

Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species

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SUMMARY

The rodent incisor grows continuously throughout its lifetime. The epithelial stem cell niche is located at the apical end of the tooth and its progeny gives rise to the ameloblasts that form the hard enamel. Previously, mesenchymal FGF10 was shown to support the niche, in conjunction with epithelial Notch signaling. Here we show that in a different continuously growing tooth type, the molar of the sibling vole, a similar regulatory system is in place. Moreover, the identical expression pattern of *Bmp4* compared to *Fgf10* suggests that BMP4 could also be involved in the regulation of the epithelial stem cell niche.

Notch and FGF10 signaling is mainly absent in the mouse molar, which stops growing and develops roots. The regulation of the epithelial stem cell niche seems to be flexible allowing for the existence of different tooth types, such as continuously growing teeth, and high and low crowned molars.

Key words: Morphogenesis, Evolution, Tooth, Stem Cell, Root Formation, Crown formation, Sibling vole, *Microtus rossiaemeridionalis*, Mouse, *Mus musculus*, FGF, BMP, Notch, Hypselodont, Hypsodont

INTRODUCTION

Adult stem cells require a special environment and are therefore usually found in the so-called stem cell niche (Spradling et al., 2001; Watt and Hogan, 2000; Nishimura et al., 2002). The niche can be defined as the environment, which allows the stem cells to function; i.e. it houses the stem cells, allows for self-renewal of the stem cells and is instructive in the generation of the differentiating progeny. Many types of stem cells have been shown to have a high degree of plasticity when transplanted, emphasizing the importance of the local environment in the regulation of differentiation (Bjornson et al., 1999; Anderson et al., 2001).

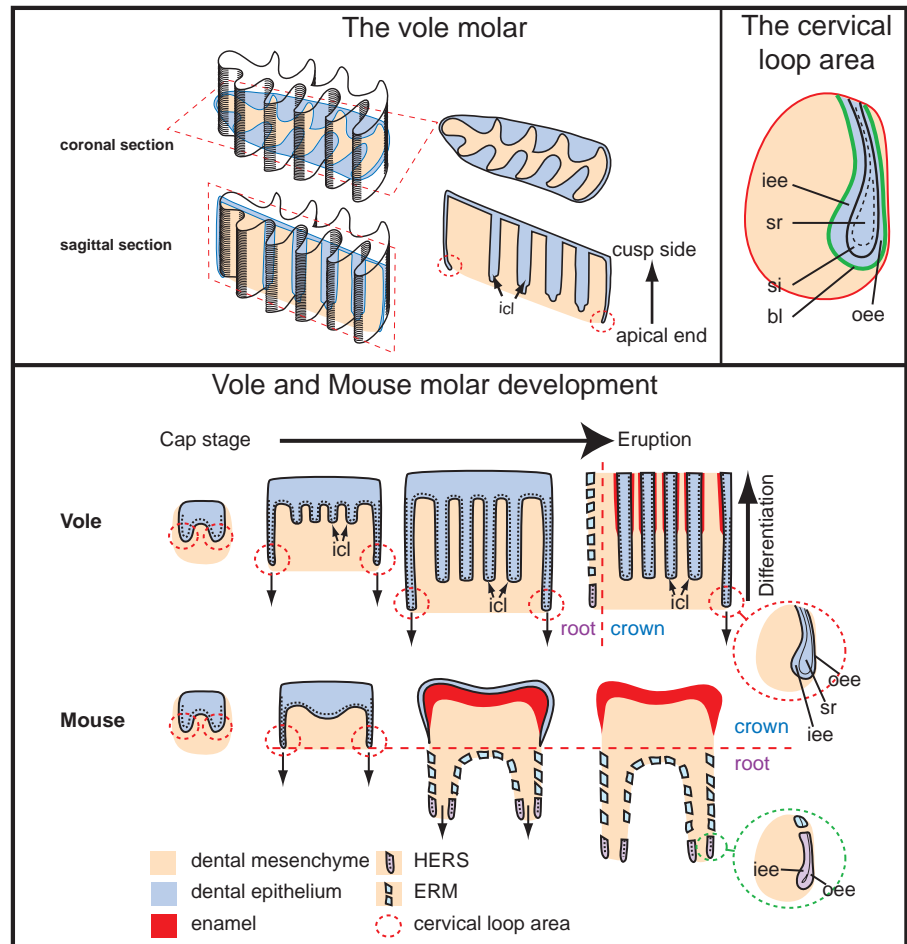
The incisor of rodents represents a special tooth type since it grows continuously throughout the lifetime of the animal. Therefore, it must possess adult mesenchymal and epithelial stem cells. The exact location of the mesenchymal stem cells in teeth is not clear, although dental mesenchymal cells have been isolated from the pulp of adult human teeth (Gronthos et al., 2000). The cervical loop area located opposite of the distal tip of the tooth, and more specifically the epithelial tissue named stellate reticulum, has been put forward as the putative site of the epithelial stem cell compartment in the mouse incisor (Harada et al., 1999). It was also suggested that Notch signaling and FGF10 are involved in the regulation of this epithelial stem cell compartment (Harada et al., 2002). In the incisor the stem cells migrate from the stellate reticulum to the inner enamel epithelium and contribute to a pool of proliferating cells, also known as transit-amplifying cells. These cells then move towards the distal tip and show

increasingly higher states of differentiation of the ameloblast cell lineage. The ameloblasts produce and deposit the enamel matrix responsible for the hardness of the tooth. The enamel is then constantly worn down at the distal tip of the incisor. In contrast, in mouse molars and all human teeth for instance, the stellate reticulum is lost after crown formation and a double layer of root sheath epithelium is left that directs root formation (Fig. 1).

The rodent incisor is not the only tooth type that shows continuous growth. Also molars of certain species grow continuously. One such species is the rabbit. Tritium labeling studies suggested that also in the rabbit molar the cervical loop area is the origin of the epithelial cell lineage (Starkey, 1963). It was concluded that the pool of transit-amplifying cells in the inner enamel epithelium cannot be sustained by itself, but probably originates from the stratum intermedium, which is the denser layer of the stellate reticulum closest to the inner enamel epithelium. A cell that is recruited into the basal layer of proliferating cells must therefore change epithelial compartments and actually re-laminate itself into the inner enamel epithelium, since the opposite side of the inner enamel epithelium faces the dental mesenchyme and therefore also borders with a basal lamina (Ten Cate, 1961).

Here we focus on a less well-known tooth system; the continuously growing molar of a vole species known as the sibling vole (*Microtus rossiaemeridionalis*). The vole and mouse are both rodents and are closely related species. Previously, the earlier stages of the vole and mouse molar were compared until the late bell stage of development (E17), and the basic aspects of morphogenesis and the distribution of

Fig. 1. Coronal and sagittal sections of the vole molar show a distinct and complex morphology. The general appearance of the sagittal section is dependent on the position it is taken, unlike most of the coronal sections. Here we used the particular sagittal section as shown that runs through the middle of the molar. The developmental histories of the vole and mouse molar start similarly and are almost identical until cap stage. After this, the actions of the enamel knots result in a different folding pattern of the epithelium. This results in the intercusp folds or loops in the vole molar that reach almost down to the base. In the mouse molar the cervical loop epithelium loses its crown fate, i.e. it loses the stellate reticulum, and switches to root. In the vole molar most cervical loop epithelium retains the crown fate except for three small areas that are converted to root fate. After completion of root formation the mouse molar has no functional cervical loop epithelium left (i.e. it is missing stellate reticulum) unlike the vole molar, where most of the cervical loop continues to generate crown. ERM, epithelial cell rests of Malassez; HERS, Hertwig's epithelial root sheath; bl, basal lamina; icl, intercusp loop; iee, inner enamel epithelium; oee, outer enamel epithelium; si, stratum intermedium; sr, stellate reticulum; dashed circles indicate the cervical loop area.



important developmental regulatory molecules were found to be almost identical (Keränen et al., 1998). Nature, therefore, provided for us the experimental setup. We have two tooth systems, the molar of the vole and the molar of the mouse, whose early development and morphogenesis are remarkably similar, but later venture on different developmental paths. The mouse molar develops roots and stops growing, the vole molar maintains the crown fate and will grow continuously. Therefore, a regulatory difference must be present and through comparison of the two phylogenetically closely related systems the developmental mechanism responsible for this divergence might be discovered.

From the incisor it is known that FGF10 and Notch signaling is important for the maintenance of the stem cell niche and the continuous growth of the mouse incisor (Harada et al., 1999; Harada et al., 2002). We compared the expression patterns of these genes between mouse and vole molar. Similarities in this regulatory system between the vole molar and mouse incisor suggested that both share a common regulatory mechanism that maintains the epithelial stem cell niche and epithelial cell lineage. In the mouse molar this regulatory system disappears and root development takes place. The analysis of the morphology of the vole molar additionally revealed the presence of three small domains lacking ameloblasts and enamel. These areas represented functional root structures and can be compared to the lingual side of the rodent incisor. This area in the incisor looks and

functions as a root, but no classic root formation takes place, and hence is known as the root analogue. The switch between root and crown epithelium can therefore occur locally during development and is flexible in nature.

MATERIALS AND METHODS

Tissues

The mouse (*Mus musculus*) tissues were obtained from CBAT6T6 × NMRI matings, the sibling vole (*Microtus rossiaemeridionalis*) tissues were obtained from a colony kept at the Department of Ecology and Systematics, Division of Population Biology, University of Helsinki. Mouse tissues were collected at 10 and 14 days after birth (dpm). Vole tissues were collected at 2, 5 and 14 dpm. Only lower jaws were used and fixed overnight in 4% PFA, decalcified in 2% PFA and 12.5% EDTA for 2-3 weeks. After dehydration and xylene treatment they were embedded in paraffin and cut serially in 7 or 10 μm thick sections.

In situ hybridization

The in situ hybridization with ³⁵S-UTP (Amersham) labeled riboprobes was performed as described previously (Wilkinson and Green, 1990). The slides from the radioactive in situ hybridization were photographed with an Olympus Provis microscope equipped with CCD camera (Photometric Ltd). Figures were processed using Adobe Photoshop (Adobe Systems, CA); the dark-field images were inverted and artificially stained red and combined with the bright-field image) and Micrographx Designer software. Every radioactive in situ

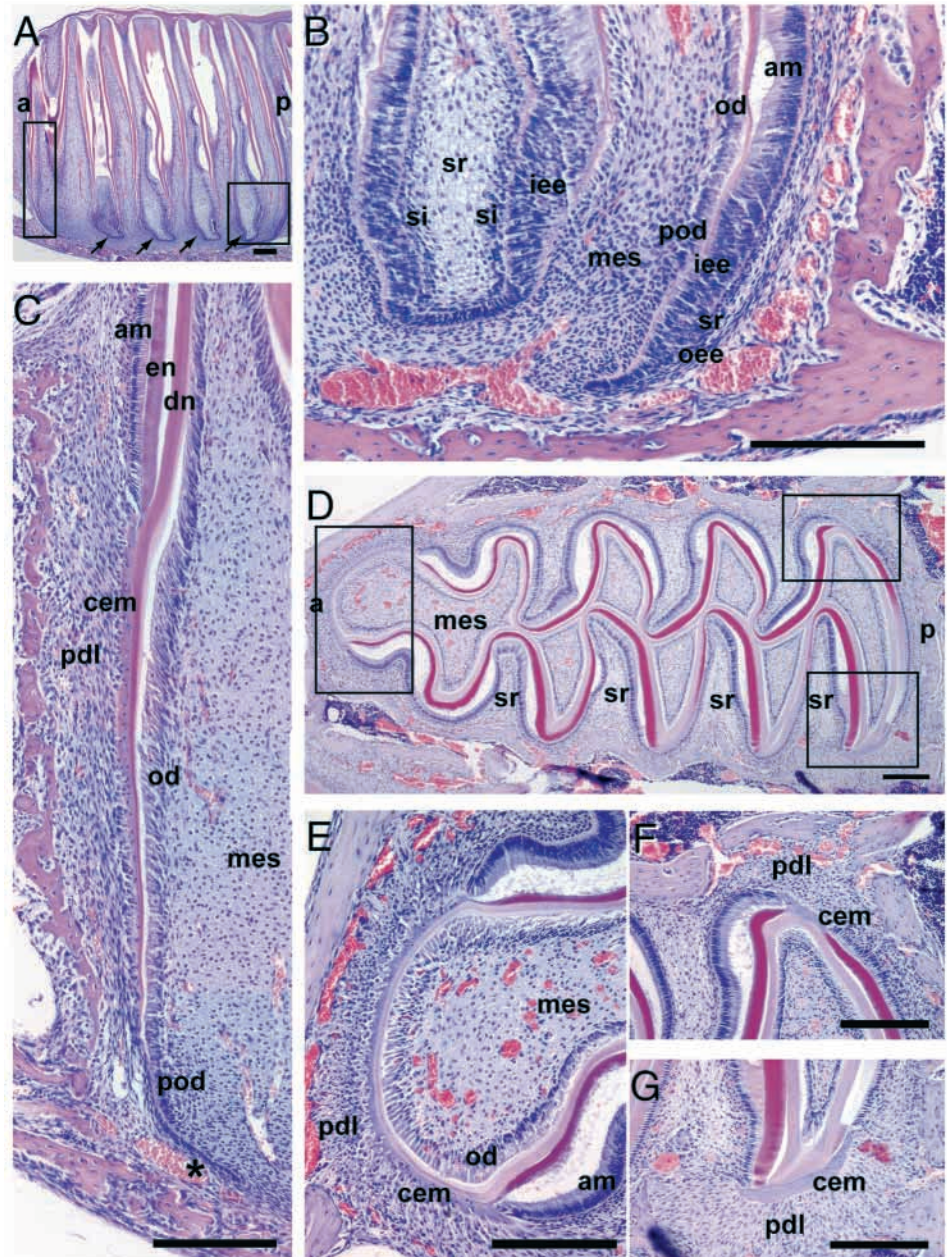


Fig. 2. Hematoxylin and eosin staining of sagittal (A-C) and coronal (D-F) sections of the vole molar. (A) Sagittal section of entire first vole molar (B) Higher magnification of the posterior cervical loop area of the vole molar. (C) Higher magnification of the anterior region showing the anterior root domain. (D) Coronal section of the vole molar showing the location of the three root domains (boxed). (E-G) Higher magnifications of the root domains. a, anterior; p, posterior; am, ameloblasts; pod, preodontoblasts; od, odontoblasts; pdl, periodontal ligament; cem, cementum; hers, hertwig's epithelial root sheath; en, enamel; dn, dentin; mes, mesenchyme; sr, stellate reticulum; si, stratum intermedium; iee, inner enamel epithelium; oee, outer enamel epithelium. Arrows in A indicate the intercusp loops. Asterisk in C indicates Hertwig's epithelial root sheath. A and C are from 5 dpn tissue. B,D,E,F,G are from 14 dpn tissue. Scale bars are 0.2 mm. The boxes in A indicate the areas magnified in B and C and the boxes in D indicate those in E-G.

hybridization was carried out at least two times, and during each experiment each individual probe was tested on several different sections. Most genes were examined more than twice and also in sections cut at different angles.

Probes

The following plasmids were used for the ^{35}S -UTP probes. All probes originated from mouse sequences except for FGF10, which originated from rat. The *Lfng* probe was a kind gift from Alan Wang (Harada et al., 1999), *jagged1* (*Jag1*)- and *delta1* (*Dll1*)-containing plasmids were a kind gift from Domingos Henrique (Mitsiadis et al., 1997; Bettenhausen et al., 1995), *Notch1*, 2, 3 probes from Urban Lendahl (Lardelli et al., 1994; Larsson et al., 1994) and *Hes1* and *Hes5* from Royuhiro Kageyama (Sasai et al., 1992). The rat *Fgf10* and murine *Fgf3* probes have been described previously (Kettunen et al., 2000). It has been shown that probes that work in the mouse generally also work in the sibling vole (Keränen et al., 1998).

RESULTS

Morphology of the crown of the vole molar

The epithelial stellate reticulum in the cervical loop area is the putative site for the location of the epithelial progenitor cells in the incisor (Harada et al., 1999). The cervical loop of the incisor contains a large amount of stellate reticulum, sandwiched in between a basal cell layer consisting of inner and outer enamel epithelium (Fig. 1). Only the outer enamel epithelium borders the dental mesenchyme and is therefore connected to a basal lamina. The stellate reticulum that borders the layer of basal epithelium is usually denser and referred to as stratum intermedium. In the vole molar there is considerably less stellate reticulum in the cervical loop area (Fig. 2B). Serial sectioning (frontal and sagittal) showed that the cervical loop is a continuous structure that spans the entire circumference of the base of the molar. A major morphological difference with

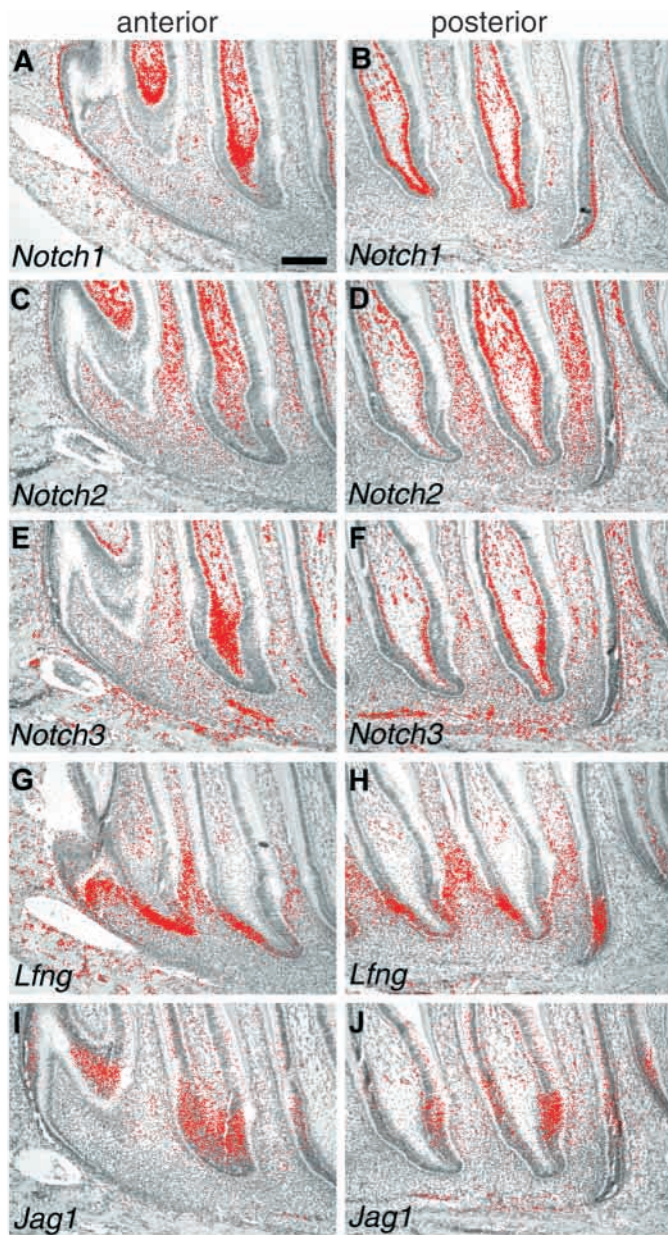


Fig. 3. Localization of *Notch1* (A,B), *Notch2* (C,D), *Notch3* (E,F), *Lfng* (G,H) and *Jag1* (I,J) in anterior and posterior sagittal sections of 2 dpn vole molars. Scale bar is 0.2 mm.

the incisor is the presence of the intercuspal folds or loops (Fig. 2A,B). To create the typical cusp pattern of the vole molar the epithelium has folded several times into the mesenchyme in a complex manner (Fig. 1). These folds run almost all the way to the base of the tooth and create large epithelial compartments that run deep down into the dental mesenchyme. These compartments consist of a basal layer of inner enamel epithelium enclosing a large compartment of stellate reticulum and stratum intermedium. The basal epithelial layer is separated from the dental mesenchyme with a basal lamina. Structurally the intercuspal loop therefore resembles the cervical loop. The intercuspal folds are not present in the rodent incisor since these lack an intricate cusp pattern.

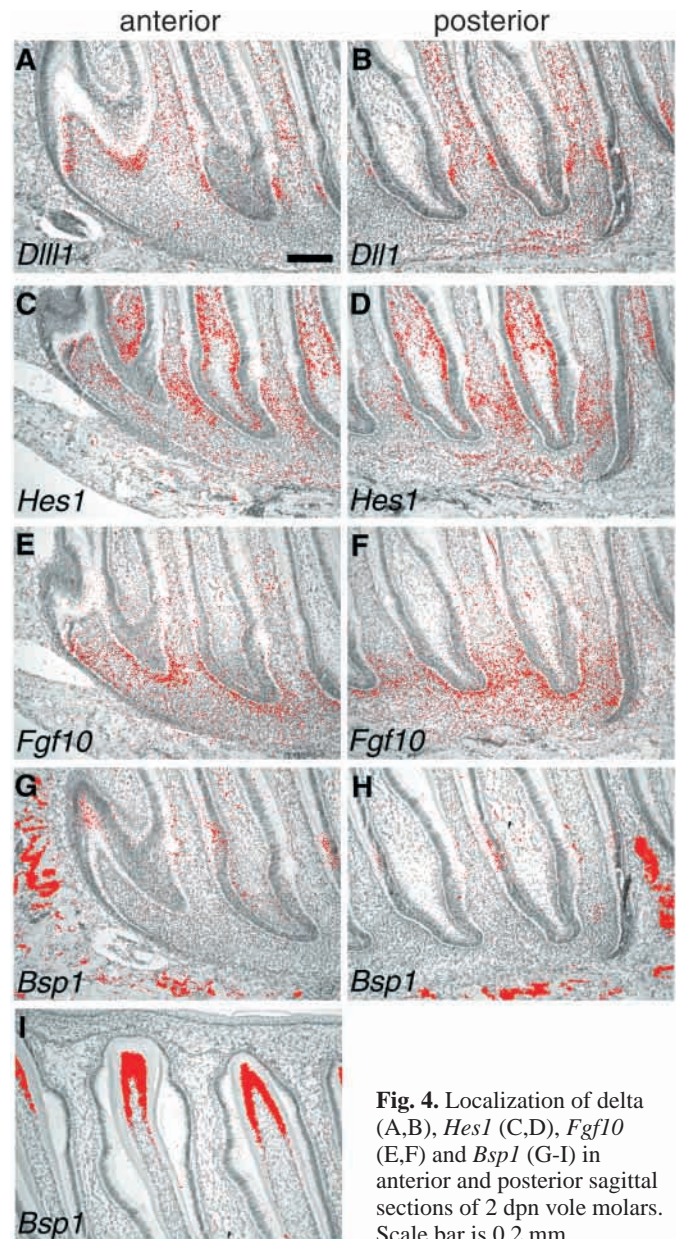


Fig. 4. Localization of delta (A,B), *Hes1* (C,D), *Fgf10* (E,F) and *Bsp1* (G-I) in anterior and posterior sagittal sections of 2 dpn vole molars. Scale bar is 0.2 mm.

Notch, BMP and FGF signaling identifies the epithelial stem cell niche in the vole molar

In the mouse incisor the Notch signaling pathway genes and *Fgf10* are expressed in a distinct pattern and are thought to be important for the regulation of the epithelial stem cell niche. In the vole molar we found a similar pattern and this pattern did not change from 2 dpn to 14 dpn in the crown area (14 dpn not shown). Here *Notch1*, 2 and 3 were expressed most strongly in the stellate reticulum compartment of the cervical and intercuspal loop, with especially strong expression in the stratum intermedium (Fig. 3B,D,F, Fig. 5B,D). *Notch2* and 3 were also expressed throughout the mesenchyme at lower levels and *Notch1* and 3 were associated with blood vessels. *Lfng* expression was present in the inner and outer enamel epithelium corresponding roughly to the regions containing the transit-amplifying cells (Fig. 3H, Fig. 5F). Above this *Lfng* domain *Jag1* was expressed in the inner enamel epithelium in

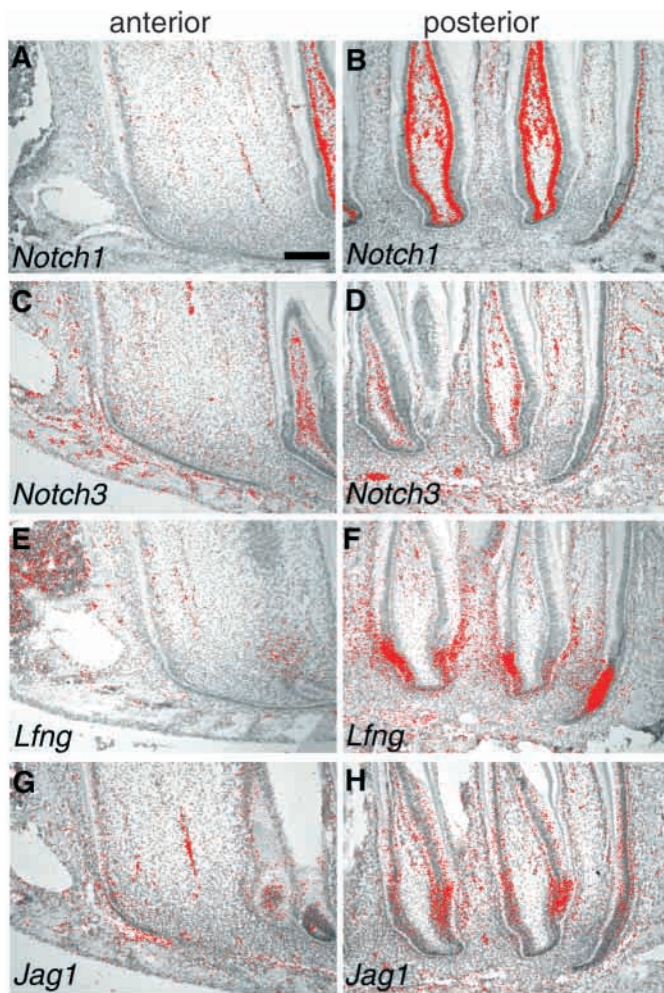


Fig. 5. Localization of *Notch1* (A,B), *Notch3* (C,D), *Lfng* (E,F) and *Jag1* (G,H) in anterior and posterior sagittal sections of 5 dpn vole molars. Scale bar is 0.2 mm.

a restricted domain corresponding to differentiating ameloblasts (Fig. 3J, Fig. 5H). The mesenchymal *Dll1* expression domain was found at the same height as the epithelial *Jag1* domain and was restricted to preodontoblasts (Fig. 4A,B). This indicated that odontoblast and ameloblast differentiation take place at the same crown level in the vole molar. *Hes5* expression could not be detected (data not shown), but *Hes1* was present throughout the stellate reticulum and mesenchyme with the highest levels of expression in the stratum intermedium, similar to the combined *Notch* expression patterns (Fig. 4D, Fig. 6D). *Bsp1* (black spleen) was used as a differentiation marker and its expression domain was exactly above that of *Jag1* in the inner and outer enamel epithelium (Fig. 4H, Fig. 6J). Near the cusps, *Bsp1* was also expressed in the coronal layer of the mesenchyme of the pulp chamber indicating a function in the closure of the pulp chambers (Fig. 4I).

Previously it has been shown that mesenchymal *Fgf10* supports the epithelial stem cell compartment in the mouse incisor (Harada et al., 2002; Harada et al., 1999). In the vole molar, *Fgf10* expression was also seen in the mesenchyme with the strongest expression near the base of the tooth, and was

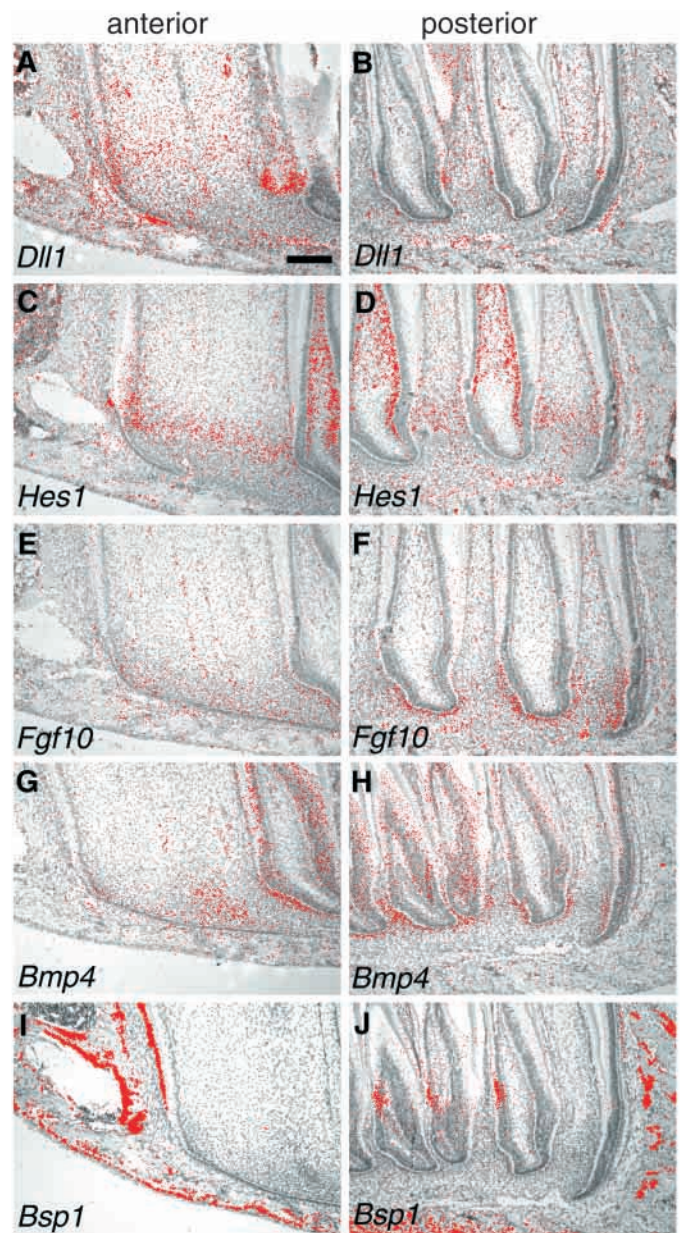


Fig. 6. Localization of delta1 (A,B), *Hes1* (C,D), *Fgf10* (E,F), *Bmp4* (G,H) and *Bsp1* (I,J) in anterior and posterior sagittal sections of 5 dpn vole molars. Scale bar is 0.2 mm.

especially strong around the intercuspal and cervical loop (Fig. 4E,F, Fig. 6E,F). These are the sites where the proliferating cells and the putative stem cells are located. *Bmp4* showed an identical mesenchymal pattern as *Fgf10* (Fig. 6G,H). *Hes1* and *Lfng* expression could both be seen as a broad band running from anterior to posterior (Fig. 3G,H, Fig. 4C,D, Fig. 5E,F, Fig. 6C,D). The band of *Lfng* expression was bordering that of *Hes1* but was located higher, nearer the cusps. Inside the *Hes1* domain, gaps were present surrounding the intercuspal folds and cervical loop, which coincided with the expression domains of *Fgf10* and *Bmp4* (Fig. 4C,D, Fig. 6C,D).

Three root analogue domains in the vole molar

Almost the entire area of cervical loop epithelium of the vole

molar maintains the crown fate throughout the life of the animal and continues to produce ameloblasts. Coronal sections revealed the presence of three small functional root domains, similar in appearance to the root of the mouse molar (Fig. 2D-G). A larger domain was present at the anterior and two smaller ones at the very ends of the crescent-shaped posterior section. None of these root domains were present at 2 days after birth, but their development had visibly started 5 days after birth. As can be seen in Fig. 2C the root domain reached only halfway up the tooth at 5 dpn. Above it there are still ameloblasts with enamel. At 14 dpn the root zone was continuous from apex to the cusps. Histological analysis showed that this enamel-free zone contained all the structures of a root, such as dentin, cementum, cementoblasts, cementocytes and periodontal ligament (Fig. 2C). Also, the apical epithelium was seen as a double layer, identical to Hertwig's epithelial root sheath, which became fragmented further away from the apex. The cementoblasts showed a strong *Bsp1* signal in the radioactive in situ hybridization (Fig. 6I).

Differential gene expression in the root and crown domain of the vole molar

In the vole molar two distinct domains are present, the crown and root domain. All the Notch signaling pathway gene expression patterns were similar at the anterior and posterior side of the vole molar in the 2 dpn tissue when the root domains are still visibly absent (Figs 3, 4). *Fgf10* was expressed at the anterior root side similarly to the posterior crown side with strong expression near the anterior cervical loop. At 5 dpn, however, the formation of the root analogue in their restricted domains is well underway and now the general trend is the absence of the Notch pathway genes in the epithelial compartments of the anterior root analogue. *Notch1* was no longer expressed in the anterior cervical loop epithelium (Fig. 5A). Epithelial *Hes1* expression was missing here, although the mesenchymal band of *Hes1* expression still ran across the entire tooth base (Fig. 6C,D). No anterior epithelial *Lfng* expression was visible and only a slight expression of mesenchymal *Lfng* was detected in the subodontoblastic layer (Fig. 5E). Anterior *Jag1* expression was weak and lacked clear boundaries (Fig. 5G). Mesenchymal *Fgf10* was still present, albeit lacking the intensive regions of expression normally found around the intercusp folds and posterior cervical loop (Fig. 6E). *Delta1* expression was found in the differentiating odontoblast similarly to the posterior side, indicating that odontoblast differentiation is not affected (Fig. 6A).

Notch and FGF10 signaling in the mouse molar root

Unlike the vole molar, the crown of the mouse molar stops growing completely and the entire epithelium switches to the root fate. On a molecular level the most striking difference is the absence of some of the key genes involved in the regulation of the epithelial stem cell compartment. *Fgf10* and *Fgf3* are absent in 10 and 14 dpn mouse molars (Fig. 7K,L: 10 dpn and *Fgf3* data not shown). *Notch1*, 3, *Hes1* and 5 also showed no expression in or near the cervical loop area at these stages (Fig. 7A,B,E,F,I,J: *Hes5* data not shown). Patches of *Notch1* and 3 were associated with blood vessels in the dental mesenchyme. Only *Notch2* and *Lfng* were expressed in the cervical loop area. *Notch2* was expressed throughout the dental mesenchyme, with the exception of the odontoblasts (Fig. 7C,D). Strong

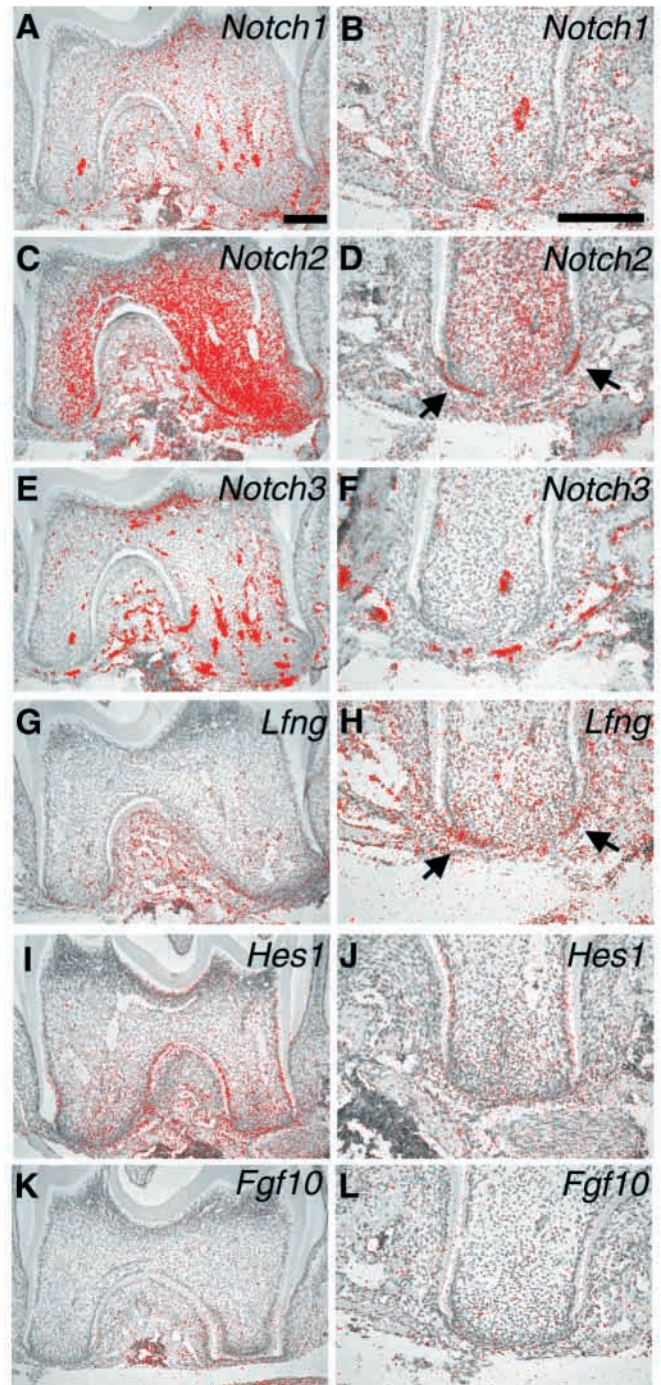


Fig. 7. Localization of *Notch1* (A,B), *Notch2* (C,D), *Notch3* (E,F), *Lfng* (G,H), *Hes1* (I,J) and *Fgf10* (K,L) in sagittal sections of 14 dpn mouse molars. B,D,F,H,J,L, higher magnification of anterior cervical loop regions in A,C,E,G,I,K, respectively. The arrows in D and H indicate the expression of *Notch2* and *Lfng* in Hertwig's epithelial root sheath. Scale bars are 0.2 mm.

expression was also found in Hertwig's epithelial root sheath, the outer enamel epithelium and the epithelium covering the crown. It was absent in the ameloblasts. *Lfng* was also expressed in Hertwig's epithelial root sheath, albeit not with the same intensity as in the transit-amplifying cells in the vole molar (Fig. 7G,H). *Lfng* expression was lacking in the

remainder of the tooth, except for some light expression in the mesenchyme near the root tips, likely representing the differentiating odontoblasts. *Hes1* was expressed in preodontoblasts and odontoblasts, and in mature ameloblasts. *Hes5* expression was not found. *Jagged1* only showed expression in ameloblasts. *Delta1* expression was mesenchymal and not very strong and showed a slight gradient, with intensity increasing towards the cusps (data not shown).

DISCUSSION

A developmental choice: root or crown

The epithelial adult stem cell niche in the tooth can only exist under special circumstances. This is because of the developmental history of the tooth. During tooth formation the crown is covered with epithelium, but when the tooth erupts the epithelium is exposed to the oral cavity and is lost (Fig. 1). At the apical end of the tooth an epithelial structure is present that is known as the cervical loop and is formed during the early morphogenesis of the crown at the cap stage. This cervical loop consists of several different epithelial layers and is present in both incisors and molars. When the crown development is quite advanced the cervical loop has two developmental fate options. It can stick to being a 'crown' and continue enamel production, or it can adopt the 'root' fate. The mouse molar is an example where the entire cervical loop switches to root, whereas the vole molar only partly makes this switch, and maintains the capacity in most of its cervical loop to produce crown (Fig. 1). There are no known examples of root epithelium switching back to crown fate, indicating that the switch is unidirectional.

If the cervical loop changes its developmental fate into a root the stellate reticulum degenerates leaving just the inner and outer enamel epithelium as a double layer of epithelium and is known as Hertwig's epithelial root sheath (HERS) (Fig. 1). This structure continues to proliferate for a limited time and directs root formation (Thomas, 1995). As the root lengthens the HERS is fragmented and invaded by cementoblast precursors and is known as the epithelial cell rests of Malassez. The limited proliferative capacity of both structures suggests that both these epithelial structures lack stem cells.

What makes a continuously growing tooth: morphology

We propose that for the hypselodont (continuously growing) teeth the cervical loop is an essential structure. We showed that the continuously growing molar of the vole maintains its cervical loop during late postnatal development, and that it is present in the entire circumference of the tooth base. It has been suggested that the bulging nature caused by the large stellate reticulum compartment of the cervical loop typical of the rodent incisor is a requirement for a functional stem cell niche (Harada et al., 2002). However, the entire cervical loop area of the vole molar is rather flat (Fig. 2B). We therefore propose that the functional essence of the cervical loop of a continuously growing tooth during development is not size, but merely the presence of the proper structural components: the inner and/or outer enamel epithelium and within it the stem cell containing stellate reticulum and/or stratum intermedium cells.

What makes a continuously growing tooth: molecular regulation

In order to keep producing crown, the original structure of the cervical loop, and therefore the stem cell niche, has to be maintained. Epithelial Notch and mesenchymal FGF10 signaling are part of the molecular regulation of the epithelial stem cell niche in the mouse incisor and the subsequent differentiation of the progeny into functional ameloblasts (Harada et al., 1999; Harada et al., 2002). We found a similar regulatory set-up in the vole molar. Based on the similar distribution of expression patterns, the epithelial cell lineage can be subdivided into several domains with increasing levels of differentiation (Fig. 8). There is the stem cell compartment characterized by *Notch* expression, a proliferation compartment characterized by *Lfng* expression and then a subset of differentiation domains each representative of a different degree of differentiation and each with their own specific marker, such as *Jag1* and *Bsp1*. It is not known how Notch regulates the stem cells, but previously Notch has been associated with stem cell differentiation in many different tissues, such as neurons and glia (Wang and Barres, 2000; Lütolf et al., 2002), lymphocytes (Anderson et al., 2001), pancreas (Apelqvist et al., 1999) and epidermis (Lowell et al., 2000). One possible function could be the regulation of stem cell division (Chenn and McConnell, 1995). *Hes1* is one of the downstream targets of Notch signaling, but its expression was almost identical to that of *Notch1*, 2 and 3, with the highest levels of expression in the stratum intermedium.

We showed that *Fgf10* was expressed in the dental mesenchyme near the cervical loop and around the base of the intercuspal folds (Fig. 4E,F, Fig. 6E,F) similar to the incisor. It has been postulated that FGF10 stimulates the division of both stem cells and transit-amplifying cells in the cervical loop (Harada et al., 1999; Harada et al., 2002). Harada and co-workers also showed that the FGF receptors FGFR1b and 2b are expressed throughout the cervical loop epithelium. The identical expression domain of *Bmp4* (Fig. 6G,H) and *Fgf10* suggests that BMP signaling could be involved in stem cell regulation, perhaps in cooperation with FGF10. We showed earlier that FGF and BMP are involved in the regulation of *Lfng* expression in the progenitors of cervical loop epithelium during early tooth development, where they have an antagonistic effect on *Lfng* and a synergistic effect on *Hes1* expression (Mustonen et al., 2002). A similar system could remain in place in the continuously growing tooth.

The intercuspal loop: a stem cell niche similar to the cervical loop

The most striking difference between mouse incisor and vole molar is the presence of the complex cusp pattern in the vole molar (Fig. 1), whereas the incisor ends in a simple cone-shaped tip. The cusp pattern is a result of the epithelial folding mediated by the actions of the enamel knots earlier during development (Jernvall et al., 2000; Vaahtokari et al., 1996). The folding leads to the formation of intercuspal loops (Fig. 1, Fig. 2A,B). These loops consist of a basal layer of inner enamel epithelium which envelopes first a layer of stratum intermedium and more to the inside the stellate reticulum. The intercuspal loop is therefore structurally comparable to the

cervical loop. Moreover, Notch signaling pathway genes, *Fgf10* and *Bmp4* were present in the intercuspal loop in a similar pattern to that in the cervical loop. The intercuspal loop could therefore function in the same way as the cervical loop, including acting as a stem cell niche.

Root analogue in the vole molar

We have shown that the vole molar possessed three small root-like domains (Fig. 2C-G). The root domains in the vole molar contained all the histological features of a root and a typical expression of *Bsp1* in the cementoblasts (D'Errico et al., 1997). We therefore propose that continuously growing teeth in general solve the problem of lack of anchorage, resulting from the absence of a classic root system, by transforming several small domains into the root fate. The presence of the anterior root analogue in the vole molar confirmed the lack of involvement of the Notch signaling pathway in root differentiation. In the anterior part of the vole molar, all Notch pathway genes were absent in the dental epithelium once root formation had been initiated (Figs 5, 6). The situation seems to be different for the dental mesenchyme since odontoblast formation is still an ongoing process, whereas the epithelial ameloblast differentiation is halted when root formation starts.

Continuous growth in vole molar versus growth arrest in mouse molar

Although the early development of the mouse molar is almost identical to that of the vole (Keränen et al., 1998), later in development an important developmental decision is made differently. The mouse molar arrests its crown development and the epithelium switches to a root fate. Therefore, it is to be expected that the molecular regulation of the cervical loop area is different in vole and mouse molars. From the viewpoint of functional morphology the vole molar resembles the mouse molar more than the mouse molar resembles the incisor. Relatively recently on the evolutionary time scale, a change occurred in the molecular regulation of the vole molar, which resulted in the extended growth period of the crown epithelium. The Notch pathway genes were not expressed in the root epithelium of the mouse molar at 10 or 14 dpn with the exception of *Notch2* and *Lfng* (Fig. 7: 10 dpn not shown). These two mRNAs were found in Herwig's epithelial root sheath. Since the HERS is the site of epithelial proliferation in the root (Kaneko et al., 1999), *Lfng* could be associated with proliferation similar to that in the cervical loop area. The Notch pathway was, however, mostly active in the mesenchyme of the crown area instead of the cervical loop area (Fig. 7).

It has been reported earlier that *Fgf10* and *Fgf3* expression is diminished in mouse molars soon after birth (Kettunen et al., 2000). We have shown that *Fgf10* and *Fgf3* are completely absent at 10 and 14 dpn when root growth still continues (Fig. 7K,L). In *Fgf10* knockout mice the early morphogenesis of the incisor is normal, but at later stages the cervical loop is greatly diminished in size and the incisor stops growing (Harada et al., 2002). Also in these mice antibodies against FGF10 halted the growth of the incisor and human FGF10 protein could rescue this phenotype. Although FGF10 controls the survival of the cervical loop area, it is unknown if FGF10 is needed for the stem cells or just the proliferation of the transit-amplifying cell

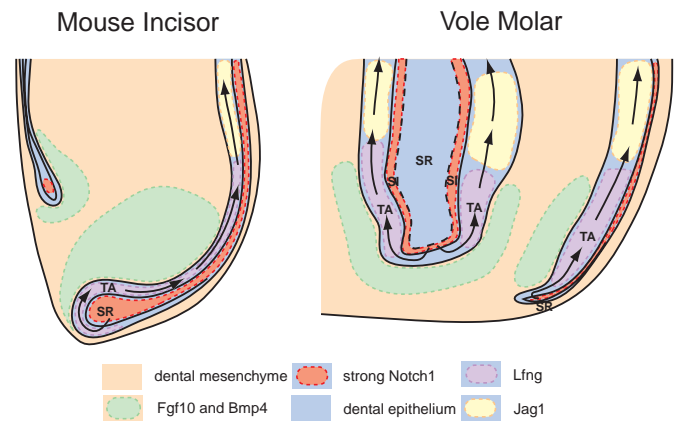


Fig. 8. Schematic representation of the epithelial stem cell compartment in mouse incisor and vole molar and the similar distribution of activities of some of the proposed key Notch signaling genes. The stellate reticulum and stratum intermedium are areas of high *Notch* expression and the putative locations of the adult stem cells. The progeny of the stem cells migrate to the inner enamel epithelium and form a population of transit-amplifying cells characterized by *Lfng* expression. The cells then start to differentiate into ameloblasts and enter the next domain characterized by *Jag1* expression. The proliferation and the stem cells are maintained by the mesenchymal *Fgf10* expression near the cervical and intercuspal loop. The identical expression of *Bmp4* and *Fgf10* indicate that BMP4 might work together with FGF10 in the modulation of the stem cells and their progeny. The main difference between vole molar and mouse incisor is the presence of intercuspal folds in the vole molar, which possess an identical distribution of the key regulators as in the cervical loop area.

pool. It is also unclear how the downregulation of Notch and FGF signaling is regulated.

Evolutionary flexibility of the regulation of the adult stem cell compartment in teeth

Not only is the regulation of the epithelial stem cell compartment conserved between hypselodont molars and incisors, it also seems that this regulation is flexible. Not all vole species have continuously growing molars. Some do develop roots, but only after an extended period of crown growth when compared to the mouse. This results in hypsodont teeth with a typical high crown. Hypsodont teeth are quite common in the animal kingdom (Janis and Fortelius, 1988), suggesting that the prolongation of crown growth and subsequent delayed switch to root fate is a flexible regulatory process. The continuously growing vole molar could therefore be seen as an extreme modification of this regulatory switch, i.e. the growth period of the crown is extended for so long that the switch is never used. We suggest that all continuously growing teeth use the same molecular pathways, FGF, Notch and perhaps BMP for the regulation of the epithelial stem cell niche (Fig. 8). These pathways can be altered spatially and temporally to fulfil the different functional requirements of teeth, be it continuous growth, root development, restricted root development, or postponement of root development.

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