CHAPTER SEVEN

Tissue Interactions Regulating Tooth Development and Renewal

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Contents

Abstract

Reciprocal interactions between epithelial and mesenchymal tissues play a fundamental role in the morphogenesis of teeth and regulate all aspects of tooth development. Extensive studies on mouse tooth development over the past 25 years have uncovered the molecular details of the signaling networks mediating these interactions (reviewed by Jussila & Thesleff, 2012; Lan, Jia, & Jiang, 2014). Five conserved signaling pathways, namely, the Wnt, BMP, FGF, Shh, and Eda, are involved in the mediation

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of the successive reciprocal epithelial–mesenchymal cross talk which follows the general principle of morphogenetic interactions (Davidson, 1993). The pathways regulate the expression of transcription factors which confer the identity of dental epithelium and mesenchyme. The signals and transcription factors are integrated in complex signaling networks whose fine-tuning allows the generation of the variation in tooth morphologies. In this review, we describe the principles and molecular mechanisms of the epithelial–mesenchymal interactions regulating successive stages of tooth formation: (i) the initiation of tooth development, with special reference to the shift of tooth-forming potential from epithelium to mesenchyme; (ii) the morphogenesis of the tooth crown, focusing on the roles of epithelial signaling centers; (iii) the differentiation of odontoblasts and ameloblasts, which produce dentin and enamel, respectively; and (iv) the maintenance of dental stem cells, which support the continuous growth of teeth.

1. INTRODUCTION

Interactions between groups of cells and different domains of the developing embryo constitute key mechanisms of morphogenesis. Hans Spemann and Hilde Mangold were the first to experimentally demonstrate the concept of "inductive interactions" between tissues in transplantation studies using tissues derived from different newt species (Spemann & Mangold, 2001). Later, transplantation experiments revealed the importance of tissue interactions in the development of a variety of morphogenetic processes in the embryos and showed them to be "the most important mechanism regulating embryonic development," as John Gurdon stated in 1971 (Gurdon, 1971).

All morphogenetic tissue interactions in the embryo follow similar general principles and key molecular players, as presented schematically by Davidson (1993) (Fig. 1). Tissue interactions are sequential and reciprocal, and are mediated by several conserved families of signaling molecules including Wnts, BMPs, Hedgehogs, and FGFs. These molecules regulate gene expression, in particular, the expression of transcription factors that are shared between various tissues and organs. Generation of tissue and organ variety is the result of unique and exclusive combinations of transcription factors that regulate cell fate and determine the identity of the target cells during the successive stages of a given morphogenetic process (Davidson, 1993). As illustrated in our review, these same principles also apply to tooth development.

The development of all organs which form from the ectodermal and endodermal sheets lining the embryo is regulated by communication

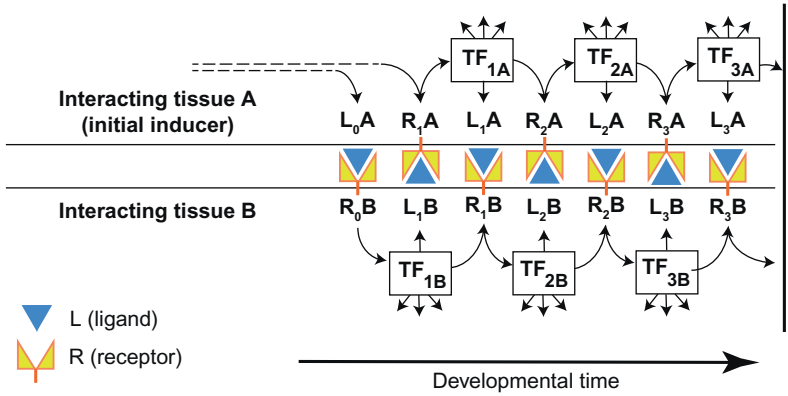

Figure 1 A chain of interactions between adjacent tissues undergoing progressive morphogenetic inductions. Release of the ligand (L) (signaling molecule) from the inductive tissue and its binding to the receptor (R) on the adjacent tissue triggers a sequence of regulatory changes in each tissue (A and B). These changes are controlled by the appearance of new transcription factors (TF) in response to signals from the adjacent tissue. New transcription factors activate a battery of genes (indicated by arrows emanating from the boxes). *Figure adapted from Davidson (1993).*

between the epithelium and underlying mesenchyme. A chain of reciprocal epithelial–mesenchymal interactions regulates all aspects of tooth development. This was first demonstrated in classic tissue recombination studies at the end of the 1960s by Edward J. Kollar, in which the epithelial and mesenchymal components of the developing mouse tooth germs were separated and combined with dental and nondental tissues, followed by intraocular engraftment in adult mice. These experiments showed that the tooth identity (molar vs. incisor) as well as the ability to reprogram nondental epithelium to tooth fate are determined by the mesenchyme (Kollar & Baird, 1969, 1970). Later tissue recombination experiments in the 1980s demonstrated that the odontogenic potential initially resides within the oral epithelium, which can induce tooth formation when recombined with neural crest-derived second branchial arch mesenchyme or with the premigratory neural crest cells (Lumsden, 1988; Mina & Kollar, 1987). These experiments indicated that the odontogenic potential shifts to the mesenchyme prior to the onset of epithelial morphogenesis at E (embryonic day) 12 (Fig. 2). In addition, tissue recombination experiments showed that epithelial–mesenchymal interactions regulate differentiation of the two major tooth-specific cell types, the odontoblasts and ameloblasts (reviewed by Hurmerinta & Thesleff, 1981; Ruch, Lesot, Karcher-Djuricic, Meyer, & Olive, 1982).

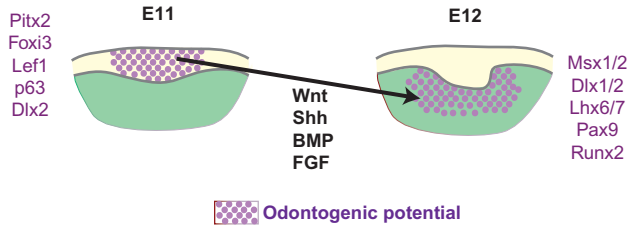

Figure 2 Shift of the odontogenic potential from epithelium to mesenchyme between the dental lamina and placode stages of tooth development. The dental lamina expresses specific transcription factors, which are associated with the dental identity and regulate the expression of signals in four conserved families. These signals mediate the shift of odontogenic potential to the mesenchyme and regulate an array of transcription factors which determine the identity and odontogenic potential of the dental mesenchyme.

In the past 25 years, great effort has been made to uncover the molecular details of the signaling networks mediating the interactions that regulate mouse tooth development (reviewed by Jussila & Thesleff, 2012; Lan et al., 2014; Thesleff, 2003). In this review, we describe the current understanding of the molecular mechanisms of the epithelial–mesenchymal interactions regulating various phases of tooth formation: (i) the initiation of tooth development, (ii) the morphogenesis of the tooth crown, (iii) the differentiation of the cells which produce mineralized dental tissues, and (iv) the maintenance of dental stem cells which support the continuous growth of teeth. The focus is on the mediation of tissue interactions by five conserved signaling pathways: the Wnt, BMP, FGF, Shh (Sonic Hedgehog), and Eda (ectodysplasin) pathways, which play fundamental roles at all stages of tooth development.

2. EPITHELIAL–MESENCHYMAL TISSUE INTERACTIONS REGULATING TOOTH INITIATION AND MORPHOGENESIS

2.1 Epithelial Signaling Centers

Teeth are initiated from the *dental lamina*, a stripe of stratified epithelium first discovered in histological sections that forms at E11 at the sites of future tooth rows (Fig. 2). Mouse embryonic dental lamina can be visualized by localized expression of several transcription factors and signaling molecules. *Pitx2* is the most specific marker of dental epithelium, and its expression persists in all epithelial cells of the developing tooth during crown

morphogenesis (Mucchielli et al., 1997). Other transcription factors in the dental lamina include *Foxi3* (Shirokova et al., 2013), *Dlx2*, *Lef1*, and *p63*, which together with *Pitx2* may well be linked to the acquisition of tooth fate and odontogenic potential in the oral epithelium. The dental lamina also expresses signaling molecules, such as *Shh*, *Bmp2*, *Bmp4*, *Bmp7*, *Fgf8*, *Fgf9*, *Wnt10a*, and *Wnt10b* (Dassule & McMahon, 1998), that likely function as mediators of the odontogenic potential from the epithelium to the mesenchyme.

The morphogenesis of the first teeth in mouse is initiated from *placodes*, which appear in E12 mouse embryos as local thickenings of the dental lamina in the incisor and molar regions (reviewed by Biggs & Mikkola, 2014; Jussila & Thesleff, 2012). Thereafter, successional tooth development occurs in molars as the second and third molars (M2 and M3) develop posterior to the first (M1). The following text refers to the development of M1. The expression of many dental lamina genes becomes restricted to the placodes, including *Pitx2* and *Foxi3* as well as several signaling pathway-associated molecules in all five signaling families. Notably, the expression of signals such as *Shh*, *Wnt10*, *Bmp2*, and *Fgf20* is further restricted to a small cluster of placodal cells called the *early signaling center* (Fig. 3; Haara et al., 2012;

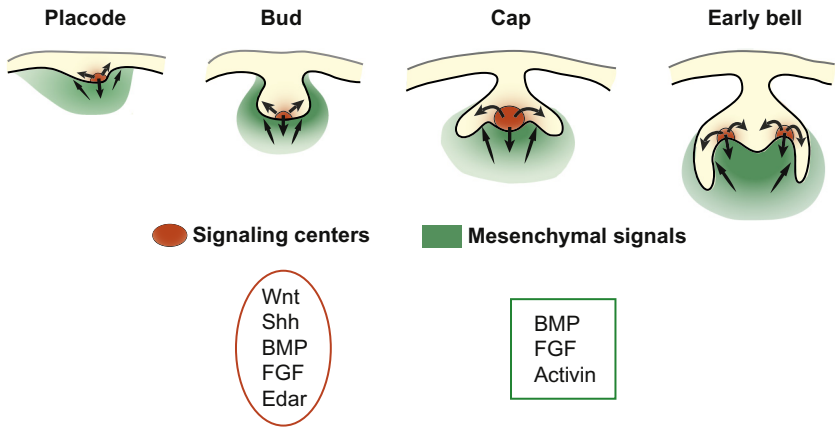

Figure 3 Epithelial–mesenchymal tissue interactions and epithelial signaling centers regulating tooth morphogenesis. Of particular significance for morphogenesis are the epithelial signaling centers which express signals of the four conserved signal families. They also express Edar, the receptor of ectodysplasin (Eda). The early signaling center in the placode regulates epithelial budding, while the primary enamel knot regulates the shift from bud to cap stage as well as the advancement of morphogenesis to bell stage. The secondary enamel knots regulate patterning of the tooth cusps in molar teeth during bell stage. The reciprocal signals expressed in mesenchyme include FGFs, BMPs, and Activin. Arrows indicate signaling across the two tissues and within the epithelium.

Jussila & Thesleff, 2012; Keranen, Kettunen, Aberg, Thesleff, & Jernvall, 1999). These signals regulate the proliferation and downgrowth of the placodal epithelium which gives rise to the tooth bud (Fig. 3).

The next signaling center, known as the (primary) *enamel knot,* forms at the tip of the bud and is characterized by the exit of cells from cell cycle (expression of *p21*) as well as localized expression of signaling molecules and signaling pathway-linked genes. The signals stimulate the growth of the flanking epithelium which gives rise to the cervical loops (CLs), and tooth morphogenesis advances from bud to cap stage. The enamel knot matures and becomes a morphologically discernible feature that is a source of many signals from the different families (reviewed by Jussila & Thesleff, 2012).

During the following bell stage, the epithelial growth and folding determine the shape and size of the tooth crown. In the molars, *secondary enamel knots* are induced in the epithelium and they act as signaling centers, expressing largely the same signals as the primary enamel knot (Fig. 3). It is the secondary enamel knots that determine the characteristic and reproducible positions and heights of tooth cusps. All five conserved signaling pathways are important for cusp patterning, as shown by the aberrant cusp patterns in mouse mutants in which signaling has been modulated (reviewed by Jernvall & Thesleff, 2012, and see below).

2.2 The Shift of Odontogenic Potential from Epithelium to Mesenchyme: Signals and Transcription Factors

The capacity to form teeth was shown to reside in the oral epithelium of E10 and E11 mouse embryos (dental lamina stage), and to shift to the underlying neural crest-derived mesenchyme by E12 (placode stage) (Fig. 2; Mina & Kollar, 1987). Signals emanating from the dental lamina are involved in transmission of the odontogenic potential to the mesenchyme and are preserved through subsequent stages of tooth development in the epithelial signaling centers, possibly contributing to the maintenance of the odontogenic potential in the mesenchyme. However, there is so far no evidence of specific signal(s) that would be sufficient to transmit the odontogenic potential. This is because its molecular basis is not known yet.

The shift of odontogenic potential coincides with the condensation of the dental mesenchymal cells, and it is therefore likely that the same epithelial signals are involved in both processes. Interestingly, epithelial *Fgf8* and *Sema3f* were shown to attract and repulse, respectively, the dental mesenchymal cells, and the mechanical stimuli associated with their compaction

were sufficient to induce the expression of some dental mesenchyme-specific genes, namely, *Msx1*, *Pax9*, and *Bmp4* (Mammoto et al., 2011).

Cellular identity is determined by the unique combination of transcription factors expressed by a cell, and many different cell types can be reprogrammed by specific sets of transcription factors (Iwafuchi-Doi & Zaret, 2014). Transcription factors induced in the mesenchyme between the dental lamina and placode stages include *Msx1*, *Msx2*, *Pax9*, *Lhx6*, *Lhx7*, *Dlx1*, *Dlx2*, and *Runx2*, and it is likely that at least some of these are responsible for the acquisition of odontogenic potential in the mesenchyme. Notably, all of the listed genes are necessary for early tooth morphogenesis, as demonstrated in single and compound knockout mice (reviewed by Bei, 2009). Tooth development is arrested at the dental lamina stage in some compound mutants, including *Msx1;Msx2, Dlx1;Dlx2*, and *Lhx6; Lhx7* (only molars arrested), while arrest at the bud stage occurs when only *Pax9*, *Msx1,* or *Runx2* is deleted. In addition, arrested development at the lamina stage in *Gli2;Gli3* compound mutants and at the bud stage in *Lef1* knockouts indicates the necessary functions of the Shh and Wnt/β-catenin pathways. It remains to be demonstrated which transcription factors can program the acquisition of dental mesenchyme identity.

The gene regulatory networks in the mesenchyme have been elucidated particularly in association with FGF and BMP signaling. Many of the transcription factors mentioned above are regulated by epithelial BMPs and FGFs during the early steps of tooth formation. *Bmp4* was the first epithelial signal associated with the shift of odontogenic potential to the mesenchyme, while epithelial *Fgf8* and *Fgf9* were shown to be necessary for tooth initiation (Trumpp, Depew, Rubenstein, Bishop, & Martin, 1999; Vainio, Karavanova, Jowett, & Thesleff, 1993). Availability of these molecules as recombinant proteins enabled the elucidation of their function in organ culture through use of beads soaked in the proteins. Collectively, these studies have unraveled signaling cascades demonstrating that epithelial *Bmp4* regulated the expression of transcription factors *Msx1* and *Msx2* as well as *Bmp4* in the dental mesenchyme, while FGF signaling exclusively regulated *Dlx1* and *Pax9* (Vainio et al., 1993). Further studies identified common targets for epithelial BMP and FGF signaling, including *Msx1*, *Dlx2*, and *Runx2* in the dental mesenchyme (Aberg et al., 2004; Bei & Maas, 1998; Neubuser, Peters, Balling, & Martin, 1997; Thomas, Liu, Rubenstein, & Sharpe, 2000; Vainio et al., 1993). Upregulation of *Msx1* by BMP and FGF signaling represents two independent signaling cascades that result in activation of mesenchymal *Bmp4* and *Fgf3*, respectively (Bei & Maas, 1998). Therefore,

the same mesenchymal transcription factors can be used to regulate different downstream targets indicating complex signaling networks formed during tooth development. Zhao, Gupta, Raj, Roussel, and Bei (2013) unraveled a complex network of transcription factors belonging to different signaling pathways (*Msx1*, *Lef1*, *Snail*, *Lhx6/8*, and *Sp3*) that regulates cell cycle. This *Msx1*-dependent signaling network was further expanded by study showing antagonistic interactions between Msx1 and Tbx2 that regulate *Bmp4* expression (Saadi et al., 2013).

2.3 Signaling Pathways and Networks in the Mediation of Epithelial–Mesenchymal Interactions During Morphogenesis

The epithelial signaling centers together with the specified dental mesenchyme are the key players in the tissue interactions which regulate tooth morphogenesis from the placode to bell stage (Fig. 3). Diagrams of proposed regulatory networks underlying epithelial–mesenchymal cross talk can be found in many publications (Aberg et al., 2004; Bei, Kratochwil, & Maas, 2000; Bei & Maas, 1998; Klein et al., 2006; Lan et al., 2014). The battery of signaling molecules expressed by the *early signaling center in the placode* and subsequently by the *primary and secondary enamel knots* regulates epithelial budding, transition to cap stage, and crown morphogenesis during bell stage. In addition to the five signaling pathways that are the focus of this review, signals in other families have significant roles in mediation of tissue interactions in the tooth, such as Activin, a TGFβ signal essential in the mesenchyme prior to bud formation (Ferguson et al., 1998), and its feedback inhibitor Follistatin, which is required in enamel knots for normal crown and cusp formation (Wang et al., 2004).

There is evidence indicating that Wnt/β-catenin may be the most upstream signal initiating the formation of signaling centers. Wnt/β-catenin signaling has been shown to be upstream of *Fgf4* and *Fgf20* as well as *Lef1* in the enamel knot (Haara et al., 2012; Kratochwil, Galceran, Tontsch, Roth, & Grosschedl, 2002). Inhibition of Wnt signaling by overexpression of the Wnt inhibitor *Dkk4* arrests tooth development at the dental lamina stage and no placodes form (Liu et al., 2008). The strongest evidence for the importance of Wnt signaling was provided by studies in which forced expression of β-catenin in the oral epithelium driven by the K14 promoter-induced continuous formation of ectopic signaling centers which gave rise to teeth (Jarvinen et al., 2006; Liu et al., 2008; Wang et al., 2009).

The ectopic signaling centers were characterized by intense focal β-catenin activity and the expression of various placode-specific molecular markers.

The morphogenesis from bud to cap stage and the formation and function of the primary enamel knot are perhaps the most actively studied topics in tooth morphogenesis research and these transitions are fully dependent on Wnt, BMP, FGF, Shh, and Eda signals. Mesenchymal *BMP4* regulates the bud-to-cap stage transition and initiates enamel knot formation through induction of cyclin-dependent kinase inhibitor *p21* in the bud stage epithelium, which probably signals the exit from the cell cycle in the forming enamel knot (Jernvall, Aberg, Kettunen, Keranen, & Thesleff, 1998). Furthermore, the arrested tooth development at bud stage in *Msx1* knockout mice was accompanied by the absence of *Bmp4* in the mesenchyme, and addition of exogenous BMP4 protein rescued the development of these teeth to cap and bell stage (Bei et al., 2000). However, conditional inactivation of *Bmp4* in dental mesenchyme in $Bmp4^{(f/f)};Wnt1Cre$ mice resulted in the arrest of the mandibular molar development at bud stage, while maxillary molars and incisors developed into mineralized teeth (Jia et al., 2013). The higher sensitivity of mandibular molars to inhibition of BMP signaling was explained by higher expression of the Wnt inhibitor *Dkk2* and the BMP inhibitor *Osr2* in the mandibular mesenchyme. This is an excellent example of the delicate fine-tuning of the signaling pathways during morphogenesis.

Of the five signaling families, only BMPs and FGFs transmit bidirectional signaling across the epithelial and mesenchymal tissues (Fig. 3). In the large *FGF signaling family,* the expression of individual signals is typically restricted to either epithelium or mesenchyme and they commonly mediate bidirectional signaling between the tissues. *Fgf3* and *Fgf10* are expressed in dental mesenchyme, while *Fgf4*, *Fgf9*, and *Fgf20* are expressed in the placodes and enamel knots during tooth morphogenesis. The analysis of the tooth phenotypes of *Fgf9;Fgf20* compound knockout mice indicated compensatory functions in the enamel knot, while *Fgf4* mutants had no tooth phenotype (Haara et al., 2012). The epithelial FGFs induce the expression of mesenchymal FGFs. For example, FGF4 in the enamel knot induces the expression of *Fgf3* in the dental mesenchyme. This induction is mediated by *Runx2*, a transcription factor required in the mesenchyme for bud-to-cap stage transition (Aberg et al., 2004).

A typical feature of FGF signaling is its feedback inhibition by Sprouty (Spry) molecules. Different *Sprouty* genes are expressed in the epithelium

(*Spry2*) and mesenchyme (*Spry4*), and molecular and genetic evidence from mouse models have indicated their importance in limiting FGF signaling in the enamel knot (Klein et al., 2006). Loss of *Spry2* function results in supernumerary tooth formation in the diastema region (in front of M1) due to the persistent enamel knot caused by hypersensitivity to FGF signaling.

Shh is the only member of the hedgehog signal family in teeth and its expression is restricted to dental epithelium. After being expressed in the dental lamina, *Shh* is restricted to the placodes and enamel knots, and during the bell stage, its expression spreads from the enamel knots to the differentiating epithelium. The expression patterns of the hedgehog targets, *Gli1* and *Ptc1*, during tooth morphogenesis indicate high Shh signaling activity throughout the dental mesenchyme and epithelium, excluding the enamel knots (Bitgood & McMahon, 1995). Conditional deletion of *Shh* from the bud stage onward using K14-Cre led to disrupted tooth morphogenesis (Dassule, Lewis, Bei, Maas, & McMahon, 2000). The mutant teeth were small and the growth of CLs and cusps was hampered. However, the enamel knots did form and they expressed all of the analyzed marker genes. Inhibition of epithelial Shh signaling through conditional ablation of *Smoothened* (*Smo*), a transducer of Shh signaling, using the same K14-Cre line resulted in a milder tooth morphogenesis phenotype (Gritli-Linde et al., 2002), indicating the importance of Shh signaling in the mesenchyme. In addition, the first and second molars were fused, and epithelial cell proliferation and ameloblast polarization were disturbed in the conditional *Smo* mutants. Taken together, the results indicated that Shh mediates both epithelial–mesenchymal and epithelial–epithelial tissue interactions (Gritli-Linde et al., 2002).

Restricted epithelial expression of *Wnt ligands* during tooth morphogenesis, with the exception of the noncanonical *Wnt5a* (Sarkar & Sharpe, 1999), indicates that canonical Wnts do not mediate signaling from mesenchyme to epithelium. Furthermore, Wnt/β-catenin reporter analyses have demonstrated localized activity predominantly in dental epithelium, and thus the majority of Wnt signaling is thought to be intraepithelial (Fujimori et al., 2010; Liu et al., 2008; Suomalainen & Thesleff, 2010). However, there is significant β-catenin activity in the dental mesenchyme during early tooth morphogenesis as well as during cell differentiation. The conditional deletion of β-catenin using *Osr2-Cre* leads to arrested tooth development at the bud stage, indicating epithelial–mesenchymal Wnt signaling (Chen, Lan,

Baek, Gao, & Jiang, 2009). In addition, conditional deletion of β-catenin in the incisor mesenchyme using *Prx1-Cre* resulted in a split incisor placode and duplication of the tooth (Fujimori et al., 2010). This phenotype was attributed to the downregulated *Bmp4* expression. A similar phenotype was observed after *ex vivo* addition of BMP inhibitor Noggin (Fujimori et al., 2010; Munne et al., 2010). These studies indicate the requirement for BMP signaling for placode integrity and exemplify the interactions between the Wnt and BMP pathways. A series of Wnt–BMP feedback circuits operating between the tooth epithelium and mesenchyme during tooth morphogenesis was uncovered through massive gene expression profiling of isolated dental epithelia and mesenchyme (O'Connell et al., 2012). It is obvious that these circuits are affected or interrupted in many mouse models in which either BMP or Wnt signaling has been targeted.

Studies using *Sosdc1* (also known as *Wise*, *Ectodin*, and *USAG1*)-null mutants and bead experiments have unraveled interactions of Wnt, BMP, FGF, and Shh pathways. *Sostdc1* is a Wnt inhibitor which in some contexts also inhibits BMP signaling (Lintern, Guidato, Rowe, Saldanha, & Itasaki, 2009). *Sostdc1* is regulated by BMP4 and is intensely expressed in teeth, except the enamel knot and its surrounding tissue (Laurikkala, Kassai, Pakkasjarvi, Thesleff, & Itoh, 2003). Supernumerary teeth as well as the fusion of molars observed in mice lacking *Sostdc1* were directly linked to its inhibitory effect on Wnt signaling (Ahn, Sanderson, Klein, & Krumlauf, 2010). Furthermore, this study demonstrated that FGF and Shh signaling are major downstream targets of *Sostdc1*.

Eda is a tumor necrosis family signaling molecule which specifically regulates the development of ectodermal appendages, including teeth. Eda was originally discovered as the causative gene for human X-linked hypohidrotic ectodermal dysplasia, a syndrome which is characterized by missing teeth, sparse hair, and deficient formation of nails and several exocrine glands (reviewed by Mikkola, 2009). The *Eda* expression domain in the early oral epithelium and dental epithelium is regulated by intraepithelial Wnt signaling, while mesenchymal Activin regulates the expression domain of its receptor, *Edar*, which is restricted to the dental lamina, placodes, and enamel knots. It has been demonstrated that Eda regulates all dental signaling centers in the dental epithelium and mediates interactions between epithelial compartments (Haara et al., 2012; Laurikkala et al., 2001). In addition, Eda signaling is integrated with all of the other four pathways as well as epithelial–mesenchymal interactions (reviewed by Mikkola, 2009). Eda

deficiency in mice decreases the size and the number of teeth, while conditional *Eda* overexpression in dental epithelium (by the K14 promoter) increases size of tooth placodes and leads to larger and supernumerary teeth (Mustonen et al., 2003; Pispa et al., 1999). Proposed Eda targets in the placodes and enamel knots include *Fgf20*, *Wnt10a*, and *Shh* as well as the signal inhibitors *Dkk4* and *Follistatin* (Haara et al., 2012; Mikkola, 2009). Notably, many of the Eda targets are shared with Wnt signaling.

The *patterning of the secondary enamel knots in* molars illustrates the significance of the delicate fine-tuning of signaling pathways and their interactions in tooth morphogenesis. This patterning process determines the locations and heights of tooth cusps and is directed by tissue interactions mediated by signals in all conserved signal families as well as their inhibitors. The process is regulated by a Turing-type reaction–diffusion mechanism which has been implicated in patterning of the placodes of ectodermal organs and involves diffusible signaling molecules, their inhibitors, and lateral inhibition (Biggs & Mikkola, 2014; Jiang et al., 2004; Salazar-Ciudad & Jernvall, 2010). The enamel knots locally produce many signaling molecules and inhibitors and are also the targets of signals. Tinkering with signaling pathways in mutant mice such as *K14-Eda, K14-Follistatin*, *Sostdc1*$^{-/-}$, and *K14Cre-Smo*$^{-/-}$ resulted in abnormal cusp patterns (Gritli-Linde et al., 2002; Laurikkala et al., 2003; Mustonen et al., 2003; Wang et al., 2004). In addition, exposing embryonic molars to different signals and their combinations in *ex vivo* cultures can have dramatic effects in cusp numbers and patterns, and interestingly, the different patterns can be generated by computer models (Harjunmaa et al., 2012; Jernvall & Thesleff, 2012; Salazar-Ciudad & Jernvall, 2010). Fine-tuning of signaling in and around the enamel knots is considered to be the most important mechanism regulating the patterning of the tooth crowns and generating the diverse cusp patterns during evolution.

Deletion of the *Foxi3* transcription factor in the dental epithelium leads to a dramatic crown phenotype featured by supernumerary, shallow, and incorrectly patterned cusps (Jussila et al., 2015). Gene expression profiling by microarray analysis placed *Foxi3* upstream of Shh, BMP, FGF, and Wnt signaling, and *ex vivo* studies demonstrated that *Foxi3* expression is regulated by BMP4, Activin, and Eda. Thus, Foxi3 links several signaling pathways during cusp patterning. Gene expression patterns in the *Foxi3* mutant teeth indicated that the intercuspal epithelium had adopted enamel knot fate, suggesting that Foxi3 functions as an inhibitor of enamel knot formation (Jussila et al., 2015).

3. EPITHELIAL–MESENCHYMAL INTERACTIONS REGULATING DENTIN AND ENAMEL FORMATION

Dentin and enamel are mineralized tissues produced at the late bell stage of tooth development by tooth-specific cells: the mesenchyme-derived odontoblasts that produce dentin and the epithelial ameloblasts that produce enamel. Formation of these tissues occurs at the interface between the epithelium and mesenchyme and is regulated by epithelial–mesenchymal interactions and the same signals as tooth morphogenesis.

At the bell stage, the enamel organ consists of two basal cell layers: (i) the inner enamel epithelium or IEE, which encloses the dental papilla and gives rise to odontoblasts and pulp cells and (ii) the outer enamel epithelium or OEE, which faces the dental follicle and later generates the cementoblasts and periodontal ligament. Together the IEE and OEE surround the loosely arranged stellate reticulum (SR) cells and a thin layer of stratum intermedium (SI) cells, which are in direct contact with the IEE (Fig. 4A).

Differentiation of the IEE cells into ameloblasts and the directly underlying dental papilla cells into odontoblasts occurs in stages that include (i) the initial or presecretory stage, (ii) secretory stage, and (iii) maturation stage. Differentiation starts from the tips of the future cusps and is marked by polarization of preodontoblasts and preameloblasts as well as matrix secretion (Fig. 4B). Secretion of collagen type I-based predentin at the epithelial–mesenchymal interface causes preodontoblasts to retreat, leaving behind a cell extension called the odontoblast process from which the noncollagenous proteins (including the most abundant, Dentin Sialophosphoprotein or DSPP) that initiate dentin mineralization are secreted. Odontoblast processes remain embedded in dentin and thus the tubular morphology of the dentin arises.

Dental epithelium and mesenchyme are separated by a basement membrane that has been suggested to support polarization of preodontoblasts, which align themselves perpendicular to it (reviewed by Hurmerinta & Thesleff, 1981; Fig. 4B). Degradation of the basement membrane subsequent to the onset of predentin formation enables direct contacts between odontoblasts and preameloblasts suggested to mediate the induction of ameloblast differentiation (Slavkin & Bringas, 1976). This was recently supported by mice lacking *EMMPRIN* (CD147), a membrane glycoprotein expressed exclusively in the preameloblasts and SI cells. The persistence of the basement membrane in *EMMPRIN*$^{-/-}$ mice leads to delayed ameloblast

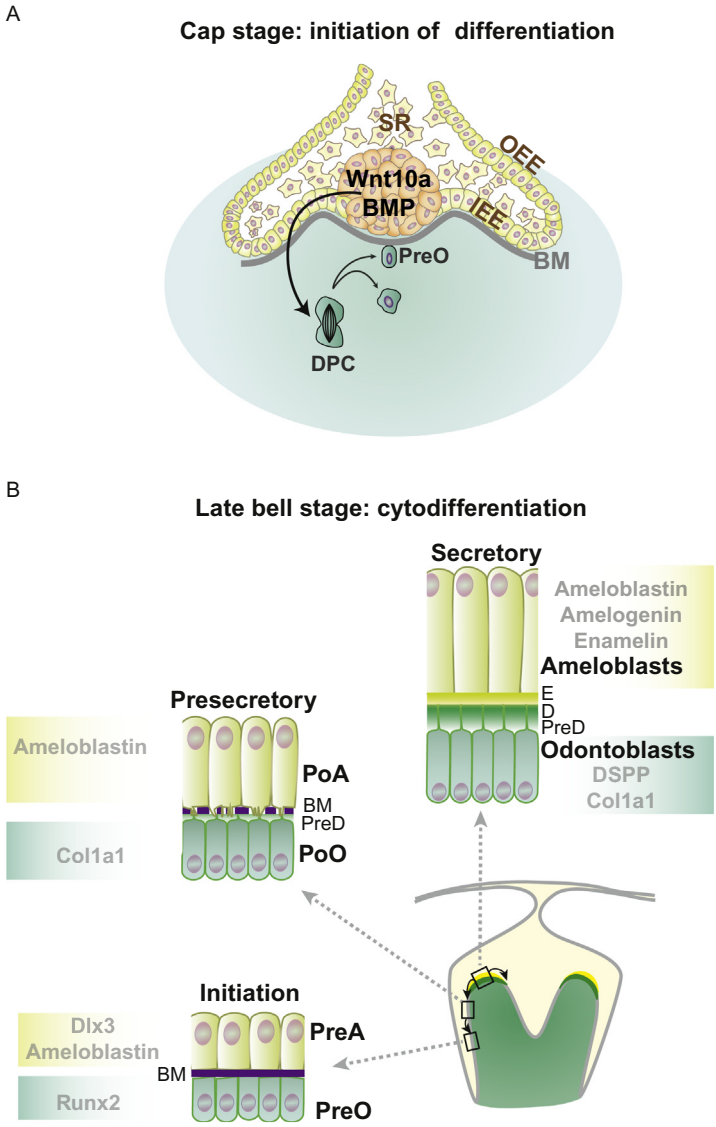

Figure 4 Epithelial–mesenchymal tissue interactions regulating differentiation of odontoblasts and ameloblasts. (A) The enamel organ during cap stage consists of inner and outer enamel epithelium (IEE and OEE, respectively) enclosing the stellate reticulum (SR) compartment. The fully developed primary enamel knot (pink cells in spherical organization) expresses Wnt10A that signals to dental papilla cells in the underlying mesenchyme (green color), which then commence odontoblast differentiation. (B) Ameloblasts and odontoblasts are generated during the bell stage of tooth development. Indicated genes represent the molecular markers that can be used to follow the progression of ameloblast (yellow boxes) and odontoblast (green boxes) lineages. *Abbreviations*: BM, basement membrane; D, dentin; E, enamel; PoA, polarized ameloblasts; PoO, polarized odontoblasts; PreA, preameloblasts; PreD, predentin; PreO, preodontoblasts; DPC, dental papilla cell.

differentiation and enamel production, and is caused by the loss of MMP-20, a matrix degrading metalloprotease that plays a role in degradation of basement membrane (Khaddam et al., 2014).

3.1 Initiation and Progression of Odontoblast Differentiation

Initiation of odontoblast differentiation starts at the tips of the future cusps where enamel knots form. The timing and the location of enamel knot formation suggest that enamel knots are the source of the first inductive signals for odontoblast differentiation (Fig. 4A; Thesleff, Keranen, & Jernvall, 2001; Yamashiro et al., 2007). *Wnt10a* is expressed in E14 mouse incisors and molars in the primary enamel knot and subsequently in the secondary enamel knots. From E14 onward the expression of *Wnt10a* shifts to the mesenchyme and becomes intense in the preodontoblasts underlying the enamel knot, which differentiate into DSPP-expressing odontoblasts (Yamashiro et al., 2007). Studies on cultured cells and tooth germs have shown that Wnt10a inhibits proliferation of dental papilla cells and initiates their differentiation by upregulating *Dspp* expression (Liu, Han, Wang, & Feng, 2013; Yamashiro et al., 2007).

BMP/TGFβ signaling has been most frequently implicated in the induction of odontoblast differentiation. *In vitro* studies and dentin regeneration experiments showed long before the development of the genetic mouse models that signals of this gene family are capable of inducing terminal differentiation of odontoblasts (Lesot et al., 2001). Mouse models lacking BMP/TGFβ signaling at earlier stages of tooth development (*Tgfβr2*$^{fl/fl}$*; Wnt1-Cre*, *Bmp4*$^{fl/fl}$*;3.6Col1a1-Cre*, and *Bmp2*$^{fl/fl}$*;Osx-Cre* null mice) display defects in dentinogenesis, namely, delayed odontoblast differentiation and reduced dentin production (Feng et al., 2011; Kim et al., 2010, 2011). Conditional ablation of BMP/TGFβ signaling at later stages of odontoblast differentiation (*DsppCre;Tgf-βr2*$^{fl/fl}$ *mice*) does not cause any noticeable dentin abnormality (Arany et al., 2014). Similarly, lack of *Smad4*, mediator of both TGFβ and BMP signaling, at an early stage of tooth development (*Smad4*$^{fl/fl}$*;Osr2-Cre* null mice) results in the generation of nonpolarized odontoblasts which deposit atubular, bone-like tissue (Bae et al., 2013). A similar phenotype was seen in transgenic mice overexpressing *Runx2* in the odontoblasts (Han et al., 2014). Lack of *Dspp* expression and production of bone matrix in teeth of both models indicate acquisition of osteoblast phenotype, suggesting that Smad4 and Runx2 are necessary for the maintenance of the odontoblast cell fate. Deletion of *Smad4* from the

mesenchyme at later stages of tooth development results in a milder phenotype, which includes various degrees of altered odontoblast polarization and thin dentin (Lian et al., 2006). Together, these studies clearly indicate that BMP/TGFβ signaling regulates odontoblast differentiation in a stage- and site-specific manner.

Changes in the morphology of *Smad4* mutant teeth were attributed to increases in Wnt signaling and indicated an inhibitory effect of BMP/TGFβ signaling on Wnt pathway activity (Bae et al., 2013). However, the published data are contradictory and it is not yet determined whether BMP and Wnt signaling interactions are synergistic or antagonistic. This can perhaps be explained by the reciprocal Wnt–BMP feedback circuits involved in the epithelial–mesenchymal interactions during tooth morphogenesis (O'Connell et al., 2012).

Furthermore, there is evidence that Wnt signaling is important for terminal odontoblast differentiation. High Wnt/β-catenin reporter activity is present in odontoblasts during differentiation (Suomalainen & Thesleff, 2010). Excessive Wnt/β-catenin activity due to constitutive stabilization of β-catenin in early odontoblasts of *OC-Cre:Catnb*$^{(+/lox(ex3))}$ mutant mice induces premature differentiation of odontoblasts that deposit high amounts of insufficiently mineralized dentin, closing the pulp chamber and expressing low levels of *DSPP* (Bae et al., 2013; Kim et al., 2011). *Smad4* is significantly upregulated in these prematurely differentiating odontoblasts, suggesting that Smad4 tunes the level of Wnt activity necessary for successful odontoblast differentiation (Bae et al., 2013). Conversely, decreased Wnt signaling activity in early odontoblasts of *OC-Cre;Wls*$^{(CO/CO)}$ mutant mice caused reduction of dentin deposition resulting in a thinner dentin wall and wider pulp chamber (Bae et al., 2015). The similarities in the dentin phenotype of the *OC-Cre;Wls*$^{(CO/CO)}$ mutant mice and mice lacking BMP/TGFβ signaling indicates the synergistic activity of the two pathways. In addition, some mouse mutants, such as mice lacking *Epiprofin* (*Epfn*) *6*, demonstrate that the defects in the terminal stages of odontoblast and ameloblast differentiation are directly related to the decreases in both β-catenin and BMP signaling (Jimenez-Rojo et al., 2010).

3.2 Ameloblast Differentiation

The majority of mouse mutants with abnormal odontoblast differentiation and dentin production described above did not display significant changes in ameloblast differentiation and enamel formation. Terminal ameloblast

differentiation is triggered by paracrine signals emanating from functional odontoblasts secreting predentin matrix. Odontoblast-derived BMPs are required for ameloblast differentiation, in particular, BMP4 that induces expression of ameloblast markers *p21* and *ameloblastin* (Wang et al., 2004). These BMP signals were shown to be fine-tuned by Follistatin, which acts as a BMP antagonist in the IEE, blocking ameloblast differentiation. The patterns of *Follistatin* expression during mouse incisor and molar development are closely related to inhibition of ameloblast differentiation and absence of enamel. *Follistatin* is expressed in the IEE at the tips of the mouse molar cusps and on the lingual aspect of the mouse incisors, which are both enamel-free areas (Wang et al., 2004).

Conditional deletion of either *Bmp4* or *Bmp2* significantly reduced enamel thickness, and changes in amelogenesis were similar to those caused by *amelogenin* mutations in mice and humans (Feng et al., 2011). The abnormal amelogenesis in these mutants was linked to decreased levels of *Dlx3* in the preameloblasts and a subsequent decrease in amelogenin production. In addition, *in vitro* studies on epithelial cell lines demonstrated that *Dlx3* positively regulates enamel matrix protein genes including *enamelin* and *amelogenin* (Zhang et al., 2015).

Enamel defects in mice carrying compound mutations of cell–cell adhesion molecules *Nectin-1* and *-3*, as well as mice lacking the cell membrane protein PERP, indicate the importance of the integrity of the ameloblast cell layer and its tight contacts with the SI cell layer (Jheon et al., 2011; Neupane et al., 2014; Yoshida, Miyoshi, Takai, & Thesleff, 2010). The SI consists of a few layers of epithelial cells adjacent to the ameloblasts. Ameloblasts and SI cells are tightly bound by desmosomes, recruited by interaction between Nectin-1 expressed in ameloblasts and Nectin-3 in SI cells (Yoshida et al., 2010).

The SI has traditionally been regarded as an important cell layer supporting ameloblast functions. The SI cells are characterized by high levels of expression of alkaline phosphatase as well as *Shh* (Koyama et al., 2001; Wise & Fan, 1989). Shh plays an important role in ameloblast polarization and secretion. Conditional deletion of either *Shh* or its downstream mediator *Smoothened* (*Smo*) in dental epithelium led to disrupted polarity and organization of the ameloblast layer and production of disorganized enamel matrix (Dassule et al., 2000; Gritli-Linde et al., 2002). The findings indicate that Shh function is required in dental epithelium and that Shh mediates the interaction between SI and (pre)ameloblasts during ameloblast polarization and secretion.

4. EPITHELIAL–MESENCHYMAL INTERACTIONS REGULATING STEM CELLS IN CONTINUOUSLY GROWING MOUSE INCISORS

4.1 Development of Cervical Loops

During the cap and bell stages of tooth development, the enamel organ extends in the apical direction to envelop the underlying dental mesenchyme. The leading edge of the extending epithelium consists of the CL where the IEE and OEE enclose loosely arranged SR cells. In teeth that form roots (e.g., all human teeth and mouse molars), the SR cells are depleted when crown development is completed and *root formation* starts. The remaining bilayered basal epithelium (IEE and OEE) is called *H*ertwig's *e*pithelial *r*oot *s*heath (HERS) (Fig. 5A; reviewed by Thesleff & Tummers, 2008). Downregulation of *Fgf10* in the mesenchyme surrounding the CL is necessary for the initiation of root formation, as shown by the addition of FGF10 to molar germ cultures prior to HERS formation. This preserved SR cells and prolonged extension of the CL in the apical direction (Yokohama-Tamaki et al., 2006). A similar phenotype was recently reported when *Smad4* was ablated in dental epithelium. The maintenance of the SR cells in the CL was attributed to a lack of the inhibitory function of mesenchymal BMP on epithelial *Shh* expression (Li et al., 2015, and see below). In continuously growing teeth-like rodent incisors, the SR cell compartment is maintained in the CLs by stem cells (Fig. 5A).

4.2 Epithelial Stem Cells

At the proximal end of the rodent incisor, two CLs flank the putative niche of mesenchymal stem cells. The bigger labial cervical loop (LaCL) contains a small population of slowly dividing label-retaining cells (LRCs) (Smith & Warshawsky, 1975). Harada et al. (1999) demonstrated that isolated LaCLs can generate new dental tissues when cultured *in vitro*, providing evidence for the presence of stem cells. The other CL, located on the lingual side (LiCL), is smaller with fewer stem cells, and the stem cells do not give rise to ameloblasts or enamel, hence the asymmetrical enamel deposition in the incisor (Fig. 5A; reviewed by Jussila & Thesleff, 2012; Kuang-Hsien Hu, Mushegyan, & Klein, 2014).

The growth of the rodent incisor is fine-tuned by a balance between the continuous enamel production (from stem cells) and tooth wear. Thus, enamel morphology and phenotype are used as a read-out to detect changes

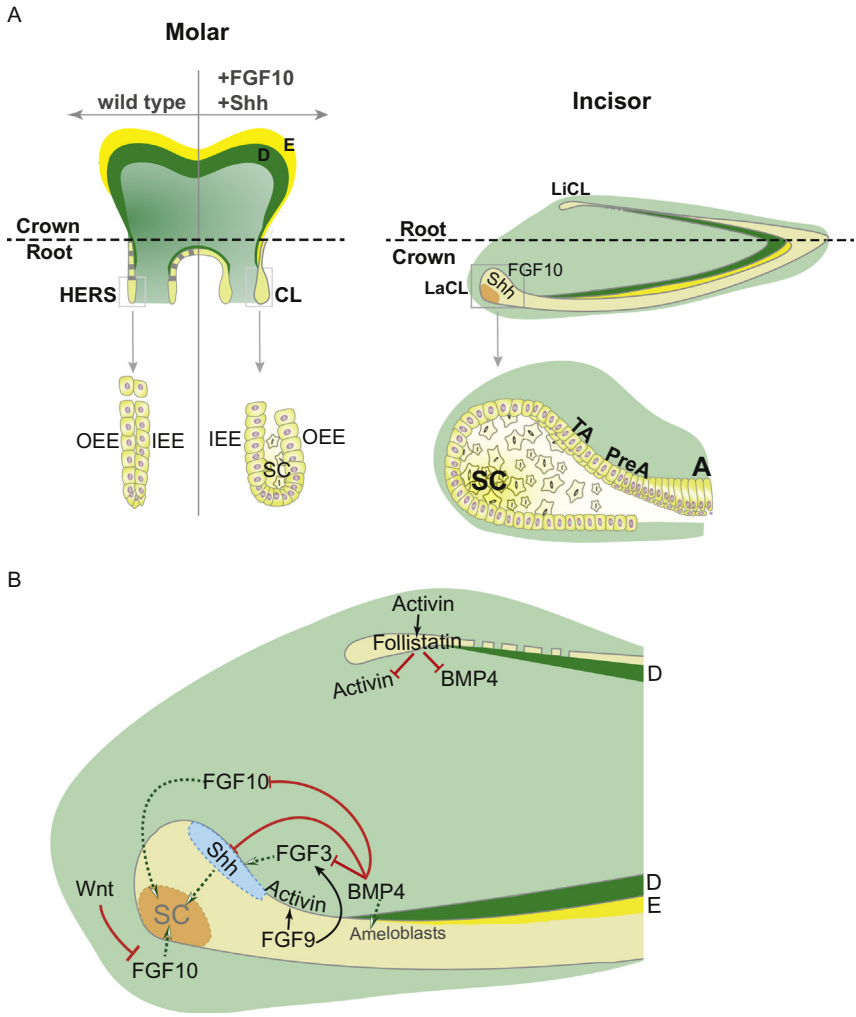

Figure 5 Cervical loops in molar root formation and continuous growth of the incisor, and the gene regulatory network that controls maintenance of epithelial stem cells. (A) In the wild-type molar tooth, disappearance of stellate reticulum (SR) cells in the cervical loop (CL) leads to formation of Hertwig's epithelial root sheath (HERS) and root formation, and is caused by downregulation of Fgf10 and Shh (illustrated in left-side root). Persistence of *Fgf10* and *Shh* expression support maintenance of the SR compartment that houses stem cells in the CL of the molar root (illustrated in right-side root) and in the LaCL of the continuously growing incisor (orange area) (Li et al., 2015; Yokohama-Tamaki et al., 2006). (B) Schematic view of the signals involved in epithelial–mesenchymal tissue interactions regulating the stem cell niche in the continuously growing incisors. Black arrows indicate positive gene regulation, red arrows indicate negative gene regulation, and green dotted arrows indicate positive effects on stem cells (orange area) and TA proliferation (blue area). *Abbreviations*: A, ameloblasts; IEE, inner enamel epithelium; OEE, outer enamel epithelium; PreA, preameloblasts; TA, transit-amplifying cells; SC, stem cells; LaCL, labial cervical loop; LiCL, lingual cervical loop.

in the stem cell niche viability, regulation, and maintenance. In addition, the labial–lingual asymmetry of the incisor can be modulated by tinkering with signaling pathways, making the incisor a valuable model to study epithelial stem cells and their regulation (reviewed by Jussila & Thesleff, 2012; Kuang-Hsien Hu et al., 2014).

Epithelial stem cells in the LaCL express molecules also found in other stem cells, such as Sox2, Lgr5, ABCG2, Bmi-1, Oct3/4, Yap, Gli1, E-cadherin, and the integrins α6 and β4 (Juuri et al., 2012; Li et al., 2011; Seidel et al., 2010; Suomalainen & Thesleff, 2010). Transgenic mouse models in which GFP expression is driven by promoters of stem cell marker genes (*Sox2-GFP*, *Bmi1-GFP*, *Lgr5-GFP*) have enabled identification and isolation of this population. Other transgenic models have provided means to genetically manipulate the stem cell niche (*Gli1-CreER*, *Ptch1-CreER*, *Bmi1-GFP*) (Biehs et al., 2013; Juuri et al., 2012; Seidel et al., 2010).

4.3 FGF Signaling and Cross Talks with BMP/TGFβ Pathways

Fgf3 and *Fgf10* are expressed intensely in the mesenchyme adjacent to the TA cells in the LaCL. In addition, *Fgf10* expression surrounds the LaCL and is also expressed at very low levels in the mesenchyme surrounding the LiCL. Fgfr1b and Fgfr2b expressing cells located within the LaCL, including the stem cell population and TA cells, respond to FGF3 and FGF10 (Harada et al., 1999, reviewed by Kuang-Hsien Hu et al., 2014).

The function of FGF10 is indispensable for stem cell maintenance in the LaCL. *Fgf10* knockout mice die at birth, but histological analysis of their incisors after development under the kidney capsule indicated that LaCL was thin and the SR cells were completely absent (Harada et al., 2002). Consistent with this data, conditional deletion or downregulation of Fgfr2b receptor led to depletion of stem cells and impaired incisor growth (Lin et al., 2009; Parsa et al., 2010). Mouse models with impaired or overactive FGF signaling have demonstrated the redundant roles of FGF3 and FGF10 signaling in the proliferation of the epithelial stem cell progeny (Klein et al., 2008; Wang et al., 2007). In addition, $Fgf3^{-/-};Fgf10^{+/-}$ mutant mice display a hypoplastic LaCL and reduced enamel formation, in contrast to the lack of phenotype in $Fgf3^{-/-}$ mice (Wang et al., 2007). Deletion of *Tbx1*, an FGF-regulated transcription factor expressed in TA cells, led to loss of enamel formation (Caton et al., 2009).

Enhanced FGF signaling through deletion of the feedback inhibitors *Spry4* and *2* ($Spry4^{-/-};Spry2^{+/-}$ mice) results in ectopic development of

enamel-producing ameloblasts on the lingual, enamel-free side. Formation of enamel on both sides prevents tooth abrasion and the incisors acquire a tusk-like phenotype, indicating the important role of fine-tuning FGF signaling in regulation of incisor asymmetry (Klein et al., 2008; Fig. 5B).

A complex signaling network involving FGF3, BMP4, Activin, and Follistatin regulates the asymmetric proliferation of stem cell progeny (Fig. 5B; Wang et al., 2007). *Bmp4* and *Activin* both overlap with *Fgf3* expression in the mesenchyme under the TA cells and the balance between these signals and their antagonists controls Fgf3 and its effects on epithelial stem cell proliferation. BMP4 negatively regulates epithelial stem cells while it promotes ameloblast differentiation (Wang et al., 2004). In line with this, mice overexpressing BMP4 inhibitor *Noggin* in the epithelium (*K14-Noggin* mice) show overgrown incisors lacking enamel (Plikus et al., 2005). Complete lack of enamel in *K14-Follistatin* mice and symmetrical formation of enamel in *Follistatin*$^{-/-}$ mice can be related to changes in *Fgf3* expression (Wang et al., 2007).

The expression of *Fgf3* and *Fgf10* in dental mesenchyme also depends on TGFβ receptors type I (Alk5) and type II. The deletion of these receptors reduces the expression of *Fgf3* and *Fgf10* and decreases the number of stem cells in the LaCL (Yang et al., 2014; Zhao, Li, Han, Kaartinen, & Chai, 2011).

4.4 Hedgehog Signaling and the Importance of Nerves

In the incisor, *Shh* expression is restricted to the TA cells and maturing ameloblasts (Bitgood & McMahon, 1995; Seidel et al., 2010; Zhao et al., 2014). Gli1+ cells and Ptch1+ cells (i.e., Hh-responsive cells) are located in the incisor stem cell niches in both the epithelium (CLs) and mesenchyme and, as demonstrated by lineage tracing, are stem cells (Seidel et al., 2010; Zhao et al., 2014). Pharmacological inhibition of Shh function *in vivo* by a Smo inhibitor demonstrated that Hh signaling is required for the maintenance of the epithelial stem cell niche and the continuous generation of ameloblasts in the adult mice (Seidel et al., 2010; Zhao et al., 2014). Most recent data demonstrated that Shh/Gli1 signaling regulates the fate of the Sox2+ epithelial stem cells in the LaCL through cross talk with the BMP/Smad4 pathway (Li et al., 2015). Loss of the inhibitory effect of mesenchymal BMP on epithelial Shh resulted in the persistence of the dental epithelial stem cell population identified by Sox2-GFP (Fig. 5B).

Hedgehog signaling is also necessary for production of differentiated odontoblasts. Interestingly, severing the inferior alveolar nerve affects dentinogenesis in a pattern similar to Shh inhibition, demonstrating that nerves provide an additional source of Shh (Zhao et al., 2014). Loss of innervation and, consequently, Shh signal initially affected only mesenchyme, but over time, it also affected the epithelium, resulting in a reduced number of LRCs and decreased cell proliferation in the CLs. Reduced *Gli1* expression in both the epithelium and the mesenchyme confirmed that the most affected populations were the dental stem cells (Zhao et al., 2014). Therefore, the proper growth and patterning of the dental sensory nerve is a requirement for the establishment of the mesenchymal stem cell niche that provides *Fgf10* expressing cells and, consequently, the signal that maintains the epithelial stem cells.

The role of the nerve bundle in the biology of the incisor tooth is dual: not only does it provide a source of Shh that regulates the stem cells in the mesenchyme, it also provides a source of mesenchymal stem cells. Lineage tracing of mesenchymal cells during development, renewal, and repair of the incisor indicated that they are in part derived from peripheral nerve-associated glial cells that differentiate into pulp cells and odontoblasts (Kaukua et al., 2014).

4.5 Wnt Signaling

Wnt signaling has been implicated as the key regulator of various epithelial stem cells, for example, in the hair follicle and intestine (Sato & Clevers, 2013; Yang & Peng, 2010). However, the epithelial stem cells in the incisor seem to lack Wnt signaling activity, as evidenced by the absence of Wnt reporters, including TOP gal, BAT gal, and *Axin2* (Juuri et al., 2012; Suomalainen & Thesleff, 2010). However, a small cell population that expresses *Lgr5* exists in the SR of the CLs (Chang et al., 2013; Suomalainen & Thesleff, 2010). In contrast to the CL epithelium, Wnt/β-catenin signaling activity is present in the mesenchyme surrounding the CL, from where it regulates the apoptosis in the CL, in particular through control of the epithelial Lgr5+ stem cells. This mesenchymal–epithelial interaction was shown to be mediated by FGF10, which was downregulated by Wnt/β-catenin signaling (Yang, Balic, Michon, Juuri, & Thesleff, 2015). In addition, this study also pointed to a role of apoptosis in the regulation of stem cell homeostasis in the continuously growing incisors (Yang et al., 2015).

5. CONCLUDING REMARKS

The epithelial–mesenchymal interactions regulate all aspects of tooth development, including initiation, morphogenesis, differentiation of hard tissue producing cells, and the maintenance of stem cells. The communication between the tissues is mediated by conserved signaling molecules which act in complex gene regulatory networks with transcription factors. Detailed understanding of developmental regulation will be crucial for bioengineering of teeth, a goal that has been set for tooth development research during the past decade. So far, the only feasible protocol for tooth bioengineering is based on the classic tissue recombination experiments demonstrating that separated dental epithelium and mesenchyme can form teeth when recombined and cultured as transplants, and that depending on timing the other tissue can be substituted with nondental tissue (Kollar & Baird, 1969; Lumsden, 1988; Mina & Kollar, 1987). These classical tissue recombination methods were refined in the past decade by utilizing dental epithelium and mesenchyme dissociated into single cells prior to recombination. The recombinants formed functional teeth when transplanted to the jaws of adult mice (Nakao et al., 2007; Oshima et al., 2011), indicating that patient-derived cells could be used for the same purpose. However, unavailability of dental progenitors requires reprogramming of patient-derived cells, either induced pluripotent stem cells or adult cells for tooth bioengineering. Therefore, deciphering the "transcription factor codes" of dental epithelial and mesenchymal stem/progenitor cells will be instrumental. Several obvious candidates for transcription factors conferring the identity of dental epithelium and mesenchyme are already known (Fig. 2). However, more work is needed to characterize the progenitor cells in the dental lamina epithelium and early tooth mesenchyme and to examine their programming as well as abilities to form teeth. The continuously growing mouse incisors are also useful models for examining dental stem and progenitor cells.

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