentinogenesis in *Trps1-Tg* mice by identifying additional genes critical for dentin formation that are affected by *Trps1* upregulation in secretory odontoblasts.

Materials and Methods

Transgenic Mice

The generation and characterization of *Trps1-Tg* and *Colla1-Dspp* transgenic (*Dspp-Tg*) mice have been described before (Napierala et al. 2012; Zhu, Gibson, et al. 2012; Zhu, Prasad, et al. 2012). *Colla1-Trps1*; *Colla1-Dspp* double transgenic (double-Tg) mice were generated by breeding *Trps1-Tg* males with *Dspp-Tg* females. *Trps1-Tg* and double-Tg mice were maintained on a soft food diet as described earlier (Napierala et al. 2012). All mice were housed in the University of Alabama at Birmingham animal facility in full compliance with all applicable federal and state guidelines. Veterinarians supervised animal care according to standard conditions of the University of Alabama at Birmingham animal care and use program, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All studies were performed with prior approval of the Institutional Animal Care and Use Committee.

Micro-Computed Tomography (μ CT) Analyses

Fixed mandibles were scanned using the Scanco μ CT40 desktop cone-beam μ CT scanner (Scanco Medical AG, Brüttisellen, Switzerland) at the following settings: 12- μ m resolution, 70 kVp, and 145 μ A with an integration time of 200 ms. Scans were automatically reconstructed into 2-dimensional slices. One hundred slices (50 slices posterior and 50 slices anterior to the mandibular bone terminus dorsal to an incisor) were analyzed using the μ CT Evaluation Program (v5.0A, Scanco Medical AG). The region of interest was drawn on each of the

100 slices to include only dentin and dental pulp. The dentin threshold was set at 195 to distinguish it from the pulp. The 3-dimensional reconstruction was performed using all the outlined slices. Enamel was not included in the analysis. Data were obtained for dentin volume (DV), total volume (TV), DV/TV, and dentin mineral density (DMD).

Histology and Immunohistochemistry (IHC)

Mandibles were collected and fixed in 4% buffered formalin for 48 h. For standard histology and IHC, mandibles were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 14 d and dehydrated through increasing concentrations of ethanol prior to paraffin embedding. 7- μ m paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) according to standard protocols. IHC was performed using heat-induced antigen retrieval in sodium citrate buffer, pH 6.0, and antibodies: anti-vitamin D receptor (1:5,000 dilution; Abcam PLC, Cambridge, UK), anti-Phex (1:200 dilution; provided by Dr. Rajiv Kumar) (Thompson et al. 2002), anti-Fam20c (1:2,000 dilution; provided by Dr. Chunlin Qin) (Wang, Wang, Lu, et al. 2012), anti-osteopontin (anti-Opn) (1:100 dilution; Abcam PLC), and anti-ASARM (1:200 dilution; provided by Dr. Peter Rowe) (Staines et al. 2012).

Fluorescent Labeling

To analyze the dentin apposition rate, mice were intraperitoneally injected with alizarin red (25 mg/kg; Sigma, St. Louis, MO, USA) followed by an injection of calcein (25 mg/kg; Sigma) 10 d later. Mice were sacrificed 48 h after the second injection, and tissues were collected for analysis. 7- μ m sections of methyl methacrylate-embedded tissues were imaged under fluorescent light using the Nikon Eclipse 90i (Tokyo, Japan) and Photometrics CoolSNAP software (Tucson, AZ, USA).

Statistical Analyses

Data are represented as the mean $\pm$ standard deviation. Probability values were calculated using the Student's *t* test. *P* < 0.1 was considered to be statistically significant.

Results

Restoring *Dspp* Expression in *Trps1-Tg* Mice Does Not Correct Dentin Structure

To determine whether the *Trps1* inhibitory effect on the secretory odontoblast function extends beyond repression of the *Dspp* gene, a genetic approach was used to restore *Dspp* expression in *Trps1*-overexpressing odontoblasts. *Dspp-Tg* mice, which have been previously characterized and validated in terms of the ability to correct the dental phenotype of *Dspp*^{-/-} mice (Gibson et al. 2013), were used to generate double-Tg mice. Overexpression of *Trps1* and *Dsp* in transgenic odontoblasts and dentin, respectively, was confirmed at the protein level by IHC (Fig. 1).

Figure 1. Immunohistochemistry analyses of Trps1 and Dsp in first mandibular molars of 1-wk-old mice. Top panel: overexpression of Trps1 in *Collal-Trps1* transgenic (Trps1-Tg) and *Collal-Trps1;Collal-Dspp* double transgenic (double-Tg) odontoblasts (arrows). Bottom panel: overexpression of Dsp in *Collal-Dspp* transgenic (Dspp-Tg) dentin in comparison with wild-type (WT) dentin. Note decreased Dsp in Trps1-Tg dentin in comparison with WT dentin and no apparent difference in Dsp between WT and double-Tg dentin. D, dentin; DP, dental pulp.

Figure 2. Representative hematoxylin and eosin-stained histological sections of first mandibular molars of 1-, 3-, and 8-wk-old mice showing a disturbed predentin-dentin organization in *Collal-Trps1* transgenic (Trps1-Tg) and *Collal-Trps1;Collal-Dspp* double transgenic (double-Tg) teeth. Distinct predentin (PD) and dentin (D) layers are present in wild-type (WT) and *Collal-Dspp* transgenic (Dspp-Tg) teeth but not in Trps1-Tg and double-Tg teeth (asterisk). Black boxes indicate areas magnified on the images.

The dentin structure was first evaluated by standard histology of first mandibular molars of 1-, 3-, and 8-wk-old mice (Fig. 2). Comparison of H&E-stained tissue sections demonstrated distinctive predentin and dentin layers in wild-type (WT) and Dspp-Tg teeth. As demonstrated previously, this well-organized structure is diminished in Trps1-Tg mice, and only 1 layer of the extracellular matrix is detectable in teeth of these mice (Napierala et al. 2012). A similar abnormal dentin structure is present in double-Tg mice and is detected in both unerupted (1 wk) and erupted (3 wk and 8 wk) teeth. This suggests that restoring Dspp is not sufficient to correct an abnormal dentin structure resulting from the overexpression of Trps1 in secretory odontoblasts.

Next, μ CT analyses of 8-wk-old mice were performed to evaluate the mineralization of dentin in double-Tg mice (Fig. 3). Two-dimensional images of molars and incisors demonstrated a thick, homogenous dentin layer in the teeth of WT and Dspp-Tg mice. As demonstrated before, the dentin thickness is severely reduced, and very little dentin was detected in the molars and incisors of Trps1-Tg mice (Napierala et al. 2012). In double-Tg mice, the dentin layer is thinner than in WT and Dspp-Tg mice, although it appears thicker than in Trps1-Tg mice. Partial improvement in the dentin layer thickness in double-Tg mice is further demonstrated by comparing 3-dimensional reconstructions of incisor dentin of WT, Dspp-Tg, Trps1-Tg, and double-Tg mice and by comparative quantitative analyses. Quantitative μ CT analyses confirmed that the contribution of the DV to TV of dentin and pulp was significantly increased in double-Tg teeth compared to Trps1-Tg teeth. However, the DV/TV in double-Tg mice is still significantly lower than in WT and Dspp-Tg mice, indicating only partial improvement in dentin formation. Interestingly, quantitative analyses of the DMD revealed that significantly decreased mineralization of Trps1-Tg dentin is not improved in double-Tg mice, as there is no difference in the DMD between Trps1-Tg and double-Tg mice, and the DV is significantly lower in these mice than in WT mice.

In summary, comparative histological and μ CT analyses of dentin revealed that

abnormalities of dentin formation and mineralization caused by the overexpression of *Trps1* in secretory odontoblasts are only partially alleviated by restoring *Dspp* expression.

Overexpression of *Trps1* in Secretory Odontoblasts Affects *Phex*, *Vdr*, and *Fam20c* Proteins

Partial improvement of dentin abnormalities in double-Tg mice suggests that *Dspp* is not the only gene affected by *Trps1* overexpression in odontoblasts. Careful analyses of μ CT images of incisors revealed the irregular mineralization of dentin in double-Tg mice, with radiolucent regions seemingly randomly distributed within dentin (Fig. 3A). Similar irregular dentin mineralization has been demonstrated in hypophosphatemic rickets (Opsahl Vital et al. 2012; McKee et al. 2013; Souza et al. 2013; Foster et al. 2014). Interestingly, we have recently identified that the overexpression of *Trps1* in a preodontoblast-derived cell line results in significant downregulation of *Phex* and *Vdr*, whose mutations cause hypophosphatemic rickets (Kuzynski et al. 2014). Considering these in vitro data and dentin mineralization defects caused by either *Phex* or *Vdr* deficiency, we hypothesized that decreased levels of either *Phex* or *Vdr* could contribute to dentin pathology in *Trps1*-Tg and double-Tg mice. Therefore, we focused our further comparative analyses on evaluating *Phex* and *Vdr* proteins in mature odontoblasts of WT, *Trps1*-Tg, and double-Tg mice. For comparison, we included *Fam20c*, which is also involved in hypophosphatemic rickets but was not affected by *Trps1* in vitro (Wang, Wang, Li, et al. 2012; Rafaelsen et al. 2013; Kuzynski et al. 2014).

Comparative IHC analyses of molars of 8-wk-old mice show no apparent difference in *Phex*, *Vdr*, and *Fam20c* protein levels or distribution between WT and *Dspp*-Tg mice (Fig. 4). In both genotypes, high levels of these proteins are detected in mature odontoblasts. As it has been demonstrated before, *Phex* is distributed along the odontoblast cell body and odontoblast processes in the predentin layer, while *Vdr* and *Fam20c* are specifically localized on the predentin side of the cell body (Davideau et al. 1996; Ruchon et al. 2000; Thompson et al. 2002; Descroix et al. 2010; Wang, Wang, Lu, et al. 2012). In contrast, in *Trps1*-Tg and double-Tg odontoblasts, *Phex* can be detected only in odontoblast cell bodies (Fig. 4). However,

even within the cell body, the *Phex* distribution is abnormal in *Trps1*-Tg and double-Tg odontoblasts, as demonstrated by a strong punctate signal instead of an evenly distributed signal as detected in WT and *Dspp*-Tg odontoblasts. Similar to the *Phex* protein, *Vdr* and *Fam20c* proteins also cluster in foci in *Trps1*-Tg odontoblasts (Fig. 4). In addition, levels of these proteins are lower in *Trps1*-Tg teeth than in WT teeth. While these protein abnormalities are not corrected in double-Tg mice, some double-Tg odontoblasts do show improved *Fam20c* expression and distribution (Fig. 4, arrowhead).

To address the potential contribution of mineralization inhibitors to the dental phenotype of *Trps1*-Tg mice, secreted

Figure 3. Restoring *Dspp* in odontoblasts partially corrects dentin defects in *Colla1*-*Trps1* transgenic (*Trps1*-Tg) mice (8-wk-old males). **(A)** Representative micro-computed tomography (μ CT) images (cross-sections) of wild-type (WT), *Trps1*-Tg, *Colla1*-*Dspp* transgenic (*Dspp*-Tg), and *Colla1*-*Trps1*;*Colla1*-*Dspp* double transgenic (double-Tg) teeth demonstrating partial correction of the dentin layer thickness in both molars and incisors of double-Tg mice in comparison with *Trps1*-Tg mice. Arrows: the dentin layer. Arrowheads: irregular dentin mineralization in *Trps1*-Tg and double transgenic mice. **(B)** Comparison of the dentin volume (DV) and dentin mineral density (DMD) in the incisors of WT, *Trps1*-Tg, *Dspp*-Tg, and double-Tg mice. Top panel: μ CT images (3-dimensional reconstruction) of dentin in the analyzed region of the incisor. Bottom panel: results of quantitative μ CT analyses demonstrating partial correction of the DV in double-Tg mice as compared to WT and *Trps1*-Tg mice. Values are expressed as a ratio of the DV in total tissue volume (TV), where the TV is a sum of dentin and pulp volume. The DMD analyses show no improvement of dentin mineralization in double-Tg mice. Data are presented as the mean $\pm$ standard deviation ($n = 5$ each genotype). Statistical significance was calculated using a paired *t* test. * $P < 0.1$, ** $P < 0.01$. NS, not significant.

Figure 4. The overexpression of *Trps1* in secretory odontoblasts affects proteins associated with hypophosphatemic rickets. Representative images of immunohistochemistry on first mandibular molars of 8-wk-old male mice showing Phex, Vdr, and Fam20c and molars of 1-wk-old mice showing osteopontin (Opn) and ASARM–matrix extracellular phosphoglycoprotein (MEPE) proteins in secretory odontoblasts (arrows). Phex, Vdr, and Fam20c proteins are highly expressed in secretory odontoblasts of wild-type (WT) and *Collal-Dspp* transgenic (*Dspp*-Tg) mice but show decreased expression in *Collal-Trps1* transgenic (*Trps1*-Tg) and *Collal-Trps1;Collal-Dspp* double transgenic (double-Tg) mice. In double-Tg mice, there are clusters of secretory odontoblasts with an expression and distribution of Fam20c similar to WT odontoblasts (arrowhead), and there are clusters of odontoblasts with an abnormal Fam20c signal, like in *Trps1*-Tg odontoblasts. Opn and ASARM-MEPE in odontoblasts are not affected by *Trps1* overexpression. D, dentin; DP, dental pulp.

phosphoprotein 1 (Spp1)/Opn and matrix extracellular phosphoglycoprotein (MEPE) were analyzed by IHC (Fig. 4). Opn and MEPE-derived ASARM peptides were detected in odontoblasts and dentin of all 4 genotypes, and we did not observe upregulation of these inhibitors in *Trps1*-Tg mice.

In summary, analyses of proteins associated with hypophosphatemic rickets and mineralization inhibitors revealed that *Trps1* affects some of the mineralization-related proteins independently of *Dspp* expression.

Effect of *Trps1* Overexpression on Formation of Mineralized Matrix Is Cell-type Specific

The lack of clear distinction between predentin and dentin in *Trps1*-Tg teeth suggests impaired secretion of the organic extracellular matrix or disorganized mineralization. To address the dynamics of dentin formation, mice were injected with alizarin red and calcein fluorescent dyes, which are incorporated into

newly formed dentin at the time of dye administration. As expected, 2 discrete lines of fluorescent labels are clearly visible in WT and *Dspp*-Tg dentin, while in *Trps1*-Tg mice, these labels overlap (Fig. 5). These overlapping lines indicate that less dentin matrix was secreted by *Trps1*-Tg odontoblasts in comparison with WT and *Dspp*-Tg odontoblasts during the 10 d between injections. In double-Tg mice, the deposition of dentin is uneven, with regions where the 2 fluorescent labels are clearly separated and regions where they are merged similar to those seen in *Trps1*-Tg mice (Fig. 5, upper panel). In addition to the reduced distance between fluorochrome labels, label lines are more diffused and wavy in *Trps1*-Tg and double-Tg dentin than in WT and *Dspp*-Tg dentin, suggesting the irregular secretion and mineralization of dentin by *Trps1*-overexpressing odontoblasts.

In *Trps1*-Tg mice, *Trps1* is overexpressed in odontoblasts and osteoblasts; therefore, the incorporation of fluorescent dyes in alveolar bone was also compared between WT and *Trps1*-Tg mice (Fig. 5, lower panel). Lines of fluorescent labels are clearly separated in *Trps1*-Tg alveolar bone, like in WT controls, indicating that impaired secretion of the mineralized matrix is restricted to odontoblasts.

Discussion

We have previously demonstrated that sustained high levels of the *Trps1* transcription factor in secretory odontoblasts

had deleterious effects on dentin formation, and this was associated with direct repression of the *Dspp* gene by *Trps1* (Napierala et al. 2012). In this study, we took a genetic approach to identify *Dspp*-independent consequences of *Trps1* upregulation in secretory odontoblasts. By crossing *Trps1*-Tg mice with *Dspp*-Tg mice, we generated double transgenic mice in which *Dspp* was restored in odontoblasts overexpressing *Trps1*. The combination of standard histological analyses and μ CT demonstrated that dentin made by double-Tg odontoblasts is improved in comparison with dentin of *Trps1*-Tg odontoblasts, but not corrected. Increased thickness of the dentin layer in double-Tg mice in comparison with *Trps1*-Tg mice indicates that *Dspp* deficiency contributes to this defect, although it is not the exclusive underlying cause. In contrast, the lack of improvement in the DMD suggests that this defect is caused predominantly by a *Dspp*-independent mechanism.

Interestingly, partial improvement of the DV in double-Tg mice revealed uneven, irregular mineralization of dentin, a

defect that is characteristic for hypophosphatemic rickets (Opsahl Vital et al. 2012; Souza et al. 2013; Foster et al. 2014). Consistently, downregulation of *Phex*, *Vdr*, and *Fam20c* proteins, whose deficiency is associated with this disorder, has been detected in secretory odontoblasts overexpressing *Trps1* in comparison with WT and *Dspp*-Tg odontoblasts. Interestingly, *Vdr*, *Phex*, and *Fam20c* are highly and specifically expressed in odontoblasts (Fig. 4), which implies a cell-autonomous function of these proteins in dentin mineralization (Ruchon et al. 2000; Bai et al. 2002; Thompson et al. 2002; Boskey et al. 2009; Wang, Wang, Lu, et al. 2012). Thus, the dentin phenotype similar to hypophosphatemic rickets is likely due to cell-autonomous consequences of the deficiency of phosphate homeostasis proteins in odontoblasts, which results in an impaired odontoblast function. Such a mechanism is additionally supported by the apparently unaffected alveolar bone in *Trps1*-Tg mice (Fig. 5), which suggests that there is no systemic effect of *Trps1* overexpression in odontoblasts and osteoblasts. Furthermore, this indicates that the overexpression of *Trps1* under the osteoblast- and odontoblast-specific fragment of the *Colla1* promoter has tissue-specific consequences, which are restricted to dentin. This, together with our recent *in vitro* data demonstrating the repression of *Phex* and *Vdr* expression in odontoblast cell lines overexpressing *Trps1* (Kuzynski et al. 2014), suggests that this inhibitory effect of *Trps1* overexpression on the function of mature odontoblasts is cell autonomous. Further, since the previous *in vitro* studies were conducted in a cell line that expresses *Dspp* only transiently during the initiation of mineralization and at a very low level, this inhibitory effect is likely to be independent of the presence of *Dsp* and *Dpp* proteins in the extracellular matrix. Whether *Trps1* directly represses the expression of these genes in secretory odontoblasts remains to be determined.

Studies of *Trps1*-Tg mice presented here, together with our previous data, revealed that *Trps1* upregulation results in impaired dentin matrix secretion as well as dentin mineralization. At the molecular level, the major dentin gene, *Dspp*, and proteins involved specifically in the regulation of mineralization and phosphate homeostasis are downregulated by *Trps1*. At the same time, *Trps1*-overexpressing odontoblasts are positive for other odontogenic markers, alkaline phosphatase and osteocalcin (Napierala et al. 2012), which suggests that *Trps1* affects the odontoblast function rather than odontoblast differentiation. Interestingly, in both *Trps1*-Tg and double-Tg mice, an irregular, wavy pattern of mineral deposition in the dentin matrix was detected (Fig. 5). This suggests that *Trps1*-overexpressing odontoblasts deposit minerals not only through

Figure 5. Cell type-specific effects of *Trps1* overexpression on tissue mineralization (8-wk-old males). Top panel: representative images of alizarin red (red) and calcein (green) double labeling of first mandibular molars showing the reduced formation of coronal dentin (the distance between fluorescent dye labels) in *Colla1*-*Trps1* transgenic (*Trps1*-Tg) mice in comparison with wild-type (WT) mice. Bottom panel: representative images of alizarin red (red) and calcein (green) double labeling of alveolar bone showing no difference in the fluorescent labeling between WT, *Trps1*-Tg, *Colla1*-*Trps1*; *Colla1*-*Dspp* double transgenic (double-Tg), and *Colla1*-*Dspp* transgenic (*Dspp*-Tg) mice. D, dentin; DP, dental pulp.

well-defined regions of their processes but possibly also through the apical and lateral membrane of the cell body. The possibility that *Trps1* overexpression disrupts organized dentin matrix secretion and mineralization is additionally suggested by histological analyses showing the dentin matrix between odontoblast cell bodies (Fig. 2). The negative effect of *Trps1* on the secretion of dentin matrix components is further supported by the abnormal distribution of *Phex*, *Vdr*, and *Fam20c* proteins within odontoblasts and dentin of *Trps1*-overexpressing odontoblasts in comparison with WT and *Dspp*-Tg odontoblasts (Fig. 4). Considering that spatially well-organized matrix secretion and mineralization are characteristic features of mature odontoblasts (Linde and Goldberg 1993; Ruch et al. 1995; Arana-Chavez and Massa 2004), these data suggest that *Trps1* must be downregulated in mature odontoblasts to allow for proper dentin formation.

In summary, results of this study provide insights into the biological significance of the dynamic *Trps1* expression pattern during dentinogenesis. *Trps1* is highly expressed in dental mesenchyme throughout all stages of tooth organ development, including preodontoblasts; however, *Trps1* is downregulated in mature secretory odontoblasts (Kantaputra et al. 2008; Napierala et al. 2012). Sustained high levels of *Trps1* in secretory odontoblasts have a dominant negative effect on their function. Thus, *Trps1* must be downregulated upon odontoblast maturation to allow for the progress of dentin formation beyond the initiation stage. Our data suggest that *Trps1* may function in maintaining the preodontoblast stage of development by restricting the expression of multiple key mineralization proteins, specifically those involved in later phases of mineralization. These data provide an *in vivo* validation of our previous *in vitro* studies and further support our conclusion that *Trps1* acts as a repressor of circumpulpal dentin formation.

Author Contributions

C.G. Mobley, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; M. Kuzynski, contributed to design, data analysis and interpretation, critically revised the manuscript; H. Zhang, P. Jani, contributed to data acquisition, critically revised the manuscript; C. Qin, contributed to conception, design, data analysis and interpretation, critically revised the manuscript; D. Napierala, contributed to conception, design, data analysis and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This study was supported by grants from the National Institutes of Health: R01DE023083 (to D.N.), F31DE024926 (to M.K.), and R01DE022549 (to C.Q.). We thank Dr. Rajiv Kumar (Mayo Clinic, Rochester, MN, USA) for kindly providing Phex antibodies and Dr. Peter Rowe (University of Kansas Medical Center, Kansas City, KS, USA) for kindly providing MEPE antibodies. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

- Arana-Chavez VE, Massa LF. 2004. Odontoblasts: the cells forming and maintaining dentine. *Int J Biochem Cell Biol.* 36(8):1367–1373.
- Bai X, Miao D, Panda D, Grady S, McKee MD, Goltzman D, Karaplis AC. 2002. Partial rescue of the Hyp phenotype by osteoblast-targeted PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) expression. *Mol Endocrinol.* 16(12):2913–2925.
- Barron MJ, McDonnell ST, Mackie I, Dixon MJ. 2008. Hereditary dentine disorders: dentinogenesis imperfecta and dentine dysplasia. *Orphanet J Rare Dis.* 3:31.
- Boskey A, Frank A, Fujimoto Y, Spevak L, Verdelis K, Ellis B, Troiano N, Philbrick W, Carpenter T. 2009. The PHEX transgene corrects mineralization defects in 9-month-old hypophosphatemic mice. *Calcif Tissue Int.* 84(2):126–137.
- Butler WT. 1998. Dentin matrix proteins. *Eur J Oral Sci.* 106 (Suppl 1):204–210.
- Butler WT, Ritchie H. 1995. The nature and functional significance of dentin extracellular matrix proteins. *Int J Dev Biol.* 39(1):169–179.
- Davideau JL, Papagerakis P, Hotton D, Lezot F, Bernal A. 1996. In situ investigation of vitamin D receptor, alkaline phosphatase, and osteocalcin gene expression in oro-facial mineralized tissues. *Endocrinology.* 137(8):3577–3585.
- Descroix V, Kato S, Lezot F, Bernal A. 2010. Physiopathology of dental rickets in vitamin D receptor-ablated mice. *J Dent Res.* 89(12):1427–1432.
- Embery G, Hall R, Waddington R, Septier D, Goldberg M. 2001. Proteoglycans in dentinogenesis. *Crit Rev Oral Biol Med.* 12(4):331–349.
- Foster BL, Ramnitz MS, Gafni RI, Burke AB, Boyce AM, Lee JS, Wright JT, Akintoye SO, Somerman MJ, Collins MT. 2014. Rare bone diseases and their dental, oral, and craniofacial manifestations. *J Dent Res.* 93(7 Suppl):7S–19S.
- Gibson MP, Zhu Q, Wang S, Liu Q, Liu Y, Wang X, Yuan B, Ruest LB, Feng JQ, D'Souza RN, et al. 2013. The rescue of dentin matrix protein 1 (DMP1)-deficient tooth defects by the transgenic expression of dentin sialophosphoprotein (DSPP) indicates that DSPP is a downstream effector molecule of DMP1 in dentinogenesis. *J Biol Chem.* 288(10):7204–7214.
- Hu JC, Simmer JP. 2007. Developmental biology and genetics of dental malformations. *Orthod Craniofac Res.* 10(2):45–52.
- Kantaputra P, Miletich I, Ludecke HJ, Suzuki EY, Praphanphoj V, Shivdasani R, Wuelling M, Vortkamp A, Napierala D, Sharpe PT. 2008. Tricho-rhino-phalangeal syndrome with supernumerary teeth. *J Dent Res.* 87(11):1027–1031.
- Kuzynski M, Goss M, Bottini M, Yadav MC, Mobley C, Winters T, Poliard A, Kellermann O, Lee B, Millan JL, et al. 2014. Dual role of the Trps1 transcription factor in dentin mineralization. *J Biol Chem.* 289(40):27481–27493.
- Linde A, Goldberg M. 1993. Dentinogenesis. *Crit Rev Oral Biol Med.* 4(5):679–728.
- MacDougall M, Dong J, Acevedo AC. 2006. Molecular basis of human dentin diseases. *Am J Med Genet A.* 140(23):2536–2546.
- Maciejewska I, Chomik E. 2012. Hereditary dentine diseases resulting from mutations in DSPP gene. *J Dent.* 40(7):542–548.
- McKee MD, Hoac B, Addison WN, Barros NM, Millan JL, Chaussain C. 2013. Extracellular matrix mineralization in periodontal tissues: noncollagenous matrix proteins, enzymes, and relationship to hypophosphatasia and X-linked hypophosphatemia. *Periodontology* 2000. 63(1):102–122.
- Napierala D, Sun Y, Maciejewska I, Bertin TK, Dawson B, D'Souza R, Qin C, Lee B. 2012. Transcriptional repression of the Dspp gene leads to dentinogenesis imperfecta phenotype in Col1a1-Trps1 transgenic mice. *J Bone Miner Res.* 27(8):1735–1745.
- Opsahl Vital S, Gaucher C, Bardet C, Rowe PS, George A, Linglart A, Chaussain C. 2012. Tooth dentin defects reflect genetic disorders affecting bone mineralization. *Bone.* 50(4):989–997.
- Rafaelen SH, Raeder H, Fagerheim AK, Knappskog P, Carpenter TO, Johansson S, Bjerknes R. 2013. Exome sequencing reveals FAM20c mutations associated with fibroblast growth factor 23-related hypophosphatemia, dental anomalies, and ectopic calcification. *J Bone Miner Res.* 28(6):1378–1385.
- Ravindran S, George A. 2014. Multifunctional ECM proteins in bone and teeth. *Exp Cell Res.* 325(2):148–154.
- Ruch JV, Lesot H, Begue-Kirn C. 1995. Odontoblast differentiation. *Int J Dev Biol.* 39(1):51–68.
- Ruchon AF, Tenenhouse HS, Marcinkiewicz M, Siegfried G, Aubin JE, DesGroseillers L, Crine P, Boileau G. 2000. Developmental expression and tissue distribution of Phex protein: effect of the Hyp mutation and relationship to bone markers. *J Bone Miner Res.* 15(8):1440–1450.
- Souza AP, Kobayashi TY, Lourenco Neto N, Silva SM, Machado MA, Oliveira TM. 2013. Dental manifestations of patient with vitamin D-resistant rickets. *J Appl Oral Sci.* 21(6):601–606.
- Souza MA, Soares Junior LA, Santos MA, Vaisbich MH. 2010. Dental abnormalities and oral health in patients with Hypophosphatemic rickets. *Clinics (Sao Paulo).* 65(10):1023–1026.
- Sreenath T, Thyagarajan T, Hall B, Longenecker G, D'Souza R, Hong S, Wright JT, MacDougall M, Sauk J, Kulkarni AB. 2003. Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. *J Biol Chem.* 278(27):24874–24880.
- Staines KA, Mackenzie NC, Clarkin CE, Zelenchuk L, Rowe PS, MacRae VE, Farquharson C. 2012. MEPE is a novel regulator of growth plate cartilage mineralization. *Bone.* 51(3):418–430.
- Sun Y, Lu Y, Chen S, Prasad M, Wang X, Zhu Q, Zhang J, Ball H, Feng J, Butler WT, et al. 2010. Key proteolytic cleavage site and full-length form of DSPP. *J Dent Res.* 89(5):498–503.
- Thompson DL, Sabbagh Y, Tenenhouse HS, Roche PC, Drezner MK, Salisbury JL, Grande JP, Poeschla EM, Kumar R. 2002. Ontogeny of Phex/PHEX protein expression in mouse embryo and subcellular localization in osteoblasts. *J Bone Miner Res.* 17(2):311–320.
- Wang X, Wang S, Li C, Gao T, Liu Y, Rangiani A, Sun Y, Hao J, George A, Lu Y, et al. 2012. Inactivation of a novel FGF23 regulator, FAM20C, leads to hypophosphatemic rickets in mice. *PLoS Genet.* 8(5):e1002708.
- Wang X, Wang S, Lu Y, Gibson MP, Liu Y, Yuan B, Feng JQ, Qin C. 2012. FAM20C plays an essential role in the formation of murine teeth. *J Biol Chem.* 287(43):35934–35942.
- Yamakoshi Y. 2009. Dentinogenesis and Dentin Sialophosphoprotein (DSPP). *J Oral Biosci.* 51(3):134.
- Zhu Q, Gibson MP, Liu Q, Liu Y, Lu Y, Wang X, Feng JQ, Qin C. 2012. Proteolytic processing of dentin sialophosphoprotein (DSPP) is essential to dentinogenesis. *J Biol Chem.* 287(36):30426–30435.
- Zhu Q, Prasad M, Kong H, Lu Y, Sun Y, Wang X, Yamoah A, Feng JQ, Qin C. 2012. Partial blocking of mouse DSPP processing by substitution of Gly(451)-Asp(452) bond suggests the presence of secondary cleavage site(s). *Connect Tissue Res.* 53(4):307–312.