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The taming of the shrew milk teeth

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SUMMARY A characteristic feature of mammalian dentition is the evolutionary reduction of tooth number and replacement. Because mice do not replace teeth, here we used *Sorex araneus*, the common shrew, as a model to investigate the loss of tooth replacement. Historically, shrews have been reported to initiate the development of several, milk or deciduous teeth but these soon become rudimentary and only the replacement teeth erupt. Shrews thus offer a living example of a derived mammalian pattern where the deciduous tooth development is being suppressed. Based on histological and gene expression analyses of serial sections, we suggest that *S. araneus* has discernible tooth replacement only in the premolar 4 (P4) position. Both generations of teeth express *Shh* in the enamel knot and in the inner enamel epithelium. Nevertheless, the deciduous P4 (dP4) is reduced in size during embryogenesis and is eventually lost without becoming functional. Analysis of growth shows that P4 replaces the dP4 in a "double-wedge" pattern indicative of competitive replacement where the suppression of the deciduous tooth coincides with the initiation of its replacement. Because activator–inhibitor mechanisms have been implicated in adjacent mouse molars and in transgenic mice with continuous tooth budding, we suggest that evolutionary suppression of deciduous teeth may involve early activation of replacement teeth, which in turn begin to suppress their deciduous predecessors.

INTRODUCTION

Tooth renewal and evolution

In most vertebrates, such as fish and reptiles, teeth are replaced continuously and tooth renewal compensates for the wear and loss of teeth throughout the life. In mammals tooth replacement is limited to one replacement and is connected to the growth of the jaw. Because the occlusal dimensions of teeth do not grow after tooth eruption, larger numbers of teeth or larger teeth are needed to replace the smaller ones of the young. In addition to limited tooth renewal, mammalian dentition is characterized by different teeth along the tooth row or heterodonty. The generalized mammalian dentition consists of anterior incisor and canine teeth and posterior premolar and molar teeth. As the jaw grows, the incisors, canines, and premolars are replaced whereas molars are added posteriorly. The evolutionary reduction in tooth replacement appears to have happened concomitantly with the evolution of complex molars and interlocking occlusion of opposing teeth (Osborn 1973; Kobayashi et al. 2002; Kielan-Jaworowska et al. 2004; Luo et al. 2004).

Several mammalian groups, such as most rodents and seals, show extreme reduction in tooth renewal and have only rudimentary milk or deciduous dentitions. Although "no single hypothesis" (van Nievelt and Smith 2005) may be adequate to explain all the cases of the loss of tooth replacement, factors proposed include small body size, short life span, reduced tooth wear, or diet (e.g., Wilson and Hill 1897; Pond 1977; Bloch et al. 1998; Godfrey et al. 2005). The general developmental process underlying loss of mammalian tooth renewal seems to be early development of permanent teeth at the expense of deciduous tooth development. The molecular nature of this process, however, remains unknown. Unlike a complete loss at tooth loci, such as premolars in murine rodents which may be linked to regulation of tooth identity (Thomas and Sharpe 1998), loss of tooth replacement can be assumed to be informative about mechanisms regulating organ renewal.

In general, the presence of embryonic rudiments might be expected because the same gene regulatory pathways are used in different organs at different stages during embryogenesis. This shared regulatory system leads to the difficulty of removing a primordium completely and there may be few selective pressures to completely eradicate an organ (Hall 2003). For example, rodents develop rudimentary tooth buds in the empty diastema region between the incisors and molars (Moss-Salentijn 1978; Peterkova et al. 1993; Keränen et al. 1999; Viriot et al. 2000). These tooth buds express several typical dental placode markers such as *Shh* (Keränen et al. 1999). In contrast, comparable evidence on rudiment formation is not

available for tooth renewal, in large part because mouse, the standard model system of tooth developmental genetics, lacks tooth replacement. Evidence on individual mouse tooth development suggest that a dynamic regulatory network of activators and inhibitors determine the initiation of individual tooth crown features (Salazar-Ciudad and Jernvall 2002), including suppression of diastema tooth rudiments in mouse (Peterkova et al. 2003) and perhaps also tooth renewal (Järvinen et al. 2006). These results raise the possibility that inhibition by replacement teeth could be a factor in the reduction of tooth renewal.

Here we investigate loss of tooth renewal in a species with rudimentary deciduous teeth, the common shrew (*Sorex araneus*). We used wild caught shrews, histological analysis, in situ hybridization analysis and 3D reconstruction to build a framework for the deciduous and permanent tooth development. We were especially interested in testing when in relation to replacement teeth is the developmental decision made for the deciduous tooth not to develop.

Shrew as a model

The development of dentition and the gene networks responsible for the morphogenesis and differentiation have been elucidated in great detail (Thesleff 2003). Tooth development has traditionally been investigated in a mouse model. Compared with the general eutherian pattern, rodent dentition is highly derived appearing in the Eocene (Flynn et al. 1985). Mice lack canines and premolars and have only one incisor and three molars in each jaw quadrant. A toothless diastema region thus separates the incisor from the molars. There is no secondary tooth formation. Thus less is known of tooth cycling and the development of the secondary (permanent) dentition due to the lack of a good model animal. There has been a growing interest in finding new model animals for the research on secondary tooth development, including in the connection to biomedical research for a model whose dentition resembles more the human dentition.

Compared with mice, shrews retain many of the more basal eutherian dental features. Shrews, now often placed in order Soricomorpha, are a highly specious group of predominantly terrestrial and insectivorous mammals. Shrew development has attracted research attention for over a century. Although shrews also lack full tooth replacement, they have been documented to have rudimentary deciduous teeth. There have been conflicting reports, however, on shrew (*Soricidae*) tooth development and particularly the secondary tooth development. The first report where microscopy sections were investigated concludes that *Soricidae* have only one dentition (Leche 1895). A second study reported that *S. araneus* develops two dentitions in positions I1, I2, I3, I4, P1, P2, P4, but that the primary dentition is rudimentary and that it disappears during embryogenesis (Ärnbäck Christie-Linde 1912). A more recent report on *S. araneus* tooth development supports this, but reports secondary tooth generation only in four positions I1, I2, C, and P4 (Kindahl 1959). According to these results, *S. araneus*, the common shrew, thus initiates the development of at least some of the primary teeth during embryogenesis, but only the secondary dentition erupts.

There has recently been a report of secondary tooth development in *Suncus murinus*, a close relative, belonging also to the *Soricidae* family of shrews. The analysis of apoptotic disappearance of primary teeth during gestation was reported (Sasaki et al. 2001), including the suggestion of incipient replacements for molars. This would contrast with the general agreement that molar teeth never develop successors in extant mammals. Additionally, it has been shown that Bmp4 expression in early *S. murinus* jaws shows a restricted and more distinct, but not contradictory expression pattern than in the mouse (Ogi et al. 2002) and *Shh* expression has been detected in the enamel knots of developing *S. murinus* tooth buds (Miyado et al. 2007; Yamanaka et al. 2007). These data support the idea that the basic aspects of molecular regulation of tooth development are similar in mouse and shrew.

Limitations of shrews as a model for tooth replacement studies include the obvious lack of complete development of deciduous teeth, and also, especially in the case of wild caught *S. araneus*, the lack of information about exact developmental age and limited access to specimens. Yet an additional challenge of *Soricidae* is their specialized anterior dentition that has made it difficult to infer tooth homologies between the first incisor and last premolar (Thenius 1989; Dannelid 1998).

Taken together, the previous evidence indicates that shrews are an example where tooth renewal is all but lost. Together with a dental formula that is less derived than that of the mouse, this suggests that shrew is an intriguing model to study secondary tooth development, its disappearance, and the evolution of tooth renewal reduction.

MATERIALS AND METHODS

Animals and preparation of embryonic tissues

Common shrews (*S. araneus*) were caught in pitfall traps dug into ground in eastern and southern Finland. Moss and fresh fish were placed at the bottom to sustain the animals and the trap was covered with wood and moss. Traps were checked twice a day. Five pregnant females were sacrificed by cervical dislocation and seven to nine embryos were collected from each female. Embryos were staged by measuring the crown to the base of the tail lengths. The smallest embryo was 7-mm long and the mean size of embryos in litters were 7.5, 9, 10, 11, and 12 mm. Heads were dissected and fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Because the embryonic stages included only early stages of tooth development, no decalcification was needed. Serial sections were taken in frontal plane at 7 μm and processed either for in situ hybridization or stained in hematoxylin–eosin for histological

analysis. Serial frontal sections of 12-mm embryos were taken at 10 μm for 3D analysis. We limited our analysis to the lower teeth because comparative information for tooth development is dominated by technically more easily studied lower teeth.

In situ hybridization and 3D reconstructions

We chose *Shh* as a marker of tooth initiation and morphogenesis. It is upregulated repeatedly during odontogenesis and *Shh* expression can be detected in the early primary epithelial band, in the early dental placodes, and later in individual teeth in the enamel knots and in the enamel secreting ameloblasts (Keränen et al. 1999; Kangas et al. 2004). Thus its expression represents most stages of early tooth development and makes it a suitable molecule for the identification of tooth buds. Radioactive in situ hybridization for paraffin sections was performed as described earlier (Wilkinson and Green 1990). *S. murinus Shh* probe was a kind gift from Atsushi Yamanaka and Masanori Uemura, Japan, and has been described earlier (Ogi et al. 2002). Probes were labeled with 35S-UTP (Amersham, Buckinghamshire, UK); exposure time was 14 days. Pictures were taken at × 4 magnification with an AX70 microscope Olympus (Melville, NY, USA) and reconstructed for 3D by the NIH image and NIH 3D programs (Apple, Cupertino, CA, USA) as described (Jernvall et al. 1998; Kassai et al. 2005).

Tooth growth rate measurements

Because we did not know the age of the embryos, we used embryo size as a proxy of developmental stage. Tooth size changes during development were examined relative to other teeth, thus these tabulations should not be sensitive to absolute time difference between different sized embryos. Note that embryos of different size can have the same absolute age, but here we assume that size manifests the stage of development. Except for a 7-mm embryo, which was from the 7.5-mm litter, all measured stages were from different litters. Tooth sizes were measured using the NIH image program. The area of tooth epithelium and *Shh* expressing field were measured by calculating the total cell number in serial histological sections (area divided by mean cell size), and the resulting values were plotted for each individual tooth at all five time points (embryonic stages). For a conservative measure of tooth initiation, clear mesenchymal extension (comparable to the early bud stage in mouse) was chosen as the starting point of individual tooth development.

RESULTS

Only dP4 of the deciduous dentition reaches cap stage in *S. araneus*

We performed a careful histological analysis from collected samples. Sample series includes five stages and extends from stages 7.5 to 12 mm providing a reasonably continuous series of developmental stages. From the deciduous dentition only primary premolar 4 (dP4) was observed reaching the cap stage. No unambiguous traits of other deciduous or permanent premolars were detected, nor any indication of deciduous incisors or canine; hence, below we focus on dP4 dynamics relative to permanent premolar (P4) and the first molar (M1).

P4 is initiated before differentiation of dP4 cervical loop

Development of dP4 was analyzed using histological sections and in situ hybridization at five different embryonic stages. Histological sections showed that at the youngest available stage, at 7.5 mm, the primary tooth bud (dP4) is at the cap stage, expressing *Shh*, indicating the formation of the primary enamel knot. The tooth anlage in the 7.5-mm shrew embryo resembles the cap stage mouse molar, with slightly more prominent lingual portion (Figs. 1A and 3) with incipient mesenchymal condensation (Fig. 3). This lingual elongation is more pronounced in the older embryos and in 9-mm embryo the tip of the down growth expresses *Shh*. Later stages indicate that this elongation is the future permanent premolar (P4). At 9 mm dP4 has proceeded into late cap stage and *Shh* expression follows the inner enamel epithelium (Figs. 1B and 2B). At 10 mm dP4 still shows the cap stage morphology, although it has now started to diminish in size. Regardless of the reduction in size *Shh* is expressed in the inner enamel epithelium (Figs. 1C and 2C). By 11 and 12 mm dP4 has almost disappeared and there is only a small round spot left. *Shh* expression can still be detected in four subsequent sections in 12-mm stage dental epithelium (Figs. 1, D and E and 2, D and E).

The developing P4 protrudes deep into the mesenchyme and at 9 mm the distal end has limited *Shh* expression in the future enamel knot area (Figs. 1B and 2B). At 10 mm P4 has proceeded into cap stage and now shows a well defined *Shh* expression in the enamel knot (Figs. 1C and 2C). At 11 and 12 mm P4 has notably grown in size and *Shh* expression extends along the inner enamel epithelium and marks the beginning of the differentiation stage (Figs. 1, D and E and 2, D and E). Taken together, the inverse growth trajectories of dP4 and P4 (and M1) suggest that the studied developmental stages reflect a true developmental series rather than variation in tooth size due to embryo size alone.

In order to ascertain the spatial organization of teeth in the jaw, morphology of dP4, P4, M1, and M2 together with *Shh* expression were reconstructed into 3D from the inner enamel epithelium at the oldest possible embryonic stage (12 mm). Compared with the adult jaw (Fig. 4, A and B), the 3D reconstruction indicates the final organization of teeth in the developing jaw. The dP4 is still present above the P4, and appears to be closest to the center of P4 enamel knot (Fig. 4C). M1 is the largest tooth already at 12-mm stage and M2 is in late cap stage. P4, M1, and M2 express *Shh* in the inner enamel epithelia. The residual dP4 anlage is almost fully covered by *Shh* expression.

Dynamics of *Shh* expression indicates suppression of dP4 development by P4 initiation

One limitation of inferring developmental processes from wild collected specimens is the uncertainty about rates. To overcome

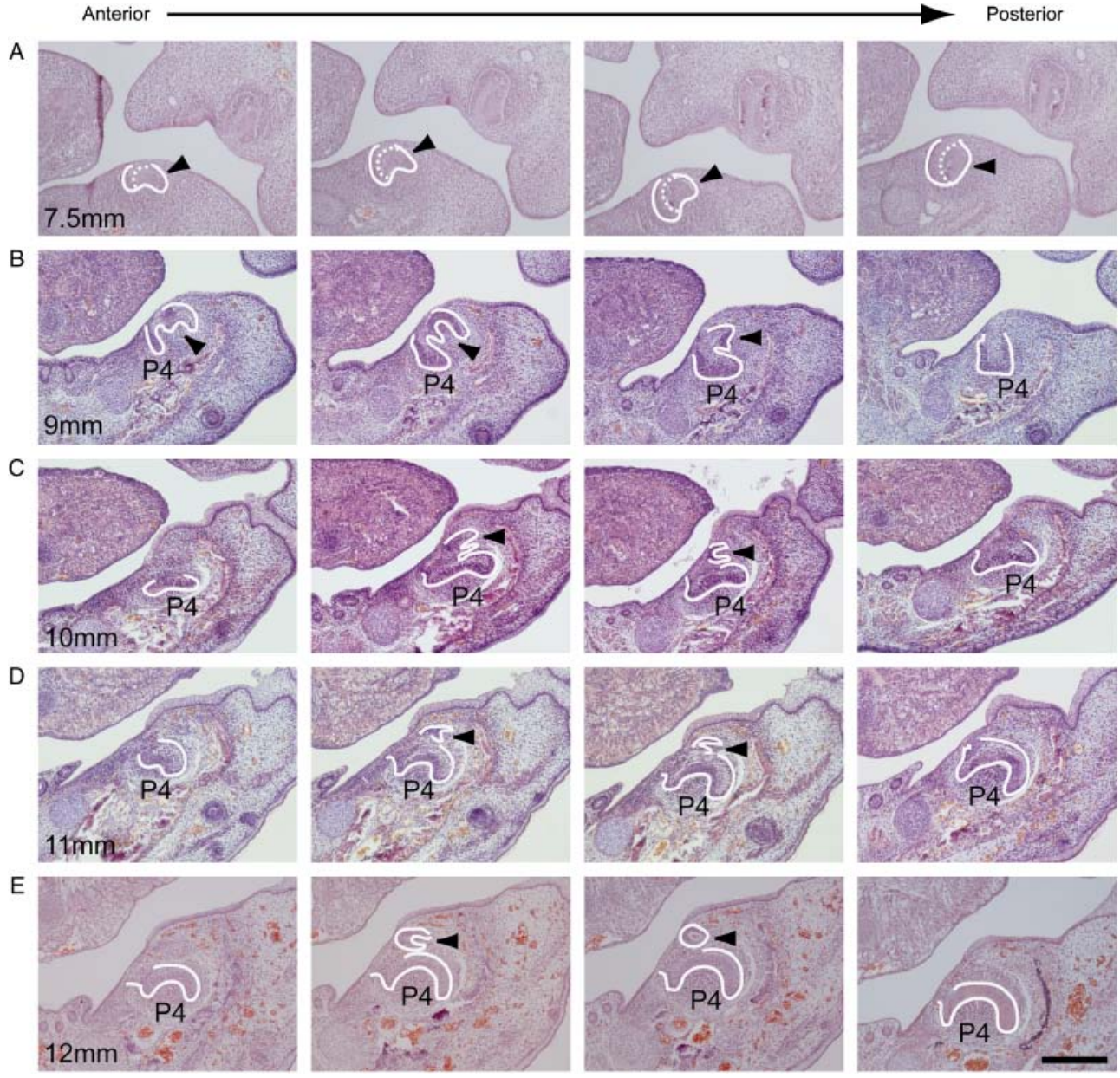

Fig. 1. Development of the deciduous and permanent premolar 4 in *Sorex araneus*. Histological sections in frontal plane are chosen from section series extending in anterior–posterior direction at each embryonic stage (mm). (A) At 7.5 mm dP4 is at the cap stage. The dashed line shows the putative border between dP4 and P4. (B) The first sign of permanent P4 is detected at 9.0 mm as P4 develops lingually to dP4. dP4 is now at the late cap stage and P4 is already at a late bud stage suggesting that the real initiation time point was between stages 7.5 and 9.0 mm. (C–E) At 10–12 mm dP4 diminishes in size and the development of P4 proceeds into late cap stage. Arrow head, dP4. Scale bar 200 μm.

these limitations, we next examined the *relative* growth of dP4 and P4, and whether these dynamics produce a double-wedge pattern of replacement (Fig. 5), classically used to infer competitive replacement of taxa (e.g., Krause 1986). We asked the question, what is the mechanism of suppression of dP4? Albeit not mutually exclusive, there are two possible modes, internal suppression and suppression by replacement. Internal suppression would occur when the replacement tooth initiation is after the deciduous tooth has started to diminish in size. On the other hand, suppression by replacement is most likely when the initiation of replacement tooth coincides with the onset of deciduous tooth size reduction (Fig. 5). Previous results indicate that P4 is initiated early relative to the development of dP4. This raises the possibility that P4 may inhibit the further development of dP4. To test this, we analyzed the dynamics of both tooth growth and the sizes of *Shh* expression domains.

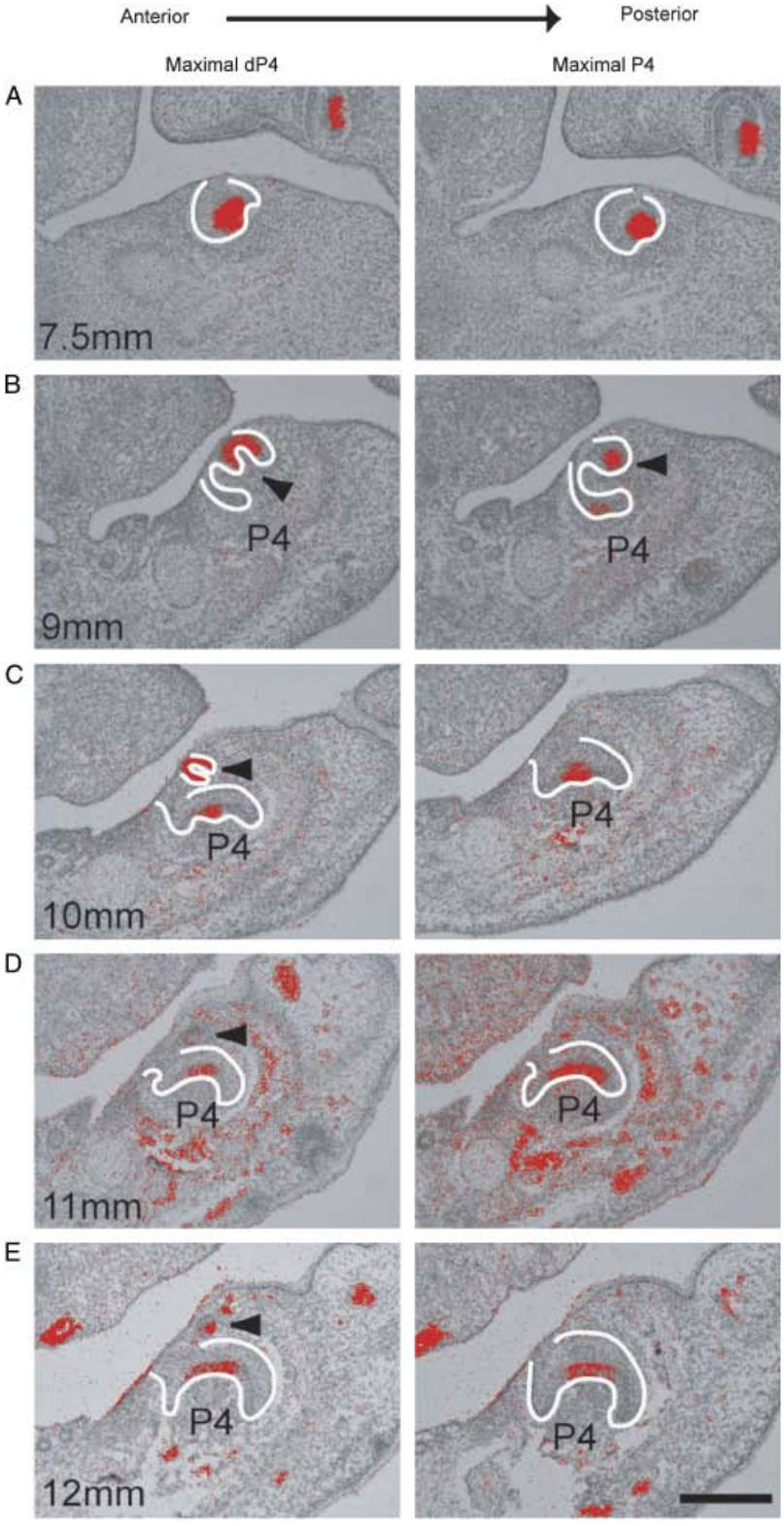

Fig. 2. Expression analysis with molecular marker *Shh* in deciduous and permanent premolar 4 shows that *Shh* is expressed in the enamel knot and in the inner enamel epithelium in both generations of teeth. Maximal *Shh* expression patterns for both dP4 and P4 are shown. (A) At 7.5 mm *Shh* is expressed in the enamel knot of dP4. (B) At 9.0 mm *Shh* expression in dP4 has expanded into the inner enamel epithelium. P4 shows weak *Shh* expression in the early enamel knot. (C) At 10 mm the P4 enamel knot is more defined and shows strong *Shh* expression. *Shh* expression persists in the deciduous dP4 even after it has started to diminish in size. (D, E) At 11 and 12 mm dP4 expresses *Shh* only weakly. In P4 the expression extends into the inner enamel epithelium. Arrow head, dP4. Scale bar 200 μm.

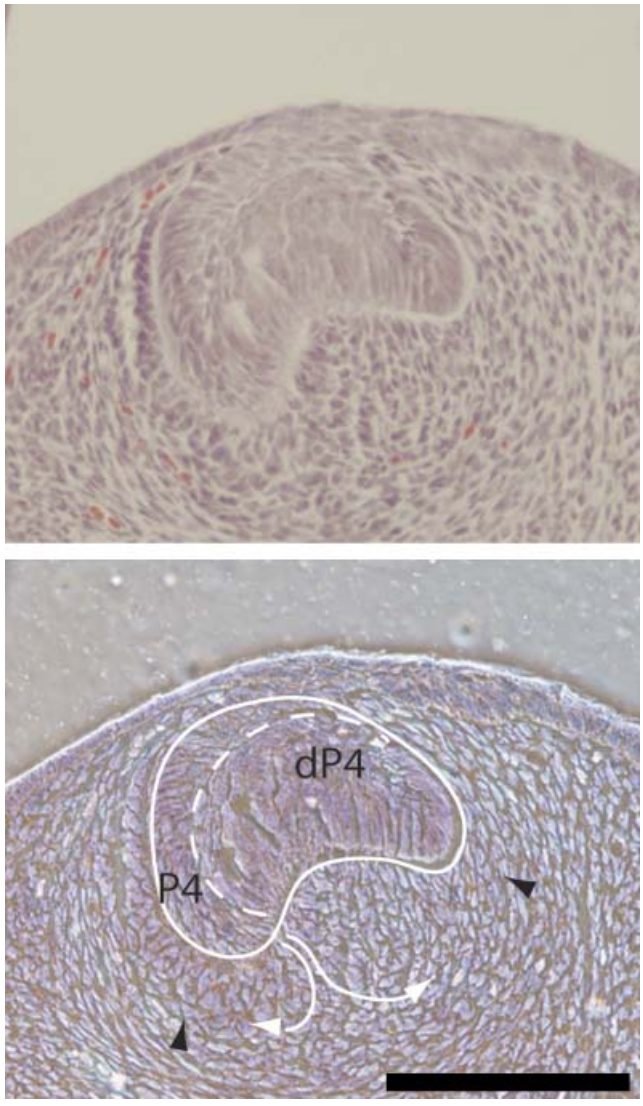

Fig. 3. The histology of the tooth bud at 7.5 mm shows differential orientation of cells in the lingual and buccal aspect of the enamel organ and in the dental mesenchyme (a normal transmission light-image and a differential interference contrast [Nomarski]-image). The dashed line and the white arrows mark the putative epithelial and mesenchymal borders, respectively, between dP4 and P4. Note the condensing mesenchyme below the P4 (black arrow head on the left) in contrast to the lack of condensing cells in the buccal side (black arrow head on the right). Scale bar 100 μm.

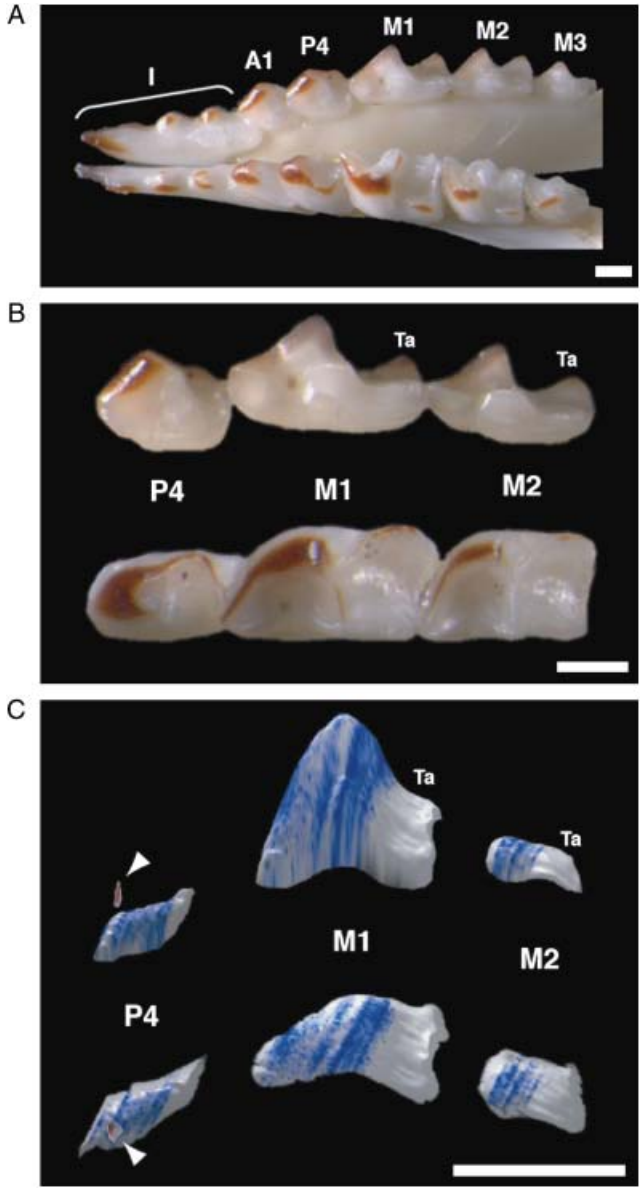

Fig. 4. (A) Lower jaw of an adult *Sorex araneus*. Typical feature of the *Sorex* teeth are the red siderose enamel (containing iron oxide). Note the large, multicusped incisor (I). Because of uncertain homologies of shrew teeth between the first incisor and fourth premolar, these teeth are called antemolars (A1). (B) Lower P4, M1 and M2 (top, obliquely lingual view; bottom, occlusal view). (C) 3D reconstruction of the developing dP4, P4, M1, and M2 at 12-mm embryonic stage (top, obliquely lingual view; bottom, occlusal view). The spatial organization of the permanent teeth is similar than in the adult jaw and the small deciduous dP4 (arrow heads) is located above and slightly lingually to the P4, closest to the middle of the P4 enamel knot area. *Shh* is expressed in the inner enamel epithelium (red in dP4 and blue in other teeth). The talonid (Ta) of shrew molars has shorter cusps than the trigonid (B), and correspodingly, *Shh* expression is not yet upregulated in the talonid of 12-mm stage M1 or M2. Scale bar 500 μm.

Size dynamics

The relative growth rates of dP4, P4, and M1 were calculated by measuring the size of the tooth from six embryonic stages (Fig. 6A). In addition to the five developmental stages, the smallest embryo (7 mm) in the 7.5-mm litter was used for the analysis of the size dynamics. The results show that dP4 is likely to peak in size at 7.5-mm stage and consecutive stages show progressively smaller dP4 until its size has decreased

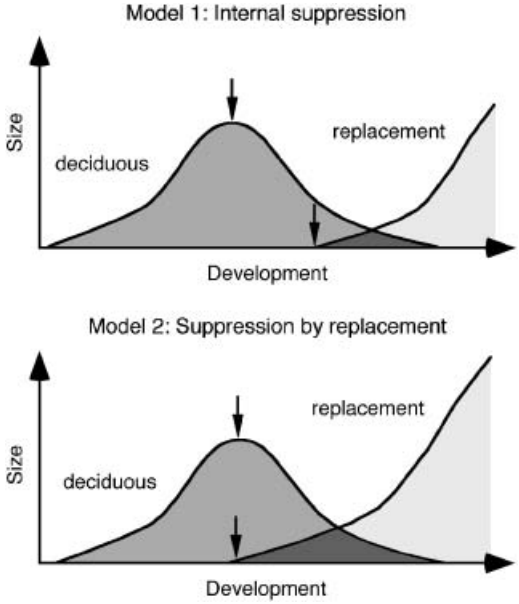

Fig. 5. There are two possible modes of suppression of dP4. Model 1 shows internal suppression. The deciduous tooth starts to diminish in size before the replacement tooth is initiated. Deciduous tooth may also inhibit the initiation of the replacement. Model 2 shows suppression by replacement. The deciduous tooth begins to decrease in size at the same time as the replacement tooth is initiated, resulting in longer temporal overlap (dark gray) and a double-wedge pattern. Arrows point to the starting points of reduction of size and to the replacement tooth initiation. In model 2 the arrows are expected to be closely aligned.

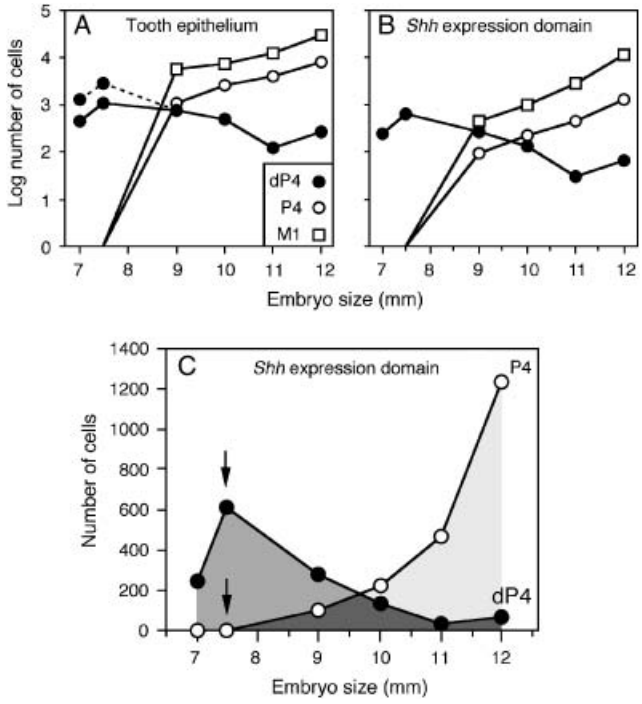

Fig. 6. Comparative analysis of dP4, P4, and M1 growth rates. (A) Size of the tooth epithelium and (B) number of cells expressing *Shh* were measured from a full series of sections in each tooth loci and plotted at each time point (embryo size). Note the higher rate of expansion of *Shh* expression in P4 and M1 compared with the growth of epithelium from 9 mm onwards (steeper lines in B than in A). For stages 7 and 7.5 mm, the dP4 and P4 sizes have been calculated using both the whole tooth bud (when P4 size is zero, dashed lines) and the minimum possible dP4 size (dashed lines, see Fig. 3). The maximum P4 size in these early stages is likely to be <300 cells. (C) Comparison of dP4 and P4 *Shh* expression domains show close temporal overlap (arrows) between the onsets of P4 development and dP4 suppression. Together with the long temporal overlap (dark gray) these results are indicative of model 2 (Fig. 5) type of regulation of deciduous teeth by their replacements.

from the peak 1000 to 100 cells. When the P4 is unambiguously detectable at 9 mm, its size already matches or exceeds that of dP4. This suggests that P4 could begin to grow slightly after dP4 is already decreasing in size. Close histological examination, however, revealed incipient changes in the lingual portion of the dP4 that gives rise to the P4 (Fig. 3), suggesting that the decline in dP4 size and the initiation of P4 are closely linked in time. To test how large an effect earlier P4 initiation could have on the patterns, we measured the smallest possible dP4 size in 7- and 7.5-mm embryos (showed by dashed line in Figs. 1A and 3). The results remained essentially the same, further supporting a temporal overlap between P4 initiation and dP4 suppression. Both permanent teeth, P4 and M1, developed approximately at the same rate, suggesting that the disappearing dP4 did not interfere with P4 growth rate (Fig. 6A).

Shh expression domain dynamics

Size of *Shh* expression domain in each tooth was similarly calculated (Fig. 6B). The *Shh* expression expands quite similarly in P4 and M1 (Fig. 6B), and in both cases faster than the growth of the teeth (Fig. 6A). Whereas this expanding *Shh* expression in P4 and M1 corresponds to the progressive differentiation of the cells in the inner dental epithelium, *Shh* expression appears to diminish in dP4 at similar or slightly faster rate than the decrease in dP4 size (Fig. 6, A and B). Although the growth rate was similar in P4 and M1, the relative expansion of *Shh* expression field in P4 was slower than in M1 (at 12-mm stage 39% of M1 and 15% of P4 express *Shh*). This suggests that dP4 might inhibit the development of P4. Also it is possible that as M1 has more cusps, the expression pattern needs to be expanded faster.

Comparison between dP4 and P4 in detail shows that the onset of decrease in dP4 *Shh* expression appears to happen when P4 *Shh* expression is first upregulated, most likely soon after 7.5-mm stage (Fig. 6C). This pattern of *Shh* expression supports the hypothesis on dP4 suppression by its replacement (Fig. 5).

DISCUSSION

We conclude that *S. araneus* develops only one secondary tooth at premolar 4 locus thus contrasting the previous reports on *S. araneus* tooth development where tooth renewal was detected in multiple positions (Ärnbäck Christie-Linde 1912; Kindahl 1959). Compared with these reports based only on histological data, we have used molecular markers to define the tooth buds. Primary dP4 could be seen in all five stages, and this primary tooth bud expressed *Shh*. Its successor P4 also showed a distinct *Shh* expression pattern. In incisors and canine *Shh* was detected only in one place in each of the other tooth positions and no indication of deciduous counterparts in these tooth loci was seen neither by histological nor molecular analysis (data not shown). The contradictory results between previous investigators and our results might be explained by various ways. As the shrews are caught in the forest the embryos have to be staged by measuring the length of the embryo, which might lead to interpretation differences. However, we had a series of samples covering most of the tooth development stages.

Another possible explanation could be that the shrews in different studies came from different populations. *S. araneus* has different karyotypic races that, together with latitudinal clines, show differences in morphology. These include changing size proportions of anterior and posterior teeth and a variably present upper tooth, so called fifth upper antemolar (Sulkava et al. 1985; Polly 2007), and it is conceivable that vestigial teeth could be variable as well. Yet another explanation is that the rudiments have been lost by evolutionary mechanisms during the 100 and 50 years, an especially unlikely explanation because genus *Sorex* dates back to the Miocene. Nevertheless, because shape differences in shrew molars have been shown to reflect the phylogenetic divergence of closely related species (Polly 2001), the question of change and variation in rudiments deserve future study. Meanwhile, we interpret the contradictory reports to indicate the difficulties of defining rudimentary tooth buds and these differences may in part reflect the advances in imaging and molecular techniques in determining subtle developmental events. To this end, our study does not obviously exclude the presence of other tooth buds that are suppressed before upregulation of *Shh* expression. We note, however, that *Shh* expression has been detected in transient diastema buds in rodents (Keränen et al. 1999), suggesting that additional shrew tooth rudiments would have to be suppressed very early in their development or, alternatively, involve specific downregulation of *Shh* expression. In general, the result that dP4 appears to be by far the best retained of the shrew deciduous dentition, fits well with the pattern of dP4 being typically the first tooth to be initiated during therian ontogeny (Luckett 1993).

The formation of secondary tooth has been traditionally thought of as a process where the free end of the lamina buds and forms the secondary tooth. However, as far as we know, it has never been shown from where the free end of lamina comes from. Our observations of *S. araneus* P4 development in relation to dP4 show that P4 is developed very early and fast. During the primary tooth development the lamina becomes part of the tooth anlage and forms the lingual cervical loop of the cap stage tooth. In the molar this cap stage tooth goes through morphogenesis and differentiation and is developed into one molar tooth as described previously (Thesleff 2003). But in the premolar area the most lingual part buds off and continues to grow deeper into the mesenchyme and forms the anlage for the future secondary tooth. The primary cervical loop continues to be the cervical loop of the deciduous tooth. These observations indicate that the dental lamina has to detach from the cervical loop of the primary tooth in order to form a new free end of lamina for the secondary tooth bud. Secondary tooth development is thus a two step process; detachment of the lamina and budding of the free end of lamina. In the *S. araneus* premolar these processes follow each other almost simultaneously and thus we cannot exclude the possibility that this feature is part of the process of dP4 becoming a rudiment. It remains to be tested if this mode of detachment and budding is also true in a model where both, the primary and secondary teeth, are functional and develop normally.

Shh has been previously shown to be a marker for enamel knots in two species of rodents (Jernvall et al. 2000) with expanding expression during differentiation into the inner enamel epithelium and the enamel-forming ameloblasts (Bitgood and McMahon 1995). Our results suggest similar conserved role for *Shh* in Soricomorpha teeth; *Shh* expression was first detected in the *Sorex* enamel knots from which the expression begun to spread in P4 and M1. In dP4 the *Shh* expression dynamics suggest that the fate of this tooth was evident with the initiation of P4 development. Interestingly, in *Shh* mutant, where *Shh* activity was removed in the epithelia, the lingual cervical loop and the dental cord were absent (Dassule et al. 2000) suggesting that there is asymmetrical signaling and growth in wild-type mouse molar. It is noteworthy that the parts that were missing in *Shh* mutants are analogous to the parts of the *Sorex* dP4 giving rise to the new cervical loop and the P4. The close association of *Shh* expression dynamics with tooth germ growth suggests that whereas *Shh* may not be the molecular trigger for dP4 suppression, it is at least one of the required factors in growth and survival of developing teeth.

The exact nature of the molecules responsible for the reduction of replacement remains to be determined. It has been shown that mammals also have the potential to activate tooth renewal when *Wnt* signaling is activated (Järvinen et al. 2006). In this work multiple enamel knots, expressing *Shh* and other knot markers, were induced by activating epithelial β-catenin. The supernumerary knots formed from new buds that developed close to each other. This mode thus resembles the mode

of secondary tooth development in the shrew where the permanent P4 is induced directly from the deciduous dP4. In humans, a loss-of-function mutation in Runt-domain transcription factor *Runx2* has been shown to induce tooth renewal (Jensen and Kreiborg 1990; Mundlos et al. 1997; Otto et al. 1997). When *Runx2* activity is reduced in *Runx2*+/− mice, the mouse molar grows a lingual extra tooth bud. This bud expresses *Shh* in the tip and thus resembles a true tooth bud (Wang et al. 2005). Since *Runx2* is expressed in dental mesenchyme and is an intracellular molecule, it may regulate a soluble signaling molecule. This could belong to *Fgf* or *Wnt* signaling families as both are affected in *Runx2* knockout mice (D'Souza et al. 1999; Aberg et al. 2004; James et al. 2006). The connection between *Wnt* signaling and *Runx2* is still fairly unknown and it remains to be tested whether they are involved in the evolutionary regulation of tooth renewal.

Additionally, previous research in mice has shown that adjacent molars inhibit the initiation of more posterior molars (Kavanagh et al. 2007). The relative size and number of molars follow a simple inhibitory cascade model in which relative strength of activation and inhibition determines the size and presence of later developing molars (Kavanagh et al. 2007). In this model weaker intermolar inhibition allows earlier initiation of adjacent molars, and we suggest that normal tooth replacement shares some aspects of the inhibitory cascade model. In species with complete tooth replacement, deciduous teeth would delay the initiation of their individual replacements. Moreover, any selective factor favoring earlier development of permanent teeth should automatically result in smaller deciduous teeth. This kind of tightly coupled developmental scenario may explain why no single evolutionary hypothesis seems to be sufficient to explain all the cases of lost tooth replacement (van Nievelt and Smith 2005). Another implication of the double-wedge replacement pattern is that it is unlikely for both the deciduous tooth and its permanent replacement to develop and erupt concomitantly, perhaps helping to exclude some of the hypothesis concerning homologies of premolars where both deciduous and replacement teeth are interspersed (reviewed in Cifelli 2000).

One intriguing consequence of the double-wedge replacement is that the earlier the permanent tooth is initiated, the less resources are allocated for the deciduous tooth. Earlier development of permanent teeth also moves the tooth making costs to the mother. For example, in indroid lemurs all permanent teeth erupt during the period of lactation (Godfrey et al. 2005). Whereas the small deciduous teeth of these lemurs may have only moderately weakened inhibition of their replacements, in shrews the inhibition is weakened to a point in which the permanent teeth are activated very early. And in the case of shrew P4, it suppresses dP4 to proceed barely beyond the cap stage. This balance between activation and inhibition will ultimately set the fate of rudimentary organs, or as Kate states in the Taming of the Shrew: *Love me or love me not, I like the cap; And it I will have, or I will have none*.

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