歯胚の上皮細胞の運命決定における転写共役因子 Smad4 の役割

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A Role of Transcriptional Co-activator Smad4 in Fate of Dental Epithelial Cells

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抄録

歯の発生における Transforming growth factor-β (TGF-β)ファミリーの役割を明らかにするため、TGF-βファミリーのシグナル伝達に必須の転写共役因子 Smad4 をタモキシフェン投与誘導性に欠失できる新しいコンディショナルノックアウト(cKO)マウスを樹立した。この成獣マウスにタモキシフェンを投与した後に切歯を解析すると、Smad4 cKO マウスでは、リン酸化 Smad1/5/9 とリン酸化 Smad2/3 に変化が認められないにも関わらず、分化後期のエナメル芽細胞を含む上皮細胞に形態異常を認めた。さらに、Smad4 cKO マウスの上皮細胞層では、アルカリホスファターゼの発現低下と鉄沈着の低下を認めた。これらの結果より、TGF-βファミリーの Smad4 依存的シグナルは、マウス切歯歯胚の上皮細胞の分化と機能発現に重要な役割を果たすと考えられた。

検索用語：TGF-βファミリー, Smad4, 歯の発生, 切歯

Abstract

To examine the role of transforming growth factor-β (TGF-β) family in tooth development, we developed a novel Smad4 conditional knockout (cKO) mouse in which Smad4 is deleted by tamoxifen treatment. We analyzed the incisors of the adult mouse after tamoxifen treatment. Smad4 cKO mouse showed ameloblastic hypoplasia and an epithelial layer at late differentiation stages, although the levels of phosphorylated Smad1/5/9 and phosphorylated Smad2/3 were unchanged. Expression levels of alkaline phosphatase and the intensity of iron pigmentation were reduced in dental epithelial cells in the Smad4 cKO mouse. These findings suggest that Smad4-dependent signaling of the TGF-β family is required for the differentiation and functions of the dental epithelial cells in adult mouse incisors.

Key words: TGF-β family signaling, Smad4, tooth development, incisor

Running title : Smad4 in dental epithelial cells of adult mouse incisors

Introduction

Ameloblasts and odontoblasts are developed from dental epithelial cells and mesenchymal cells, respectively, to form enamel and dentin during tooth development[1)]. Rodent incisor is a useful experimental model to study molecular mechanisms of the tooth development because it continuously grows after birth (even in adults) and shows continuous tooth development[2-4)]. The incisors contain a stem cell pool of the dental epithelium and mesenchyme, called cervical loop, at the proximal end of the labial side[2-4)]. The mesenchymal stem cells differentiate into dental pulp and odontoblasts. In contrast, the epithelial stem cells differentiate into secretory ameloblasts, stratum intermedium (SI) and stellate reticulum (SR), which are outer cell layers of ameloblasts. Ameloblasts, which are polarized columnar cells aligned on enamel, secrete matrix proteins and enzymes such as amelogenin, ameloblastin, enamelin, enamelysin/MMP-20, kallikrein/KLK4 and alkaline phosphatase (ALP) to deposit and mineralize enamel during differentiation at secretory and maturation stages[5)]. The SI and SR form papillary layer, characterized by its papillary form and the deep invagination with capillary network in the maturation stage. The papillary layer regulates the enamel mineralization in cooperation with ameloblasts[6)]. The ameloblasts decrease in height and lose their characteristic Tomes' process through the maturation stage. These cells progress becoming cuboidal and lose secretory characteristics as they entered the post-maturation stage[7-8)].

The process of dental cell proliferation and differentiation is regulated by various signaling molecules. Members of the transforming growth factor-β (TGF-β) family are key regulators of the tooth development[1)]. The TGF-β family ligands bind to two types of transmembrane serine/threonine kinase receptors, type I and type II receptors. Type II receptors (constitutively active kinases) phosphorylate type I receptors as substrates[9,10)]. Type I receptors are inactive kinases but get activated through phosphorylation by type II receptor

kinases. Then, the type I receptors phosphorylate downstream effectors, such as transcription factors Smads. The TGF-β family are classified into osteogenic and non-osteogenic members, based on their biological activity in an ectopic bone-formation assay. The osteogenic members induce phosphorylation of Smad1, Smad5 and Smad9 in target cells. In contrast, the non-osteogenic members induce phosphorylation of Smad2 and Smad3[9,10)].

Smad4 is an essential co-activator for transcriptional activity of all phosphorylated Smad proteins in the TGF-β family signaling. Smad4 forms complexes with the phosphorylated Smad proteins and move into the nucleus to regulate transcription of target genes[11)]. Smad4-null mouse (global knockout (KO) mouse) dies at about 7-8 days during embryonic development (E7-8)[12)]. Therefore, it is hard to investigate the role of TGF-β family in mature tissues using a global Smad4 KO mouse. Tissue-specific cKO of Smad4 has been developed to study a role of the TGF-β family during tooth development[13-18)]. Lack of Smad4 in mesenchymal cells induced morphological and functional defects in odontoblasts leading to abnormalities in dentin and root formation[13-15)]. Loss of Smad4 in epithelial specific cKO causes decreased enamel formation[16-18)]. However, it is still unknown the role of TGF-β family signaling through Smad4 in tooth development, especially in dental epithelial cells, because global KO and epithelial specific cKO mouse of Smad4 died soon after birth or earlier. In the previous study, examination of later stages of tooth formation was impossible using embryonic molar tooth germ [12,16-18)]. In the present study, we developed novel Smad4 cKO mouse, in which Smad4 is deleted in response to tamoxifen treatment in adults and analyzed focusing on incisors. These mice allow us to study the role of Smad4 in tooth development. Furthermore, mouse incisors provided us continuous observation of dental epithelial cells. Using this model, we found that Smad4 is required for differentiation and function in dental epithelial cells at later stages in an adult mouse incisor.

Materials and Methods

Animals

Smad4:floxed (*Smad4$^{f/f}$*) mice[12)] and *CAGGCre-ER^{TM}* (*CAG-CreERt$^{TG/-}$*) mice[19)] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *Smad4$^{f/f}$* mice were crossed with *CAG-CreERt$^{TG/-}$* mice to develop *Smad4$^{f/f}$*; *CAG-CreERt$^{TG/-}$* (Smad4 cKO) mice, in which exon 8 of *Smad4* was deleted in response to tamoxifen-treatment using Cre-*loxP* system (Fig. 1A). *Smad4$^{f/f}$*; *CAG-CreERt$^{-/-}$* mice were used as control. The male mice were injected with tamoxifen (Sigma-Aldrich, St Louis, MO, USA) (40 mg/kg/day) intraperitoneally (i.p.) daily for 5 days at 10-week-old. On day 23 after the first injection, the mice were sacrificed, and parts of the mandible were carefully dissected to be used for further analysis. The mice were housed in a 12 hour light/dark cycle. This study was approved by the Institutional Animal Care and Use Committee (Permission number: 2046) and was conducted according to the Saitama Medical University Animal Experimentation Regulations.

μCT analysis

Mandibles were scanned using the micro-computed tomography (μCT) (CosmoScan GX; Rigaku, Tokyo, Japan) to produce 60 μm isotropic voxel spacing, using X-ray setting of 90 kV, 88 μA, high resolution mode at 4 min. The 3D images of the mandible were reconstructed from the scanning slice data.

Histological analysis

Mandibles were dissected and fixed with 4% paraformaldehyde for 2 days at 4°C, and subsequently decalcified with Osteosoft (Merck Millipore, Darmstadt, Germany) for 10 days at room temperature (RT). Paraffin sections were made at 4 μm thickness on sagittal and

coronal planes. Some of the sections were stained with hematoxylin-eosin (HE) staining according to the standard method.

For Azan staining, the sections were deparaffinized using xylene and hydrated with descending alcohol series (100%, 70%). Thereafter, they were immersed in 0.1% azocarmine G solution containing 1% acetic acid for 1 hour and rinsed with tap water. Differentiation by aniline-alcohol stopped by adding 95% alcohol with 1% acetic acid for 1 min. Subsequently, slides were incubated in 5% phosphotungstic acid solution for 1 hour, treated with Aniline Blue-Orange G solution for 30 min and differentiated by 95% alcohol. Slides were dehydrated and mounted.

For Berlin Blue staining, after deparaffinization and rehydration, slides were stained with the mixture of 2% ferrocyan-kalium solution and 1% hydrochloric acid for 30 min and then rinsed with distilled water. For counterstaining of the nucleus, slides were treated by nuclear fast red for 5 min, dehydrated and mounted.

For immunohistochemical analysis, the sections were dried at RT and treated with 3% H_2O_2 for 5 min to inhibit endogenous peroxidase activity. After been washed with PBS, the sections were treated with 10% goat serum in PBST at RT for 1 hour and then sequentially stained with a rabbit anti-mouse Smad4 monoclonal antibody (ab40759, 1/400 ; Abcam, Cambridge, UK), a rabbit anti-phosphorylated Smad1/5/9 polyclonal antibody (9511, 1/200 ; Cell signaling technology, Danvers, MA, USA), a rabbit anti-mouse phosphorylated Smad2/3 polyclonal antibody (sc-11769, 1/200 ; Santa Cruz Biotechnology, Dallas, TX, USA) or a rabbit anti-rat bone-specific alkaline phosphatase polyclonal antibody (M190, 1/800 ; Takara Bio., Shiga, Japan) over night at 4°C. After washing with PBS, the sections were reacted with the anti-rabbit antibody (Nichirei Bio., Tokyo, Japan) at RT for 60 min. Visualization was made using 3,3′-diaminobenzidine tetrahydrochloride (DAB) horseradish peroxidase (HRP) substrate kit (Vector laboratories, Burlingame, CA, USA), and sections were counterstained

with hematoxylin for 1 min and dehydrated and mounted. For the negative control, immunostaining procedures were made with an anti-normal rabbit IgG polyclonal antibody (2729, Cell signaling technology) instead of the primary antibody; this confirmed the absence of nonspecific immunoreactions. They were analyzed using a microscope BioRevo BZ-9000 (Keyence, Osaka, Japan).

Results

Loss of Smad4 leads to hypoplasia of dental epithelial cells at the maturation and post-maturation stages

To examine the role of TGF-β family signaling through Smad4 in incisors after birth, we established a mouse line of Smad4 cKO in response to tamoxifen-treatment (Fig. 1A). After the experiment period, DNA were isolated from the multiple tissues/organs (spleen, lung, liver, and kidney) of Smad4 cKO mice. In the previous study[12)], the mice were indicated lack of exon 8 of *Smad4* by carrying out the reverse transcription polymerase chain reaction (RT-PCR) technique in which primers were used to distinguish between the conditional allele and the wild-type allele (data not shown). Mineralization level of incisors of control and Smad4 cKO mice were not distinguishable in μCT images (Fig. 1B). However, in the histological analysis, we noticed that a dental epithelial layer (stained in red) in incisors of Smad4 cKO mice was thicker than control mice on their labial side after Azan staining (Fig. 1C).

To examine the role of Smad4 in the dental epithelial cells, we analyzed longitudinal sections of the incisors of the control and Smad4 cKO mice (Fig 1D). A series of differentiation process in dental epithelium were classified into four stages according to their morphological changes: a cervical loop (which is a pool of dental stem cells) secretory, maturation and post-maturation stages (Fig 1D). The morphology of dental epithelial cells in the cervical loop, secretory and maturation stages were indistinguishable in control and Smad4 cKO mice (the panels a, b, c, e, f and g in Fig. 1D). However, at the post-maturation stage, ameloblasts were flattened and depolarized in Smad4 cKO mice in contrast to the polarized columnar and cuboidal morphology in control mice (the panels d and h in Fig. 1D). In addition, the papillary layer, an outer epithelial cell layer of ameloblasts, was disorganized with broken microvascular structure in Smad4 cKO mice. The boundary between ameloblasts and the papillary layer was unclear in Smad4 cKO mice (the panels c, d, g and h in Fig. 1D).

Immunohistochemical analysis of Smad4 and phosphorylated Smad1/5/9 and Smad2/3 in incisors

We examined the localization of Smad4 and transcriptional partners, such as phosphorylated Smad1/5/9 and Smad2/3, in incisors by immunohistochemical analysis (Fig. 2). High levels of Smad4 were detected throughout in dental epithelial and mesenchymal cells, such as ameloblasts, papillary layer, odontoblasts and pulp in the control mice (panels a-d in Fig. 2A). The deletion of Smad4 was confirmed by a marked reduction of the protein levels of Smad4 in the cKO mice in a broad range of cell types. Additionally, the expression pattern of Smad4 protein depended on the area in Smad4 cKO mice (panels e-h in Fig. 2A). Immunohistochemical analysis of Smad4 displayed a mosaic pattern of expression, especially in an immature area (panels e' in Fig. 2A). Both phosphorylated Smad1/5/9 and Smad2/3 were detected in various types of dental cells in control mice (panels a-d in Fig. 2B and C, respectively). As seen in control mice, the phosphorylated Smad1/5/9 and Smad2/3 were seen in the dental epithelial and mesenchymal cells in Smad4 cKO mice, confirming that phosphorylation of Smad1/5/9 and Smad2/3 is an event independent from Smad4 (Fig. 2A, 2B and 2C).

Smad4-dependent signaling is required for the maintenance of normal function in dental epithelial cells

To examine the changes in the dental epithelial cells in the Smad4 cKO mice, we analyzed the localization of ALP, which is an enzyme highly expressed in the dental epithelial cells, SI layer, maturation ameloblasts, papillary layer and odontoblasts (panels a-d in Fig. 3A)[20,21]. The protein levels of ALP were reduced or negligible in the dental epithelial cells in the Smad4 cKO mice (panels e-h in Fig. 3A). Furthermore, we stained the sections with Berlin

blue, which detects iron, to analyze the ability of iron transportation in the dental epithelial cells. In agreement with the previous reports [22,23)], the iron pigmentation with Berlin blue staining was detected in ameloblasts and microvascular structure of papillary layer at the maturation and post-maturation stages in control mice (panels c, d in Fig. 3B). However, in Smad4 cKO mice, the pigmentation in ameloblasts and papillary layer was almost negligible especially at the maturation stage, suggesting that the iron transporting ability was reduced in the dental epithelial cells (panels g and h in Fig. 3B). Taken together, these findings suggest that Smad4 is required for the normal development of dental epithelial cells, such as ameloblasts and SI layer, at the maturation and post-maturation stages of adult mouse incisors.

Discussion

In the present study, we examined the role of TGF-β family signaling in adult mouse incisors by establishing a mouse line of cKO of Smad4 in response to tamoxifen-treatment using Cre-*loxP* system, a site-specific recombinase technology[12,28)]. In Smad4 cKO mice, recombination between two *loxP* sequence include the target DNA sequences deletes exon8 contained in the MH2 domain of *Smad4*[5,12)]. The *Smad4* MH2 domain plays a dominant role in the interaction with transcriptional co-activators and is directly associated with the basal transcriptional mechanisms[5)]. In the previous study, inactivation of the Smad4 was determined in the Cre-mediated recombination removing exon 8 of *Smad4*.

Mouse incisor is a useful model for studying molecular mechanisms of dental development in the place of embryonic molar, because incisors are continuously formed and provide us with successive observations of tooth formation even in adulthood. The TGF-β family signaling through Smad4 plays important roles in embryonic molar crown formation[13-18)]. However, the role of Smad4 was still unclear in the whole stage from dental stem cells to tooth eruption. Since global KO and epithelial specific cKO of Smad4 are lethal during embryonic development and soon after birth, respectively, further examination of the later stage of tooth formation was impossible[12,16-18)]. This is the first study to develop a cKO mouse of Smad4 in response to a tamoxifen-treatment in the adult mouse. These mice allowed us to study the role of Smad4 in development of incisors after birth.

Using the Smad4 cKO mice, we found that Smad4 is required for normal development of dental epithelial cells in adult mouse incisors, especially in ameloblasts and papillary layer cells at the late differentiation stages. Ameloblasts are polarized columnar cells, which secrete enamel matrix proteins and proteinases[5)]. The cooperation of ameloblasts and papillary layer regulates enamel microenvironment, enables degradation of enamel matrix proteins, dehydration and influx of calcium, phosphate and iron ions[6,22,23)]. The expression

levels of ALP and the iron transport ability evaluated by Berlin blue staining were markedly reduced in ameloblasts and papillary layer in Smad4 cKO mice. ALP is highly expressed in dental epithelial cells, such as ameloblasts at maturation stages, SI layer and papillary layer to regulate mineralization of teeth[20,21)]. Previous studies reported that the lack of ALP causes an enamel defect[24,25)]. Additionally, mutations in ALP gene has been associated with hypophosphatasia (HPP), which leads to a lack of ALP and defects in teeth. [24,25)] In mouse mature osteoblast, deletion of Smad4 significantly diminished the bone volume and the expression of ALP in vivo[26)]. Considering previous researches, Smad4 and ALP are essential for the bone and teeth formation. Further consideration will be needed to yield any findings about the enamel formation and reduction of ALP. Regarding the iron transport ability, ameloblasts at maturation stage are limited to rodent incisors, but not humans, and involve the ferritin (iron-binding protein) based pigmentation. Therefore, rodent incisors exhibit deep-orange surface color[27)]. Iron incorporation in enamel provides resistance against cracking and abrasion[28)]. The ability of iron transport was also reported to be involved in mineralization of enamel[22,23)]. These findings suggest that the mineralization levels of enamel should be reduced in Smad4 cKO mice, although they did not show an abnormality of mineralization in neither enamel nor dentin evaluated by μCT analysis. This was because the experimental period used in the present study (3-4 weeks after tamoxifen injection) might be too short to detect any abnormality in mineralization in adult mouse incisors by μCT. Additionally, as shown in Fig2A, Smad4 cKO mice did not show total deletion of Smad4. This was likely caused due to low rate of recombination. It is known that higher dose of tamoxifen can induce recombination efficiency, but also results in the lethal effect to the mouse[12,19)]. Moreover, Smad4 cKO in the younger mice was still more lethal (data not shown), therefore, in this study, Smad4 was deleted in the 10-week-old mice, which appeared fully grown regarding body weight and sexual maturation. Further studies using epithelial cell-specific Cre-mediated

mouse would be needed to clarify this possibility. However, it deserved more than passing a notice that the partial reduction of Smad4 indicated a correlation among phenotype of dental epithelial cells in Smad4 cKO mice.

Smad4 is an essential co-activator for the transcriptional activity of phosphorylated Smad1/5/9 and Smad2/3 in intracellular signaling of osteogenic BMPs and non-osteogenic TGF-βs of the TGF-β family, respectively[9-11)]. Both phosphorylated Smad1/5/9 and Smad2/3 were detected in various types of dental cells even in the Smad4 cKO mice (present study), indicating that both signaling pathways are activated by osteogenic and non-osteogenic ligands of the TGF-β family in the dental cells. Moreover, these results were expected because Smad4 is required in downstream events of the phosphorylation of Smad1/5/9 and Smad2/3 by receptor kinases and Smad4 does not affect the phosphorylation levels of Smad proteins. However, it was still unclear which type of ligand(s), osteogenic or non-osteogenic members of the TGF-β family, was/were involved in the regulation of the dental epithelial cell function in adult incisors. Treatment of the Smad4 cKO mice with specific inhibitors for the osteogenic and non-osteogenic members of the TGF-β would elucidate this question.

Conclusions

Adult Smad4 cKO mice showed abnormalities in dental epithelial cells, such as ameloblasts and papillary layer. In these cells, the expression levels of ALP and the ability of iron transport were reduced than those in control mice. Our findings suggest that the Smad4-dependent intracellular signaling of the TGF-β family is required for differentiation and function of dental epithelial cells in the adult mouse incisors.

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References

1) Thesleff I : Epithelial-mesenchymal signalling regulating tooth morphogenesis. J Cell Sci **116**, 1647-1648, 2003

2) Harada H, Kettunen P, Jung H S, Mustonen T, Wang YA and Thesleff I : Localization of putative stem cells in dental epithelium and their association with Notch and Fgf signaling. J Cell Biol **147**, 105-120, 1999

3) Harada H, Toyono T, Toyoshima K, Yamasaki M, Itoh N, Kato S, Sekine K and Ohuchi H : FGF10 maintains stem cell compartment in developing mouse incisors. Development **129**, 1533-1541, 2002

4) Harada H and Ohshima H : New perspectives on tooth development and the dental stem cell niche. Arch Histol Cytol **67**, 1-11, 2004

5) Lu Y, Papagerakis P, Yamakoshi Y, Hu JC, Bartlett JD and Simmer JP : Functions of KLK4 and MMP-20 in dental enamel formation. Biol Chem **389**, 695-700, 2008

6) Josephsen K, Takano Y, Frische S, Praetorius J, Nielsen S, Aoba T and Fejerskov O : Ion transporters in secretory and cyclically modulating ameloblasts: a new hypothesis for cellular control of preeruptive enamel maturation. Am J Physiol Cell Physiol **299**, C1299-C1307, 2010

7) Barron MJ, Brookes SJ, Kirkham J, Shore RC, Hunt C, Mironov A, Kingswell NJ, Maycock J, Shuttleworth CA and Dixon MJ : A mutation in the mouse Amelx tri-tyrosyl domain results in impaired secretion of amelogenin and phenocopies human X-linked amelogenesis imperfecta. Hum Mol Genet **19**, 1230-1247, 2010

8) Smith CEL, Poulter JA, Antanaviciute A, Kirkham J, Brookes SJ, Inglehearn CF and Mighell AJ : Amelogenesis imperfecta; genes, proteins, and pathways. Front Physiol **8**, 435, 2017

9) Katagiri T and Watabe T : Bone morphogenetic proteins. Cold Spring Harb Perspect Biol **8**, a021899, 2016

10) Katagiri T, Tsukamoto S and Kuratani M : Heterotopic bone induction via BMP signaling: Potential therapeutic targets for fibrodysplasia ossificans progressiva. Bone, doi:10.1016/j.bone.2017.07.024., 2017

11) Shi Y and Massague J : Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell **113**, 685-700, 2003

12) Yang X, Li C, Herrera PL and Deng CX : Generation of Smad4/Dpc4 conditional knockout mice. Genesis **32**, 80-81, 2002

13) Li J, Huang X, Xu X, Mayo J, Bringas P Jr, Jiang R, Wang S and Chai Y : SMAD4-mediated WNT signaling controls the fate of cranial neural crest cells during tooth morphogenesis. Development **138**, 1977-1989, 2011

14) Kim TH, Bae CH, Lee JY, Lee JC, Ko SO, Chai Y and Cho ES : Temporo-spatial requirement of Smad4 in dentin formation. Biochem Biophys Res Commun **459**, 706-712, 2015

15) Yun CY, Choi H, You YJ, Yang JY, Baek JA, Cho ES : Requirement of Smad4-mediated signaling in odontoblast differentiation and dentin matrix formation. Anat Cell Biol **49**, 199-205, 2016

16) Huang X, Xu X, Bringas P Jr, Hung YP and Chai Y : Smad4-Shh-Nfic signaling cascade-mediated epithelial-mesenchymal interaction is crucial in regulating tooth root development. J Bone Miner Res **25**, 1167-1178, 2010

17) Li J, Feng J, Liu Y, Ho TV, Grimes W, Ho HA, Park S, Wang S and Chai Y : BMP-SHH signaling network controls epithelial stem cell fate via regulation of its niche in the developing tooth. Dev Cell **33**, 125–135, 2015

18) Xu X, Han J, Ito Y, Bringas P Jr, Deng C and Chai Y : Ectodermal Smad4 and p38MAPK are functionally redundant in mediating TGF-beta/BMP signaling during tooth and palate development. Dev Cell **12**, 322-329, 2008

19) Hayashi S and McMahon AP : Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol **244**, 305-318, 2002

20) Nakano Y, Le MH, Abduweli D, Ho SP, Ryazanova LV, Hu Z, Ryazanov AG, Den Besten PK and Zhang Y : A critical role of TRPM7 as an ion channel protein in mediating the mineralization of the craniofacial hard tissues. Front Physiol **7**, 258, 2016

21) Yadav MC, de Oliveira RC, Foster BL, Fong H, Cory E, Narisawa S, Sah RL, Somerman M, Whyte MP and Millán JL : Enzyme replacement prevents enamel defects in hypophosphatasia mice. J Bone Miner Res **27**, 1722-1734, 2012

22) Yanagawa T, Itoh K, Uwayama J, Shibata Y, Yamaguchi A, Sano T, Ishii T, Yoshida H and Yamamoto M : Nrf2 deficiency causes tooth decolourization due to iron transport disorder in enamel organ. Genes Cells **9**, 641-651, 2004

23) Yoshioka H, Yoshiko Y, Minamizaki T, Suzuki S, Koma Y, Nobukiyo A, Sotomaru Y, Suzuki A, Itoh M and Maeda N : Incisor enamel formation is impaired in transgenic rats overexpressing the type III NaPi transporter Slc20a1. Calcif Tissue Int **89**, 192-202, 2011

24) McKee MD, Yadav MC, Foster BL, Somerman MJ, Farquharson C and Millán JL : Compounded PHOSPHO1/ALPL deficiencies reduce dentin mineralization. J Dent Res **92**, 721-727, 2013

25) Gasque KC, Foster BL, Kuss P, Yadav MC, Liu J, Kiffer-Moreira T, van Elsas A, Hatch N, Somerman MJ and Millán JL : Improvement of the skeletal and dental hypophosphatasia phenotype in Alpl-/- mice by administration of soluble (non-targeted) chimeric alkaline phosphatase. Bone **72**, 137-147, 2015

26) Tan X, Weng T, Zhang J, Wang J, Li W, Wan H, Lan Y, Cheng X, Hou N, Liu H, Ding J, Lin F, Yang R, Gao X, Chen D and Yang X : Smad4 is required for maintaining normal murine postnatal bone homeostasis. J Cell Sci. **120**, 2162-2170, 2007.

27) Smith CE : Cellular and chemical events during enamel maturation. Crit Rev Oral Biol Med **9**, 128-161, 1998

28) Pugach MK and Gibson CW : Analysis of enamel development using murine model systems: approaches and limitations. Front Physiol **5**, 313, 2014

Figure legends

Figure 1. Ablation of Smad4 induces an abnormality in dental epithelial cell layers

(A) Male *Smad4*$^{f/f}$*; CAG-CreERt*$^{-/-}$ (Control) and *Smad4*$^{f/f}$; *CAG-CreERt* $^{TG/-}$ (Smad4 cKO) mice were injected with 40 mg/kg of tamoxifen i.p. daily for 5 days at 10-week-old. On day 23 after the first tamoxifen injection, the mice were harvested for μCT and histological analysis.

(B) μCT images of control (upper panels) and Smad4 cKO mice (lower panels). Left: 3D model of mandibles. Right: coronal sections of mandibles at the tooth cervix in a gray scale, respectively (n = 2), Scale bars indicate 1.0mm.

(C) Coronal sections of mandibular incisors on the labial side prepared from control (panels a and b) and Smad4 cKO mice (panels c and d). The sections were examined with Azan staining (n = 3). Coronal sections of mandibular incisors. Scale bars indicate 100 μm (panels a and c), High-magnification views in the boxed areas in a and c are shown in b and d respectively. Scale bars indicate 10 μm (panels b and d). De: dentin, En: enamel, AB: alveolar bone and CT: connective tissue.

(D) Sagittal paraffin sections of mandibular incisors prepared from control (panels a-d) and Smad4 cKO mice (panels e-h) stain by Hematoxylin and Eosin. Ep: epithelial cells, Me: mesenchymal cells, Am: Ameloblasts, SI: stratum intermedium, SR: stellate reticulum, En: enamel, PL: papillary layer and *: microvascular. Scale bars indicate 50 μm (n = 4).

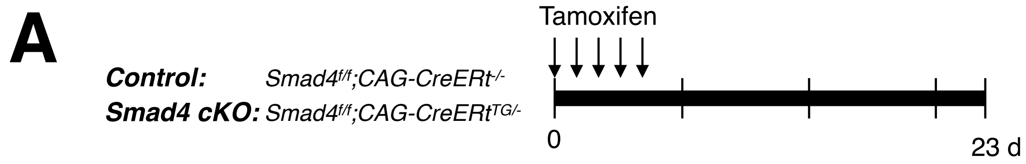
Figure 2. Immunohistochemical analysis of Smad4, phosphorylated Smad1/5/9 and phosphorylated Smad2/3 in control and Smad4 cKO incisors

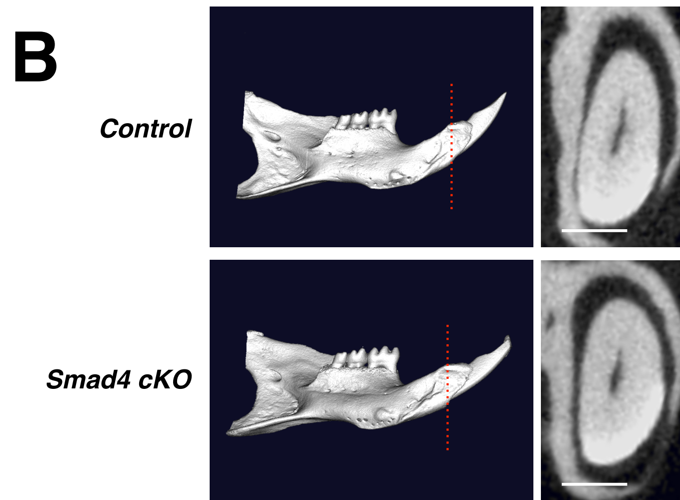
Sagittal paraffin sections of mandibular incisors prepared from control (panels a-d) and Smad4 cKO mice (panels e-h) were stained with a rabbit anti-mouse Smad4 monoclonal

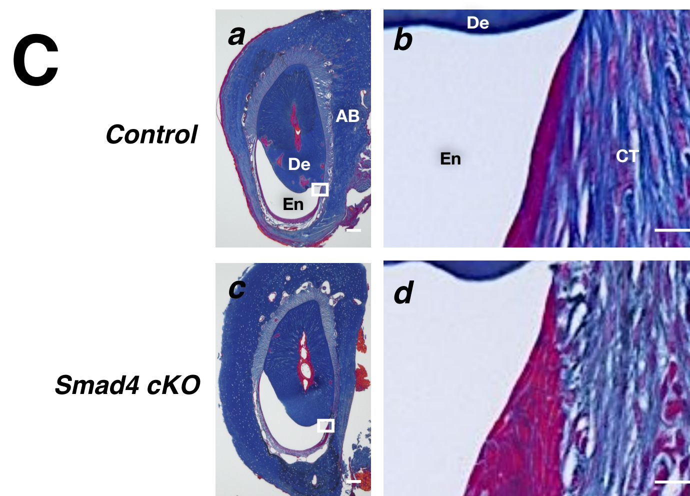
antibody(A), a rabbit anti-phosphorylated Smad1/5/9 polyclonal antibody and a rabbit anti-mouse phosphorylated Smad2/3 polyclonal antibody(C). The antigen-antibody complexes were visualized by DAB. Scale bars indicate 50 μm (panels a-h in Fig. 2A-C). High-magnification views in the boxed areas in a and e are shown in a' and e' respectively. Scale bars indicate 50 μm. (panels a' and e' in Fig. 2A) (n = 3).

Figure 3. Functional abnormalities of dental epithelial layers observed in Smad4 cKO mice

Sagittal paraffin sections of mandibular incisors prepared from control (panels a-d) and Smad4 cKO mice (panels e-h) were stained with a rabbit anti-rat bone-specific alkaline phosphatase polyclonal antibody (A) (n = 4) and Berlin blue (B) (n = 2), respectively. The antigen-antibody complex was visualized by DAB. Scale bars indicate 50 μm.







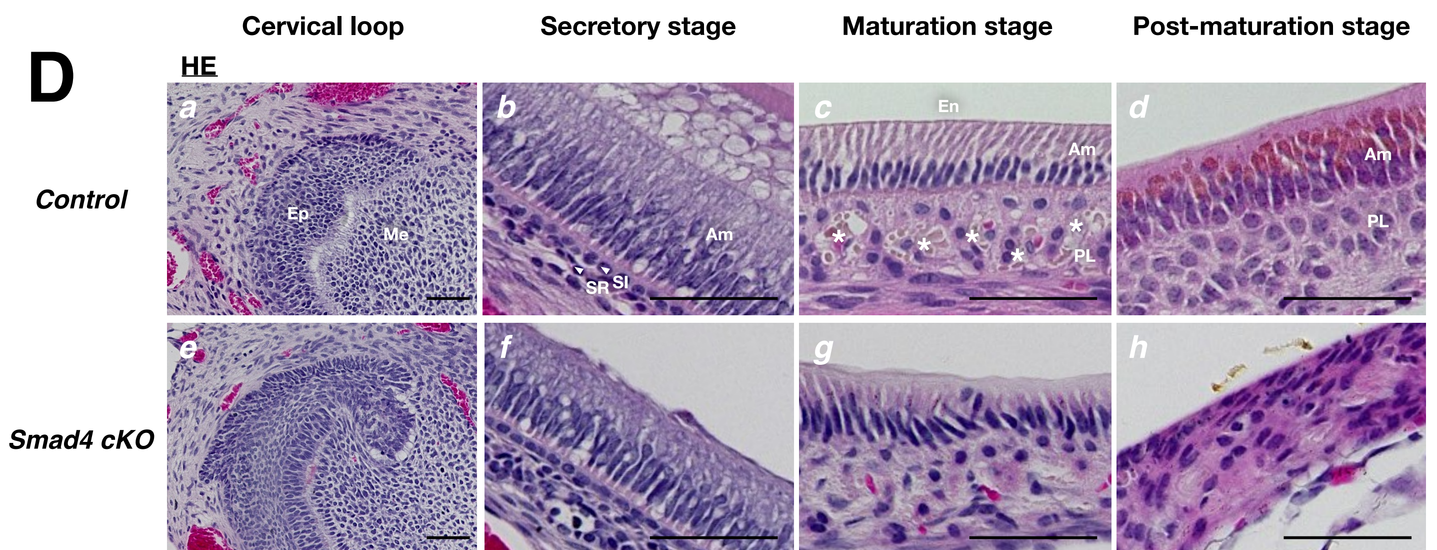


Figure 1

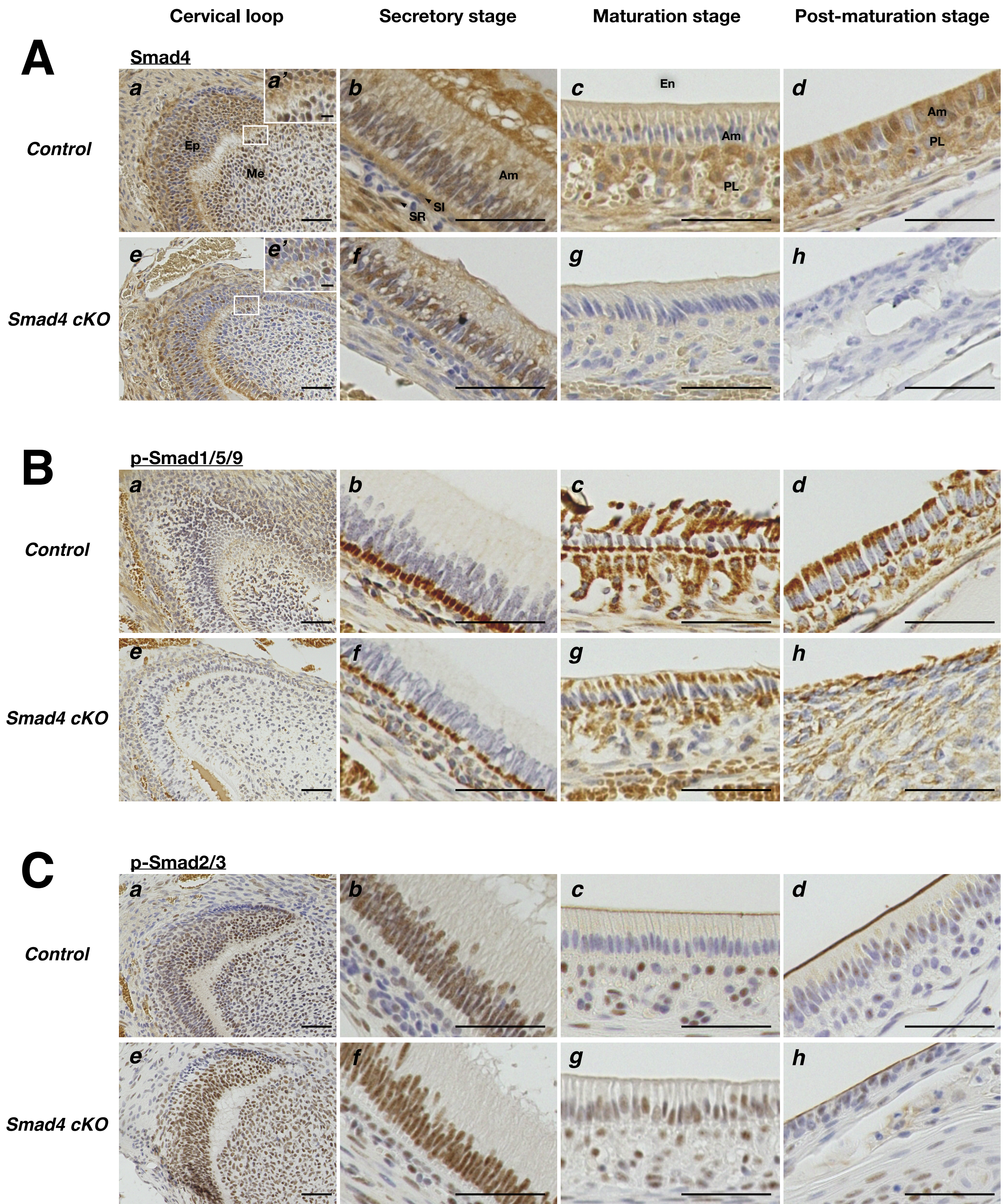


Figure 2

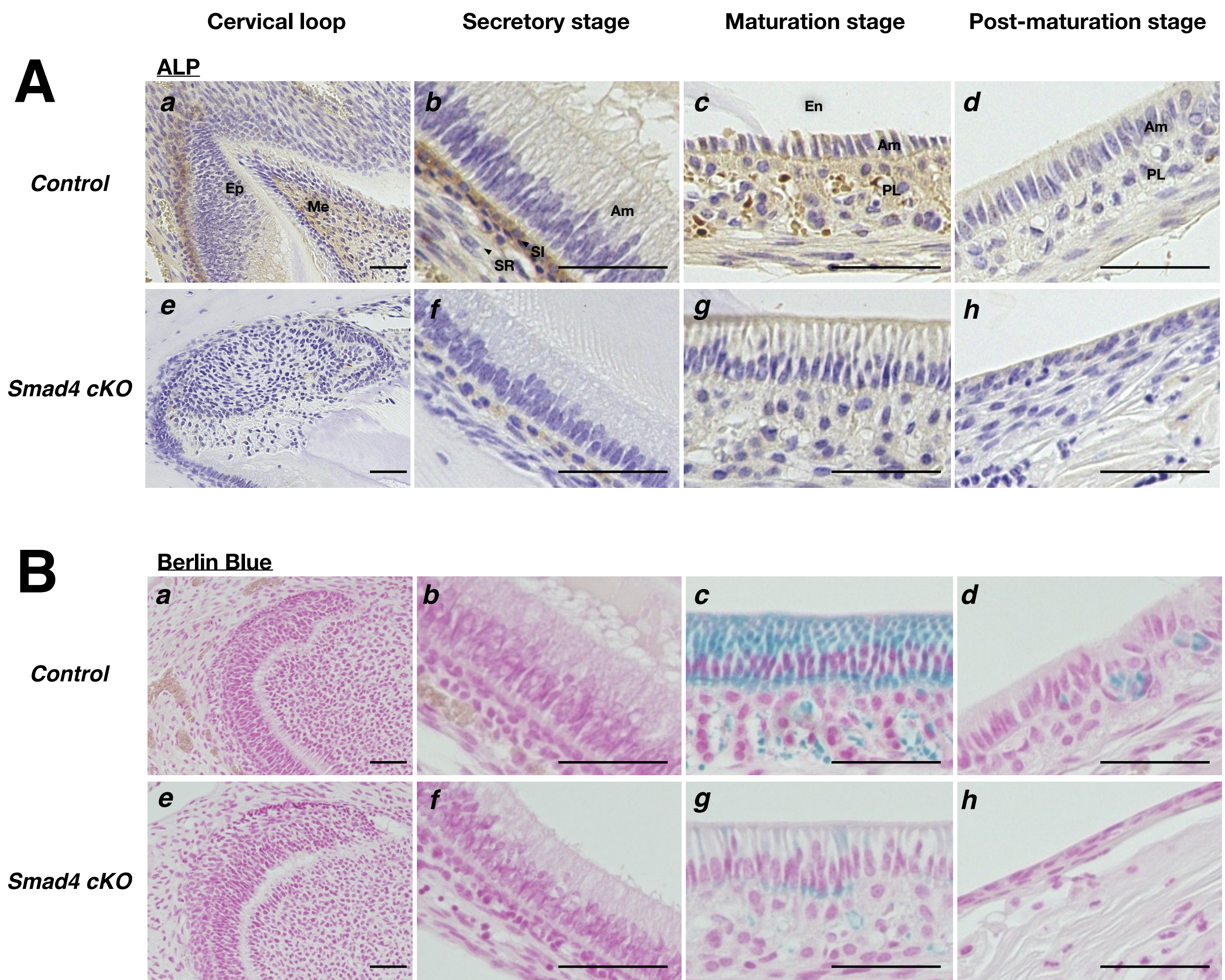


Figure 3