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Establishment of crown–root domain borders in mouse incisor

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ABSTRACT

Teeth are composed of two domains, the enamel-covered crown and the enamel-free root. The understanding of the initiation and regulation of crown and root domain formation is important for the development of bioengineered teeth. In most teeth the crown develops before the root, and erupts to the oral cavity whereas the root anchors the tooth to the jawbone. However, in the continuously growing mouse incisor the crown and root domains form simultaneously, the crown domain forming the labial and the root domain the lingual part of the tooth. While the crown–root border on the incisor distal side supports the distal enamel extent, reflecting an evolutionary diet adaptation, on the incisor mesial side the root-like surface is necessary for the attachment of the interdental ligament between the two incisors. Therefore, the mouse incisor exhibits a functional distal–mesial asymmetry. Here, we used the mouse incisor as a model to understand the mechanisms involved in the crown–root border formation. We analyzed the cellular origins and gene expression patterns leading to the development of the mesial and distal crown–root borders. We discovered that *Barx2*, *En1*, *Wnt11*, and *Runx3* were exclusively expressed on the mesial crown–root border. In addition, the distal border of the crown–root domain might be established by cells from a different origin and by an early *Follistatin* expression, factor known to be involved in the root domain formation. The use of different mechanisms to establish domain borders gives indications of the incisor functional asymmetry.

Most mammalian teeth are composed of two domains: crown and root. The crown domain appears first during tooth morphogenesis. It develops from the epithelial component known as the enamel organ. The enamel organ is composed of distinct cell layers, including the inner and outer enamel epithelium (IEE, OEE) surrounding the stellate reticulum (SR). The IEE differentiates into enamel-secreting ameloblasts (for review, (Jussila and Thesleff, 2012)) covering the crown domain with enamel, whereas the SR cells are thought to fuel the growth of the crown. The transition from crown to root domain begins when the crown has achieved its shape and the SR cells disappear from the cervical loops (CL) (Tummers and Thesleff, 2003; Tummers et al., 2007). The loss of SR cells leads to the formation of a double-layered epithelium called Hertwig's epithelial root sheath (HERS). The HERS guides root development and is crucial to the proper differentiation of odontoblasts. Moreover, the HERS gives rise to a fenestrated network of cells on the root surface known as epithelial cell rests of Malassez (ERM). Because of its open structure ERM allows mesenchymal cells to reach the root surface and differentiate into cementoblasts. The resulting cementum is a key element that anchors the tooth within its socket (Bosshardt and Nanci, 1997). While the main part of the periodontal ligament insures the junction between the tooth root and the jawbone, the transseptal fibers guarantee a proper tooth alignment by attaching together the cementum of adjacent teeth.

Despite its continuous growth, the mouse incisor contains the root and crown domains. The root domain is situated on the lingual and mesial sides, while the crown domain is situated mainly on the labial side of the tooth (Fig. 1). Due to its simple tubular shape the crown and root domains form concomitantly and no crown-to-root transition takes place. The simultaneous growth of the domains is supplied by specific stem cells located in CLs at the base of the incisor (Harada et al., 1999; Seidel et al., 2010; Juuri et al., 2012). While the labial CL is thick and contains a sizable SR cell population, the lingual CL is narrow and encloses only a few SR cells. Therefore, the lingual CL resembles CLs of the molar after the crown-to-root transition (Tummers and Thesleff, 2003). The morphological labial–lingual asymmetry of the incisor forms as it rotates within the jaw at embryonic day (E) 13 (Mucchielli and Mitsiadis, 2000). The lingual–labial asymmetry of the tooth is functionally important for rodents and different strength of the labial and lingual surface leads, after abrasion, to a cutting edge adapted to rodents' diet (Amar et al., 1986).

Simultaneously forming crown and root domains of the mouse incisor appear to limit one another. The mesial surface of the mouse incisor is enamel-free and therefore allows the attachment of the transseptal fibers that are necessary for the adjacent incisor alignment (Johnson, 2006). However, the distal surface of the mouse incisor is partly covered with enamel, forming the distal

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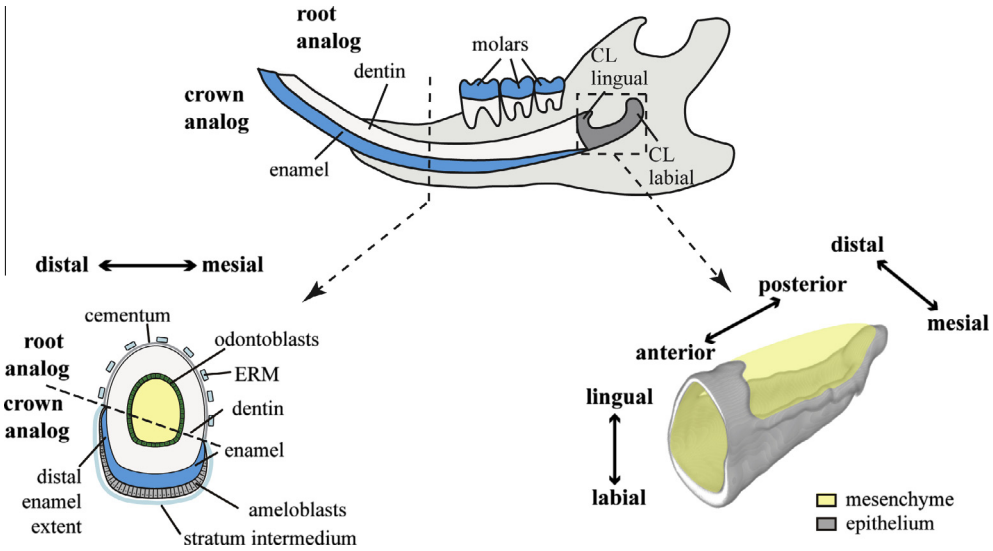

Fig. 1. Schematic illustrations of the mouse incisor. While dentin is deposited on the labial and lingual surfaces of the incisor, enamel is found only on the labial surface. The presence of ameloblasts distinguishes the crown analog of the mouse incisor, as opposed to the root analog. The schematic frontal section of the tooth shows the asymmetry between the root (lingual) and crown analogs (labial). The layer of ameloblasts between the stratum intermedium and the enamel covers the crown analog and forms the distal enamel extent on the distal side. The root analog is covered with cementum and epithelial cell rests of Malassez (ERM). The 3D representation of the proximal region of a mouse incisor displays the cervical loops, containing the stem cells fueling incisor renewal.

enamel extent (DE) (Fig. 1). It has been suggested earlier that the size of the DE is one of the parameters reflecting the tooth evolutionary adaptation to specific diets (Croft et al., 2011). From these observations, it can be concluded that while the mesial surface has to remain enamel-free due to its mechanical attachment function, the size of the DE on the distal surface can vary. Therefore, a more adaptive system may establish the crown–root border on the distal than on the mesial side.

Most of the studies visualize the labial–lingual asymmetry of the ameloblast differentiation by sagittal sections of the mouse incisor (Suomalainen and Thesleff, 2010). Indeed, the sagittal asymmetric gene expression pattern can demonstrate the genetic interactions required for ameloblast formation only on the labial side of the incisor (Klein et al., 2008). For instance, the asymmetric Follistatin expression was demonstrated to be necessary for enamel patterning, and therefore root domain formation (Wang et al., 2004). The establishment of the crown–root border on the mesial and distal sides of the incisor has, however, remained unclear. This question is of importance from the perspective of *in vitro* tooth construction using regenerative medicine protocols. The possibility to form crown and root domains on a synthetic scaffold from the same cells requires the knowledge of the mechanism of crown and root fate induction. Moreover, a proper location of the cementoenamel junction, determined by the crown–root border, would insure a proper periodontal ligament formation.

In order to visualize mandibular mouse incisor morphogenesis, we made 3D reconstructions of the epithelial shape from cap stage (E14) to the nearly fully-grown tooth (P2) (Fig. 2). We used these reconstructions to understand the precise location of the crown and root domains during the incisor formation, and to analyze our subsequent results.

Here, we analyzed ectopic enamel formation in Follistatin KO mouse incisor and discovered a distal–mesial asymmetry in this ectopic formation. In order to understand the asymmetry, we used genetic fate mapping and frontal sections of the incisor and gene expression patterns that could explain the formation of crown–root border on the distal and mesial sides. We investigated expression patterns of 32 genes and among them discovered 7 exhibiting a distal–mesial asymmetry, namely Follistatin, CDK6, CxCR4, En1, Wnt11, Barx2, and Runx3. The gene expression patterns were asymmetric during incisor morphogenesis and reflected a difference in the process of domain border formation on the distal compared to the mesial side of the incisor. In addition, we discovered that most of the epithelial cells that form the incisor originated from the *Shh*+ epithelial cells whereas part of the distal side of the incisor originated from *Shh*-negative epithelial cells. We concluded that the crown–root borders might result from a different mechanism on the distal and mesial side, and we suggested that this difference might have lead to the specific mesial and distal function of the incisor.

1. Results

1.1. Follistatin represses ameloblast fate on the distal side

The activity of the signaling molecule Activin A is necessary for proper ameloblast differentiation. On the lingual side of the mouse incisor, Activin A activity is inhibited by *Follistatin* and thus ameloblast differentiation is prevented, leading to a root-like structure (Wang et al., 2007). *Follistatin* KO (FSKO) mutant mice exhibit an ectopic ameloblast differentiation on the lingual root domain of the incisor. Therefore, *Activin A* and *Follistatin* expression are among the key factors in the patterning of the lingual–labial asymmetry during mouse incisor morphogenesis. However, their involvement in crown–root border formation remains unclear.

To assess the involvement of the Activin/Follistatin signaling in the establishment of the crown–root border, we checked *Follistatin* and *Activin A* expression on incisor frontal sections (Fig. 3A). At E13, *Follistatin* was expressed mainly in the epithelial cells while mesenchymal expression was detected only on the distal side of the incisor (Fig. 3Aa and b, arrow). While at E16, *Follistatin* expression was faint and symmetrical (Fig. 3Ac and d), later stage (P2) displayed asymmetrical epithelial expression (Fig. 3Ae, arrows). Interestingly, *Activin A* expression was symmetrical during incisor morphogenesis from E13 to P2. At E13, *Activin A* expression was mainly found in the mesenchyme that gives rise to the dental pulp (Fig. 3Ag and h). Later, at E16 and P2, *Activin A* was expressed in the