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Molecular mechanisms underlying tooth root development

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Root is a crucial and functional part of tooth for mammals, and tooth root development begins after the completion of tooth crown. Although bioengineered tooth has been extensively researched in animal studies recently, it is still impossible to regenerate new tooth in humans. Moreover, the intricate molecular networks that regulate root development, which are the cornerstone of tooth regeneration, still remain largely unknown. Therefore, fully understanding tooth root development is of great interest to aid further research in the field of regenerative medicine. Herein, we review the advances in the understanding of processes and mechanisms of root development using different mouse models. In this review, we start with the Hertwig's epithelial root sheath (HERS) formation, which marks the initiation of root development. We then highlight root dentinogenesis and cementogenesis with a focus on the molecules and signaling networks mediating root development. In addition, we summarize the complicated molecular interactions between epithelium and mesenchyme during root development. Finally, this review also features a list of various root abnormalities using gene deletion or overexpression mouse models to provide an overview of root malformations.

Keywords: root development, epithelium, mesenchyme, dentinogenesis, cementogenesis

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Introduction

In mammals, root is an indispensable part of the whole tooth. In mice, crown continues to develop after birth and reaches to its full size until postnatal day 4 (P4). Then root starts to form and lasts 3 weeks[1]. At P4, the outer enamel epithelium (OEE) and inner enamel epithelium (IEE) at the cervical loop elongate and form a continuous double layer structure named Hertwig's epithelial root sheath (HERS), which marks the commencement of the root development[2]. Morphologically, HERS is a structural boundary of two dental ectomesenchymal tissues: dental papilla and dental follicle which will form the pulp, dentin and periodontium. Following tooth root development and elongation, tooth finally erupts into the oral cavity and then establishes occlusal contacts with opposing teeth.

During the whole process of root development, epithelium and mesenchyme is tightly interacted and mutual influenced. Until now, there have been a lot of studies related to root development defects by application of transgenic mouse models, but most of these studies focused on a few molecules or signaling pathways and still we did not elucidate the mechanism of root development. Here, in this review, we divide the completed root development into three main parts: (1) The formation of HERS associated with the transition from crown to root; (2) Apically growth of the HERS and dentinogenesis; (3) The disintegration of the HERS and cementogenesis. This review will provide an overview of recent studies related to root development by using mouse models, in order to get a more comprehensive understanding of the regulatory mechanisms for root development.

HERS formation

In mice, during molar root development, the transition from dental cervical loop to HERS is regarded as the initiation of root formation. And it was generally accepted that root development proceeds under the control of HERS. Hence, it is of particular importance to figure out the formation and development of HERS since the precise mechanism underlying HERS development and growth remain to be elucidated. HERS originated from the stellate reticulum and/or OEE, and HERS formation is regulated by sequential and reciprocal interactions between epithelium and mesenchyme[3]. Similar to other organogenesis, the functional form of HERS is the result of precise coordination between the processes of cell proliferation, differentiation and apoptosis[4]. A widely accepted theory of HERS formation is the differences of cell dynamics between IEE and OEE. At beginning, growth factors like insulin-like growth factor I and hepatocyte growth factor (Hgf) secreted by the surrounding mesenchymal tissues are capable of increasing cell proliferation in the OEE but not IEE[5, 6], so it was believed that more active cell proliferation in the outer epithelium layer of HERS results in the HERS elongation. In consistent with this theory, disordered cell proliferative activity in IEE and OEE due to sustained Egf signaling and Fgf3/10 expression result in inhibited HERS formation[7, 8]. Subsequently, HERS formed a continuous bi-layer of flat, cuboidal cells, with extension in root apical direction. During this process, transforming growth factor-β *(*TGF-*β)*/BMP signaling is especially crucial for the fate of HERS. It has been demonstrated that exogenous Bmp4, a member of TGF-*β* superfamily, inhibits HERS development by preventing its

proliferation and elongation during root formation[9]. Furthermore, deletion of epithelial BMP signaling by Bmp receptor1a (*Bmpr1a*) conditional knockout mice promotes the differentiation of crown epithelial into HERS[10]. So this may suggest that BMP signaling pathway control the transition from crown to root. Smad4 is a central intracellular effector of both TGF-β and BMP signaling. Inactivation of TGF-β/Smad4 signaling by using *K14-Cre; Smad4*$^{fl/fl}$ mice also resulted in abnormal HERS which enlarges but could not elongate to guide root development, so root development halts at the initiation step[11]. In addition, Shh, a member of the hedgehog family, is also expressed in dental epithelium, has been suggested to be induced or inhibited by BMP/TGF-β/Smad4 signaling[12]. Shh is a secreted protein which has been showed to be an important regulator for the initiation of tooth formation at the early stage of tooth development. At the beginning of root development, Shh transcripts were restricted to the HERS cells and were related to the regulation of proliferative activity of HERS cells[13]. Moreover, the expression of Shh needs Smad4 in the dental epithelium during the epithelial-mesenchymal interaction, and either deletion or activation of the hedgehog signaling in the dental mesenchyme results in shorter molars. Therefore, HERS-derived Shh is important not only in HERS formation but also in root elongation.

Different from incisor which has only one root, molars have two or three roots. Over the past decades, most of the researches were related with root elongation area, but little was known about the root furcation formation. In mouse mandibular molar, at P4, two tongue-shaped epithelial protrusions grow toward each other and at P8, both the lingual and buccal epithelial protrusions were almost in contact. Until now, the epithelial protrusions were considered to be HERS since the structure and morphology is same as that in elongation area. During the process of furcation development, there were mainly three different activities of HERS observed. Firstly, invagination of epithelial protrusions, then horizontally extension, and finally connection and dissociation of HERS. Recently, the formation of furcation was contributed to proliferative differences between mesenchymal cells in different areas. By comparing cell proliferation of the mesenchyme in the mesial-root-forming (MRF) regions and the bifurcation-forming regions, one study found that the mesenchymal cells in the MRF showed relatively higher proliferation[14]. So it may indicate that the bifurcation-formation region was passively formed. Consistent with this view, other researchers also found that the occurrence of taurodont teeth which is characterized by elongated root trunk or unformed root trunk at furcation area is related to changed proliferative activity in the neighboring root mesenchyme[15, 16, 17]. Although these studies may suggest that the mesenchyme is the driver of single- and multi-rooted root patterning, the molecule or signaling pathway which regulate the proliferative activity is still undecided. Since the interaction between epithelium and mesenchyme is especially important for root development, the furcation development is most likely regulated by both. Our point of view is that the formation of furcation is mainly controlled by epithelium. Firstly, it has been reported that deletion of *Smo* or *Smad4* by *K14-Cre* in dental epithelium made the HERS fail to form, resulting in rootless molar. Secondly, during the horizontally extension of HERS, we found that

more proliferative activity around HERS, but less or even no proliferative cells in the mesenchyme between the HERS. So it is likely that more active HERS and passive mesenchyme allow the HERS to invaginate toward the center of teeth. Thirdly, signaling factors secreted from HERS are expressed in the mesenchyme around HERS during the furcation development. For example, Axin2 which is a target gene of Wnt signaling, is highly express in the mesenchyme around HERS, and our research found that loss and activation of Wnt signaling pathway lead to root defects[18,19]. In keeping with this idea, loss of *Wnt10a* both in mice and human can also lead to taurodontism[20].

Root elongation and dentinogenesis

After formation, HERS then elongates apically and guides root formation under the sequential and reciprocal regulation of epithelium and mesenchyme. During root elongation, the epithelial cells of HERS gradually enclose the expanding dental papilla. Then, HERS starts to induce the differentiation of odontoblasts from ectomesenchymal cells at the periphery of the dental papilla, resulting in pre-dentin and dentin formation [21]. The basement membrane of HERS is secreted by both dental epithelial and mesenchymal cells and acts as an inducer for odontoblast differentiation. HERS secretes extracellular matrix components, such as laminin 5 and TGF-β to induce differentiation of dental papilla into root odontoblasts. In turn, odontoblasts produce signaling molecules such as BMPs to regulate the growth and morphogenesis of HERS[22, 23, 24, 25]. In the developing root, odontoblast lineage cells have been traditionally classified as odontoblasts and preodontoblasts according to their status of differentiation. Odontoblasts are a type of terminally differentiated matrix secreting and mineralizing cells with a tall, columnar shape. Preodontoblasts are the precursors of odontoblasts with a cuboidal shape. Particularly, our lab firstly identified and characterized the "apical odontoblasts" (Aods) as new population of odontoblasts which were responsible for root elongation[26]. Aods were always present on the apical side of developing roots and moved downward together with HERS. Normal differentiation and maintenance of odontoblasts is the prerequisite for root development and disturbed odontoblast differentiation will result in various root abnormalities. Analysis of the expression patterns of growth factors during odontogenesis suggests that members of the TGF-β superfamily, IGFs, WNTs, FGFs and other molecules contribute to odontoblast terminal differentiation. During root development, Bmp 2, 3 and 7 are expressed in early odontoblasts in the apical region, but Bmp4 is expressed in pre-odontoblasts and its expression is down-regulated after odontoblasts differentiation[27]. Inhibition of TGF-β signaling in *Wnt1-Cre;Tgfbr2*$^{fl/fl}$ mice and *Osterix-Cre;Tgfbr2*$^{fl/fl}$ mice results in abnormal dentin formation [25, 28]. Ablation of *Smad4* in the dental mesenchyme by using *Osr2-Ires* Cre and odontoblast specific knockout of Smad4 resulted in disturbed odontoblast differentiation and abnormal root development[29]. This disruption not only impaired the mesenchymal BMP signaling pathways but also consequently altered the fate of HERS. So intact TGF-β/BMP communication between dental epithelial and ectomesenchymal tissues was required for the normal development of tooth roots.

Besides, canonical Wnt/β-catenin signaling pathway is of great importance in developing molar roots mainly based on the observation that *Axin2*, a direct target of canonical Wnt/ β-catenin signaling pathway, highly expressed in the root odontoblasts[30]. Also, some Wnts, such as Wnt5a and Wnt10a, as well as Wnt signaling mediator, *Lef1*, are expressed in developing odontoblasts. Dkk1, an inhibitor of Wnt signaling, is strongly expressed in pre-odontoblasts. The Wntless, is specially required for the secretion of Wnt proteins, and tissue-specific deleted in odontoblasts leads to impaired molar root elongation[31, 32, 33]. Moreover, disruption of Wnt signaling in mesenchyme either by *β-catenin* deletion or overexpression of *Dkk1* resulted in disrupted differentiation of root odontoblasts and severely retarded molar root[34]. However, constitutive stabilization of *β-catenin* leads to shortened roots with excessively deposited dentin[18]. These reports therefore suggest that modulation of Wnt signaling play an important role during odontoblast differentiation and dentin formation.

Other transcriptional factors such as Nuclear Factor I C (Nfic) and Osterix (Osx) also play important role in odontoblast differentiation. Nfic, which belongs to the nuclear factor I family of transcriptional factors, is primarily expressed in odontoblasts. Deletion of *Nfic* has been shown to leads to short and abnormal root due to suppression of odontoblast proliferation and differentiation by activating Shh signaling pathway. However, no major changes were noticed in molar crown formation in the *Nfic*-deficient mice, which indicating that Nfic is a transcription factor exclusively related to root development [35, 36, 37]. Osx, a key mesenchymal transcriptional factor participating in the differentiation and mineralization of odontoblasts. Recent studies revealed that Osx is capable of promoting expression and secretion of dentine sialophosphoprotein (DSPP), contributing to dentinogenesis. Additionally, a series of reports have uncovered site-specific function of Osx in tooth root development. Both odontoblastic conditional *Osx* knockout mice and conventional *Osx* knockout mice showed severe disrupted odontoblast differentiation and finally resulted in short molars[38, 39, 40].

It is worth mentioning that normal initiation of HERS can be followed by disturbed root elongation because of abnormal mesenchymal modulation. Our previous data showed that disrupted differentiation of apical odontoblasts lead to rootless molar in the *OC-Cre; Ctnnb1*$^{co/co}$ mouse with normal initiation extension of HERS[19]. In addition, it is still questionable whether the signaling molecules and growth factors remain effective during root elongation process in light of the observation that HERS structures appeared in impaired molar roots of several gene target mice[19, 41, 42]. Therefore, although HERS maybe indispensable for root initiation, optimal differentiation of odontoblasts is necessary for root elongation and formation.

Cementogenesis

At P10, HERS started to dissociate. Subsequently, the root continued development and HERS cells became increasingly dissociated. If the epithelial root sheath fails to become interrupted at the correct developmental stage (postnatal day 7 or later in mice) and remains attached to the surface of the root, the dental follicle mesenchymal cells cannot penetrate the HERS to contact the dentin and cannot be in-

duced to become cementoblasts to form cementum.

As root formation is completed, HERS divides into epithelial nests and cords and remains quiescent in the periodontal ligament, known as epithelial rests of Malassez (ERM). According to observations in histological sections, the number of epithelial cells decreases during the epithelial sheath fragmentation. So this phenomenon arouses the thinking of the reason or the fates of the disappeared epithelial cells. It has been controversial for decades about the relative contribution of HERS cells and dental follicle cells to cementogenesis. Previously, the widely accepted theory of cementogenesis suggests that cementum is a dental follicle-derived tissue that the dental follicle cells become cementoblasts and secret cementum subsequent to HERS disintegration[43]. However, now more and more researchers suggested that HERS cells underwent epithelial-mesenchymal transition (EMT) to differentiate into cementoblasts, directly participating in the formation of cementum[11]. Additionally, some researchers also proposed an epithelial origin for acellular cementum and a mesenchymal origin for cellular cementum[44, 45]. EMT was believed to be the conversion of epithelial cells into mesenchymal cells, and it is indispensable for embryogenesis and tissue development. Meanwhile, EMT can also explain why HERS cells decrease in number even though some cells may die by apoptosis. Some in vitro experiments by direct co-culture of HERS cells and dental follicle cells showed that HERS cells were corroborated to own the ability of mineralization and they acted as an indispensable inductive factor for the differentiation of dental follicle cells during formation of the periodontal structures[46]. By using *K14-Cre;R26R* mouse, Huang et al found that HERS cells on the surface of the root can also express cementoblast markers, such as collagen I and bone sialoprotein. In the root apical region, HERS cells are embedded in the cellular cementum, which indicated that HERS cells might participate in the formation of the cementum[2]. Moreover, some more recent findings showed that activation of TGF-β signaling induces HERS fragmentation through EMT and the fragmented HERS cells contribute to the formation of PDL and acellular cementum through periostin and fibronectin expression[47].

The mature mammalian tooth root is covered by cementum on its surface and is stabilized by periodontal ligament fibers which are embedded in both the cementum and alveolar bone. The cementum can be broadly classified into acellular and cellular cementum based on its cellular components. The thin and flat acellular cementum covers the cervical portion of the root, which is essential for tooth attachment. And the thicker and rough cellular cementum occupies the apical portion of the root. According to the functionality of cementum in the periodontal ligament attachment, the acellular type is more important for the cementum regeneration. Nevertheless, for decades, the regulation and formation of cementum types during cementum development are largely unknown. Our findings suggest that cementum type is not determined by its specific location, and Wnt signaling might be important for the determination of cementum type during cementum formation[48]. In addition, compared to cellular cementum, acellular cementum is more hypersensitive to regulators of mineralization which mainly refer to extracellular levels of inorganic phosphate (P_i) and pyrophosphate (PP_i)[49].

Conclusions

Unlike crown formation that has been studied with a substantial progress, root development remains poorly understood. All the recent studies we mentioned above are still far enough to reveal the intricate regulations during root development. It is though clear that reciprocal and sequential interactions between the epithelium and mesenchyme eventually leads to the formation of root dentin, cementum and other periodontal tissues during root development (Fig. 1). To date, there has been considerable studies with the application of gain- or loss-of-function of different genes which have made great progress in the root development field (Table 1). However, several important questions related to the mechanisms of root development are still controversial and unsolved. (1) How does the change in expression of genes result in the transition from crown morphogenesis to root development? (2) What is the mechanism that regulates the furcation development and how is the number of tooth roots determined? (3) What are drive factors for root elongation? (4) What is the origin of cementoblasts? In this review, we focus on molecule regulations during the progress of root development, hoping that knowledge related to the mechanisms of root development will pave the way to the development of tooth regeneration in the future.

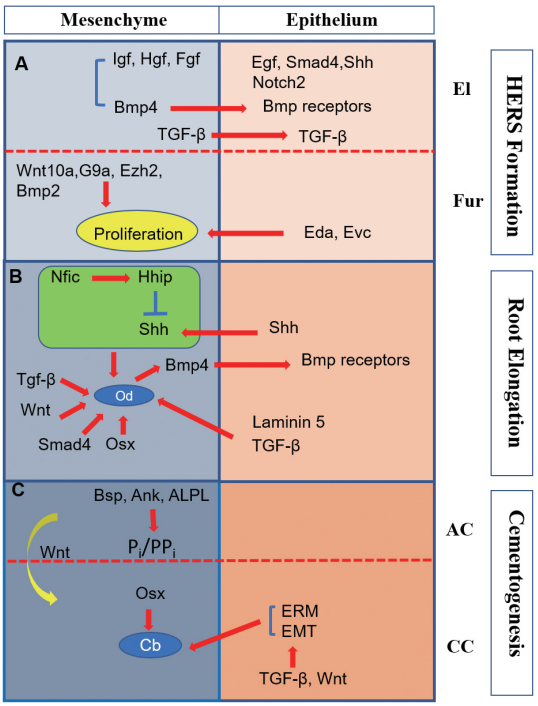

Figure 1. Schematic of main molecules and signaling pathway between epithelium and mesenchyme during root development. (A) At HERS formation stage, *Egf, Smad4, Shh, Notch2* express in the epithelium, and *Igf, Hgf, Fgf, Bmp4, TGF-β* secreted from mesenchyme to epithelium also take part in the formation of HERS. For the furcation formation, *Eda* and *Evc* from the epithelium, and *Wnt10a, G9a, Ezh2, Bmp2* from the mesenchyme can regulate the proliferation of mesenchymal cells around HERS, leading to the Furcation formation. (B) During root elongation, HERS secreted *laminin 5*, *Shh* and *TGF-β* to mesenchyme and induce the differentiation of odontoblasts. In turn, odontoblasts can produce *BMPs* to regulate the growth of HERS. The other molecules like *Nfic,* and *Osx* and signaling pathway like *TGF-β* and *Wnt/β-Catenin* in mesenchyme also regulate the odontoblast differentiation. (C) During cementogenesis, HERS on the one side divides into ERM, and on the other side undergoes EMT to differentiate into cementoblasts under the regulation of *TGF-β* and *Wnt* signaling for the cellular cementum formation. Another key transcription factor for the differentiation of cementoblast is *Osx* during cellular cementum formation. For acellular cementum, it is more

sensitive to the regulation of Pi/PPi under the control of *Bsp, ANK* and *ALPL*. And *Wnt* signaling may be an important determination of cementum type. EI: elongation area; Fur: Furcation; AC: Acellular cementum; CC: Cellular cementum.

Table 1. Current mouse models of root abnormalities

Signaling pathway	Genotype	Root abnormalities	References
Wnt signaling	*OC-Cre; Ctnnb1$^{co/co}$*	No root	Kim TH et al, 2013[19]
	2.3kb Col1a-Dkk1	Short root	Han XL et al, 2011[50]
	OC-Cre:Catnb$^{+/lox(ex3)}$	Short root, cementum hyperplasia	Bae CH et al, 2013[18]
	Oc-Cre; Wls$^{fl/fl}$	Short root	Bae CH et al, 2015[51]
	Wnt10a$^{-/-}$	Low or no furcation	Yang J et al, 2015[20]
	Sclerostin$^{-/-}$	Increased cementum	Kuchler U et al, 2014[52]
	K14-Cre; Wnt10$^{fl/fl}$	Low or no furcation	M.Yu et al, 2020[53]
Bmp/Tgf-β signaling	*K14-Cre; Smad4$^{fl/fl}$*	No root	Huang X et al, 2010[11]
	Oc-Cre; Smad4$^{co/co}$	Short root	Gao Y et al, 2009[41]
	Osx-Cre; *Tgfbr2$^{fl/fl}$*	Short root	Wang Y et al, 2013[27]
	K14-rtTA; tetO-Cre; Bmpr1a$^{fl/fl}$	Short root	Li J et al, 2015[54]
	Bmp9$^{-/-}$	Short root	Huang X et al,2019[55]
	3.6kb Col1-Cre; Bmp4$^{fl/fl}$	Decreased dentin thickness	Gluhak-Heinrich J et al, 2010[56]
	Krt5-rtTA/tetO-Cre/Alk3(Bmpr1a)$^{fl/fl}$	Ectopic cementogenesis	Yang Z et al, 2013[10]
	OC-Cre; Tgfbr2$^{fl/fl}$	Dysplastic dentin	Ahn YH et al,2015[57]
Others	*c-Fos$^{-/-}$*	No root	Alfaqeeh S et al, 2015[58]
	CaR$^{-/-}$	Short root	Sun W et al, 2010[59]
	Wnt1-Cre; Dlx3$^{F/lacZ}$	Short root	Duverger O et al, 2012[60]
	Ring1a-/-; Ring1b$^{cko/cko}$	Short root	Lapthanasupkul P et al, 2012[61]
	Sox2-Cre; Fam20C$^{co/co}$	Short root	Wang X et al, 2012 [62]
	Lhx6$^{-/-}$	Short root, No furcation	Zhang Z et al, 2013[63]
	2.3-kb Col1-Cre; Osx$^{fl/fl}$	short root	Zhang H et al, 2015[39]
	3.6kb Col1a1-Cre Osx$^{fl/fl}$; OC-Cre; Osx$^{fl/fl}$	Short root	Kim TH et al, 2015[40]
	Osx-Cre;PPR$^{fl/fl}$	Short root	Ono W et al, 2016[64]
	Wnt1-Cre; iZEG-Dlx2	Short root	Dai J et al, 2017[65]
	Osx-Cre; Raptor$^{fl/fl}$	Short root	Xie F et al, 2019[66]
	Evc$^{-/-}$	No furcation	Nakatomi M et al,2013[16]
	Pitx2-Cre; Irf6$^{fl/fl}$	No furcation	Chu EY et al, 2016[67]
	Edar$^{-/-}$	No furcation	Fons Romero JM et al, 2017[15]
	2.3kb Col1-Cre; Osx$^{fl/fl}$	Reduced cellular cementum	Cao, Z et al, 2012[68]
	Bsp$^{-/-}$	Decreased acellular cementum	Foster BL, 2015[69]
	Phospho1$^{-/-}$	Increased cellular cementum	Zweifler LE. 2016[70]
	Npp1$^{asj/asj}$	Ectopic acellular cementum	Bae CH et al, 2017[48]
	Osr2-Cre; Ezh2$^{fl/fl}$	No furcation	Jing J et al. 2019[17]

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한글초록

치아뿌리 형성과정에 대한 이해

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최근 바이오치아에 대한 연구는 동물실험을 통해 광범위하게 연구되고 있으나 사람에게 적용할 수 있는 치아를 재생하는 것은 여전히 불가능한 상태이다. 특히 치아뿌리는 치아를 턱뼈에 유지시키는데 있어서 핵심적인 역할을 수행한다. 치아뿌리의 형성과정에 대한 이해의 증진은 치아재생을 달성하는데 있어서 필수적인 부분이나 이에 대한 이해는 부족한 실정이다. 본 논문에서는 문헌고찰을 통해 치아뿌리의 발생을 조절하는 기전에 대한 최근 연구동향에 대해 살펴보았다. 치아뿌리의 형성을 개시하는 Hertwig 상피치근집의 형성과 치아뿌리 상아질의 형성과정 그리고 시멘트질의 형성을 조절하는 기전에 대해 조사하고, 치아뿌리 형성과정에서 상피와 간엽간의 상호작용에 대한 최신 연구 동향을 조사하였다. 또한 치아뿌리 형성이상을 표현형으로 나타내는 다양한 유전자변형 동물모델에 대해 살펴보았다. 치아뿌리 형성과정에 대한 이해는 향후 치아재생을 달성하는데 있어서 필수적인 지식기반을 제공할 것이다.

주제어: 치아뿌리형성, 상피, 간엽, 상아질형성, 시멘트질형성