Symposium Article

The dental lamina: An essential structure for perpetual tooth regeneration in sharks

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Keywords: Tooth Regeneration; Shark; Dental Lamina; Tooth Development; EvoDevo

ABSTRACT: In recent years non-classical models have emerged as mainstays for studies of evolutionary, developmental and regenerative biology. Genomic advances have promoted the use of alternative taxa for the study of developmental biology, and the shark is one such emerging model vertebrate. Our research utilizes the embryonic shark (*Scyliorhinus canicula*) to characterize key developmental and regenerative processes that have been overlooked or not possible to study with more classic developmental models. Tooth development is a major event in the construction of the vertebrate body plan, linked in part with the emergence of jaws. Early development of the teeth and morphogenesis is well known from the murine model, but the process of tooth redevelopment and regeneration is less well known. Here we explore the role of the dental lamina in the development of a highly regenerative dentition in sharks. The shark represents a polyphyodont vertebrate with continuously repeated whole tooth regeneration.

This is presented as a major developmental shift from the more derived renewal process that the murine model offers, where incisors exhibit continuous renewal and growth of the same tooth. Not only does the shark offer a study system for whole unit dental regeneration, it also represents an important model for understanding the evolutionary context of vertebrate tooth regeneration.

Introduction

The evolutionary origin of teeth has long been debated, as their potential divergence from skin-borne odontodes remains unclear (Fraser et al. 2010; Donoghue and Rücklin 2016; Martin et al. 2016). Given the structural similarity between odontode types, it has recently been suggested that a defining feature of true teeth from alternative odontodes (i.e. skin odontodes) is their successional regeneration within defined families, linked via a continuous dental lamina (Martin et al. 2016). This is in contrast to the sequential addition and propagation of dermal denticles in elasmobranchs, within the epidermis during development and growth (Reif 1978; Reif 1980; Cooper et al. 2017; Cooper et al. 2018). The dental lamina is a dynamic oral epithelial outgrowth present in all vertebrates that develop repeated tooth generations, which includes diphyodont dentitions with two generations or polyphyodont dentitions with multiple tooth generations (Tucker and Fraser, 2014; Figure 1).

Regeneration of the vertebrate dentition is inextricably linked to the presence and maintenance of the epithelial dental lamina, which houses the important stock of stem/progenitor cells that

enable to the progression of tooth supply. We now know that stem/progenitor cell clusters are contained in the dental lamina of various vertebrate groups, despite their diverse dental regenerative properties (Tucker and Fraser, 2014). However, less is known about the role of the underlying ectomesenchymal condensate which belies an important collaboration with the epithelium for the regeneration and continued production of teeth. Recently, Hh signaling has been shown to control the maintenance of mesenchymal dental stem niches associated with the continuously growing incisors of mice, via associated neurovascular bundles (Zhao et al. 2014). However, more study is required to understand (i) the role of mesenchymal regulation of tooth regeneration, and (ii) the importance of the collaborative association between the epithelium and mesenchyme in maintaining tooth regenerative capacity, given that both are essential to the formation of the unit tooth.

Our understanding of dental development has been greatly enhanced by detailed developmental studies of sequentially initiated mammalian molars and the continuously growing mouse incisor (Jarvinen et al. 2006; Järvinen et al. 2009; Juuri et al. 2012). In recent years, a plethora of new information has highlighted the vast conservation of both the developmental process and the genetic regulation of tooth development and regeneration across the vertebrate clade. Except for a few inconsistencies in mineral composition, teeth are incredibly similar from fishes to mammals (Tucker and Fraser, 2014), suggesting that this developmental conservation could be useful when comparing across divergent groups of vertebrates. What is quite intriguing for evolutionary developmental biologists is why, if this conservation is so prevalent, do teeth differ so greatly in form, function and pattern? This in turn directly relates tooth shape and function to the trophic ecology of a species. In contrast to

most mammals (which are diphyodonts), the majority of vertebrates undergo multiple rounds of successional dental regeneration (polyphyodonty; Figure 1A-E), often continuing throughout life (Tucker and Fraser 2014; Rasch et al. 2016). There is, however, substantial evidence to suggest that polyphyodonty depends not only on the preservation of the dental lamina (DL) but specifically upon the maintenance of an epithelial dental stem niche within the DL (Smith et al. 2009; Martin et al. 2016).

Early Tooth Development and Conservation

In vertebrates, prior to the development of the first teeth, a field of dental competence known as the odontogenic band (OB) is established within the oral epithelium (Tucker and Fraser 2014; Rasch et al. 2016). The combined expression of sonic hedgehog (*shh*), paired-like homeodomain 2 (*pitx2*), and SRY (sex determining region Y)-box 2 (*sox2*) demarcates the position of the first dental generation. An initial thickening of the OB is followed by the emergence of the dental placodes which undergo morphogenesis and give rise to the dentition (Keränen et al. 1999; Fraser et al. 2004; Jussila et al. 2014; Rasch et al. 2016).

The initial signals that likely direct the formation of the odontogenic band and the early dental lamina are not necessarily the earliest signs of odontogenetic activity. For many years we have known about the now classic scenario of the odontogenic homeobox code (Tucker and Sharpe 2004), wherein early epithelial (specifically, *Fgf8* and *Bmp4*) and ectomesenchymal (e.g. *Barx1*, *Dlx2*; *Msx1/2*) signal combinations reflect a patterning disparity along the proximal and distal regions of the jaw (i.e. in mice) that translate to regional specification of tooth types, e.g.

proximal molars and distal incisors. This dental pre-pattern or homeobox code is an intriguing idea that warrants further investigation, based on the fact that this hypothesis was first described from observations in a highly derived mammal, the mouse. The murine dentition houses a peculiar heterodont dental series with distinct distal incisor and proximal molar territories, separated by a vast toothless diastema (Keränen et al. 1999).

Other vertebrates with distinct heterodonty throughout the jaw arc may also rely on a coded prepattern, perhaps earlier than the emergence of the odontogenic band (Tucker and Sharpe 2004). Some sharks show a great shift in the shape of teeth along the jaw from distal to proximal regions. Most however, show a graded shift in tooth shape along the jaw, which can be seen to an extreme in some groups. The Bullhead sharks (Family Heterodontidae), for example, show a severe shift in tooth morphology from the distal multi-cuspid pointed, grasping teeth to the proximal crushing, molariform teeth (Summers et al. 2004; Berkovitz and Shellis 2017; Smith et al. 2018; Jambura et al. 2020); with a similar shift from cuspate to molariform teeth occurring throughout ontogeny (Reif 1976). However, in Heterodondus there is no physical separation between the tooth types (although some sharks do show a diastema-like separation of tooth type, see Smith et al. 2018), and the continuous tooth row shows an impressive gradation from one type into the other from distal to proximal positions. How this occurs is unknown but must reflect some differential shifts in the early developmental signaling. This tooth-type gradation could also appear as a result of graded expression profiles or signaling concentrations both along the jaw and importantly within the dental lamina. Whether an odontogenic homeobox code is conserved and exists beyond mammals is yet to be determined.

Successive dental regeneration requires sources of multipotent progenitor cells capable of differentiating into all the cell lineages of the developed tooth. Epithelial progenitors which differentiate into the ameloblast lineages are housed within the dental lamina (DL). The DL is an invaginated epithelial sheet which derives from the OB and extends into the underlying dental mesenchyme, and in polyphyodonts gives rise to successive dental generations (Tucker and Fraser 2014). In sharks and rays, the DL is continuous and can cover the entire length of each jaw, connecting teeth to successive dental generations (Figure 3). Alongside morphological diversification, vertebrates have evolved great diversity in their dental regenerative capacity (Jernvall and Thesleff 2012; Tucker and Fraser 2014; Figure 1). This ability to regenerate the dentition has in turn facilitated morphological diversification between species, as well as between life stages. From the crushing beak-like dentition of the pufferfish (Thiery et al. 2017) to the venomous fangs of snakes (Figure 1E) (Zahradnicek et al. 2008), and the vastly diverse monophyodont (e.g. mouse; Figure 1F), diphyodont (e.g. Human; Figure 1G) or polyphyodont dentitions in mammals (e.g. rock wallaby and manatees), modifications to the vertebrate dentition has facilitated niche specialization (Van Valkenburgh 1989; Holliday and Steppan 2004). Despite this morphological diversification, the development and regulation of DL stem cells remains highly conserved.

Numerous studies have recently implicated the sex-determining region Y-related box 2 (Sox2) transcription factor as a primary marker of epithelial dental stem cells (Juuri et al. 2012; Gaete and Tucker 2013; Juuri et al. 2013; Martin et al. 2016), with Wnt/ß-catenin signaling playing a

critical role in initiating dental development from these cells (Gaete and Tucker 2013; Martin et al. 2016). Sox2 negatively regulates Wnt/ß-catenin signaling in osteoblasts (Mansukhani et al. 2005), whilst the expression of constitutively-activated ß-catenin specifically within Sox2+ cells is sufficient to induce dental initiation in mice (Xavier et al. 2015). Research into successional dental regeneration in a diverse range of polyphyodont vertebrates is beginning to highlight the mechanisms through which lifelong dental regeneration is regulated (Fraser et al. 2013; Gaete and Tucker 2013; Wu et al. 2013; Martin et al. 2016; Rasch et al. 2016; Thiery et al. 2017; Salomies et al. 2019). A detailed understanding of the evolution of successional dental regeneration is required if we are to fully understand secondary loss of polyphyodonty in derived vertebrate lineages (i.e. mammals).

Animals with the potential for dental regeneration, whether continuously growing incisors in mice, natural diphyodont replacement in humans, or multi-generational replacement in reptiles and fishes, likely share genetic and cellular components of the DL. Conversely, dentitions with reduced regenerative capacity may have lost certain genetic components necessary for the continued production of teeth. At least in mammals, the selection pressure to produce intricate tooth shapes necessary for occlusion and efficient food processing is thought to have played a role in the reduction of tooth generations (Jernvall and Thesleff 2012; Tucker and Fraser 2014). This trade-off has led to an almost complete loss of dental lamina activity to make way for a more permanent dental set. These genetic and cellular constituents of the dental lamina are likely present in all toothed vertebrates, however following the active odontogenic phase of most mammals, the DL breaks down and dental production ceases. Cell death, cell movement away from the epithelial dental lamina, and epithelial to mesenchymal transitioning of DL cells

all contribute to the subsequent break down of the DL in mammals, (e.g. as observed in the diphyodont pig; (Buchtová et al. 2012)). Premature degradation of the DL in mammals could be the result of the overwhelming inhibitory signaling from the predecessor tooth. In contrast, polyphyodont vertebrates, including the shark, seem to maintain the DL, perhaps by preventing this potentially overwhelming inhibition and allowing the repeated and controlled dental initiation to continue.

The degradation of the DL in diphyodont animals does not lead to the complete loss of the dental lamina. In humans, the degradation of the dental lamina following the initiation of the second generation and the formation of the molar series, results in isolation of epithelial cell clusters within the soft tissue of jaw (Buchtová et al. 2012; Fraser et al. 2019). These rested, lamina remnants (lamina rests) are quiescent, but retain regenerative potential to some capacity, visible through the expression of proliferative and stem-associated markers (Fraser et al. 2019). Occasionally, these rested epithelia become active and tumorigenic (e.g. odontomas and ameloblastomas). The triggers of these pathologies seem to be related to Wnt/ß-catenin signaling (Fujii et al. 2019), which interestingly is the key regulator of dental regeneration initiation in polyphyodonts.

The shark dentition: a model for continuous tooth replenishment

The phylogenetic position of chondrichthyans renders them ideal models for comparing with osteichthyan successional dental regeneration (Martin et al. 2016; Rasch et al. 2016). Extant chondrichthyans include sharks, rays and holocephalans (chimaeras), and exhibit highly

diverse dental morphologies (Johanson et al, this volume.; Smith et al. 2013; Underwood et al. 2015). Elasmobranchs (only sharks and rays) possess a rapid rate of dental turnover, best described as a dental 'conveyor belt' (Tucker and Fraser 2014; Rasch et al. 2016). Within the sharks, many teeth develop ahead of function, aligned in discrete family units (Figure 2) (Smith 2003). In some species, tooth families can be separated by an enlarged inter-dental region (space between tooth families within the DL) with the relative positions of adjacent teeth within neighboring families being unclear (e.g. the frilled shark, *Chlamydoselachus anguineus)* (Figure 2C) (Smith et al. 2018). However, in the majority of sharks and rays it is clear that adjacent tooth families develop alternate tooth positions but with more or less separated inter-dental regions (e.g. the Porbeagle shark *Lamna nasus*) (Figure 2B) (Underwood et al. 2016; Smith et al. 2018). In the case of the small-spotted catshark, *Scyliorhinus canicula,* (Figure 2A) as with many shark and ray species (Rasch et al. 2016; Smith et al. 2017), the DL is continuous, with no inter-dental region separating tooth families. Instead, adjacent tooth families overlap and only remain identifiable due to their highly organized staggered arrangement, maintained by the alternate initiation of adjacent tooth positions (Figure 2A, and Figure 3). Even following substantial growth of the jaw and multiple rounds of regeneration, this pattern is exceptionally well maintained as a result of seamless developmental coordination between adjacent tooth families. However, how this coordinated and extended developmental process is regulated is currently unknown.

A focus on different shark species with variations in the inter-dental space (e.g. Figure 2), and species with breaks in the organization of tooth families (i.e. diastema) might be an interesting for future investigation and modelling. We can hypothesize that enlarged inter-dental regions,

in sharks, could arise from an increase in inhibitory signaling from developing tooth buds either at the jaw margin during first tooth formation or within the SL during initiation of subsequent generations (Figure 2C). Closely packed tooth buds are off-set and alternate (e.g. in *Scyliorhinus canicula*) due to the close proximity of teeth combined with relatively smaller inhibitory zones (ZOI; Figure 2B; Figure 3). We can speculate that larger inter-dental regions result from a larger inhibitory zone around the developing tooth buds in the SL, thus forcing newly formed tooth buds in the SL to be more widely spaced (e.g. in *C. anguineus*; Figure 2C) or even separated by a more prominent diastema. It is clear, however, that sharks already employ this type of inhibitory zone patterning during the development of the skin denticles (Cooper et al. 2018) in the epidermis, and the similarity between the units of the skin versus the oral cavity make this a potentially interesting comparison.

The inhibition of tooth generation between teeth within the developing dentition is not, or at periods within the evolution of the group has not been, orthogonal, as evidenced by tooth fusion and fission. The earliest and most basal chondrichthyans with known dentitions, such as *Doliodus*, have separate tooth families present as whorls within which the crowns of the teeth are separate, but the roots fused (Maisey et al. 2014). This state is also present within a number of more derived chondrichthyan groups, largely within the holocephalan clade (total group) (Johanson, *et al.* this volume). Indeed, the evolution of the continuously growing tooth plates in chimaeroids is the result of fusion of successive teeth within a tooth family (Johanson, *et al.* this volume, and references therein). This would suggest that, in early chondrichthyan evolution, the inhibition of tooth development between successive teeth within a family was weak and incomplete, with tooth roots and sometimes crowns becoming fused into a continuously

growing sheet. Conversely, the inhibition between adjacent families appears far more conserved with unambiguous evidence of fusion of adjacent tooth families not known. Indeed, in rays with extreme reduction of numbers of tooth families and extemely laterally expanded individual teeth, there is no evidence of fusion, but instead the teeth themselves become wider through ontogeny and adjacent families are strongly sutured together (Underwood et al. 2015). It would therefore appear that the zone of inhibition surounding developing teeth has at times been breached during evolution, whereas the inhibition to tooth formation in the interdental region is fundamental, and the inhibition mechanism has been retained through the entirety of chondrichthyan evolution.

Regenerative cyclicity and stop-start initiation of the shark successional lamina

All teeth on each jaw in the catshark are interconnected via a deep lying, continuous jaw length dental lamina (Figure 3) (Martin et al. 2016; Rasch et al. 2016). Sox2 is concomitant with maintenance of stem/progenitor states within the dental lamina but is not expressed at the site of successional tooth initiation. However, as a stem/progenitor marker it has received a great degree of attention in recent years (Juuri et al. 2012; Gaete and Tucker 2013; Juuri et al. 2013; Martin et al. 2016). In sharks, Sox2+ dental progenitors initially associated with an embryonic-stage progenitor cell cluster known as the tooth/taste junction (TTJ; Martin *et al*., 2016), a region of the oral surface where cells, are either directed to establish a taste territory or migrate to form a discrete niche within the distal tip of the dental lamina (the successional lamina (SL)) – the site of dental initiation. Cyclical upregulation of Wnt/ß-catenin signaling within this Sox2+ progenitor niche is associated with enhanced proliferation of the SL and the onset of dental

initiation (Martin et al. 2016)(Figure 5 and 6). Each tooth family in the catshark jaw shows a staggered, alternate system (Figure 2, 3, and 6); when one family is initiating a new tooth ('*start'* phase) in the SL, the neighboring families (either side) are in a '*stop*', or '*pause*' phase – a period where the lamina continues to pool cells toward the point of initiation after delay (Figure 5 and 6). This shift from a '*stop*' or '*pause*' phase of the successional lamina is followed by the upregulation of Wnt/ß-catenin, thus initiating the start of a new successive tooth generation (Martin et al. 2016).

A number of genes have been implicated in the regulation of tooth regeneration in elasmobranchs, or other polyphyodont and diphyodont dentitions (Figure 4). Expression of these genes e.g. *sox2* (Rasch et al. 2016; Martin et a. 2016), *β-catenin*, *lef1*, *pitx1* and *2*, Fgf and Bmp genes (Figure 4), can be used to highlight particular compartments of the dental lamina that may (i) house stem cells, and (ii) show activity toward tooth initiation. However, understanding the genetic trigger that forces daughter cells away from regions of slow cycling (progenitors) is invaluable in the quest for the origin of the repeated initiatory signals, in contrast to the inhibitory action of molecules involved in promoting the cyclical sequence. Interestingly, some genes associated with early OB/DL establishment and first-generation initiation are not always involved in the redevelopment of later generations within the successional lamina e.g. Shh (Seppala et al. 2017). It appears that after the establishment of the dental lamina and the initiation of the first tooth generation, elements of the lamina are now required for maintenance, not completely new development, suggesting that competence of the successional lamina is maintained and constantly active. This makes the cyclical activity of SL intriguing – what genes assist in the stop/pause phase versus genes that reactivate the cycle?

One potentially crucial distinction that may permit the continuation of tooth production in polyphyodont dentitions is the supply of new competent epithelial cells that feed the successional lamina from the TTJ niche (Martin et al. 2016; Figure 2) – an important connection between the SL and the oral epithelium. This continuous provision of competent dental epithelial cells to the SL appears to be a common thread among polyphyodont dentitions. Cell lineage tracing studies in a range of divergent vertebrates (e.g. *DiI*; Martin et al. 2016; Salomies et al. 2019) show the passage of labeled cells from the TTJ at the oral surface to the deeply invaginated SL, then finally contributing to the formation of new teeth. This supply of new cells from the surface niche could be unique to multi-generational dentitions, and perhaps offers a contrasting scenario to dentitions with more limited regenerative capacity e.g. mammals. Therefore, mammals may not have a viable supply of new DL cells, and instead may rely on resident cells within the lamina for a second generation before termination of the process. The genetic control of both the continuous supply of competence and the termination of regeneration is unknown, yet an important focus for future studies. However, it is intriguing to suppose the trigger for this supply of new epithelial cells could come from the association of the DL with the TTJ, a sox2+ progenitor cell cluster that also supplies the oral taste buds closely linked to the dentition (Figure 2, and 3; Martin et al. 2016).

Cyclical stabilization of Wnt/β-catenin signaling may permit the cyclical progression of continuous tooth regeneration in sharks. Simply put, the basis of this *start*/*stop* cycle of tooth initiation could in fact be a Wnt-on/Wnt-off cycle. Importantly, transient cells (presumptive transit-amplifying cells) emanating from the DL and TTJ (tooth/taste junction) that pool into the

SL (Martin et al. 2016) likely influence the cyclical nature of these inhibitory (pre-initiatory) and activatory (initiation) signals (Figure 5). Thus particular competence factors (Figure 4) combined with adequate spacing behind (aboral; Figure 5) the predecessor, beyond the zone of inhibition (ZOI; Figure 6) allows the 'Wnt-on' phase to overwhelm the inhibition (pre-initiation stage of the SL; Figure 5) to activate a precisely timed sequence of tooth initiation (Figure 5 and 6).

The canonical Wnt/β-catenin pathway is an ancient signaling network which allows communication between cells across short spatially restricted distances. In an activated state, β-catenin translocates to the nucleus co-activating TCF/LEF directed transcription (MacDonald et al. 2009). Wnt signaling organizes an extremely wide range of developmental processes from early embryogenesis to dental development (Järvinen et al., 2006; Gaete and Tucker, 2013; Wu *et al.*, 2013; Martin *et al.*, 2016; Rasch *et al.*, 2016; Salomies *et al.*, 2019;), reviewed in (Tamura and Nemoto 2016); and has been linked to a number of cellular processes such as: proliferation, migration fate specification and stemness (Logan and Nusse 2004), and regeneration (MacDonald et al. 2009). Given its competence as a master regulator in a wide range of cell fate decisions, its activation/downstream effects are tightly regulated. Importantly, many components in the Wnt-pathway affect their own expression levels (e.g. Axin, Dishevelled, Dickkopf (Lustig et al. 2002; Jung et al. 2009; Hu et al. 2014; Bernkopf et al. 2015; Larraguibel et al. 2015), reviewed in (MacDonald et al. 2009)); which could translate to cyclical waves of activation and suppression seen in cyclical tooth regeneration. Furthermore, as an ancient developmental pathway, Wnt/β-catenin pathway activities are highly integrated with other signaling networks; for instance its interaction with the hedgehog pathway (Sarkar et al. 2000;

Ahn et al. 2010; Bernkopf et al. 2015); providing enhanced environmental cues, in turn leading to heightened positional specificity.

This *stop/start* process of repeated tooth initiation is not only contained by the cellular restriction of the SL (Figure 5), but additionally there is physical constraint of this tissue by the limit of the deep furrow of the Meckel's and Palatoquadrate cartilages. Therefore, this physical restriction implies that the SL has to rely on a pausing period and subsequent movement of the preceding tooth in order for space to become available and for the cycle to continue. This cycle and subsequent conveyor belt transition from initiation to functional teeth rely on a stable supply of progenitor cells from the oral surface, in order to maintain the cell population necessary for repeated tooth development in each tooth file/family (Figure 2). In sharks, new teeth physically translocate orally though the 'conveyor belt' (DL), away from the growing SL (Figure 5 and 6). If developing teeth secrete signaling molecules which inhibit dental initiation in the SL, then the movement of teeth through the conveyor belt system may dilute this inhibitory signal due to an increased distance between the tooth and SL. This in turn would trigger a subsequent round of dental initiation (Figure 5 and 6). This sequence sets up the stop/start or pre-initiation/initiation cycle of regeneration (Martin et al. 2016; Fraser and Thiery 2019).

In mouse molars, the inhibitory signals from the predecessor and permanent first-generation tooth may be too strong to permit the normal progression of a second-generation. Recent work by Popa et al. (Popa et al. 2019) uncovered a fascinating relationship between predecessor teeth and the activity and competence of the closely associated SL, although this work was focused on non-regenerative murine molars. Popa and colleagues (2019) discovered that in fact these

molars do develop a small, rudimentary SL that fails to initiate a second dental generation. The reason, it seems, is that the close proximity of the predecessor tooth acts as an inhibitory influence on successional tooth formation. This is in contrast to the shark SL where the close proximity of developing predecessor teeth in the conveyor belt has little effect on the overall competence of the SL beyond the temporary inhibition of the cycle (Figures 5 and 6). Thus, new teeth initiate in a cyclical manner, even though the generations share a close connection within the same DL epithelium (Figure 2, 3, and 5). The contrasting competence of these successional laminae based on proximity could underlie a common mechanism for both regenerative loss and continuity. Ultimately, both of these successional laminae stop the regenerative program, whether temporarily (paused) in the case of the shark, or permanently as the case in mice. It would be intriguing to investigate the genetic and physical differences between a monophyodont and diphyodont dental system (e.g. Popa et al. 2019) and to compare these to a polyphyodont model, to determine how the proximity of initiation events may impact the competence, progression or inhibition of new tooth generations. The mouse and shark dental systems offer two deviations of SL competence – (i) close proximity of SL to predecessor teeth from closely-linked epithelia (i.e. sharks) and (ii) rudimentary SL present (e.g. in mice molars) in close proximity to the predecessor tooth (therefore the SL is likely under the influence of the predecessor ZOI) but separated by a vast laminae connection. Whether the predecessor tooth and its ZOI, in mice, alone invokes enough of an inhibitory influence on the SL (and therefore a loss of the second generation) is likely, however, we know little about the competence of a greatly extended DL, forcing connectivity between the predecessor tooth and the SL to be distant. It is interesting to note that even in the shark, inter-dental regions are maintained along with the active sites of the SL where new teeth initiate (Figure 6). Why these inter-dental

regions within the continuous dental lamina in sharks do not break-down is unknown, however, these inter-tooth regions might be crucial for the maintenance of repetitive dental patterning.

Future prospects in the field of dental regeneration

Although we have recently seen an increase in dental regenerative research on non-mammalian models, the focus has remained centered on candidate markers initially identified in non-polyphyodont models (Handrigan et al. 2010; Handrigan and Richman 2010; Gaete and Tucker 2013; Weeks et al. 2013). There is a need to move away from the candidate approach, in order to identify novel markers regulating polyphyodonty (Salomies et al. 2019) or mammalian tooth renewal (Jheon AH et al. 2011; Seidel et al. 2017). Previous studies of dental regeneration in the catshark (Martin et al. 2016) has laid the foundations for transcriptome analyses of the rapidly regenerating SL, which will ultimately lead to novel insights into the mechanisms underpinning both the initiation and pause in the perpetual cycle. Importantly, understanding the genetic mechanisms of the pause-phase of a regenerative dental cycle may shed light more generally upon how dental competence can be lost, and potential regained.

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Acknowledgements

We would like to thank Zerina Johanson (Natural History Museum, London, UK), Brett Clark (Imaging and Anaysis Centre, Natural History Museum, London, UK) for MicroCT scanning and AVIZO segmentation of *Lamna nasus* and *Chlamydoselachus anguineus*. We thank James Maclaine and Ollie Crimmen for access to the adult *C. anguineus* specimen (Natural History Museum London, UK: BMNH 2016.4.11.1).

Figure Legends

Figure 1. Diversity of the vertebrate dental lamina. The comparative active dental lamina (orange) related to dental regeneration or renewal, in Fish (**A**, Shark; **B**, Cichlid), Reptiles (**C**, Gecko; **D**, Alligator; **E**, Snake) and Mammals (**F**, Mouse; and **G**, Human). Each image represents teeth (blue) under active regeneration, renewal or replacement within jaws in scheme-section.

The shark (**A**), Gecko (**C**), Alligator (**D**), and Snake (**E**) all have many-for-one dental regeneration, i.e. many teeth are formed in a regenerative series for a single functional position in the jaw. The cichlid fish (**B**) and the human (**G**) both have a one-for-one system of tooth regeneration, however the cichlid like the rest of the fish and reptiles (represented) have continuous multi-generational replacement (polyphyodonty), and the human only regenerates some tooth positions a single time (diphyodonty). The mouse (**F**) has a unique renewal system where the incisor only, is capable of continuous renewal of a single tooth and no true replacement of the initial and single generation of teeth (monophyodonty). Arrowheads in **A-E**, and **G** point to the successional lamina associated with development of multiple tooth generations. In **F**, the arrowhead points to the murine

cervical loops that house epithelial stem cells responsible for incisor renewal.

Figure 2. Diversity of dental patterning in sharks. MicroCT scans of three shark lower jaws (A, Small Spotted Catshark, *Scyliorhinus canicula*; B, Porbeagle Shark, *Lamna nasus*; and C, Frilled Shark, *Chlamydoselachus anguineus*) to show the variety in the patterning of dental spacing. Tooth families are either crowded and staggered (alternate) as in *S. canicula* (A) or non-staggered (non-alternate) with some inter-dental space in *L. nasus* (B), and non-staggered, (non-alternate) with large inter-dental space as in *C. anguineus* (C). Soft tissues not shown but developing teeth (in all but *C. anguineus*) would be covered in the epithelial dental lamina, in life, and the regulation of this spacing is governed by an interplay of both inhibitory and activatory signals associated with the first tooth positions and within the successional lamina for later maintenance during regeneration. H&E Histology of the first developing teeth and dental lamina in *Scyliorhinus canicula* (D, E). D, Early growth of the dental lamina after first tooth formation (T1). The

successional lamina (arrowhead) is the region for new tooth formation, after the pooling of new competent cells from the oral epithelial (oe) tooth/taste junction niche (ttj). E, the second-generation tooth (T2) in the family initiates within the SL (arrowhead) after a pause to allow sufficient spacing between generational units. F, Schematic of E with orientation, and annotation. tb, tastebud; dp, dental papilla; oe, oral epithelium; ttj, tooth/taste junction progenitor niche; dl, dental lamina; dm, condensing dental mesenchyme; de, dental epithelium; mde, middle dental epithelium; sl, successional lamina. Orientation: ora=oral, abo=aboral, lab=labial, lin=lingual. Scale bars: A=5mm; B=2cm; C=1cm; D=100μm.

Figure 3. The catshark dental lamina in 3D. Segmentations of microCT scans of the lower jaw of a stage 33 catshark (*S. canicula*). **A**, dorsal view of the developing lower jaw dentition. Mineralised teeth (yellow) are embedded within the continuous dental lamina (red; a, anterior; p, posterior). **B**, Dorso-lateral view of the lower jaw showing position of the dental lamina *in situ*. **C**, segmented dental lamina (red; anterior=up) showing the aboral undulations of the successional lamina (green arrowhead) and developing teeth (not yet mineralized; dotted lines) within the lamina. D, virtual section in sagittal view showing the deep invagination of the dental lamina (red) with mineralized teeth (yellow), note that the non-mineralized developing tooth units within the lamina are not visible, but the successional lamina extends deep into the jaw toward the cartilaginous furrow of the mandible (blue). tb, tastebuds overlay the invaginated dental lamina. Scale bar in A=500μm.

Figure 4. Gene expression during cyclical tooth initiation in a shark (Stage 32; *Scyliorhinus canicula,* sagittal thin paraffin sections). The early forming dentition of the lower jaw (A, B, C, E,

G, I) and upper jaw (D, F, H, I) during first to second-generation transition, and initiation within the successional lamina. Early epithelial signals *fgf10* (B), *pitx2* (D), *lef1* (F) *bmp4* (G) and *b-cat* (I) highlight the earliest initiation phase of second generation after the pause (in the start/stop cycle) with expression associated with the extended SL. During early morphogenesis of the second-generation teeth, expression of epithelial (*lef1* (E)), or both epithelial and mesenchymal markers (*fgf3* (A), *pitx2* (C), bmp4 (H), *b-cat* (J)) show progression of generational development within the DL, leaving space within the regions behind the tooth in the aboral free-end of the SL for the subsequent generation. DAPI nuclear stain (white) with false colored expression (magenta) from section in situ hybridization. Oral, left; aboral, right. Scale bar in A=100μm.

Figure 5. Four stages of regenerative initiation in *Scyliorhinus canicula* (stage 33). DAPI (white) stained histology of the upper (A-D) and lower jaw (E-H) catshark dentition. The dental lamina plus the developing teeth are highlighted in red (false colour). Green arrowheads (A, B, F, G) indicate the initiation phase of the stop-start regeneration of the shark dentition. Thickening of the successional lamina, equivalent to the inner dental epithelium of the initiating tooth. Red arrowheads (C, D, E, G, H) indicate the period following initiation and subsequent pause or stop phase of the regenerative cycle to include inhibition, growth, cell pooling within the successional lamina. Oral, left; aboral, right. Scale bar in E=100μm.

Figure 6. The cyclical stop-start hypothesis of tooth pattern initiation and regeneration in sharks. (A) The hatchling (stage 34) catshark dentition (microCT scan) showing the pattern of the dentition as a result of the alternate pattern that sets-up the regenerative dentition (red). (B) A model of the alternate regenerative dental pattern based on simplified hexagonal packing

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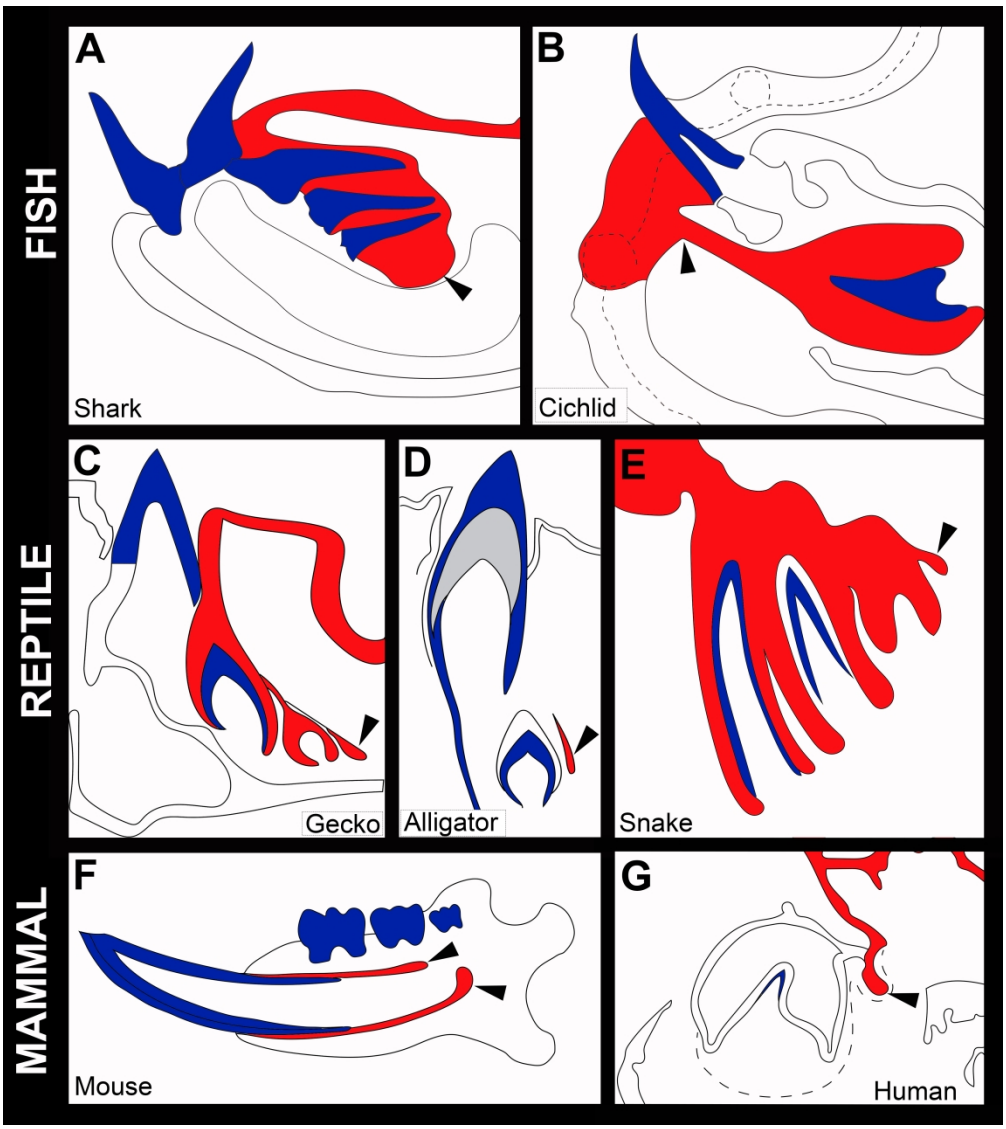

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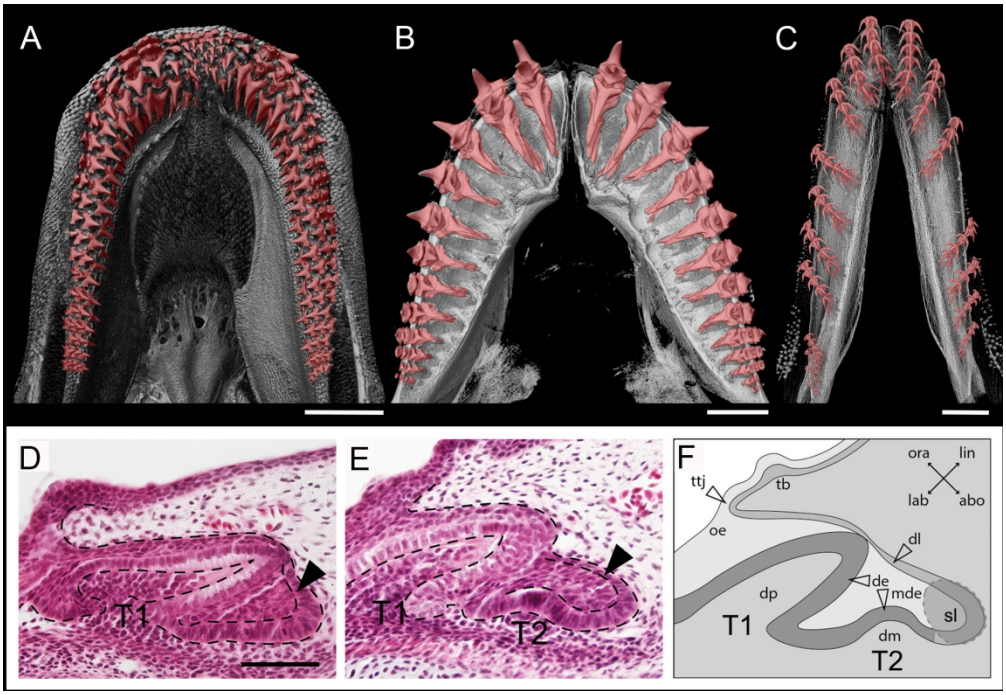

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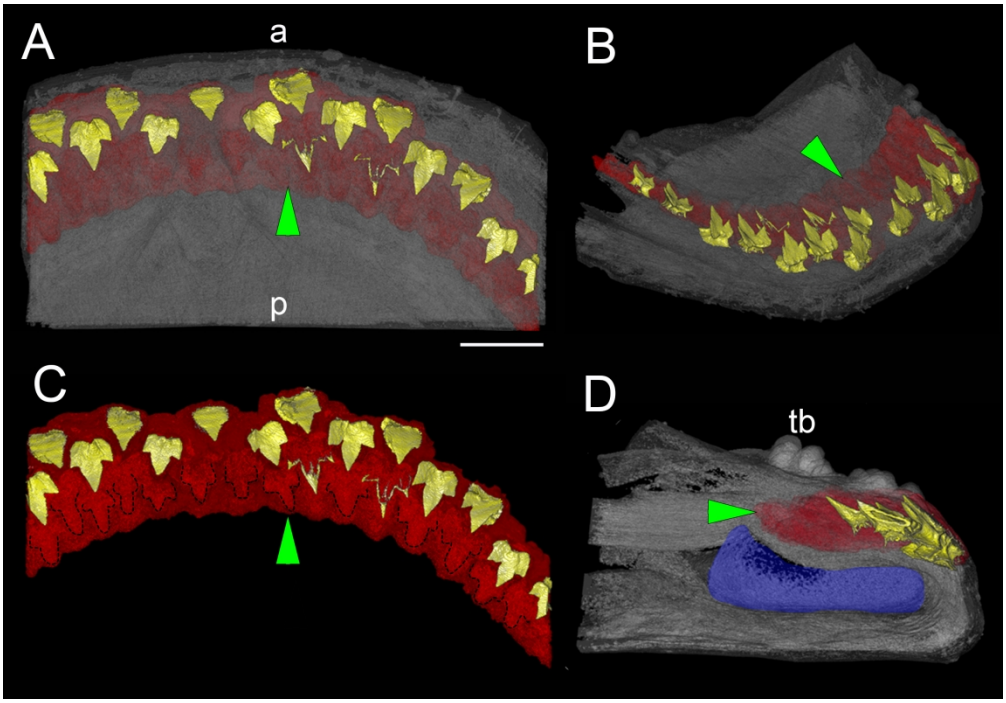

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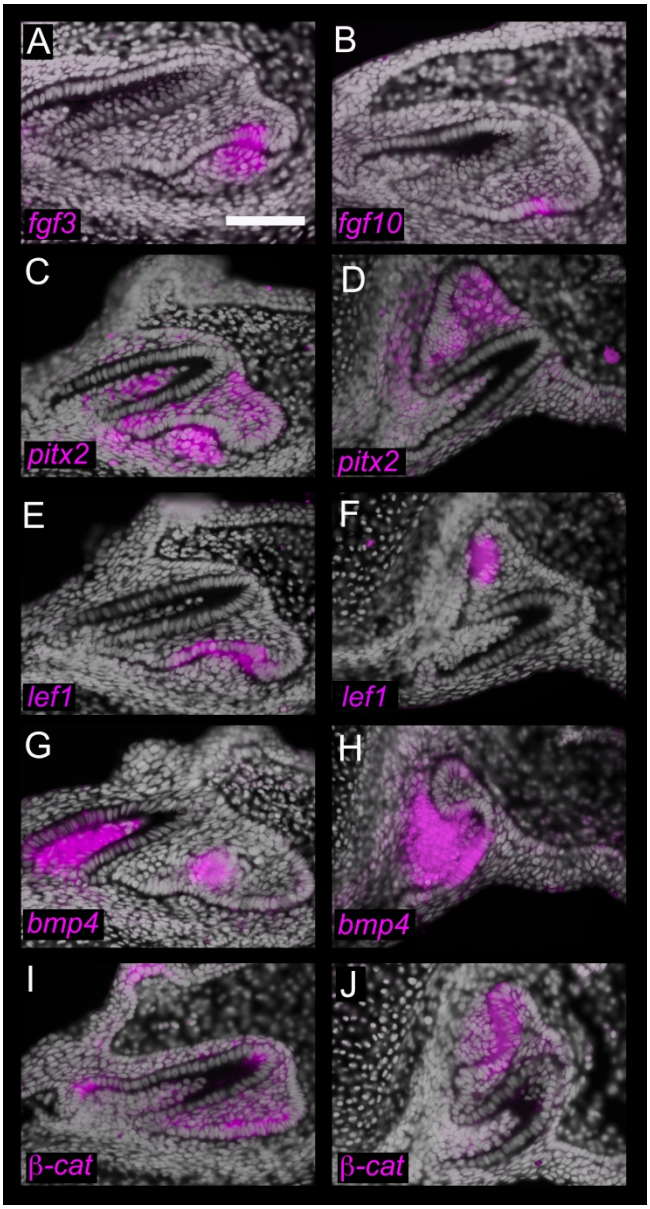

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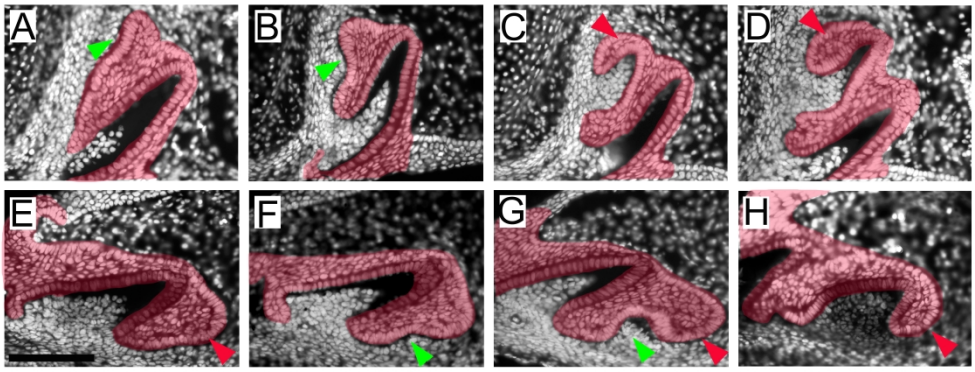

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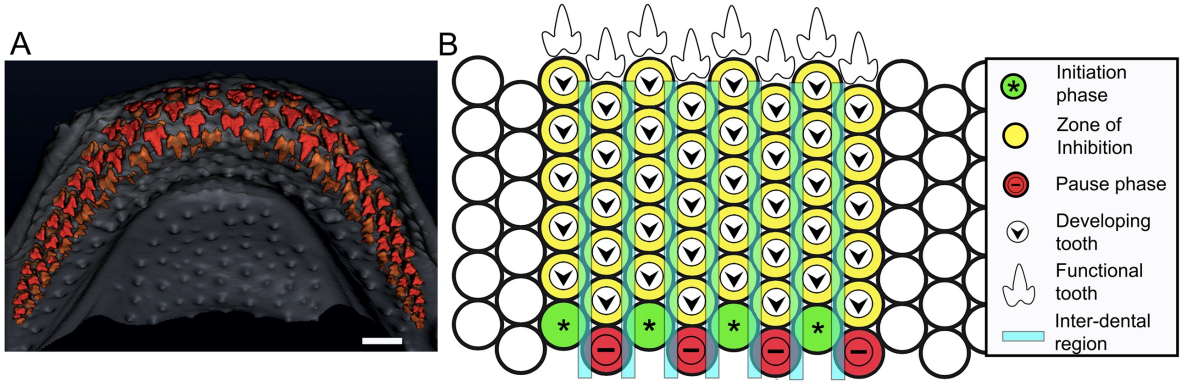

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