To the root of the stem cell problem

The evolutionary importance of the epithelial stem cell niche during tooth development

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following articles, which are referred to in the text by their roman numerals. In addition, unpublished data are presented.

I Mustonen T., Tummers M., Mikami T., Itoh N., Zhang N., Gridley T., and Thesleff I.. (2002) Lunatic fringe, FGF, and BMP regulate the Notch pathway during epithelial morphogenesis of teeth. Dev Biol. 248(2):281-93.

II Yamashiro T., Tummers M., and Thesleff I. (2003) Expression of bone morphogenetic proteins and Msx genes during root formation. J. Dent. Res. 82(3), 172-176.

III Tummers M. and Thesleff I. (2003) Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche. Development 130, 1049-1057.

IV Tummers M. and Thesleff I. (submitted) Teeth with continuously growing roots - from a rodent incisor to a sloth molar.

ABBREVIATIONS

bHLH – basic Helix Loop Helix
BMP – Bone Morphogenetic protein
BrDU - 5-bromo-2-deoxyuridine
BSA – Bovine serum albumin
Bsp – Bone sialo protein
DNA – Deoxyribonucleic Acid
DPN – Days Post Natal/ Days after birth
Eda – TNF Ligand Ectodyplasin
Edar – TNF receptor for ectodysplasin
ERM – Epithelial cell rests of Malassez
FGF – Fibroblast Growth Factor
FGFR – Fibroblast Growth Factor Receptor
GFP - Green Fluorescent Protein
HERS – Hertwig's Epithelial Root Sheath
HES – Hairy/Enhancer of Split
Msx – Vertebrate homologue of Drosophila muscle (Msh) segment gene
k14-Eda – transgenic mouse line in which Eda is expressed under the keratin 14 promotor
MT-MMP – Membrane type matrix metalloproteinase
PDL – Periodontal Ligament
TGFβ– Transforming Growth Factor beta
TNF – Tumor Necrosis Factor
Wnt – Vertebrate homologue of the *Drosophila* Wingless gene

SUMMARY

A tooth is an ectodermal organ and its development relies on epithelial-mesenchymal interactions that are mediated by conserved signalling pathways common to other developmental processes. During the transition from the bud to the cap stage the cervical loop is formed. This structure will later become the adult epithelial stem cell niche in continuously growing teeth, such as the mouse incisor. Notch signalling is involved in demarcating the boundary between the enamel knot, the signalling center of the tooth, and the remainder of the epithelial compartment. There is a sharp boundary of *Lunatic fringe* (a Notch receptor modulator) first at the lingual side and later also at the buccal side of the enamel knot. Lunatic fringe may play a role in boundary formation of the enamel knot and Notch signalling in the epithelium is regulated by mesenchymal FGFs and BMPs. The enamel knot subsequently directs the formation of the cervical loop.

The cervical loop has the specific structure of centrally located stellate reticulum surrounded by a basal layer of epithelium. The stellate reticulum is the putative site for the adult stem cells. In continuously growing teeth such as the mouse incisor and the sibling vole molar this structure and the regulatory system of epithelial Notch and mesenchymal FGF signalling is maintained. In non-continuously growing teeth, such as the low-crowned molar of the mouse, the stellate reticulum disappears and FGF and Notch signalling are downregulated. BMP signalling plays an important role in the epithelial-mesenchymal interactions during early tooth development. However, neither BMPs nor any of the MSX transcription factors seem to have an important role in guiding root formation. It is therefore not clear how the growth of the root is directed after losing the stem cell niche.

The regulation of the epithelial stem cell niche seems highly flexible and allows for evolutionary novelty. Different tooth types can be generated by merely extending the maintenance of the stem cell niche: in low-crowned teeth the stem cell niche is maintained shortly resulting in a low crown, in high-crowned teeth the maintenance is extended resulting in a longer growth period of the crown, and in continuously growing teeth the stem cell niche is maintained indefinitely.

There are two major types of continuously growing teeth: continuously growing ‘crowns’ and ‘roots’. The continuously growing root is a rare tooth found in the Edentates, such as the sloth. In the transgenic k14-Eda overexpressing mouse the continuously growing incisor is transformed from the ‘crown’ type into the ‘root’ type, and is similar to the sloth in structure and histology. The stem cell niche does not adopt the typical root structure known as Hertwig’s epithelial root sheath (HERS) as can be seen for instance in mouse molars. Instead it maintains its stellate reticulum and the typical molecular regulatory setup of Notch and FGF signalling that is found in continuously growing crowns. The root fate is therefore not automatically linked to HERS, and a functional stem cell niche is not necessarily associated with crown formation. This regulatory flexibility allows for patterning flexibility of the proximal-distal axis of the tooth. Teeth are flexible in their regulation of crown height, resulting in different ratios of crown and root surface. And teeth can also do a merely partial conversion of the crown in a root surface, creating teeth like the rodent incisor with enamel on the front (crown) and dentin on the back (root) of the tooth. The independence of the regulation of the stem cell niche and of differentiation has allowed for developmental flexibility and evolutionary variation in tooth character.

INTRODUCTION

Ectodermal organs and early tooth development

This thesis focuses on the role that the epithelial stem cell niche plays in development and in evolution of teeth. The epithelial stem cell niche is a structure that is formed during development and different regulatory decisions during the formation of this structure result in different tooth types. The emergence of new tooth types is a natural feature of evolution. Developmental decisions can be directly linked to evolutionary adaptations and the emergence of evolutionary novelties, such as the rodent incisor. I will not focus on any medical or practical applications of dental stem cells in this thesis since I do not believe that pure science should be polluted with practical rhetoric no matter how interesting it might seem to society.

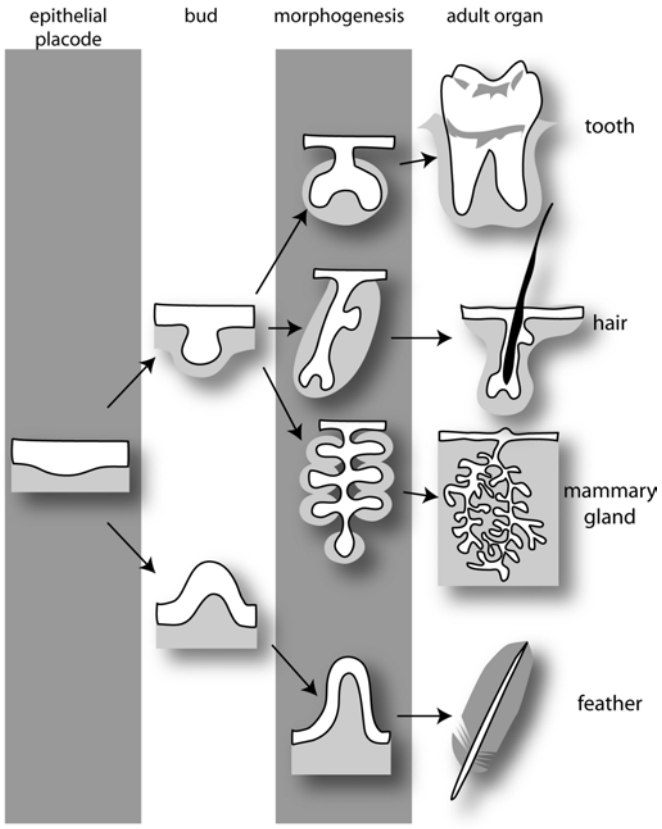

Figure 1. *The development of ectodermal organs. Most ectodermal organs start their development in a similar fashion with an epithelial thickening. The epithelium then buds into or protrudes out from the mesenchyme with mesenchyme condensing around the epithelium. From that moment on the development of different organs take on different shapes during morphogenesis resulting in quite unique adult forms that perform radically different functions.*

An introduction to the early development is necessary to understand the developmental history of the stem cell niche and the nature of the tooth as an organ. Teeth are ectodermal organs similar to hair, scales, nails, feathers, and mammary glands (figure 1). They develop as a combination of ectoderm and mesenchyme with a continuous and dynamic reciprocal signalling interaction between them (Pispa and Thesleff, 2003). The epithelium and mesenchyme both instruct and receive information which guides the morphogenesis and identity of the ectodermal organ.

The development of all ectodermal organs starts in a similar fashion and deviates during later stages in order to give rise to different adult structures (figure 1). The first morphological sign during the development of all ectodermal organs is an epithelial thickening at the initiation stage

resulting in the formation of an epithelial placode. During the bud stage the epithelium of the placode invaginates into the mesenchyme (our outwards in the case of feathers) and forms an epithelial bud surrounded by condensed dental mesenchyme. From this moment onwards the morphogenesis of different ectodermal organs becomes specific.

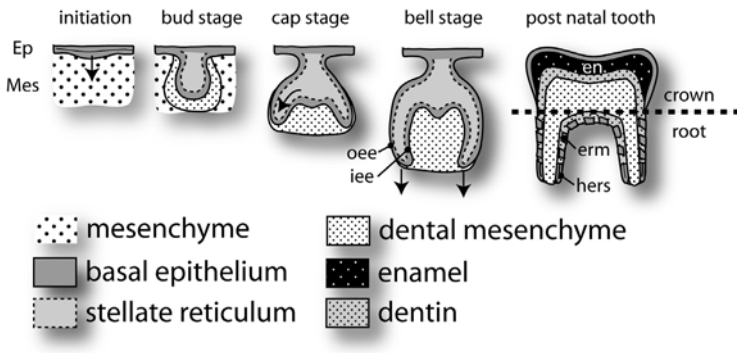

Figure 2. *Tooth development. The tooth is an ectodermal organ and shares the first stages (initiation and bud stage) with other ectodermal organs. Tooth development is characterized by reciprocal dynamic signalling between epithelium and mesenchyme during all stages. The instructive capacity switches between these tissues. During initiation the oral epithelium thickens and forms the dental placode. The then epithelium buds into the mesenchyme and neural crest derived mesenchyme condensates around the epithelium (bud stage). During the cap stage the lateral cervical loops are formed which will form the epithelial stem cell niche in continuously growing teeth and Hertwig's epithelial root sheath (HERS) in teeth that have limited growth and form a root. During bell stage the cervical loops keep extending and the epithelium near the oral side folds to an intricate pattern that corresponds to the later cusps of the tooth. At this point all structures are still part of the crown. During post natal development the cervical loop can switch fate and the HERS is formed. At this point root formation starts. The HERS fragments into a fenestrated network of epithelial cells named epithelial cell rests of Malassez (ERM). Oee, outer enamel epithelium; iee, inner enamel epithelium.*

In teeth the bud stage is followed by the cap stage where the cervical loop is formed and the bell stage in which more extensive growth and folding occurs of the dental epithelium. Also a start is made with the differentiation of both mesenchymal and epithelial tissues into their terminal cell fates (figure 2). This folding pattern will later transform into the species specific occlusal pattern of cusps, which determines the function of the tooth. At this point in time only the foundations for the crown are established. During post natal development the roots are formed preceded by structural changes in the cervical loop area. The epithelium of the root no longer differentiates into ameloblasts that produce the hard enamel matrix. Instead the root epithelium forms two structures, Hertwig's epithelial root sheath in the cervical loop area and epithelial cell rests of Malassez further up the root. In short, the crown is always formed first and then only the root.

The concept of Stem cells

The stem cell concept can easily be summarized in a simple diagram (figure 3). In reality the concept of the stem cell is not so clear. The whole subject is enveloped in a cloud of rhetoric and mystification. One could even claim that stem cells are actually nothing more than new semantics. There is however a general consensus on the definition of stem cell. A stem cell has certain properties that distinguish it from other cells. It is capable of renewing itself and can give rise to a selection of differentiated progeny. These general characteristics are not so easily defined on a molecular level. In general there is a lack of studies on the nature of stem cells in teeth and hence the dental stem cell is still quite a mysterious cell. This thesis does not unveil the secret identity and characteristics of the dental stem cells, but will show the functional and

especially the evolutionary relevance of the epithelial stem cell niche in the tooth in light of the existence of different tooth types.

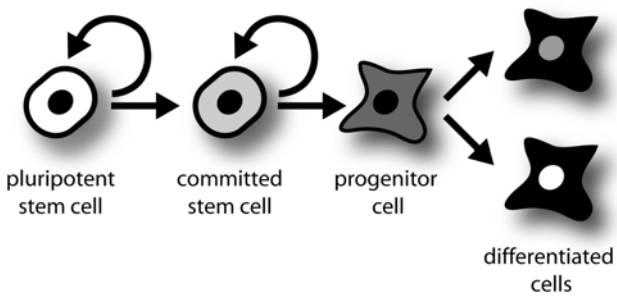

Figure 3. *The stem cell concept. A pluripotent stem cell gives rise to different lines of committed stem cells. These produce line specific progenitors which can give rise to usually several differentiated cells - after Gilbert (Gilbert, 2000).*

In general it is problematic to study stem cells in vivo because there are no general markers for stem cells. One might expect that all stem cells have common properties that are reflected on a phenotypical level of gene expression considering their plasticity and interchangeable nature. In two independent studies the gene expression of two different stem cell populations were studied with DNA microarray (Ramalho-Santos et al., 2002; Ivanova et al., 2002). Despite some overlap in gene expression between the two stem cell populations nothing jumps out as an obvious stem cell marker. It is possible that the very nature of stem cells, pluripotency and an undifferentiated state, is caused by not expressing anything in particular? Maybe we have to acknowledge the fact that there will never be a general marker for stem cells. Recently, there was a more elegant study on the stem cells of the hair follicle (Tumbar et al., 2004). Here the stem cells were labelled in an ingenious way with GFP in the stem cell niche and a microarray was done on stem cells derived directly from an in vivo niche instead of cell culture. In the stem cell niche lie the answers and I will approach this problem here from a developmental and evolutionary perspective.

Morphogenesis versus cell differentiation in teeth

A major focus of much of the stem cell research is on differentiation of stem cells into various cell types or fates. Naturally this could be of potential interest for tissue replacement therapies and other stem cell related therapies (Thesleff and Tummers, 2004; Chai and Slavkin, 2003). In dental research an obvious aim is the production of the hard matrix that covers our teeth, enamel and dentin, but also the regeneration of the periodontal ligament is a potential target. Mesenchymal stem cells can be isolated from the adult dental pulp (Gronthos et al., 2000). The cultured dental pulp cells differentiated into odontoblasts forming dentin when transplanted into muscle. Also these dental pulp stem cells can generate a hierarchy of progenitors, ranging from a small population of self-renewing cells to a larger population of committed progenitors (Gronthos et al., 2002). The adult dental pulp is therefore not a uniform collection of differentiated cells, but represents stem cells and a range of differentiated progeny. It is unknown if the mesenchymal stem cells are located in a niche or what the structure of this niche should look like.

Notch signalling is involved in the differentiation of the mesenchymal stem cells in the adult dental pulp. A ‘regeneration’ model for the mesenchymal compartment in a tooth can be achieved by simply drilling a hole in the tooth. When a hole is drilled in the molar of the rat Notch signalling is activated and *Delta1*, a Notch ligand, is upregulated in the odontoblasts near the injury (Mitsiadis et al., 1999). The injury is partially repaired with dentin produced by odontoblasts.

The Hat-7 cell line is an epithelial stem cell line that originates from the cervical loop of the murine incisor (Kawano et al., 2003). It is capable of producing differentiated cells that express

several markers characteristic for ameloblasts, the main epithelial cell lineage. The other main epithelial cell lineage is the root epithelium. Regeneration of dental tissues and matrix might be very well feasible in the far future, but practical procedures are still very much beyond us. However, to obtain a differentiated cell from a cell line is a problem that operates on a different order than the formation of a complex three dimensional shape, i.e. organogenesis. The stem cell niche plays a central role in organogenesis. The function of a stem cell niche goes beyond providing a specific differentiated tissue. It is an intrinsical structural part of the tooth and helps to shape this organ.

Are morphogenesis and differentiation linked in ectodermal organs?

The path from a stem cell to a complex organ is an interesting problem. During the development of ectodermal organs either the mesenchyme or epithelium instructs the fate of the neighbouring tissue. This was shown by tissue recombination studies in which mesenchymal and epithelial tissue of different developing teeth and other ectodermal organs were combined. The instructive capacity to determine fate between compartment switches back and forth between mesenchyme and epithelium during development (Lumsden, 1988; Mina and Kollar, 1987; Hardy, 1992; Dhouailly, 1975). Many of these instructive signals emanating from either epithelium or mesenchyme are transient in nature and depend on reciprocal signalling. Therefore the instructions for identity of the organ switch back and forth between epithelium and mesenchyme. As for tooth shape, the enamel knot, a signalling center, is created in the epithelium during bud stage (Vaahtokari et al., 1996; Jernvall and Thesleff, 2000). This transient structure and the later secondary enamel knots will direct the growth of the dental tissues and therefore determine the shape of the tooth.

However, this does not answer the question whether the differentiation of the stem cell progeny is linked with morphogenesis. Heterotopic recombination between salivary gland mesenchyme and mammary gland epithelium results in a salivary-like branching pattern (Kratochwil, 1969). Although morphogenesis occurs in a salivary-gland manner, the differentiation of the cells does not. It produces mammary gland-like cells. Morphogenesis and differentiation are therefore not necessarily linked together on a regulatory level.

Early tooth development and the formation of the adult epithelial stem cell niche.

The environment of the stem cell, the stem cell niche, defines the properties of the stem cell. The stem cell niche can be defined as the environment that sustains the stem cell population and is instructive in the differentiation and proliferation of its progeny (Watt and Hogan, 2000; Spradling et al., 2001; Nishimura et al., 2002). The importance of the niche is shown by transplantation experiments of stem cells after which the transplanted stem cell adapts their fate according to the niche and not their original lineage (Bjornson et al., 1999; Anderson et al., 2001). This also suggests that the different stem cells niches in an organism are in essence very different since they produce different progeny from the a similar stem cell. There must also be similarities between them since it could be expected that the maintenance of a stem cell requires similar conditions. To better understand the stem cell niche a closer look at its developmental history is in order. The stem cell niche is a structured arrangement of tissues and therefore has to be formed.

The standard model for epithelial stem cells in teeth is the continuously growing incisor of the mouse (Harada et al., 1999) (figure 4). The stem cells are associated with a tissue layer named stellate reticulum at the core of the stem cell niche and they express *Notch1*. The stellate reticulum is first seen during the transition from initiation phase of tooth development to bud

stage (figure 2, 5). The epithelium buds into the mesenchyme and the central core forms the stellate reticulum and is surrounded by a basal layer of epithelium. This is a basic configuration that we can also find in the adult stem cell niche. During the initiation phase *Notch* is already expressed throughout the simple oral epithelium (Mitsiadis et al., 1995). This layer thickens after receiving proper signals from the underlying mesenchyme and the stellate reticulum is created from the suprabasal cells. The question arises if these suprabasal cells are the first representation of stem cells. Is the 'stemcellness' depended on the lineage, or alternatively on the environment or stem cell niche? *Notch* is down-regulated in the basal epithelium that makes direct contact with the mesenchyme, a process possibly regulated by the mesenchyme (Mitsiadis et al., 1995). If the stem cell identity is lineage dependent then maintenance of *Notch* in the suprabasal cells could function to maintain the stem cell fate of these cells. The formation of the stem cell niche, which is only visible in later stages, could therefore already be intimately linked with the initiation events of tooth development.

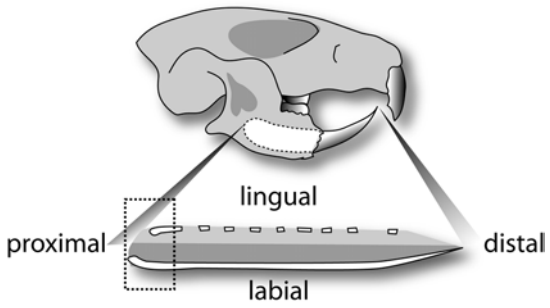

Figure 4. *The lower rodent incisor. This incisor is quite long and runs almost through the entire lower jaw. The cervical loop is located at the proximal end (box) and new tissue is produced there. At the distal tip the tooth is worn down. The lingual side is the root analogue and covered with dentin and cementum. The labial side is the crown analogue and covered with enamel.*

In teeth, the final form of the epithelial stem cell niche has its origin in the cap stage of development (figure 2, 5). At this stage the signalling center of the tooth, the enamel knot, secretes growth factors that regulate the growth and expression of other regulatory molecules. The cusp pattern which is specific for every tooth is the ultimate end product of the regulatory actions on tooth shape by the enamel knots at the cap stage and the secondary enamel knots which appear later. One of the morphogenetic effects of the actions of the enamel knot is the creation of the so called cervical loop (figure 5). This epithelial structure contains the epithelial stem cells in mature adult teeth.

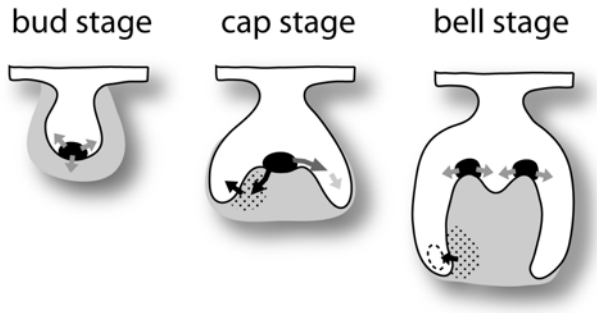

Figure 5. *The formation and independence of the stem cell niche. During the bud stage the enamel knot (black area) directs the morphogenesis of the surrounding tissue. During cap stage the cervical loops are formed. The epithelium grows 'out' of the bud. This is a crucial stage for the formation of the stem cell niche. Until now the enamel knot directed the growth of the neighbouring epithelium either through direct signalling through the epithelium (arrows on the right) or indirectly by setting up a compartment in the mesenchyme that provides the necessary signals for the growth of the nearby epithelium (arrows and dotted area on the left). A possible signal emanating from the mesenchymal compartment would be FGF10. During bell stage the cervical loop has escaped from the sphere of direct influence of the secondary enamel knots. At this point the epithelial stem cell niche can be considered independent as a regulatory unit.*

This however does not mean that all adult teeth contain epithelial stem cells. On the contrary, the cervical loop undergoes some radical changes in many teeth during root formation, and it is

thought to lose the stem cells during this process. Hence adult epithelial stem cells can only be studied in teeth that have a special adaptation to wear: continuous growth. A stem cell niche with adult stem cells is required to replenish lost tissue because the tooth wears down constantly at the tip due to wear.

During cap stage the enamel knot signals to the cervical loop which extends from the budding epithelium laterally (figure 5). At this point its structure is similar to the adult stem cell niche. There is a loosely aggregated stellate reticulum in the centre surrounded by a slightly denser stratum intermedium. Both these tissues are surrounded by basal layers of epithelium, the outer enamel epithelium and the inner enamel epithelium. The cervical loop epithelium is flanked on the inner side with dental mesenchyme and on the outer side with the dental sac tissue. The cervical loop will extend towards the base and quickly escape the regulatory influence of the enamel knot. The primary enamel knot largely disappears by means of apoptosis after the cap stage (Jernvall et al., 1998). New enamel knots, the secondary enamel knots, are formed at the bell stage. It is unlikely that the secondary enamel knots function in the maintenance of the epithelial stem cell niche. Instead species-specific tooth shapes are determined by these secondary enamel knots which are located and produce signalling molecules at the sites of the future cusps (Keränen et al., 1998; Salazar-Ciudad and Jernvall, 2002).

This point could be considered as the birth of the adult stem cell niche. From this moment on it is regulated as an independent structural entity. To understand its function and regulation it is better to focus on the standard model for this stem cell niche, the mouse incisor.

The rodent incisor

The rodent incisor is one of those classic adaptations which made an evolutionary success story possible. Although the rodents also have other rather successful adaptations the chisel like incisor gave it an edge on its competition. The rodent incisor is a continuously growing tooth. Its shape is a fairly simple cone which narrows at the tip. This narrowing is not a result of growth. It is the result of a special feature of the incisor. Its labial half (towards the lip) consists of the hard enamel typical of the crown (figure 4). However, its lingual half (towards the tongue) consists of the slightly softer dentin typical of root. The incisor is longitudinally divided in a labial crown analogue, and a lingual root analogue. The rodent incisor can be sharpened by simply moving the hard enamel covered crown side of the upper incisor over the soft dentin covered root side of the lower incisor and vice versa (See for instance: Life of Mammals, David Attenborough). This results in a self-sharpening system with two sets of opposing chisel-like incisors. These teeth can bite through materials other animals cannot, and any wear of the teeth is compensated by new growth on the base, self-sharpening the tooth in the process.

The molecular regulation of the stem cell niche has been mostly studied in the mouse incisor. The usual time to look at the incisor is two days post natal (2DPN) because all the structures have been formed and the tooth is still easily excised from the jaw. It is clear that the cervical loop area has vast regenerative capabilities as has been shown by experiments in which only cervical loop epithelium was left. It is able to regenerate the lost epithelium quickly (Harada et al., 1999). The same paper proposed that the stellate reticulum is the putative site for epithelial stem cells and that Notch and FGF signalling are involved in the regulation of the epithelial stem cell compartment. Slowly dividing cells (a characteristic of stem cells) were identified by pulse-chase BrdU labelling in the stellate reticulum. The stellate reticulum specifically expresses *Notch1* (and *Notch2* and *3*, but in slightly different patterns). Other components of the Notch signalling pathway are expressed in different epithelial compartments of the stem cell niche regulating the differentiation of the stem cell progeny. *Fgf3* and *Fgf10* are expressed in the mesenchyme bordering the cervical loop in slightly different patterns and support the stem

cell niche, because the corresponding FGF receptors for *Fgf3* and *Fgf10*, *Fgfr1b* and *Fgfr2b*, are expressed in the epithelium. This conclusion is confirmed by functional studies in which *Fgf10* was knocked out or the cervical loop was incubated with FGF10 antibodies. The cervical loop failed to develop properly (Harada et al., 2002). The origin of the stem cells was also confirmed by much older studies. In the continuously growing molar of the rabbit it was shown with tritium labelling studies that the inner enamel epithelium could not be sustained by itself but that the stratum intermedium must contribute cells to it (Starkey, 1963).

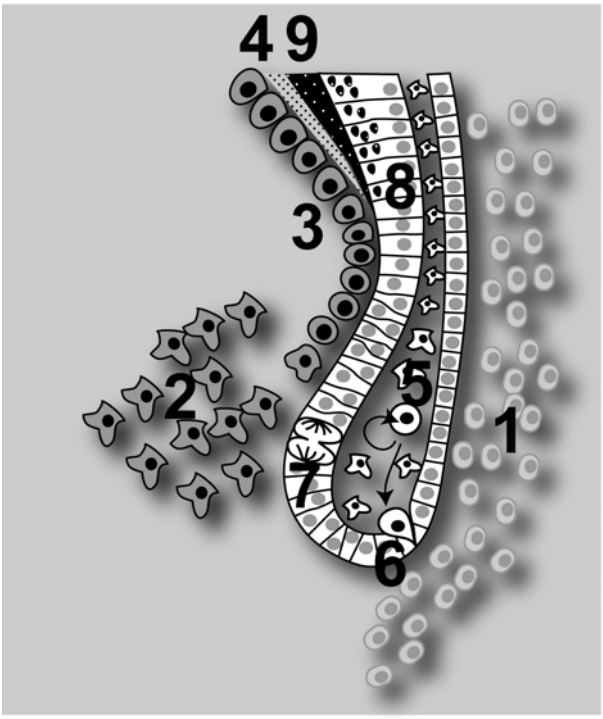

Figure 6. *The epithelial stem cell niche and the long road of the stem cell progeny. A stem cell in the stellate reticulum divides (5) and gives rise to a progenitor which inserts itself in the basal layer of epithelium 6). It proliferates (7) and forms the pool of transit-amplifying cells. Further away from the cervical loop the progeny starts differentiating into ameloblasts (8) and deposit enamel matrix (9).The dental mesenchyme (2) differentiates along the ameloblasts into odontoblasts (3) and deposit dentin (4). It is unknown if the mesenchymal dental follicle cells (1) play a role in the regulation of the stem cell niche, or if they differentiate into different cells near the cervical loop.*

The structure of the cervical loop

In many stem cell niches the stem cells are actually part of a basal layer attached to a basal lamina (Watt and Hogan, 2000). They divide asymmetrically and the stem cells remain in the basal layer. The daughter cell that gives rise to the differentiating progeny delaminates from the basal lamina and moves away from the basal layer. In the cervical loop the opposite seems to be the case. Pulse-chase BrdU experiments showed that slowly dividing cells (typical of stem cells) are present in the stellate reticulum and not in the basal layer (Harada et al., 1999). The progeny of the stem cell, the proliferating and differentiating cells, however, are all located in the basal layer. A putative model for the dynamics of the stem cell and its lineage shows therefore the stem cell centrally in the stellate reticulum or in the layer between stellate reticulum and the basal layer known as stratum intermedium (figure 6). It is unknown if it divides symmetrically or asymmetrically. One daughter cell remains and keeps the stem cell fate, the other forms the progenitor cell for the proliferating cells. Via an unknown mechanism this progenitor 'relaminates' itself into the basal layer and under the influence of FGF proliferates and becomes part of the pool of transit-amplifying cells. These 'move' around the cervical loop and as part of the inner enamel epithelium start differentiating into ameloblasts. Once terminally differentiated they will start laying down the enamel matrix. The alternative model is that the stem cells are present in the outer enamel epithelium and give rise to proliferating and differentiating progeny

in the inner enamel epithelium. The stellate reticulum will have its own population of stem cells or derive new tissue from delaminating progenitors originating from the basal layer.

The transformation of the cervical loop during root formation

The concept of a continuously growing tooth is rather unfamiliar to most of us because our own teeth are very different. Our teeth stop growing relatively early on and then erupt into the oral cavity fully formed. This kind of tooth consists of a clearly separated crown and root part. The mouse molar is the model system for this kind of tooth development. It follows a similar developmental history as our own teeth. I will refer here therefore to the mouse molar as a representative of the so called low crowned, or brachydont teeth to which also all our teeth belong.

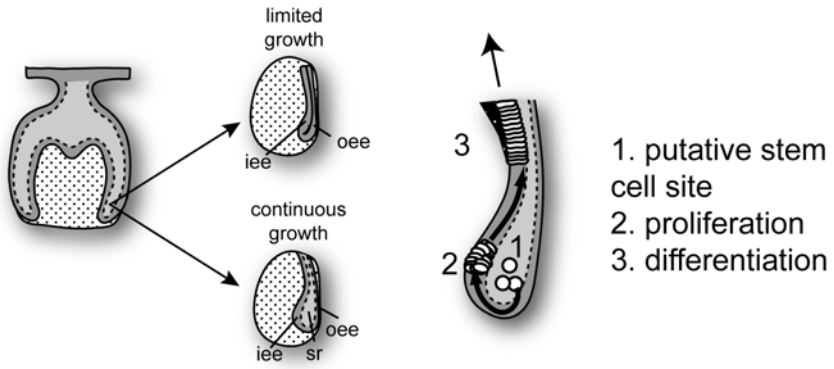

Figure 7. *The stem cell niche faces two developmental choices. Either it maintains the stellate reticulum and prolongs growth, or it loses the stellate reticulum and forms the HERS and exhibits limited growth. sr, stellate reticulum; iee, inner enamel epithelium; oee, outer enamel epithelium.*

The early development of the continuously growing incisor and the low crowned molar of the mouse are very similar. Until the cap stage there is hardly any difference. But once the cervical loops are formed during the cap stage the differences start to accumulate. The lower incisor actually rotates so it is aligned longitudinally with the jaw instead of being perpendicular to it. This event seems to be at least partly regulated by Notch signalling (Mucchielli and Mitsiadis, 2000). Both the cervical loops of the molar and incisor continue to grow and extend. In the molar the crown is formed first. In the incisor there is no clear separation of these events, since the crown and root analogue form almost simultaneously. After crown formation the cervical loop of the molar undergoes a radical transformation (figure 7, 8). The stellate reticulum and stratum intermedium between the inner and outer enamel epithelium disappears. What is left is a double layer of basal epithelium known as Hertwig's epithelial root sheath (HERS). The epithelium above HERS fragments and forms a fenestrated network of epithelial cells known as the epithelial cell rests of Malassez (ERM). Through this network mesenchymal components can migrate from outside the tooth and form the periodontal ligament which attaches the root surface to the jaw bone. With the formation of the HERS no ameloblast differentiation occurs anymore and this structure together with the ERM is thought to be typical for a root (Ten Cate, 1998). Therefore, the formation of the HERS, which coincides with the loss of the putative site, signifies the initiation of root formation. The conclusion that arises from these observations is that initiation of root formation and loss of stem cells are intimately linked during development. However, we will later see that his conclusion is not quite right.

The root and its supporting tissues

There are two major epithelial structures in the root, the HERS and the ERM. The main function of the root is to keep the tooth firmly attached to the jaw. This is mainly accomplished by the formation of the periodontal ligament (PDL). So far we have mainly focussed on two compartments of the tooth, the dental epithelium and the dental papilla (mesenchyme). The tooth is surrounded by another mesenchymal compartment known as the dental follicle. It is this layer that will give rise to most of the supporting structures of the periodontal ligament (Ten Cate, 1998; Thomas, 1995)(figure 8).

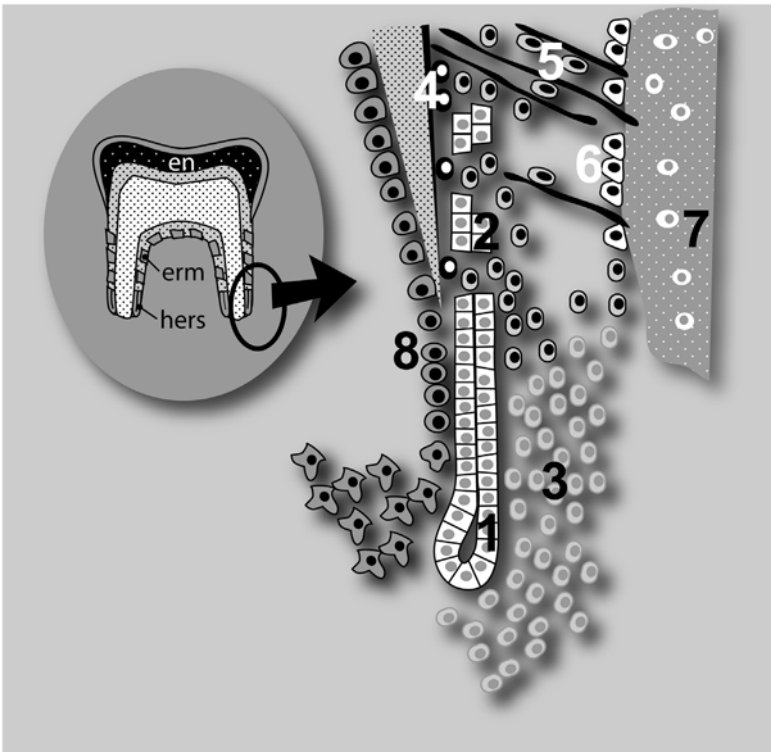

Figure 8. *The structure of the root. The HERS (1) fragments and forms islands of epithelial cells, the ERM (2). The follicular cells surrounding the tooth (3) can now reach the surface of the dentin and form cementoblasts (4) that deposit cementum on the top of the mesenchymal dentin. The follicular cells also contribute to the formation of fiber bundles (5) typical of the periodontal ligament. These connect the cementum to the surface of the surrounding bone (7). Follicular cells also give rise to the alveolar lining (6) of the bone that forms new bone. Dentin is formed by dental mesenchyme differentiating into odontoblasts (8).*

As the epithelium fragments cells of follicular origin can migrate through the gaps in the epithelium and reach the dentin surface. These differentiate into cementoblasts that produce the cementum although there is some controversy since it is also suggested that epithelial cells themselves can differentiate into cementoblasts. Immortalized HERS cells first produce enamel-related proteins such as ameloblastin and then change their morphology and produce an extracellular matrix similar to acellular cementum (Zeichner-David, 2003) It is therefore possible that cementum is produced by two different cell types; first by cementoblasts of epithelial origin, and later by neural crest derived cementoblasts. This is however still quite controversial. Other studies report that the differentiation of dental sac cells is dependent on making a close connection with HERS cells (Suzuki et al., 2002; Kagayama et al., 1998). The view that HERS cells do not contribute to the direct production of cementum seems to be more general (Diekwisch, 2001) but it seems clear that the HERS has some kind of active role in cementum formation (Hammarstrom et al., 1996).

The fiber bundles and cells of the PDL are also of follicular origin. The fiber bundles connect the cementum surface with the surface of the jawbone, which also becomes lined with cells of follicular origin. Besides the primary cementum some teeth form also secondary cementum.

This usually occurs late in root formation and seems to be expendable to some degree for tooth support, since not all teeth form it.

The initiation of root formation is still quite a mysterious event. For instance the enzyme MT1-MMP is involved in the eruption of teeth and probably in root extension (Beertsen et al., 2004). But eruption is a late event in root formation and teeth are even known to erupt without a root. A transgenic mouse lacking *NFI-C/CTF* transcription/replication factor however has molars whose crowns grow normal, but lack root formation (Steele-Perkins et al., 2003). This could for the first time indicate a gene that is clearly involved and essential for early events in root formation.

Notch signalling

Notch signalling has been associated with many functions during development. It has been associated with stem cell differentiation in many different tissues such as neurons and glia (Wang and Barres, 2000; Lutolf et al., 2002), lymphocytes (Anderson et al., 2001), pancreas (Apelqvist et al., 1999) and epidermis (Lowell et al., 2000). It might also be involved in the regulation of stem cell division (Chenn and McConnell, 1995). But stem cells are hardly the only area Notch signalling is involved in. In fact it would be difficult to mention a developmental event in which Notch signalling is not seen.

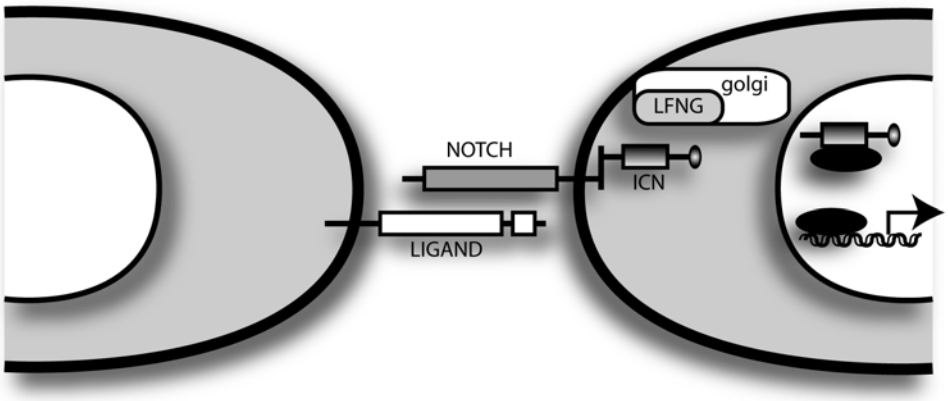

Figure 9. *Notch signalling. The Notch receptor and its ligand are both membrane bound proteins. Cell signalling is therefore restricted to neighbourin g cells. Once activated the intracellular part of notch (ICN) is cleaved off and moves to the nucleus where can induce the transcription of bHLH genes such as Hes1. Notch signalling is modulated by lunatic fringe (LFNG).*

One of the more closely studied process in which Notch signalling is involved is segmentation and more specifically somitogenesis. Notch signalling has a crucial role in the so-called clock-wave model which determines the boundaries of the somites in a dynamic manner. With this in mind we have to remember however that the modularity of developmental regulatory networks gives a great evolutionary flexibility. One would for instance expect that Notch also has an important role in the segmentation of other species. This is however not always the case. During the segmentation of the fruit fly *Drosophila melanogaster* Notch signalling has no function whatsoever. However in another arthropod relative the Central American wandering spider Notch signalling is crucial during segmentation (Stollewerk et al., 2003). This example teaches us a lesson we have to keep in mind when looking at teeth. The modularity and redundancy can cause what first seem great differences between teeth between different species and differences between individual teeth within a species. Or a specific signalling network might be present

without an obvious function, but nonetheless even if it might actually be without a function it could be a sign of a lost function, be it a historical loss on the species scale or on the scale of loss in individual teeth within a single dentition. In article II we can see a possible example of this. Here the function of Lunatic fringe during early development is examined and it shows that the knock out mouse (which has no *Lunatic fringe* expression) has no tooth phenotype.

Notch signalling is special in the sense that its effects are spatially very limited. This is because both the ligands (Jagged and Delta) and receptors (Notch) are membrane bound (figure 9). Therefore a cell bearing the ligand has to make contact with a cell bearing the receptor if a signal is to be transduced. To complicate matters there are several ligands (Jagged1-4, Delta, Deltalike), several receptors (Notch 1-4), and several modulators of Notch signalling (Lunatic fringe, Radical fringe, Manic fringe) in the mouse. This increases the range of possible interactions and the possible outcomes considerably. Furthermore the cellular context (other signals it is receiving, or the environment of the cell) and the history (the cell lineage of the cell) will determine also the outcome of the Notch signalling interactions. And the Fringes, the modulators of Notch signalling, have different effect on different Notch receptors (Hicks et al., 2000). In all we have a highly complex system, but at the same time also a very robust one, as development proves time after time during each generation. Downstream of Notch signalling there are many targets, but the most recognized are the bHLH (basic Helix Loop Helix) molecules, such as Hairy and Hes (Hairy/enhancer of split). These proteins contain a basic helix loop helix motive which in turn can bind to enhancer regions of other genes and therefore modulate their expression.

Ectodysplasin signalling

Article IV in this thesis deals with a phenotype caused by over-expression of one of the splice variants of *ectodysplasin* (Eda), a TNF ligand. I will not go into the details of ectodysplasin signalling, since the main topic of this thesis is the nature of the stem cell niche. However a short introduction into the nature of this signalling is in order.

A mutation in the *ectodysplasin* gene causes the congenital defect known as ectodermal dysplasia. Generally in ectodermal dysplasias the development of 2 or more ectodermal organs is abnormal. The most common ectodermal dysplasia is the X-linked hypohydrotic ectodermal dysplasia. This disease is re-enacted in 3 mouse models which phenotypes are caused by mutations in different genes; *tabby*, *downless* and *crinkled*. These mice have an obvious tooth phenotype in which incisors and third molars are often missing, and the number of cusps on their molars is generally reduced (Grüneberg, 1965). They also have defects in hair and gland development (Grüneberg, 1971; Blecher et al., 1983; Laurikkala et al., 2002).

The *Eda* (tabby) gene encodes for a ligand of the Tumor Necrosis Factor (TNF) family. *Edar* (Downless) encodes the corresponding TNF receptor. In the tooth *Edar* is expressed in the primary and secondary enamel knots (Tucker et al., 2000; Laurikkala et al., 2001). *Eda* is expressed in the epithelium more or less flanking the enamel knots in the outer enamel epithelium. The signalling therefore occurs within the epithelial compartment and not between epithelium and mesenchyme. *Ectodysplasin* expression is induced by Wnt signals and *Edar* is induced by activinßA, a TGFß signal coming from the underlying mesenchyme (Laurikkala et al., 2001; Laurikkala et al., 2002).

Evolutionary origin of teeth

The origin of teeth is still controversial (Smith, 2003). It has been originally thought that vertebrate teeth evolved from modified parts of the mineralized dermal skeleton. However more recent data suggests that teeth might have evolved before the mineralized skeletons had evolved during vertebrate evolution (Holland and Chen, 2001). Euconodonts are the first vertebrates to have a mineralized skeleton. Their oropharyngeal cavity possessed tooth-like structures with enamel. In fact these tooth-like structures seem to be the only structures that were mineralized in the eucondonts. Also in very early euconodont fossils such as Yunnanozoon (Chen et al., 1995) and Haikouella (Holland and Chen, 2001) mineralised pharyngeal denticles were found. Could this all suggest that teeth might have evolved first? The Haikouella is a soft-bodied fish-like animal that might give a glimpse on the ancestral state of the Yunnanozoon fossil (Shu et al., 1999). In both specimens we see examples of the earliest biomineralization in chordates and this might not reflect the appearance of such hard elements in a defensive function as armour, but the appearance of these kinds of elements in the mouth region allowed for an active hunting lifestyle. This would weaken the theory that dermal denticles, or odontodes became modified in the jaw region with the evolutionary invention of the jaw, although there does seems to be a strong morphogenetic link between the evolution of teeth and bones (Butler, 1995).

In a way trying to find the very first teeth is the same as trying to catch the very first fish (Janvier, 1999). The debate on the evolution of fish follows the debate on the evolution of teeth. A question remains if the conodont 'teeth' are truly homologous to mammalian teeth despite their similarities (Smith and Hall, 1990).

With the evolutionary establishment of teeth came the proliferation in tooth patterns and tooth shapes reflecting functional adaptations. Although it must be said that teeth not need to be present in the oral cavity, since ectopic teeth, or denticles are present outside the mouth in teleost fishes (Sire, 2001). These denticles have acquired also the completely different function of improving hydrodynamics, similar to the odontodes covering the skin of sharks. As for dental patterning the old views on evolutionary developmental models of this patterning are challenged by new ideas (Smith, 2003). Teeth might have evolved quite independent from jaws which has implications for their developmental regulation, suggesting that maybe embryonic endoderm instead of skin ectoderm had genetic control over patterning of the dentition.

Tooth shape determines the functionality of the individual tooth, whereas pattern determines the functionality of the entire oral apparatus. The enamel knots play a crucial role in the regulation of the shape of the occlusal surface. The enamel knots function through a 'simple' dynamic network of activators and inhibitors (Salazar-Ciudad and Jernvall, 2002). In practical terms these kinds of models could explain also pattern differences between different closely related species by small variations in these kinds of systems between individual teeth. Moreover, the relationship could be even on a deeper level as can be seen in the evolution of tooth shape in cichlids, where there is a positive relationship between number of teeth in a row and tooth shape (Streelman et al., 2003).

Tooth patterns are group specific resulting in for instance the exotic dental pattern in lungfish consisting of palatal dental plates that have remained unchanged for millions of years (Reisz and Smith, 2001) or the more familiar pattern of mammalians which is more variable. Mammalians have the characteristic dental formula with four different groups; incisors, canines, premolars and molars, although there is quite a lot of variation within this theme due to variations in tooth number. Changes in tooth numbers have affected the functionality of different tooth patterns and this phenomenon is under the influence of genetic variations present in a population resulting in the generation of morphological diversity (Line, 2003). In the model system of the mouse, the

premolars are missing as are the lateral incisors and canines. The only remaining teeth in the sloth are some of its cheek teeth. Obviously general dental patterns can have a great influence on functionality. Another lesson we must take in account is that most of our knowledge is based on murine dentition, but the enormous diversity of teeth, tooth shape, and tooth pattern in vertebrates must caution us in making general conclusions based on this data (Butler, 1995). We must look at different species and different kinds of teeth.

Mammalian evolution and the rodent dental pattern

The class of Mammalians can be divided into subclasses: prototheria and theria (for general literature on mammals, their evolution and their teeth I would like to refer to 'Life of Mammals' , David Attenborough, and 'Mammal Evolution' by R.J.G. Savage and M.R. Long). The Prototherians are mostly extinct except for the egg-laying platypus and exchidnas of Australasia. Most of the current mammalian species belong to the subclass of the Theria, which contains three major groups; the Pantotheria, Marsupialia and Eutheria. We will be mostly looking at eutheria, which fall into two groups, the Edentata, of which we will look into deeper into the teeth of the sloths in article IV, and of some rodents which belong to the Epitheria. One of the key factors to the success of the mammals is the heterodont patterning of their teeth. There are functional differences between different teeth in the mouth which is reflected in different shapes. The functional subdivision of teeth made individual specialization of teeth possible.

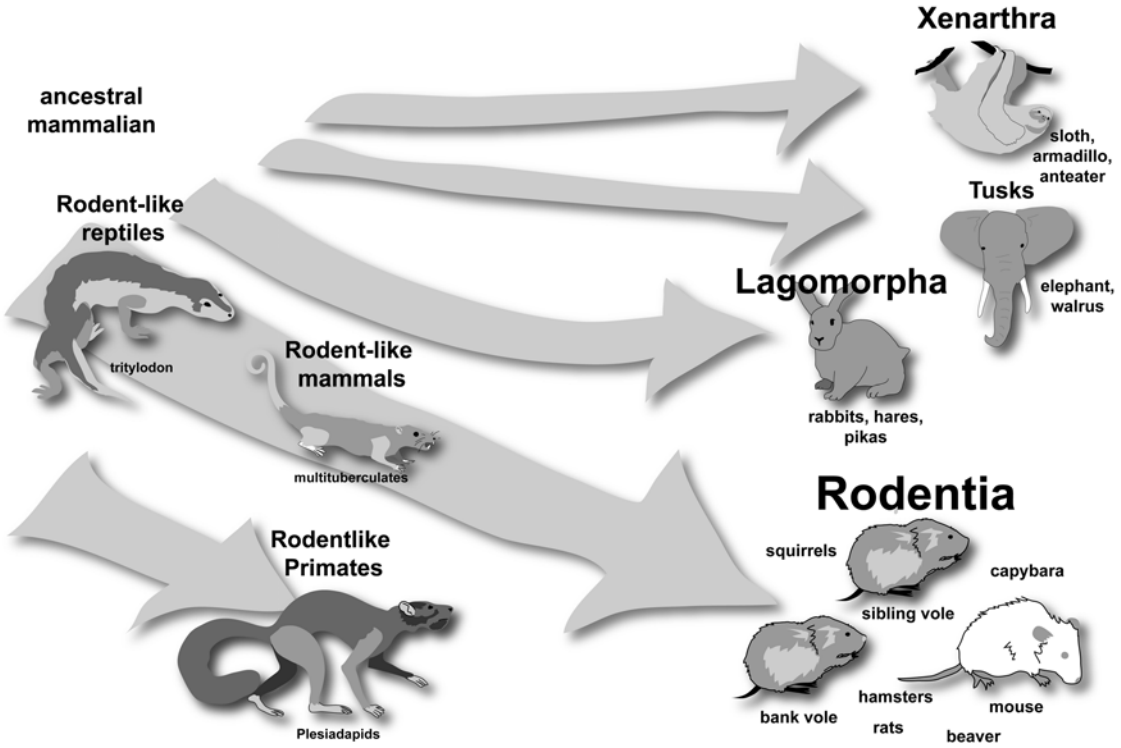

Figure 10. *The abundance of 'strange' teeth in the mammalian group. The rodent pattern of enlarged incisors-diastema-molars is rather common and has evolved on separate occasions many times. Continuously growing roots as can be found in the group of Xenarthra are also well represented. Other mammalian species also show continuously growing roots such as the tusks of the elephant.*

The modern rodents are one of the most successful mammalian groups around. They have radiated into a large and diverse group of species. There are currently about 1600 species of

rodents compared to a total of 4000 mammalian species. Needles to say they are an evolutionary success story. The diversity in lifestyle and habitat is great among the rodents, which reflects on their worldwide distribution.

One key to the success of rodents is their dentition. All rodents have one pair of incisors in the upper and lower jaw, which are curved and grow continuously. The rate of growth equals that of wear. The incisors only have enamel on the front face which ensures self-sharpening of these teeth in conjunction with their triangular shape. The incisor region is followed by a diastema region with no teeth. In the back of the jaw a variable amount of grinding molars are found, usually 4 of them on each side.

The myomorphs (hamsters, mice, rats and voles) are the most numerous group of rodents with about a 1000 different species. The number of cheek teeth is often reduced, with premolars frequently missing, usually resulting in 3 molars in row or even less. There were three great waves of speciation within the myomorphs. Firstly the cricetids appeared (hamsters and new world mice). Their dentition is characterized by retaining cuspidate teeth. The second wave was formed by the microtids and voles. Their dentition becomes progressively more hypsodont and teeth acquire continuously open roots, and the typical zig-zag enamel pattern of the crown. The microtids probably originated from cricetids in the late Pliocene. Thirdly the murids (rats and mice) appeared. Their teeth are cuspidate and rooted. The murids probably originated in south-east-Asia from late Miocene cricetids.

The evolutionary origin of this classic rodent dental pattern is not completely clear, but during the late Triassic the Tritylodonts appeared which thrived until the mid Jurassic. These proto-mammals had enlarged incisors but were lacking canines. A diastema region separated the rodent-like incisors with square cheek teeth. Their lifestyle and dietary habits were probably very similar to some of our modern rodents.

During the Jurassic the Tritylodonts were replaced by the Multituberculates, which were rodent-like mammals and are now extinct. They were present from at least the late Jurassic and maybe even late Triassic. The Multituberculates also had enlarged incisors, followed by the characteristic diastema region lacking teeth, and then several multicusped teeth in the cheek region. The Multituberculates were present until into the Tertiary, becoming extinct in late Eocene times. Rodents became abundant when the Multituberculates disappeared suggesting that they took over the niche previously occupied by the Multituberculates.

The incisor teeth of early fossil rodents (early paramyids) are rounded in a section instead of the typical triangular form of modern rodents, and the enamel extends around much of the tooth. In their evolution towards modern rodents they acquired the triangular shape and the enamel became restricted to the front face.

However, the ‘rodent’ pattern is not restricted to rodents. In a case of classic convergent evolution the lagomorphs also display this pattern. The lagomorphs are made up from rabbits, hares and pikas and are limited to about 80 living species. They too have the typical set of incisors, a diastema region following by a row of grinding molars. However, they do not seem to be related to rodents in any way besides this superficial similarity. Interestingly the enamel of the incisors is not restricted to the front face but can be found around the entire tooth. The ratio of enamel and dentin surface can therefore vary greatly in continuously growing teeth.

There are many more examples of the occurrence of the rodent pattern, but I will restrict myself to giving one more example here. The Plesiadapids were squirrel-like primates with rodent-like incisors, followed by a long diastema. In a way the Plesiadapids were highly specialized

primates with claws instead of nails and their rodent-like dental pattern. They went extinct about at the same time as the rodents were undergoing their own radiation, possibly due to competition with the rodents.

On conclusion there is an abundance of 'strange' teeth in the animal kingdom, a subject we only touched briefly here, and that their commonness in actuality represents normality instead of strangeness (figure 10). The discovery of the true nature of teeth must lie in the study of all these different kinds of teeth, their evolutionary adaptations, and developmental regulation of their morphogenesis.

Proximal-distal patterning of the tooth

Patterning of dentition can be viewed from several angles and all are important. The patterning of the occlusal surface of the tooth resulting in specific cusp patterns of individual teeth and species is well studied. The cusp pattern influences the function of the tooth greatly and an enormous variety has been recorded. The cusps are the result of folding of the dental epithelium. The cause of this folding can be traced back to the actions of the enamel knot, the signalling center of the tooth (Vaahtokari et al., 1996). It produces signalling molecules and directs the morphogenesis of the epithelium. During later stages the secondary enamel knots take over and they are already more indicative of the future cusp pattern. Differences in these cusp patterns are the result of small changes in the distribution of inhibitors and activators of epithelial growth and different cusp patterns and intermediate evolutionary stages can accurately be predicted by a model that links morphogenesis and pattern formation (Salazar-Ciudad and Jernvall, 2002). However, the cusp pattern is not the only patterning surface that defines a tooth.

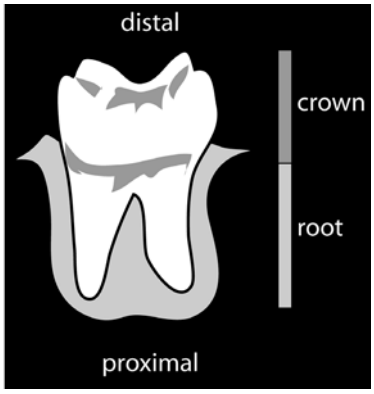

Figure 11. *Proximal-distal patterning of the tooth subdivides it in a crown and a root domain. The distribution of these domains along the proximal-distal axis determines largely the functionality of a tooth. The distal end equals the occlusal surface where normally the cusps are located and proximal refers to the apical end where the root tip can be found.*

The proximal-distal (apical end – occlusal surface) patterning of the tooth determines to a great degree the functionality of a tooth (figure 11). It determines the crown height, which influences how much wear a tooth can endure; it determines the root length, which influences the anchorage of the tooth to the jaw bone; it determines the distribution of root and crown domains, which allow for novel inventions such as the rodent incisor. Overall, the proximal-distal patterning of a tooth determines some general characteristics of a tooth. This is unlike cusp shape which allows for narrow specializations of tooth functionality, although we have to keep in mind that there is a distinction between several broad cusp shape patterns, which also give more general characteristics to teeth. To complete our knowledge on developmental characteristics of tooth patterning we therefore have to consider the proximal-distal patterning of the tooth.

AIMS OF THE STUDY

In order to understand the function, the structural changes within, and the evolutionary importance of the epithelial stem cell niche of the tooth, I examined in this thesis the different aspects of these problems. Meanwhile this thesis tries to distil a general picture of the regulatory and developmental flexibility of the stem cell niche and the subsequent differentiation of its progeny and its evolutionary significance. More specific aims were:

- To elucidate the relationship between root development and continuous growth.

- To study the molecular mechanism regulating the epithelial stem cell niche in different teeth of different species to gain an overall view.

- To study root development and its relationship to loss of epithelial stem cells.

MATERIALS AND METHODS

The sloth sections, Bradypus tridactylus, are of an embryo of unknown but advanced age. The teeth have already erupted. The sections were collected and processed by Van den Broek in 1913 and are now part of the historical collection of the Hubrecht Laboratorium in Utrecht (contact person: Jenny Narraway, Hubrecht laboratorium, Utrecht, the Netherlands).

The mouse (Mus musculus) tissues were NMRI mice, 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.

Tissues were collected and fixed overnight in 4% PFA, decalcified in 2% PFA and 12.5% EDTA for post natal tissues for 2-3 weeks. After dehydration and xylene treatment the tissues were embedded in paraffin and cut serially in 7 or 10 μm thick sections.

Radioactive in situ hybridization with ^{35}S labelled RNA probes is identical as described previously (Wilkinson and Green, 1990). The probes and their origin can be found in article III, with the exception of the probes for *Eda* and *Edar*. They were described previously (Mustonen et al., 2003). And the reference to the *Bmp*, *Msx* and *Bsp1* probes can be found in article II.

K14-Eda mouse is a transgenic mouse that overexpresses the ligand *Eda* under the keratin14 promoter and has been described previously (Mustonen et al., 2003).
Immunohistochemistry was performed on 7 μm thick paraffin sections. After deparaffination the sections were pre-treated with 10 minutes of microwave treatment in 10 mM Natrium Citrate buffer, pH 6.0 and a subsequent Proteinase K treatment 7 μg/ml in PBS for 20 minutes. After washes in PBS the tissue was blocked for 1 hour in 3% BSA in PBS and incubated in polyclonal rabbit anti-human keratin (Dako, A0575) 1:250 overnight at 4^{o}C. The Vectastain ABC kit (Vector laboratories) was used for detection and stained with DAB (Vector laboratories).

The preparation of explant cultures and bead implantation experiments, and the preparation of recombinant rat lunatic fringe protein can be found in article I.

RESULTS AND DISCUSSION

The developmental history of the epithelial stem cell niche and notch signalling during tooth development (article I)

Notch expression is associated with the putative stem cell compartment in the adult stem cell niche (Harada et al., 1999; Tummers and Thesleff, 2003). If we trace the origin of *Notch* expression throughout the developmental history of the tooth then we see the first expression already before there is any sign of a tooth. *Notch* is expressed throughout the oral epithelium (Mitsiadis et al., 1995). After initiation of tooth development the oral epithelium locally thickens into a dental placode which still expresses *Notch*. In the bud stage the epithelium buds into the dental mesenchyme and now we see the first subdivision in the epithelium of *Notch* expression. The basal layer loses its expression and *Notch* is only maintained in the central stellate reticulum and stratum intermedium. During cap stage the cervical loops form and *Notch* shows a similar expression as in the bud stage.

The bud and cap stage seem crucial for the development of the stem cell niche. The question is however if *Notch* signalling has a similar role during these early stages as it has in the adult stem cell niche. Not to mention the question if these early stages actually represent a stem cell niche?

During early bud stage (E13) *Notch* expression is not identical for *Notch1*, *2* and *3*. *Notch3* is not expressed, and *Notch1* and *2* are expressed in the stellate reticulum and not in the basal epithelium (article I). At the cap stage (E14) *Notch1* is still expressed throughout the stellate reticulum, although *Notch2* is now more restricted and hardly visible near the enamel knot. What could be the function of this *Notch* distribution at this early stage?

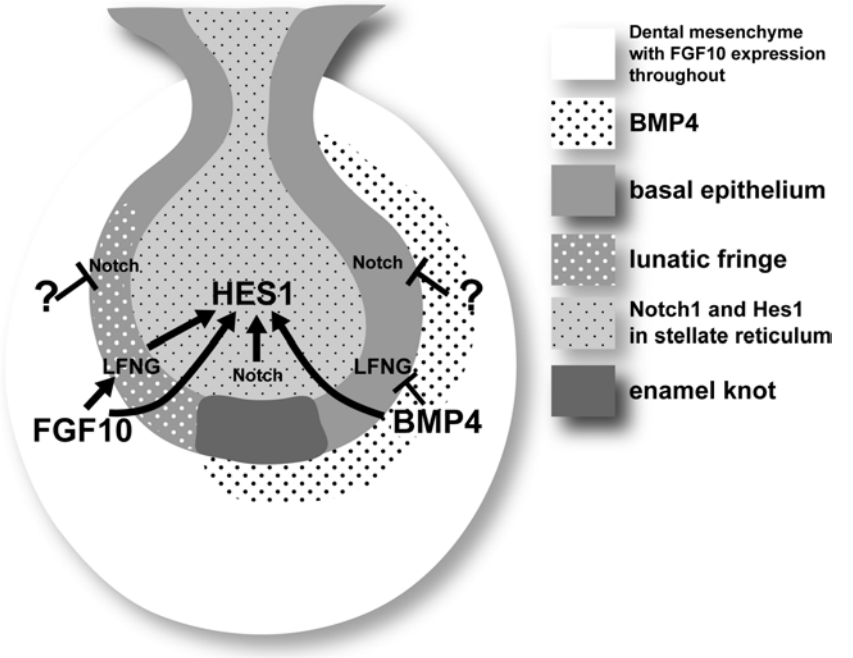

Figure 12. *The regulation of Lunatic fringe, Notch1, and Hes1 in the dental epithelium during the advanced bud stage of tooth development. Based on a figure in article I.*

The answer to that question may lie in *Lunatic fringe*, the modulator of Notch signalling. *Lunatic fringe* expression borders the enamel knot during these early stages first at the lingual side, and later it also engulfs the enamel knot on the buccal side. *Lunatic fringe* expression then marks the boundary between enamel knot epithelium and the remainder of the dental

epithelium. *Notch* expression is associated with boundary formation (de Celis et al., 1996), but in the tooth bud there is no sharp boundary of *Notch* expression with the enamel knot. Lunatic fringe can modulate the activity of Notch signalling which is reflected in the transcription of its downstream targets. *Hes1* is one of those downstream targets and has been reported as a marker for Notch activity (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). However there were no sharp boundaries of *Hes1* expression either, although *Hes1* does show higher expression levels at the lingual side than on the buccal side at the early stages corresponding to the distribution of *Lunatic fringe*. There may be therefore another target of Notch signalling involved.

Epithelial *Lunatic fringe* needs a mesenchymal signal to be transcribed. Bead experiments on tissue culture in vitro shows that *Lunatic fringe* expression is induced by FGF10 and FGF4 protein, which are respectively expressed in the mesenchyme and enamel knot. The induction could be blocked by simultaneously adding a BMP4 bead. This corresponds in vivo to buccal *Bmp4* expression which blocks the induction of *Lunatic fringe* at an early stage. In short, FGFs from both mesenchyme and the enamel knot induce the expression of *Lunatic fringe* around the enamel knot. *Lunatic fringe* expression is delayed at the buccal side because of the transient expression of *Bmp4* (figure 12).

Could Lunatic fringe have a similar function here as in the adult stem cell niche such as is present in the continuously growing incisor? Notch signalling requires cell-cell contact. The effect of Lunatic fringe is therefore limited. The highest expression of the ligand *Jagged1* is restricted to the enamel knot area and the region of the dental lamina connecting the tooth germ to the oral epithelium. *Jagged2* had only weak expression, and *Delta1* was not detected. If *Jagged1* is the main ligand in this system then the effects on *Notch* and *Lunatic fringe* must be limited to the areas of joint expression and therefore the boundaries with the enamel knot and the dental lamina. Similar to the incisor this area around the enamel knot could be responsible for the recruitment of stem cells out of the stellate reticulum and turn them into the pool of proliferating cells that are known later as the transit-amplifying cells in the incisor (Harada et al., 1999).

Either Lunatic fringe signalling is involved in setting up the boundary of the enamel knot, or the enamel knot cells are unable to express *Lunatic fringe* due to cellular conditions and the enamel knot actually defines the sharp *Lunatic fringe* boundary. If the latter is true then the function of Lunatic fringe must lie with the recruitment of stem cell progeny or another process. However, *Lunatic fringe* mutant mice do not show a tooth phenotype. There doesn't seem to be any redundancy with other fringe genes such as *Manic* and *Radical fringe* since their expression was normal and do not overlap with *Lunatic fringe*. Moreover, different fringes have different effects on Notch2 (Shimizu et al., 2001) and Lunatic fringe can have a different effect on Notch1 and Notch2 activity in the presence of the same ligands (Hicks et al., 2000) There must be other regulatory mechanisms in place to take over the function of Lunatic fringe in the *Lunatic fringe* knockout mice. Or the expression of *Lunatic fringe* during early tooth morphogenesis is a leftover of a previously active regulatory process; a fossil of a developmental past. Or is the striking pattern of *Lunatic fringe* a reflection of an active role in a different tooth of the same dentition?

In conclusion, during the transition from bud to cap stage the cervical loop, which represents the epithelial stem cell niche, is formed. Notch signalling plays a role during this early phase of tooth development by demarcating the boundary between the signalling center in the tooth, the enamel knot, and the stellate reticulum, the putative site for epithelial stem cells.

Root development (article II)

A root is not a passive tissue. A root consists of a complex composition of tissues and matrices and a root must grow. This all suggests that there must be some active signalling between different tissues to regulate these processes similar to all developmental processes. However, little is known about regulation of root development.

An important group of signalling molecules during early tooth development are the bone morphogenetic proteins (BMPs). BMPs are members of the TGFβ superfamily of growth and differentiation factors. In early tooth development BMPs are associated with epithelial-mesenchymal interactions (Åberg et al., 1997; Thesleff et al., 1995; Vainio et al., 1993) and are known to induce the expression of Msx1 and Msx2 (Vainio et a., 1993) and the formation of the enamel knot (Jernvall et al., 1998). Msx1 and Msx2 are transcription factors containing a homeobox. *Bmps* are also downstream of *Msx* and can even rescue the phenotype of a *Msx1* null mutant mouse (Bei et al., 2000). Therefore it is clear that BMP-Msx interactions are involved in reciprocal interactions during early tooth development.

root					
	Pre-odontoblasts	Early odontoblasts	cementoblasts	HERS	ERM
Bmp2	-	+	-	-	-
Bmp3	-	+	++	-	-
Bmp4	+	-	-	-	-
Bmp7	-	+	-	-	-
Msx1	-	±	-	-	-
Msx2	-	±	-	+	+

Table 1. *Expression of several Bmps and Msx in the root.*

Also during root formation *Bmps*, *Msx1* and *Msx2* are active. As mentioned in the introduction the structure of the root is quite different from that of the early tooth germ. The major epithelial structures are the HERS (derivative of the cervical loop) and the ERM (fragmented epithelium further away from the cervical loop). Any reciprocal signalling should therefore occur between these epithelial compartments and the mesenchymal ones. It is not so clear what these mesenchymal compartments are exactly. The most obvious compartment is that of differentiating odontoblasts that reside directly next to the epithelial compartments. These can be subdivided into smaller compartments based upon state of differentiation (preodontoblasts and mature odontoblasts, with possibly a gradient of different states between these). Reciprocal signalling can be expected between these epithelial and mesenchymal compartments.

The results are summarized in Table 1 and more details can be found in article II. The results show that it is unlikely that BMP and Msx signalling have a similar function during early and late tooth development. Interestingly *Bmp4* was expressed in the early pre-odontoblasts that line the root sheath epithelium (HERS). The HERS expressed *Msx2*. Most Bmp expression was associated with odontoblast differentiation in a sequential manner. *Bmp4* was in the early-preodontoblasts, *Bmp2* and *Bmp7* in the preodontoblasts and odontoblasts but not in the mature odontoblasts of the crown and root. *Bmp3* is expressed in the pre-odontoblasts and odontoblasts of the root but is absent in the crown.

Another important area of expression was the dental follicle cells in the root area. *Bmp3* was strongly expressed in the cementoblast which are responsible for depositing cementum on the surface of the root dentin. It is unknown if it functions in an inhibitory fashion similar to the

inhibitory role of BMP3 in the differentiation of the osteoprogenitor cells into osteoblasts (Daluiski et al., 2001).

In conclusion, during root formation BMP and Msx signalling fulfil a different function than during early tooth development. The tissues of the root require rather specific signalling to function which identity is so far still unknown.

Proximal distal patterning of the tooth (article III)

The significance of the epithelial stem cell niche in the tooth goes deeper than just providing a source of renewable tissue. The theory of evolution by means of natural selection has been the great backbone and stimulus for biological research since its conception (Darwin, 1859). Similarly evolution can give us an insight into the biological significance of the stem cell niche that no practical or medical application of stem cells could ever do. The epithelial stem cell niche could be manipulated during evolution to create various categories of teeth. This is because it plays an important role in the patterning of the proximal distal patterning of the tooth (figure 11).

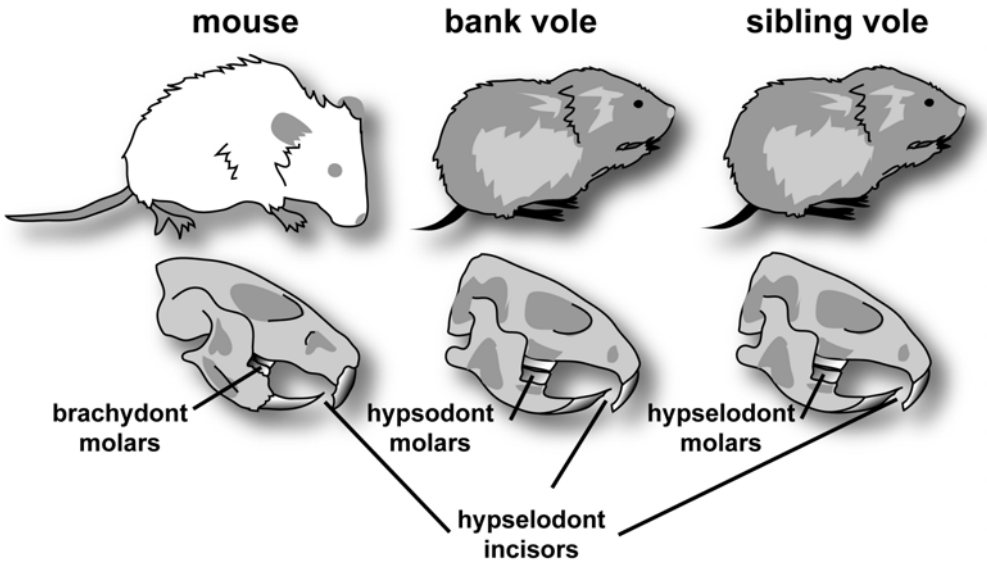

Figure 12. *Closely related rodent species can show different tooth types (brachydont, hypsodont and hypselodont) in the molar region, but share the characteristic hypselodont incisor of all rodents.*

Teeth come in many different shapes and forms. The most striking variation of form under the influence of proximal distal patterning is tooth height (figure 12). The typical human tooth belongs to the category of low crowned or brachydont teeth. They have a low crown and a high root. The next category would be that of mesodont teeth in which the crown and root are of equal high, but these teeth are relatively rare. The next category is that of high crowned or hypsodont teeth. Here the crown is much higher than the root. This tooth type is abundant and often an evolutionary drive is seen from low crowned to high crowned teeth. For instance, a significant increase in the prevalence of hypsodonty occurred during the Neogene due to changes in the environment (Jernvall and Fortelius, 2002) showing the evolutionary importance of this adaptation. A drier climate can result in more fibrous vegetation which increases the wear on teeth. A higher crown allows for more wear, and therefore extends the duration in

which a tooth is functional. Continuously growing or hypselodont teeth could be considered as an extreme form of hypsodonty. It has been proposed that increased crown height is a relatively simple matter of delayed termination of morphogenesis/cytodifferentiation and that hypselodonty is simply the extreme outcome of such a delay (von Koenigswald, 1982). In hypselodont teeth the crown formation never stops, although the crown of the tooth is partially converted to the root fate. This possibly occurs to solve the problem of anchoring a 'rootless' tooth to the jawbone, and sometimes as in the continuously growing incisor it causes mechanistic properties that help keep the incisor sharpened.

Although the continuously growing incisor of the mouse is a convenient model, it might not be the best model to study continuously growing teeth. The incisor is a common property of all rodents and therefore a relatively old evolutionary adaptation. Many rodents and other species have also continuously growing molars. Between closely related species there can be the difference between brachydont, hypsodont and hypselodont teeth (figure 13). For instance if we look at rodents, the mouse (*Mus musculus*) has brachydont molars, the bank vole (*Clethrionomys glareolus*) has hypsodont molars, and another vole species, the sibling vole (*Microtus rossiaemeridionalis*) has hypselodont molars. The switch to hypsodont and hypselodont molars is therefore a more recent one than the generally shared switch to continuously growing incisors.

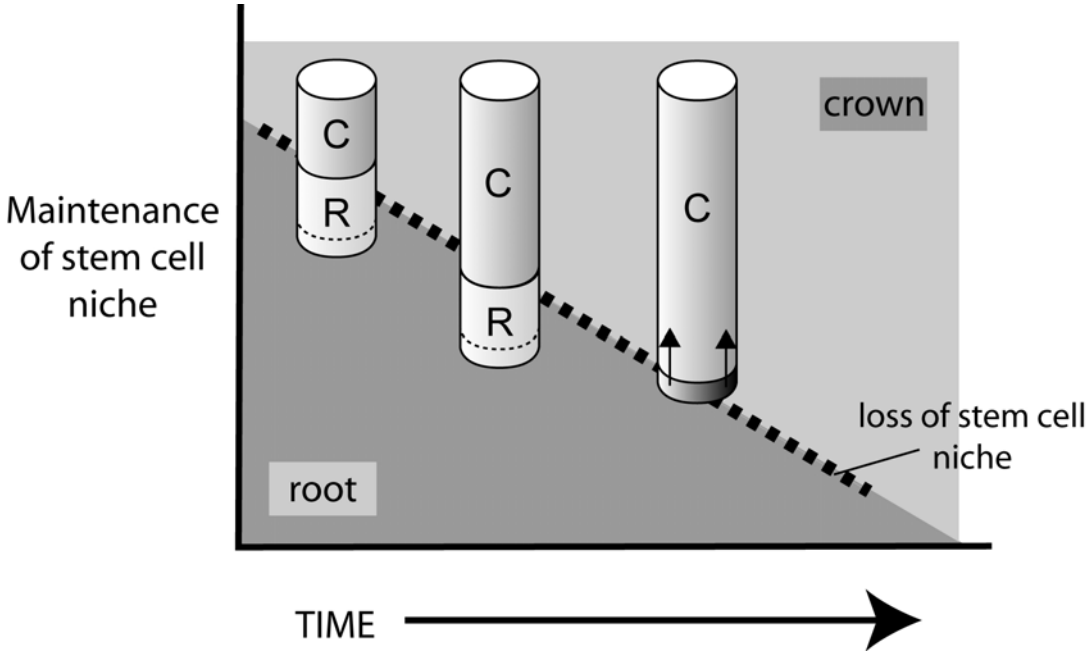

Figure 14. *From brachydont to hypsodont to hypselodont. The evolutionary step from brachydont to hypsodont and hypselodont can be represented as a extension of the regulation of the stem cell niche. The first tube represents a brachydont tooth. The stem cell niche is maintained only shortly resulting in a low crown. In the second example the stem cell niche is maintained longer during the life of the animal and a higher crown is the result. Hypselodont teeth are represented in the last category. The stem cell niche is maintained at least as long as the duration of the lifespan of the animal or at least close to this duration.*

We could view the progression of brachydonty, to hypsodonty and finally to hypselodonty as a regulatory event caused by regulation of the stem cell niche and the patterning of the proximal distal axis (article III and IV) (figure 14). In brachydont teeth the stem cell niche is maintained only shortly and the stem cells are lost early. This results in only a short period of growth of the crown and a subsequent relatively long growth of the root. In hypsodont teeth the stem cell niche is maintained longer and crown formation enjoys a similarly long period of growth, before

the stem cell niche is lost and root formation starts. This results in a high crown with a relatively short root. In hypselodont teeth the stem cell niche is maintained indefinitely and similarly also crown formation and root formation is postponed. Because there is still a need to anchor the tooth to the jaw bone a smaller part of the tooth is converted into root and therefore crown and root formation can occur simultaneously in continuously growing teeth. Possibly a similar phenomenon occurs in hypsodont teeth since root formation can be postponed for a relative long period of time.

The regulation of the stem cell niche in the sibling vole and mouse molar (Article III)

The interest in the continuously growing molar of the sibling vole is twofold. Firstly, it is important to establish if the stem cell niche of all continuously growing teeth is similar in structure and molecular regulation (figure 15). Secondly, by comparing it with a non-continuously growing molar in a related species we can make deductions on the fate of the stem cell niche and regulatory events that are associated with the demise of the stem cell niche.

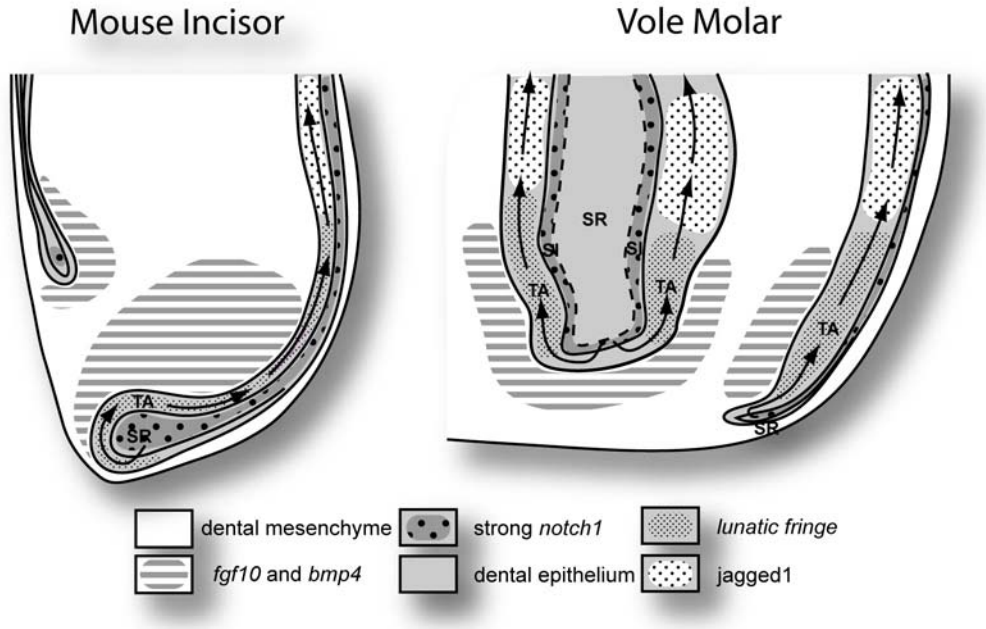

Figure 15. *Conservation of molecular regulation between different continuously growing teeth.The stellate reticulum and stratum intermedium are areas of high levels of notch expression and the putative location of the epithelial stem cells. The progeny of these stem cells migrate to the inner enamel epithelium and give rise to a population of transit-amplifying cells characterized by Lunatic fringe expression. The cells then differentiate into ameloblasts and express Jagged1. The stem cells and proliferating cells are maintained by mesenchymal Fgf10 that flanks the cervical loop. The vole molar has intercuspal folds which have an identical regulatory setup as the cervical loops.*

The continuously growing molar of the sibling vole has a complex structure (figure 16). There is nothing as obvious as 'the' cervical loop. The cervical loop is a structure that is not restricted to one position but is found in the circumference of the entire base of the tooth. During later stages there are some local changes in the structure of the cervical loop in the vole molar. Three smaller domains, one anterior and two posterior lateral, have lost the stellate reticulum typical of a functional stem cell niche and now resemble the HERS of a root. Similarly to the root analogue of the rodent incisor this continuously growing molar has formed partial root domains,

but instead of a single larger root domain it has three smaller ones orientated in a triangular pattern.

Because of the folding pattern of the epithelium, which creates the specific cusp pattern, intercuspal folds or loops are formed. These compartments are similar in structure to the cervical loop. There is a core of stellate reticulum and stratum intermedium enveloped by inner enamel epithelium. The intercuspal loop represents a continuation of the stem cell niche from the cervical loop rather than a separate structure.

The idea that the cervical loop and intercuspal loops represent functional stem cell niches comes from comparison of the molecular regulation of the stem cell niche. The major components of the Notch signalling pathway and FGF10 are expressed in identical patterns compared to the mouse incisor (Article III)(Harada et al., 1999). *Notch1* is expressed in the stellate reticulum, *Lunatic fringe* in the transit-amplifying cells of the inner enamel epithelium, *Jagged1* in the preameloblasts, and *Bsp1* in the ameloblasts. *Fgf10* can be seen in the dental mesenchyme of the base near the cervical loop and possibly functions in a similar manner as in the mouse incisor: as a maintenance factor for the stellate reticulum and inducing factor of proliferation (Harada et al., 2002).

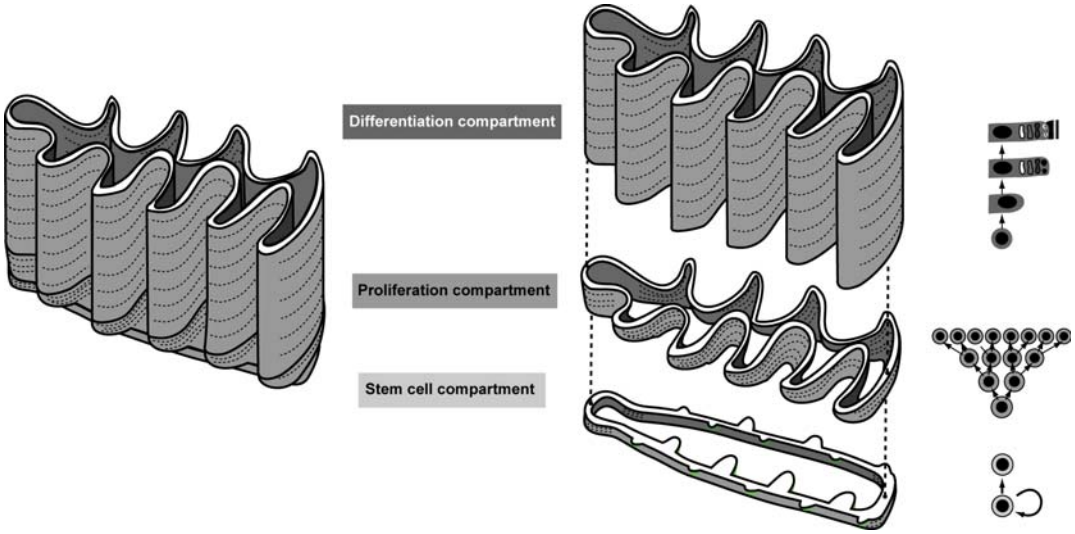

Figure 16. *The continuously growing molar of the vole subdivided in its different functional compartments; the stem cell compartment at the base, above it the proliferation compartment, and on top the differentiation compartment. As can be seen from this representation the vole molar has a complex shape.*

At 5 DPN we can see the occurrence of the root domains in the molar of the sibling vole. Since there is an obvious change in structure it is expected to have a similarly obvious change in regulation of the stem cell niche. In the anterior root domain *Notch1* is no longer expressed at 5 DPN although it was earlier. This coincides with a loss of the stellate reticulum. *Lunatic fringe* is also no longer expressed indicating at least a string reduction in proliferating cells. Mesenchymal *Fgf10* was still expressed but less intense as compared to normal around other cervical loop area.

Similar regulatory changes occur in the mouse molar, a brachydont tooth. Root formation in the mouse starts at 7 DPN when the HERS is first seen, although of course it is questionable if this is the first regulatory event leading to root formation. *Fgf3* and *Fgf10* are down-regulated soon after birth in the mouse molar (Kettunen et al., 2000). This event therefore precedes the formation of the HERS and could be one of the causes of the loss of stellate reticulum similar as

has been seen in the incisor of a *Fgf10* knockout mouse (Harada et al., 2002). At 10 and 14 DPN when root formation is in full swing no *Fgf3* or *Fgf10* is found. Interestingly, only *Notch2* and *Lunatic fringe* are expressed in the HERS of the 14 DPN root, instead Notch signalling seems to be mostly associated with the dental mesenchyme and is mostly active in the crown area. Therefore Notch and FGF signalling seem to be lost when the switch is made between maintenance of the epithelial stem cell niche and transition to the HERS typical of the root.

In conclusion there is an evolutionary trend from brachydonty to hypsodonty and hypselodonty which is made possibly by prolonging or maintaining indefinitely a status quo in the molecular regulation of the epithelial stem cell niche.

Evolutionary flexibility of the regulation of the adult stem cell niche and the progression in crown height (article IV)

Let me summarize the main conclusion so far before we go any further. The stem cell niche and its regulation are conserved between different continuously growing teeth. The presence of smaller root domains with a crown domain indicates the flexibility of the switch between crown and root and change between stem cell niche and HERS. This suggests that on an evolutionary scale the diversity in tooth types, such as brachydont, hypsodont and hypselodont teeth which in itself is an old idea, is due to the differential regulation of the stem cell niche (Article III), specifically in a temporal manner. The transition from brachydont to hypsodont occurred when the stem cell niche is maintained longer and root development is postponed. Closely related species as the bank vole with its hypsodont molars, and the sibling vole with its hypselodont molars, indicate that hypselodonty is an extreme form of hypsodonty. The stem cell niche is maintained indefinitely and root development is postponed as a whole. Instead some smaller domains are converted to the root fate. We have therefore a simple progression of low to high crowned molar and from high crowned molar to a molar in which the crown will grow throughout the lifetime of the animal.

However, to complicate matters there exists a rather unusual type of tooth found in an unusual group of animals; the Edentates (meaning without teeth), nowadays usually referred to as the order of Xenarthra, because of the odd number of vertebrae in this group. Although their old classification of Edentates might suggest that these animals do not have teeth this is not the case for all members of the group. Anteaters lack teeth, but armadillos and sloths just lack incisors and canines, but possess cheek teeth. Their teeth are described as continuously growing pegs covered with dentin, but this type of tooth is actually not even that uncommon. For instance, an elephants tusk could be described in a similar manner. Both the sloth tooth and the elephant tusk start with an animal cap, but lose it soon and only dentin surface remains. The sloth teeth and elephant tusks could therefore be considered to be hypselodont teeth without a proper crown (since it is lost rather early). This would however be impossible if crown formation is associated with the stem cell niche and root formation with HERS.

Separation of the regulation of stem cell niche and the regulation of fate decision between crown and root (article IV)

Can a root exist without the HERS? Beautifully preserved sections of the sloth molar prepared by a Dutch researcher named Van Den Broek in 1913 show no presence of HERS (Article IV). These teeth lack ameloblasts, but have odontoblasts covered with a dentin layer onto which a layer of cementum is deposited. These are clearly root structures. The cervical loop structure is that of a continuously growing tooth with a core of stellate reticulum surrounded by inner and outer enamel epithelium. Therefore the sloth molar has a root without a HERS.

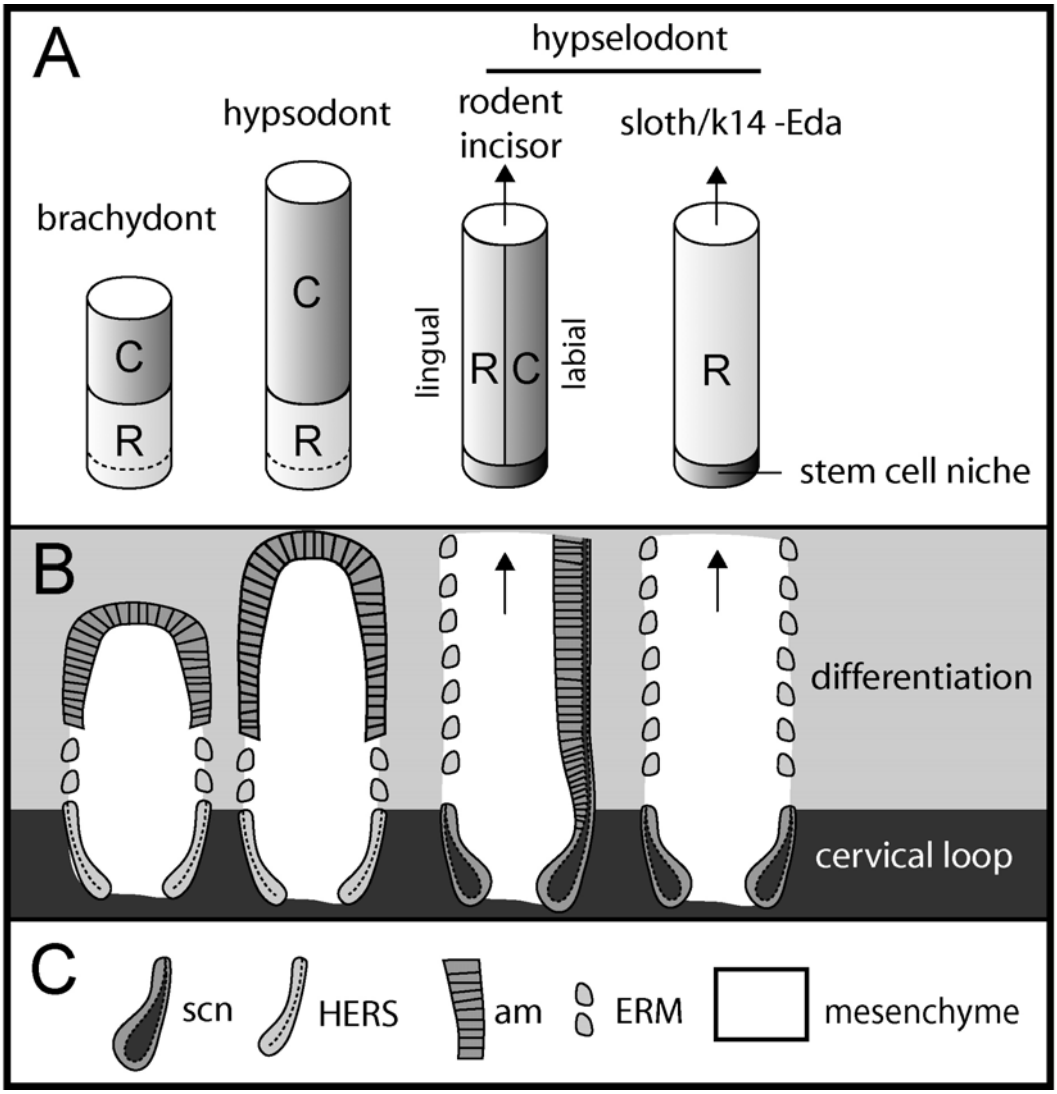

Figure 17. *How to pattern a tooth by means of independent regulation of the stem cell niche and the differentiation of the stem cell progeny. By maintaining the stem cell niche for a longer time the height of the tooth is increased. This caused the transition from brachydont, to hypsodont. Here root formation is delayed and crown formation is extended. This would indicate that the loss of the stem cell niche automatically dictates the initiation of root formation and the fate switch of the stem cell progeny to the root pathway. The situation is however more complex. If the stem cell niche is maintained indefinitely root formation can still be initiated in local domains. In the rodent incisor half of the tooth maintains the crown fate, the other half the root fate. Since the root side of the tooth also has to grow continuously, it is sometimes suggested that the root half derives the stem cells from the crown side. Continuously growing roots such as the sloth molar and the k14-eda incisor show that this does not have to be the case. Here no crown domain is present whatsoever. There is no HERS structure, and instead the cervical loop is identical to that of a hypselodont crown. This suggests that HERS is not a necessary structure for root formation and the maintenance of stem cell niche does not imply progeny with crown fate. Am, ameloblasts (crown epithelium); ERM, epithelial cell rests of Malassez (root epithelium); scn, stem cell niche; HERS, Hertwig's epithelial root sheath; C, crown; R, root.*

A molecular analysis of these antique slides is unfortunately impossible. However, a transgenic mouse line in which the TNF ligand *ectodysplasin* (Eda) is constitutively expressed under the

ectodermal keratin 14 promoter shows an incisor phenotype that resembles that of the sloth molar. Normally only the root analogue shows the root phenotype, but in the k14-Eda incisor the root analogue is also converted into the root fate (Article IV)(Mustonen et al., 2003). A frontal section only shows dentin instead of the normal deposit of enamel on the labial side. A typical root structure is the epithelial cell rests of Malassez (ERM). A keratin staining shows the presence of these structures at both the lingual and labial side in the k14-Eda incisor. Also *Bsp1* expression can be found at both sides and is typical of cementoblasts, a root specific cell line. Both the lingual and labial side of the k14-Eda incisor therefore express markers of a root and show the presence of root structures, instead of normally only the lingual side.

The stem cell niche of the k14-Eda incisor seems largely unaffected. There is a normal expression of most markers of the Notch signalling pathway in the epithelium of the cervical loop, and the mesenchymal *Fgf3* and *Fgf10* when compared to the wild type incisor (Article IV)(Harada et al., 1999). *Notch1* is still expressed in the stellate reticulum and *Lunatic fringe* in the transit-amplifying cells. *Jagged1* is normally expressed in the preameloblasts, and is not expressed in the cervical loop epithelium of the k14-Eda incisor. There is also no histological indication of the presence of ameloblasts. The epithelium of the inner enamel epithelium is cuboidal when it leaves the loop and brakes up in a fenestrated network towards the tip (as shown by the anti-keratin staining).

The k14-Eda incisor and the sloth molar are continuously growing roots. They should not exist if the survival of the stem cell compartment is coupled to the maintenance of the crown fate of the epithelium. A functional stem cell niche is present, but no ameloblasts are formed. This also means that the HERS is not a special morphological structure for root formation. The regulation of epithelial fate is one that cannot be found in these kinds of structural characteristics but must be sought on a molecular level instead. It is unknown what the exact molecular mechanisms are that can cause the switch between crown and root fate. It is clear that the stem cell niche and differentiation compartment are not coupled to each other on a strict one to one level; stem cell niche means ameloblasts, and HERS means root epithelium (figure 17). Moreover, the flexibility of the regulatory connection between stem cell niche and differentiation compartment allows for the phenomenon of a mixed pattern of crown and root in continuously growing teeth.

CONCLUDING REMARKS

The epithelial stem cell niche can be studied from many perspectives. The evolutionary perspective shows the epithelial stem cell niche as a developmental regulatory tool used for creating tooth diversity. These adaptations are simple in nature, but can have a great impact on the functionality of a tooth; a higher tooth that can withstand more wear; a tooth that never stops growing.

The regulatory components of epithelial stem cell niche are conserved between different continuously growing teeth, as can be seen by the similarities in FGF and Notch signalling between the mouse incisor and the molar of the sibling vole. It raises the question if all continuously growing teeth have evolved by the developmental adjustment of the same regulatory molecular networks of signalling molecules.

A stem cell niche is a structure and like all structures it has a developmental history. The same signalling pathways may be used for different functions during the development of the stem cell niche as can be seen for Notch signalling. It can be used in demarcating the boundary of the enamel knot, and later function in the differentiation of the stem cell progeny.

The proximal-distal patterning is flexible. A major determinant in this patterning is the subdivision of the tooth in a crown and root domain each with a different function. Variation in this patterning can lead to novel evolutionary inventions such as the rodent incisor

Different species and tooth types need to be analyzed to understand the nature of teeth. It is all too easy to focus on one type of tooth, the brachydont tooth, and draw general conclusions from studies on these kinds of teeth. However, the true nature of the tooth lies in its morphogenetic flexibility as a response to adjustments to environmental changes. The tooth pattern in the jaw is highly flexible, the cusp patterns and shapes of individual teeth are highly flexible, and the proximal-distal patterning of the tooth is highly flexible, giving rise to highly changeable shapes and forms. A combination of all three aspects of form would be necessary to understand the nature of the tooth as a developing organ and an adaptive evolutionary tool. Proximal-distal patterning is apparently regulated by the same signalling pathways in different kinds of continuously growing teeth. Changes in this regulatory setup precede the loss of the epithelial stem cells in the stem cell niche.

A transgenic model such as the incisor of the k14-Eda overexpressing mice can give us insight in the nature of extraordinary tooth type; the continuously growing root. In conclusion I would like to point out this extraordinary tooth type actually represents a rather common tooth type. The anthropomorphic view on tooth development which is reflected in the study of mainly the brachydont molar tooth model in the mouse gives a distorted view. Hypsodonty is common amongst mammalian species. Hypselodonty is also a rule rather than an exception. The rodent incisor is a rather shining example of hypselodonty being a rule rather than something exotic with 1500 species alone carrying this trademark! A brief survey of mammalian tooth types shows even more examples. And it also shows that the continuously growing root is not a freak accident, but rather a normal tooth on the far end of the spectrum. The lagomorphs are unrelated to the rodents but show the same dental pattern of continuously growing incisors, a diastema and molars in the back. The incisors of the rodent, however, do not have a lingual root analogue. In a cross section there is enamel around the entire tooth. Early rodents had a greater area of enamel than modern ones placing them in between the lagomorphs and the modern rodents. The sloth has no enamel in a cross section and forms the far end of this spectrum. All are continuously growing teeth, but together they show a gradual change from all enamel to no enamel. Therefore, to characterize tooth development based on the brachydont model neglects the existence of tooth variety

The ‘exotic’ and ‘strange’ teeth that can be found in the dentition of the sloth can give us general insight because they form a normal part of the tooth character spectrum. In fact the continuously growing root is not such a rare phenomenon as many people would like to think. In this particular case it teaches us that stem cell niche maintenance and crown fate are not necessarily linked, nor is the root fate with the presence of HERS. In conclusion, the independence in the regulation of the stem cell niche and differentiation of the stem cell progeny allowed for emergence of new tooth characters during evolution. The great variety in tooth character created by developmental regulatory independence should be considered when drawing general conclusions based on research on a brachydont tooth model, such as the mouse molar. To understand the tooth we have to research the teeth in all their variety of shape and function on a developmental and evolutionary level.

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REFERENCES

Anderson, A. C., Robey, E. A. and Huang, Y. H. (2001) Notch signaling in lymphocyte development. *Curr. Opin. Genet Dev* **11**, 554-560.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe, D. A., Lendahl, U. and Edlund, H. (1999) Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-881.

Attenborough, D. Life of Mammals. Attenborough, D. 2003. DVD. BBC/Discovery Channel.

Bailey, A. M. and Posakony, J. W. (1995) Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Dev* **9**, 2609-2622.

Beertsen, W., Holmbeck, K., Niehof, A., Bianco, P., Chrysovergis, K., Birkedal-Hansen, H. and Everts, V. (1904) Inhibition of molar eruption and root elongation in MT1-MMP-deficient mice. *Connect. Tissue Res.* **44 Suppl 1**, 298-299.

Bei, M., Kratochwil, K. and Maas, R. L. (2000) BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development. *Development* **127**, 4711-4718.

Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C. and Vescovi, A. L. (1999) Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* **283**, 534-537.

Blecher, S. R., Debertin, M. and Murphy, J. S. (1983) Pleiotropic effect of Tabby gene on epidermal growth factor-containing cells of mouse submandibular gland. *Anat. Rec.* **207**, 25-29.

Butler, P. M. (1995) Ontogenetic aspects of dental evolution. *Int. J. Dev. Biol.* **39**, 25-34.

Chai, Y. and Slavkin, H. C. (2003) Prospects for tooth regeneration in the 21st century: a perspective. *Microscopy Research & Technique* **60**, 469-479.

Chen, Y. L., Dzik, J., Edgecombe, G. D., Ramsköld, L. and Zhou, G. Q. (1995) A possible Early Cambrian chordate. *Nature* **377**, 720-722.

Chenn, A. and McConnell, S. K. (1995) Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82**, 631-641.

Daluiski, A., Engstrand, T., Bahamonde, M. E., Gamer, L. W., Agius, Stevenson, S. L., Cox, K., Rosen, V. and Lyons, K. M. (2001) Bone morphogenetic protein-3 is a negative regulator of bone density. *Nat. Genet.* **27**, 84-88.

Darwin, C. (1859) *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life.*, Viking Press.

de Celis, J. F., Garcia-Bellido, A. and Bray, S. J. (1996) Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**, 359-369.

Dhouailly, D. (1975) Formation of cutaneous appendages in dermo-epidermal recombinations between reptiles, birds and mammals. *Wilhelm Roux Arch.* **177**, 323-340.

Diekwisch, T. G. (2001) The developmental biology of cementum. *Int. J. Dev. Biol.* **45**, 695-706.

Gilbert, S.F. (2000) *Developmental biology*, Sinauer.

Gronthos, S., Brahim, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., DenBesten, P., Robey, P. G. and Shi, S. (2002) Stem cell properties of human dental pulp stem cells. *J. Dent. Res.* **81**, 531-535.

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G. and Shi, S. (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc. Natl. Acad. Sci. USA.* **97** , 13625-13630.

Grüneberg, H. (1965) Genes and genotypes affecting the teeth of the mouse. *J. Embryol. exp. Morph.* **14**, 137-159.

Grüneberg, H. (1971) The glandular aspects of the tabby syndrome in the mouse. *J. Embryol. exp. Morph.* **25**, 1-19.

Hammarstrom, L., Alatli, I. and Fong, C. D. (1996) Origins of cementum. *Oral Diseases* **2**, 63-69.

Harada, H., Kettunen, P., Jung, H. S., Mustonen, T., Wang, Y. A. and Thesleff, I. (1999) Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *J. Cell Biol.* **147**, 105-120.

Harada, H., Toyono, T., Toyoshima, K., Yamasaki, M., Itoh, N., Kato, S., Sekine, K. and Ohuchi, H. (2002) FGF10 maintains stem cell compartment in developing mouse incisors. *Development* **129**, 1533-1541.

Hardy, M. H. (1992) The secret life of the hair follicle. *Trends Genet.* **8**, 55-61.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000) Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat. Cell Biol.* **2**, 515-520.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000) Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat. Cell Biol.* **2**, 515-520.

Holland, N. D. and Chen, J. (2001) Origin and early evolution of the vertebrates: new insights from advances in molecular biology, anatomy, and palaeontology. *Bioessays* **23**, 142-151.

Ivanova, N. B., Dimos, J. T., Schaniel, C., Hackney, J. A., Moore, K. A. and Lemischka, I. R. (2002) A stem cell molecular signature. *Science* **298**, 601-604.

Janvier, P. (1999) Catching the first fish. *Nature* **402**, 21-22.

Jernvall, J. and Fortelius, M. (2002) Common mammals drive the evolutionary increase of hypsodonty in the Neogene. *Nature* **417**, 538-540.

Jernvall, J. and Thesleff, I. (2000) Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech. Dev.* **92**, 19-29.

Jernvall, J., Åberg, T., Kettunen, P., Keränen, S. and Thesleff, I. (1998) The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. *Development* **125**, 161-169.

Kagayama, M., Sasano, Y., Zhu, J., Hirata, M., Mizoguchi, I. and Kamakura, S. (1998) Epithelial rests colocalize with cementoblasts forming acellular cementum but not with cementoblasts forming cellular cementum. *Acta Anat.* **163**, 1-9.

Kawano, S., Morotomi, T., Toyono, T., Nakamura, N., Uchida, T., Ohishi, M., Toyoshima, K. and Harada, H. (2003) Establishment of dental epithelial cell line (HAT-7) and the cell differentiation dependent on Notch signaling pathway. *Connect. Tissue Res.* **43**, 409-412.

Keränen, S. V., Åberg, T., Kettunen, P., Thesleff, I. and Jernvall, J. (1998) Association of developmental regulatory genes with the development of different molar tooth shapes in two species of rodents. *Dev. Genes Evol.* **208**, 477-486.

Kettunen, P., Laurikkala, J., Itäranta, P., Vainio, S., Itoh, N. and Thesleff, I. (2000) Associations of FGF-3 and FGF-10 with signaling networks regulating tooth morphogenesis. *Dev. Dyn.* **219**, 322-332.

Kratochwil, K. (1969) Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. *Dev. Biol.* **20**, 46-71.

Laurikkala, J., Mikkola, M., Mustonen, T., Åberg, T., Koppinen, P., Pispa, J., Nieminen, P., Galceran, J., Grosschedl, R. and Thesleff, I. (2001) TNF signaling via the ligand-receptor pair ectodysplasin and edar controls the function of epithelial signaling centers and is regulated by Wnt and activin during tooth organogenesis. *Dev. Biol.* **229**, 443-455.

Laurikkala, J., Pispa, J., Jung, H. S., Nieminen, P., Mikkola, M., Wang, X., Saarialho-Kere, U., Galceran, J., Grosschedl, R. and Thesleff, I. (2002) Regulation of hair follicle development by the TNF signal ectodysplasin and its receptor Edar. *Development* **129**, 2541-2553.

Lecourtois, M. and Schweisguth, F. (1995) The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. *Genes Dev* **9**, 2598-2608.

Line, S. R. (2003) Variation of tooth number in mammalian dentition: connecting genetics, development, and evolution. *Evolution & Development* **5**, 295-304.

Lowell, S., Jones, P., Le Roux, I., Dunne, J. and Watt, F. M. (2000) Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. *Curr. Biol.* **10**, 491-500.

Lumsden, A. G. (1988) Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development Suppl.* **103**, 155-169.

Lutolf, S., Radtke, F., Aguet, M., Suter, U. and Taylor, V. (2002) Notch1 is required for neuronal and glial differentiation in the cerebellum. *Development* **129**, 373-385.

Mina, M. and Kollar, E. J. (1987) The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. *Arch. Oral. Biol.* **32**, 123-127.

Mitsiadis, T., Lardelli, M., Lendahl, U. and Thesleff, I. (1995) Expression of Notch 1, 2 and 3 is regulated by epithelial- mesenchymal interactions and retinoic acid in the developing mouse tooth and associated with determination of ameloblast cell fate. *J Cell Biol* **130**, 407-418.

Mitsiadis, T. A., Fried, K. and Goridis, C. (1999) Reactivation of Delta-Notch signaling after injury: complementary expression patterns of ligand and receptor in dental pulp. *Exp. Cell Res.* **246**, 312-318.

Mucchielli, M. L. and Mitsiadis, T. A. (2000) Correlation of asymmetric Notch2 expression and mouse incisor rotation. *Mech. Dev.* **91**, 379-382.

Mustonen, T., Pispa, J., Mikkola, M. L., Pummila, M., Kangas, A. T., Jaatinen, R. and Thesleff, I. (2003) Stimulation of ectodermal organ development by ectodysplasin-A1. *Dev. Biol.* **259**, 123-136.

Nishimura, E. K., Jordan, S. A., Oshima, H., Yoshida, H., Osawa, M., Moriyama, M., Jackson, I. J., Barrandon, Y., Miyachi, Y. and Nishikawa, S. (2002) Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* **416**, 854-860.

Pispa, J. and Thesleff, I. (2003) Mechanisms of ectodermal organogenesis. *Dev. Biol.* **262**, 195-205.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C. and Melton, D. A. (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* **298** , 597-600.

Reisz, R. R. and Smith, M. M. (2001) Developmental biology. Lungfish dental pattern conserved for 360 Myr. *Nature* **411**, 548.

Salazar-Ciudad, I. and Jernvall, J. (2002) A gene network model accounting for development and evolution of mammalian teeth. *Proc. Natl. Acad. Sci. USA* **99**, 8116-8120.

Savage, R.J.G. and Long, M.R. (1986) *Mammal evolution - an illustrated guide*, British Museum (Natural History).

Shimizu, K., Chiba, S., Saito, T., Kumano, K., Takahashi, T. and Hirai, H. (2001) Manic fringe and lunatic fringe modify different sites of the Notch2 extracellular region, resulting in different signaling modulation. *J. Biol. Chem.* **276**, 25753-25758.

Shu, D. G., Chen, L., Conway Morris, S., Zhang, X. L., Hu, S. X., Han, J., Zhu, M., Li, Y. and Chen, L. Z. (1999) Lower cambrian vertebrates from south china. *Nature* **402**, 42-46.

Sire, J. Y. (2001) Teeth outside the mouth in teleost fishes: how to benefit from a developmental accident. *Evolution & Development* **3**, 104-108.

Smith, M. M. (2003) Vertebrate dentitions at the origin of jaws: when and how pattern evolved. *Evolution & Development* **5**, 394-413.

Smith, M. M. and Hall, B. K. (1990) Development and evolutionary origins of vertebrate skeletogenic and odontogenic tissues. *Biol. Rev. Camb. Philos. Soc.* **65**, 277-373.

Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001) Stem cells find their niche. *Nature* **414**, 98-104.

Starkey, W. E. (1963) The Migration and Renewal of Tritium Labelled Cells in the Developing Enamel Organ of Rabbits. *Br. Dent. J.* **115**, 143-153.

Steele-Perkins, G., Butz, K. G., Lyons, G. E., Zeichner-David, M., Kim, H. J., Cho, M. I. and Gronostajski, R. M. (2003) Essential role for NFI-C/CTF transcription-replication factor in tooth root development. *Mol. Cell Biol.* **23**, 1075-1084.

Stollewerk, A., Schoppmeier, M. and Damen, W. G. (2003) Involvement of Notch and Delta genes in spider segmentation. *Nature* **423**, 863-865.

Streelman JT.Webb JF.Albertson RC.Kocher TD. (2003) The cusp of evolution and development: a model of cichlid tooth shape diversity. *Evolution & Development* **5**, 600-608.

Suzuki, M., Inoue, T., Shimono, M. and Yamada, S. (2002) Behavior of epithelial root sheath during tooth root formation in porcine molars: TUNEL, TEM, and immunohistochemical studies. *Anat Embryol* **206**, 13-20.

Ten Cate, A.R. (1998) *Oral Histology. Development, structure and function.*, Mosby, St Louis

Thesleff, I. and Tummers, M. (1904) Stem cells and tissue engineering: prospects for regenerating tissues in dental practice. *Medical Principles & Practice* **12 Suppl 1**, 43-50.

Thesleff, I., Vaahtokari, A., Kettunen, P. and Åberg, T. (1995) Epithelial-mesenchymal signaling during tooth development. *Connect. Tissue Res.* **32**, 9-15.

Thomas, H. F. (1995) Root formation. *Int. J. Dev. Biol.* **39**, 231-237.

Tucker, A. S., Headon, D. J., Schneider, P., Ferguson, B. M., Overbeek, P., Tschopp, J. and Sharpe, P. T. (2000) Edar/Eda interactions regulate enamel knot formation in tooth morphogenesis. *Development* **127**, 4691-4700.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M. and Fuchs, E. (2004) Defining the epithelial stem cell niche in skin. *Science* **303**, 359-363.

Tummers, M. and Thesleff, I. (2003) Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species. *Development* **130**, 1049-1057.

Vaahtokari, A., Åberg, T., Jernvall, J., Keränen, S. and Thesleff, I. (1996) The enamel knot as a signaling center in the developing mouse tooth. *Mech. Dev.* **54**, 39-43.

Vainio, S., Karavanova, I., Jowett, A. and Thesleff, I. (1993) Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75**, 45-58.

von Koenigswald, W. (1982) Zum verstandnis der Morphologie der wuhlmausmolaren. *Z Geol Wiss Berlin* **10**, 951-962.

Wang, S. and Barres, B. A. (2000) Up a notch: instructing gliogenesis. *Neuron* **27**, 197-200.

Watt, F. M. and Hogan, B. L. (2000) Out of Eden: stem cells and their niches. *Science* **287**, 1427-1430.

Wilkinson, D. and Green, J. (1990) *In situ* hybridization and the three-dimensional reconstruction of serial sections. in *Postimplantation Mammalian Embryos* (Copp, A.J. and Cole, D.E., eds) pp. 155-171, Oxford University Press, London.

Zeichner-David, M. O. (2003) Role of Hertwig's epithelial root sheath cells in tooth root development. *Dev. Dyn.* **228**, 651-663.

Åberg, T., Wozney, J. and Thesleff, I. (1997) Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev. Dyn.* **210**, 383-396.