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REVIEW

Molecular regulatory mechanism of tooth root development

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The root is crucial for the physiological function of the tooth, and a healthy root allows an artificial crown to function as required clinically. Tooth crown development has been studied intensively during the last few decades, but root development remains not well understood. Here we review the root development processes, including cell fate determination, induction of odontoblast and cementoblast differentiation, interaction of root epithelium and mesenchyme, and other molecular mechanisms. This review summarizes our current understanding of the signaling cascades and mechanisms involved in root development. It also sets the stage for *de novo* tooth regeneration.

INTRODUCTION

Human diseases of the tooth root have been long recognized. In 1944, Brown first reported one case of hypoplasia of the tooth root.[1] In 1972, Lind introduced the concept of short root anomaly disease.[2] The etiology of tooth root hypoplasia or short root anomaly disease is complicated and includes autosomal dominant inherited diseases (Singleton–Mertern syndrome, Ehlers–Danlos syndrome, etc.), Osteopetrosis (CLCN7 mutations) and hypophosphatasis.[3–5] The developmental processes of root formation are affected in these diseases.

The tooth is one of the best models for developmental biology and organ regeneration studies. The process of tooth development, the critical molecules involved in tooth crown formation and its reciprocal induction events have been addressed by multiple studies in recent years.[6–12] In mammals, the root is an important part of the whole tooth. Root development follows crown formation. Research on root development has made great progress in recent years.[13–21] A biological root has also been regenerated and functions in animal studies.[22–25]

Here, we review the tooth root development literature and describe the dental epithelial and mensenchymal contributions to root development with a detailed morphological and molecular analysis. This review will highlight the research on root development in recent years and will promote the understanding of processes required for tooth regeneration in the future.

PROCESSES OF ROOT DEVELOPMENT

Tooth development is a complex physiological process that includes the bud, cap and bell stages, tooth root development and tooth eruption.[6–12]

The interaction between dental epithelial and neural crest derived-mesenchymal cells is essential for tooth development.[7,26] After the crown formation is nearly complete, the tooth root begins to develop with the guidance of the double layer of the epithelial sheath, called the Hertwig's epithelial root sheath (HERS). The HERS is formed from the outer and inner enamel epithelium at the neck ring of the crown and grows in the apical direction. Morphologically, the epithelial root sheath is located between the two regions of neural crest-derived mesenchyme: the dental papilla and the dental follicle. When the HERS grows apically, the dental papilla cells adjacent to the inner epithelial layer of the HERS and the epithelial basement membrane are induced to become odontoblasts, and later to form root dentin. After root dentin formation, the epithelial root sheath enveloping the root begins to be interrupted or perforated. The formation of a mesh-like structure in the HERS allows dental follicle cells to contact the newly formed root dentin surface through the epithelial root sheath. These dental follicle cells differentiate into cementoblasts to form cementum. In addition, some of the HERS cells undergo epithelial-to-mesenchymal transition to become cementoblasts and form cementum. At the same time, collagen fibers secreted by dental follicle cells are embedded into the new cementum matrix and fix the root in the jaw bone. Following tooth root development and elongation, the tooth erupts into the oral cavity to establish occlusal contacts with opposing teeth and performs its physiological function.[19,21,27–28]

CELL DIFFERENTIATION OF THE TOOTH ROOT

During tooth root development, all functional hard tissues are formed by three kinds of cells: HERS, dental papilla mesenchymal and dental

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follicle cells, which form developing apical complexes.[19,21,29] Developing apical complexes are located in the apical region of the developing tooth and can develop an entire tooth root *in vitro*, independently without a crown. The hard tissues in the root, dentin and cementum, are formed by odontoblasts and cementoblasts,[30–31] which are derived from the dental mesenchyme.

Dentin formation
Dentin is a pale yellow substance that is the major component of the tooth hard structure covering the dental pulp. At the beginning of root development, the two layers of the HERS form before the appearance of root dentin. The basement membrane of the HERS is secreted by both dental epithelial and mesenchymal cells and acts as an inducer for odontoblast differentiation. Laminin 5 is secreted by the HERS and can induce dental papilla cell attachment, growth, migration and differentiation.[32] The HERS also secretes transforming growth factor-beta (TGF-β), which induces the differentiation of dental papilla cells into odontoblasts.[33–36] If the continuity of the HERS is disrupted, the dental papilla cells fail to differentiate. Thus, the HERS is critical for root dentin formation.

Although the structure and composition of dentin in the crown and the root are similar, their development varies. Morphologically, mature odontoblasts in the crown are elongated and stylolitic, but they are cubic in the root region. At the molecular level, nuclear factor Ic (Nfic) appears to play an important role in root, but not crown, dentin formation. If Nfic is knocked out in the dental mesenchyme, dentin fails to form, but instead bone-like mineral material is detectable, with odontoblasts that are not polarized or embedded in the cellular dentin. In contrast, odontoblasts in the crown pulp differentiate and dentin develops in Nfic mutant mice.[17–18,20,37] Therefore, the mechanism of dentin formation in the crown and the root must be different.

Cementum formation
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 contains acellular and cellular cementum. In most of the teeth, cellular cementum covers the apical one-third of the root, and acellular cementum covers the rest. Cellular intrinsic fiber cementum appears after injury of the root and is called repaired cementum. Cellular cementum is a bone-like mineralized tissue attached on the surface of root dentin or, in some animals, crown enamel. Cementum formation begins when both epithelial cells of the HERS and mesenchymal cells of the dental follicle are in proximity to the developing root surface. The relative contribution of epithelial and mesenchymal components to cementogenesis remains an area of debate. Previous studies have suggested that cementum is a dental follicle-derived connective tissue that forms subsequent to HERS disintegration. In 2009, we reported 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 and express ALPase. Some of the HERS cells on the surface of the acellular cementum also express ALPase. Our results are consistent with epithelial–mesenchymal transformation during root development, which was suggested by several previous studies showing that dental follicle as well as dental epithelial cells participates in cementogenesis.[14,19,38–42]

Periodontal ligament formation
Periodontal ligaments are derived from the dental follicle. The initiation of dental ligament formation begins at the stage when HERS interruption is detectable. After interacting with the HERS, pro-fibroblasts migrate to the root surface and alveolar bone surface. The tiny and short collagen fibers attach to the root surface and grow into the periodontal space. The fibers embedded in the cementum are called Sharpey's fibers. The arrangement of these fibers at the early root developmental stage is disordered. Later, the fibroblasts, pre-fibroblasts and stem cells in the dental follicle are activated and the fibers grow thick and well-organized. These strong fibers are important for linking the root and alveolar bone and to stabilize the tooth in the alveolar bone securely for the function of mastication.[14,31,43]

INTERACTION BETWEEN THE EPITHELIUM AND MESENCHYME DURING ROOT DEVELOPMENT
The interaction between the epithelium and mesenchyme plays an essential role in ectodermal organogenesis, such as that in the lung, kidney, glands and hair.[44–48] During tooth root development, the epithelium is located between the neural crest-derived mesenchyme tissues, the dental papilla and dental follicle. The sandwich-like arrangement of the developing root apical structure suggests that there is interaction between the dental epithelium and mesenchyme during root formation.

Function of the dental epithelia during root development
The dental epithelium in the root is different from that of the crown. Unlike epithelial cells in the crown, HERS cells do not respond to certain signals from the dental mesenchyme and do not differentiate into ameloblasts. The function of the HERS is not the same as the crown epithelium either. The HERS functions as an inducer of odontoblast and cementoblast differentiation, root growth and division of dental apical foramen, and it may help to determine root number. Some of the epithelial root sheath cells directly differentiate into cementoblasts or cementocytes during root development.[21]

If the continuity of the epithelial root sheath is interrupted too early during root dentin formation, odontoblast differentiation will be affected and the dental pulp and periodontal ligament will connect together without dentin in the affected region, where the collateral root canals are present. In contrast, 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 induced to become cementoblasts to form cementum. Then, the root dentin surface, in particular the cervical region of the tooth, is exposed, causing tooth sensitivity.[49] Therefore, the HERS is essential for the formation of both dentin and cementum during root development and may be considered the developmental center for root formation.

HERS also plays an essential role in determining root number. At approximately postnatal day 12, the buccal and lingual processes of the first mouse molar—the multiroot tooth—grow horizontally and contact each other to form the furcation of the tooth and give rise to the root contour. After segregation into multiroot canals, each root continues its elongation to complete root formation.[21,50]

Fate of the HERS
Previously, the fate of HERS cells was evaluated by continuous histological observations. Recently, we traced HERS cells using the Cre-loxp system (Keratin-14 (K14)-Cre; R26R-transgenic mice) and found that HERS cells are detectable on the surface of the root throughout root formation and do not disappear. Most of the HERS cells are attached to the surface of the cementum and others separate to become the epithelial rest of Malassez. HERS cells secrete extracellular matrix

components onto the surface of the dentin before dental follicle cells penetrate the HERS network to contact dentin. HERS cells also participate in cementum development and may differentiate into cementocytes. Therefore, based on our findings and that of others, at least six possible outcomes for HERS cells have been proposed: (i) becoming the epithelial rest of Malassez; (ii) apoptosis; (iii) incorporation into the advancing cementum front; (iv) epithelial–mesenchymal transformation; (v) migration toward the periodontal ligament; and (vi) differentiation into cementoblasts.[15,51–57]

Function of the dental mesenchyme during root development

The role of the dental mesenchyme in HERS induction during root development is not so clear. The primary hard tissue of root dentin is formed by odontoblasts, which have differentiated from dental papilla cells. Cementum is mostly formed by cementoblasts, which have differentiated from dental follicle cells. Molecules such as TGF-β, Nfic, insulin-like growth factors, WNTs and fibroblast growth factors (FGFs) contribute to odontoblast and cementoblast terminal differentiation.[16,58–63] Bone morphogenetic proteins (BMPs) and Fgf signaling in the dental mesenchyme may participate in the induction of the HERS, because Bmp3, Bmp4 and Fgf10 are expressed strongly before root development. The number of roots and direction of root formation may also be influenced by the mesenchyme and structures derived from the mesenchyme.

MOLECULAR MECHANISMS OF ROOT DEVELOPMENT

Gene expression during root development

Various genes, including TGF-β, BMPs, FGFs, Sonic hedgehog (Shh), Notch, Gli, Msx1, Msx2, Nfic and others, are involved in the process of root development.[16,58–63] We list the expression of various genes in different structures of the developing tooth root, including the dental epithelium (HERS), dental papilla and dental follicle (Table 1).

Tgf-β/Bmp signaling

The TGF-β superfamily of cytokines is comprised of TGF-βs, BMPs, activins and related proteins. TGF-β signaling plays an important role in developmental biology, disease, and regeneration.[64–66] The canonical TGF-β signaling pathway involves the TGF-β ligand binding to the type II and type I receptors. The activated receptor complex phosphorylates Smad proteins (R-Smads), which form a complex with the common Smad (Smad4). The Smad complex then translocates into the nucleus to regulate the expression of an array of target genes.[67–69] Tgf-β signaling is important for cementum formation. In cellular cementum, Tgf-β is strongly expressed in cementoblasts. Tgf-β also induces the expression of Timp, inhibiting metal proteinases and inducing periodontal ligament cell proliferation and synthesis of extracellular matrix.

Tgf-β signaling in both the dental epithelium and mesenchyme also plays essential roles in root dentin formation and root development.[20,36,70]

Smad4 is a central mediator of the canonical TGF-β signaling pathway. Deletion of Smad4 results in blockage of TGF-β/Bmp signaling. Ablation of *Smad4* in the dental mesenchyme (*Osr2-IresCre;Smad4^{fl/fl}* mice) results in short root formation and defects in odontoblast differentiation and dentin formation. Moreover, ectopic bone-like structures replaced normal dentin in the teeth of *Osr2-IresCre;Smad4^{fl/fl}* mutant mice. Loss of *Smad4* results in upregulation of canonical WNT signaling, and downregulation of *Dkk1* and *Sfrp1*, which are WNT pathway inhibitors.[70] Comparing different animal models provides more detail about Tgf-β signaling during root dentin forma-

Table 1 Gene expression during root development

Items	Dental epithelium	Dental papilla	Dental follicle	Cementoblast
Tgf-beta1	+			
Bmp2	+	+	+*	
Bmp3		+		+
Bmp4	+	+		
Bmp7	+	+		+
Egf			+*	
Egfr	+			
Fgf1	+			
Fgf2	+			
Fgfr1	+			
Fgfr2	+			
Notch1	+			
Notch2	+			
Notch3	+			
Shh	+			
Ctgf	+		+*	
Timp1		+		
Timp2	+			
Timp3	+			
IGF	+			
HGF	+			
Msx1		+		
Msx2	+	+	+	+
Runx2		+	+*	
Nfic		+		
Smad4	+	+	+	+

BMP, bone morphogenetic protein; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; Nfic, nuclear factor Ic; Shh, Sonic hedgehog.
* Our unpublished data.

tion. In *Osr2-IresCre;Smad4^{fl/fl}* mice, dental mesenchyme differentiation is arrested at the late bell stage and secretory stage, with no detectable expression of *Dspp*. Odontoblast differentiation is delayed and *Dspp* expression is eventually detectable in mice lacking *Tgfbr2* (*Wnt1-Cre;Tgfbr2^{fl/fl}*).[36] In contrast, odontoblast cell polarization is not affected in newborn *Osr2-IresCre;Bmpr1a^{fl/fl}* mice, in which BMP signaling is blocked in the dental mesenchyme. These results demonstrate that Tgf-β, but not Bmp, plays important roles in root dentin formation. Moreover, exogenous TGF-β1 can induce odontoblast differentiation and dentin formation in dental papilla cells *in vitro*.[71–73] Following inactivation of *Smad4* in dental epithelial cells (HERS) in *K14-Cre;Smad4^{fl/fl}* mutant mice, the development of molar roots is arrested and the formation of dentin is also severely affected. Smad4-mediated TGF-β/BMP signaling is required in the dental epithelium for *Shh* expression in the HERS and *Nfic* expression in the CNC-derived dental mesenchyme. Ectopic Shh induces *Nfic* expression in the dental mesenchyme and partially rescues root development in *K14-Cre;Smad4^{fl/fl}* mice.[20] Thus, we conclude that TGF-β/BMP signaling in the HERS relies on a Smad4-dependent mechanism that regulates *Nfic* expression *via* Shh signaling in the dental papilla.

Nfic

Nfic is a member of the nuclear factor I family, which includes Nfia, Nfib, Nfic and Nfix.[74] The four nuclear factor I members function independently. *Nfic* has a specific function as a key regulator of root dentin formation. In molars of Nfic mutant mice, the crown develops normally, but molar root development is defective due to abnormal dentin formation.[17–18,20] The defective dentin in Nfic mutant mice is

similar to that of Tgf-β1-overexpressing transgenic mice.[37] As mentioned above, Nfic is a downstream target of TGF-β1 signaling during tooth root development. Tgf-β1 induces odontoblast differentiation through the Smad pathway by increasing p21 and other Tgf-β-responsive gene expression levels *via* the degradation of Nfic, which suppresses p21 expression. During early odontoblast differentiation, Tgf-β1 and MAPK activation enhances the formation of a Smad2/3-Nfic-Smurf1/2 complex and results in the degradation of Nfic. During late odontoblast differentiation and mineralization, Nfic signaling results in the dephosphorylation of p-Smad2/3. Consequently, Tgf-β1 induces odontoblast differentiation through the Smad signaling pathway in early odontoblast differentiation, whereas Nfic signaling modulates late odontoblast differentiation and mineralization.[37]

Shh

Shh, a member of the hedgehog signaling family, is expressed in the dental epithelium and plays an essential role during tooth development.[15] During root development, Shh is strongly expressed in the HERS, which suggests a function in root formation. Patched, the membrane receptor of Shh, and Gli1, a transcript activated by Shh, are also detectable in the root epithelium (HERS) and mesenchyme.[61–62] The partial rescue of root development by Shh in rootless *K14-Cre;Smad4^{fl/fl}* mutant mice indicates the essential function of Tgf-β-mediated Shh signaling in regulating root formation.[20]

Fgf3 and Fgf10

Fgfs are expressed in the dental epithelium and mesenchyme during tooth crown formation. However after birth, their expression changes. For example, Fgf3 and Fgf10 are expressed in the dental mesenchyme during the bud and bell stages of tooth crown development, but after birth, their expression is dramatically reduced. In contrast, Fgf3 and Fgf10 are expressed in the dental mesenchyme and help to maintain stem cell proliferation in the cervical loop of the incisors, which continue to grow throughout life in the mouse. In molar development, however, Fgf10 is turned off prior to root development. If Fgf10 remains active in the dental papilla of the molars during root development, the HERS will be enlarged and the root will fail to form. In voles, the molar continues to grow throughout life, and Fgf10 expression is detectable in the dental pulp adjacent to the enlarged HERS.[75–77] Taken together, these data suggest that Fgf10 is an important regulator for controlling the switch from crown to root formation.

Wnt

Wnt is also important for tooth crown development, but Wnt expression is barely detectable during root development. If Wnt signaling is upregulated *via* inhibition of Bmp signaling, dentin formation is replaced by bone formation. Therefore, Wnt may help to control cell fate decision during mineral tissue formation.[70]

Hepatocyte growth factor

Hepatocyte growth factor (Hgf) is one of the mediators of epithelial–mesenchymal interactions in rodent tooth. Hgf receptors are expressed in the enamel epithelium of molar germs as well as in HERS cells. Hgf stimulates root development in transplanted tooth germs, proliferation of HERS cells in culture and HERS elongation in an organ-culture system. Moreover, cell proliferation in the HERS increases after treatment with Hgf. This effect is downregulated when an antibody against the Hgf receptor is added to the culture medium. Thus, Hgf signaling controls root formation *via* the development of the HERS.[78]

CONCLUSION

We have made important great progress in studies of tooth root development in the last 10 years. Development of ectodermal organs involves similar processes and, in some cases, the same genes as those used earlier in development.[79–81] Like the tooth crown, tooth root development involves the interaction of the dental epithelium and the cranial neural crest-derived mesenchyme. These reciprocal interactions are mediated by a series of signals, including from Tgf-β, Bmp, Wnt, Shh, Fgf and other molecules, and by the function of homebox genes, such as Nfic and Msx1/2. This review provides an overview of root development and highlights the developmental biology of the tooth root. We hope that knowledge about the cell fate of the HERS, interaction of dental epithelial and mesenchymal cells, and other molecular mechanisms during tooth root development will facilitate tooth regeneration studies in the future.

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