Development 139, 3487-3497 (2012) doi:10.1242/dev.085084

Tooth shape formation and tooth renewal: evolving with the same signals

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Summary

Teeth are found in almost all vertebrates, and they therefore provide a general paradigm for the study of epithelial organ development and evolution. Here, we review the developmental mechanisms underlying changes in tooth complexity and tooth renewal during evolution, focusing on recent studies of fish, reptiles and mammals. Mammals differ from other living vertebrates in that they have the most complex teeth with restricted capacity for tooth renewal. As we discuss, however, limited tooth replacement in mammals has been compensated for in some taxa by the evolution of continuously growing teeth, the development of which appears to reuse the regulatory pathways of tooth replacement.

Key words: Evo-devo, Patterning, Teeth, Tooth shape, Tooth replacement

Introduction

For the past 20 years, teeth have been used extensively as models in analyses related to developmental patterning, signaling and evolution. During mouse embryogenesis, the late onset of tooth development (odontogenesis) makes the mouse dentition an accessible model system for diverse types of developmental studies. As a result, teeth constitute a developmental module that can be studied in relative isolation. The wealth of data on tooth development is rivaled by the central role of dentitions in documenting the evolutionary history of vertebrates. The relative completeness of the vertebrate fossil record is due in no small part to the hardness, and hence preservability, of teeth. Furthermore, unlike other epithelial-based organs, such as hair, scales and feathers, teeth are found throughout vertebrate groups, thus providing a general paradigm for studying the evolution and development of epithelial organs.

The basic steps of tooth morphogenesis were described well over 100 years ago and are basically similar in all vertebrates (e.g. Owen, 1845; Leche, 1895) (Fig. 1). Briefly, tooth formation is regulated by epithelial-mesenchymal interactions; the mesenchyme derives from the neural crest, whereas the epithelium may be ectodermal or endodermal (Soukup et al., 2008; Fraser et al., 2009). The establishment of the dental lamina, the area that forms teeth, precedes the initiation of individual teeth. Teeth become visible during the following stages of development, called the bud and the cap stages, in which the initial epithelial invagination and the tooth crown area, respectively, appear. The cap stage is followed by the bell stage, during which species-specific cusp patterns emerge. After the formation of the cusp pattern, the tooth grows to its final size, and mesenchymal odontoblasts and epithelial ameloblasts differentiate at the epithelial-mesenchymal interface to form dentin and enamel, respectively. These hard dental tissues, together with cementum, which is made by cementoblasts, have largely similar compositions in all vertebrates, with enamel being up to 98% hydroxyapatite.

Research during the past 20 years has uncovered the iterative use of largely the same molecular pathways throughout the various stages of tooth development (Jernvall and Thesleff, 2000; Bei, 2009; Tummers and Thesleff, 2009). Members of the transforming growth factor β (TGFβ), fibroblast growth factor (FGF), sonic hedgehog (Shh) and Wnt signaling pathways are all required repeatedly during tooth development, and the abolition of any of these pathways results in a developmental arrest of teeth, most commonly at the bud stage. These different pathways are integrated at several stages of the tooth development process and the overall regulatory network is highly conserved in evolution (Jernvall and Thesleff, 2000; Fraser et al., 2009).

Much of the past research in this field has focused on four 'life history' stages of the tooth: determination of tooth-forming regions, initiation of tooth formation, determination of tooth shape and regulation of tooth renewal. Whereas the number of tooth-forming regions and the number of teeth show an evolutionary tendency to decrease (reviewed by Davit-Béal et al., 2009), tooth shape complexity and tooth renewal increase through time (Fig. 2). Below, we focus on these increases in dental complexity and tooth renewing capacity, and review both the evolutionary patterns and developmental mechanisms underlying these changes in fish, reptiles and mammals.

Making a tooth: shape formation and shape modification

Prior to the initiation of tooth development, the dental lamina, which is also known as the odontogenic band, appears within the dental epithelium. The lamina forms as a thickening of the epithelium and restricts the tooth-forming area laterally (Fig. 1). Even though the molecular mechanisms of dental lamina formation are currently unknown, several studies have shown that sonic hedgehog (*Shh*) and pituitary homeobox 2 (*Pitx2*) are expressed in the dental lamina in several taxa (Keränen et al., 1999; Fraser et al., 2006; Smith et al., 2009; Buchtova et al., 2008; Vonk et al., 2008). From within the lamina, individual teeth are initiated, and this process can be seen, at least in mammals, by a gradual restriction of *Shh* and *Pitx2* expression to the specific domains, referred to as placodes, that give rise to teeth.

After the initiation of tooth development, the morphology of individual teeth unfolds during the bud, cap and bell stages (Fig. 1). These developmental stages are crucial for the survival of the animal because the correct function of teeth depends almost entirely on the patterns established prior to tooth eruption. Once erupted, the tooth morphology changes only through wear or damage, and there is no remodeling of the mineralized tissue, unlike the rest of the skeleton. The lack of remodeling also implies that the evolutionary modification of tooth morphology happens during its ontogeny.

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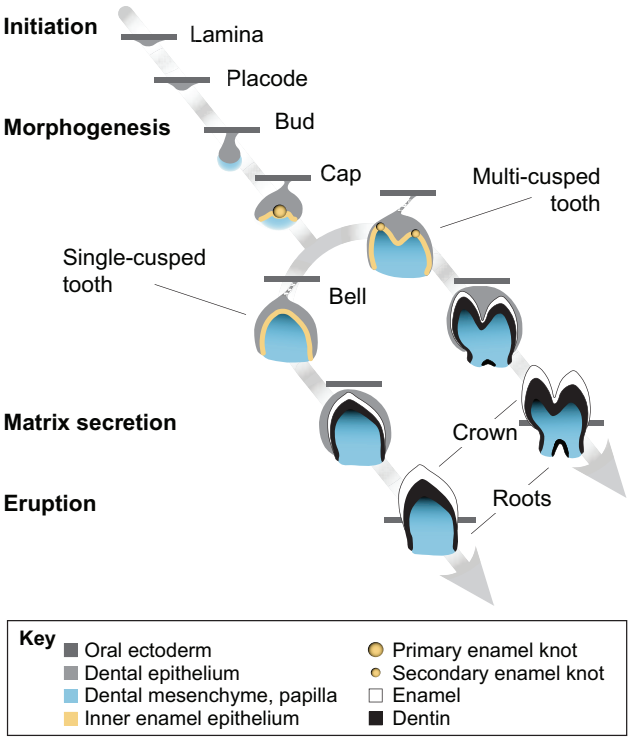

Fig. 1. The principal stages of tooth formation. Prior to the initiation of tooth development, the tooth-forming region (the dental lamina) appears within the dental epithelium. The development of individual teeth is then initiated within specific domains of the lamina, referred to as placodes. During the bud stage, the dental epithelium invaginates into the dental mesenchyme, which condenses around the epithelium to form a bud. Then, during the cap stage, the epithelium extends further into the mesenchymal tissue and wraps itself around the condensing mesenchyme. The cap stage is followed by the bell stage, during which species-specific cusp patterns emerge: in a single-cusped tooth, a primary enamel knot, which first appears at the cap stage, gives rise to the tip of the crown; in multicusped mammalian teeth, secondary enamel knots form at the places of future cusps. This stage is then followed by final growth and matrix secretion, during which time the inner enamel epithelium differentiates into ameloblasts, which produce enamel, and the adjacent mesenchymal cells differentiate into odontoblasts that secrete dentin. Roots continue to develop during eruption.

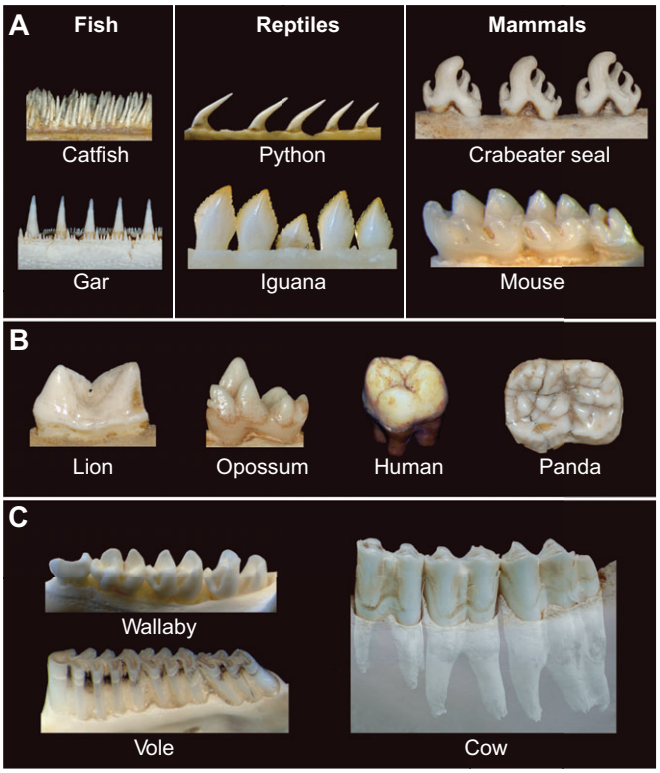

Fig. 2. Evolutionary diversity within the teeth of living species. (**A**) Teeth are found across the vertebrates but mammalian teeth tend to have more cusps and are generally more complex, whereas fish and reptiles tend to have larger numbers of simple teeth. (**B**) Among mammals, molars are generally simpler in animal-eating species (e.g. lions), whereas plant-eating species (e.g. pandas) have complex crown topography. (**C**) Mammals have limited tooth renewal capacity and only a few groups of mammals (e.g. wallabies) have species that can develop new molars posteriorly. A more common solution is tall (hyposdont) teeth, where only part of the crown is visible outside the jaw (note that the parts inside the jaw are made visible in the image of cow molars). Some mammalian species (e.g. the vole) have ever-growing (hypselodont) molars. By contrast, fish and reptiles have continuously replaced teeth (e.g. see tooth being replaced in iguana, in A). Anterior is towards the left. Images are not to the same scale.

The evolution of tooth morphologies shows a general trend towards higher complexity, and this is achieved by the addition of cusps, the major surface features on teeth (Fig. 2A). Cusps can have the shape of individual bumps but can also have elongated blade-like shapes. In many species, cusps are joined together to form complex crest patterns (Fig. 2B). Whereas multicusped teeth occur in most taxonomic groups, the highest complexity and most diverse tooth shapes have evolved in mammals (Fig. 2B). Together with a general decrease in tooth number, mammals have evolved a progressively more regionalized dentition, known as heterodonty (Luo, 2007). The basal mammalian dentition thus consists of different tooth types: incisors, canines, premolars and molars. Of these, the premolars and molars have evolved the highest complexity, especially in mammals that are specialized to eat fibrous vegetation (Evans et al., 2007). The adaptive link with diet also means that additional cusps have evolved repeatedly in mammalian lineages that are specialized to eat plants. For example, the evolution of the four-cusped upper molar from a three-cusped predecessor by the addition of a specific cusp, the hypocone, has happened at least 20 times independently (Hunter and Jernvall, 1995). Ecologically, evolution of the hypocone is associated with an adaptive shift from animal-only to animal- and plant-based diets. In the case of humans, the molar pattern conforms to the basic four-cusped pattern, which is the typical tooth morphology of most primates.

Developmentally, multicusped teeth form in mammals by the repeated appearance of the epithelial signaling centers, known as enamel knots (Fig. 3A). During the bud stage, a primary enamel knot forms at the tip of the tooth bud, and the non-dividing cells of the knot begin to express several signaling molecules. These include members of the FGF, bone morphogenetic protein (BMP) and Wnt families, as well as Shh (Fig. 3A). In teeth with a single cusp, no additional enamel knots form; however, in multicusped teeth, a secondary enamel knot appears at the future location of each cusp (Fig. 1; Fig. 3A,B). The formation of secondary enamel knots, which are non-proliferative, is thought to lead to the folding of the inner enamel epithelium, owing to spatial differences in cell

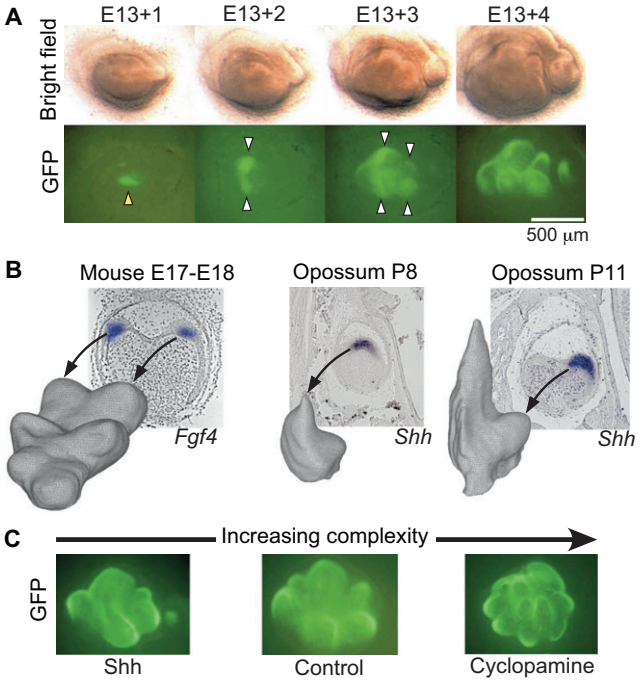

Fig. 3. Cusp formation in mammals is regulated by secondary enamel knots. (**A**) A molar from a heterozygous *Shh*-GFP transgenic mouse, which expresses green fluorescent protein (GFP) under a *Shh* promoter. Bright-field and fluorescence images show the primary enamel knot (yellow arrowhead) at E13+1 and secondary enamel knots of the main cusps (white arrowheads) at E13+2 and E13+3. Later (at E13+4), GFP, and hence Shh, is expressed in the differentiating ameloblasts throughout the crown. (**B**) Histological sections (top) stained for *Fgf4* and *Shh* expression and three-dimensional renderings of the epithelial-mesenchymal interface (bottom) of developing mouse and opossum teeth show the appearance of cusps. Mouse cusps are initiated very close in time to each other and the cusps become close to equal in height. By contrast, opossum cusps are initiated several days apart resulting in cusps that are unequally tall (as also seen in the opossum tooth shown in Fig. 2B). (**C**) Mouse molars cultured with Shh (left) exhibit a delay in cusp formation and a reduction in cusps (compared with control molars; centre), whereas culture with the Shh inhibitor cyclopamine (right) increases cusp formation, allowing them to form close to each other. Molars were treated for 4 days and the images are at 8 days. The sections of opossum teeth are modified, with permission, from Moustakas et al. (Moustakas et al., 2011); the section of mouse teeth is modified, with permission, from Jernvall et al. (Jernvall et al., 1994); and Shh treatments in C are modified, with permission, from Harjunmaa et al. (Harjunmaa et al., 2012).

proliferation (Jernvall et al., 1994). The sequence of appearance of individual secondary enamel knots and cusps closely corresponds to the relative height of individual cusps and the order in which they begin to mineralize (Fig. 3B). Dentin and enamel matrices are secreted, starting from the tallest cusp tips and proceeding to the base of the crown from which point the roots start to develop.

Because teeth are present across the vertebrates, next we review comparative evidence on tooth shape development in fish, reptiles and mammals to highlight similarities and differences in the regulation of shape.

Tooth shape development in fish

Most of the research on tooth development in fish has focused on regional determination rather than on tooth shape. Currently, it is not known whether fish teeth have enamel knots or comparable signaling centers that are involved in the formation of additional cusps. Streelman et al. (Streelman et al., 2003) examined in cichlid fish how single-cusped first-generation teeth are replaced with two- and three-cusped teeth. Morphological variation of these cichlid teeth was shown to be compatible with activator–inhibitor-like dynamics found in the regulation of enamel knots in mammalian teeth (Streelman et al., 2003). A second study examined tooth development in the Mexican tetra *Astyanax*, which can typically have up to six cusps in a tooth (Trapani et al., 2005). These multicusped teeth form from single tooth germs (a group of cells committed to form a tooth) and not by a fusion of several unicusped tooth germs, as is known to be the case in some fish (reviewed by Trapani et al., 2005). The results from *Astyanax* suggest that, in principle, aspects of enamel knot-like regulation of many cusps may be present in fish. One feature that differs from mammalian teeth, however, is the diminutive size of multicusped teeth in many fish. Nevertheless, asymmetric growth of the inner enamel epithelium modulated by asymmetric inhibition has been postulated to cause shape changes in growing fish teeth (Vandervennet et al., 2006). Yet another special case is the formation of the beak in pufferfish. Fraser et al. (Fraser et al., 2012) showed that the beak appears during development through the elongation of a single tooth in each jaw quadrant, and multiple generations of these teeth are then stacked together to form the parrot-like beak.

Tooth shape development in reptiles

Recent evidence from studies of tooth development in snakes and lizards (reptiles belonging to order Squamata) shows that the cap-bell staged tooth germ in these reptiles is histologically relatively simple compared with that of mammals, with no easily identifiable enamel knots (Handrigan and Richman, 2010a; Richman and Handrigan, 2011). Molecular analyses show that *Shh* is expressed in the inner enamel epithelium, but the expression pattern is more diffuse with no localized knot-like domains (Handrigan and Richman, 2010a; Richman and Handrigan, 2011). Nevertheless, tooth morphogenesis is arrested in lizards and snakes when Shh signaling is inhibited by cyclopamine (Buchtova et al., 2008; Handrigan and Richman, 2010a). In addition to *Shh*, it was shown that *Wnt6*, *Wnt7a*, *Bmp4* and *Edar* are expressed in the inner enamel epithelium of the developing teeth of snakes and lizards, suggesting that the regulatory networks of reptilian tooth development are largely shared with those of mammals (Handrigan and Richman, 2010b; Richman and Handrigan, 2011).

The issue remains, however, how multicusped teeth could develop in reptiles. Handrigan and Richman (Handrigan and Richman, 2011) examined development of small bicusped teeth in leopard gecko. They discovered an absence of mammal-like folding of the inner enamel epithelium; instead, differential deposition of enamel matrix formed cusps. Expression of *Bmp2* in ‘enamel epithelial bulge’ cells may play a role in stopping enamel formation centrally, whereas, either side of the bulge, enamel continues to form, resulting in two cusps (Handrigan and Richman, 2011). Whereas relatively few living reptiles have multicusped teeth (such as geckos and perhaps iguanas, see Fig. 2A), several extinct groups show highly elaborated dental patterns. The most mammal-like morphologies can be found in terrestrial notosuchian crocodilians, which show both heterodonty and fully multicusped molariform teeth (O’Connor et al., 2010). No living crocodiles have multicusped teeth, but histological studies (Westergaard and Ferguson, 1987) have reported an enamel knot-like structure, which

is presumed to be the primary enamel knot. It would be interesting to determine whether this structure exhibits properties of a true enamel knot. Others have reported localized patterns of *Shh* expression along the margins of the alligator jaw (Harris et al., 2006), suggesting that, in contrast to snakes and lizards, current crocodylian reptiles may posses the potential to form mammal-like secondary enamel knots. If this were the case, it could be hypothesized that multicusped crocodilian teeth could be produced in vitro by adjusting the activator–inhibitor dynamics of the enamel knots. A thorough inventory into variation in crocodilian dentition could shed light onto the issue of multicusped teeth.

A special morphology present in snakes is the venom-delivering fangs, which have been proposed to develop from their own dental lamina in advanced groups of snakes (Vonk et al., 2008). Most elaborated fangs have an enclosed canal, and Zahradnicek et al. (Zahradnicek et al., 2008) have shown that this canal forms during development by proliferation-induced invagination of the epithelium, resulting in a groove that eventually closes off from the surface. The molecular regulation of this epithelial folding seen in fangs, however, remains unknown.

Tooth shape development in mammals

Since the discovery of the secondary enamel knots in mouse (Jernvall et al., 1994), they have been described in other taxa. Similar histological and molecular markers manifesting enamel knots have been reported in voles, shrews, ferrets and opossums (Jernvall et al., 2000; Miyado et al., 2007; Järvinen et al., 2008; Järvinen et al., 2009; Moustakas et al., 2011). The presence of secondary enamel knots in the gray short-tailed opossum (Moustakas et al., 2011), which is a marsupial, is significant because it indicates that all mammals are likely to share the same principle of regulating cusp development (Fig. 3B). Histologically, enamel knots consist of tightly packed epithelial cells that are removed apoptotically after cusp development commences. Cell proliferation kinetics have shown that the secondary enamel knots form from non-proliferative or slowly cycling cells previously belonging to the primary enamel knot (Coin et al., 1999), suggesting that not all the cells of the primary enamel knot are removed apoptotically. It remains to be shown whether the identity of these slowly cycling cells is consequential for cusp development per se, or whether these cells reflect the spatiotemporal dynamics of cusp patterning. One molecular difference between the primary and the secondary enamel knots is *Fgf10* expression. *Fgf10* is expressed in dental mesenchyme in the mouse and opossum, but in addition it is expressed in the primary enamel knot of the opossum (Moustakas et al., 2011). Moustakas et al. (Moustakas et al., 2011) suggested that the epithelial *Fgf10* expression promotes the development of the tall and sharp crown topography found in opossums (Fig. 1B, Fig. 3B).

The number of identified mutations causing a modification in cusp number has increased over the past 10 years. At least 16 individual gene mutations have been reported to alter cusp patterns in the mouse (Bei, 2009; Harjunmaa et al., 2012). Of these, the effects of ectodysplasin (*Eda*), sprouty (*Spry2* and *Spry4*), *Fgf3* and sclerostin domain containing 1 gene (*Sostdc1*) have been characterized in the greatest detail.

The *Eda*-null mutant Tabby was probably the first mouse mutant in which a tooth phenotype was described and genetically identified (Falconer, 1952; Srivastava et al., 1997). The lack of functional ectodysplasin (encoded by *EDA*) in humans results in hypohidrotic ectodermal dysplasia (HED), a syndrome that affects ectodermal organs, including teeth, hair and skin glands. Multiple teeth are missing and the crowns of the remaining teeth typically lack cusps and are conical in shape. Tooth morphology in the *Eda* mouse mutant is characterized by missing or fused cusps. Comparison of *Eda*-null mutant teeth with teeth from mice that overexpress *Eda* under the keratin 14 promoter show that most crown features are affected in an additive manner. Cusp spacing and height are positively regulated by the amount of available Eda (Kangas et al., 2004). Increasing Eda signaling even further by simultaneously increasing *Eda* and its receptor *Edar* produces further separation of cusps laterally together with a girdle like cingulid, which is present in many mammals but normally missing in the mouse molars. Cusp number, however, is not markedly increased in molars with increased Eda signaling (Harjunmaa et al., 2012).

Eda is a tumor necrosis factor (TNF) family signal and binds to its receptor Edar, which is expressed in the enamel knots. In mice in which Eda signaling is affected, the size and shape of enamel knots are modified. It is plausible that most morphological effects of Eda signaling on tooth shape are exerted through the regulation of enamel knot size. At the level of signaling, mutations in *Eda*, *Edar* and the intracelluar mediator *Edaradd* can cause HED individually by blocking the activation of nuclear factor κB (NF-κB) in enamel knot cells (reviewed by Mikkola, 2008). The downstream targets of NF-κB appear to include at least *Dkk4*, follistatin and *Shh* (Fliniaux et al., 2008), suggesting that Eda may be involved in the negative regulation of Wnt, BMP and activin signaling, in addition to upregulating *Shh*. Eda signaling, through modulation of multiple pathways, appears to have a ‘tinkering’ role in tooth shape development such that it balances signaling from multiple pathways. This kind of tinkering can be hypothesized to make Eda signaling a common target in phenotypic evolution. Indeed, allelic variation in *Eda* has been associated with the evolution of stickleback fish armor plates (Colosimo et al., 2005), and single nucleotide polymorphisms of *Edar* have been associated with human hair morphology (Fujimoto et al., 2008). Recently, *Edar* polymorphism was linked to shovel-shaped incisor shapes (Kimura et al., 2009) and *Eda* polymorphism to rates of deciduous tooth eruption in humans (Pillas et al., 2010), providing evidence for the role of Eda signaling in tooth evolution.

The loss of function in Eda signaling causes simplification of tooth morphology, whereas loss of function of *Spry2* and *Spry4* or *Sostdc1* results in morphologies that are more complex. In the case of *Spry2* and *Spry4*, which are intracellular feedback regulators of FGFs, cusp morphology is subtly altered (Klein et al., 2006). By contrast, analyses of the effects of loss of function of *Fgf3* signaling in mouse and human molars show that the last developing cusps are reduced or missing (Charles et al., 2009). In the case of human upper molars, the last developing and evolutionarily newest cusp in the ancestry of primates, the hypocone, is missing (Charles et al., 2009). *Fgf3* is expressed in the developing mouse molars in the primary enamel knot and in the underlying dental mesenchyme, whereas *Spry2* is expressed in the dental epithelium, including the enamel knot and Spry4 in the mesenchyme (Klein et al., 2006). In addition to *Fgf3*, several other FGF genes are expressed in the developing tooth (Kettunen et al., 2000). Because FGF receptors are not expressed in the enamel knots, whereas they are intensely expressed in the mesenchyme and the epithelium flanking the enamel knots (Kettunen et al., 1998), FGF signaling appears to provide a mechanism by which the inner epithelium folds around the enamel knots, thereby forming cusps. Therefore, tinkering with

the overall balance of FGF signaling through Spry genes or individual FGF genes, such as *Fgf3*, results in a modification of cusp patterns.

The protein encoded by *Sostdc1* (also known as ectodin, *Wise* and *Usag1*) has been described as an antagonist of both BMP and Wnt signaling (Laurikkala et al., 2003; Itasaki et al., 2003; Yanagita et al., 2004). The *Sostdc1*-null mutation causes an expansion of enamel knots, which results in altered crown features, such as crests joining cusps, in addition to frequently fused teeth (Kassai et al., 2005). Sostdc1 has been reported to inhibit BMP signaling by binding to BMPs (Laurikkala et al., 2003; Yanagita et al., 2004), and the lack of functional Sostdc1 makes teeth hypersensitive to superfluous BMP in vitro by accelerating tissue differentiation (Kassai et al., 2005). Because *Sostdc1* can be induced by BMP4, one role for Sostdc1 may be to limit the differentiation of enamel knots, a role supported by the expression of *Sostdc1* outside the enamel knots. Increasingly, however, Sostdc1 has been suggested to regulate principally Wnt signaling in tooth development. Ahn et al. (Ahn et al., 2010) showed that reducing the dose of the Wnt co-receptors Lrp5 and or Lrp6 (low-density lipoprotein receptor-related proteins) in *Sostdc1* mutants rescues the wild-type tooth morphology. A null mutation of another Wnt receptor, *Lrp4*, has been shown to produce a change in tooth phenotype very similar to that generated by the *Sostdc1*-null mutation (Ohazama et al., 2008). In a study to examine further the molecular mechanisms of Sostdc1, Lintern et al. (Lintern et al., 2009) suggested that separate domains exist for Lrp6 and Bmp4 binding, but with a preference for Sostdc1 to bind Lrp6 over Bmp4 (Lintern et al., 2009). Lrp6 interferes with Bmp4 inhibition by Sostdc1, whereas Bmp4 does not interfere with Wnt inhibition by Sostdc1 (Lintern et al., 2009). Therefore, Sostdc1 may integrate BMP and Wnt signaling pathways in a context-dependent manner. Yet another proposed mode of regulation is Shh-mediated inhibition of Wnt by Sostdc1 (Cho et al., 2011). Finally, *Lrp4* is upregulated by Eda signaling (Fliniaux et al., 2008), underscoring the breadth of the integration of signaling pathways.

The involvement of multiple signal pathways in the regulation of tooth shape suggests that no single factor is sufficient to explain the dental diversity observed in mammals. Furthermore, present evidence shows that each secondary enamel knot that gives rise to individual cusps expresses the same set of genes (Jernvall et al., 2000) [but see Moustakas et al. (Moustakas et al., 2011) and above on the primary and the secondary enamel knots]. Computational modeling has been used to study how complex networks regulate the iterative formation of each enamel knot and make different tooth shapes. These models have shown that simple activator–inhibitor feedback loops are able to generate many kinds of cusp patterns (Salazar-Ciudad and Jernvall, 2002; Salazar-Ciudad and Jernvall, 2010). One requirement, however, for making diverse shapes is the coupling of growth with signaling. Hence, rather than forming a prepattern of enamel knots for each tooth shape, patterns are realized as the tooth germ grows. Activators of enamel knot differentiation include Wnt and BMP proteins, whereas inhibitors of differentiation, and hence factors promoting growth, include FGFs and Shh (Fig. 3C). Experimental evidence has implicated Wnt and BMP proteins in the regulation of *Shh* (Zhang et al., 2000; Ahn et al., 2010), which in turn has been proposed to inhibit Wnt signaling through Dkk1 (Ahn et al., 2010). The requirement for multiple factors is underscored by the effect of multiple pathways to substantially increase cusps number in mouse molars (Harjunmaa et al., 2012). Simultaneously administered superfluous Eda and activin A proteins, together with cyclopamine, which inhibits Shh signaling, doubles cusp number in cultured molars (Harjunmaa et al., 2012). This result suggests that the cusp number, and crown complexity in general, is a polygenic trait and multiple pathways need to be 'tinkered' with to generate mammalian dental diversity.

In conclusion, although multicusped teeth have evolved in many vertebrates, they are most diverse in mammals. By contrast, the players responsible for the developmental regulation of tooth shape appear to be largely conserved throughout the vertebrates. The same pathways are found to be active during tooth formation in fish, reptiles and mammals. A defining feature of mammals may be the presence of secondary enamel knots. Compared with the more diffuse expression domains found in reptiles, mammalian enamel knots appear to be a manifestation of more localized expression domains of the shared regulatory genes. The spatially localized signaling activity of the secondary enamel knots may be hypothesized to have allowed a highly refined regulation of cusp patterns, a requisite for exact occlusion in mammals. However, more comparative studies are required to show whether there are other differences in the regulation of tooth shape, such as subtly different expression domains of some signaling molecules. Regardless, it is likely that the repeated evolution of tooth crown features, such as the hypocone, stems from changes in gene regulation associated with enamel knot formation. To that end, the study of teeth offers multiple opportunities for testing the extent to which the regulation of the same genes is involved in the evolution of similar morphologies across species.

Tooth renewal: successional tooth replacement and continuous tooth growth

Enamel is resistant to abrasion, and both the thickness and microstructure of enamel have evolved to prolong the life of a tooth. Nevertheless, tooth wear causes a gradual loss of crown morphology and eventually the loss of the tooth. When teeth are lost at a faster rate than the life span of an animal, solutions to preserve dental function have evolved. The prevalent solution is tooth replacement, in which a worn out or lost tooth is replaced with a new one (Fig. 4) that develops from the so-called successional dental lamina at the lingual side of the preceding tooth. Continuous replacement of teeth throughout the life span of an individual is likely to be basal for all vertebrates and is found from fish to reptiles. Mammals differ from other vertebrates in that they have a limited capacity for tooth renewal, and the majority of extant mammals replace teeth maximally once. The so-called diphyodont dentition – that is, having deciduous (first set, 'milk teeth') and then permanent incisors, canines and premolars – is found in humans. Almost 50% of orders have species that are not fully diphyodont, and many taxa, such as muroid rodents, bats, shrews and the striped skunk, do not replace any of their teeth; hence, they are monophyodont (van Nievelt and Smith, 2005). The fossil record of early mammals shows a gradual reduction in tooth replacement, usually attributed to the evolution of exact occlusion, which in turn evolved with an increase in dental complexity (Kielan-Jaworowska et al., 2004).

An exceedingly rare mode of tooth regeneration found in mammals is the addition of new molars distally (behind the last molar, Fig. 2C). The little rock wallaby (*Peradorcas concinna*), the manatee (*Trichechus*) and the silvery mole rat (*Heliophobius argenteocinereus*) are currently the only known species in which

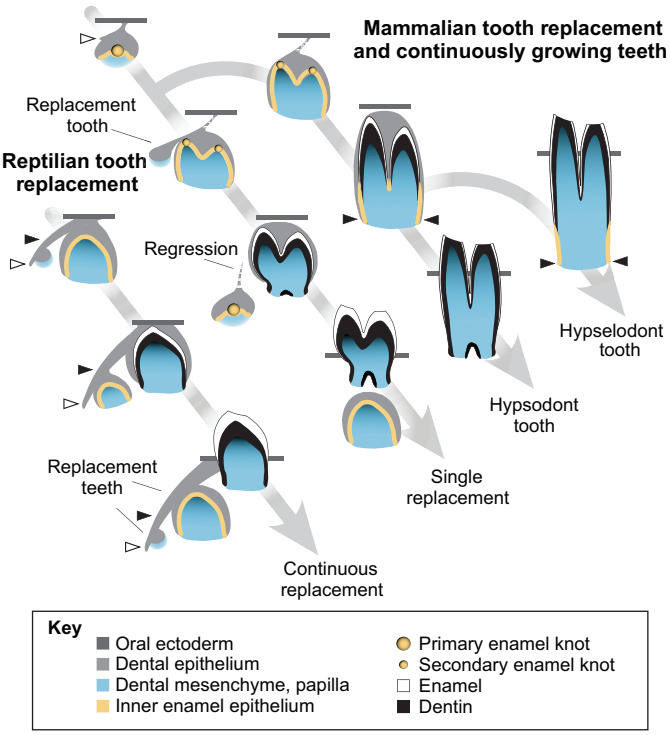

Fig. 4. Tooth renewal differs among species. The reptilian mode of continuous tooth replacement (shown on the left) involves a dental lamina that extends to form a successional lamina (white arrowheads). A stem cell niche is retained in the dental lamina (black arrowheads). This form of replacement is also likely to be present in many fish. In mammals, the replacement tooth bud develops from the successional lamina as in reptiles, but the lamina regresses and continuous tooth replacement does not occur. Instead, in many mammalian lineages teeth have become tall (hypsodont) by delaying root formation; hence, the teeth can wear more (also see Fig. 2C). The most-derived stage of tooth regeneration is hypselodonty, which is found, for example, in rodent incisors and vole molars, where the tooth retains stem cells at its base and continues to grow throughout the life of the individual.

new molars develop behind the distalmost molar (Rodrigues et al., 2011). As the anterior molars wear down, they are shed and all the teeth move gradually forwards, thereby making room for the new molars.

Another distinct but frequent solution to prevent tooth loss due to tooth wear in mammals is the evolution of tall, or hypsodont, teeth (Fig. 2C, Fig. 4). Hypsodont molars have evolved multiple times, and can be found in horses, cows and rodents (von Koenigswald, 2011). A derived case of hypsodonty is hypselodonty, in which teeth continue to grow throughout the life of an animal to compensate for wear. Hypselodont molars are found in many rodents, such as voles, and hares, but an extinct rhinoceros species has also been inferred to have been hypselodont (Janis and Fortelius, 1988). In addition to rodents, continuously growing incisors are found in one primate species, the aye aye (*Daubentonia*). Both tooth replacement and continuous tooth growth require the availability of competent stem and progenitor cells, and an increasing research effort has been dedicated to identifying such cells in teeth and analyzing their regulation. In this respect, the recently reported *Heliophobius* mole rat offers a unique example of having hypsodont molars that are continuously added distally (Rodrigues et al., 2011).

Tooth replacement in fish

There is considerable variation in the replacement patterns in fish. In cichlids, the replacement teeth bud off directly from oral epithelium (Vandervennet and Huysseune, 2005). In trout and salmon, replacement teeth form from a thickening in the outer enamel epithelium of the unerupted predecessor tooth (Fraser et al., 2006; Huysseune and Witten, 2006; Smith et al., 2009). In the pharyngeal dentition of zebrafish, the replacement tooth forms from a successional dental lamina that buds from the crypt epithelium of the already erupted and functional tooth (Huysseune, 2006). The zebrafish successional dental lamina grows in length and the tooth forms later at its tip. Because this lamina can persist for a while as such, without giving rise to a replacement tooth at its tip, it was suggested that the two events are under separate developmental control. Each tooth in zebrafish has a distinct successional lamina, thereby differentiating this lamina from the permanent and continuous lamina of reptiles (see below). Gene expression studies in trout show that *Pitx2* and *Bmp4* are expressed during tooth replacement (Fraser et al., 2006), but functional molecular data are not yet available.

Tooth replacement in reptiles

Most information on tooth replacement has historically, as well as recently, come from studies of reptiles. The detailed examination of lizards and alligators indicated that their tooth replacement is a regulated process that is not dependent upon tooth wear or loss, and that replacement teeth form from a successional dental lamina (Fig. 4) (Osborn, 1971; Westergaard and Ferguson, 1987). This lamina buds from the outer enamel epithelium of the preceding tooth, grows and generates the next tooth at its tip. The consecutive generations of replacement teeth develop sequentially from the successional dental lamina. This characteristic reptile pattern of tooth replacement was also recently described in snakes and geckos, together with the dynamics of gene expression during tooth development in these animals. Activities of both Wnt and BMP signaling were detected during the initiation of successional lamina, whereas *Shh* expression was absent (Handrigan and Richman, 2010b; Richman and Handrigan, 2011). In the adult leopard gecko (*Eublepharis macularius*), putative stem cells have been found in the lingual dental lamina but not within the highly proliferative successional dental lamina (Handrigan et al., 2010). These cells are slow-cycling and express known stem cell markers, including *Lgr5*, *Dkk1* and *Igfp5*. Because stimulation of the Wnt/β-catenin pathway caused proliferation in the dental lamina, Handrigan et al. (Handrigan et al., 2010) suggested that the regulation of the gecko stem cells is quite similar to that of mammals.

Tooth replacement in mammals

Relatively little is known about the mechanisms of tooth replacement in mammals, largely because the most used model species, the mouse, has lost the capacity to replace its teeth. Histological analyses of tooth replacement in several species have indicated that the secondary teeth originate from the free end of the dental lamina lingual to their deciduous counterpart, and this is basically a similar process to that operating in reptiles (Berkowitz, 1973; Lucket, 1993). More recently, ferret tooth replacement was revisited through the three-dimensional reconstruction of developing teeth (Järvinen et al., 2009). Resembling tooth replacement in reptiles, the permanent teeth of ferret develop lingually from a successional dental lamina. This lamina elongates and forms a bud at the tip, which in turn gives rise to the replacement tooth. The timing of lamina elongation and bud

initiation appears to differ between different teeth. This heterogeneity between elongation and budding suggests that there are distinct control mechanisms for the initiation of replacement and for the initiation of the morphogenesis of the replacement tooth (Järvinen et al., 2009).

Gene expression analysis of tooth replacement in ferrets showed localized intense expression of *Sosdc1* in the lingual aspect of the deciduous tooth enamel organ at the onset of successional lamina formation (Järvinen et al., 2009). *Axin2*, which is indicative of active Wnt/β-catenin signaling, was expressed in the mesenchyme, whereas *Shh* expression was not associated with the replacement process at any stage, although it was intensely expressed in the enamel knots. These expression patterns in ferrets are in line with the findings in reptiles (Handrigan and Richman, 2010a; Handrigan and Richman, 2010b) and suggest the involvement of Wnt and Bmp, but not Shh signaling in tooth replacement. Functional evidence for the role of Wnts in mammalian tooth regeneration is provided by experiments in which multiple teeth develop successionally in vitro after Wnt/β-catenin signaling is activated in the oral epithelium (Fig. 5) (Järvinen et al., 2006). This raises the possibility that Wnt signaling can reactivate or 'unlock' tooth replacement that has been lost in evolution. An additional, atavistic-like effect in these Wnt-based experiments is the simplification of teeth to be principally conical in shape (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009). Although the molecular level interactions are not yet known, a loss-of-function of transcription factor *Sp6* (epiprofin) also produces multiple supernumerary but simple teeth in mouse (Nakamura et al., 2008). As in cusp patterning, adjusting the balance between activation and inhibition can be used to model the effects of Wnt signaling on patterning (Fig. 5) (Järvinen et al., 2006).

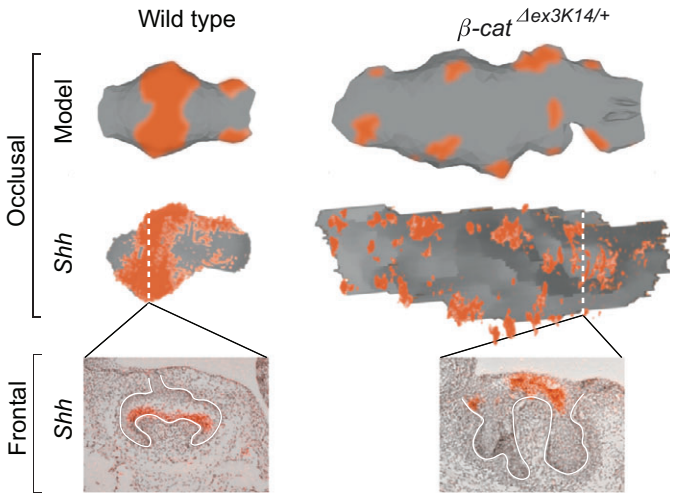

Fig. 5. Computational and experimental tooth renewal. A computational model implementing activator–inhibitor-like dynamics in the regulation of enamel knots and growth reproduces many of the effects of stabilized Wnt signaling in the epithelium (Järvinen et al., 2006). The teeth, shown from an occlusal view (top), are at E16, and the 'wild-type' and 'mutant' model simulations are run for the same number of iterations; only the activator–inhibitor interaction has been altered. Model images show simulated Shh expression (orange) over shape; *Shh* images show real Shh expression (orange) over shape. Orange indicates xxxxxx. Dashed white lines in the three-dimensional renderings show the approximate locations of the histological sections, which are shown below. Model and data are based on results from Järvinen et al. (Järvinen et al., 2006) and Salazar-Ciudad and Jernvall (Salazar-Ciudad and Jernvall, 2010).

Mammalian molars do not have replacement teeth and individual molars develop from distal extension of the dental lamina. Molars develop sequentially, as do other teeth of the primary dentition; thus, in mice and humans the third molar (the wisdom tooth) is the last to develop (Fig. 6A). Whereas the first mouse molar develops quite normally when cultured in vitro, the second and third molars are frequently delayed or missing in culture conditions. Kavanagh et al. (Kavanagh et al., 2007) were, however, able to rescue the development of distal teeth by dissecting the posterior extension of dental lamina and culturing it separately from the first molar. This was interpreted to be due to inter-molar inhibition, where the size and number of distal molars depend on the balance between mesenchymal activators that promote enamel knot induction and inhibitor that are expressed in the previously initiated molars. These inhibitors have not yet been identified, but Bmp4 and activin A were shown to be able to accelerate posterior molar development (Kavanagh et al., 2007). Furthermore, the relationship between the inhibition and activation of molar development can be formulated as a quantitative inhibitory cascade model that predicts molar proportions in a wide range of rodent species (Kavanagh et al., 2007) (Fig. 6A). Accordingly, many supernumerary molars that are equal in size are most likely to appear when activation and inhibition are in balance. Indeed, in species with continuous addition of molars distally, individual teeth are approximately equal in size (Rodrigues et al., 2011), and it can be hypothesized that the distal lamina of the molars behaves much like the successional lamina in tooth replacement (Järvinen et al., 2008).

Continuously growing teeth in mammals

The most frequently observed evolutionary solution to prevent tooth loss due to wear in mammals is the development of high crowned (hypsodont) and continuously growing (hypselodont) teeth. Developmentally, hypsodont teeth are attained by delaying the onset of root formation, which then can lead to the formation of hypselodonty when roots fail to develop entirely (Fig. 4). Normally, roots begin to develop from the cervical loop when the enamel organ epithelium stops differentiating into the enamel-forming ameloblasts and instead forms the bilayered Hertwig's epithelial root sheath (HERS). HERS directs tooth formation but has a limited capacity to proliferate, and therefore root growth eventually stops. *Fgf10* is downregulated in the cervical loop mesenchyme in molars (Kettunen et al., 2000) and this is apparently a prerequisite for root development as the addition of recombinant Fgf10 to cultured molars maintains the stellate reticulum cells in cervical loops and inhibits HERS formation (Yokohama-Tamaki et al., 2006). Epidermal growth factor (EGF) has effects similar to those of Fgf10, while insulin-like growth factor 1 (Igf1) stimulates HERS proliferation and the growth of the root itself (Fujiwara et al., 2005; Fujiwara et al., 2009). When teeth are cultured in vitro long enough for roots to form, cervical loops with stellate reticulum cells are maintained on the lateral sides of the root, whereas HERS forms between the root and the filter, suggesting that the cervical loop-to-HERS transition is sensitive to environmental factors (Tummers et al., 2007).

Whereas continuously growing (hypselodont) molars are found in many mammalian species, the principal model system with which to study hypselodonty has been the mouse incisor. Rodent incisors are evolutionarily derived teeth in which the sharpness of the cutting edge is formed by continuous growth and the asymmetric deposition of the hard enamel at the anterior side,

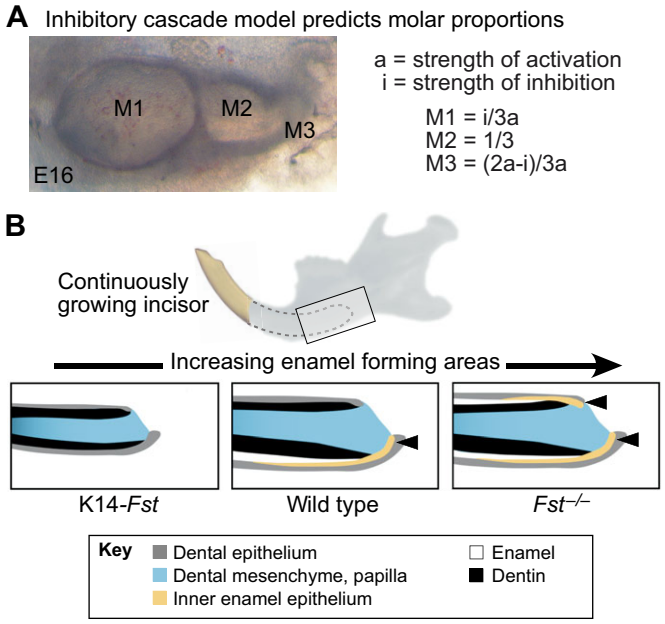

Fig. 6. Sequential initiation of molars and continuous growth of rodent incisors. (**A**) Molar teeth (M1, M2 and M3) develop sequentially. Many species have the potential to develop a fourth molar after the formation of the third molar and a few species have continuous generation of new molars. An inhibitory cascade model, derived from experimental manipulation of mouse molars, predicts molar proportions and number. When supernumerary molars form, the molars should be roughly the same size. In this model, 'a' denotes strength of the activator and 'i' denotes strength of the inhibitor. Candidate activators include BMPs and activin A; candidate inhibitors include Shh. (**B**) Continuously growing incisors of a wild-type mouse retain epithelial stem cells (black arrowheads) on their labial (outer) side. The lingual side lacks enamel. Deletion of follistatin (*Fst*$^{-/-}$) results in ectopic lingual enamel, whereas the expression of follistatin throughout the epithelium under the control of the keratin 14 promoter (K14-*Fst*) causes a loss of enamel and of the stem cell niche. Follistatin is not normally expressed in the labial inner enamel epithelium. Molar culture image in A is modified, with permission, from Kavanagh et al. (Kavanagh et al., 2007).

while the lingual side remains covered with dentin. The large cervical loop on the labial side houses the stem cells for enamel-forming ameloblasts.

The cervical loop stem cell niche of incisors was first identified when label-retaining cells were found to be localized inside the stellate reticulum (Smith, 1980; Harada et al., 1999) and more recently by using in vivo genetic lineage tracing (Seidel et al., 2010; Juuri et al., 2012). This work also shows that the progeny of the stem cells invade the loop epithelium, proliferate as transit amplifying cells and eventually differentiate into ameloblasts. Additionally, *Sox2* marks the incisor stem cells and *Sox2*-expressing cells contribute not only to ameloblasts but also to all other epithelial cell lineages of the tooth (Juuri et al., 2012). The cervical loop resembles other stem cell niches, such as the crypts of the intestine and the bulge of the hair follicle. All of these niches are surrounded by mesenchymal tissue, providing cues to the epithelium that are necessary for the self-renewal and differentiation of epithelial cells. In the incisor, the maintenance and differentiation of the stem cells are regulated by signals of many protein families. The requirement for FGFs has been repeatedly demonstrated; in particular, mesenchymally expressed *Fgf10* and its receptor *Fgfr2b* in the epithelium have been shown to be necessary for stem cell maintenance and proliferation (Harada et al., 2002; Wang et al., 2007; Klein et al., 2008; Parsa et al., 2010). *Bmp4* and activin βA are expressed in mesenchyme and also act in signal networks with FGFs to inhibit and stimulate stem cells, respectively (Wang et al., 2007). Shh is produced by the preameloblasts, which are the progeny of the epithelial stem cells. Seidel et al. (Seidel et al., 2010) showed that Shh is required for the generation of stem cell progeny but not for the survival of stem cells themselves. Wnt signaling seems to be absent in the cervical loop stem cell compartment (Suomalainen and Thesleff, 2010).

The modulation of signal pathways in transgenic mice has provided further insights into the regulation of the epithelial stem cells and their differentiation into enamel-forming ameloblasts. Deletion of the genes encoding the BMP inhibitor follistatin results in ectopic enamel formation on the lingual surface of the incisor and stimulates growth of the lingual cervical loop, resulting in a symmetric incisor (Fig. 6B) (Wang et al., 2004; Wang et al., 2007); similarly, inhibition of the function of Spry genes results in large incisors with enamel also covering the lingual side (Klein et al., 2008). Furthermore, overexpression of the BMP inhibitor Noggin results in stimulated growth of the incisor as well as a total absence of enamel (Plikus et al., 2005). These results underscore the delicate balance of signaling to regulate enamel formation and the development of the asymmetric incisor.

The same signal networks that regulate the incisor stem cells are also active in the stem cell niche discovered in the continuously growing vole molars (Tummers and Thesleff, 2003), indicating a conservation of the regulation of continuous growth of different teeth. Because there are both hypsodont and hypselodont vole species, comparisons of otherwise similar vole molars may yet be useful in determining how open-ended teeth grow.

In conclusion, as in the case of tooth shape formation, largely the same regulatory pathways that govern tooth renewal are found across vertebrates. Interestingly, Shh appears not to be active either in tooth replacement or stem cell survival but is required for the later steps of stem cell recruitment to the growing tooth. Analogously, the role of Shh in crown formation appears not to be the induction of the enamel knots but the following spatial regulation of knots and cusps (Fig. 3C). The limited capacity for tooth replacement in mammals has been compensated for by the evolution of continuously growing teeth, the development of which appears to reuse the regulatory pathways of tooth replacement. Furthermore, it can be hypothesized that, similar to other types of tooth renewal, development of supernumerary molars at the distal end of the tooth row may also depend on stem cells. It remains to be tested whether mouse molar development could be made open ended as in wallabies and manatees. Regardless, the possibility to regenerate adult teeth from in vitro cultured explants (Nakao et al., 2007) makes the potential for therapeutic tooth regeneration a relatively tangible goal.

Conclusions

In summary, recent studies of tooth development in various vertebrate species have shown that many of the same molecular pathways appear to be involved in the determination of both tooth shape and tooth renewal. Furthermore, the same molecular tools, or modules, appear to have been retained throughout evolution. The complex teeth of mammals use many of the same molecular modules as fish teeth. What appears to have changed during evolution is the refinement of the spatial and temporal use of the

shared signaling pathways. Similarities observed between species in signaling are undoubtedly partly due to the currently limited inventory of genes. To this end, there may yet be other components of signaling involved in tooth development, of which some may be active only in some taxonomic groups. For example, inhibition of microRNAs in mice affects cusp patterning and the incisor stem cell niche (Cao et al., 2010; Michon et al., 2010), and it will be interesting to examine microRNA function in fish and reptile tooth development. In general, genomic tools are likely to make data from multiple new species readily available. Questions concerning the repeated evolution of individual cusps, the role of stem cells in the formation of hypselodont teeth or the regulation of tooth replacement may benefit from comparative approaches that take advantage of the diversity of genomes and teeth. Comparative approaches will also help to identify regulatory cis-regions that are specific to teeth. Computational tools used in connection with genetic tools should offer a more refined understanding of the regulation of tooth shape and the detailed morphologies such as cusp shape and size, which are all crucial for correct occlusion. In addition to signaling, the study of tissue properties such as cell adhesion, proliferation and migration are likely to provide new insights into what exactly is regulated when cusp shape or number is changed.

Acknowledgements

We are grateful to Alistair Evans, Ian Corfe, Janne Granroth, Enni Harjunmaa, Teemu Häkkinen, Elina Järvinen, Tuomas Kankaanpää, Juha Laakkonen, Jackie Moustakas, Elodie Renvoisé and Susanna Sova for help with the illustration materials. We thank Jackie Moustakas, Ian Corfe, Isaac Salazar-Ciudad and three anonymous referees for comments and discussions.

Funding

This work was supported by the Academy of Finland and the Sigrid Juselius Foundation.

Competing interests statement

The authors declare no competing financial interests.

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