Journal of Dental Research
2017, Vol. 96(4) 421–429

DOI: 10.1177/0022034516683674
journals.sagepub.com/home/jdr

Loss of Function of *Evc2* in Dental Mesenchyme Leads to Hypomorphic Enamel

H. Zhang[1], H. Takeda[2], T. Tsuji[3], N. Kamiya[1*], T. Kunieda[3], Y. Mochida[4], and Y. Mishina[1]

Abstract

Ellis-van Creveld (EvC) syndrome is an autosomal-recessive skeletal dysplasia, characterized by short stature and postaxial polydactyly. A series of dental abnormalities, including hypomorphic enamel formation, has been reported in patients with EvC. Despite previous studies that attempted to uncover the mechanism leading to abnormal tooth development, little is known regarding how hypomorphic enamel is formed in patients with EvC. In the current study, using *Evc2*/*Limbin* mutant mice we recently generated, we analyzed enamel formation in the mouse incisor. Consistent with symptoms in human patients, we observed that *Evc2* mutant mice had smaller incisors with enamel hypoplasia. Histologic observations coupled with ameloblast marker analyses suggested that *Evc2* mutant preameloblasts were capable of differentiating to secretory ameloblasts; this process, however, was apparently delayed, due to delayed odontoblast differentiation, mediated by a limited number of dental mesenchymal stem cells in *Evc2* mutant mice. This concept was further supported by the observation that dental mesenchymal-specific deletion of *Evc2* phenocopied the tooth abnormalities in *Evc2* mutants. Overall, our findings suggest that mutations in *Evc2* affect dental mesenchymal stem cell homeostasis, which further leads to hypomorphic enamel formation.

Keywords: cilium, Hedgehog signaling, ameloblast, odontoblast, *Limbin*, stem cells

Introduction

Ellis-van Creveld (EvC) syndrome is an autosomal-recessive chondrodysplasia (McKusick et al. 1964). Typical symptoms of EvC include shorter limbs and ribs, postaxial polydactyly, dysplastic nails, and cardiovascular defects (McKusick et al. 1964; Baujat and Le Merrer 2007). Genetic studies have linked around two-thirds of EvC-affected patients with mutations in either *EVC* or *EVC2* (Ruiz-Perez et al. 2000, 2003). Interestingly, our previous work identified *EVC2* genetic mutations (also known as *LIMBIN*) in Japanese brown cattle (Takeda et al. 2002). A recent study also identified *EVC2* mutations in Tyrolean Grey cattle (Murgiano et al. 2014). Both cases suggest a conserved function of *EVC2* across different species. In addition to the skeletal and cardiovascular symptoms, several dental abnormalities were documented in patients with EvC, including neonatal teeth, delayed eruption, hypodontia, supernumerary teeth, conical teeth, taurodontism, and hypomorphic enamel formation (Mostafa et al. 2005; Veena et al. 2011). The variable dental symptoms in these patients suggest that *EVC2* plays important roles during different stages of tooth organogenesis.

Tooth development is a sequential process requiring a series of reciprocal interactions between dental epithelium and dental mesenchyme (Thesleff and Hurmerinta 1981). Despite dentition differences between mice and humans, the mouse model has been primarily used for the study of tooth development. During mouse embryonic tooth development, the tooth undergoes a series of morphological differentiations initiated from the thickening of dental epithelium to the bud stage, cap stage,

[1]Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI, USA
[2]Unit of Animal Genomics, GIGA Research Center and Faculty of Veterinary Medicine, University of Liège, 1 Avenue de l'Hôpital, Liège, Belgium
[3]Graduate School of Environmental and Life Science, Okayama University, Okayama City, Japan
[4]Department of Molecular and Cell Biology, Henry M. Goldman School of Dental Medicine, Boston University, Boston, MA, USA
*Current address: Faculty of Budo and Sport Studies, Tenri University, Nara, Japan.

A supplemental appendix to this article is available online.

Corresponding Authors:
Y. Mochida, Department of Molecular and cell Biology, Henry M. Goldman School of Dental Medicine, Boston University, 72 E. Concord St., Boston, MA 02118, USA.
Email: mochida@bu.edu
Y. Mishina, Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, 1011 N. University Avenue, Ann Arbor, MI 48109-1078, USA.
Email: mishina@umich.edu

and bell stage (Jussila and Thesleff 2012; Lan et al. 2014). Later in the bell stage, cells originating from the dental mesenchyme and cells originating from the dental epithelium differentiate into odontoblasts and ameloblasts, both of which will secrete dentin and enamel matrix proteins, respectively. The differentiations of odontoblasts and ameloblasts are processes that require reciprocal interactions between these 2 cell types. Initially, signaling molecules from preameloblasts induce the differentiation of mesenchymal cells into odontoblast cells. The differentiated odontoblasts will then deposit dentin matrix to further induce differentiation of preameloblasts to secretory ameloblasts (Thesleff and Hurmerinta 1981; Bei 2009).

In addition to reciprocal interactions between dental epithelium and mesenchyme, cell-autonomous factors also play important roles during ameloblast differentiation. Of many growth factors, Sonic Hedgehog (SHH)–mediated Hedgehog signaling is required for ameloblast differentiation and maturation. *Shh* is highly expressed in the preameloblast. Genetic ablation of *Shh* in the dental epithelium has resulted in limited numbers of secretory ameloblasts and limited enamel deposition (Dassule et al. 2000). Similarly, specific ablation of *Smo*, the Hedgehog effector protein, in dental epithelium leads to similarly defective ameloblast differentiation (Gritli-Linde et al. 2002).

EvC syndrome has been categorized as ciliopathy due to the intracellular ciliary localization of EVC and EVC2 (Baujat and Le Merrer 2007). In vitro mechanistic studies have demonstrated that EVC and EVC2 form a protein complex at the bottoms of primary cilia, a complex required for transducing Hedgehog signaling (Dorn et al. 2012; Caparrós-Martin et al. 2013). A recent report also indicated that *Evc* is involved in regulating symmetric responses to Hedgehog signaling during molar development (Nakatomi et al. 2013). We recently generated *Evc2/Limbin* mutant mice and identified abnormal tooth phenotypes therein (Zhang et al. 2015), along with other craniofacial abnormalities (Badri, Zhang, Ohyama, Venkitapathi, Alamoudi, et al. 2016; Badri, Zhang, Ohyama, Venkitapathi, Kamiya, et al. 2016). Despite these findings, the pathophysiologic mechanism leading to hypomorphic enamel formation in patients with EvC remains unknown. In the current studies, we used the mouse incisor as a model to investigate the pathophysiologic mechanism leading to hypomorphic enamel formation in the *Evc2/Limbin* mutant mice we generated. The results of our in vivo studies strongly suggest that these mice showed reduced numbers of dental mesenchymal stem cells and a subsequent delay in odontoblast differentiation that, secondarily, led to hypomorphic enamel formation.

Materials and Methods

Animals

Generations of *Evc2*/*Limbin* global mutant mice and *Evc2*/*Limbin* floxed mice were reported previously (Zhang et al. 2015). *Evc2*/*Limbin* homozygous mutant embryos and pups ($Evc2^{ex12/ex12}$) were obtained through the intercrossing of $Evc2^{ex12/+}$ mice. $Evc2^{dE/dE}$ mice are the Cre recombined allele for *Evc2*/*Limbin* mice, which do not have a lacZ expression cassette but show the same phenotype as $Evc2^{ex12/ex12}$ mice (Zhang et al. 2015). Noon of the date when the vaginal plug was observed was designated as embryonic day 0.5 (E0.5). Mice homozygous for the *Evc2*/*Limbin* floxed allele ($Evc2^{fx/fx}$) were bred with heterozygous *Evc2*/*Limbin* floxed mice carrying a *Wnt1-Cre* transgene ($Evc2^{ex12/+}$;*Wnt1*-Cre) (Danielian et al. 1998) to generate specific deletion of *Evc2*/*Limbin* in neural crest derivatives, including dental mesenchyme. All animal experiments were performed in accordance with the policies and federal laws for the judicious use of vertebrate animals, as approved by the University Committee on Use and Care of Animals at the University of Michigan.

Histology, X-Ray, Beta-Gal Staining, Immunohistochemistry, and Alkaline Phosphatase Staining

Mouse mandibles were dissected out and fixed in 4% paraformaldehyde (PFA). Subsequently, they were embedded in paraffin, sectioned parasagittally, and stained with hematoxylin and eosin (H&E) for histologic observations. At least 4 pairs of mutants and littermate controls were used in histologic observations and immunohistochemistry analysis. X-ray images were taken by means of a Faxitron X-ray system (Faxitron). For histologic analysis of undecalcified incisors, P8 mandibles were fixed, cryoprotected, and embedded for cryosection and H&E staining. For beta-gal activity staining and immunohistochemistry, mandibles were fixed with 4% PFA and cryoprotected by 30% sucrose in phosphate-buffered saline (PBS) before being embedded for cryosection; specimens were cut into 10-μm sections. Sections were stained with 1 mg/mL X-gal at 37°C for up to 2 d for beta-gal activity. For immunohistochemistry, sections were incubated overnight at 4°C with antibody against amelogenin (C-19, 1:100; Santa Cruz Biotechnology), Ki-67 (D3B5, 1:200; Cell Signaling), Osterix (OSX, ab22552, 1:500; Abcam), and Phospho-Histone 3 (06-570, 1:500; Millipore). Mouse anti-DSP was a kind gift from Dr. Chunlin Qin (Qin et al. 2003). For immunohistochemistry, a series of cryosections was made from the buccal to the lingual side. Sections containing at least 80% of incisors were used for immunohistochemistry. Quantifications were done with an average of 4 sections used to represent 1 biological sample. The average of 4 different biological samples was then taken for the comparison of differences between controls and mutants. For alkaline phosphatase (ALP) staining, sectioned mandibles were directly stained with BM purple solution (11442074001; Roche).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

E18.5 incisor tooth germs were dissected out from mandibles. Subsequently, dental epithelium and mesenchyme were separated after Dispase (2 U/mL, 04942078001; Roche) treatment and were subjected to RNA isolation with TRIzol reagent (Life

Technology) according to the manufacturer's instructions (Thomas et al. 2000). Quantitative real-time polymerase chain reaction (PCR) was performed with Applied Biosystems ViiA7, with the following TaqMan probes: Mm00494645_m1 for *Gli1*, mm00436026_m1 for *Ptch1*, mm99999915_g1 for *Gapdh*, and Mm00507596-m1 for *Evc2* (ex13 and ex14).

Results

Enamel Hypoplasia Is Observed in Evc2 *Mutant Incisors*

To understand the pathophysiologic mechanism of hypomorphic enamel formation in the EvC syndrome, we studied the enamel formation using global and tissue-specific *Evc2/Limbin* mutant mouse lines generated in our laboratory (Zhang et al. 2015) (Fig. 1A). The mouse incisor grows continuously and showcases all stages of tooth development from the posterior to the anterior region, which makes it a convenient model for the study of epithelial and mesenchymal interactions during enamel formation (Møinichen et al. 1996). Taking advantage of the lacZ expression cassette knocked into the *Evc2* locus in the global heterozygous mutant mice ($Evc2^{ex12/+}$), we identified *Evc2* expression in both the dental epithelium and the dental mesenchyme (Fig. 1B). X-ray radiography of 2-mo-old heads demonstrated that $Evc2^{ex12/ex12}$ mice showed a decreased length of incisor roots compared with those of wild-type and *Evc2* heterozygous ($Evc2^{ex12/+}$) mice (Fig. 1C). Thereafter, wild-type and *Evc2* heterozygous mice were used as controls in comparison with *Evc2* homozygous mutants ($Evc2^{ex12/ex12}$). Molars in *Evc2* mutants also showed a decreased root length, depicted in X-ray radiograms, after removal of the maxillae and mandibles from the rest of the head (Fig. 1D). In addition to the decreased root length, *Evc2* mutant incisors showed less pigmentation compared with littermate controls, suggesting hypomorphic enamel formation in *Evc2* mutants (Fig. 1C).

Enamel Hypoplasia in Evc2 *Mutant Is Due to Delayed Ameloblast Differentiation*

Well-orchestrated ameloblast proliferation, differentiation, and maturation are critical for enamel formation (Thesleff and Hurmerinta 1981). To determine if hypomorphic enamel formation in *Evc2* homozygous mutant mice is due to defective ameloblast differentiation, we examined embryonic mandibular incisors at E18.5, the earliest stage at which secretory ameloblasts can be detected. Compared with controls, in which we observed gradual maturation of ameloblasts from the cervical

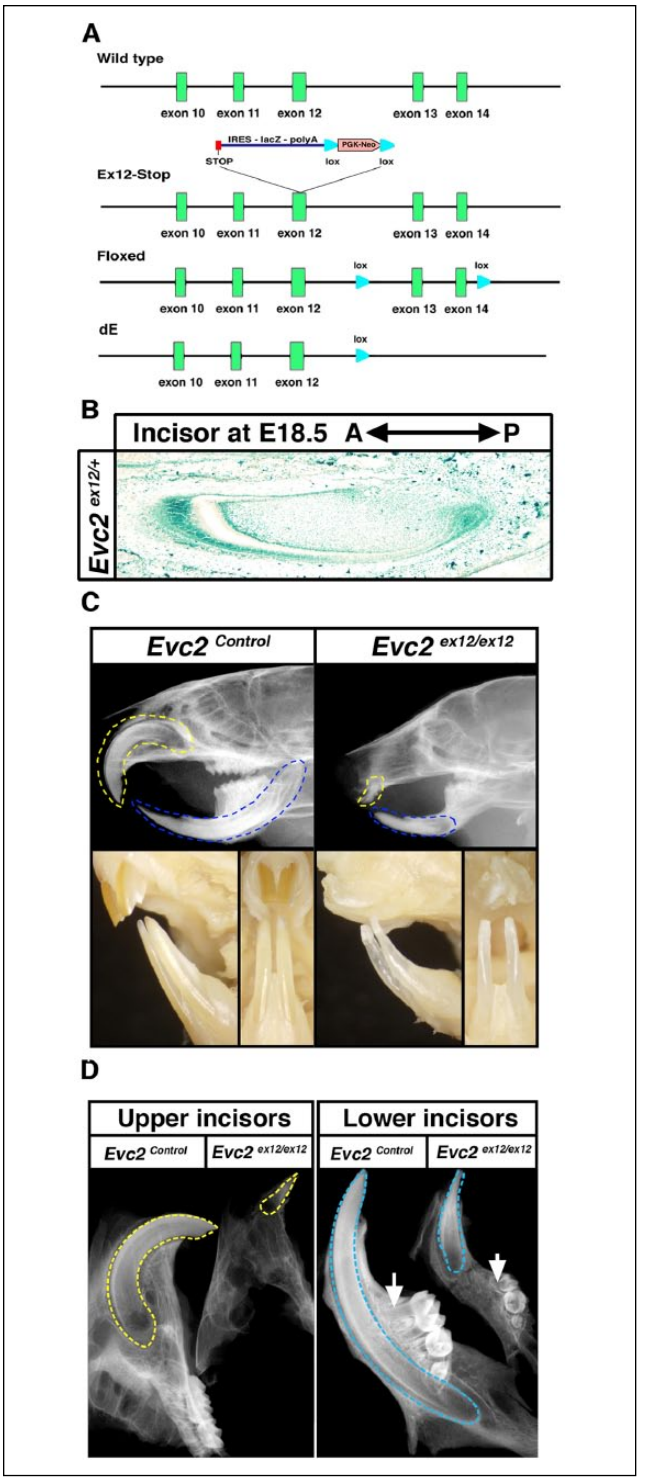

Figure 1. *Evc2* mutant mice showed hypomorphic enamel formation. (**A**) Mutant alleles of *Evc2*/*Limbin* used in this study. Ex12-Stop (a global knockout allele), a stop codon, along with an IRES-LacZ cassette has been inserted into exon 12 to mimic a nonsense mutation identified in human patients. Floxed (a conditional allele) and 2 loxP sites have been introduced to flank exon 13 and exon 14. A Cre-mediated recombination results in a frame shift downstream of exon 12 of *Evc2*. dE, a Cre recombined allele, produces a similarly truncated EVC2 protein with the one from Ex12-Stop allele, if any. (**B**) LacZ staining of $Evc2^{ex12/+}$ incisor indicates that *Evc2* is expressed in dental epithelium, mesenchyme, and surrounding bone tissue. (**C**) Lateral X-ray radiogram of 2-mo-old *Evc2* mutant mice shows hypomorphic maxilla and mandible incisor formations (top). Frontal view and side view of the same mice demonstrate possible hypomorphic enamel in *Evc2* mutant mice (bottom). Yellow dashed lines indicate maxilla incisors and blue dashed lines indicate mandible incisors. (**D**) Lateral X-ray radiogram of 2-mo-old dissected maxilla and mandible shows hypomorphic incisor and molar formation in *Evc2* mutant. Yellow dashed lines indicate maxilla incisors and blue dashed lines indicate mandible incisors.

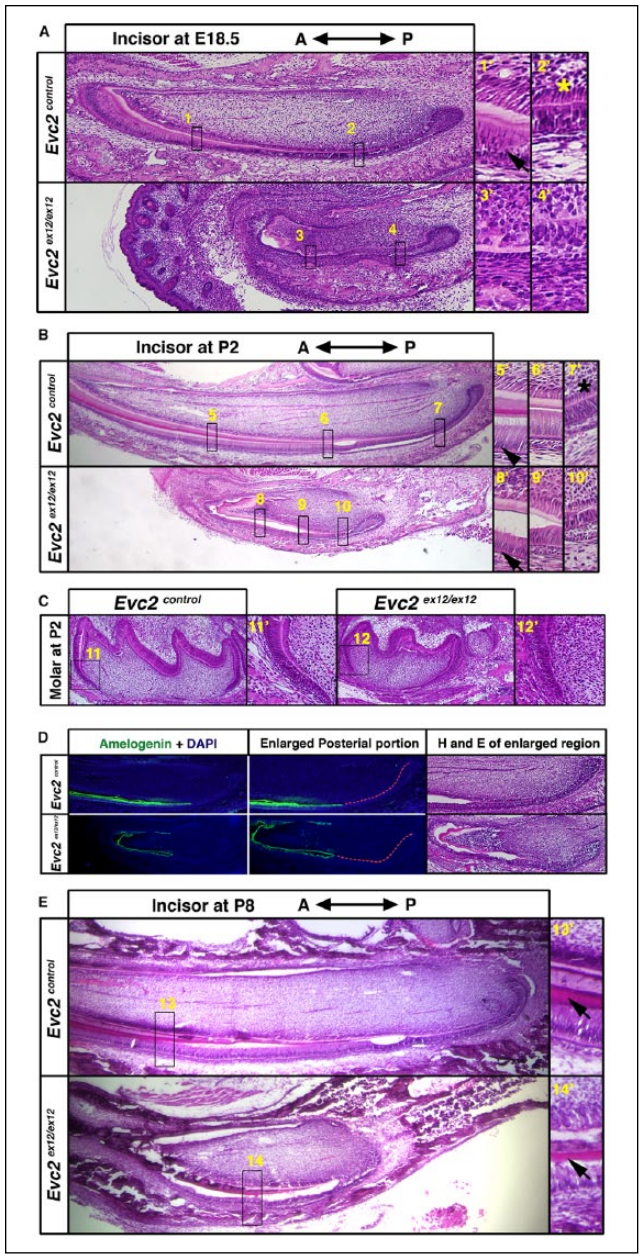

Figure 2. *Evc2* mutant incisors showed delayed ameloblast differentiation and hypomorphic enamel formation. (**A**) Sagittal sections of embryonic day 18.5 (E18.5) embryonic mandible incisors. Regions in boxes in both controls and mutants were enlarged and shown on the right. Arrows indicate the ameloblasts with polarized nuclei and * indicates the odontoblasts with polarized nuclei. (**B**) Sagittal sections of postnatal day 2 (P2) mandible incisors. Regions in boxes in both control and mutant were enlarged and shown on the right. Arrowheads indicate ameloblasts with polarized nuclei in both controls and *Evc2* mutants and * indicates the odontoblasts with polarized nuclei. (**C**) Sagittal sections of postnatal day 1 molar. Regions in boxes in both control and mutant were enlarged and shown. (**D**) Immunohistochemistry of amelogenin in mandible incisors at P2 indicates a delayed ameloblast differentiation. Posterior regions of incisors of both genotypes were enlarged and shown. Red dashed lines indicate the epithelial cells without amelogenin immunosignals. The immediate next section was processed for hematoxylin and eosin staining. (**E**) Sagittal sections of postnatal day 8 (P8) mandible incisors. Regions in boxes in both control and mutant were enlarged and shown on the right. Arrows indicate enamel formation in both control and *Evc2* mutants.

loop to the anterior tip, we could not detect any ameloblasts with polarized nuclei in *Evc2* homozygous mutant incisors (Fig. 2A). At E12.5, when tooth development was just initiated, we detected thickened epithelia in both controls and *Evc2* mutants (Appendix Fig. 1A), suggesting initiation of incisor development is not affected. In contrast, starting from E15.5, we observed a smaller tooth germ with a smaller cervical loop (Appendix Fig. 1B). At postnatal day 2 (P2), we observed ameloblasts with polarized nuclei in both controls and *Evc2* homozygous mutant mice (Fig. 2B). The ameloblasts with polarized nuclei were found at a more anterior region in *Evc2* mutants than in controls, suggesting that maturation of ameloblasts was delayed but not absent in *Evc2* homozygous mutant mice. Similarly, we also detected delayed ameloblast maturation in *Evc2* mutant molars at P2 (Fig. 2C). Delayed ameloblast differentiation is supported by an observation that more cells not producing amelogenin were observed in *Evc2* mutant incisors at P2 (Fig. 2D, Appendix Fig. 1C). To assess if delayed ameloblast maturation is still capable of supporting enamel formation in *Evc2* mutants, we examined undecalcified incisors at postnatal day 8 (P8). Both control and *Evc2* mutant incisors showed enamel formation, further supporting the hypothesis that *Evc2* mutation results in delayed enamel formation in comparison with that in controls (Fig. 2E).

Odontoblast Differentiation Is Delayed in Evc2 *Mutants*

Mutual interactions between ameloblasts and odontoblasts are required for the maturation of both cell types. Particularly, the maturation of ameloblasts depends on dentin secreted by odontoblasts (Linde and Granstrom 1978; Thesleff and Hurmerinta 1981). Along with delayed ameloblast maturation in *Evc2* homozygous mutant mice, we detected delayed odontoblast maturation as well, evidenced by the presence of more preodontoblasts with nonpolarized nuclei in the mesenchyme of *Evc2* homozygous mutant mice at P2 (Fig. 2B). ALP and OSX (aka SP-7) are marker proteins highly produced in differentiated odontoblasts (Linde and Granstrom 1978; Chen et al. 2009). ALP staining results indicated more non–ALP-producing cells in the dental mesenchyme of *Evc2* homozygous mutant mice (Fig. 3A). Similarly, more non–OSX-producing cells were detected in the dental mesenchyme of *Evc2* homozygous mutants (Fig. 3B, C). In summary, along with delayed ameloblast differentiation, we also detected delayed odontoblast differentiation in *Evc2* homozygous mutant incisors.

Delayed Odontoblast Differentiation in Evc2 *Mutant Is Due to Limited Numbers of Dental Mesenchymal Stem Cells*

In mouse incisors, *Gli1* has been reported as a marker for both epithelial and mesenchymal stem cells, which give rise to both ameloblasts and odontoblasts during incisor growth (Seidel et al. 2010; Zhao et al. 2014). To examine if *Evc2* mutation has an impact on maintaining stem cells in both dental epithelium and mesenchyme, we introduced *Gli1-lacZ* into the *Evc2*

mutant background by breeding them with mice carrying the Cre recombined allele for *Evc2*/*Limbin* ($Evc2^{dE/+}$). $Evc2^{dE/dE}$ mice, which do not have a lacZ expression cassette, showed the same phenotype as $Evc2^{ex12/ex12}$ mice (Zhang et al. 2015) (Fig. 1A). The resulting mice ($Evc2^{dE/dE}$; *Gli1-lacZ*) showed a significantly decreased number of lacZ-positive cells in both dental epithelium and mesenchyme (Fig. 4A, B). The mutant incisors appeared to show less intense lacZ staining compared with controls (Fig. 4A), suggesting decreased Hedgehog signaling in the mutant incisors. Previous reports indicated that dental epithelial stem cells are located in the cervical loop, while dental mesenchymal stem cells are located in the mesenchyme next to the cervical loops (Seidel et al. 2010; Zhao et al. 2014). Both groups of stem cells give rise to transient amplifying (TA) cells in the dental epithelium and the mesenchyme, respectively. The TA cells in both the epithelium and the mesenchyme will undergo further differentiation and eventually become postmitotic cells responsible for secreting enamel and dentin. Since Ki-67 is present in the cells within active cell cycles but is absent in the cells out of cell cycles, we used Ki-67 to label the TA cells and to differentiate them from the postmitotic population. In *Evc2* mutant incisors, we observed significantly increased Ki-67–positive cells in the odontoblast layer compared with controls, suggesting that the loss of *Evc2* keeps cells in the mitotic state, resulting in delayed odontoblast differentiation (Fig. 4C, red dashed lines, 4D). Conversely, no significant difference was observed in epithelial TA cells in the ameloblast layer in *Evc2* mutant incisors (Fig. 4C, orange dashed lines, 4D). To examine if the small incisors in *Evc2* mutants are due to differential cell proliferation or cell death, we further examined cell proliferation using antibody against phospho-histone H3 (P-H3) and cell death using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. In *Evc2* mutant incisors at E18.5, we did not detect obvious differences in the numbers of P-H3- and TUNEL-positive cells compared with those in controls (Fig. 4E–G). Given the previous reports that Hedgehog signaling positively regulates odontoblast differentiation (Zhao et al. 2014), the compromised Hedgehog signaling detected in *Evc2* mutant incisors is the likely reason for the delayed odontoblast differentiation observed. Since no differential cell proliferation and apoptosis were detected in Evc2 mutant incisors, we concluded that the decreased numbers of stem cells in the dental mesenchyme led to subsequent shorter incisors.

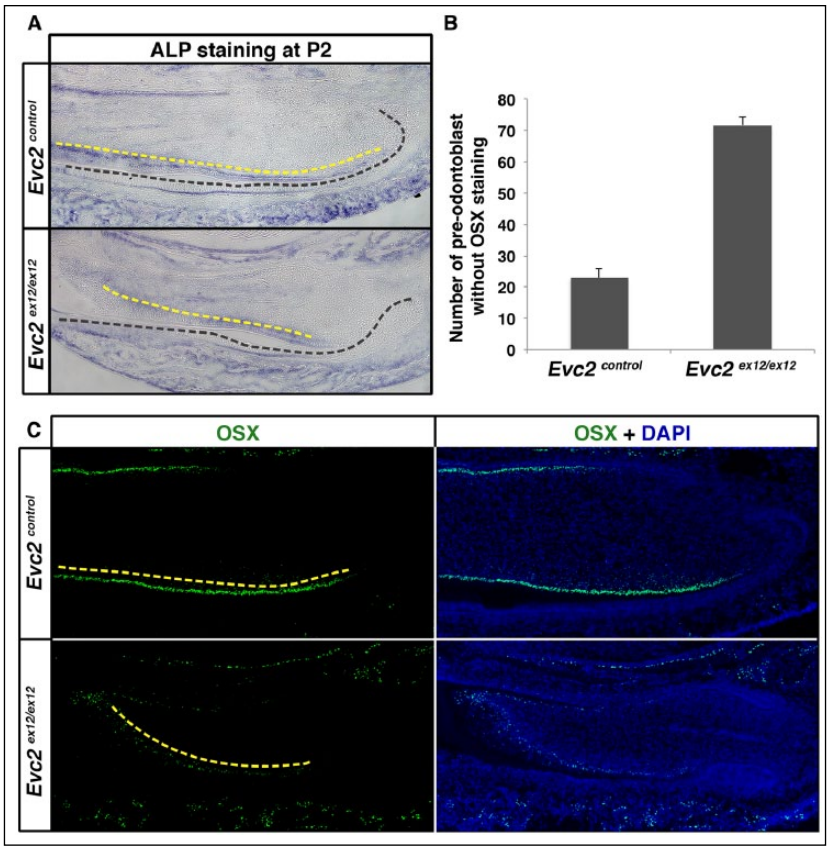

Figure 3. *Evc2* mutant incisors showed delayed odontoblast differentiation. (**A**) Mandible incisors from postnatal day 2 (P2) were sectioned and processed for alkaline phosphatase (ALP) staining. Black dashed lines indicate a boundary between dental epithelium and mesenchyme. Yellow dashed lines indicate ALP-stained cells in the odontoblast layer. (**B, C**) Mandible incisors from P2 were sectioned and processed for immunohistochemistry for Osterix (OSX). White dashed lines separate dental epithelium and mesenchyme. Red dashed lines indicate OSX-expressing dental mesenchymal cells. Yellow dashed lines indicate OSX-stained cells in the odontoblast layer. The number of OSX-expressing dental mesenchymal cells was quantified and is shown in B ($n = 4$, $P < 0.01$).

Mesenchymal-Specific Deletion of Evc2 *Phenocopies Hypomorphic Enamel in* Evc2 *Global Mutant*

Our analysis demonstrated decreased Hedgehog signaling in dental epithelium in *Evc2* mutant incisors (Fig. 4A), which may lead to hypomorphic enamel formation through affecting ameloblast differentiation. Our analysis also demonstrated evidence of delayed odontoblast differentiation in association with a limited number of dental mesenchymal stem cells, which may also affect ameloblast differentiation and lead to subsequent hypomorphic enamel formation. To determine which alteration(s) critically contribute to hypomorphic enamel formation in *Evc2* mutants, we took advantage of the conditional *Evc2* allele with *Wnt1*-Cre to specifically delete *Evc2* in the dental mesenchyme (Chai et al. 2000). At P28, similar to what was observed in $Evc2^{ex12/ex12}$ mice, incisors from the $Evc2^{fx/fx}$;*Wnt1*-Cre mice (conditional mutants) showed less pigmentation (Fig. 5A). Mandibular radiography clearly demonstrated a decreased length of the incisor root (Fig. 5B).

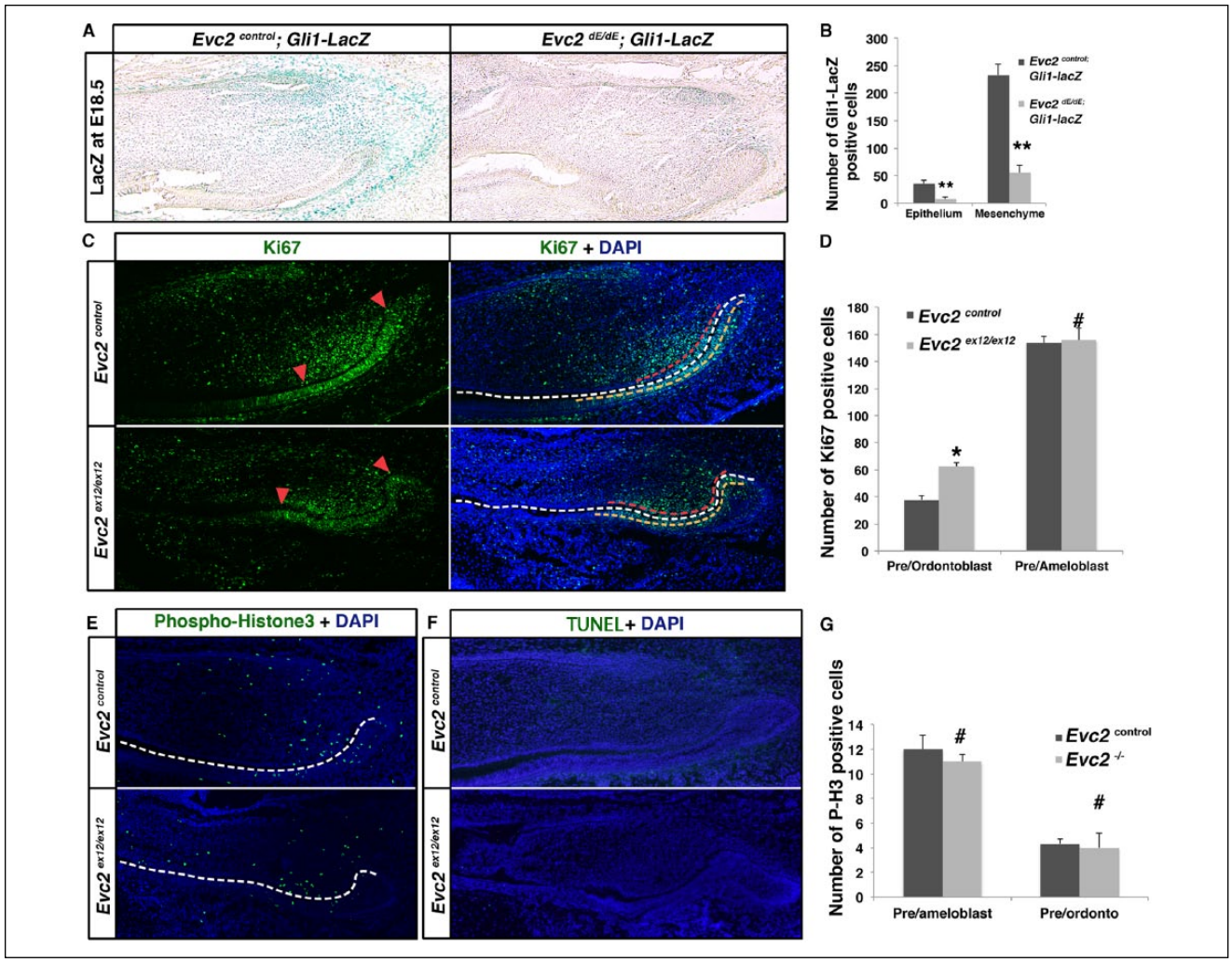

Figure 4. *Evc2* mutant incisors showed decreased dental epithelial and mesenchymal stem cells. (**A**) A *Gli1*-LacZ reporter was introduced to label dental epithelial and mesenchymal stem cells in the incisor. E18.5 mandible incisors of indicated genotypes were processed for LacZ staining. (**B**) Quantification of *Gli1*-LacZ–positive cells in A. $n = 3$, $^{**}P < 0.01$. (**C**) Immunohistochemistry of Ki-67 was applied to mark the transient amplifying (TA) cells in controls and *Evc2* mutants. Red arrowheads in the left panels indicate the 2 ends of the zone of Ki-67–positive cells in the odontoblast layers. White dashed lines in the right panels indicate a boundary between dental epithelium and mesenchyme. Red dashed lines indicate areas of Ki-67–positive cells in preodontoblasts (areas between 2 red arrowheads shown in the left panels). Yellow dashed lines indicate Ki-67–positive cells in preameloblasts. (**D**) Quantification of Ki-67–positive cells in B. $n = 4$, $^{*}P < 0.05$; $^{\#}$not significant. (**E**) Immunohistochemistry of phospho-histone3 (P-H3) depicts proliferating cells in control and *Evc2* mutant incisors at postnatal day 2 (P2). White dashed line indicates a boundary between dental epithelium and dental mesenchyme. (**F**) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining indicates apoptotic cells in control and Evc2 mutant incisor at P2. (**G**) Quantification of P-H3–positive cells in C. $n = 4$, $^{\#}$not significant.

Micro–computed tomography (CT) of molars clearly indicated hypomorphic enamel in *Evc2* conditional mutants. Histologic assessment of conditional mutants at the newborn stage consistently showed delayed ameloblast and odontoblast differentiation (Fig. 5C). We isolated an embryonic incisor at postnatal day 0 (NB) and physically separated dental epithelium (negative for Cre activity) from mesenchyme (positive for Cre activity). As expected, levels of *Evc2* expression clearly indicated that is *Wnt1*-Cre–mediated deletion is restricted in the dental mesenchyme (Fig. 5D). We also confirmed a reduction of *Gli1* expression only in the dental mesenchyme but not in the epithelium (Fig. 5D), suggesting that Hedgehog signaling activity is compromised in a mesenchyme-specific manner. Conversely, specific deletion of *Evc2* in dental epithelium mediated by *K14*-Cre did not lead to overt abnormalities in incisors (data not shown). These results strongly suggest that in *Evc2* mutant incisors, delayed odontoblast differentiation in dental mesenchyme secondarily leads to hypomorphic enamel formation.

Discussion

A wide spectrum of dental abnormalities can be found in patients with EvC. In the current study, we used both global and conditional mutant mouse lines for *Evc2*/*Limbin* as models to investigate the pathophysiologic mechanism leading to hypomorphic enamel formation. Our in vivo studies on the $Evc2^{ex12/ex12}$ mutant mice and mesenchymal-specific knockout of *Evc2* suggested that loss of *Evc2* in the dental mesenchyme

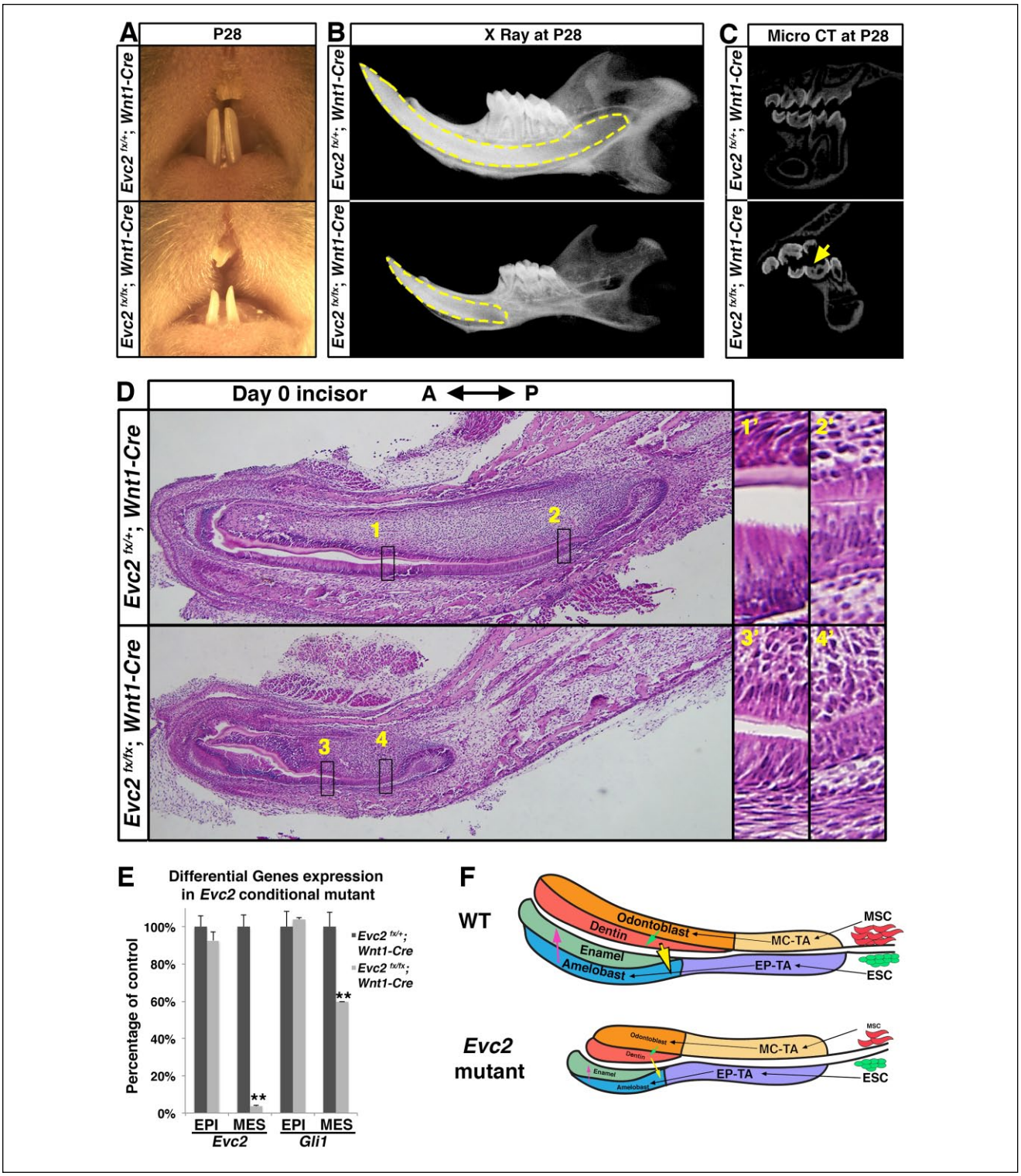

Figure 5. Mesenchyme-specific deletion of *Evc2* phenocopied hypomorphic tooth formation found in *Evc2* global knockout incisors. (**A**) Frontal view of maxilla and mandible incisor of *Evc2* conditional knockout mice at postnatal day 28 (P28). (**B**) X-ray radiogram of *Evc2* conditional mutants showed hypomorphic tooth formation. Mandibles of *Evc2* conditional mutants and littermate controls at P28 were dissected and sagittal X-ray radiography was performed. Yellow dashed lines indicate incisors. (**C**) Micro–computed tomography (CT) of Evc2 conditional mutants and littermate controls at P28 indicates hypomorphic enamel in molars. (**D**) Mandible incisors of mesenchyme-specific *Evc2* mutants and littermate controls were sagittally sectioned and processed for hematoxylin and eosin staining. Regions in boxes in both control and mutant were enlarged and shown on the right. (**E**) Quantitative reverse transcribed polymerase chain reaction from incisor epithelium and mesenchyme indicated that *Wnt1-Cre*–mediated deletion of *Evc2* is specific for dental mesenchyme. EPI and MES stand for epithelial and mesenchymal tissues, respectively. $n = 3$, $^{**}P < 0.001$. (**F**) A model to explain how mesenchymal function of *Evc2* secondarily affects enamel formation. EP-TA, epithelial transient amplifying cells; ESC, dental epithelial stem cells; MC-TA, mesenchymal transient amplifying cells; MSC, dental mesenchymal stem cells. See text for details.

resulted in decreased numbers of dental mesenchymal stem cells, leading to delayed odontoblast differentiation, which secondarily leads to hypomorphic enamel formation through delayed ameloblast differentiation. Overall, our work elucidated, for the first time, the pathophysiologic mechanism leading to hypomorphic enamel formation in patients with EvC and suggested a mechanism whereby *Evc2* mutations lead to hypomorphic tooth development through compromised dental mesenchymal stem cells (Fig. 5E).

In vitro mechanistic studies have suggested that EVC and EVC2 can form a protein complex, which is required for their mutual localization at the bottoms of the primary cilia (Dorn et al. 2012; Caparrós-Martin et al. 2013). This is consistent with genetic observations that a homozygous mutation of either of them leads to similar mouse phenotypes and that genetic deletion of both of them does not increase the severity of the phenotypes (Caparrós-Martin et al. 2013). In our $Evc2^{ex12/ex12}$ mutant mice, we observed pale mandibular incisors and decreased root size (Fig. 1C), which were also observed in *Evc* mutant mice (Nakatomi et al. 2013). These observations are consistent with what was demonstrated in mechanistic studies that EVC and EVC2 are reciprocally required to form a protein complex at the bottoms of primary cilia.

Results from previous studies suggested that the EVC/EVC2 protein complex is required for transducing Hedgehog signaling (Dorn et al. 2012; Nakatomi et al. 2013). Mutation in *Evc* or *Evc2* makes Hedgehog responding cells bear a compromised but not abolished response to the Hedgehog ligand (Zhang et al. 2015). Hedgehog signaling mediated by SHH is required for ameloblast differentiation and maturation and for maintenance of dental epithelial stem cells (Dassule et al. 2000; Gritli-Linde et al. 2002; Seidel et al. 2010). In the *Evc2* mutant mice, we also detected decreased numbers of dental epithelial stem cells. A logical speculation is that decreased Hedgehog signaling in the dental epithelium leads to delayed ameloblast differentiation and hypomorphic enamel formation and that specific deletion of *Evc2* in dental epithelium would phenocopy the tooth abnormalities in *Evc2* mutant mice. In contrast, results from our studies demonstrated that a dental epithelial-specific deletion of *Evc2* does not lead to overt tooth abnormalities. This is likely due to the fact that the remaining Hedgehog signaling in the *Evc2* mutant dental epithelium is still sufficient to maintain dental epithelial stem cells and to drive ameloblast differentiation.

In *Evc2* mutant mice, associated with delayed ameloblast differentiation, we also detected delayed odontoblast differentiation. Since ameloblast differentiation depends on the dentin secreted by odontoblasts (Thesleff and Hurmerinta 1981), the delayed ameloblast differentiation in *Evc2* mutant mice likely results from delayed odontoblast differentiation. This speculation is also supported by our observation that $Evc2^{fx/fx}$*;Wnt1*-Cre mice phenocopy the tooth abnormalities in *Evc2* mutant mice (Fig. 5A, C, D).

Evc2 mutants apparently feature short incisors compared with those of controls, which can be detected at as early as E15.5. It is possible that at cup stage or earlier, compromised Hedgehog signaling in the tooth mesenchyme leads to this small incisor germ. However, currently we are not able to rule out the possibility that *Evc2* loss of function leads to abnormal *Shh* expression at early stages. During minimization stages, our analysis indicated no apparent differences in cell proliferation and cell death in *Evc2* mutant incisors. The enlarged TA zones observed in dental mesenchyme in *Evc2* global mutant mice represented delayed transition into the postmitotic stages of preodontoblasts. A recent report indicated that Hedgehog ligands secreted from neural bundles support the maintenance of dental mesenchymal stem cells (Zhao et al. 2014), which give rise to odontoblasts in adult mouse incisors. Similarly, in our studies, we detected fewer *Gli1*-lacZ–labeled cells in the dental mesenchyme. Limited availability of the dental mesenchymal stem cells may account for delayed odontoblast differentiation and thus the shorter incisor length, since no apparent differential cell proliferation and apoptosis have been detected in *Evc2* mutant incisors.

In this study, we used mouse incisors as a model to investigate the pathophysiologic mechanism leading to hypomorphic enamel formation in EvC patients. Our in vivo data from genetic studies are summarized in Figure 5E. Compared with controls, *Evc2* mutations lead to a limited number of dental mesenchymal stem cells, in association with delayed odontoblast differentiation. The underlying mechanism relating these 2 phenotypes may be an interesting topic for future study. Subsequently, delayed odontoblast differentiation leads to reduced deposition of dentin (Fig. 5E, green arrow), which may result in delayed ameloblast differentiation (Fig. 5E, yellow arrow) and subsequent hypomorphic enamel formation (Fig. 5E, pink arrow).

Author Contributions

H. Zhang, Y. Mochida, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; H. Takeda, T. Tsuji, T. Kunieda, contributed to data analysis, critically revised the manuscript; N. Kamiya, Y. Mishina, contributed to conception, design, and data analysis, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

We gratefully acknowledge Drs. Ray Manas, Scott Greg, Sudha Rajderkar, Ke'Ale Louie, and Crystal Collier for their contributions during earlier stages of this work; Drs. Satoru Hayano, Ce Shi, and Jingwen Yang for advice on research strategy; Drs. Satoru Hayano, Ce Shi, and Wanida Ono for critical reading of this manuscript; and Dr. Chunlin Qin for generous help on DSPP antibody. This study is supported by the National Institutes of Health (R01DE019527 to Y. Mochida and R01DE020843 to Y. Mishina). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

Badri MK, Zhang H, Ohyama Y, Venkitapathi S, Alamoudi A, Kamiya N, Takeda H, Ray M, Scott G, Tsuji T, et al. 2016. Expression of Evc2 in

craniofacial tissues and craniofacial bone defects in Evc2 knockout mouse. Arch Oral Biol. 68:142–152.

Badri MK, Zhang H, Ohyama Y, Venkitapathi S, Kamiya N, Takeda H, Ray M, Scott G, Tsuji T, Kunieda T, et al. 2016. Ellis van Creveld2 is required for postnatal craniofacial bone development. Anat Rec (Hoboken). 299(8):1110–1120.

Baujat G, Le Merrer M. 2007. Ellis-van Creveld syndrome. Orphanet J Rare Dis. 2:27.

Bei M. 2009. Molecular genetics of ameloblast cell lineage. J Exp Zool B Mol Dev Evol. 312B(5):437–444.

Caparrós-Martin JA, Valencia M, Reytor E, Pacheco M, Fernandez M, Perez-Aytes A, Gean E, Lapunzina P, Peters H, Goodship JA, et al. 2013. The ciliary Evc/Evc2 complex interacts with Smo and controls Hedgehog pathway activity in chondrocytes by regulating Sufu/Gli3 dissociation and Gli3 trafficking in primary cilia. Hum Mol Genet. 22(1):124–139.

Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development. 127(8):1671–1679.

Chen S, Gluhak-Heinrich J, Wang YH, Wu YM, Chuang HH, Chen L, Yuan GH, Dong J, Gay I, MacDougall M. 2009. Runx2, osx, and dspp in tooth development. J Dent Res. 88(10):904–909.

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol. 8(24):1323–1326.

Dassule HR, Lewis P, Bei M, Maas R, McMahon AP. 2000. Sonic hedgehog regulates growth and morphogenesis of the tooth. Development. 127(22):4775–4785.

Dorn KV, Hughes CE, Rohatgi R. 2012. A smoothened-Evc2 complex transduces the Hedgehog signal at primary cilia. Dev Cell. 23(4):823–835.

Gritli-Linde A, Bei M, Maas R, Zhang XM, Linde A, McMahon AP. 2002. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. Development. 129(23):5323–5337.

Jussila M, Thesleff I. 2012. Signaling networks regulating tooth organogenesis and regeneration, and the specification of dental mesenchymal and epithelial cell lineages. Cold Spring Harb Perspect Biol. 4(4):a008425.

Lan Y, Jia S, Jiang R. 2014. Molecular patterning of the mammalian dentition. Semin Cell Dev Biol. 25–26:61–70.

Linde A, Granstrom G. 1978. Odontoblast alkaline phosphatases and Ca2+ transport. J Biol Buccale. 6(4):293–308.

McKusick VA, Egeland JA, Eldridge R, Krusen DE. 1964. Dwarfism in the Amish I. The Ellis-van Creveld syndrome. Bull Johns Hopkins Hosp. 115:306–336.

Møinichen CB, Lyngstadaas SP, Risnes S. 1996. Morphological characteristics of mouse incisor enamel. J Anat. 189(Pt 2):325–333.

Mostafa MI, Temtamy SA, el-Gammal MA, Mazen IM. 2005. Unusual pattern of inheritance and orodental changes in the Ellis-van Creveld syndrome. Genet Couns. 16(1):75–83.

Murgiano L, Jagannathan V, Benazzi C, Bolcato M, Brunetti B, Muscatello LV, Dittmer K, Piffer C, Gentile A, Drögemüller C. 2014. Deletion in the EVC2 gene causes chondrodysplastic dwarfism in Tyrolean Grey Cattle. PLoS One. 9(4):e94861.

Nakatomi M, Hovorakova M, Gritli-Linde A, Blair HJ, MacArthur K, Peterka M, Lesot H, Peterkova R, Ruiz-Perez VL, Goodship JA, et al. 2013. Evc regulates a symmetrical response to Shh signaling in molar development. J Dent Res. 92(3):222–228.

Qin C, Brunn JC, Baba O, Wygant JN, McIntyre BW, Butler WT. 2003. Dentin sialoprotein isoforms: detection and characterization of a high molecular weight dentin sialoprotein. Eur J Oral Sci. 111(3):235–242.

Ruiz-Perez VL, Ide SE, Strom TM, Lorenz B, Wilson D, Woods K, King L, Francomano C, Freisinger P, Spranger S, et al. 2000. Mutations in a new gene in Ellis-van Creveld syndrome and Weyers acrodental dysostosis. Nat Genet. 24(3):283–286.

Ruiz-Perez VL, Tompson SW, Blair HJ, Espinoza-Valdez C, Lapunzina P, Silva EO, Hamel B, Gibbs JL, Young ID, Wright MJ, et al. 2003. Mutations in two nonhomologous genes in a head-to-head configuration cause Ellis-van Creveld syndrome. Am J Hum Genet. 72(3):728–732.

Seidel K, Ahn CP, Lyons D, Nee A, Ting K, Brownell I, Cao T, Carano RA, Curran T, Schober M, et al. 2010. Hedgehog signaling regulates the generation of ameloblast progenitors in the continuously growing mouse incisor. Development. 137(22):3753–3761.

Takeda H, Takami M, Oguni T, Tsuji T, Yoneda K, Sato H, Ihara N, Itoh T, Kata SR, Mishina Y, et al. 2002. Positional cloning of the gene LIMBIN responsible for bovine chondrodysplastic dwarfism. Proc Natl Acad Sci U S A. 99(16):10549–10554.

Thesleff I, Hurmerinta K. 1981. Tissue interactions in tooth development. Differentiation. 18(2):75–88.

Thomas BL, Liu JK, Rubenstein JL, Sharpe PT. 2000. Independent regulation of Dlx2 expression in the epithelium and mesenchyme of the first branchial arch. Development. 127(2):217–224.

Veena KM, Jagadishchandra H, Rao PK, Chatra L. 2011. Ellis-van Creveld syndrome in an Indian child: a case report. Imaging Sci Dent. 41(4):167–170.

Zhang H, Takeda H, Tsuji T, Kamiya N, Rajderkar S, Louie KA, Collier C, Scott G, Ray M, Mochida Y, et al. 2015. Generation of Evc2/Limbin global and conditional KO mice and its roles during mineralized tissue formation. Genesis. 53(9):612–626.

Zhao H, Feng J, Seidel K, Shi S, Klein O, Sharpe P, Chai Y. 2014. Secretion of shh by a neurovascular bundle niche supports mesenchymal stem cell homeostasis in the adult mouse incisor. Cell Stem Cell. 14(2):160–173.