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Requirement of Smad4-mediated signaling in odontoblast differentiation and dentin matrix formation

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Abstract: Dentin is the major part of tooth and formed by odontoblasts. Under the influence of the inner enamel epithelium, odontoblasts differentiate from ectomesenchymal cells of the dental papilla and secrete pre-dentin which then undergo mineralization into dentin. Transforming growth factor-beta (TGF-β)/bone morphogenetic protein (BMP) signaling is essential for dentinogenesis; however, the precise molecular mechanisms remain unclear. To understand the role of TGF-β/BMP signaling in odontoblast differentiation and dentin formation, we generated mice with conditional ablation of *Smad4*, a key intracellular mediator of TGF-β/BMP signaling, using *Osr2* or *OC-Cre* mice. Here we found the molars of *Osr2*^{Cre}*Smad4* mutant mice exhibited impaired odontoblast differentiation, and normal dentin was replaced by ectopic bone-like structure. In *OC*^{Cre}*Smad4* mutant mice, cell polarity of odontoblast was lost, and the thickness of crown dentin was decreased in later stage compared to wild type. Moreover, the root dentin was also impaired and showed ectopic bone-like structure similar to *Osr2*^{Cre}*Smad4* mutant mice. Taken together, our results suggest that Smad4-dependent TGF-β/BMP signaling plays a critical role in odontoblast differentiation and dentin formation during tooth development.

Key words: TGF-β/BMP signaling, Smad4, Odontoblasts, Dentin

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Introduction

During dentinogenesis, cranial neural crest cell–derived odontoblasts play a role in the secretion of pre-dentin and dentin following terminal differentiation [1]. Dentin is a major component of tooth and is produced by odontoblasts. During the late bell stage of tooth development, odontoblasts differentiate from ectomesenchymal cells of the dental papilla under the influence of the inner dental epithelium [2]. Moreover, 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 [3, 4].

Transforming growth factor-beta (TGF-β) superfamily, insulin-like growth factors, WNTs, fibroblast growth factors, and other kinds of growth factors are involved in differentiation of dental papilla cells into odontoblasts during tooth development [5]. TGF-β is a multifunctional regulator of a variety of cellular functions, including cell proliferation, differentiation, apoptosis and matrix synthesis [6]. TGF-β/bone morphogenetic protein (BMP) signaling has been implicated to have specific roles during tooth development as seen vari-

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ous disturbances with its disruption by tissue-specific targeting of odontoblast and dentin formation. In a previous study, *Wnt1-Cre;Tgfbr2* mice have shown delayed odontoblast differentiation and decreased dentin thickness [7]. Moreover, targeted inactivation of Bmp2 or Bmp4 also showed phenotypes of impaired odontoblast maturation and tooth root defects [8, 9]. These data suggested that TGF-β/BMP signaling plays critical role in odontoblast differentiation and dentin formation.

TGF-β superfamily consists of TGF-βs, BMPs, activins, and other related proteins, and TGF-β/BMP signaling transduction pathway maintains cell proliferation, differentiation, apoptosis, migration, and reconstruction of proteins [10]. Smad4, a key mediator of TGF-β/BMP signaling, functions as a multifunctional regulator for cranial neural crest cell migration, proliferation, differentiation, protein reconstruction, and immune response, or other physiological functions [10, 11] and expresses in oral epithelium and dental mesenchyme during tooth development [12]. To study the functions of Smad4 during full tooth development, tissue-specific gene targeting technology like conditional knockout strategy is essentially required to overcome disadvantage of *Smad4*-null mice, which is arrested at E7.5–E8.5 with early stage of tooth development [13].

Here, we generated and analyzed the tissue-specific conditional *Smad4* disruption mice under the control of *Osr2* and *OC* promoter to investigate the role of TGF-β/BMP signaling in odontoblast differentiation and dentin formation.

Materials and Methods

Mouse strains and tissue preparation

All experimental procedures were approved by the animal Welfare Committee of Chonbuk National University. *Smad4*-floxed allele (*Smad4*$^{fl/fl}$), *Osr2Ires-Cre*, and *OC-Cre* mice have been previously described [14-16]. Tissue specific activities of *Osr2Ires-Cre* and *OC-Cre* have been reported in dental mesenchyme and odontoblasts [15, 16]. Rosa26 (*R26R*) reporter mice [17] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To generate *Osr2Ires-Cre:Smad4*$^{fl/fl}$ (*Osr2*^{Cre}*Smad4*) and *OC-Cre:Smad4*$^{fl/fl}$ (*OC*^{Cre}*Smad4*) mice, *Osr2Ires-Cre:Smad4*$^{fl/+}$ and *OC-Cre:Smad4*$^{fl/+}$ (control) mice were crossed with *Smad4*$^{fl/fl}$ mice, respectively. Genotyping of mice was carried out by allele-specific polymerase chain reaction as previously described using the following oligonucleotide primers: *Smad4 floxed* (*Smad4a*, 5′-AAG AGC CAC AGG TCA AGC AG-3′; *Smad4b*, 5′-GGG CAG CGT AGC ATA TAA GA-3'; *Smad4c*, 5′-GAC CCA AAC GTC ACC TTC AC-3'), *OC-Cre* (*Cre1*, 5'-ATC CGA AAA GAA AAC GTT GA-3'; *Cre2*, 5'-ATC CAG GTT ACG GAT ATA GT-3'). *Osr2Ires-Cre* (*Osr2Ires-Cre1*, 5'-GAA TTC GCC AAT GAC AAG ACG CTG-3'; *Osr2Ires-Cre2*, 5'-CTA CAA GGA TCT AGC ACA TGC TG-3'), *Rosa26R* (*R1295*, 5'-GCG AAG AGT TTG TCC TCA ACC-3'; *R523*, 5'-GGA GCG GGA GAA ATG GAT-3'; *R26F2*, 5'-AAA GTC GCT CTG AGT TGT TAT-3'). To analyze the level of Cre activity, *Osr2Ires-Cre*, *OC-Cre* mice were crossed with *Rosa26R* mice, and the mandibles (P0 and P8) of the double-transgenic mice were processed for X-gal staining, as described previously [18].

Tissue preparation and histology

For histology analysis, the mice at the age of P0 to P28 were sacrificed and their heads and mandibles were carefully dissected. Tissues were fixed in 4% paraformaldehyde and decalcified in 10% ethylenediaminetetraacetic acid/phosphate buffered saline solution for 1 to 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.

Immunohistochemistry

For immunohistochemistry, sections were treated with 3% hydrogen peroxide and incubated with rabbit polyclonal antibodies against osterix (Osx; 1:200, Abcam Inc., Cambridge, MA, USA), Dspp (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phosphate regulating endopeptidase homology on the X chromosome (Phex; 1:50, Sigma-Aldrich, St. Louis, MO, USA), Dmp1 (1:750, Takara Bio Inc., Shiga, Japan), biglycan (Bgn; 1:800, Dr. Larry Fisher). Histostain Plus primary kit (Zymed Laboratories, San Francisco, CA, USA) was used according to the manufacturer's instructions.

Kidney capsule transplantation

The tooth germ of the mandibular at E14.5 was dissected from embryo. The explants were placed on filters supported by metal grid in a tissue culture dish and cultured for 1 day during genotyping. The host mouse was anesthetized using pentobarbital (0.5 mg/10 g body weight) and the explants were grafted under the kidney capsule according to standard procedure. Two weeks after transplantation, the host mice were sacrificed and the grafts were processed for histological analysis.

Results

Localization of Cre recombinase activity for gene targeting during tooth development

The *Osr2Ires-Cre* and *OC-Cre* transgene directed their specific activity of Cre recombinase at dental mesenchyme and odontoblasts respectively, as β-galactosidase activity was observed in the dental mesenchyme of *Osr2^{Cre}R26R* double transgenic mouse at E14.5 (Fig. 1A) and in odontoblasts of mandibular molars in *OC^{Cre}R26R* double transgenic mouse at P8 (Fig. 1B, C). Based on these results, we crossed *Osr2Ires-Cre* transgenic mice with *Smad4$^{fl/fl}$* mice for targeting *Smad4* ablation at the stage of initial coronal dentin formation. *OC-Cre* transgenic mice were used for targeting the stage of initial root dentin formation and long term observation of coronal dentin formation.

Impaired odontoblast differentiation with Smad4 disruption in dental mesenchyme

Since odontoblast differentiation was impaired following the disruption of *Smad4* in dental mesenchyme, histological differences in odontoblast were compared with the mandibular molar of *Osr2^{Cre}Smad4* and control mice (Fig. 2A, D). At newborn stage, *Osr2^{Cre}Smad4* mice have a layer of non-polarized cuboidal cells with centrally located nucleus during coronal dentin formation while control mice have polarized odontoblasts. In addition, dentin matrix was absent in *Osr2^{Cre}Smad4* mice (Fig. 2D). Immunohistochemistry of mandibular molars revealed that lower or no Osx and Phex were expressed in the odontoblasts of *Osr2^{Cre}Smad4* mice (Fig. 2E, F) while highly expressed in differentiating odontoblast and odontoblastic processes in control mice (Fig. 2B, C). *Osr2^{Cre}Smad4* mice die within a day after birth, precluding investigation of tooth development at later stage. To determine whether the abnormal odontoblast differentiation in *Osr2^{Cre}Smad4* is due to delayed development, we performed kidney capsule transplantation. Mandibular first molars of embryo at E14.5 were collected and cultured for one day. By following the genotype results, it was transplanted on the kidney capsule of wild type mice and observed after 2 weeks (Fig. 2G–L). Interestingly, the tooth development of *Osr2^{Cre}Smad4* mice was delayed even with normal enamel formation with well differentiated ameloblasts. However, enamel and dentin were formed with well differentiated ameloblasts and odontoblasts in control mice. Interestingly, ectopic hard tissue with cellular inclusion was formed by odontoblasts with a distinct difference from former produced dentin *Osr2^{Cre}Smad4* mice (Fig 2G, J). Cellular inclusion was commonly detected in ectopic hard tissue at cervical region of root dentin in the *OC^{Cre}Smad4* mice targeting at the stage of initial root dentin formation (Fig. 2M, N). Immunohistochemistry after kidney capsule transplantation revealed that Osx was highly expressed in differentiating polarized odontoblasts in control mice while expressed in matrix forming cells and the cells trapped in dentin matrix in *Osr2^{Cre}Smad4* mice (Fig. 2H, K). However, the expression of Phex was obviously decreased in the odontoblast of *Osr2^{Cre}Smad4* mice when compared to control mice after kidney capsule transplantation (Fig. 2I, L). These results indicated that inactivation of TGF-β/BMP signaling in odontoblasts may disturb the differentiation processes of odontoblasts during tooth development.

Targeted ablation of Smad4 in odontoblasts leads a disturbance of dentin formation

Ablation of *Smad4* in odontoblasts was established in *OC^{Cre}Smad4* mice as seen the specific activity of Cre activity in odontoblasts of mandibular molars by *OC^{Cre}R26R* double

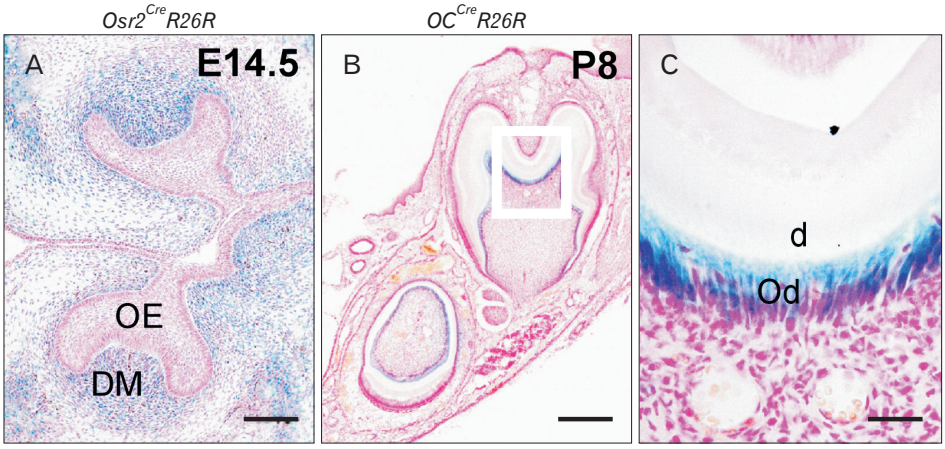

Fig. 1. Localization of Cre recombinase for conditional inactivation of *Smad4* in dental mesenchyme. β-Galactosidase activities are shown in the dental mesenchyme of *Osr2^{Cre}R26R* at embryo 14.5 (E14.5) (A) and in coronal odontoblasts of *OC^{Cre}R26R* mice at postnatal 8 (P8) (B), respectively. (C) Enlarged white-boxed area in panel B is shown. d, dentin; DM, dental mesenchyme; Od, odontoblasts; OE, oral epithelium. Scale bars=100 μm (A), 200 μm (B), 25 μm (C).

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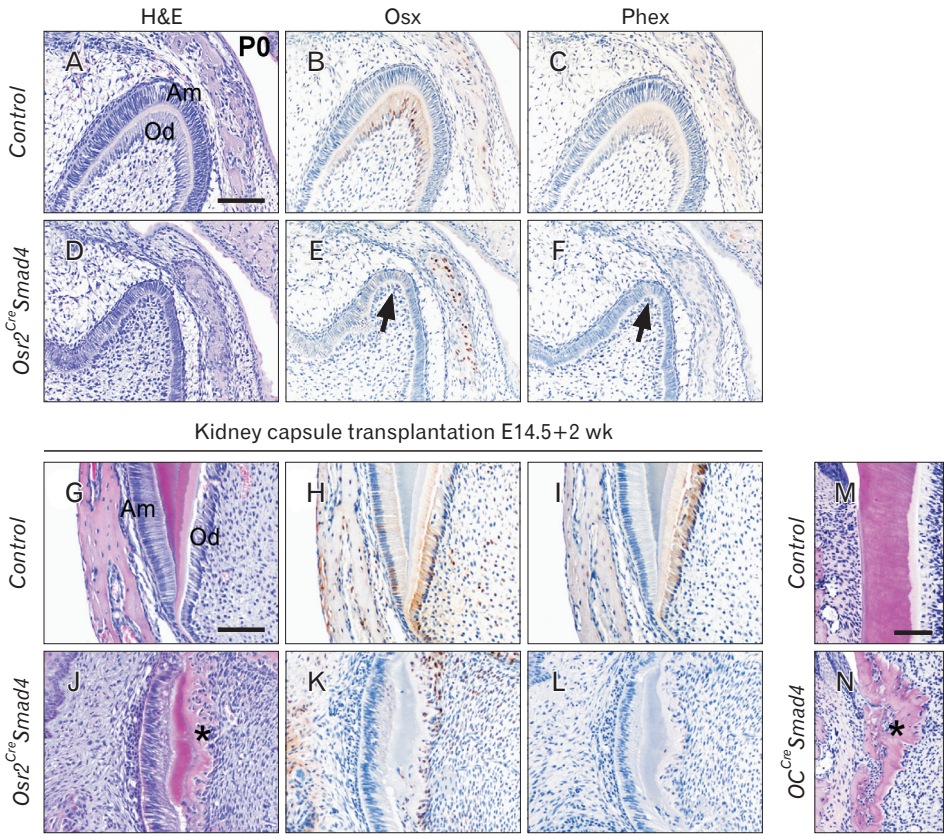

Fig. 2. Impairment of odontoblast differentiation and dentin formation by *Smad4* inactivation during dentinogenesis. H&E staining of lower molar tooth germs of control and *Osr2*^{Cre}*Smad4* mice at newborn stage (A, D). Immunohistochemical staining for Osx (B, E), Phex (C, F) with mandibular molar tooth germs of control and *Osr2*^{Cre}*Smad4* mice at postnatal 0 (P0). Osx and Phex were highly expressed in coronal odontoblasts in control mice (B, C) while lower expression of Osx and Phex were detected in mesenchymal cells (black arrows) in *Osr2*^{Cre}*Smad4* mice (E, F). Root forming area of mandibular molar tooth germs from control and *Osr2*^{Cre}*Smad4* mice after kidney capsule transplantation was histologically analyzed at embryo (E) 14.5+1+2 wk (G–L). Immunohistochemical staining for Osx (H, K), Phex (I, L) with mandibular molar tooth germs of control and *Osr2*^{Cre}*Smad4* mice. The same area of mandibular molar tooth germs from control and *OC*^{Cre}*Smad4* mice at P21 was analyzed by H&E staining (M, N). Osx and Phex were highly expressed in differentiating odontoblasts at cervical dentin in control mice (H, I) while the lower expression of Osx was observed in entrapped cells in a bone-like tissue of *Osr2*^{Cre}*Smad4* mice (K). The expression of Phex were not detected in the entrapped cells in *Osr2*^{Cre}*Smad4* mice (L). The asterisks in panels J and N indicate bone-like structure of ectopic hard tissue formation. Am, ameloblasts; H&E, hematoxylin and eosin; Od, odontoblasts; Osx, osterix; Phex, phosphate regulating endopeptidase homology on the X chromosome. Scale bar=50 μm.

transgenic mouse (Fig. 1). Histological analysis of mandibular molars revealed that dentin thickness was definitely decreased in *OC*^{Cre}*Smad4* mice compared to control mice. In addition, the odontoblasts have shorter height and flattened morphology in *OC*^{Cre}*Smad4* mice than in control mice, implying the loss of odontoblast polarity by ablation of *Smad4* in odontoblasts. At the age of P10, coronal dentin thickness did not show any remarkable difference between control and *OC*^{Cre}*Smad4* mice (Fig. 3A, E, I). Interestingly, coronal dentin thickness was slightly increased in the *OC*^{Cre}*Smad4* mice while dramatically increased in control mice at the age of P14, P21, and P28 (Fig. 3B–D, F–H). These results indicate that inactivation of TGF-β/BMP signaling in odontoblasts may disturb dentin matrix production during dentin formation.

Molecular changes in odontoblasts and dentin matrix with disruption of Smad4

To determine molecular changes in odontoblasts and dentin formation following ablation of *Smad4*, immunohistochemical staining was performed with mandibular tissue sections of control and *OC*^{Cre}*Smad4* mice at P10. Dspp was slightly expressed in odontoblasts and dentinal tubules in *OC*^{Cre}*Smad4* mice while highly expressed in control mice (Fig. 4A, D). Dmp1 was observed in dentin matrix of *OC*^{Cre}*Smad4* mice with strong

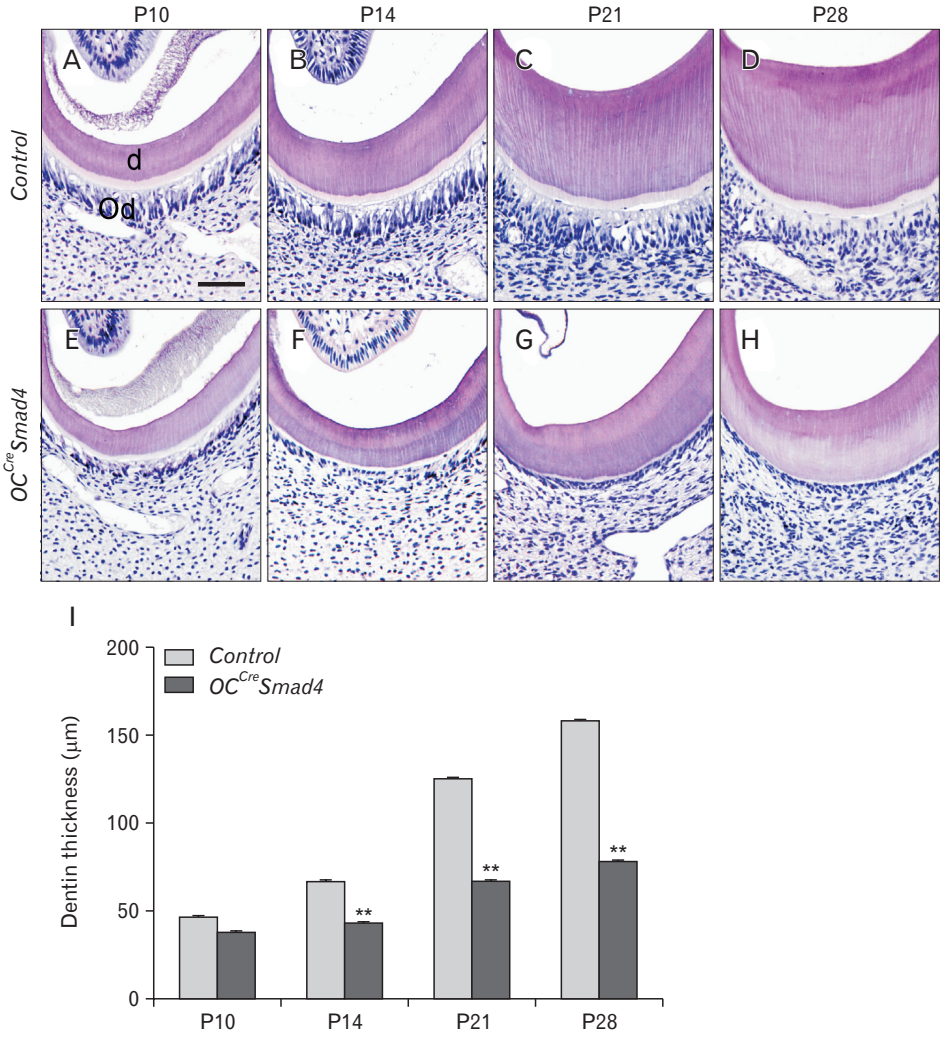

Fig. 3. Reduced dentin matrix apposition in *OC*^{Cre}*Smad4* mice. Hematoxylin and eosin staining of crown dentin of control (A–D) and *OC*^{Cre}*Smad4* mice (E–H) at postnal (P) 10, P14, P21, and P28. Dentin thickness was compared after the measurement of crown dentin thickness with control and *OC*^{Cre}*Smad4* mice at P10, P14, P21, and P28 (n=5, each genotype for stage, respectively) (I). d, dentin; Od, odontoblasts. $^{**}P<0.01$. Scale bar=50 μm.

expression while weak expression in control mice (Fig. 4B, E). Bgn, a proteoglycan, exhibited specific expression at pre-dentin in both control and *OC*^{Cre}*Smad4* mice (Fig. 4C, F).

Discussion

In this study, we investigated the significance of Smad4, a key intracellular signaling mediator of TGF-β/BMP signaling, by tissue-specific ablation under the control of *Osr2* or *OC* promoter during tooth development. We found that during dentinogenesis the ablation of *Smad4* with two types of gene targeting by different Cre activation commonly results in disturbed differentiation of odontoblasts and abnormal bone-like dentin formation, which observed at the stage of initial coronal dentin formation as well as initial root dentin formation.

TGF-β superfamily is comprised of many members, such as TGF-βs, Nodal, Activin, and BMPs. TGF-β/BMP signaling transmits initial signals across the plasma membrane through the formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors. The type I receptor is phosphorylated and then activates specific type II receptor. Activated type I receptors initiate intracellular signaling through phosphorylation of specific Smad proteins, R-Smads. Activated R-Smads form a complex with Co-Smad and Smad4 and then translocate into the nucleus to direct transcriptional response [19]. TGF-β/BMP signaling plays an important role in regulating extensive processes including cell proliferation, differentiation, apoptosis, migration, and extracellular matrix remodeling [6, 20]. From long ago, TGF-β/BMP signaling has been implicated to have an important role during dentin formation as seen that cranial neural crest cell–derived odontoblasts secret pre-dentin and dentin following terminal differentiation [21]. Odontoblasts are differentiated

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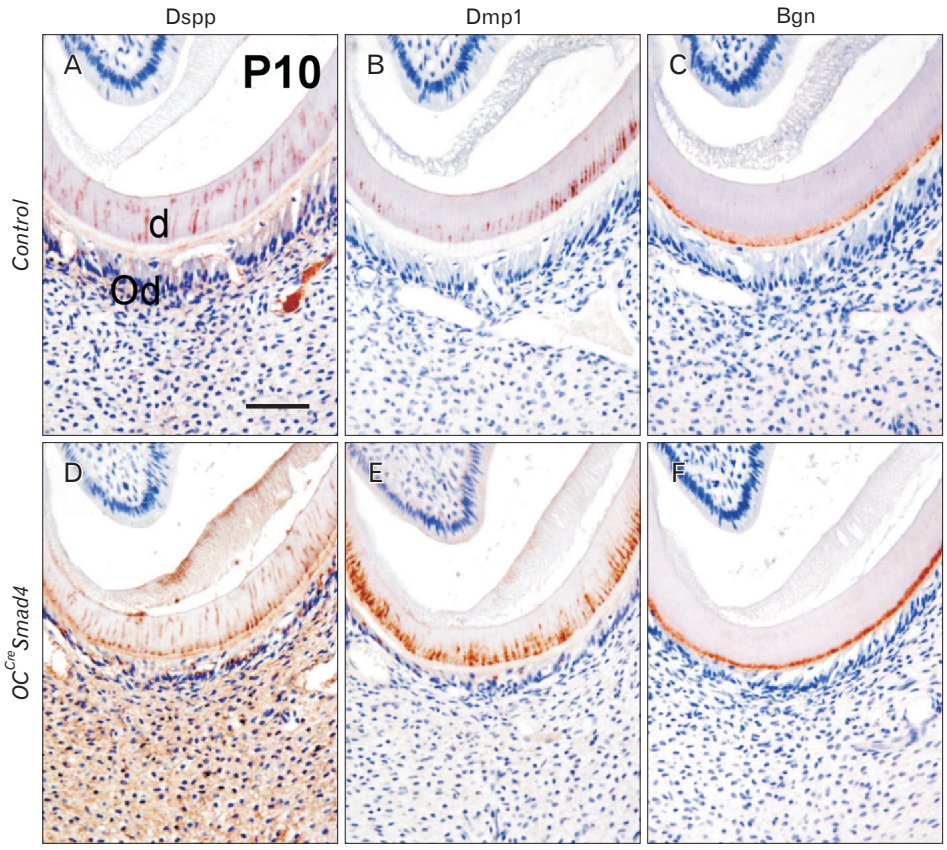

Fig. 4. Molecular changes in odontoblasts and dentin matrix with disruption of *Smad4* in odontoblasts. Immunohistochemical staining of mandibular molar crown dentin in control (A–C) and OC^{Cre}*Smad4* mice (D–F) at P10. Immunohistochemical staining for Dspp (A, D), Dmp1 (B, E), and Bgn (C, F) with mandibular molar of control and OC^{Cre}*Smad4* mice. The expression of Dspp was detected in odontoblast and dentinal tubules in control mice (A) while broad expression in pre-dentin and pulp of OC^{Cre}*Smad4* mice (D). The expression of Dmp1 was increased in OC^{Cre}*Smad4* mice (E) compared to control (B). The expression of Bgn was not changed (C, F). Bgn, biglycan; d, dentin; Dmp1, dentin matrix protein 1; Dspp, dentin sialophosphoprotein; Od, odontoblasts. Scale bar=50 μm.

from the dental mesenchymal cells in dental papilla under the influence of the inner dental epithelium. Odontoblasts become tall and in columnar shape with differentiation [21]. Smad4, the intracellular mediator for the TGF-β/BMP signaling, plays important role in regulating early tooth development [22]. Among the TGF-β superfamily, TGFβ-1, TGFβ-2, TGFβ-3, BMP2, BMP4, BMP7, and follistatin are expressed in the inner enamel epithelium, dental papilla and in polarizing and functional odontoblasts. Exogenous TGFβ-1, BMP2, BMP4, and BMP7 can induce odontoblast differentiation and dentin formation in dental papilla cells *in vitro* [23-26]. These data indicate that TGF-β/BMP signaling play critical roles in odontoblast differentiation and dentin formation. In our data, $Osr2^{Cre}$*Smad4* mice exhibited disturbed differentiation of odontoblasts and abnormal bone-like structures instead of dentin formation. The results suggest that conditional inactivation of *Smad4* cause morphological and functional deficiency in odontoblasts during odontoblast differentiation and dentin formation, implying that *Smad4*-dependent TGF-β/BMP signaling plays a significant role in odontoblast differentiation and dentin formation during tooth development. In addition, impaired dentin formation was commonly exhibited in different types of *Smad4* ablation models such as *Dspp-Cre;Smad4*, *OC-Cre;Smad4*, and *Col-Cre;Smad4* at the crown, cervix, and root furcation area of molars respectively [27]. These abnormalities suggest the requirement of Smad4-dependent TGF-β/BMP signaling for appropriate dentin formation. Also, exogenous TGF-β1 regulates Dspp and Dmp1 expression in odontoblast cell lines [28, 29], indicating that TGF-β/BMP signaling regulates dentin matrix secretion. In our results, OC^{Cre}*Smad4* mice exhibited thinner crown dentin and bone-like root dentin structures in the cervical region when compared to control mice. This abnormality suggests the failure of appropriate odontoblast differentiation and resultant reduction of dentin matrix apposition.

Taken together, our results suggest that Smad4-dependent TGF-β/BMP signaling plays a critical role in odontoblast differentiation and dentin formation during tooth development.

Acknowledgements

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