Cellular and molecular mechanisms of tooth root development

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ABSTRACT

The tooth root is an integral, functionally important part of our dentition. The formation of a functional root depends on epithelial-mesenchymal interactions and integration of the root with the jaw bone, blood supply and nerve innervations. The root development process therefore offers an attractive model for investigating organogenesis. Understanding how roots develop and how they can be bioengineered is also of great interest in the field of regenerative medicine. Here, we discuss recent advances in understanding the cellular and molecular mechanisms underlying tooth root formation. We review the function of cellular structure and components such as Hertwig’s epithelial root sheath, cranial neural crest cells and stem cells residing in developing and adult teeth. We also highlight how complex signaling networks together with multiple transcription factors mediate tissue-tissue interactions that guide root development. Finally, we discuss the possible role of stem cells in establishing the crown-to-root transition, and provide an overview of root malformations and diseases in humans.

KEY WORDS: Tooth, Root, Odontogenesis, Signaling network, Stem cells and tissue regeneration

Introduction

Teeth perform important physiological functions in our daily life, playing roles in mastication and speech. Each tooth has two main anatomical components: the crown and the root (Fig. 1). Whereas the crown is mostly made up of highly vascularized dental pulp surrounded by dentin and enamel on the outer surface (for anatomical terms, see the Glossary, Box 1), the dentin of the root is covered by cementum, rather than enamel. The root is also surrounded by the periodontal ligament (PDL; see Glossary, Box 1), a fibrous connective tissue structure that connects the root cementum to the alveolar bone (Fig. 1). Importantly, the tooth root is an essential element in the function of the dentition because it anchors teeth to the maxilla or mandible. Accordingly, the loss of roots leads to diminished bone support and, hence, perturbed tooth function. In addition, during mastication and resting states, the root helps transmit and balance occlusal forces through PDLs to the jaw bone, blood supply and nerve innervations. The root development therefore initiates with a thickening of the oral epithelium, which eventually becomes the dental lamina. At this stage, the epithelium exhibits so-called odontogenic inductive potential and is able to induce the initiation of tooth development when recombined with non-odontogenic neural crest derived-mesenchyme (Lumsden, 1988; Mina and Kollar, 1987). The dental lamina then invaginates into the underlying cranial neural crest (CNC)-derived mesenchyme to form the tooth bud. The mesenchyme condenses around the epithelial tooth bud and, via the expression of a particular set of transcription factors and signaling molecules, gains the ability to instruct tooth morphogenesis (Kollar and Baird, 1970a,b; Thesleff and Sharpe, 1997). Subsequently, the epithelium undergoes folding that determines the shape and number of cusps, with additional factors secreted by the enamel knot (see Glossary, Box 1) regulating these events (Jernvall and Thesleff, 2000; Thesleff and Sharpe, 1997). Finally, the epithelium differentiates into ameloblasts and the mesenchyme differentiates into odontoblasts; ameloblasts deposit enamel, which is the hard outermost layer of the tooth crown, whereas odontoblasts secrete dentin matrix, which hardens into dentin surrounding the dental pulp (Fig. 1) (Thesleff and Sharpe, 1997). Together, these cells help to build the crown portion of the tooth.

The molecular regulation of early tooth morphogenesis leading to the formation of the crown has been studied extensively. Indeed, a large body of work has shown that the network controlling crown development encompasses the action of major signaling pathways, including the Tgfβ, Bmp, Fgf, Wnt and Shh pathways, which act recurrently at various stages (Chai and Maxson, 2006; Tucker and Sharpe, 2004). However, the precise molecular network controlling late stages of tooth development, including the crown-to-root transition and root formation, is yet to be determined. Indeed, most of our knowledge about root development is based on histology and 3D imaging analyses, and although some studies have shown that many of the same signaling molecules involved in regulating crown formation are also involved in controlling root formation, how these molecules achieve their signaling specificity during root formation is only just beginning to be understood. In addition, although multiple mutant animal models have been shown to exhibit root development defects, most studies of these animals have simply highlighted root developmental defect phenotypes, but have yet to elucidate the molecular regulatory network for root development. There is thus a need to integrate these studies to gain a more comprehensive understanding of root development. Furthermore, it is not yet known what determines the number of roots per tooth and the direction of root formation, although they are likely to be influenced by the Hertwig’s epithelial root sheath (HERS; see Glossary, Box 1), CNC-derived mesenchyme and adjacent anatomical structures. Future studies will help us to gain a better understanding of the integration between dental roots and jaw bones required for proper function.

In this Review, we explore some of the unique transcriptional regulatory and signaling networks that may play key roles in regulating root formation. Based on advances in our understanding...
of how these networks function during tooth morphogenesis, we review what is currently known about the cellular and molecular mechanisms involved in the formation of tooth roots and their potential implications in stem cell-mediated tissue regeneration, as well as their relevance in human disease. Finally, we suggest some future directions for investigating the molecular and cellular regulatory mechanism of root development and stem cell-mediated tissue regeneration.

**An overview of the tooth root and its morphogenesis**

Development of the tooth root starts following crown formation, once the enamel tissue has reached the future cementoenamel junction (see Glossary, Box 1), which is the point at which the enamel and cementum meet and that defines the anatomical boundary between the crown and the root (Fig. 1). The apical region of the enamel organ (see Glossary, Box 1) elongates and gives rise to the HERS (Fig. 2), a bilayer epithelial structure between the dental papilla (see Glossary, Box 1) and dental follicle (Orban, 1980) (see Glossary, Box 1). The HERS then grows apically and guides root formation (Fig. 2), determining the size, shape and number of tooth roots (Cate, 1996). Any disturbance in the formation of the HERS leads to malformations affecting root structure, shape, number, length and other features (reviewed by Luder, 2015). The CNC-derived mesenchyme condenses around and continuously interacts with the HERS and, subsequently, the mesenchyme of the apical papilla comes into contact with the inner layer of the HERS and undergoes differentiation into odontoblasts, which secrete radicular (i.e. root-covering) dentin. If the continuity of the HERS is disturbed prematurely, the differentiation of root odontoblasts is compromised (Kim et al., 2013). This function of the HERS in regulating odontoblast differentiation requires direct contact; laminin 5 secreted by the HERS induces the growth, migration and differentiation of mesenchymal cells in the dental papilla (Mullen et al., 1999). Moreover, the HERS produces growth factors that contribute to the induction of odontoblast differentiation, suggesting that the HERS functions as a signaling center to guide root formation (Huang et al., 2009, 2010).

The HERS also plays a role in controlling cementum formation. After its dynamic movement towards the apical region of the tooth, the HERS becomes perforated via localized apoptosis or epithelial-to-mesenchymal transition (EMT), giving rise to a mesh network structure (Fig. 2C) (Huang et al., 2009; Luan et al., 2006). This network might facilitate the continuous interaction among epithelial cells and their collective interaction with the CNC-derived mesenchyme, as well as contact between dental follicle cells and newly formed dentin. After they initiate contact with dentin, cells in the dental follicle differentiate into cementoblasts, which produce cementum-specific extracellular matrix proteins, including collagen fibers (Zeichner-David, 2006). In the apical region of the root, cementoblasts remain embedded in the matrix, giving rise to cellular cementum. By contrast, the rest of the root is covered by acellular cementum. Both cementoblasts and the HERS are indispensable for the formation of cementum. For example, if the HERS is not perforated at the correct developmental stage, dental follicle cells cannot contact dentin and, consequently, cementoblast differentiation is altered and cementum formation is abnormal (Luan et al., 2006).

The dental follicle is not the only source of cementoblasts; the HERS expresses collagen I, bone sialoprotein and ALPase, all of which are typical cementoblast markers (Huang et al., 2009), and it is now clear that the HERS contributes directly to the pool of cementoblasts in the root via EMT (Bosshardt et al., 2015; Huang et al., 2009; Xiong et al., 2013). Indeed, the HERS does not completely degenerate during root formation; besides undergoing EMT, some fragments of the HERS become the epithelial cell rests of Malassez (ERM; see Glossary, Box 1), which are quiescent residues that contribute to cementum regeneration and repair (Xiong et al., 2013).

In addition to contributing to cell differentiation, the HERS helps to determine the number of tooth roots that form (Fig. 3) (Cate, 1996). As it forms, the HERS develops tongue-shaped epithelial protrusions that join horizontally to form a bridge, called the furcation, where the root becomes divided, constituting the base of the pulp cavity (Orban, 1980). After the furcation forms, root development in multi-rooted teeth is driven by the apical growth of the ERM.

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**Box 1. Glossary**

**Cementoenamel junction**: The point at which the enamel and cementum meet, which defines the anatomical boundary between the crown and the root.

**Cementum**: Specialized hard tissue covering the root that provides attachment for periodontal ligaments.

**Dental follicle**: A population of cells around both the enamel organ and dental papilla that gives rise to the cementum, periodontal ligament and alveolar bone.

**Dental papilla**: A group of cranial neural crest-derived cells under the enamel organ. It gives rise to dental pulp, including odontoblasts.

**Dental pulp**: Soft tissue comprising the central part of the tooth.

**Dentin**: Mineralized tissue secreted by odontoblasts adjacent to the enamel.

**Enamel**: Highly mineralized tissue covering the crown.

**Enamel knot**: A signaling center during tooth development. It secretes signaling molecules to guide tooth morphogenesis.

**Enamel organ**: A complex epithelial structure located above the dental papilla that produces enamel for the developing tooth.

**Epithelial cell rests of Malassez (ERM)**: Residual cells from HERS, found within the periodontal space.

**Hertwig’s epithelial root sheath (HERS)**: A bilayered epithelial structure extending from the apical region of the enamel organ, growing apically, and guiding tooth root formation.

**Neurovascular bundle**: Anatomical structure consisting of nerves, arteries and veins in close proximity.

**Periodontal ligament (PDL)**: Connective tissue between the tooth root and the alveolar bone.
the HERS, in the same way as occurs in single-rooted teeth (Orban, 1980). Thus, the growth of the HERS in different orientations contributes to the formation of multi-rooted teeth, including two-rooted lower molars and three-rooted upper molars (Fig. 3) (Orban, 1980). The differential proliferation of mesenchymal cells in furcation-forming and root-forming regions may also be involved in determining root number through regulation of the directionality of HERS growth (Sohn et al., 2014).

The correct formation and degeneration of the HERS are also crucial for PDL development (Cho and Garant, 2000). PDL formation starts with the migration of dental follicle cells in contact with the HERS in between the root and alveolar bone. This event coincides with the beginning of HERS perforation. During migration, a number of cytoplasmic processes project from the leading edges of the CNC-derived dental follicle cells and begin secreting collagen fibers. Initially, these collagen fibers are disorganized, but as development progresses they thicken and become arranged in a structured manner. The proper secretion and distribution of these collagen fibers contribute to the correct orientation and attachment of the PDL, which is crucial for its ability to connect the root and alveolar bone, stabilizing and preparing the tooth for mastication (Cho and Garant, 2000; Palmer and Lumsden, 1987).

The CNC-derived mesenchyme also contributes to the formation of many cell types during root formation, including odontoblasts, dental pulp cells, cementoblasts and PDL cells. Previous studies have indicated that CNC-derived cells contribute to mesenchymal tissues during early development (Chai et al., 2000), although there have been limited studies using genetic cell lineage tracing to demonstrate the dynamic contribution of CNC-derived cells specifically during root formation. Recently, however, using an inducible Cre line, researchers have found that osterix (Sp7)-positive mesenchymal cells contain progenitor cells that contribute to different mesenchymal cell types during root development (Ono et al., 2016). We have also recently generated a Pax9-CreER line that can specifically target the CNC-derived mesenchyme in order to follow its contribution to different mesenchymal cell types during root formation (Feng et al., 2016). These genetic tools will be highly useful for targeting the CNC-derived cell population and will offer the opportunity to investigate how these CNC-derived cells may interact with the HERS to contribute to root formation.

Signaling networks that regulate tooth root development

An array of growth and transcription factors is expressed during the initiation of root formation, suggesting that these factors might perform crucial functions in regulating the epithelial-mesenchymal interactions that are involved in all steps of tooth development. For example, Bmp, Tgfβ, and their mediator Smad4, as well as Shh, Msh2 and Dlx2, are expressed in HERS cells (Äberg et al., 1997; Huang et al., 2010; Lezot et al., 2000; Nakatomi et al., 2006; Yamashiro et al., 2003). In the CNC-derived dental mesenchyme adjacent to the HERS, there is expression of Gli1, Nfic, Fgf, Tgfβ, Bmp, Wnt and its inhibitors, as well as PTHrP/PPR (Pthlh/Pth1r) (Huang et al., 2010; Ono et al., 2016; Steele-Perkins et al., 2003; Wang et al., 2013). In addition, some signaling molecules are expressed in both the HERS and the CNC-derived dental mesenchyme (Liu et al., 2015). To date, multiple mutant animal models have been generated to elucidate the importance of these signaling molecules and their downstream target genes in regulating root formation (summarized in Table 1). Below, we discuss these approaches and highlight our current knowledge of how these key signaling molecules and their networks regulate tooth root development.

Bmp/Tgfβ signaling

In root development, Bmp signaling is actively involved in regulating cell fate decisions during the formation of the HERS and the differentiation of odontoblasts (Fig. 4). Bmp2, 3, 4 and 7 are expressed during the initiation of tooth root development...
Epithelium/enamel role in the mesenchyme during root development because ablation of mesenchyme, it appears that Tgf signalling in odontoblasts and bone-producing mesenchyme results in failure of root elongation, reduced radicular dentin matrix density, and delayed molar eruption (Wang et al., 2013). Furthermore, Smad4, a central mediator of the canonical Bmp/Tgfβ signalling pathway, controls the size of roots; ablation of Smad4 in odontoblasts results in short roots and defects in odontoblast differentiation and dentin formation (Gao et al., 2009). Moreover, loss of Smad4 in odontoblasts leads to the failure of HERS dissociation through mesenchymal-epithelial interaction (Gao et al., 2009). This phenotype is similar to that of Tgfr2 mutant mice, suggesting that one of the important functions of Smad4 is to mediate Tgfβ signaling in the dental mesenchyme in the regulation of root formation (Fig. 4).

Smad-mediated Tgfβ1 signaling appears to act together with Nfic to regulate both the early and late stages of odontoblast differentiation. Nfic is a member of the nuclear factor I family and functions as a key regulator of root dentin formation. In humans and mice, Nfic expression is restricted to odontoblasts and preodontoblasts in developing molars (Gao et al., 2014). In Nfic knockout mice, molars develop a normal crown but root development is affected due to alterations in patterning, growth and dentin formation (Steele-Perkins et al., 2003). Nfic signaling modulates Tgfβ signaling via dephosphorylation of phospho-Smad2/3 during late odontoblast differentiation, maturation and mineralization (Lee et al., 2009). Similarly, Nfic antagonizes the effects of Tgfβ1 on stem cells from the apical papilla (SCAPs) in vitro (He et al., 2014).

Wnt signaling
Wnt signaling is also important in regulating root formation (Lohi et al., 2010). Although it is well known that Wnt ligands are expressed during the early stages of tooth development and that dental mesenchyme-specific deletion of the Wnt signaling mediator β-catenin leads to tooth development arrest at the bud stage (Chen et al., 2009; Sarkar and Sharpe, 1999), the precise functional role of Wnt signaling in regulating tooth root development is only now emerging. The canonical Wnt pathway is highly active in developing molar roots, based on the expression of Axin2 in root odontoblasts and preodontoblasts (Lohi et al., 2010) and the expression pattern of Wnt10a during root development (Yamashiro et al., 2007). Moreover, Wnt10a can induce the expression of Dspp, a gene associated with dentinogenesis, in odontoblasts (Yamashiro et al., 2007). These studies suggest that canonical Wnt activity might play a role in the regulation of root dentinogenesis. Furthermore, Wnt10a null mice exhibit taurodontism, which is characterized by elongated root trunks and a low or absent furcation, as do many patients with Wnt10a mutations (Yang et al., 2015). Strikingly, odontoblast differentiation and root dentin formation are not affected in Wnt10a null mice (Yang et al., 2015), indicating that other Wnt ligands are likely to contribute to root dentinogenesis and that Wnt10a must be specifically involved in the formation of the root furcation. Moreover, deletion of the Wntless (Wls) gene in odontoblasts reduces canonical Wnt activity. This gene encodes a chaperone protein that regulates all Wnt ligands. Its loss leads to inhibition of odontoblast maturation and therefore to root elongation (Bae et al., 2015). Consistently, Wnt10a and Axin2 expression of Tgfbr2 in the HERS does not affect root formation (Li et al., 2015), whereas loss of Tgfbr2 in the CNC-derived mesenchyme leads to a root development defect (Oka et al., 2007). More recently, it was shown that Tgfβ signaling within the CNC-derived dental mesenchyme can indirectly regulate HERS cells and control root development through tissue-tissue interactions (Wang et al., 2013). Specifically, loss of Tgfβ signaling in odontoblasts and bone-producing mesenchyme results in failure of root elongation, reduced radicular dentin matrix density, and delayed molar eruption (Wang et al., 2013). Furthermore, Smad4, a central mediator of the canonical Bmp/Tgfβ signalling pathway, controls the size of roots; ablation of Smad4 in odontoblasts results in short roots and defects in odontoblast differentiation and dentin formation (Gao et al., 2009). Moreover, loss of Smad4 in odontoblasts leads to the failure of HERS dissociation through mesenchymal-epithelial interaction (Gao et al., 2009). This phenotype is similar to that of Tgfr2 mutant mice, suggesting that one of the important functions of Smad4 is to mediate Tgfβ signaling in the dental mesenchyme in the regulation of root formation (Fig. 4).

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levels are also dramatically decreased in the odontoblasts of Wls mutant roots (Bae et al., 2015). Additionally, β-catenin-mediated Wnt signaling is required to regulate the differentiation of CNC-derived odontoblast cells during tooth formation, because tissue-specific inactivation of β-catenin in developing odontoblasts results in molars that completely lack roots (Kim et al., 2013). This study also shows that compromised Wnt signaling in the CNC-derived mesenchyme can adversely affect the formation of HERS cells and may interrupt the epithelial-mesenchymal interactions that are crucial for root formation (Fig. 4). Similarly, tissue-specific overexpression of Dkk1, an inhibitor of Wnt/β-catenin signaling, in odontoblasts leads to short roots and dentin defects in mandibular molars (Han et al., 2011). Together, these studies suggest that Wnt signaling must be tightly controlled during root formation, and it is thus not surprising that the overactivation of Wnt signaling can also lead to root development defects (Bae et al., 2013; Kim et al., 2012).

Wnt signaling also interacts with other signaling pathways to control root formation. For example, canonical Bmp signaling is required for maintaining the expression of Wnt signaling inhibitors, such as Dkk1 and Sfrp1, in odontoblasts to regulate dentin formation; accordingly, loss of Bmp signaling in the dental mesenchyme leads to elevated Wnt signaling, resulting from downregulation of Dkk1 and Sfrp1, and the formation of ectopic bone-like structures in the dentin region (Fig. 4) (Li et al., 2011). HERS cells also require β-catenin-mediated Wnt signaling to initiate root cementum formation following the cessation of Bmp signaling as tooth crown formation concludes (Yang et al., 2013). When Bmp signaling is blocked via tissue-specific depletions of Bmpr1a in the crown epithelia at the end of tooth crown formation, tooth root development initiates earlier than normal, as indicated by the premature switch in cell fate of crown epithelia from ameloblasts to cementoblasts, caused by the upregulation of Wnt/β-catenin signaling (Yang et al., 2013). This study suggests that the interaction between Bmp and Wnt signaling determines the transition between crown and root formation during tooth development.

**Fgf and Hh signaling**

The Fgf and Hh signaling pathways are involved in mediating epithelial-mesenchymal interactions that are crucial for organogenesis. For example, Fgf10 signaling in the dental mesenchyme plays a key role in controlling the crown-to-root transition via the regulation of HERS formation during molar development. Indeed, the disappearance of Fgf10 expression in the dental mesenchyme near the epithelial cervical loop is required for the initiation of root formation in mouse, rat and human molars, and the persistence of Fgf10 expression is required for the continuous growth of crowns in vole molars (Tummers and Thesleff, 2003; Yokohama-Tamaki et al., 2006). Furthermore, treatment of molar tooth germs with exogenous Fgf10 at the crown-to-root transition stage leads to the inhibition of HERS formation in vitro (Yokohama-Tamaki et al., 2006). These studies suggest that Fgf10 controls the switch between crown and root formation during molar tooth development by regulating epithelial-mesenchymal interactions (Fig. 4). In the developing tooth, Fgf2 is expressed in differentiating odontoblasts at the apical end and in the furcation zone of the root, development. Indeed, the disappearance of Fgf10 expression in the dental mesenchyme near the epithelial cervical loop is required for the continuous growth of crowns in vole molars (Tummers and Thesleff, 2003; Yokohama-Tamaki et al., 2006). Furthermore, treatment of molar tooth germs with exogenous Fgf10 at the crown-to-root transition stage leads to the inhibition of HERS formation in vitro (Yokohama-Tamaki et al., 2006). These studies suggest that Fgf10 controls the switch between crown and root formation during molar tooth development by regulating epithelial-mesenchymal interactions (Fig. 4). In the developing tooth, Fgf2 is expressed in differentiating odontoblasts at the apical end and in the furcation zone of the root, as well as in cementoblasts and fibroblasts of the PDL, suggesting that Fgf2 might be involved in regulating various aspects of root development (Gao et al., 1996; Madan and Kramer, 2005).

Similarly, the Shh signaling pathway is clearly involved in regulating the interactions between the HERS and the CNC-derived mesenchyme during root formation (Fig. 4). Shh ligand secreted by dental epithelial cells in the apical region of molar roots functions in

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**Table 1. Mouse mutants exhibiting root development defects**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Genotype</th>
<th>Root defect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp/Tgfβ signaling</td>
<td>K14-Cre;Smad4fl/fl</td>
<td>No roots</td>
<td>Huang et al., 2010</td>
</tr>
<tr>
<td></td>
<td>K14-rtTA;tetO-Cre;Bmpr1afl/fl</td>
<td>Short roots</td>
<td>Li et al., 2015</td>
</tr>
<tr>
<td></td>
<td>OC-Cre;Smad4fl/fl</td>
<td>Short roots</td>
<td>Gao et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Bmp2-cre/O2+/Cre-EGFP</td>
<td>Short roots</td>
<td>Rakian et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Wnt1-Cre;Bmpr1afl/fl</td>
<td>Fewer roots</td>
<td>Our unpublished data</td>
</tr>
<tr>
<td></td>
<td>Dspp-Tgfb1</td>
<td>Short roots</td>
<td>Thyagarajan et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Osr2-Cre;Tgfb1/2</td>
<td>Short roots</td>
<td>Wang et al., 2013</td>
</tr>
<tr>
<td>Wnt signaling</td>
<td>Wnt10a+/−</td>
<td>Elongated root trunks and a low or absent furcation</td>
<td>Yang et al., 2015</td>
</tr>
<tr>
<td></td>
<td>OC-Cre;WlsCICCO</td>
<td>Short roots</td>
<td>Bae et al., 2015</td>
</tr>
<tr>
<td></td>
<td>OC-Cre;CtnnbFICICO</td>
<td>Short roots</td>
<td>Kim et al., 2013</td>
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<tr>
<td></td>
<td>2.3 kb Col1a1-Dkk1</td>
<td>Short roots</td>
<td>Han et al., 2011</td>
</tr>
<tr>
<td></td>
<td>OC-Cre;CatnbFICICO</td>
<td>Short roots</td>
<td>Bae et al., 2013</td>
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<tr>
<td></td>
<td>Col1a1-Cre;CatnbFICICO</td>
<td>Short roots</td>
<td>Kim et al., 2012</td>
</tr>
<tr>
<td>Fgf signaling</td>
<td>Osr2-Cre;Fgf10fl/fl</td>
<td>Short roots</td>
<td>Nakatomi et al., 2006</td>
</tr>
<tr>
<td>Shh signaling</td>
<td>Ptgfl/fl</td>
<td>Short roots</td>
<td>Liu et al., 2015</td>
</tr>
<tr>
<td>Others</td>
<td>Gli1-CreERT2;R26SmoM2fl/fl</td>
<td>Short/no roots</td>
<td>Steele-Perkins et al., 2003</td>
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<tr>
<td></td>
<td>Nfic−/−</td>
<td>Short roots</td>
<td>Ono et al., 2016</td>
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<td></td>
<td>PTHrP/PPR</td>
<td>No roots</td>
<td>Lu et al., 2009</td>
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<td></td>
<td>Nif−/−</td>
<td>No roots</td>
<td>Afqaeeh et al., 2015</td>
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<td>c-Fos−/−</td>
<td>Short roots</td>
<td>Zhang et al., 2013</td>
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<td>Lhx6−/−</td>
<td>Misshappen/short roots</td>
<td>Aloubi et al., 2007</td>
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<td>Mscx−/−</td>
<td>Fused roots</td>
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<td>Evc−/−</td>
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<td>Duverger et al., 2012</td>
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<td>Wnt1-Cre;Dlx3FloxZ</td>
<td>Short roots</td>
<td>Laphanasaupkul et al., 2012</td>
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<td></td>
<td>Ring1a−/−;Ring1bFloxZ</td>
<td>Short roots</td>
<td>Kim et al., 2015</td>
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<td>3.6 kb Col1a1-Cre;OsrXfl/fl</td>
<td>Short roots</td>
<td>Kim et al., 2015</td>
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<td>OC-Cre;OsrXfl/fl</td>
<td>Short roots</td>
<td>Zhang et al., 2015</td>
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<td></td>
<td>2.3 kb Col1a1-Cre;OsrXfl/fl</td>
<td>Short roots</td>
<td>Cao et al., 2012</td>
</tr>
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</table>
|                  | 3.6 kb Col1a1-Osx | Acceleration of cellular cementum formation |}
both the HERS and nearby dental mesenchymal cells during root formation, as indicated by the expression patterns of genes encoding the Shh ligand, the Hh receptors Ptc1 and Ptc2, the transcriptional regulator Smo, and the transcription factor Gli1 (Nakatomi et al., 2006). As root development proceeds, Shh signaling activity is gradually reduced in both the epithelium and mesenchyme of the molar root (Li et al., 2015; Nakatomi et al., 2006). Overactivation of the Shh pathway before the initiation of the molar root, either by disruption of Ptc1 or constitutive activation of Smo, results in reduced proliferation in dental mesenchymal cells near the HERS and shortened roots (Liu et al., 2015). Furthermore, loss of Ptc1 signaling in the dental epithelium results in persistence of Shh-Gli1 signaling activity in the dental mesenchyme and an eventual lack of roots, which can be partially rescued by restoring Nfic expression by addition of exogenous Shh protein (Huang et al., 2010). Significantly, Nfic also regulates the expression of Hh-interacting protein (Hhip) to provide negative feedback to Shh signaling (Fig. 4) through mesenchymal-epithelial interactions during root formation (Liu et al., 2015). A recent study has shown that PTHrP/PPR inhibits Nfic expression in the CNC-derived mesenchyme during root formation and that loss of this inhibition leads to a root development defect (Ono et al., 2016), suggesting that Nfic is another factor that must be regulated precisely in order to ensure normal root formation.

In addition to the signaling molecules mentioned above, studies have shown that IGF1 and HGF may also be involved in regulating root formation (Fujinawa et al., 2005; Sakuraba et al., 2012), although their precise function in regulating epithelial or mesenchymal cells is yet to be elucidated. Nonetheless, taking all of these studies together, we are beginning to understand the complex signaling network that regulates epithelial-mesenchymal interactions during root formation (summarized in Fig. 4). These studies suggest that there are multiple activators and inhibitors that appear to work together to achieve a balanced signaling outcome and produce the proper patterning, number and length of dental roots during later stages of tooth morphogenesis. Clearly, studies of mutant animal models have provided important insights into the regulatory mechanisms of tooth root development. However, in parallel, we have also learned that tooth root development defects are part of monogenic, multifactorial inheritance and chromosomal disorders (discussed below). Moving forward, it will be important to explore how well these mutant animal models reflect the tooth developmental defects seen in humans, and whether these animal models can be used to discover potential therapeutic solutions for patients.

**Dental stem cells and the transition from crown to root development**

The crown/root ratio is one of the parameters used to classify teeth among different species. Teeth are classified into three categories based on this parameter: (1) brachydonts, in which the root is longer than the crown; (2) hypsodonts, where the crown is longer than the root; and (3) hypselodonts, which grow continuously during the lifetime of the animal and typically do not include a classic root (Tummers and Thesleff, 2003, 2008). Recent studies suggest that modulation of stem cell populations might explain, at least in part, the differences between these tooth types.

Members of the third group, especially the mouse incisor, are used to study stem cell biology because they harbor both epithelial and mesenchymal stem cells in the proximal region of the tooth that allow them to grow continuously (Fig. 5A) (Kuang-Hsien Hu et al., 2014; Zhao et al., 2014). In the mouse incisor, continuous growth via formation of enamel by the epithelium only occurs on the labial side. The lingual side of the incisor exhibits characteristics distinct from the labial side and is thought to function as a root, although because no classic root formation takes place this region is known as the root analog. Study of the root analog, by investigation of the
Previous studies have shown that peripheral nerve-associated cells and their niche during tooth development. Epithelial stem cells (dark blue) expressing Sox2, Bmi1 and/or Gli1 are located in the proximalmost region of the cervical loop. Mesenchymal stem cells (orange) expressing Gli1 reside in the proximalmost region of the incisor pulp, in a periarterial niche that responds to signals from the adjacent sensory nerve. Together, these stem cells fuel the continuous growth of the mouse incisor.

From an evo-devo perspective, the phenotype of Smad4 mutant mice is reminiscent of vole, rabbit and guinea pig molars, which also grow continuously and harbor a population of epithelial stem cells at their apical ends. Interestingly, the expression patterns of genes that control epithelial stem cell behavior are similar in mouse incisors and vole molars. For instance, Notch genes and Fgf10 are expressed in both mouse incisors and vole molars but are absent in mouse molars (Harada et al., 2002; Tummers and Thesleff, 2003). These genes, together with the Shh and Bmp pathways, are likely to constitute a molecular network that regulates the niche and epithelial stem cell compartments in mammals. Consequently, this network differentially controls the transition from crown to root during tooth development, depending on the species, producing the observed variety of different tooth types.

**Root developmental defects in humans**

As highlighted above, a number of signaling pathways and transcriptional regulators have been identified as important for tooth root development in animal models. However, how well these animal models reflect the causes of root developmental defects in humans remains to be determined. Notably, human dentition is more complex than that in any of the animal models available and includes single-rooted incisors, canines and bicuspids, as well as multi-rooted molars. Furthermore, the direction and number of dental roots are different in the upper and lower molars; these properties together represent an important anatomical feature supporting the functions performed by human dentition.
In humans, certain genetic mutations have been linked to root development defects (Dong et al., 2005; Yang et al., 2015). However, the majority of root defects observed in humans are associated with complex genetic disorders that lead to multiple developmental defects (reviewed by Luder, 2015). For example, BCOR mutations are responsible for causing oculofaciocardiodental (OFCD) syndrome, in which patients suffer from multiple craniofacial and cardiac anomalies but also have canines with extremely long roots (Fan et al., 2009; Gorlin, 1998). In these patients, BCOR mutations cause abnormal activation of AP-2α (TFAP2A), which in turn promotes the osteodentinogenic capacity of mesenchymal stem cells and hence root malformation (Fan et al., 2009). Studies of OFCD syndrome serve as a perfect example of how studying a rare genetic disorder can help reveal the molecular mechanisms of congenital malformation.

Among the disorders that only affect root development, the premature arrest of root development is common. These disorders are usually associated with trauma that affects the HERS and neighboring neurovascular structures, which disturbs elongation of the root and dentin formation (Andreasen and Kahler, 2015). A similar phenotype is observed in patients exposed to radiation or chemotherapy (Pedersen et al., 2012). This finding is consistent with recent work demonstrating that the neurovascular bundle secretes factors that contribute to stem cell maintenance and homeostasis (Zhao et al., 2014). Dilaceration is another malformation of the tooth root, typically observed as part of an eruption disorder (Topouzelis et al., 2010). It is characterized by a sharp curvature in the apex of the tooth that is frequently the consequence of indirect trauma to the primary teeth.

Taurodontism, another disorder affecting roots only, is specific to primary and permanent multirooted teeth and is characterized by apical displacement of the bi- or trifurcation (Dineshshankar et al., 2014). It is often associated with reduced constriction at the cementoenamel junction and an increased occluso-apical height of the crown and pulp cavity due to a delay in the development of the epithelial bridges in the presumptive area of the furcation (Dineshshankar et al., 2014). Taurodontism may have a genetic component; for example, in tricho-dento-osseous syndrome, which is associated with DLX3 mutations (Wright et al., 2008). Mutations in DLX3 are also associated with amelogenesis imperfecta hypoplastic-hypomaturation with taurodontism in humans (Dong et al., 2005). Of note, the ablation of Smad4, Nf1c or Wnt10a in mice leads to similar phenotypes (Li et al., 2015; Liu et al., 2015; Yang et al., 2015).

There are also a number of tooth development defects that affect both the crown and root. These include double teeth, regional odontodysplasia, hypophosphatasia, dentin dysplasia type I, dentinogenesis imperfecta types I, II and III, and X-linked hypophosphatemia. Double teeth result from the merging of two adjacent tooth germs during odontogenesis (Hattab, 2014). A related but distinct disorder is concrescence, in which two adjacent teeth are joined by means of only radicular cementum (Romito, 2004). In regional odontodysplasia, root formation often ends prematurely, leaving a wide-open apex (Hamdan et al., 2004). Hypophosphatasia, which is characterized by defective mineralization and sometimes skeletal abnormalities, is caused by loss-of-function mutations in the ALPL gene, which encodes tissue-nonspecific alkaline phosphatase in humans and mice (Foster et al., 2013). Dentin dysplasia type I (DDI) is an infrequent disorder that affects the formation of the dentin in both the crown and root; in the root, dysplastic hard tissue and scattered soft tissue fill the central space. DDI is transmitted as an autosomal dominant trait but its specific genetic cause is unknown (Kim and Simmer, 2007). The tooth of dentinogenesis imperfecta type I and II patients exhibit early and complete obliteration of the pulp cavity, which is instead filled with dentin. By contrast, dentinogenesis imperfecta type III patients exhibit excessively large pulp cavities (Kim and Simmer, 2007).

Again, the genetic causes of these dentinogenesis imperfecta types are unclear, although some studies have linked certain genes to particular types. For example, dentinogenesis imperfecta type I, which is associated with osteogenesis imperfecta, is attributed to mutations in COL1A1 and COL1A2, whereas mutations in DSPP are responsible for dentinogenesis imperfecta type II. Finally, X-linked hypophosphatemia, which is characterized by hypomineralized dentin and enlarged pulp cavities, similar to taurodontism except that the furcation is not displaced apically (Fong et al., 2009), is linked to mutations in PHEX.

Overall, disruptions of root development are rather frequent and are commonly associated with the early loss of teeth, with deleterious consequences for oral health. Importantly, we need to link animal models with human tooth root development defects in order to gain a better understanding of the etiology of these disorders as well as of the normal development of roots.

Conclusions and future directions

Despite the significant recent progress that we have discussed above, the overall regulatory mechanisms of dental root formation remain poorly understood. It is clear that, during early tooth development, reciprocal and sequential interactions between the epithelium and mesenchyme eventually lead to the formation of root dentin, cementum and periodontal tissues. The major signaling pathways involved in these processes are the Tgfβ/Bmp, Wnt, Fgf and Shh pathways, which work together with multiple transcription factors to mediate tissue-tissue interactions that guide root development. However, there are still several important unanswered questions concerning root development, including how root patterning is established. Among the features that are decided during development are the number, size, direction and fine morphology of the roots. Intrinsic elements such as combinatorial gene expression and the activity of complex molecular networks in the root epithelium and mesenchyme have been shown to control tissue-tissue interactions during root development (Fig. 4). However, several anatomical features suggest that other craniofacial structures can also influence the root pattern. For instance, in humans and mice, maxillary first molars have three roots whereas mandibular first molars only have two. The density of the bone in the maxilla and mandible differs significantly, being lower in the maxilla (Devlin et al., 1998), and researchers and clinicians have thus speculated that maxillary molars possess three roots to increase the stability of these teeth in a bone of low density. Other factors that may potentially affect molar root development are vascularization and nerve innervation. Indeed, recent studies have shown that the neurovascular bundle serves as a niche environment for mesenchymal stem cells in mouse incisors (Kaukua et al., 2014; Zhao et al., 2014). However, the mouse incisor does not have a traditional root, making it difficult to study how the neurovascular bundle may influence root development. Future studies might shed light on whether molar mesenchymal stem cells are also influenced by the neurovascular bundle during root development. Understanding all these aspects of root development will improve our knowledge of the factors that regulate tooth development. From a clinical perspective, understanding how roots develop, how they might be affected by periodontal diseases and how they could be bioengineered using regenerative approaches (see Box 2) is also crucial for the oral health field.
Box 2. Bio-roots: engineered tooth roots and their applications

Although studies using animal models have shown that it is indeed possible to regenerate a whole tooth (Ikeda et al., 2009), clinicians have questioned whether such a newly formed tooth will have the proper size, shape, color and occlusion necessary to function with the rest of the natural dentition, especially in humans. By contrast, the regeneration of a ‘bio-root’ using stem cells and a scaffold is more feasible and, hence, a more attractive approach. Furthermore, if restored with a porcelain crown, a bio-root functions as a natural tooth (Wei et al., 2013). Two general approaches have been explored in the tooth regeneration field: (1) recapitulating embryonic development to produce a tooth/root; and (2) using adult stem cells combined with scaffolding biomaterials to produce a bio-root (reviewed by Dadu, 2009). Taking the first approach, several studies have shown that dental cells obtained from dissociated porcine or rat tooth buds can generate multiple small, organized tooth crowns with root-like structures (e.g. Young et al., 2005). More recently, a study reported that functional teeth with roots could be generated after the transplantation of tooth buds formed from reggenerated mouse tooth bud cells (Ikeda et al., 2009). However, the regeneration of functional human teeth from human tooth germ transplanted into an immunocompromised mouse model cannot be clinically feasible or practical. With regard to the second approach, the assembly of stem cell-derived tissues into a functional root has proven to be challenging. To date, PDL, cementum and dentin have been bioengineered using different sources of adult stem cells, including human dental pulp stem cells (DPSCs), human periodontal ligament stem cells (PDLSCs), and stem cells from human exfoliated deciduous teeth (SHED) (Granthos et al., 2000; Miura et al., 2003; Seo et al., 2004). Most recently, using a combination of scaffold, DPSCs and PDLSCs, a bio-root has been developed in miniature pigs (Wei et al., 2013). Notably, the regenerated bio-root restored with a porcelain crown exhibited characteristics of a normal tooth after 6 months of use, including dentinal tubule-like and functional PDL-like structures (Wei et al., 2013). Although the success rate of bio-root-supported restorations is less than that of restorations supported by implants (Gao et al., 2016), we are confident that bio-root-supported restoration will ultimately offer an important biological solution for patients as tissue engineering procedures improve.

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Competing interests

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