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# Sox2 marks epithelial competence to generate teeth in mammals and reptiles

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## SUMMARY

Tooth renewal is initiated from epithelium associated with existing teeth. The development of new teeth requires dental epithelial cells that have competence for tooth formation, but specific marker genes for these cells have not been identified. Here, we analyzed expression patterns of the transcription factor Sox2 in two different modes of successional tooth formation: tooth replacement and serial addition of primary teeth. We observed specific Sox2 expression in the dental lamina that gives rise to successional teeth in mammals with one round of tooth replacement as well as in reptiles with continuous tooth replacement. Sox2 was also expressed in the dental lamina during serial addition of mammalian molars, and genetic lineage tracing indicated that Sox2$^+$ cells of the first molar give rise to the epithelial cell lineages of the second and third molars. Moreover, conditional deletion of *Sox2* resulted in hyperplastic epithelium in the forming posterior molars. Our results indicate that the Sox2$^+$ dental epithelium has competence for successional tooth formation and that Sox2 regulates the progenitor state of dental epithelial cells. The findings imply that the function of Sox2 has been conserved during evolution and that tooth replacement and serial addition of primary teeth represent variations of the same developmental process. The expression patterns of Sox2 support the hypothesis that dormant capacity for continuous tooth renewal exists in mammals.

**KEY WORDS: Dental lamina, Ferret, Mouse, Reptile, Stem cells, Successional tooth formation, Tooth replacement**

## INTRODUCTION

Tissue renewal requires cells that can produce differentiating progeny. Many organs, including skin, hair and intestine, renew throughout the life of an individual. Interestingly, some organs, such as teeth, differ in their regenerative capacities between species. For example, reptiles replace their teeth continuously throughout life, whereas in mammals tooth replacement is restricted to one round (Fig. 1A). The numbers and shapes of teeth also vary between species. Dentitions of reptiles are generally homodont (all teeth have a similar shape) whereas mammals have a heterodont dentition (differently shaped teeth that belong to several tooth classes: incisors, canine, premolars and molars) (Fig. 1A). During mammalian evolution, replacement capacity has been reduced, whereas complexity of tooth shapes has increased. Most mammalian species replace their deciduous teeth (incisors, canine and premolars) once. The permanent molars form posterior to the deciduous teeth and are part of the primary dentition (Osborn, 1893), but they are not replaced in any mammal (Fig. 1A).

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The main features of tooth morphogenesis have been conserved throughout evolution. All teeth form from surface oral epithelium and underlying neural crest-derived mesenchyme. Interactions between the tissues, mediated by conserved signaling pathways, control epithelial morphogenesis and cell differentiation (Tummers and Thesleff, 2009). In all vertebrates, the first sign of tooth development is the formation of an epithelial primary dental lamina. It can be recognized as a horseshoe-shaped thickening and as a localized band of gene expression in the embryonic oral cavity or pharynx that marks the future tooth rows (Fraser et al., 2009). In mammals, development of the primary dentition starts from epithelial placodes forming within the dental lamina. Knowledge concerning the role of placodes in the initiation of different types of teeth is still limited because they have been studied mainly in mice, which have only a single continuously growing incisor and three molars in each jaw quadrant (Fig. 1A).

Likewise, the mechanisms of tooth replacement have remained poorly understood because mouse teeth are not replaced. The single replacement of teeth in other mammals and the continuous replacement in some reptiles share similar morphological and molecular features (Järvinen et al., 2009; Leche, 1895; Ooë, 1981; Richman and Handrigan, 2011; Smith et al., 2009b). In both cases, the replacement teeth develop successionally from epithelium associated with the preceding tooth, and are initiated during an early stage of morphogenesis of the preceding tooth. In most vertebrates, the enamel organs of deciduous tooth germs are connected with a dental lamina running on the lingual side and linking the tooth germs to oral epithelium (Fig. 1B). The individual replacement teeth are initiated as an extension of the dental lamina, which is called the successional dental lamina (Järvinen et al., 2009; Leche, 1895; Ooë, 1981; Philipsen and Reichart, 2004; Richman and Handrigan, 2011; Smith et al., 2009b) (Fig. 1B). The capacity for tooth replacement is believed to reside in the dental lamina and

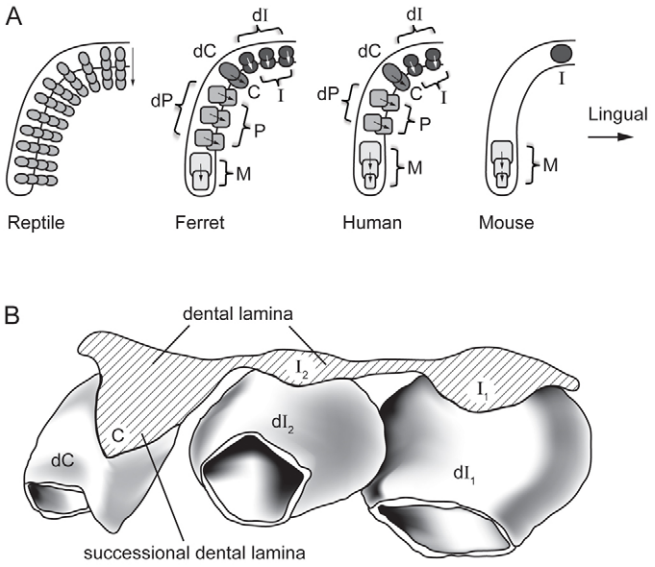


**Fig. 1. Variations in tooth replacement in reptiles and mammals, and serial addition of molars in mammals.** (**A**) Schematics of dentitions of different vertebrates showing the left half of the lower jaws. Replacement teeth form at the lingual side of the dental arch. Mammalian molars are added serially in the posterior direction. Most reptiles have a homodont dentition, which is continuously replaced. Humans and ferrets represent typical mammals with a heterodont dentition composed of incisors, one canine, premolars and molars, and all teeth except molars are replaced once. Mice have one continuously growing incisor, a toothless diastema region and three molars. Mouse teeth are not replaced. (**B**) Reconstruction of human deciduous tooth germs illustrating their connection by the continuous dental lamina and the initiation of replacement tooth formation by budding of the successional dental lamina. Lingual view of anterior tooth germs in the lower jaw. A similar dental lamina is present in most other mammals and squamate reptiles. C, permanent canine; dC, deciduous canine; dI, deciduous incisor; dP, deciduous premolar; I, permanent incisor; M, molar; P, permanent premolar.

successional dental lamina. Recently, label-retaining putative stem cells have been localized in the successional dental lamina in species with life-long tooth replacement, the leopard gecko (*Eublepharis macularius*) (Handrigan et al., 2010) and American alligator (*Alligator mississippiensis*) (P.W. and C.-M.C., unpublished).

Successional formation of teeth is observed, in addition to tooth replacement, when new primary teeth are added within a tooth class. This process is characteristic of the heterodont dentitions of mammals, which usually have more than one incisor, premolar and molar (Fig. 1A). In general, the primary teeth within one class erupt in a specific sequence. However, their early development has not been described in detail. The three mouse molars offer a model system for the analysis of successional tooth formation, as the second (M2) and third molars (M3) are added posteriorly along the tooth row in the back of the jaw (Fig. 1A; Fig. 5A). A dissected first molar (M1) tooth germ gives rise to M2 and M3 when transplanted to the anterior chamber of the eye, but the mechanism of the initiation of M2 and M3 has remained elusive. It appears that all successionally forming teeth, including replacement teeth and serially added teeth in tooth classes, develop from dental epithelium associated with preceding teeth, which has maintained the competence for tooth development (Järvinen et al., 2006). Understanding the mechanisms of successional tooth formation requires the molecular characterization of the tooth-forming epithelium and identification of progenitor cells. Specific markers for epithelial tissue with tooth-forming capacity are so far unknown.

We recently discovered that Sox2 marks the epithelial stem cells in the continuously growing mouse incisor and demonstrated that *Sox2*-positive ($Sox2^+$) stem cells give rise to all epithelial cell lineages of the incisor (Juuri et al., 2012). These findings led us to explore whether *Sox2* expression is associated with tooth renewal in general. In the present study, we have localized Sox2 expression during the two modes of successional tooth formation in different species: tooth replacement in ferret, human and five reptiles; and serial posterior addition of molars in mouse and ferret. The results indicate that $Sox2^+$ progenitors are already present in the primary dental lamina during tooth initiation, and later reside in the dental lamina in both modes of successional tooth formation. Genetic fate mapping of *Sox2*-expressing cells of mouse M1 showed that they give rise to the sequentially developing M2 and M3. Conditional deletion of *Sox2* resulted in expansion of dental lamina epithelium associated with M2 and M3. Our results indicate that Sox2 marks the epithelial competence to generate teeth and suggest that it acts as a negative regulator of successional tooth formation.

## MATERIALS AND METHODS

### Animals and tissue processing

Wild-type NMRI mice were used at various embryonic stages. Plug day was counted as embryonic day (E) 0 and embryos were staged according to morphological criteria. *Sox2-GFP* mice (D'Amour and Gage, 2003) were a kind gift from Fred H. Gage (Salk Institute, CA, USA). For genetic fate mapping, *Sox2CreERT2* (Arnold et al., 2011) and $R26R^{lacZ}$ (Soriano, 1999) mice were crossed and genotyped as described previously. *Shh::GFPCre;*$Sox2^{fl/fl}$ mouse line (*Sox2*cKO) was generated by crossing *Shh::GFPCre* (Harfe et al., 2004) with $Sox2^{fl/fl}$ mice (Smith et al., 2009a) (The Jackson Laboratory, stock 013093). For histology, radioactive *in situ* hybridization and immunohistochemistry, mouse tissues were fixed in 4% paraformaldehyde (PFA), dehydrated, and embedded in paraffin. Sections from 4.5-µm to 7-µm thickness were cut in frontal and sagittal planes. All aspects of mouse care and experimental protocols were approved by the National Board of Animal Experimentation.

Pregnant ferret females (gestation period is 42 days) were euthanized according to the guidelines for fur animals in fur farming research station of Agricultural Research Centre of Finland (Kannus, Finland) and embryos were collected at E28, E32 and E34. Heads were fixed in 4% PFA and the E34 heads were decalcified in Morse's solution for one week before paraffin embedding. Sections of 7-µm thickness were cut in frontal plane.

Human jaw sections from 13-gestational-weeks-old fetuses were obtained from the collection owned by the University of Turku, Finland. The samples had been fixed in 4% PFA, decalcified, and embedded in paraffin. The ethical approval was obtained from the Joint Authority for the Hospital District of Southwest Finland Ethics Committee, Turku, Finland (1/11 March 2007) and from the National Supervisory Authority for Welfare and Health of Finland (VALVIRA 648/32/300/05).

Corn snake, ball python and gecko embryos were provided by Triple-R-Corns (Aldergrove, BC, Canada). Gecko embryos were decalcified in 7% EDTA in 2% PFA for 8 weeks whereas snakes were fixed in 4% PFA overnight, embedded in paraffin and sectioned. Animals were sacrificed according to procedures approved by the University of British Columbia animal ethics committee, protocol number A11-0352.

Fertilized alligator eggs were collected in the Rockefeller Wildlife Refuge, Louisiana. Eggs were incubated at 30°C and staged according to Ferguson (Ferguson, 1985). One-year-old iguanas were from a local vendor and kept in the University of Southern California (USC) animal facility. Animals were sacrificed according to the procedure approved by the Institutional Animal Care and Use Committee (IACUC) at USC.

### Organ culture

E14.5 *Sox2-GFP* mouse molars were dissected and cultured in Trowell type organ culture (Sahlberg et al., 2002). The medium contained DMEM and

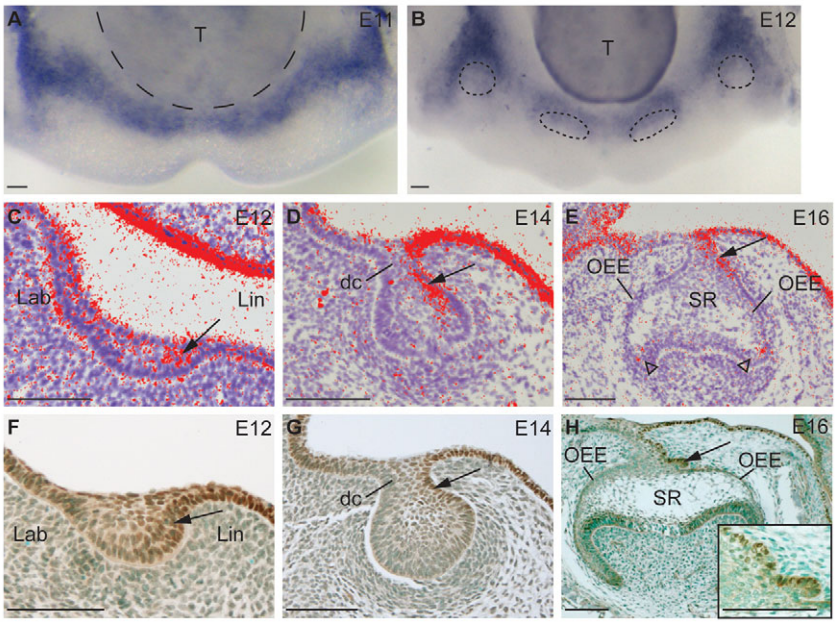


**Fig. 2. Sox2 is localized to primary dental lamina and to lingual dental epithelium during mouse molar development.** (**A**,**B**) Whole-mount *in situ* hybridization showing expression of *Sox2* mRNA (purple) in mouse lower jaw at E11 in the primary dental lamina (A), and at E12 in oral epithelium and lingual to the tooth placodes (B, dashed circles). (**C-H**) The expression of *Sox2* mRNA (red, C-E) and protein (brown, F-H) in the lower molar from E12 to E16 is gradually restricted to lingual dental epithelium (arrows). Arrowheads in E point to *Sox2* expression in E16 cervical loops. Arrow in H points to budding of dental epithelium at the lingual side of molar at E16 (inset shows higher magnification). dc, dental cord; Lab, labial; Lin, lingual; OEE, outer enamel epithelium; SR, stellate reticulum; T, tongue. Scale bars: 100 μm.

F12 (Ham's Nutrient Mix: Life Technologies) (1:1) supplemented with 10% fetal calf serum (PAA Laboratories), 150 mg/ml ascorbic acid, glutamine and penicillin-streptomycin and the medium was changed every second day.

### In situ hybridization and immunohistochemistry

Radioactive *in situ* hybridization on paraffin sections was carried out according to standard protocols (Wilkinson and Green, 1990). [35S]-UTP (Amersham)-labeled RNA probes were used to detect expression of mouse *Sox2* (Ferri et al., 2004). Whole-mount *in situ* hybridization was performed on mouse lower jaws fixed with 4% PFA using InSituPro robot (Intavis AG). BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim) was used to visualize the digoxigenin-labeled probe. For immunostaining, sections were rehydrated and heated in a microwave in 10 mM sodium-citrate buffer (pH 6.0). Immunostaining was performed using the Ultravision Large Volume Detection System Anti-Rabbit, HRP Kit (Thermo Scientific) and the DAB Peroxidase Substrate Kit (Vector Laboratories, SK4100) using rabbit anti-Sox2 antibody (1:500-1:2000, Millipore) or goat anti-Sox2 antibody (R&D Systems).

### Genetic fate mapping

For genetic fate mapping of $Sox2^{+}$ cells, 10 mg tamoxifen (Sigma T-5648; Sigma-Aldrich) in corn oil was given by oral gavage to pregnant females carrying *Sox2CreERT2;R26R*$^{lacZ}$ embryos and control embryos lacking the Cre-driver at E13. Whole-mount X-Gal staining was performed as previously described (Seidel et al., 2010). Tissues were processed into paraffin, sectioned at 4.5-μm thickness and counterstained with Fast Red (Sigma-Aldrich).

## RESULTS

### *Sox2* localizes to the primary dental lamina and to lingual dental epithelium during mouse molar development

*Sox2* is expressed in the mouse incisor during morphogenesis and becomes gradually restricted to the epithelial stem cell niche (Juuri et al., 2012). Here, we examined *Sox2* expression during the initiation of mouse dentition and during morphogenesis of the mouse molar, which does not grow continuously and lacks the epithelial stem cell niche found in the incisor. The primary dental lamina gives rise to teeth in all vertebrates and thus probably represents the origin of all dental epithelia. In mouse, both incisor and molar placodes form from the dental lamina. We localized *Sox2* expression by whole-mount *in situ* hybridization specifically to the dental lamina in mouse lower jaw at E11 (Fig. 2A). At E12, *Sox2* was strongly expressed at the lingual side of the molar and incisor placodes, but the expression had decreased between placodes (Fig. 2B).

We next localized *Sox2* expression by radioactive *in situ* hybridization in frontal sections of E12-E16 mouse M1 (Fig. 2C-E). Throughout this period, *Sox2* was expressed in the oral epithelium, and faint or no expression was observed in mesenchymal tissues. At E12, *Sox2* expression was strongest at the lingual aspect of placode epithelium (Fig. 2C). Subsequently, the dental epithelium undergoes morphogenesis into a cap-shaped enamel organ composed of distinct cell layers, including outer and inner enamel epithelium (OEE and IEE, respectively) surrounding a core of stellate reticulum (SR) cells. The enamel organ is connected to the oral epithelium by a dental cord, which becomes disrupted during later stages. At E14 and E16, *Sox2* was expressed in the oral epithelium at the lingual side of the tooth germ and continued from there to the lingual cells of the dental cord and enamel organ, including OEE and SR cells (Fig. 2D,E). Faint expression was also detected in the cervical loops at E16 (Fig. 2E). The OEE on the buccal aspect of the tooth was negative for *Sox2*. Similar expression patterns were recently reported (Zhang et al., 2012).

For comparison, we detected Sox2 protein from E12 to E16 (Fig. 2F-H) and found expression in a similar pattern to *Sox2* mRNA at all stages. At E16, an epithelial protrusion expressing Sox2 was seen at the junction between the dental cord and OEE at the lingual aspect of the molar (Fig. 2H). This small lingual bud can also be observed in frontal sections of E17 and E18 mouse molars (data not shown). This bud of the dental lamina is reminiscent of the initiation of replacement tooth formation in the ferret (Fig. 3A) and might represent the rudiment of the successional dental lamina for molar replacement teeth, which mammals lost >200 million years ago in the course of evolution (Kielan-Jaworowska et al., 2004; Ungar, 2010).

### Sox2 expression marks the dental lamina during replacement tooth formation in ferret and human

The finding that Sox2 was expressed during mouse molar morphogenesis in the lingual aspect of dental epithelium, which is the location of replacement tooth initiation in other species,

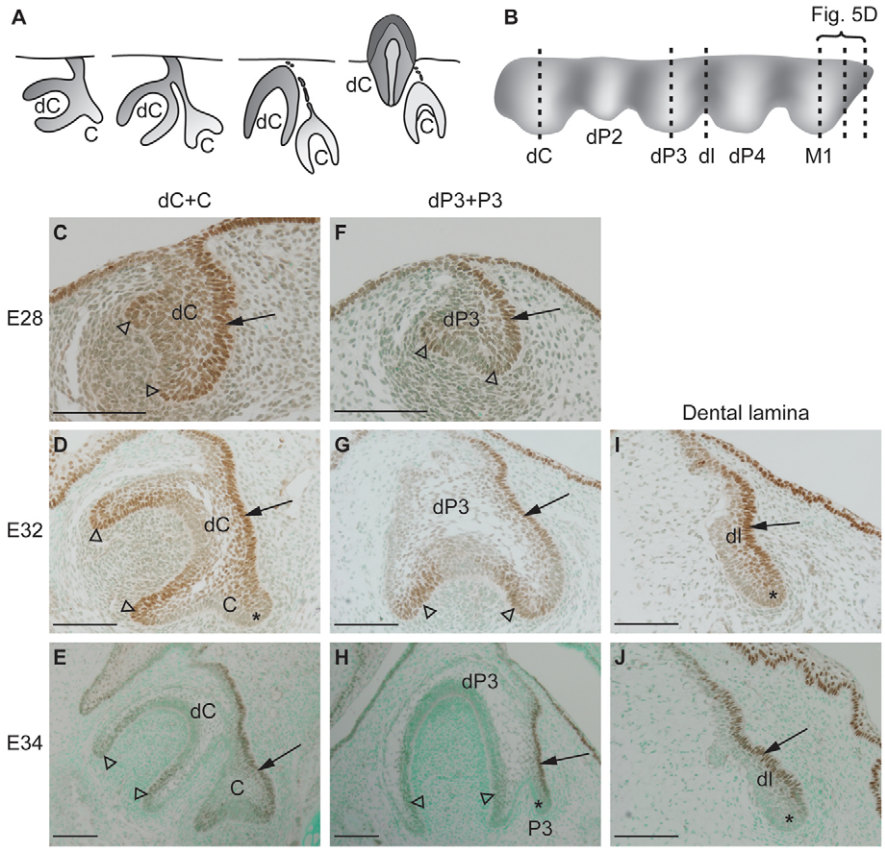


**Fig. 3. Sox2 expression localizes to dental lamina and successional dental lamina during ferret tooth replacement.** (**A**) Schematic of frontal sections showing development of the ferret permanent canine (C), which will later replace the deciduous canine (dC). (**B**) Schematic sagittal and buccal view of the developing ferret tooth row showing the deciduous canine (dC), deciduous second (dP2), third (dP3) and fourth premolar (dP4), and first molar (M1) connected by the dental lamina (dl). Dashed lines in B indicate the sites of sections in C-E (dC), F-H (dP3) and I,J (dl) and in Fig. 5D (M1). (**C-J**) Localization of Sox2 protein (brown) during ferret tooth replacement. Arrows point to Sox2 expression in lingual dental epithelium. Asterisks indicate the Sox2-negative free end in the successional dental lamina, and in the dental lamina between deciduous teeth. Sox2 localizes also to the cervical loops and inner enamel epithelium (C-H, arrowheads). A and C-J are frontal sections, lingual to the right; B is a sagittal view, posterior to the right. Scale bars: 100 μm.

prompted us to examine Sox2 expression during replacement of ferret and human teeth. Ferret (*Mustela putorius furo*) dentition resembles that of human (Fig. 1A). We have previously described the process of tooth replacement in ferret embryos (Järvinen et al., 2009) and demonstrated that replacement is initiated from the free edge of the dental lamina on the lingual side of each deciduous tooth (Fig. 3A). We selected three stages of canine and third premolar development to investigate the localization of Sox2 protein during replacement: (1) before the initiation of replacement tooth development, (2) during the splitting of the successional dental lamina from the deciduous tooth enamel organ and (3) after the initiation of replacement tooth morphogenesis.

At all three stages studied, $Sox2^+$ cells were observed on the lingual aspect of the dental epithelium both in the deciduous canine (dC) and in the deciduous third premolar (dP3) (Fig. 3C-H). As the successional dental lamina detached from the OEE and started to grow down from the deciduous tooth to form the permanent canine (C) and the third premolar (P3), the free end of the budding lamina was negative for Sox2 (Fig. 3D,H). When the permanent canine had reached early cap stage, continuous lingual Sox2 expression extended from the oral epithelium to the successional dental lamina connecting the dC and C, and continued in the OEE of C to its cervical loop (Fig. 3E). A similar pattern of Sox2 expression persisted in the bell stage enamel organ of C (data not shown). The dental lamina between the deciduous tooth germs (dP3 and dP4) expressed Sox2 in the lingual epithelial cells (Fig. 3I-J). The free end of the dental lamina was, however, negative for Sox2. A similar lingual localization of Sox2 was observed during replacement of the dP2 and dP4 and in the dental lamina between all teeth (data not shown). We detected Sox2 also in the cervical loops and IEE of each tooth, as well as in the oral epithelium, but not in mesenchymal cells.

As is typical of mammalian teeth, human teeth are replaced once. We studied Sox2 localization during the initiation of permanent premolars in sections of a human fetus at 13 weeks of gestation. Sox2 was expressed in the lingual side of the dental lamina of a deciduous premolar (supplementary material Fig. S1). The free end of the successional dental lamina of the budding permanent premolar was negative for Sox2, similar to the ferret (Fig. 3D,H).

## Sox2 localizes to the dental lamina during continuous tooth replacement in reptiles

Continuous tooth replacement in reptiles has been characterized at both the morphological and the molecular level (Osborn, 1974; Richman and Handrigan, 2011), and putative stem cells have been localized in the dental lamina of the leopard gecko and American alligator (Handrigan et al., 2010) (P.W. and C.-M. C., unpublished). We used three lizards, American alligator, green iguana (*Iguana iguana*) and leopard gecko, and two non-venomous snakes, ball python (*Python regius*) and corn snake (*Elaphe guttata*), as models of continuous tooth replacement to study Sox2 localization.

In all species, Sox2 was expressed in the dental lamina connecting the teeth to the oral epithelium (Fig. 4). In alligator, Sox2 was detected in the dental lamina as well as in the lingual side of the developing first-generation tooth (surface tooth) (Fig. 4A). The forming successional dental lamina showed strong Sox2 expression on its lingual side (Fig. 4B). Lingual Sox2 expression continued in the OEE of the second-generation tooth (submerged tooth) (Fig. 4C, 2°). The successional dental lamina further detaches from the OEE of the second-generation tooth, and $Sox2^+$ cells were detected in the whole successional dental lamina except the free end (Fig. 4D). A similar Sox2 expression pattern was observed in the successional

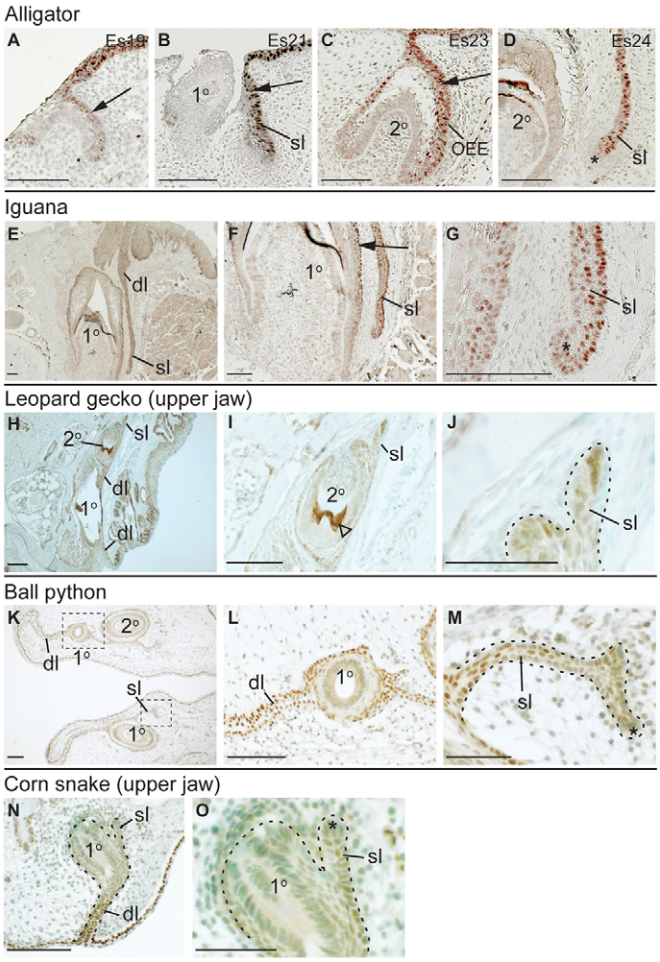


**Fig. 4. Sox2 expression localizes to dental lamina and successional dental lamina during continuous tooth replacement in five reptile species.** (**A-O**) Localization of Sox2 protein (brown) in American alligator [embryonic stages (Es) 19-24] (A-D), green iguana (juvenile) (E-G), leopard gecko (post-hatching juvenile) (H-J), 60-day post-oviposition ball python (K-M) and 30-day post-oviposition corn snake (N,O). Arrows in alligator and iguana point to Sox2 expression in the lingual dental epithelium (A,B,C,F). Note that there is no lingual asymmetry in Sox2 expression in snakes (L,N). Sox2 expression is absent from the free end of the successional dental lamina (asterisks) in all species except gecko. Staining in the deposited enamel in gecko is non-specific (l, arrowhead). Boxed areas in K represent higher magnifications in L and M. Dashed lines outline the dental epithelium. 1°, first generation tooth; 2°, second generation tooth; dl, dental lamina; OEE, outer enamel epithelium; sl, successional dental lamina. Scale bars: 100 μm.

dental lamina extending from the second-generation tooth of the juvenile iguana (Fig. 4E-G) as well as in the successional dental lamina extending from the second-generation teeth of the post-hatching juvenile gecko (Fig. 4H-J).

In pre-hatching snakes, we examined the mirror image rows of palatal and marginal teeth to look for the labial-lingual asymmetry in Sox2 expression that was observed in the ferret and human. In contrast to mammals, staining was equivalent on both sides of the dental lamina and was similar in marginal and palatal teeth (Fig. 4K,L,N). As in other species studied, the free end of the successional dental lamina was negative for Sox2 (Fig. 4M,O).

In summary, Sox2 was associated with tooth replacement in all species studied, and the expression patterns showed shared features. We detected Sox2 expression exclusively in epithelial dental cells as well as in the oral epithelium in all species, and Sox2 specifically marked the dental lamina and successional dental lamina in both mammals and reptiles. The free end of the successional lamina, which actively proliferates to produce the next-generation tooth, was negative for Sox2. In addition, regional differences in Sox2 expression appeared in the dental lamina. Its lingual aspect showed intense Sox2 expression in all species, but the labial expression was absent in mammals. The lack of this lingual bias in reptiles might reflect higher competence in their successional dental lamina epithelium.

## Dynamic *Sox2* expression is associated with serial addition of molars in mouse and ferret

Although mice have lost the capacity to replace teeth, their posterior molars develop in succession, as in other mammals. The M2 and M3 develop sequentially from M1 along the anterior-posterior axis of the jaw (Fig. 5A). When M1 reaches the cap stage the dental epithelium buds from its posterior end. This bud increases in size in posterior direction and develops into M2. Later, when M2 reaches the cap stage, the dental epithelium gives rise to a posterior bud, which will form M3. We used the developing mouse molars as a model for successional tooth formation and examined the role of Sox2 in this process.

At E18, M1 and M2 have developed to the bell stage, and the bud of M3 has been initiated from the posterior end of M2 (Fig. 5B). In a sagittal section of E18 lower jaw, the continuation of Sox2 expression from the dental cord to the dental lamina connecting M1 and M2 was evident. As at E16, the Sox2$^+$ rudimental dental lamina budding from the OEE epithelium was noticed above M1 (Fig. 2H; Fig. 5B). Sox2 expression was observed also in the developing M3.

To follow the dynamics of *Sox2* expression during molar addition, we dissected E14.5 molars from the *Sox2-GFP* reporter mouse and monitored GFP expression in culture (Fig. 5C). Although the Sox2$^+$ oral epithelium had been removed from the dissected M1, some GFP expression was present on the oral surface, representing the dental cord. GFP expression was localized in the posterior end of the M1, which represents the dental lamina generating the M2. After 6 days, the M1 crown had advanced in morphogenesis and was GFP negative, whereas the M2 bud had grown in size and was positive for GFP. This pattern was repeated, and after 9 days the crown of M2 had developed and was GFP negative, whereas GFP expression was observed posterior to M2. The GFP$^+$ tooth bud of M3 had formed after 14 days. A domain of GFP$^+$ tissue appeared next to the teeth during extended culture and is likely to represent oral epithelium.

In the ferret, at the posterior end of M1, from where M2 develops, the majority of dental epithelial cells expressed Sox2 (Fig. 5D), which is in line with observations of mouse M2 formation. Taken together, the locations of the most intense Sox2 expression in both mouse and ferret molars occurred in the dental lamina where new molars are added.

## Genetic fate mapping demonstrates that serially added molars form from Sox2$^+$ cells

To test whether the Sox2$^+$ cells of the mouse M1 give rise to M2 and M3, we utilized *Sox2-CreER;R26R$^{lacZ}$* mice for genetic fate mapping (Arnold et al., 2011). We genetically labeled *Sox2*-expressing cells by administering tamoxifen *in vivo* to pregnant females at E13, collected embryos one day later, and dissected M1s for organ culture (Fig. 6A). The descendants of the Sox2$^+$ cells were identified after 0.5, 6 and 12 days by detecting *lacZ* expression using X-gal staining of whole-mount samples (Fig. 6B-D) and paraffin

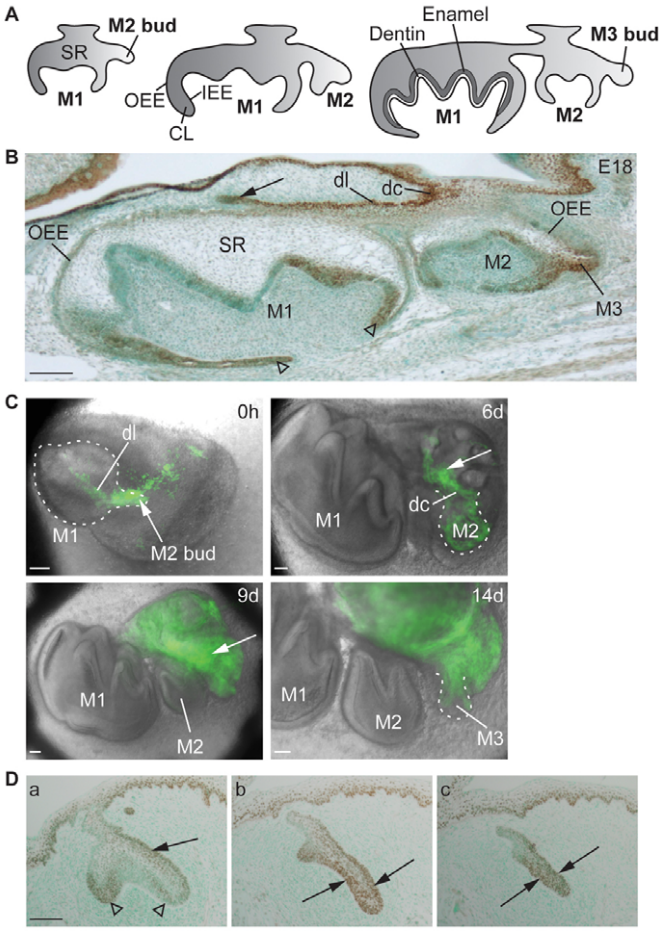


**Fig. 5. Sox2 expression is associated with successional formation of posterior molars in mouse and ferret.** (**A**) Schematic of successional development of molars (see text for details). (**B**) Localization of Sox2 protein (brown) in E18 mouse molars. Sox2 is expressed in the dental cord and dental lamina connecting M1 to M2, and in the rudimentary dental lamina bud above M1 (arrow). Sox2 is expressed in the bud, which will form M3. Arrowheads point to Sox2 expression in M1 cervical loops. (**C**) Dynamics of *Sox2* expression (green) during successional addition of molars. A dissected E14.5 M1 of a *Sox2-GFP* reporter mouse gives rise to M2 and M3 during 14 days of culture. The buds of M2 and M3 express *Sox2-GFP* but the completed crowns of M1 after 6 days (6d) and M2 after 9 days (9d) do not express *Sox2-GFP*. Arrows point to the GFP$^+$ dental lamina, which will give rise to a new tooth. Dashed lines outline the tooth germs. (**D**) Localization of Sox2 protein (brown) in ferret M1 at E34 in frontal sections from anterior (a) to posterior (c). The planes of sections are shown by dashed lines in Fig. 3B. Sox2 localizes to the lingual OEE (a, arrow) and cervical loops of M1 (a, arrowheads). In the posterior end of M1 from where M2 develops, Sox2 localizes both to lingual and labial sides of M1 epithelium (b,c). Lingual is towards the right. CL, cervical loop; dc, dental cord; dl, dental lamina; IEE, inner enamel epithelium; M, molar; OEE, outer enamel epithelium; SR, stellate reticulum. Scale bars: 100 μm.

sections (Fig. 6E-H). After 0.5 day, M1 was at the late bud stage and *lacZ*$^+$ cells were detected in the lingual side of dental epithelium including OEE and some SR cells (Fig. 6E). X-gal staining increased towards the posterior end of M1 corresponding to the areas of the highest *Sox2-GFP* expression (compare Fig. 6F and Fig. 5C, 0h). After 6 days, M1 had reached the bell stage of morphogenesis and the majority of its epithelial cells were *lacZ* negative. However, prominent clusters of *lacZ*$^+$ cells were present in the cervical loop at its posterior end, indicating that E13 Sox2$^+$ cells contribute predominantly to the posterior part of M1 (Fig. 6G). M2 development had advanced to the bud stage, and the bud epithelium, as well as the dental lamina, were composed almost entirely of *lacZ*$^+$ cells (Fig. 6C,G). After 12 days, M2 had developed to early bell stage and *lacZ*$^+$ cells were detected in all epithelial layers of the molar crown: ameloblasts, SR, IEE and OEE cells (Fig. 6H,H′). At this time, the M3 bud had formed, and it was also largely composed of *lacZ*$^+$ cells (Fig. 6D; data not shown). Few or no *lacZ*$^+$ cells were detected at any time point in the mesenchyme. *lacZ*-expressing cells were absent in *R26R*$^{lacZ/+}$ embryos lacking the *Sox2CreER* allele, which were used as control (data not shown).

These results demonstrate that Sox2$^+$ cells associated with the M1 at E13 give rise to successional molars, and that Sox2$^+$ cells contribute to all epithelial cell lineages of the mouse molar crown.

### Loss of *Sox2* leads to abnormal epithelial growth of successionally developing mouse molars

To investigate the role of *Sox2* in the dental epithelium, we examined the morphology of lower molars of *Shh::GFPCre;Sox2*$^{fl/fl}$ mutants (hereafter called *Sox2*cKO), in which *Sox2* is conditionally inactivated in the epithelium. Because of early postnatal mortality of the mutants, the analysis was performed between E17 and postnatal day (P) 0. The epithelial morphology of M2 and M3 in *Sox2*cKO differed from littermate controls as the dental cord connecting M2 to oral epithelium was markedly expanded and hyperplastic (Fig. 7; supplementary material Fig. S2) and the dental lamina connecting M3 to the oral epithelium was elongated. No morphological abnormalities were observed in the mutant M1 (Fig. 7).

We detected no obvious differences in proliferation in the dental epithelium of *Sox2*cKOs and controls (data not shown). Nevertheless, it is possible that very small changes in proliferation can result in differences in epithelial volume. Sox2 has been shown to inhibit canonical Wnt signaling (Mansukhani et al., 2005), and increased Wnt signaling has been linked to supernumerary tooth formation both in human and transgenic mice (Wang and Fan, 2011). Therefore, we examined expression of the Wnt target gene *Axin2* in control and *Sox2*cKO embryos, but expression in the mutant epithelium was unchanged (data not shown).

To assess the efficiency of *Sox2* inactivation by Cre recombinase, we examined *lacZ* expression in lower molars of *Shh::GFPCre;R26R*$^{lacZ}$ embryos. At E13, the M1 showed a mosaic pattern of *lacZ* expression (supplementary material Fig. S3). At P5, recombinase efficiency was very high in M1 and M2, but a more mosaic recombination pattern was observed in the dental cord and M3 (supplementary material Fig. S3). We also detected some remaining Sox2 protein expression in the *Sox2*cKO embryos at E17 and E18 (supplementary material Fig. S4). This incomplete deletion of *Sox2* might explain the mild molar phenotype of the mutants.

## DISCUSSION

We have localized Sox2 in dental epithelia at the sites that are either known or proposed to possess capacity for tooth renewal in several mammalian and reptilian species. By genetic fate mapping we demonstrated that Sox2$^+$ cells of the mouse M1 give rise to the successionally developing M2 and M3. In addition, conditional deletion of *Sox2* indicated that Sox2 regulates the amount of dental epithelium.

*Sox2* was expressed in the primary dental lamina, which marks the future dental arches in vertebrates and has been proposed to house stem cells for all dental epithelial tissues (Fig. 8) (Smith et al., 2009b). Importantly, *Sox2* was subsequently expressed in the dental

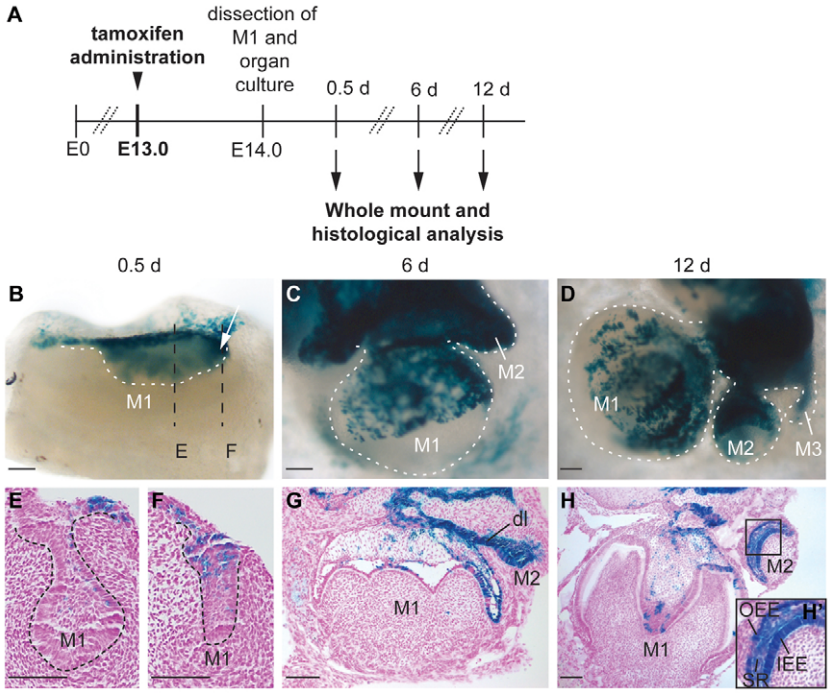


**Fig. 6. Genetic fate mapping demonstrates that successional molars derive from Sox2$^+$ cells.** (**A**) Timing of tamoxifen administration and analysis of *Sox2Cre-ER;R26R$^{lacZ}$* molars. (**B-D**) *lacZ* expression (blue) in X-Gal-stained whole-mount samples of M1 cultured for 0.5, 6 and 12 days after tamoxifen administration at E13.0. Arrow in B points to the posterior end of the M1 from where the M2 develops. Dashed line outlines the dental epithelium in B and the whole tooth germs in C and D. (**E-H′**) Histological sections from whole-mount samples shown in B-D. The sections in E and F were cut in the frontal plane at positions indicated by black dashed lines in B, other sections were cut in the sagittal plane. Dashed lines in E and F mark the border between epithelium and mesenchyme. Boxed area of M2 shown at higher magnification in H′ shows *lacZ* expression in all dental epithelial cell layers. dl, dental lamina; IEE, inner enamel epithelium; M, molar; OEE, outer enamel epithelium; SR, stellate reticulum. Scale bars: 100 μm.

lamina (Fig. 8), which connects the deciduous tooth germs as an epithelial sheet and is embedded on their lingual side, i.e. the side where the replacement teeth form in all animals (Fig. 1B) (Järvinen et al., 2009; Ooë, 1981; Richman and Handrigan, 2011; Smith et al., 2009b). This dental lamina is generally assumed to contribute progenitors for tooth replacement but no markers have been identified to differentiate the dental lamina from the flanking dental epithelium. In all species studied, a continuous stripe of Sox2$^+$ cells extended from the oral epithelium through the dental cord and enamel organ of the first-generation tooth to the successional dental lamina. This pattern of gene expression is unique and comparison of replacement of mammalian and reptile teeth indicated that most aspects of *Sox2* expression have been conserved during evolution. We conclude that *Sox2* is the first known gene that marks the dental lamina and successional dental lamina.

In reptiles, Sox2 was expressed in all successional teeth and in the successional lamina. Interestingly, in the ferret a stripe of Sox2$^+$ cells continued to the permanent tooth germ, indicating the presence of a dental lamina at the lingual aspect of the enamel organ. Although ferret tooth replacement is limited to one round, the dental lamina appears to be maintained as part of the developing permanent tooth, similar to reptiles. We did not have human material of advanced enough stages to see the permanent tooth germs, but a successional dental lamina extending from the developing human permanent teeth exists (Ooë, 1981). This, together with our observations, is in line with the hypothesis that there may be dormant capacity for further rounds of replacement in the mammalian teeth (Järvinen et al., 2006; Jensen and Kreiborg, 1990; Richman and Handrigan, 2011). Additionally, our findings indicate that even mouse molars might possess the competence for generation of replacement teeth, although mouse teeth are normally not replaced and no mammals replace molars. *Sox2* was expressed in mouse in a similar location as that observed in the ferret, suggesting that mouse molar epithelium might have an embedded dental lamina. Additionally, a Sox2$^+$ epithelial bud was observed protruding from the cord near the junction to the OEE, perhaps representing aborted initiation of a replacement tooth.

Serial horizontal addition of primary teeth within a tooth class represents another example of successional tooth formation. We observed that dynamic Sox2 expression was repeated when mouse molars were added to the tooth row, indicating that Sox2 expression is associated with the posterior extension of dental epithelium where the addition of new teeth takes place (Fig. 8). By genetic fate mapping, we demonstrated that the Sox2$^+$ cells of the mouse M1 gave rise to all epithelial cell lineages of the successionally developing M2 and to the bud of M3. Therefore, we propose that the Sox2$^+$ cells have the capacity to form the epithelial component of a new tooth.

Continuous horizontal addition of molars exists in some mammals, such as the silvery mole rat (*Heliophobius argenteocinereus*), and this resembles the continuous tooth replacement in reptiles (Rodrigues et al., 2011). Supernumerary posterior molars are occasionally present in humans (Shahzad and Roth, 2012) and also in wild-type mice (our unpublished observations). These examples support the notion of a sustained epithelial competence for tooth formation. Our observations pointed out striking similarities between the processes of tooth replacement and serial addition of primary teeth in mammals. First, when mouse molars and ferret replacement teeth are initiated, the preceding tooth has reached the early cap or early bell stage of development. Second, in both cases the new tooth formed successionally from Sox2$^+$ dental epithelial tissue associated with the dental cord and enamel organ of the preceding tooth, i.e. the dental lamina (Fig. 8). The two processes differ in the orientation and direction of new tooth formation: replacement tooth formation is initiated from the lingual side of the preceding tooth and occurs in a vertical direction, whereas addition of primary teeth within a tooth class is initiated from the posterior (sometimes anterior) aspect of the preceding tooth and takes place horizontally (Fig. 8). The resemblance between the formation of replacement teeth and posterior molars has been noted previously (Järvinen et al., 2008; Jensen and Kreiborg, 1990) and, based on the apparent similarities in morphology, developmental timing and *Sox2* expression patterns, we suggest that the two modes of successional tooth formation actually represent variations of the same developmental process.

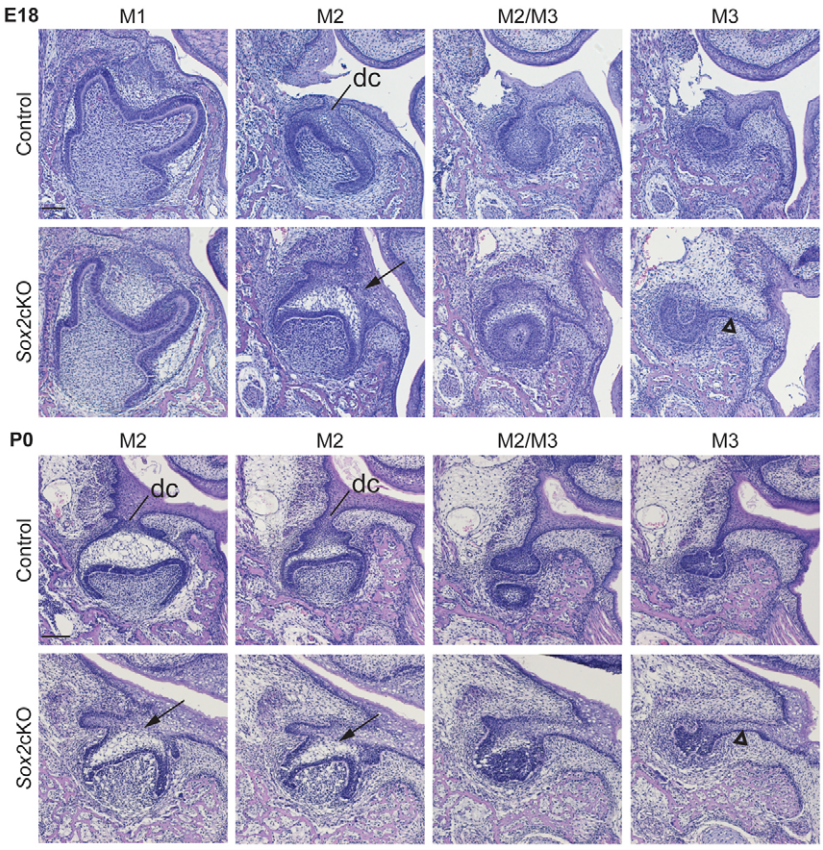


**Fig. 7. Conditional deletion of *Sox2* leads to hyperplastic dental epithelium of M2 and M3.** Hematoxylin and Eosin-stained serial frontal sections of mandibular molars of control and *Sox2*cKO mice at E18 and P0. *Sox2*cKO shows no obvious phenotype in M1 (data for P0 not shown), whereas in M2 the dental cord is expanded (arrows) and dental lamina between M2 and M3 is expanded. M3 is attached to oral epithelium by an extended sheet of dental lamina (arrowheads). See also supplementary material Fig. S2. dc, dental cord. Lingual is towards the right. Scale bars: 100 µm.

Based on our genetic fate mapping result and on the morphological and molecular similarities between posterior molar addition and tooth replacement, we propose that $Sox2^{+}$ cells in the dental lamina, which contributed to the successive molars, also give rise to replacement tooth epithelium. This, however, needs to be experimentally confirmed using a genetic fate mapping approach in a species with continuous replacement.

Interestingly, supernumerary tooth formation was reported in a human patient carrying a heterozygous loss-of-function mutation in the *SOX2* gene (Numakura et al., 2010). The supernumerary tooth phenotype resembles that observed in two other syndromes, cleidocranial dysplasia (CCD) and the craniosynostosis and dental anomalies syndrome (CRDSA) (Jensen and Kreiborg, 1990; Nieminen et al., 2011). Clinical follow-up of the development of extra teeth in CCD patients indicated that they form in succession as part of an additional replacement and as supernumerary posterior molars (Jensen and Kreiborg, 1990), and histological examination of the tissue associated with the CCD supernumerary teeth revealed an abundance of dental epithelium (Lukinmaa et al., 1995). *Sox2*cKO mice developed hyperplastic dental epithelium associated with the developing M2 and M3, which is in line with the supernumerary tooth phenotype of the human patient with *SOX2* mutation and indicates that Sox2 prevents the expansion of dental epithelium. Increased canonical Wnt signaling induces supernumerary tooth formation in familial adenomatous polyposis syndrome in human and the capacity for continuous formation of teeth is unlocked in mice in which the Wnt pathway is activated by stabilization of β-catenin in the oral epithelium (Wang and Fan, 2011). Indeed, Sox2 can function as an inhibitor of canonical Wnt signaling (Mansukhani et al., 2005). Thus, Sox2 might function as an inhibitor of the formation of replacement teeth and supernumerary molars by inhibiting epithelial growth. We did not observe changes in proliferation or *Axin2* expression in the *Sox2*cKOs, but this may result from an incomplete deletion of *Sox2* in the mutants.

Continuous tooth renewal and replacement require a source of stem cells that can self-renew and produce progeny. In the reptiles studied,

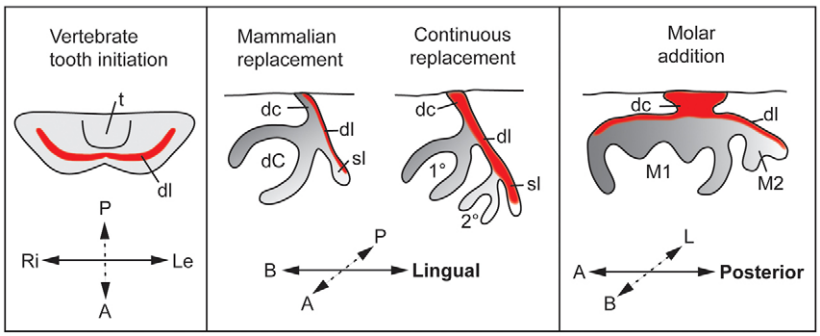


**Fig. 8. Sox2 expression is associated with epithelial competence of dental lamina in different modes of successional tooth formation.** Schematic of the localization of Sox2 (red) in the primary dental lamina in the lower jaw, and in the dental lamina during different types of successional tooth formation: mammalian tooth replacement, continuous replacement and molar addition. The drawings also illustrate the morphological similarity between the two modes of successional tooth formation. 1°, first generation tooth; 2°, second generation tooth; A, anterior; B, buccal; dc, dental cord; dC, deciduous canine; dl, dental lamina; L, lingual; Le, left; M, molar; P, posterior; Ri, right; sl, successional dental lamina; t, tongue.

*Sox2* expression overlapped the regions in the dental lamina where putative stem cells have been localized (Handrigan et al., 2010) (P.W. and C.-M.C., unpublished). Sox2 might play an important role in the maintenance of the dental lamina, which is likely to be a prerequisite for successional tooth formation. The termination of successional tooth formation in mammals might, however, not be the result of depletion of the $Sox2^+$ cells because Sox2 expression was maintained in ferret secondary tooth germs, which do not give rise to replacement teeth. One plausible reason for the discontinued successional tooth formation may be lack of signaling that induces tooth initiation from the $Sox2^+$ cells in the dental lamina. In reptiles, mesenchymal signals have been suggested to initiate this process (Handrigan and Richman, 2010). The genetic fate mapping experiments reported here demonstrated that the $Sox2^+$ cells contribute to all epithelial cell types of the successional molars, which is similar to our observations in incisors (Juuri et al., 2012). Therefore, *Sox2* might be a marker for naive dental progenitor cells, which have the capacity for new tooth generation. The role of Sox2 in the dental lamina might be to inhibit proliferation of the progenitors and/or to maintain their progenitor state. Further investigation with functional experiments is required to elucidate the specific function of Sox2 in the dental lamina cells.

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
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