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From Snapshots to Movies: Understanding Early Tooth Development in Four Dimensions

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Abstract

The developing tooth offers a model for the study of ectodermal appendage organogenesis. The signaling networks that regulate tooth development have been intensively investigated, but how cell biological responses to signaling pathways regulate dental morphogenesis remains an open question. The increasing use of *ex vivo* imaging techniques has enabled live tracking of cell behaviors over time in high resolution. While recent studies using these techniques have improved our understanding of tooth morphogenesis, important gaps remain that require additional investigation. In addition, some discrepancies have arisen between recent studies, and resolving these will advance our knowledge of tooth development.

Keywords

tooth development; epithelial dynamics; cell behavior; morphogenesis; molecular signaling

Introduction

Ectodermal appendages, including the hair follicle, mammary gland, nail, and tooth, initiate development through a shared program that involves induction and placode formation (reviewed in Biggs and Mikkola, 2014). The mouse tooth provides an excellent model system to investigate the development of these ectodermally-derived organs, as it is relatively large and can be easily accessed and manipulated. Importantly, the signaling pathways that regulate tooth development are well conserved in different tooth types and between species, as well as during the development of related organs (Fraser et al., 2009; Tummers and Thesleff, 2009).

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In mice, the first morphological sign of tooth development is observed at embryonic day (E) 11.5, with the appearance of localized thickenings of the oral epithelium known as dental placodes (Fig. 1; reviewed in Tucker and Sharpe, 2004). The instructive information for tooth development resides in the dental epithelium at this stage, and the epithelium has the potential to induce odontogenic fate in any neural crest-derived mesenchyme to form tooth (Mina and Kollar, 1987; Lumsden, 1988). By the early bud stage (E12.5), the placode continues to stratify and invaginate to form the dental lamina, and underlying mesenchyme begins to condense. At this stage, the odontogenic potential shifts to the mesenchyme. *Pax9* and *Msx1* expression are hallmarks of the dental mesenchyme in the bud and are required for mesenchymal *Bmp4* expression, which is important for progression through the subsequent odontogenic stages (Chen et al., 1996; Neubüser et al., 1997; Tucker et al., 1998). By E14.5, the tooth bud extends around the dental mesenchyme (also called the dental papilla) to form a cap-shaped epithelium also known as the enamel organ. A group of post-mitotic cells called the primary enamel knot that forms within the dental epithelium regulates tooth crown development at the cap stage. Signaling molecules secreted by the enamel knot, which include members of the Hh, Wnt, Bmp and Fgf families, play a crucial role in shaping the tooth by maintaining the enamel knot and regulating cell proliferation in adjacent cells that is thought to drive the folding of the epithelium into cusps (Jernvall et al., 1994; Jernvall et al., 2000; Harjunmaa et al., 2014). In addition, the enamel knot expresses *p21*, *Msx2* and *Bmp2* to induce growth arrest and subsequently apoptosis of the signaling center (Vaahtokari et al., 1996; Aberg et al., 1997; Jernvall et al., 1998; Thesleff et al., 2001). At E15.5–17.5, the epithelium continues to invaginate, and concurrently, secondary enamel knots form at a distance from the disintegrating primary enamel knot and control the shape of the tooth cusps (Thesleff et al., 2001; Matalova et al., 2005). The shape and size of the tooth is determined throughout these stages, and thus, the early odontogenic stages are critical for determining adult dental morphology. During late odontogenic stages, dental epithelial and mesenchymal cells differentiate into ameloblasts and odontoblasts that lay down enamel and dentin, respectively. Other cell types, such as cementoblasts and cells of the periodontal ligament, differentiate at these stages as well.

While the field has accumulated a wealth of knowledge regarding the molecular regulation of tooth development, how individual cells translate these molecular signals into an integrated morphological output remains unclear. One constraint has been that, until recently, the cellular behaviors that drive tooth morphogenesis have been studied using static histological analyses. While such an approach offers us clues about the cellular basis of morphogenesis, it limits our understanding of tissue behaviors, as we are limited to deducing highly dynamic, three-dimensional cellular activities from sequential, often two-dimensional, snapshot analyses. For instance, rigorous histological analysis of the developing incisor placode led to the proposal that an early signaling center located within the incisor field is a rudimentary tooth that subsequently undergoes apoptosis and disintegrates (Hovorakova et al., 2011). However, a recent study using *ex vivo* imaging suggested that this early signaling center may function as an integral part of the developing incisor (Ahtiainen et al., 2016). In addition, two-dimensional analysis often involves tissue treatments that could introduce artifacts. For example, it was only recently discovered that the molar epithelial progenitor cells initially arrange into a rosette-like structure at the

placode stage of tooth development. This is because the structure is sensitive to formaldehyde treatment and thus becomes disrupted upon fixation (Prochazka et al., 2015). For these reasons, the field has moved towards investigating cellular processes under conditions that better resemble *in vivo* development.

Recently, several groups have proposed different models for how a tooth germ develops using *ex vivo* imaging techniques on live tissue to link genetics and signaling with the physical morphogenesis of the tooth. Importantly, live explants faithfully recapitulate normal development in terms of tissue morphogenesis, and many gene expression and differentiation markers appear as normal. Below, we review these new findings and propose potential future directions.

Molar Signaling Centers

The textbook version of the signals governing molar tooth development is that the process is initiated and organized by a single signaling center, the initiation center or primary enamel knot precursor, in the middle of each placode. However, recently more detailed and dynamic analysis has revealed a more complicated picture. The Fibroblast growth factor (Fgf), Sonic Hedgehog (Shh), Wnt and BMP signaling pathways all play multiple roles. In particular, Fgfs, including *Fgf8* and *9*, are expressed in the dental placode, and *Fgf10* is expressed in the mesenchyme from the placode's earliest appearance. *Fgf8* expression appears even earlier in the entire proximal half of the jaw epithelium (Neubüser et al., 1997; Kettunen and Thesleff, 1998; Prochazka et al., 2015; Kettunen et al., 2000). *Fgf9*- or *Fgf10*-null mice form mineralized teeth with mild phenotypes, suggesting redundancy of FGF ligands (Yokohama-Tamaki et al., 2006; Haara et al., 2012). However, tissue-specific deletion of *Fgf8* arrested tooth development before the early bud stage, indicating that *Fgf8* cannot be compensated for by other Fgf family members during early tooth development (Prochazka et al., 2015).

It has been recently reported that the conventionally understood signaling center for the molar placode, as marked by expression of *Shh*, is at least transiently located distally to the molar placode itself, the latter being defined by cell movements, epithelial thickening and up-regulated *Fgf8* expression; this was detected by genetic labeling of the *Shh*- and *Fgf8*-expressing populations in a mouse embryo using the double driver strain *Shh^{Egfp/Cre};Fgf8^{lacZ}* (Prochazka et al., 2015). Surprisingly, the proximal *Fgf8* center also expresses *Shh* target genes, despite not apparently expressing *Shh* itself. It still remains unclear at what points the *Fgf8*-expressing molar placode and the *Shh*-expressing signaling center are distinct and whether, somehow, these coalesce. Interestingly, expression of *Etv4*, which is considered to be a direct transcriptional target of the Fgf pathway, is restricted to the molar placode, despite the broad expression of *Fgfr2* across the oral epithelium. This suggests selective activation of canonical Fgf signaling in dental epithelium that is under tight temporospatial control. To confirm this, it would be important to analyze the expression of additional Fgf pathway targets, as some of the target genes may differentially respond to signaling inputs (Roehl and Nüsslein-Volhard, 2001). In addition, future experiments involving assessment of temporospatial expression patterns as well as conditional inactivation of Fgf pathway regulators will improve our understanding of the dynamic regulation of Fgf signaling in the molar placode. Several negative feedback regulators of Fgf

signaling are expressed in the developing tooth, and these are prime candidates for control of these processes (reviewed in Thisse and Thisse, 2005). For instance, the Sprouty (Spry) gene family has been shown to regulate Fgf signaling in odontogenesis, and loss of *Spry2* and *Spry4* both result in persistence of Fgf signaling during early tooth development (Klein et al., 2006). *Mkp3* is another negative feedback regulator that is expressed adjacent to the *Fgf8*-expressing domain in the developing limb, at the midbrain/hindbrain boundary, and in the maxillary, mandibular and frontonasal prominence (Klock and Herrmann, 2002). *Mkp3* mRNA is also detected in the oral cavity at E11.5, and thus it will be interesting to test if the *Mkp3* expression pattern complements *Fgf8* expression within the *Fgfr2*-expressing domain. In addition, a large suite of other negative and positive regulators of the Fgf pathway exists that merit further study.

Molar Progenitor Cell Migration

A very early event in tooth development was studied by Prochazka et al., who proposed that directed cell migration of transiently *Fgf8*-expressing cells is involved in formation of the tooth germ (Fig. 2A and B). The authors discovered that the expression domain of *Fgf8* in the mandibular epithelium is predominantly in the proximal region of the mandible at E11.5, the placode stage of tooth development (Prochazka et al., 2015). Genetic lineage tracing together with ablation of this population revealed that *Fgf8*-expressing cells are progenitors that give rise to the entire molar epithelium and thus are required for progression through subsequent odontogenic stages. Building on this knowledge, the authors used *Fgf8*-driven tamoxifen-dependent Cre recombinase to study the cellular dynamics in the molar placode at E11.5. They showed that, at E11.5, the dental epithelial progenitor cells at the proximal region adopt elongated shapes and arrange into a large (perhaps 100–200 μm diameter), rosette-like structure. Such anterior movement would bring these cells towards the above-mentioned *Shh*-expressing center. Analyses of cells labeled using *Fgf8^{ires}-Cre* revealed that the cells within the rosette-like structure underwent more directed and dynamic cell migration, compared to cells that are located posteriorly to the rosette-like structure (Fig. 3A and B). Genetic and pharmacological perturbation of the Fgf and Shh pathways resulted in deregulated migration of these dental epithelial cells. However, the two pathways may control different aspects of cell migration. Upon chemical inhibition of the Fgf pathway, using a relatively low dose of the inhibitor SU5402, cell migration was no longer visible; whether this was due to arrest of migration as such, or to the non-formation of a migratory suprabasal layer of cells, remains an open question, as discussed below. Upon chemical inhibition of the Shh pathway, the placodal cells still moved in the general distal-ward direction of the future tooth germ but failed subsequently to converge towards the center of the future tooth. Interestingly, this low-dose pharmacological inhibition of either Fgf or Shh signaling had no apparent effects on cell proliferation and apoptosis. However, as discussed next, the role of these pathways is still somewhat unresolved.

Prochazka et al. also observed that genetically hyperactivating Shh signaling, using *Fgf8^{CreER};R26R^{SmoM2}*, resulted in distal expansion of the tooth germ into the normally toothless diastema region that separates the incisor from the molars. It has been previously reported that two successive and transient bud-like structures in the diastema may be the rudimentary precursors of the premolars present in ancestral species (Prochazka et al.,

2010). These rudimentary buds can develop into a supernumerary tooth in several mutants (Klein et al., 2006; Lochovska et al., 2015). It will be interesting to further investigate if the expanded tooth germ upon increased Shh activity can contribute to supernumerary tooth formation.

Molar Epithelial Stratification and Bending

A second recent paper, by Li et al., demonstrates regulatory roles for the Fgf and Shh pathways in early tooth morphogenesis in addition to instructing epithelial cell migration (Li et al., 2016b). In this paper, it was reported that chemical inhibition of the Fgf pathway in E11.5 mandibles significantly impaired the proliferation of the dental epithelial cells, resulting in failure of epithelial stratification of the molar tooth germ (Figs. 4B). However, inhibiting the Fgf pathway after E11.5, when some stratification had already occurred, arrested further proliferation but did not prevent invagination of the developing tooth germ (Fig. 4E). In a complementary experiment, the authors ectopically activated the Fgf pathway by introducing FGF10-soaked beads to single-layered tongue epithelium. Exposure to recombinant FGF10 promoted the stratification of tongue epithelium, suggesting that Fgf signaling is both necessary and sufficient to induce epithelial stratification.

The effects of FGF inhibition on proliferation were thus different from those reported by Prochazka et al. One possible explanation for the discrepancies could be the different level of pharmacological treatment. Prochazka et al. exposed the whole mandible explant to 2.5 μM of Fgf pathway inhibitor (SU5402), whereas Li et al. treated mandibular tissue slice culture with 20 μM SU5402. Clearly, the higher dose applied to a smaller piece of tissue could have had different effects, although it was found still to be well below that needed to produce cell death (Li et al., 2016). Several studies have shown that the Fgf pathway can elicit differential cellular responses, including cell proliferation, migration, and differentiation, through activation of different downstream pathways (reviewed in Boilly et al., 2000). For instance, half maximal activity of cell migration required higher FGF concentrations compared to cell proliferation in rat lens epithelial explants (McAvoy and Chamberlain, 1989). It is possible that the dosage used by Prochazka et al. was sufficient to perturb the cell migration, while having a negligible effect on cell proliferation. It is also possible that different durations of drug exposure could have led to different cellular responses, or that differences in experimental setup could explain the distinct outcomes. Further investigation of dose- and duration-dependent response of the dental placode cells to SU5402 through analyzing different downstream pathways would help resolve this discrepancy. Also, the role of Fgf signaling in the dental mesenchyme should be investigated, as *Fgfr1IIIc* is expressed in the dental mesenchyme at the earliest stages of tooth development (Kettunen and Thesleff, 1998). Reconciling the observations this way would also require that inhibition of the early migration not affect the later cell movements associated with deepening invagination. A recent study showed that the inhibitory effect of SU5402 may not be limited to Fgf signaling, but rather can extend to other tyrosine kinases, and differential dosage and duration of SU5402 treatment may also elicit distinct off-target effects (Gudernova et al., 2016). Therefore, more specific suppression of Fgf signaling, perhaps through genetic deletion of Fgf receptors, may help to address this issue.

Interestingly, the dental placode increased in width at the expense of the depth of invagination upon chemical inhibition of Shh signaling (Figs. 4D and G). The resulting shallower, wider invagination has also been observed in Shh and Hedgehog pathway genetic hypomorphs and knockouts, not only in tooth but also in hair follicles, which are thought to be formed at these early stages by much the same signaling and morphogenetic processes (reviewed in Pispas and Theis, 2003; St-Jacques et al., 1998; Gritli-Linde, 2002; Prochazka et al., 2015). In the Li et al. study, hyperactivation of the pathway resulted in the opposite phenotype, with accelerated narrowing and deepening of the placode invagination. In both cases, careful quantification showed that there was no significant change in overall cell proliferation rate and apoptosis. These results collectively indicate that the Shh pathway has a regulatory role in epithelial cell rearrangement. The Fgf and Shh pathways together may promote asymmetric cell proliferation that leads to local epithelial stratification and regulation of cell tension and/or motility that leads to epithelial bending. The authors suggest that these processes jointly drive the invagination of the molar epithelium into the underlying mesenchyme.

The Li et al. study demonstrated that Fgf and Shh signaling can promote cell proliferation and epithelial bending, respectively, through gain- and loss-of-function approaches. One important point that could be followed up in the future is that the experimental framework used in the gain-of-function experiments exposed the entire explant to chemical Shh agonist. The lack of a restricted or graded spatial distribution of signal and the degree of signaling activity that may be outside the physiological range may have led to cells not responding in a physiological manner, although a similar effect was seen with SHH protein applied via a protein-soaked bead (Cobourne et al., 2004). It is also worth noting that the experiments could not distinguish between roles of the Shh signal in orienting cell migration versus triggering or maintaining migration as such. Because expression of *Shh* in the tooth germ is spatially separated from the early molar placode and localized to the distal tip of the early tooth bud, it is even possible that tissue slices, which would normally successfully have undergone invagination, did not contain the endogenous source of *Shh* (Prochazka et al., 2015). Verifying the endogenous *Shh* expression within the tissue slice culture would strengthen the relationship between the experimental observations and events that occur *in vivo*.

Suprabasal Cell Intercalation-Mediated Contraction

One possible cellular mechanism through which Shh signaling promotes epithelial bending is through interaction of cells in the suprabasal layer, as demonstrated by the Li et al. paper described above and another from the same group (Panousopoulou and Green, 2016). Using the molar placode as a model, these authors showed that invagination of the placode is driven by *Shh*-dependent horizontal intercalation of suprabasal cells (Fig. 2C and D). These suprabasal layers of cells in the tooth germ form a shrinking and thickening canopy over underlying epithelial cells. Experimental incision through the epithelium and mesenchyme that flank the molar placode in jaw slice explants resulted in elastic recoil from the incision site (Fig. 5A). When the incision was introduced specifically in the suprabasal layer, but not the basal layer, the suprabasal layer recoiled laterally from the cut. When an initial incision through the suprabasal cell layer was followed by lateral incision of the flanking tissues, this

essentially abolished the recoil of the molar placode (Fig. 5B). These data suggest that the suprabasal cells, but not the basal cell layers, generate a tensile force sufficient and necessary to invaginate the molar placode. The intercalating tissue is, critically, anchored at its edges to the basal lamina by shoulder cells that both contact the lamina basally and intercalate apically. Crucially, Hedgehog pathway inhibition arrested the suprabasal canopy contraction and abolished the intercalation-associated planar cell elongation in both suprabasal and shoulder cells (Li et al., 2016b).

Given that the above experiments were done in slice explants, effects of the inhibition on long-range planar migration, of the kind observed by Prochazka et al., could not be tested. As with the Fgf inhibition, there might seem to be a discrepancy between the two sets of Shh inhibition experiments. The Prochazka et al. work showed little effect of Hedgehog pathway inhibition on cell motility as such, and the Li et al. treatments in effect arrested cell movement. However, in the both cases there was a definite inhibition of cell convergence, and this may indicate that the Shh signal provides a directional cue rather than a migratory signal as such, and that the later intercalation of cells in the invaginated tooth bud may be more sensitive to its absence than the earlier superficial migration. It would be interesting to see how Shh signaling inhibition affects cell movement and intercalation in real time using time-lapse confocal imaging on pharmacologically treated tissue. Further insights into the cellular response to Shh may be needed, since *Gli1* expression is widespread in the tooth and gives few clues as to how it might provide directionality.

Panousopoulou and Green observed enriched actomyosin complex and E-cadherin punctae at the surfaces of the suprabasal cells. They proposed that E-cadherin-based spot adherens junctions enable the cell intercalation, which in turn pulls the tissue towards the midline to hold its morphology. The authors suggested that this process also seals the top of the tooth germ so that cell proliferation and delamination below the intercalating layer further forces the underlying epithelial cells to invaginate into the mesenchyme. The intercalating suprabasal cells are replenished, in part by the incorporation of delaminating basal cells. The authors 3D-rendered the nuclear morphology in the molar placode, using deformation from a spherical shape to reveal the external forces experienced by the cells. They showed that the tension in the suprabasal layer stretched the nuclei from the default sphere shape of unstressed cells into a flattened “lentil” shape, indicating the equal magnitude of force exerted in all directions in the epithelial plane. It is apparent that the cells experience a tensile force along the lingual-buccal axis that results in the most commonly depicted epithelial invagination, but that a similar force in the proximo-distal axes of the jaws sustains invagination in that aspect to form the front and rear of the elongated pit or “boat” shape of the early molar tooth. It will be interesting to further assess which cell behaviors contribute to this proximo-distal force.

Cell Behaviors Along Different Axes

The Prochazka et al. and the Panousopoulou, Li and Green experiments describe two quite different-seeming cell migrations. One involves distal migration of cells distally from the jaw hinge while the other is a convergence of cells into the invaginating tooth bud. One is apparently FGF-dependent, the other FGF-independent. One is apparently Hedgehog

pathway oriented, the other, Hedgehog pathway dependent. How can these discrepancies be accounted for? One potential explanation involves the different explant culture techniques used to image the tissue. The directed cell migration events proposed by Prochazka et al. are supported by live-tissue imaging of E11.5 hemi-mandible using spinning disk confocal microscopy. The entire hemi-mandible was imaged *en face* from the oral surface, and the images obtained were processed by maximal intensity projection for cell tracking analysis. This computational method projects the three-dimensional information into two dimensions, and the subsequent analysis does not focus on the tracking information along the rostral-caudal axis. In contrast, Panousopoulou and Green's suprabasal cell intercalation hypothesis is based on imaging of tooth slices. This method allows observation of the cell behavior that occurs along the vertical (superficial-to-deep) axis without compromising the resolution. However, the tissue slice preparation would likely remove more proximal tissue. Since Prochazka et al. propose that cell migration towards the *Shh*-signaling center comes from more proximal tissue, this would be largely or entirely absent in the slice cultures. The successful invagination of the placode, which apparently occurs normally in slice cultures, despite the likely absent proximal tissue (near the jaw hinge), suggests that the cell migration and cell intercalation are independent events. It is even possible that slice culture removes critical distal signals or perturbs proximo-distal signals in other ways. Another possibility is that the two events take place in a sequential manner: the dental epithelial cells first migrate distally and define the position and the size of the future tooth germ along the proximo-distal axis, and then they start to proliferate and intercalate to grow and invaginate into the underlying mesenchyme. Various factors can influence the developmental schedule of embryogenesis (*e.g.* genetic background, transgenic and mutant alleles, and tamoxifen administration) (Naiche and Papaioannou, 2007; Dandekar and Glass, 1987). Therefore, it is possible that the staging of embryos in the two studies were slightly different. One conceptually simple experiment to test this would be to perform live imaging on intact jaw explants (*i.e.* not slices) in a single genetic background using multiphoton microscopy, which may allow for greater imaging depth compared to traditional spinning disk confocal microscopy, and then analyzing the cell behaviors in four-dimension.

Formation of Signaling Centers in Early Incisor Development

Many signaling cascades, including the *Shh* and *Fgf* pathways, are iteratively activated in both the incisor and the molar placodes. A recent study by Ahtiainen et al. performed *ex vivo* analysis of the developing incisor placode in a fluorescent ubiquitination-based cell cycle indicator (Fucci) mouse to understand the molecular regulation of cell behaviors in the incisor placode (Ahtiainen et al., 2016). The authors demonstrate that there is a population of non-mitotic cells that resides in the incisor placode near the oral surface. Time-lapse confocal imaging reveals that while these cells actively migrate towards the midline of the tooth bud to condense, the total number of cells remains relatively constant. Once condensed, these cells may act as a signaling center to regulate the early stages of incisor development (Fig. 2E), as the location of the non-mitotic cells coincides with the expression of signaling molecules that regulate tooth development.

The authors define this early population of quiescent, morphogen-secreting cells as the “initiation knot”. Cell quiescence is a common feature of signaling centers in various organs,

including the apical ectodermal ridge of the developing limb bud, the developing hair placode, and the enamel knot in the developing tooth germ (e.g., Martin, 1998; Ahtiainen et al., 2014; Jernvall et al., 1994). Notably, the authors reported that the initiation knot, which appears very early, and the enamel knot, which appears later during odontogenesis, are distinct populations. Tracking individual cells in the initiation knot of the mandibular explant revealed that these cells remained within clear boundaries near the oral surface, whereas the enamel knot appeared *de novo* at the tip of the mature bud (Fig. 1F). This observation is consistent with a previous study that demonstrated, using finely-staged embryos, that the two spatially distinct signaling centers appear sequentially (Hovorakova et al., 2011; Hovorakova et al., 2013).

While the cells in the initiation knot remain quiescent, they may promote proliferation in the neighboring cells to drive tooth morphogenesis. To test this possibility, the authors use *Tabby* mice, which exhibit smaller tooth and cusp patterning defect due to mutations in the *Eda/Edar/NF- κ B* pathway (Pispa et al., 1999; Harjunmaa et al., 2014). The authors found that the overall number of quiescent cells and the volume of the initiation knot were reduced in *Tabby* mice. In turn, the smaller initiation knot resulted in reduced expression of genes that are important for progression of tooth development, suggesting a link between the size of the initiation knot and that of the resulting tooth bud. One alternative explanation would be that the smaller initiation knot and tooth bud are independently initiated but sequential events. While it is likely that the tooth bud depends on specific molecular signals from the initiation knot, it could also be that the *Eda/Edar/NF- κ B* pathway determines the size of the dental anlage, and the smaller initiation knot would be a secondary result of a hypomorphic tooth germ. One way to address this would be to perform spatiotemporal ablation of the initiation knot cells. Another remaining question is whether the smaller tooth germ in *Tabby* mice results from reduced mitogenic signal in the initiation knot. This can be addressed in the future by unbiased molecular characterization of the initiation knot and quantitative analysis of cell proliferation in the tooth germ of *Tabby* mice.

Cell Behaviors in Molar vs. Incisor Epithelium

Cell migration has been observed in various morphogenetic contexts (reviewed in Aman and Piotrowski, 2010). As described above, molar placode cells migrate and this is essential for formation of the tooth germ; however, in the incisor, as observed by Ahtiainen et al., the migration seems to be limited to the cells in the initiation knot, whereas cell proliferation seems to be the main driving force for subsequent invagination. One possible reason for this discrepancy is the difference in the developmental timing of incisor and molar. Ahtiainen et al. studied similar embryonic stages (E11.5 and E12.0) to those in the molar-focused papers reviewed above. However, because development of the incisor is faster than that of molar, it is likely that Ahtiainen et al. analyzed the cell behaviors at more advanced stages of tooth development. Later stages in molar morphogenesis were not analyzed by either Prochazka et al. or the Green group, and it would be interesting to see if later stages of invagination are, like the incisor, dominated more by proliferation processes than by cell movement. Conversely, an examination of incisor at earlier stages and at higher resolution might reveal similarities to the molar, since Ahtiainen et al. made their observations at relatively low magnification. Another possibility is that signaling centers in incisors and molars have

qualitatively different instructive roles. It is important to note that the molar signaling center is spatially distinct from the molar placode, whereas the incisor signaling center (*i.e.* initiation knot) is located within the incisor field. Moreover, morphogenesis of the incisor and the molar placode differ on a three-dimensional level. The incisor placode maintains its location and adopts a relatively rounder shape as it develops, whereas the molar placode undergoes significant expansion along the proximo-distal axis to adopt its cylindrical shape. Therefore, it is conceivable that cells respond differently to the same signals to contribute to the morphogenesis of the two tooth types.

Conclusions and Future Prospects

The studies discussed in this review have enhanced our understanding of how molecular signaling translates into dental epithelial cell dynamics. However, many mysteries remain, and it is certain that tooth morphogenesis is more complex than our current understanding. Most studies of tooth development have focused on the epithelial contribution due to the availability of genetic tools and the tissue's well-defined structure. However, it is well known that epithelium and mesenchyme exchange highly dynamic and reciprocal signaling to orchestrate tooth development. The dental mesenchyme takes on an instructive role between E11.5 and E12.5, during which placodal invagination occurs, suggesting that mesenchymal signaling is critical for directing epithelial invagination (Mina and Kollar, 1987). Panousopoulou and Green showed by explantation that the initial invagination forces in the molar were autonomous to the epithelium, but it is theoretically possible that the dental mesenchyme coordinately undergoes remodeling to facilitate invasion by the invaginating epithelium (Panousopoulou and Green, 2016). The presence of abundant F-actin and phosphomyosin in jaw mesenchyme also allows for the possibility of a role of mesenchyme during tooth development. Therefore, investigating dental mesenchymal dynamics would be a logical next step to further our understanding of tooth morphogenesis. Recently, tamoxifen-inducible Cre lines under control of promoters of early dental mesenchymal markers, such as *Fgf10* and *Msx1*, have been generated, and these may be useful for future studies (El Agha et al., 2012; Lallemand et al., 2013). In parallel, the field has also begun to explore dental mesenchymal cell dynamics through combining *ex vivo* imaging with tissue recombination techniques (Li et al., 2016a; Mammoto et al., 2011).

In addition, there are a number of exciting studies that investigate cellular behaviors underlying different processes of tooth morphogenesis which have not been highlighted in this review. While this review focuses on dental placodal invagination, it is equally interesting to understand the process through which placodes are formed. Li et al. suggest that the vertical mitotic angle of placodal cells relative to the basement membrane may promote the thickening, suggesting that perturbation of genes known to be involved in regulating mitotic orientation could further this line of investigation (Li et al., 2016b). Another group explored the mechanism through which bud-to-cap transition occurs. The tooth germ at E12.5, which is essentially an invaginated bud of epithelium, deforms into a distinct shape that resembles a cap by E14.5 (Fig. 1). To understand this process, Morita et al. combined long-term live imaging with Fucci cell cycle analysis, correlating the tissue growth pattern with mitotic orientation and frequency in the tooth germ at E14.5 (Morita et al., 2016). The authors reinforced previous studies showing that differential cell proliferation

is one of the driving forces of tooth morphogenesis (Jernvall et al., 1994). In addition, through quantitative analysis of fluorescence recovery of YFP-actin after photobleaching, the authors showed that dynamic actin remodeling might play a role in tooth germ morphogenesis.

Another interesting question is the mechanism through which the second and third molars develop. Both humans and mice develop three molars per jaw quadrant, with each subsequent molar forming at the posterior of the previous tooth germ. Gaete et al. explored this phenomenon at tissue and cellular level. Through combining explant culture with DiI labeling and tissue truncation, they found that second and third molars are derived from progenitors that reside in the “tail” of the first molar tooth germ, a structure more superficial than the bulk of the tooth germ (Gaete et al., 2015). This finding complements previous work examining development of molars (Juuri et al., 2013). Investigating different stages of tooth morphogenesis will provide us with an understanding of how the dental organ derives its characteristic 3D shape from a flat epithelial sheet.

The tooth has long been an important model for developmental biologists. A deeper understanding of the morphogenetic events in tooth development will be of broad significance to the field of organogenesis, as many signaling pathways and cellular events in tooth development are not restricted to odontogenesis, but rather are deployed in various developmental contexts.

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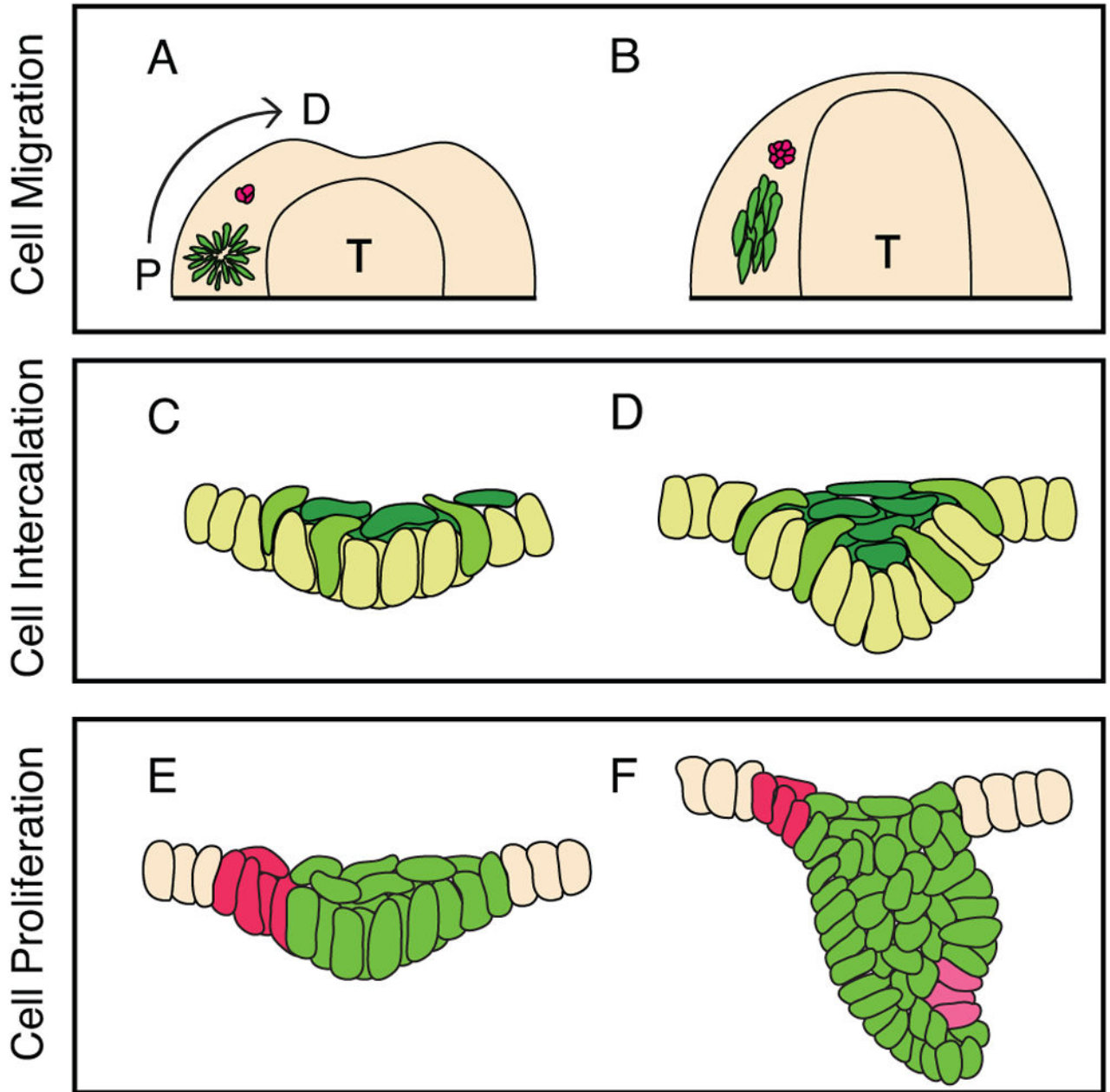


Figure 2.

Cell movements in early tooth development. **A,B:** Prochazka et al. proposed that, viewing the mandible from above, progenitor cells in the molar placode (purple) arrange into a rosette-like structure, then subsequently dissociate to migrate towards a *Shh*-expressing signaling center (red). **C,D:** In the model developed by Panousopoulou and Green, shown in frontal aspect, basal cells in the molar placode delaminate towards the center of the placode (yellow-green). The delaminated cells form the suprabaasal tissue canopy (dark green), which intercalate along the midline to drive epithelial invagination. **E,F:** Ahtiainen et al. proposed that, in incisors, a population of non-proliferating cells that co-localizes with morphogen

expression (red) instructs the neighboring epithelial cells (green) to proliferate, and this leads to tooth morphogenesis. The enamel knot forms *de novo* at the tip of the tooth germ (pink). T: tongue. A and C are E11.5. B and D are E12.5. E is E12.0. F is E13.5.

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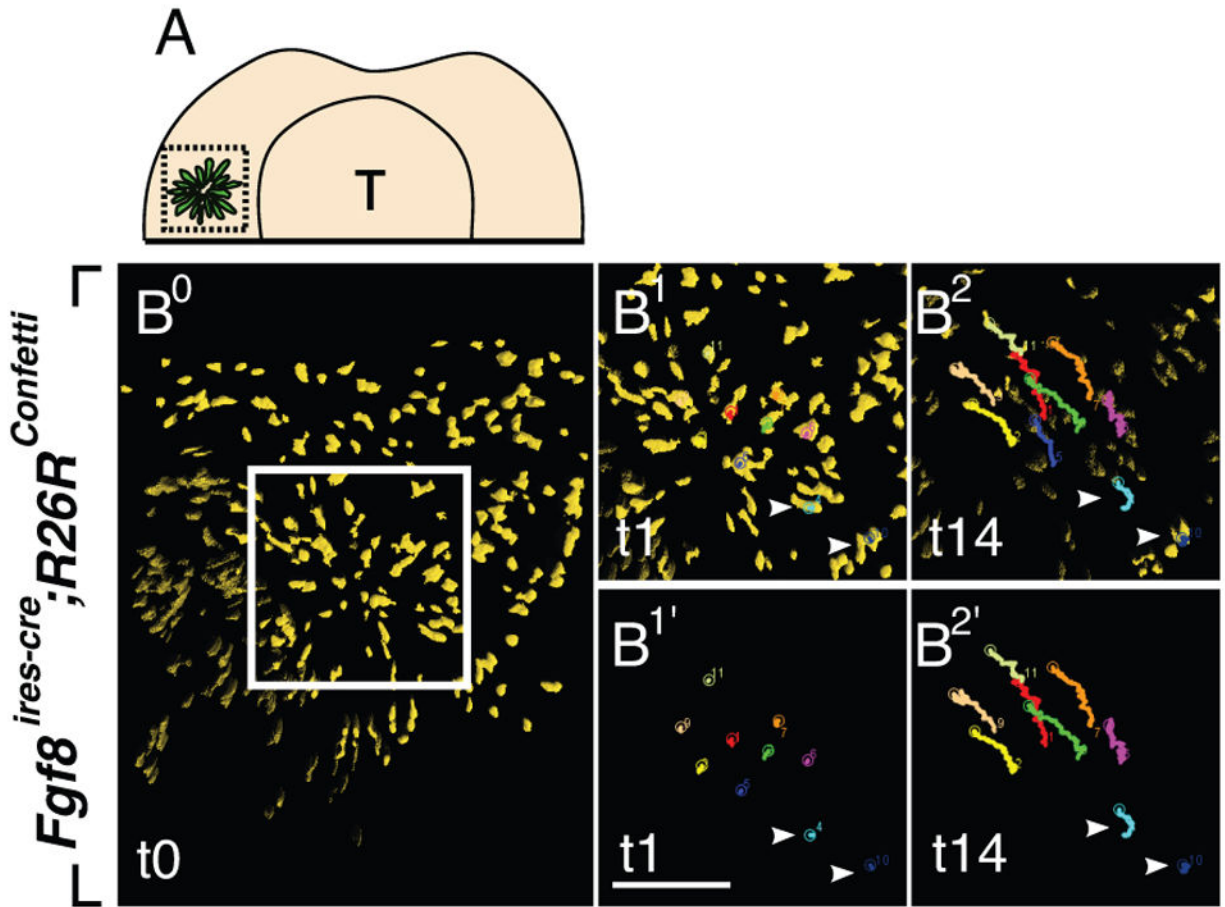


Figure 3. Fgf and Shh signaling instruct molar placodal cells to undergo directed cell migration. **A:** Schematic presentation of *Fgf8*-expressing molar placodal cells from oral surface view at E11.5. Dotted square demarcates the molar placode region. **B-B^{2'}:** Time-lapse imaging *via* spinning disk confocal microscopy reveals that *Fgf8*-expressing cells within the rosette-like structure are released and migrate distally. Tracking of the cells within the rosette-like structure (white box) over time (B¹-B² and B^{1'}-B^{2'}). Arrowheads (lower right) indicate posterior cells that do not undergo dynamic migration. Scale bar: 100µm. Reproduced with permission from Elsevier.

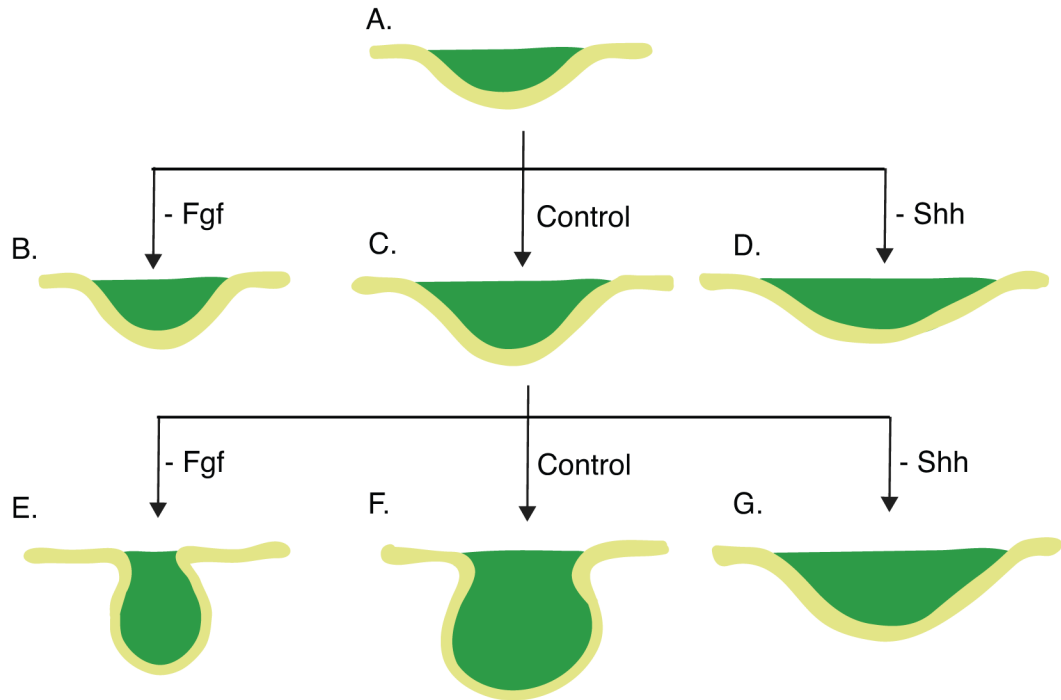


Figure 4.

Fgf and Shh signaling promote stratification and epithelial bending, respectively. **A–D:** The molar placode (A) at E11.5 stratifies and bends into early tooth bud (C). However, inhibition of Fgf signaling results in significantly shallower tooth bud (B). Inhibiting the Shh pathway results in a shallower and wider tooth bud (D). **E–G:** Similar observation is made when the early tooth bud (C, E12.5) is treated with Shh and Fgf inhibitors. Early tooth bud further stratifies and invaginates into late tooth bud (F). However, inhibition of Fgf results in narrower tooth germ (G), while inhibiting Shh results in shallower and wider tooth germ (E). A: E11.5. B–D: E12.5. E–G: E13.5.

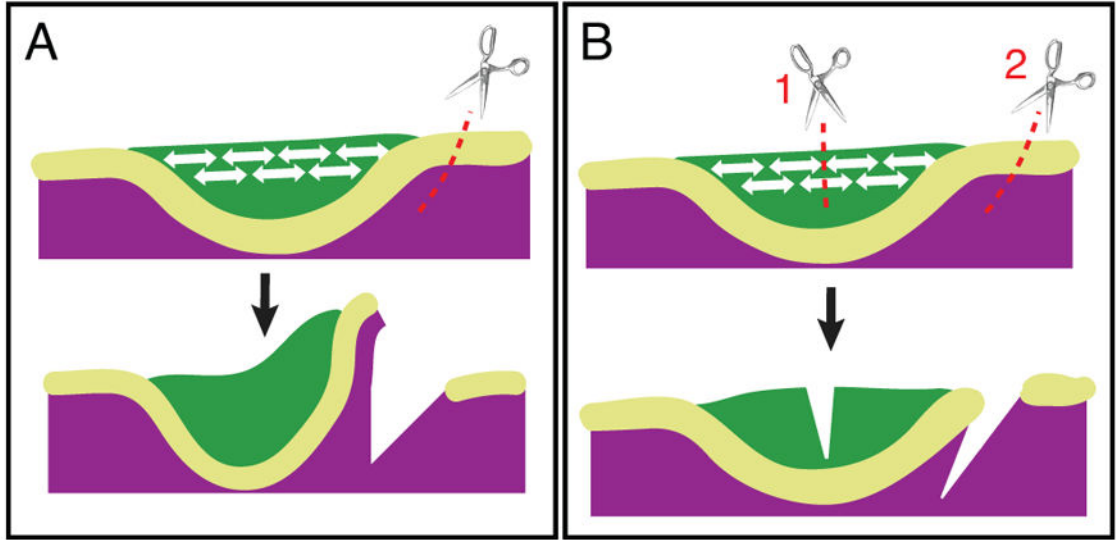


Figure 5.

Contractile suprabasal tissue promotes bending of molar placode. **A:** Single lateral incision in the epithelium that flanks the molar placode at E11.5 allows rapid bending of the molar placode. **B:** Incision through the suprabasal layer (cut 1) abolishes the lateral cut- (cut 2) induced bending, showing that bending depends on suprabasal tension. Green: suprabasal layer. Yellow-green: basal layer. Purple: mesenchyme.