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Temporo-spatial requirement of Smad4 in dentin formation

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ABSTRACT

The TGF-β/BMP family plays an important role in multiple stages of tooth development. TGF-β/BMP signaling is required for odontoblast differentiation and dentin formation; however, the precise molecular mechanisms underlying dentin formation remain unclear. To address the role of TGF-β/BMP signaling in dentin formation, we analyzed mice in which *Smad4*, a key intracellular mediator of TGF-β/BMP signaling, was subjected to tissue-specific ablation under the control of *Dspp*, *OC*, or *Col1a1* promoters. Three independent *Smad4* conditional knockout mice exhibited various dentin defects in the crowns and roots of their molars depending on the transactivator. In all mutant molars, crown dentin thickness was thinner than that of the control. In addition, impaired dentin was found in the cervical region and root furcation area. Although the initial differentiation of odontoblasts was normal, odontoblast polarity abruptly decreased and the expression of *Col1a1*, *OC*, and *Dspp* was reduced in the odontoblasts of mutant molars. In *Dspp-Cre*-mediated *Smad4* disruption mice, primary dentin formation was slightly delayed, while secondary dentin formation was severely affected in the cervical region of the molars. These results indicate that TGF-β/BMP signaling is required for odontoblast maturation and dentin formation in a stage- and site-dependent manner.

1. Introduction

Dentin is a major component of teeth and is produced by odontoblasts. During the late bell stage of tooth development, odontoblasts differentiate from ectomesenchymal cells in the dental papilla under the influence of the inner dental epithelium [1]. Odontoblasts then produce extracellular matrix, including collagen type I as well as non-collagenous proteins such as dentin sialophosphoprotein (DSPP), osteocalcin (OC), and dentin matrix protein-1 (Dmp1), which facilitate the mineralization of the dentin matrix.

The transforming growth factor-β (TGF-β) superfamily is composed of TGF-βs, BMPs, activins, and related proteins. TGF-β signaling plays an important role in regulating a variety of cellular functions, including cell proliferation, differentiation, apoptosis, and matrix synthesis [2]. To date, many genetic studies have reported various disturbances in odontoblasts and dentin formation following the disruption of the TGF-β/BMP signaling pathway. Delayed odontoblast differentiation and decreased dentin thickness has been found in *Wnt1-Cre;Tgfbr2* mice [3]. In *Osx-Cre;Tgfbr2* mice, significant root abnormalities were observed, whereas notable dentin abnormalities were not observed in *Dspp-Cre;Tgfbr2* mice [4,5]. In addition, conditional disruption of *Bmp2* or *Bmp4* also leads to decreased mature odontoblast differentiation and root defects due to the improper maturation of odontoblast and dentin formation, respectively [6–8]. From these reports, it is suggested that TGF-β/BMP signaling may play key roles in odontoblast differentiation and dentin formation.

Moreover, the deletion of *Smad4*, a common mediator for the TGF-β/BMP signaling pathway, causes morphological and functional defects in odontoblasts. Gao et al. [9] reported that *OC-Cre*-mediated *Smad4* disruption in mice resulted in delayed odontoblast differentiation and irregular dentin formation only in the root region. Ablation of *Smad4* in the dental mesenchyme results in defects in odontoblast differentiation and ectopic bone-like structures instead of normal dentin [10]. These results strongly suggest that TGF-β/BMP signaling plays a crucial role in odontoblast

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differentiation and dentin formation in a stage- and site-dependent manner. However, the precise molecular mechanisms underlying odontoblast differentiation and dentin formation remain unclear. Here, we investigate the roles of TGF-β/BMP signaling in dentin formation by analyzing three independent mouse lines with tissue-specific inactivation of *Smad4* using Cre transactivators (*Dspp-Cre*, *OC-Cre*, and *Col1a1-Cre*) under the control of each matrix protein gene promoter.

2. Materials and methods

2.1. Mouse strains

All experimental procedures were approved by the Animal Welfare Committee of Chonbuk National University. *Smad4*-floxed allele (*Smad4$^{fl/fl}$*), *Col1a1-Cre*, *OC-Cre*, and *Dspp-Cre* mice have been previously described [11–14]. *Dspp-Cre* and *Rosa26 (R26R)* reporter mice [15] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

To generate *Col1a1-Cre;Smad4$^{fl/fl}$* (*Smad4^{Col}*), *OC-Cre;Smad4$^{fl/fl}$* (*Smad4^{OC}*), and *Dspp-Cre;Smad4$^{fl/fl}$* (*Smad4^{Dspp}*) mice, *Col1a1-Cre;Smad4$^{fl/+}$*, *OC-Cre;Smad4$^{fl/+}$*, or *Dspp-Cre;Smad4$^{fl/+}$* (control) mice were crossed with *Smad4$^{fl/fl}$* mice as appropriate. Mouse offspring were genotyped by polymerase chain reaction analysis using previously described primers [11–14]. To analyze the level of Cre activity, *Col1a1-Cre*, *OC-Cre*, and *Dspp-Cre* mice were crossed with *R26R* mice, and the mandibles of the double-transgenic mice were processed for *X-gal* staining, as described previously [16].

2.2. Tissue preparation and histology

For histological analysis, 2- to 16-week-old mice were sacrificed and their mandibles were carefully dissected. Tissues were fixed in 4% paraformaldehyde (PFA) and decalcified in 10% EDTA solutions for 2–4 weeks at 4 °C. The decalcified tissues were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5 μm thickness. Slides were stained with hematoxylin and eosin (H–E).

2.3. Immunohistochemistry and in situ hybridization

For immunostaining, sections were treated with 3% hydrogen peroxide, and incubated with rabbit monoclonal anti-Smad4 antibody (1:200; Abcam Inc., Cambridge, MA, USA). The Histostain Plus rabbit primary (DAB) kit (Zymed laboratories, San Francisco, CA, USA) was used according to the manufacturer's instructions. *In situ* hybridization of tissue sections was performed using standard procedures [16]. We prepared digoxigenin-labeled probes for *Col1a1*, *Dsp*, *OC*, and *Bsp* as described previously [16,17].

2.4. Micro-computed tomography

Mandibles were dissected from 1- to 12-month-old mice and fixed in 4% PFA. The specimens were scanned in a desktop scanner (1076 Skyscan Micro-CT, Skyscan, Kontich, Belgium). They were subsequently reconstructed and analyzed with CTscan software (Skyscan).

3. Results

3.1. Targeted disruption of Smad4 in odontoblasts leads to impaired dentin formation

To confirm the localization of Smad4 in odontoblasts, we performed immunohistochemistry in the mandibular molar of newborn mice. Smad4 was specifically localized in the nuclei and cytoplasm of differentiating odontoblasts (SFig. 1A). We next confirmed that Cre recombination at the *Dspp-Cre*, *OC-Cre*, and *Col1a1-Cre* promoters was active in the odontoblasts. In the mouse molars at P3, strong β-galactosidase activity was observed in the odontoblasts of *Dspp-Cre;R26R*, *OC-Cre;R26R*, and *Col1a1-Cre;R26R* double transgenic mice (SFig. 1B–D). Micro-computed tomography revealed that the mandibular molars of *Smad4^{Dspp}*, *Smad4^{OC}*, and *Smad4^{Col}* mice at 4 weeks exhibited various degrees of thin crown dentin and root abnormalities (SFig. 1E–H). These disturbances in dentin formation were confirmed in H–E-stained tissue sections of the mandibular first molars. In the molars of *Smad4^{Dspp}* and *Smad4^{OC}* mice, the dentin thickness was slightly thinner than that of the control (SFig. 1I–K). The roots of the *Smad4^{OC}* mice were slightly shorter than the others and were composed of irregular dentin with cellular inclusion (SFig. 1K). In the molars of *Smad4^{Col}* mice, the crown and root dentin were extremely thin, but root length did not notably differ. In addition, their pulp chambers and root canals were filled with necrotic pulp and inflammatory cells due to pulpal exposure (SFig. 1L).

3.2. Impaired dentin formation is caused by disturbances in odontoblast maturation and matrix production

Since dentin formation was impaired following the disruption of *Smad4* in odontoblasts, histological differences in odontoblasts and dentin were compared in the mandibular first molars of *Smad4^{Dspp}*, *Smad4^{OC}*, *Smad4^{Col}*, and control mice. At 2 weeks old, odontoblasts were well differentiated with a tall columnar shape in the control mice (Fig. 1A). In contrast, odontoblast heights decreased in *Smad4^{Dspp}*, *Smad4^{OC}*, and *Smad4^{Col}* mice (Fig. 1A–D). In these mice, coronal dentin thickness was slightly thinner than that of control mice (Fig. 1A–D). At 4 weeks old, dentin apposition remarkably increased in control mice. However, the coronal dentin thickness of *Smad4^{Dspp}*, *Smad4^{OC}*, and *Smad4^{Col}* mice were less than half of that in the control mice (Fig. 1E–H). In both the *Smad4^{Dspp}* and control mice, dentin thickness slightly increased with age, but differences in coronal dentin thickness remained at 8 weeks and 16 weeks (data not shown). To assess the gene expression changes in the crown odontoblasts following disruption of *Smad4*, *in situ* hybridization was performed. In the control, *Col1a1*, *OC*, and *Dsp* were highly expressed in the odontoblasts (Fig. 1I, M, Q). In *Smad4^{Dspp}* mice, the expression of *Col1a1*, *OC*, and *Dsp* were slightly down-regulated, whereas they were significantly reduced in *Smad4^{OC}* and *Smad4^{Col}* mice (Fig. 1J–L, N–P, R–T).

3.3. Smad4-mediated signaling is involved in the regulation of dentin formation in a stage- and site-specific manner

Besides coronal dentin, impaired dentin formation was also found in the cervical region of the molars in *Smad4^{Dspp}*, *Smad4^{OC}*, and *Smad4^{Col}* mice. At 2 weeks old, odontoblasts in the cervical region were well differentiated with columnar shapes in control mice (Fig. 2A). In contrast, odontoblast heights slightly decreased in *Smad4^{Dspp}* mice and severely decreased in *Smad4^{OC}* and *Smad4^{Col}* mice (Fig. 2B–D). At 4 weeks old, dentin in the cervical region was remarkably thickened in control mice (Fig. 2E). However, dentin thickness in the cervical region of *Smad4^{Dspp}*, *Smad4^{OC}*, and *Smad4^{Col}* mice was thinner than that of the control mice (Fig. 2F–H). At 8 weeks old, hypoplastic dentin defects were found in the cervical region of *Smad4^{Dspp}* mice in contrast to the normally formed dentin in the control mice (Fig. 2I, J). At 16 weeks old, hypoplastic dentin defects in the cervical region were prominent and looked like a cave in *Smad4^{Dspp}* mice. In addition, some odontoblasts were entrapped in the dentin matrix of the cervical

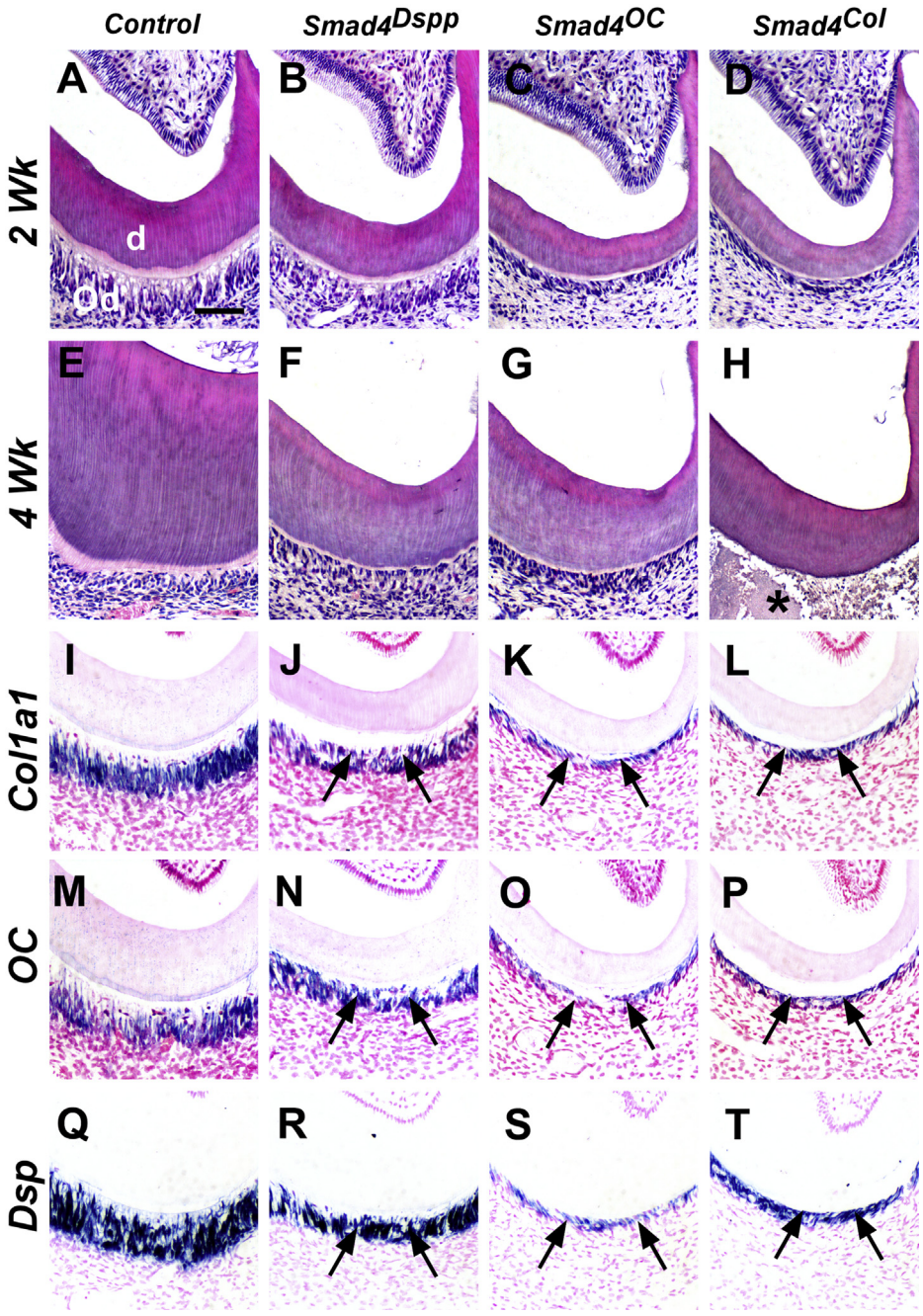

Fig. 1. Crown dentin impairment in odontoblast-specific *Smad4*-inactivated mice. (A–L) Crown dentin formation in molars from $Smad4^{Dspp}$, $Smad4^{OC}$, $Smad4^{Col}$, and control mice from 2 to 4 weeks of age. In the control, dentin thickness gradually increased with age. However, the gain of dentin and the polarity of the odontoblasts were remarkably reduced with age in $Smad4^{Dspp}$, $Smad4^{OC}$, and $Smad4^{Col}$ mice. (I–T) Down-regulation of major dentin matrix protein expression in the crown odontoblasts of the *Smad4* gene-targeted mice at 2 weeks-old. In the control, *Col1a1*, *OC*, and *Dsp* were highly expressed in the odontoblasts. However, this gene expression was down-regulated and restricted in the thin layer of odontoblasts in the $Smad4^{Dspp}$, $Smad4^{OC}$, and $Smad4^{Col}$ mice. Od, odontoblast; d, dentin. Black arrows indicate decreased gene expressions in odontoblasts and * indicates necrotic pulp. Scale bar: 100 μm (A–T).

region (Fig. 2K, L). The cave-like hypoplastic dentin defect in the cervical region was also observed in micro-computed tomography of 12-month-old $Smad4^{Dspp}$ mice (Fig. 2M). Gene expression changes were observed in the odontoblasts of cervical region following disruption of *Smad4* in odontoblasts. In the control, *Col1a1*, *OC*, and *Dsp* were highly expressed in odontoblasts (Fig. 3A, E, I). In $Smad4^{Dspp}$ mice, expression of *Col1a1*, *OC*, and *Dsp* were slightly down-regulated, whereas they were significantly reduced in $Smad4^{OC}$ and $Smad4^{Col}$ mice (Fig. 3B–D, F–H, J–L). On the other hand, *Bsp* was not expressed in odontoblasts, but rather expressed in the cementoblasts and osteoblasts of control mice (Fig. 3M). In $Smad4^{OC}$ mice, *Bsp* was expressed in odontoblasts, but was not expressed in the odontoblasts of $Smad4^{Dspp}$ and $Smad4^{Col}$ mice (Fig. 3N–P).

Impaired dentin formation was also found in the root furcation area of molars from $Smad4^{OC}$ and $Smad4^{Col}$ mice. In the root furcation area of the molar at 2 weeks old, odontoblasts were poorly differentiated in $Smad4^{OC}$ mice, whereas they were well

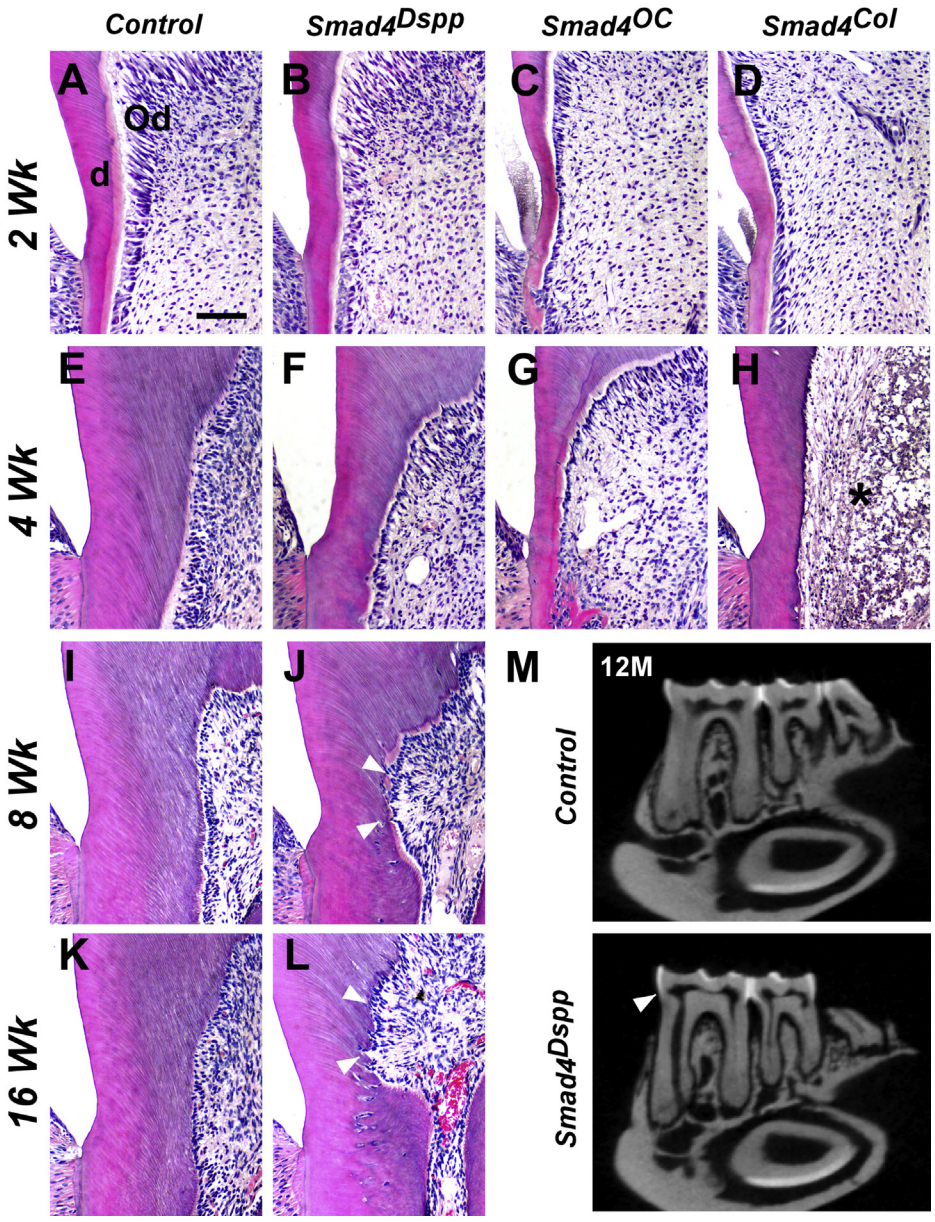

Fig. 2. Highly responsive odontoblasts to Smad4 signaling in the cervical crown. (A–L) Dentin formation in the cervical region of the molars in *Smad4*^{Dspp}, *Smad4*^{OC}, *Smad4*^{Col}, and control mice from 2 to 16 weeks of age. With age, cervical dentin was gradually deposited and continuous with other part of crown dentin in control. However, impaired dentin formation was observed in the cervical region of the molars in *Smad4*^{Dspp}, *Smad4*^{OC}, and *Smad4*^{Col} mice. In the molars of 8-week-old and 16-week-old *Smad4*^{Dspp} mice, severe hypoplastic dentin was observed in the cervical region and some odontoblasts were entrapped in dentin matrix. (M) Micro-computed tomography of the mandibles obtained from *Smad4*^{Dspp} and control mice at 12 months old. As indicated with a white arrowhead, impaired secondary dentin formation was apparently found in the cervical region of the mutant molar. Od, odontoblast; d, dentin. * indicates necrotic pulp. Scale bar: 100 μm (A–L).

differentiated in *Smad4*^{Dspp}, *Smad4*^{Col}, and control mice (Fig. 4A–D). At 4 weeks old, the dentin thickness of the furcation area of *Smad4*^{Dspp} mice was similar to that of the control, but was very thin in *Smad4*^{Col} mice. In *Smad4*^{OC} mice, dentin in the furcation area was apparently thin and irregular (Fig. 4E–H). In *Smad4*^{Dspp}, *Smad4*^{Col}, and control mice, *Col1a1*, *OC*, and *Dsp* were specifically expressed in the odontoblasts of the furcation area (Fig. 4I, J, L, M, N, P, Q, R, T). However, these genes were significantly down-regulated in the odontoblasts of *Smad4*^{OC} mice (Fig. 4K, O, S). In the control, *Bsp* was specifically expressed in the cementoblasts and osteoblasts aligned root dentin and alveolar bone. *Bsp* was similarly expressed in *Smad4*^{Dspp} and *Smad4*^{Col} mice, but was down-regulated in *Smad4*^{OC} mice (Fig. 4U–X).

4. Discussion

In this study, we investigated the role of TGF-β/BMP signaling in odontoblasts during dentin formation. Three independent Smad4 gene-targeted mouse lines, *Smad4*^{Dspp}, *Smad4*^{OC}, and *Smad4*^{Col}, exhibited impaired dentin formation in the crown, cervix, and root furcation area of molars. These abnormalities were closely associated with the temporo-spatial requirement of TGF-β/BMP signaling during odontoblast differentiation and dentin formation.

Odontoblasts are differentiated from the ectomesenchymal cells in the dental papilla under the influence of the inner dental epithelium. With differentiation, odontoblasts become tall columnar cells and sequentially synthesize collagen type I,

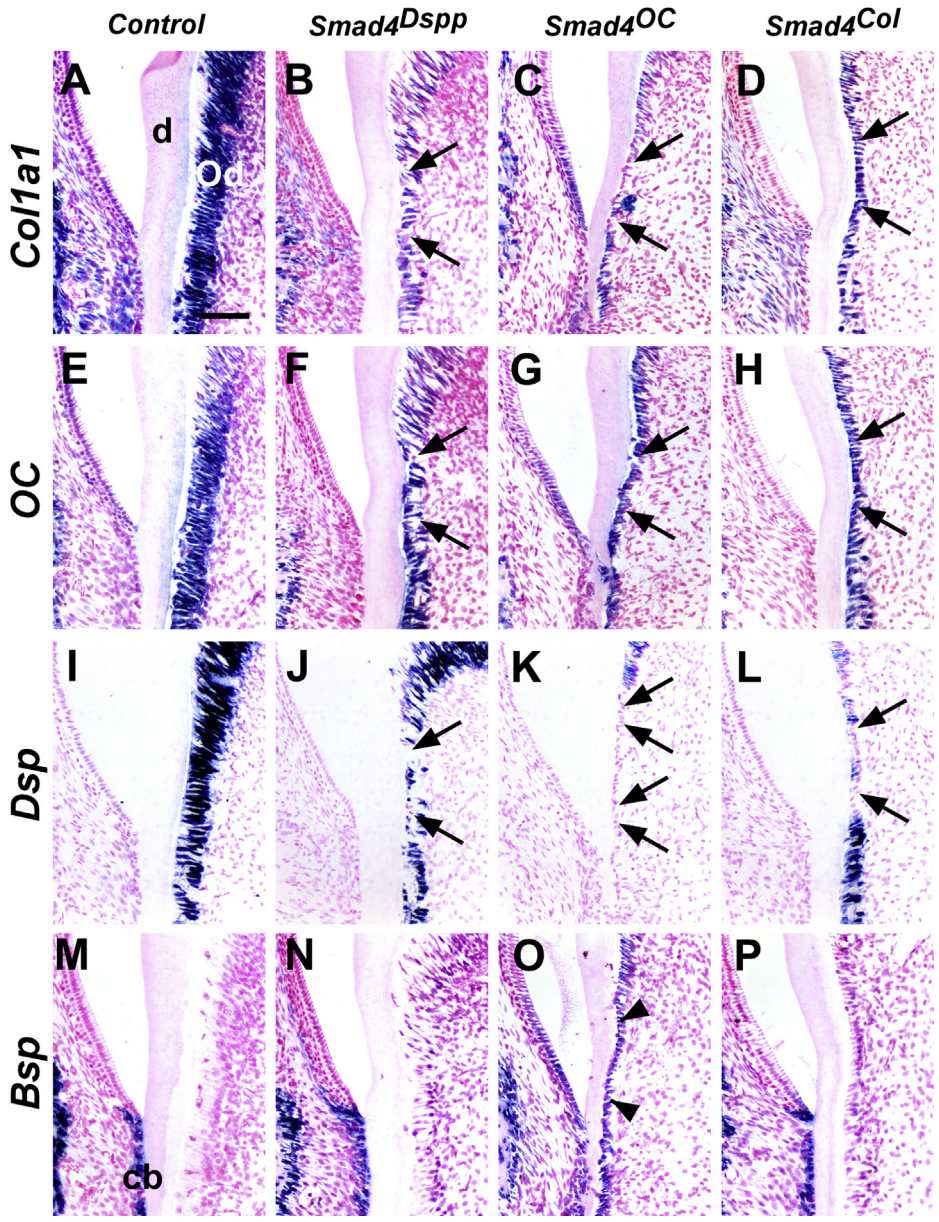

Fig. 3. Gene expression changes in the odontoblasts of the cervical region in *Smad4* gene-targeted mouse molars. Expression of *Col1a1* (A–D), *OC* (E–H), *Dsp* (I–L), and *Bsp* (M–P) in the odontoblasts of the mandibular molars from 2-week-old $Smad4^{Dspp}$, $Smad4^{OC}$, $Smad4^{Col}$, and control mice. In the control, *Col1a1*, *OC*, and *Dsp* were strongly expressed in the odontoblasts of the cervical regions. In contrast, *Col1a1* and *OC* were down-regulated in the odontoblasts of the cervical region in mutant molars. Moreover, *Dsp* was severely down-regulated in the mutant mice, while *Bsp* was not expressed in odontoblasts but expressed in the cementoblasts and osteoblasts of control mice. In $Smad4^{OC}$mice, *Bsp* was expressed in odontoblasts (black arrowheads), but was not expressed in the odontoblasts of $Smad4^{Dspp}$ and $Smad4^{Col}$ mice. Od, odontoblast; d, dentin; cb, cementoblast. Black arrows indicate decreased gene expressions in odontoblasts. Scale bar: 100 μm (A–P).

osteocalcin, and then Dspp [18]. We used Cre transactivators under the control of each matrix protein gene promoter to disrupt the *Smad4* gene in the odontoblasts during dentin formation. Odontoblast height and dentin thickness was significantly reduced in the crown of mutant molars. In addition, *Col1a1*, *OC*, and *Dsp* expression were down-regulated in mutant odontoblasts. These changes were prominent in $Smad4^{OC}$ and $Smad4^{Col}$ mice, and mild in $Smad4^{Dspp}$. These findings indicate that phenotypic differences between mutants may result from the stage of gene disruption depending on Cre promoter activity. This is strongly supported by previous reports suggesting that *Col1a1* and *OC* were early marker genes for odontoblasts during differentiation, whereas *Dspp* was a late marker gene [18,19]. Therefore, our results suggest that TGF-β/BMP signaling is required for odontoblast differentiation and dentin formation in a stage-dependent manner.

Fundamental differences between crown and root dentin have been reported previously [20–22]. These may be come from cellular and molecular differences in odontoblast differentiation and dentin formation. In our results, various degrees of dentin defects were also observed in the root furcation area of mutant molars. Although odontoblast differentiation and marker gene expression was not changed significantly in $Smad4^{Dspp}$ and $Smad4^{Col}$ mice, odontoblasts were poorly differentiated and marker gene expression was apparently down-regulated in $Smad4^{OC}$ mice. Dentin also formed smoothly in $Smad4^{Dspp}$ and $Smad4^{Col}$ mice, but irregular dentin was found in $Smad4^{OC}$ mice. These findings are in

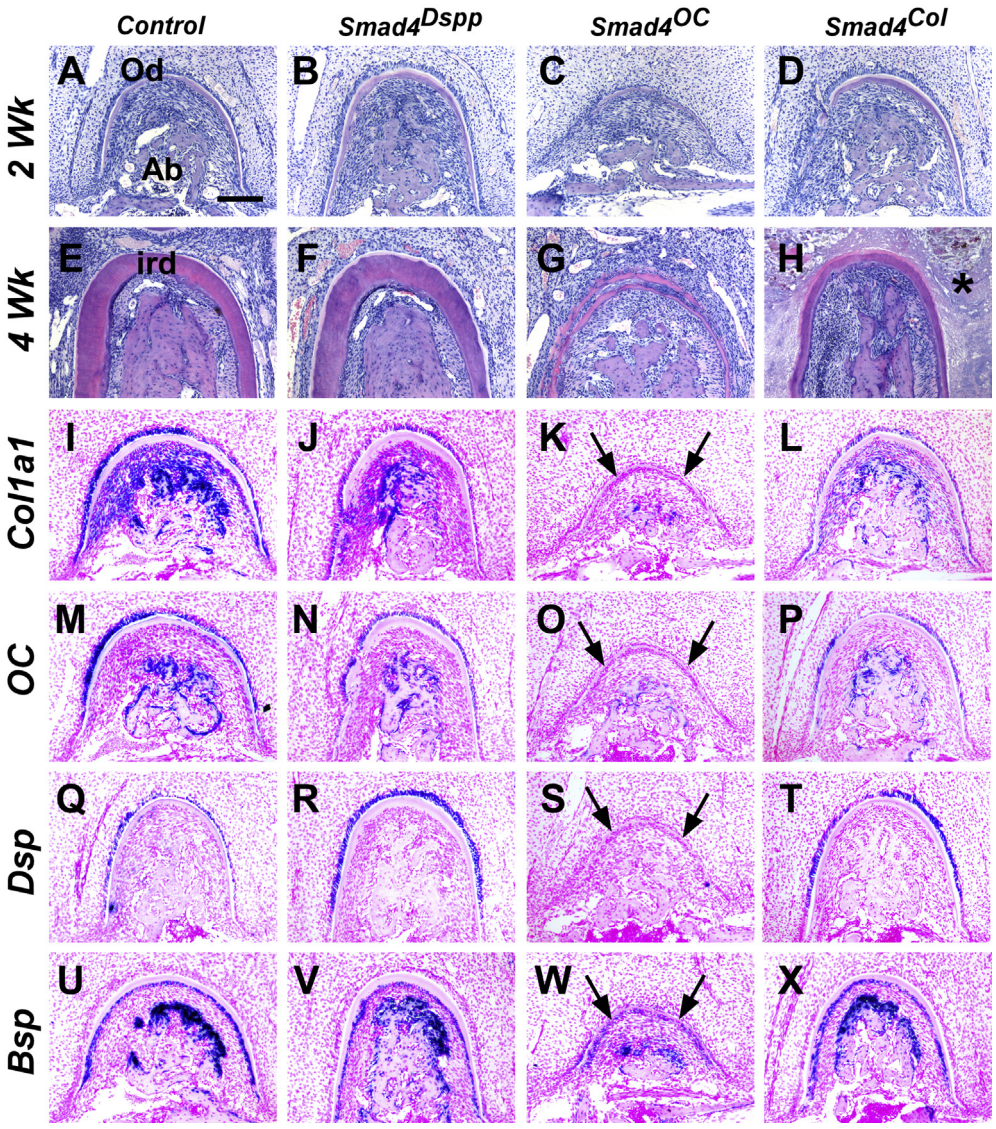

Fig. 4. Impairment of odontoblast differentiation and dentin formation in the root furcation region of *Smad4* gene targeted mice. (A–D) 2 weeks old. (E–H) 4 weeks old. (I–L) *Col1a1*. (M–P) *OC*. (Q–T) *Dsp*. (U–X) *Bsp* expression. Odontoblasts were well differentiated in 2-week-old $Smad4^{Dspp}$, $Smad4^{Col}$, and control mice; however, odontoblasts were poorly differentiated in $Smad4^{OC}$ mice. At 4 weeks old, the dentin thickness of $Smad4^{Dspp}$ was similar to that of the control, but was very thin in $Smad4^{Col}$. In $Smad4^{OC}$, apparently thin dentin was irregularly formed and many cells were entrapped in dentin matrix. *Col1a1*, *OC*, and *Dsp* were specifically expressed in the odontoblasts of 2-week-old $Smad4^{Dspp}$, $Smad4^{Col}$, and control mice. In contrast, these were down-regulated in the odontoblasts of $Smad4^{OC}$. In the control, *Bsp* was specifically expressed in the cementoblasts and osteoblasts aligned outside the root dentin and alveolar bone. *Bsp* was similarly expressed in $Smad4^{Dspp}$ and $Smad4^{Col}$ mice, but was also down-regulated in $Smad4^{OC}$. Ab, alveolar bone; Od, odontoblast; ird, inter-radicular dentin. Black arrows indicate decreased gene expressions in odontoblasts and * indicates necrotic pulp. Scale bars: 200 μm (A–X).

agreement with previous reports of irregular dentin formation in the molar roots following *OC-Cre* mediated *Smad4* disruption [9]. However, it is not clear that restricted dentin defects are associated with root-specific requirements of TGF-β/BMP signaling in odontoblast differentiation and dentin formation. Recently, Li et al. [10] reported that interference in the terminal differentiation of odontoblasts resulted in the formation of ectopic bone-like structures in the developing crown of *Osr2Ires-Cre* mediated *Smad4*-disrupted mice. From these reports, it is suggested that TGF-β/BMP signaling may be required for the initial differentiation of odontoblasts both in the crown and roots.

Interestingly, cave-like dentin defects were observed in the cervical region of aged $Smad4^{Dspp}$ mouse molars (Fig. 2). These defects were not prominent in mutant mice at 4 weeks of age, but apparent dentin defects appeared in mutant mice from 8 weeks and persisted until 12 months. These data indicate that secondary dentin formation was permanently disrupted in the cervical region following *Smad4* ablation in odontoblasts, and that odontoblasts in cervical region may be highly responsive to Smad4 signaling during secondary dentin formation.

In conclusion, our results suggest that TGF-β/BMP signaling is required for odontoblast differentiation and dentin formation in a stage and site dependent manner.

Conflict of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.014.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.014.

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