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ORIGINAL RESEARCH

Deficiency of Trps1 in Cementoblasts Impairs Cementogenesis and Tooth Root Formation

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Abstract

Cementum is the least studied of all mineralized tissues and little is known about mechanisms regulating its formation. Therefore, the goal of this study was to provide new insights into the transcriptional regulation of cementum formation by determining the consequences of the deficiency of the Trps1 transcription factor in cementoblasts. We used *Trps1*^{Col1a1} cKO (*2.3Co1a1-Cre*^{ERT2}*;Trps1*$^{fl/fl}$) mice, in which *Trps1* is deleted in cementoblasts. Micro-computed tomography analyses of molars of 4-week-old males and females demonstrated significantly shorter roots with thinner mineralized tissues (root dentin and cementum) in *Trps1*^{Col1a1} cKO compared to WT mice. Semi-quantitative histological analyses revealed a significantly reduced area of cellular cementum and localized deficiencies of acellular cementum in *Trps1*^{Col1a1} cKO mice. Immunohistochemical analyses revealed clustering of cementoblasts at the apex of roots, and intermittent absence of cementoblasts on *Trps1*^{Col1a1} cKO cementum surfaces. Fewer Osterix-positive cells adjacent to cellular cementum were also detected in *Trps1*^{Col1a1} cKO compared to WT mice. Decreased levels of tissue-nonspecific alkaline phosphatase (TNAP), an enzyme required for proper cementogenesis, were apparent in cementum, periodontal ligament, and alveolar bone of *Trps1*^{Col1a1} cKO. There were no apparent differences in levels of bone sialoprotein (Bsp) in cementum. Quantitative analyses of picrosirius red-stained periodontal ligament revealed shorter and disorganized collagen fibers in *Trps1*^{Col1a1} cKO mice demonstrating impaired periodontal structure. In conclusion, this study has identified Trps1 transcription factor as one of the important regulators of cellular and acellular cementum formation. Furthermore, this study suggests that Trps1 supports the function of cementoblasts by upregulating expression of the major proteins required for cementogenesis, such as Osterix and TNAP.

Keywords TRPS1 · Cementum · Cementoblast · Tooth root · Periodontium · Conditional knockout mice

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Introduction

Cementum is a mineralized tissue that covers the root of the tooth. It serves as the attachment site for the periodontal ligament that anchors the tooth to the alveolar bone, hence it is crucial for the structural integrity of the periodontium. The cementum at the apical portion of the root contains cementocytes and it is called the cellular cementum, while the acellular cementum is a thin layer of the extracellular matrix (ECM) covering the cervical portion of the root. In comparison with other mineralized tissues, the cementum and the cells that make it–cementoblasts–are far less characterized. The molecular composition of the cementum is similar to the bone and dentin ECM. It consists of the collagen type I network and is enriched in tissue-nonspecific alkaline phosphatase (TNAP), bone sialoprotein (Bsp), and dentin matrix protein 1 (Dmp1). Due to the similarity of the cementum and the bone tissue, transcriptional mechanisms regulating cementum formation likely share similarities with those in the bone. Accordingly, the major osteogenic transcription factor Osterix (Osx/Sp7) is critical not only for osteogenesis but also for cementogenesis as demonstrated by significantly decreased cellular cementum formation in mice with conditional deletion of Osx/Sp7 in cementoblasts [1]. The acellular cementum, on the other hand, has been shown to be affected by disturbed phosphate/pyrophosphate (P_i/PP_i) balance [2–7]. This has been demonstrated, for example, in human patients with hypophosphatemic rickets and hypophosphatasia, and was observed in mouse models of these disorders [2, 8–11]. Even the acellular cementum deficiency in *Bsp* KO mice has been attributed to the secondary dysregulation of genes controlling P_i/PP_i ratio [4]. While the human patients' and animal models' studies provide strong evidence that the formation and homeostasis of cementum rely on the adequate levels of the Osx/Sp7 transcription factor and proteins regulating P_i and PP_i balance, the transcriptional regulation of the cementum is poorly understood.

In our previous in vitro studies of mineralizing cells, we uncovered that the Trps1 transcription factor supports expression of several genes important for cementogenesis, including Osx/Sp7, TNAP, and Bsp [12]. Trps1 is a GATA-type transcription factor involved in endochondral bone development, tooth formation and regulation of the mineralization process [13–16]. In humans, heterozygous mutations in the *TRPS1* gene cause trichorhinophalangeal syndrome – a skeletal dysplasia with low bone mass and dental abnormalities [14]. The broad array of dental phenotypes presented by TRPS patients, which includes supernumerary teeth, microdontia, predisposition to severe dental caries, and delayed tooth eruption, indicates that this transcription factor is involved in multiple aspects of tooth formation [13, 17]. During tooth development, Trps1 is highly expressed in dental mesenchymal cells, including dental follicle cells, which give rise to cementoblasts [13]. Our previous studies of $Trps1^{-/-}$ mice revealed that Trps1 supports proliferation of cells in the developing tooth organ [18], which may contribute to the microdontia phenotype frequently presented in TRPS patients. However, due to neonatal lethality of $Trps1^{-/-}$ mice and mild phenotypes of $Trps1^{+/-}$ mice, the studies addressing the role of Trps1 in formation of the tooth crown and root, and dental mineralized tissues have been very limited.

Recently, we have generated *Trps1* conditional knockout (cKO) mice with postnatal deletion of *Trps1* driven by tamoxifen-inducible Cre expressed under the control of the *2.3kbCol1a1* promoter ($2.3kbCol1a1\text{-}Cre^{ERT2}$;$Trps1^{fl/fl}$ a.k.a. $Trps1^{Col1a1}$ cKO mice). In $Trps1^{Col1a1}$ cKO mice, *Trps1* is deleted in osteoblasts, odontoblasts, and cementoblasts. Analyses of the tooth crown revealed wider predentin, decreased volume of dentin, globular pattern of dentin mineralization, as well as localized enamel defects [19], which together suggest that compromised quality of the dentin and enamel contributes to increased susceptibility and severity of dental caries in TRPS patients. Exposed tooth roots were also observed in $Trps1^{Col1a1}$ cKO mice, which may be indicative of disrupted periodontal structure.

Based on the previous studies implicating Trps1 in the regulation of the mineralization process [12, 16, 19, 20] and high and specific *Trps1* expression in cells giving rise to cementoblasts [13], we hypothesize that Trps1 is involved in cementum formation through regulation of gene expression in cementoblasts. The goal of this study was to determine the consequences of the *Trps1* deficiency in cementoblasts on the molecular characteristics of these cells, and their function, i.e., the formation of the cementum in vivo. To this end, we used previously generated $Trps1^{Col1a1}$ cKO mice [19] and a set of complementary approaches assessing the quantity and mineralization of the cementum, and expression of proteins characteristic for cementoblasts.

Results

Expression of Trps1 in Cementum and Alveolar Bone

In our previous studies, we observed exposed tooth roots in $Trps1^{Col1a1}$ cKO mice [19], which suggests the compromised structure of the periodontal complex. Since the formation of the sound periodontal structure depends on the formation of the cementum as well as alveolar bone, first, we analyzed whether Trps1 is expressed in cells of these tissues. Immunohistochemical staining of the mandibles of 4 wk old WT mice detected the presence of the Trps1 protein in the nuclei

of cementocytes and cementoblasts in the cellular cementum, as well as osteoblasts and osteocytes in the alveolar bone (Fig. 1 left panel). In the *Trps1*^{Col1a1} cKO mice, no specific immunostaining signal was detected in these cells indicating the effective deletion of *Trps1* by tamoxifen-induced Cre recombinase (Fig. 1 right panel). In conclusion, these IHC analyses demonstrate high Trps1 expression in cells contributing to the formation and homeostasis of the periodontal complex. Taking into consideration that cementum is a poorly studied tissue, and little is known about the transcriptional regulation of the formation and homeostasis of this tissue, we focused the studies of *Trps1*^{Col1a1} cKO mice on the characterization of the cementum.

Impaired Root Formation in *Trps1*^{Col1a1} cKO Mice

To evaluate the consequences of the *Trps1* deficiency in cementoblasts and odontoblasts on tooth root formation, we performed quantitative µCT analyses comparing roots of mandibular first molars of 4 wk old WT and *Trps1*^{Col1a1} cKO mice. These analyses revealed that *Trps1*^{Col1a1} cKO males and females have significantly shorter (Fig. 2A) and overall smaller (Fig. 2B, total root volume) roots than WT mice. Of note, there is no difference in the overall body size and tooth crown size between WT and *Trps1*^{Col1a1} cKO females [19]. The mineralized tissue (cementum and root dentin) fraction in the roots of *Trps1*^{Col1a1}cKO males and females was also significantly lower than in WT mice (Fig. 2B). However, there was no difference in the overall mineral density of the root tissues (Fig. 2B). More detailed analyses revealed that the mineralized root tissue thickness ranged from 24 µm to 120 µm with most of the tissue thicker than 60 µm in WT females, while in the *Trps1*^{Col1a1} cKO females, the mineralized root tissue thickness ranged from 12 µm to 84 µm with most of the tissue thickness below 60 µm (Fig. 2C). A similar shift of the root mineralized tissue thickness was detected in *Trps1*^{Col1a1} cKO males. In *Trps1*^{Col1a1} cKO males, most of the root mineralized tissues was thinner than 36 µm, and the thickness ranged from 12 µm to 48 µm, while in the WT males, the tissue thickness ranged from 24 µm to 102 µm with most of the tissue thicker than 36 µm (Fig. 2C). Thus, the quantitative µCT analyses revealed that *Trps1* deficiency in odontoblasts and cementoblasts significantly impairs the size of the roots as well as the formation of root mineralized tissues in both male and female mice.

Reduced Cementum Formation and Abnormal Distribution of Cementoblasts in *Trps1*^{Col1a1} cKO Mice

It is difficult to distinguish cementum from dentin using µCT, therefore to determine whether the deficiency of cementum contributes to the decreased mineralized tissue thickness in *Trps1*^{Col1a1} cKO mice, we used histological analyses of root sections stained with toluidine blue, which differentiates between cementum and dentin. These analyses were done in females, because µCT results showed that both sexes are affected in the same way, but the phenotype is more severe in females. Toluidine blue staining of root histological sections was used for semi-quantitative histological analyses of

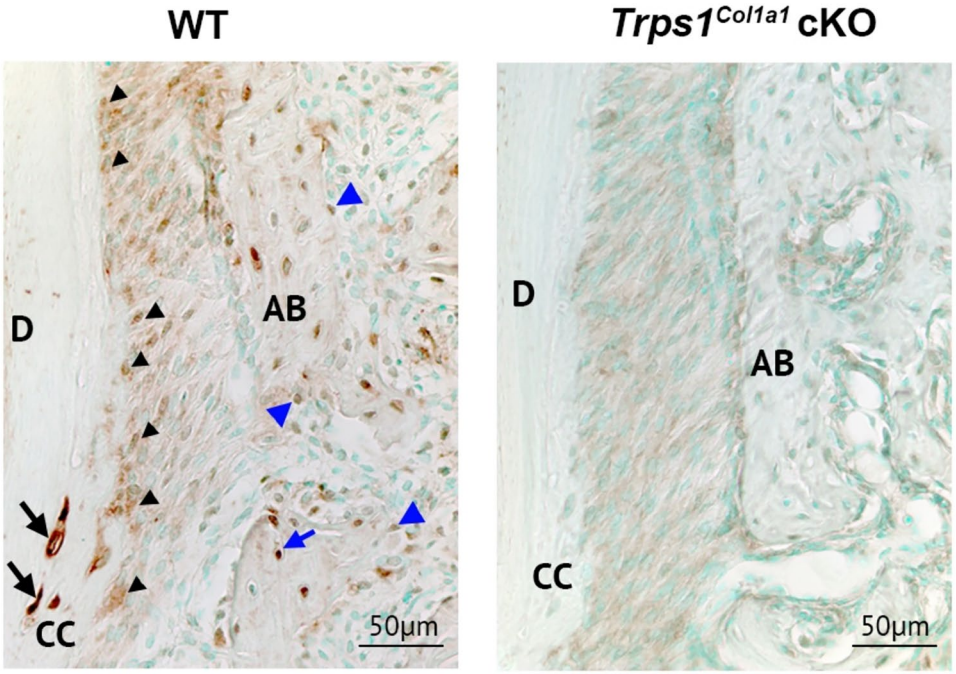
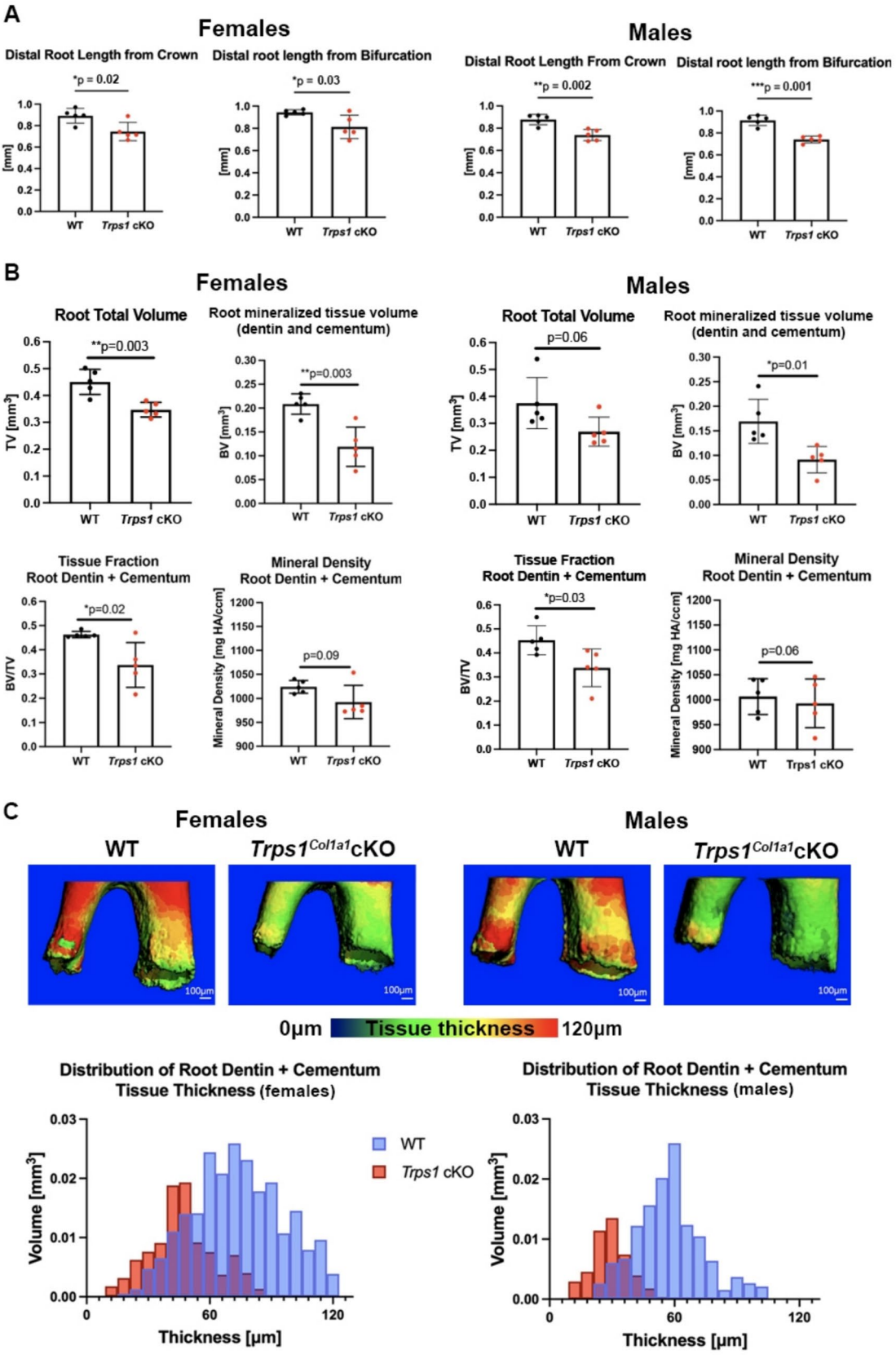


Fig. 1 Efficient depletion of Trps1 from cementoblasts and cementocytes, as well as osteoblasts and osteocytes of the alveolar bone in *Trps1*^{Col1a1} cKO mice. Trps1 immunostaining images showing localization of the Trps1 protein in tooth root and alveolar bone of mandibular first molars in 4 wk old WT and *Trps1*^{Col1a1} cKO mice. In WT mice, Trps1 is highly expressed in cementoblasts (black arrowheads), cementocytes (black arrows), osteoblasts (blue arrowheads), and osteocytes (blue arrows). *Trps1*^{Col1a1} cKO mice have diminished expression of Trps1 in root and alveolar bone. CC–cellular cementum; D–dentin; AB–alveolar bone


A
Females
Distal Root Length from Crown
*p = 0.02
[mm]
WT
Trps1 cKO
Distal root length from Bifurcation
*p = 0.03
Males
Distal Root Length From Crown
**p = 0.002
Distal root length from Bifurcation
***p = 0.001
B
Females
Root Total Volume
**p=0.003
TV [mm³]
Root mineralized tissue volume (dentin and cementum)
**p=0.003
BV [mm³]
Males
Root Total Volume
p=0.06
Root mineralized tissue volume (dentin and cementum)
*p=0.01
Tissue Fraction Root Dentin + Cementum
*p=0.02
BV/TV
Mineral Density Root Dentin + Cementum
p=0.09
Mineral Density [mg HA/ccm]
*p=0.03
p=0.06
Trps1 cKO
C
Females
Males
WT
Trps1Col1a1cKO
100µm
0µm
Tissue thickness
120µm
Distribution of Root Dentin + Cementum Tissue Thickness (females)
Distribution of Root Dentin + Cementum Tissue Thickness (males)
Volume [mm³]
Thickness [µm]
WT
Trps1 cKO

◂**Fig. 2** Shorter and thinner tooth roots in *Trps1*$^{Col1a\ 1}$cKO compared with WT mice. Results of quantitative μCT analyses of tooth roots in mandibular first molars 4 wk old WT and *Trps1*^{Col1a1} cKO mice ($n=5$/genotype/sex; *$p<0.05$, **$p<0.01$, ***$p<0.001$). **A** Measurements of the distal root length. **B** Measurements of the root total volume, volume of root dentin and cementum, tissue fraction of root dentin and cementum, and mineral density of root dentin and cementum. **C** Representative 3D μCT images of the roots. The mineralized tissue was pseudo-colored based on the tissue thickness; the tissue thickness color scale is shown below the images. The graphs below the 3D images show the comparison of the quantity (volume) of the root mineralized tissue with specific thickness in WT and *Trps1*^{Col1a1} cKO mice

cementum. Low-magnification microscopic images showed shorter distal roots in *Trps1*^{Col1a1} cKO mice, consistent with the μCT results (Fig. 3A). Measurements of the area of the cellular cementum demonstrated significantly reduced area in *Trps1*^{Col1a1} cKO compared with WT mice (Fig. 3A). While the measurements of the acellular cementum area did not show the statistically significant difference between WT and *Trps1*^{Col1a1} cKO mice, an irregular thickness and partial deficit of the acellular cementum in *Trps1*^{Col1a1} cKO mice was apparent on high magnification images (Fig. 3A, white arrows). Hence, the histological analyses demonstrated that the *Trps1* deficiency in cementoblasts affects both the cellular and acellular cementum.

To gain insights about the cause of the cementum defects in *Trps1*^{Col1a1} cKO mice, we first assessed localization of cementoblasts using immunohistostaining detecting Tubb3 (a member of the β tubulin protein family) that has been shown to be a cementoblasts marker [21]. As expected for cementoblasts, Tubb3-positive cells were uniformly lining up along the cellular cementum in WT mice (Fig. 3B). In contrast, in *Trps1*^{Col1a1} cKO mice, cementoblasts were unevenly distributed and clustered at the apical portion of the cementum; at some places, the cementum surface was not covered by cementoblasts (Fig. 3B). In comparison with WT mice, in *Trps1*^{Col1a1} cKO, fewer Tubb3-positive cells were observed on the cellular cementum surface beyond the apical end of the root. These analyses suggest that *Trps1* deficiency in cementoblasts leads to either defective cementoblast differentiation or premature loss of cementoblasts.

To assess the cementoblast function–the secretion of the organic components of the cementum ECM and its mineralization–we used fluorescent double-labeling (alizarin red followed by calcein) and microscopic imaging of fluorochromes incorporated into mineralizing ECM in a 7-days interval. We observed the decreased distance between the fluorescent labels in the cellular cementum and root dentin of *Trps1*^{Col1a1} cKO mice in comparison with WT (Fig. 3C), which indicates that less mineralized ECM was formed in *Trps1*^{Col1a1} cKO mice than in WT mice in the same period of time. Hence, the *Trps1* deficiency in cementoblasts impairs the secretion of the cementum ECM and its mineralization.

Decreased Formation of the Alveolar Bone in *Trps1*^{Col1a1} cKO Mice

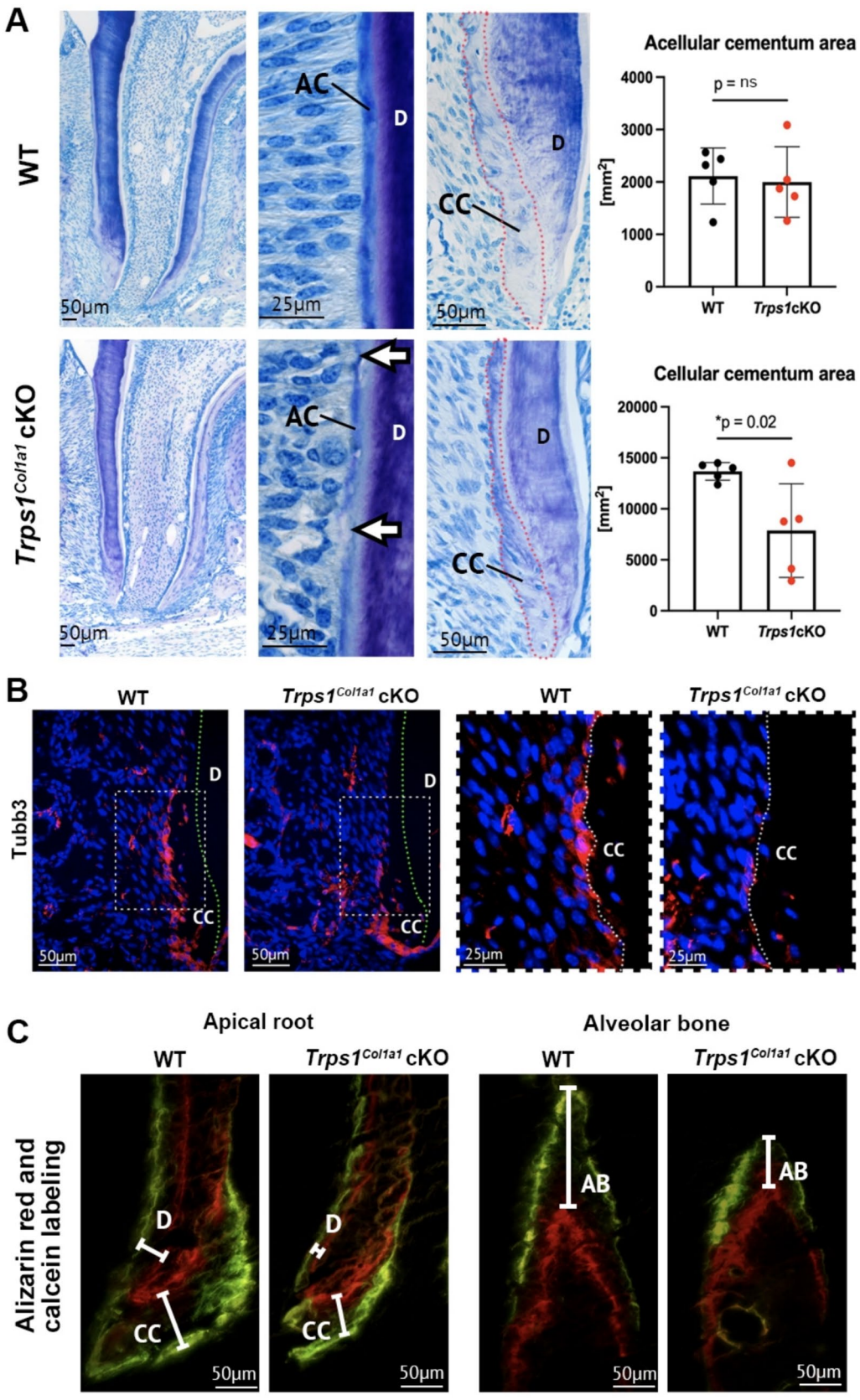
The fluorescent double-labeling analyses also revealed the decreased mineral apposition in the alveolar bone of *Trps1*^{Col1a1} cKO mice (Fig. 3C). Following on this observation, we performed μCT analyses of the alveolar bone of 4wk old male and female mice. These analyses revealed significantly smaller alveolar bones (Total Tissue Volume—TV) and decreased Bone Volume Fraction (BV/TV) in *Trps1*^{Col1a1} cKO males and females in comparison with WT mice (Fig. 4). These results suggest that deficiency of the alveolar bone in *Trps1*^{Col1a1} cKO mice may contribute to the exposed roots phenotype that we reported in our previous study [19].

Differential Effect of *Trps1* Deficiency on Expression of the Cementum and Alveolar Bone Proteins

The cementum and bone ECM have similar molecular composition, and Osx has been shown to be a major transcription factor regulating formation of these mineralized tissues. Since our previous in vitro studies demonstrated that *Trps1* deficiency in cells producing mineralized ECM results in dramatic downregulation of Osx [12], we first investigated the changes in the Osx protein levels using IHC. Consistent with the previous in vitro data, IHC analyses detected fewer Osx-positive cells in the periodontal ligament area adjacent to the acellular cementum as well as in the cellular cementum of *Trps1*^{Col1a1} cKO mice in comparison with WT (Fig. 5A). Furthermore, decreased expression of Osx was also noticeable in osteoblasts on the alveolar bone surface (Fig. 5A). These results demonstrate that Trps1 supports expression of Osx in cementoblasts and osteoblasts of the alveolar bone.

Next, we analyzed expression of TNAP, a major enzyme supporting formation of both cementum and bone. In particular, the expression of TNAP in the early root formation stage is essential for formation of the acellular cementum and sound cementum-periodontal ligament interface [22]. As expected, in WT mice, TNAP was widely detected in the periodontal ligament adjacent to acellular and cellular cementum (Fig. 5B). High levels of TNAP were also detected on the surface of the alveolar bone of WT mice. In contrast, the levels of TNAP in the periodontal ligament and alveolar bone were noticeably lower in *Trps1*^{Col1a1} cKO mice (Fig. 5B). This effect of *Trps1* deficiency on TNAP expression is also consistent with the previously published in vitro studies [12]. Hence, our data show that Trps1 supports expression of TNAP in the dentoalveolar structure.

Bsp is commonly used as a marker of the acellular cementum, but it is also highly expressed in bone. Bsp expression is also critical for formation of the acellular cementum [23,


A
WT
Trps1Col1a1 cKO
AC
D
CC
50μm
25μm
Acellular cementum area
p = ns
[mm2]
4000
3000
2000
1000
0
WT
Trps1cKO
Cellular cementum area
*p = 0.02
20000
15000
10000
5000
B
Tubb3
WT
Trps1Col1a1 cKO
C
Apical root
Alveolar bone
Alizarin red and calcein labeling
AB

◂**Fig. 3** Histological analyses of cementum in mandibular first molars of 4wk old WT and *Trps1*^{Col1a1} cKO females demonstrating impaired formation of the cellular and acellular cementum, and uneven distribution of cementoblasts in *Trps1*^{Col1a1} cKO mice. **A** Representative images of toluidine blue-stained root sections and results of semi-quantitative analyses of the cementum area. Left panel: low magnification images showing the general appearance of the roots; middle panel: higher magnification images showing the irregular thickness and partial absence (white arrows) of the acellular cementum (AC) in *Trps1*^{Col1a1} cKO mice; right panel: higher magnification images showing decreased area of the cellular cementum (CC, outlined by red dotted lines) in *Trps1*^{Col1a1} cKO mice compared to WT. The graphs show the results of semi-quantitative analyses of the acellular (top) and cellular (bottom) cementum area ($n = 5$ females/genotype; $^{*}p < 0.05$). **B** Results of the IHC for Tubb3 (a cementoblasts marker) in tooth roots showing uneven distribution of cementoblasts in *Trps1*^{Col1a1} cKO mice. The dotted areas on the left-side images indicate the areas showed in higher magnification on the right-side images. Note the surfaces of cellular cementum not covered by Tubb3-positive cells in *Trps1*^{Col1a1} cKO mice. **C** Fluorescent microscopy images showing mineralized tissues of the tooth root (CC–cellular cementum and D–dentin) and the alveolar bone (AB). The distance between alizarin red and calcein lines indicates the mineralized tissue formed within 7 days. Note the decreased distance between the fluorescent labels in cementum, dentin, and alveolar bone in *Trps1*^{Col1a1} cKO compared with WT mice. CC–cellular cementum; AB–alveolar bone; D–dentin

24]. Since defects in the acellular cementum were detected in *Trps1*^{Col1a1} cKO mice (Fig. 3A), we compared Bsp levels in *Trps1*^{Col1a1} cKO and WT mice. Although the IHC analyses did not show an apparent difference in Bsp levels in the acellular and cellular cementum of WT and *Trps1*^{Col1a1} cKO mice, the distribution of the Bsp protein accentuated the irregularity of the acellular cementum in *Trps1*^{Col1a1} cKO mice (Fig. 5C). Notably, the IHC analyses detected a striking difference in Bsp levels in the alveolar bone of WT mice and *Trps1*^{Col1a1} cKO mice (Fig. 5C) with overall lower levels of Bsp in *Trps1*^{Col1a1} cKO alveolar bone. Moreover, while high levels of Bsp were detected throughout the alveolar bone ECM of WT mice, only the margins of the alveolar bone ECM were positive for the Bsp in *Trps1*^{Col1a1} cKO mice (Fig. 5C). Hence, *Trps1* deficiency affects Bsp expression differently in the cementum and alveolar bone tissues. In summary, the results of IHC analyses of major proteins important for cementum and alveolar bone formation revealed tissue-specific consequences of *Trps1* deficiency.

Impaired Organization of Collagen Fibers in Periodontal Ligament in *Trps1*^{Col1a1} cKO Mice

Cementum serves as the attachment site for the periodontal ligament anchoring the tooth to the alveolar bone. Based on the results showing irregular acellular cementum and decreased levels of TNAP, which causes loss of attachment in hypophosphatasia patients, we speculated that the periodontal ligament may also be impaired in *Trps1*^{Col1a1} cKO mice. To assess the organization of collagen fibers in the periodontal ligament, Picrosirius red staining was performed. Picrosirius red-stained collagen fibers appear as bright yellow or orange under polarized light when densely packed and thick, while thin collagen fibers are green. Microscopic imaging under polarized light revealed that in WT mice, the periodontal ligament was composed of regularly oriented dense orange/yellow collagen fibers connecting the root to the alveolar bone (Fig. 6B, D). On the other hand, there were fewer yellow collagen fibers in *Trps1*^{Col1a1} cKO mice (Fig. 6B, D), indicating thinner and less organized collagen. Quantitative analyses demonstrated a significantly reduced area occupied by well-organized thick alveolar crest fibers in the cervical portion of the periodontal ligament (Fig. 6B, C) as well as the oblique fibers in the apical portion of the periodontal ligament (Fig. 6D, E) in *Trps1*^{Col1a1} cKO mice in comparison with WT.

Discussion

Cementum is the least studied tissue of all mammalian mineralized tissues. Although, its molecular composition is fairly well characterized, little is known about the transcriptional regulation of the cementum formation. Here, using a conditional KO mouse approach with *Trps1* deletion in cementoblasts (Fig. 1), we demonstrate that the Trps1 transcription factor is required for the proper formation of the tooth root and cementum in both male and female mice, as well as for the distribution and function of cementoblasts. Our study revealed that deletion of *Trps1* in cementoblasts impairs formation of the cellular and acellular cementum, and results in compromised periodontal structure with less organized collagen fibers. Furthermore, our data suggest that this function of Trps1 is executed, at least in part, through supporting the expression of Osx – the major transcription factor involved in cementogenesis, and TNAP – the enzyme regulating P_i/PP_i balance in the local, cellular environment. Hence, this study broadens the knowledge on the regulation of function of cementoblasts, which are the least studied cells producing mineralized ECM. In addition, this study provides some insights into reported association of *Trps1* with colonization of periodontal pathogens [25] by demonstrating that *Trps1* deficiency results in defective structure of periodontium. Such compromised structure may make the periodontium susceptible to oral bacteria invasion.

The molecular composition of the cementum ECM shares some similarities with bone and dentin suggesting that similar molecular mechanisms govern the formation of these tissues. Recently, we have demonstrated that Trps1 is required for the formation of the sound dentin—teeth of *Trps1*^{Col1a1} cKO mice have reduced dentin layer, expanded predentin, and globular dentin mineralization pattern, which is characteristic for disturbed phosphate

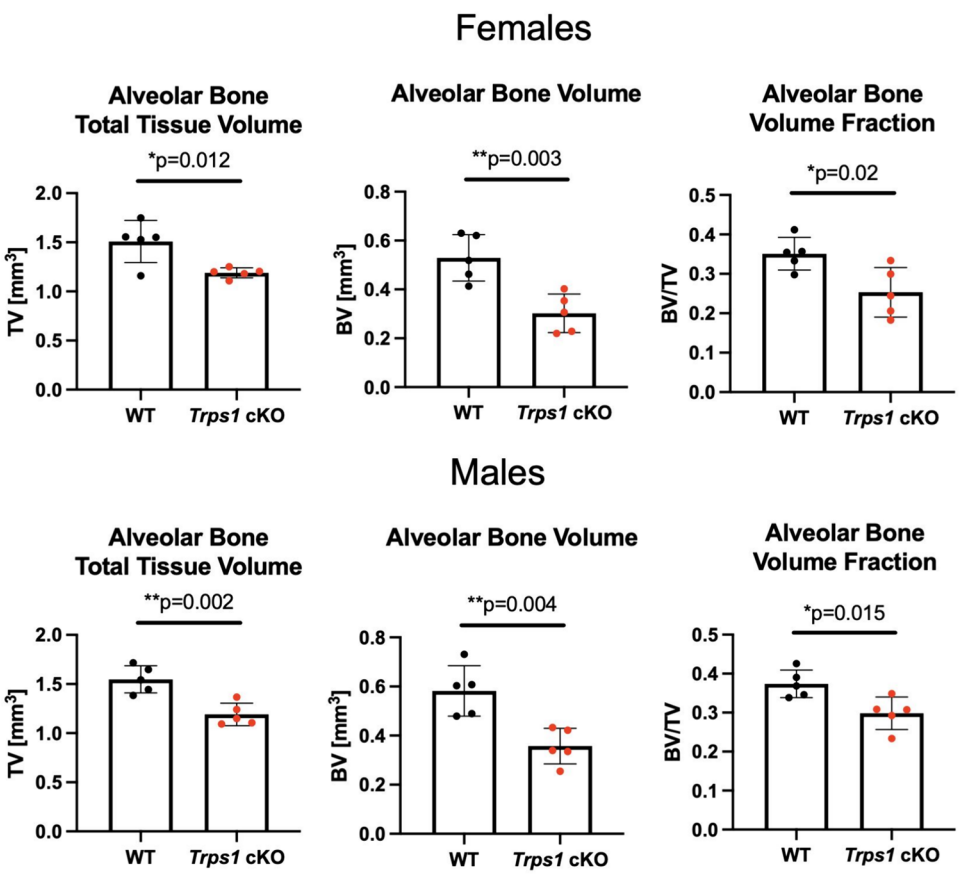


Fig. 4 Results of μCT analyses of alveolar bone of 4 wk old female and male mice ($n=5$ mice/genotype/sex; $*p<0.05$, $**p<0.005$) demonstrating significantly smaller alveolar bones (TV) and lower bone volume fraction (BV/TV) in *Trps1*^{Col1a1} cKO males and females in comparison with WT mice

homeostasis [19]. Furthermore, our previous in vitro data from the analyses of cells derived from preodontoblasts revealed that expression of many genes required for formation of mineralized ECM of dentin and bone is supported by Trps1 [12]. Since many of these genes are also important for the cementum formation, and this tissue is highly sensitive to P_i/PP_i balance, these data prompted us to examine the consequences of *Trps1* deficiency on cementum. Indeed, the μCT analyses of tooth roots of 4 wk old mice revealed significantly reduced thickness of mineralized tissue and the length of the roots in *Trps1*^{Col1a1}cKO males and females (Fig. 2). These data, together with the fluorescent double-labeling of mineralized tissues (Fig. 3C) showing less cementum deposited within the 7 days period in *Trps1*^{Col1a1} cKO mice, demonstrate the importance of Trps1 for the formation of mineralized tissues in tooth roots. The subsequent histological analyses that allowed for a detailed examination of cementum revealed significantly reduced area of the cellular cementum and irregular thickness of a thin layer of the acellular cementum (Fig. 3). Hence, these data together with our previously published dentin phenotype in *Trps1*^{Col1a1} cKO mice, establish Trps1 as one of the transcription factors important for both dentin and cementum formation. It is important to note that, while *Trps1*^{Col1a1} cKO males are significantly smaller than WT, there is no significant difference in the overall body size and crown size between WT and *Trps1*^{Col1a1} cKO females as we reported previously [19]. This indicates that the effect of *Trps1* deficiency on the tooth root size reflects the role of Trps1 specifically in tooth root formation. This is further supported by reduced number of cementoblasts (Fig. 3B) and reduced cementum formation in *Trps1*^{Col1a1} cKO mice (Fig. 3C).

In mice, the formation of cementum continues after 4wk of age (the age analyzed in this study). Although we have not analyzed older mice, the cementoblasts abnormalities (Fig. 3B), and reduced cementum formation rate (Fig. 3C) suggest that the deficiencies in cementum in 4wk old Trps1^{Col1a1} cKO mice do not reflect a simple delay of the tissue formation. The uneven distribution of cementoblasts with their clustering at the apex of the root and their deficit beyond the apical portion of the root (Fig. 3B) suggest that *Trps1* deficiency in cementoblasts leads to their premature loss. Alternatively to the premature cementoblasts apoptosis hypothesis, and based on well demonstrated (in many cell types) function of Trps1 as a pro-proliferative transcription factor [16, 18, 26, 27], it is possible that Trps1-deficient cementoblasts prematurely cease the proliferation. Regardless of the mechanism underlying the cementoblast deficit, it is likely that the deficiency of cementum and shorter roots

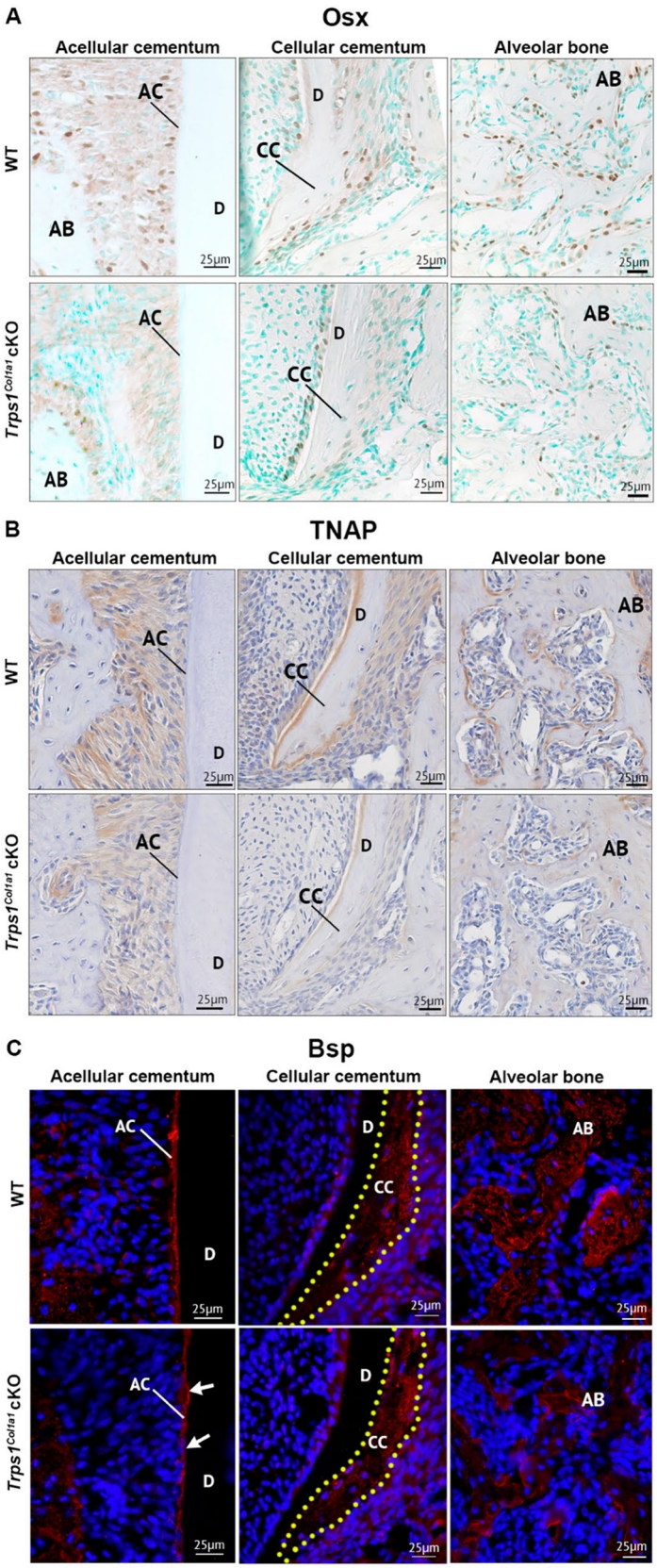


Fig. 5 Tissue-specific consequences of *Trps1* deficiency on expression of proteins important for cementum formation and alveolar bone. Representative microscopic images of IHC on the tooth root with adjacent periodontal ligament and alveolar bone in 4wk old WT and $Trps1^{Col1a1}$ cKO mice. (**A**) IHC for Osx showing fewer Osx-positive cells around the acellular and cellular cementum, and on the alveolar bone surface in $Trps1^{Col1a1}$ cKO mice compared to WT mice. (**B**) IHC for TNAP showing lower TNAP levels around both acellular and cellular cementum and on the alveolar bone surface in $Trps1^{Col1a1}$ cKO mice compared to the WT. (**C**) IHC for Bsp showing comparable levels of Bsp in acellular and cellular cementum of WT and $Trps1^{Col1a1}$ cKO mice, and notably lower levels of Bsp in the alveolar bone of $Trps1^{Col1a1}$cKO mice in comparison with WT. Arrows point at the irregularity of the acellular cementum in the $Trps1^{Col1a1}$ cKO mice. AC–acellular cementum; CC–cellular cementum; D–dentin; AB–alveolar bone

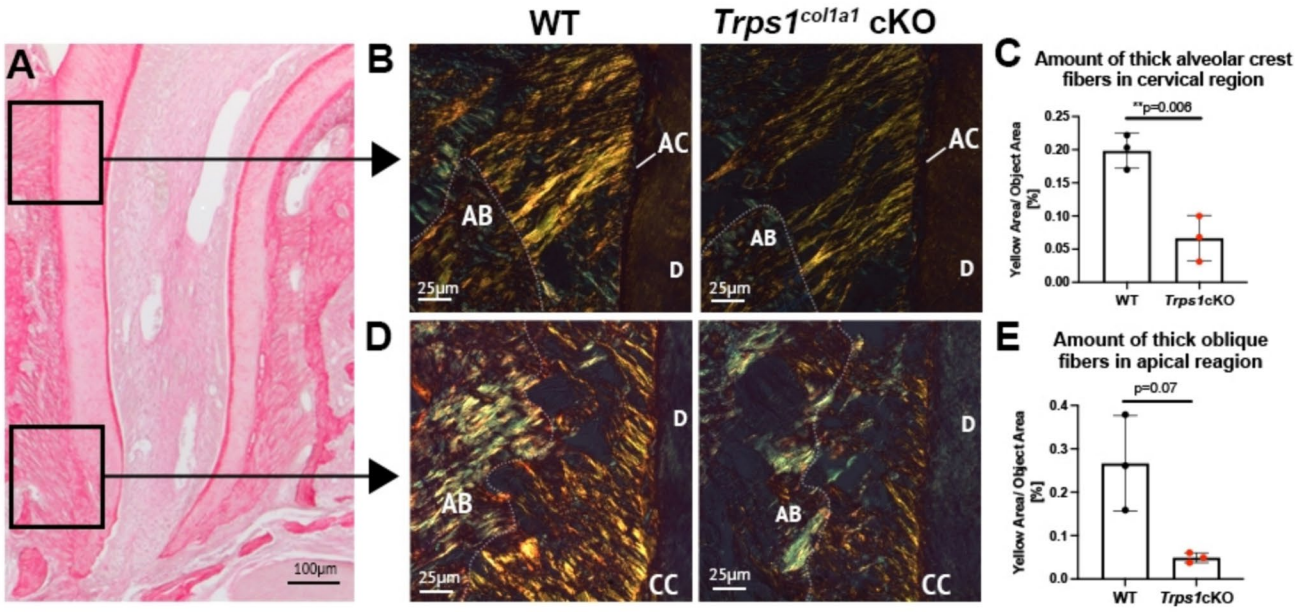


Fig. 6 Impaired organization of collagen fibers in periodontal ligament of *Trps1*^{Col1a1} cKO mice. **A** A representative image of Picrosirius red-stained a molar root viewed under a light microscope (bright field). The black boxes indicate areas imaged under the polarized light in the comparative analyses of WT vs. *Trps1*^{Col1a1} cKO mice. **B** Images of the Picrosirius red-stained cervical region of the periodontal ligament of WT and *Trps1*^{Col1a1} cKO mice viewed under the polarized light. The dotted white line demarcates the alveolar bone/periodontal ligament interface. **C** Quantification of thick collagen fibers in the cervical region expressed as the percentage of the yellow area in the total periodontal ligament area in the analyzed region. **D** Images of the Picrosirius red-stained apical region of the periodontal ligament of WT and *Trps1*^{Col1a1} cKO mice viewed under the polarized light. The dotted white line demarcates the alveolar bone/periodontal ligament interface. **E** Quantification of thick collagen fibers in the apical region expressed as the percentage of the yellow area in the total periodontal ligament area in the analyzed region. Data were obtained from analyses of 3 females/genotype; results from each mouse and mean values ± standard deviation are shown. AC–acellular cementum; CC–cellular cementum; D–dentin; AB–alveolar bone

phenotype would persist at later age in *Trps1*^{Col1a1} cKO mice.

In this study, we also provide some insights into the molecular pathology underlying the tooth root abnormalities in *Trps1*^{Col1a1} cKO mice—likely, the reduced expression of Osx (Fig. 5A) likely is a contributor to the root phenotype in *Trps1*^{Col1a1} cKO mice. Osx has been identified as one of the major transcription factors supporting cementogenesis and tooth root formation as demonstrated by studies of cKO mice with deletion of Osx in cementoblasts [1, 28]. Osx cKO mice have significantly shorter roots due to failed root elongation and deficiency of cellular cementum, but no obvious defects in the tooth crown. Osx regulates the cellular cementum formation through activation of Dkk and subsequent downregulation of Wnt/βcatenin signaling [29]. Importantly, Wnt signaling is important for cementum and root formation [30, 31], and Trps1 has been shown to regulate Wnt signaling in endochondral ossification and hair follicles [32, 33]. Further studies are needed to determine whether Trps1 regulates Osx expression directly or indirectly in cementoblasts, and if Wnt signaling pathway is regulated by Trps1 during cementogenesis. However, the striking similarities between *Trps1*^{Col1a1} cKO and Osx cKO tooth root abnormalities and lower than in WT expression of Osx in *Trps1*-deficient cementoblasts strongly suggests that the tooth root phenotype in *Trps1*^{Col1a1} cKO mice is at least partly caused by the Osx deficiency. Thus, these data together with our previous in vitro studies of odontoblast-like cells [12], place Trps1 upstream of the Osx transcription factor and demonstrate the cell-autonomous requirement of Trps1 for Osx expression in cells committed to formation of mineralized ECM.

Human patients and animal models studies suggest that acellular cementum is more sensitive than cellular cementum to changes in the P_i/PP_i balance [2, 10, 22, 34–36]. For example, acellular cementum deficiency with a concomitant loss of continuity between the root and periodontal ligament have been reported in hypophosphatasia patients [10, 37] and in *Alpl*$^{-/-}$ mice [22, 35, 38] with genetic deficiency of TNAP enzymatic activity, which is a major regulator of the P_i/PP_i ratio in the local extracellular compartment. Similarly, reduced acellular cementum thickness and detachment of periodontal ligament have been described in individuals with genetic forms of hypophosphatemia and in Hyp mice (a model of X-linked hypophosphatemia) [34, 40]. Acellular cementum is also compromised by *Trps1*

deficiency in cementoblasts (Fig. 3A). This is accompanied by reduced TNAP levels throughout the periodontal ligament as well as the cementum and alveolar bone surfaces (Fig. 5B). Interestingly, levels of Bsp in cementum do not seem to be affected by *Trps1* deficiency (Fig. 5C). Since Bsp supports cementum formation through mechanisms independent of P_i/PP_i balance, the results of immunohistochemical analyses suggest that the disturbed P_i/PP_i balance due to TNAP deficiency is likely one of the underlying causes of the acellular cementum pathology in *Trps1*^{Col1a1} cKO mice. Of note unlike in TNAP KO and Bsp KO mice, no detachment of the periodontal ligament was detected in *Trps1*^{Col1a1} cKO mice, demonstrating a milder periodontal structure impairment than in the cases of TNAP or Bsp deficiency.

The importance of the cementum for the function and homeostasis of the periodontium is underscored by periodontal pathologies in individuals with impaired or deficient cementum. For example, individuals with hypophosphatasia demonstrate insufficient periodontal attachment and premature tooth loss due to acellular cementum hypoplasia [8–11]. Consistently, disorganization of periodontal ligament is also apparent in *Trps1*^{Col1a1} cKO mice (Fig. 6). Although no ligament detachment has been observed in *Trps1*^{Col1a1} cKO mice, the disorganization of collagen fibers suggests a compromised periodontal structure, which may predispose to accumulation of oral bacteria. Interestingly, GWAS studies identified *TRPS1* as one of the loci associated with periodontal pathogen colonization [25]. The impaired organization of collagen fibers in *Trps1*^{Col1a1} cKO periodontal ligament suggests that this association may, in part, reflect the role of Trps1 in the structural integrity of the periodontium. However, recent studies have identified Trps1 as one of the key transcription factors regulating TI-Treg cells in the context of cancer malignancy [41]. This points to a new interesting function of Trps1 in the immune response, which may contribute to the periodontal health.

Our analyses of alveolar bone along with cementum revealed differences in the regulation of these two mineralized tissues. Deposition of mineralized extracellular matrix and tissue volume was decreased in both tissues by *Trps1* deficiency (Figs. 3 and 4), so were the levels of Osx and TNAP (Fig. 5A, B). However, changes in the distribution of Bsp in the extracellular matrix were apparent only in alveolar bone of *Trps1*^{Col1a1} cKO mice in comparison with WT (Fig. 5C). While cementum and bone share many similarities, some features are found in bone only. For example, cementum remodeling does not occur under physiological conditions, however it can occur in response to excessive occlusal stress. The observed changes of Bsp distribution in alveolar bone of *Trps1*^{Col1a1} cKO mice highlight the differences between formation and homeostasis of cementum and bone.

In summary, this study identifies the Trps1 transcription factor as a major regulator of the cementoblast function and cementum formation. Furthermore, it expands the knowledge on the role of Trps1 in formation of dental mineralized tissues revealing that not only the proper formation of the tooth crown [19] but also root mineralized tissues require Trps1. Furthermore, the differential effect of *Trps1* deficiency on Bsp levels in cementum and alveolar bone revealed differences in transcriptional regulation of cementum and alveolar bone formation. Importantly, we have demonstrated that expression of Trps1 in cementoblasts supports expression of another major cementoblast-regulating transcription factor (Osterix), and a major enzyme involved in cementum and periodontal ligament formation (TNAP). Hence, this study provides a better understanding of the transcriptional regulation of cellular and acellular cementum formation, which has been very poorly understood so far.

Materials and Methods

Animals

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (protocol #22091787) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Preparation and genotyping of *2.3kbCol1a1-Cre*^{ERT2}*;Trps1*$^{fl/fl}$ conditional knockout (*Trps1*^{Col1a1} cKO) was described previously [19]. Experimental *Trps1*^{Col1a1} cKO and control WT (*Trps1*$^{fl/fl}$) mice were administered with tamoxifen (0.1 mg/g body weight/day, via intraperitoneal injection) at postnatal days (P)1, P2, P9, P16 and P23, and P30 to induce deletion of *Trps1* in specific cells. Mice were euthanized by CO_2 inhalation for the collection of tissues for the analyses. Male and female mice were used in this study, and analyzed separately.

Micro-Computed Tomography (μCT) Analysis

Hemimandibles ($n = 5$/ genotype/sex) of 4 wk old mice were imaged using the Scanco μCT 50 (Scanco Medical, Brüttisellen, Switzerland) system. Specimens were scanned in 70% ethanol at 55 KVp, 0.36 degrees rotation step (180 degrees angular range) and a 1,500 ms exposure per view, and 6-μm voxel size. Measurements of tissue mineral density (TMD), thickness (Th), volume (BV), dentin and cementum tissue fraction (BV/TV) in the total tooth root volume (TV), and alveolar bone were done as described before [19].

Fluorescent Double-Labeling

For the analyses of the cementum formation, P27 mice were intraperitoneally injected with alizarin red (25 mg/kg; A3882 Sigma) followed by an injection of calcein (25 mg/kg; C0875 Sigma) 6 days later. Mice were sacrificed 48 h after the second injection, and hemimandibles were collected for analysis. Hemimandibles were fixed with 10% formalin (Fisher, #S7100-4) overnight, dehydrated with ethanol followed by xylene, and then embedded in methyl methacrylate. Seven µm sections of embedded tissues were imaged under fluorescent microscope (Zeiss AxioCam on a Zeiss Axioskop A1 microscope) and images were processed by ZEN software.

Histology and Semi-Quantitative Analyses of Cementum and Periodontal Ligament

Hemimandibles ($n = 5$/genotype/sex) were dissected, fixed with 10% formalin overnight, decalcified with 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4) for 14 days, and embedded in paraffin. Seven µm sagittal sections were stained with toluidine blue (TB, Fisher, AC348600250) or picrosirius red (PR, Fisher, NC9908782) according to the manufacturer's instructions. Images of TB- and PR-stained sections were captured by Zeiss AxioCam on a Zeiss Axioskop A1 microscope and Nikon TE2000 microscope with polarizing filters, respectively. BIOQUANT software (BIOQUANT Image Analysis Corporation, Nashville, TN USA) was used to measure cementum area and alignment of collagen fibers. The area of the acellular and cellular cementum was measured on tissue sections ($n = 5$ mice/females/genotype) stained with TB, which allows to distinguish cementum (light blue color) from dentin (darker blue color) [40, 42–45]. The organization of the collagen fibers in periodontal ligament was assessed in the area between 200 µm from cemento-enamel junction (CEJ) and 200 µm from the root apex, on tissue sections stained with picrosirius red. The organization of the collagen fibers was expressed as the percentage of red/orange area (labeling long well-organized fibers) in the total analyzed periodontal ligament area ($n = 3$ females/genotype).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin sections using heat-induced antigen retrieval in sodium citrate buffer (pH 6.0). The following primary antibodies were used: anti-TRPS1 (1:100, OriGene, TA314642), anti-βIII Tubulin (Tubb3, 1:500, Abcam, ab18207), anti-Osx (1:500, Abcam, ab22552), anti-TNAP (1:100, Abcam, ab65834), anti-Bsp (1:500, kindly provided by Dr. Renny Franceschi, University of Michigan). Sections for diaminobenzidine visualizing system were immersed in methanol containing 1% of hydrogen peroxide. The Vectastatin Elite ABC kit (Vector Laboratories Inc., PK-6101) was used for secondary antibody and avidin–biotin peroxidase system, followed by visualization with DAB Substrate Kit (Vector Laboratories Inc., SK-4100). Counterstaining was performed using hematoxylin or methyl green staining. For immunofluorescence, sections were incubated with Alexa Fluor 546-conjugated secondary antibody (1:500, Thermo Fisher Scientific, A-11035) and DAPI. Images were captured with Zeiss AxioCam on a Zeiss Axioskop A1 microscope and ZEN software.

Statistical Analyses

Five mice/genotype/sex were used in the experiments, unless otherwise stated. Males and females were analyzed separately. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA). Statistically significant differences were determined using the Student's t-test. A p-value of < 0.05 was considered statistically significant. All values are shown as mean ± standard deviation (SD).

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Author Contributions K.F. contributed to the design of the study, performed most of the experiments, analyzed and interpreted the data, drafted and revised the manuscript. M.S. contributed to the design of the study, performed µCT analysis, interpreted the data and revised the manuscript. L.L. and K.V. contributed to µCT data acquisition, analysis and interpretation. P.H. and P.K contributed to the analyses of the alveolar bone µCT data, D.N contributed to conception, design, data analyses and interpretation, drafted and revised the manuscript. The authors gave final approval and agreed to be accountable for all aspects of the work.

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Declarations

Conflict of interest Kaoru Fujikawa, Mairobys Socorro, Lyudmila Lukashova, Priyanka Hoskere, Paulina Keskinidis, Kostas Verdelis, Dobrawa Napierala, there is no conflict of interest

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