Sox2+ progenitors in sharks link taste development with the evolution of regenerative teeth from denticles

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Teeth and denticles belong to a specialized class of mineralizing epithelial appendages called odontodes. Although homology of oral teeth in jawed vertebrates is well supported, the evolutionary origin of teeth and their relationship with other odontode types is less clear. We compared the cellular and molecular mechanisms directing development of teeth and skin denticles in sharks, where both odontode types are retained. We show that teeth and denticles are deeply homologous developmental modules with equivalent underlying odontode gene regulatory networks (GRNs). Notably, the expression of the epithelial progenitor and stem cell marker sex-determining region Y-related box 2 (*sox2*) was tooth-specific and this correlates with notable differences in odontode regenerative ability. Whereas shark teeth retain the ancestral gnathostome character of continuous successional regeneration, new denticles arise only asynchronously with growth or after wounding. Sox2+ putative stem cells associated with the shark dental lamina (DL) emerge from a field of epithelial progenitors shared with anterior-most taste buds, before establishing within slow-cycling cell niches at the (*i*) superficial taste/tooth junction (T/TJ), and (*ii*) deep successional lamina (SL) where tooth regeneration initiates. Furthermore, during regeneration, cells from the superficial T/TJ migrate into the SL and contribute to new teeth, demonstrating persistent contribution of taste-associated progenitors to tooth regeneration in vivo. This data suggests a trajectory for tooth evolution involving cooption of the odontode GRN from nonregenerating denticles by *sox2*+ progenitors native to the oral taste epithelium, facilitating the evolution of a novel regenerative module of odontodes in the mouth of early jawed vertebrates: the teeth.

regeneration | stem cells | odontogenesis | chondrichthyes | evolutionary novelty

The evolutionary origin of teeth, a key vertebrate innovation, is a long-disputed question in vertebrate biology (1–3). Anatomical and developmental similarities between teeth and denticles, both consisting of basal bone, dentine, and a hypermineralized enamel-related tissue surrounding a pulp cavity, have long been recognized (4, 5). Despite these similarities, teeth in extant vertebrates are functionally, topographically, and organizationally distinct from the rest of the exoskeleton and considered a largely independent developmental module (6, 7). Current hypotheses posit that oral teeth evolved either from odontodes in the external dermal skeleton (outside-in) or deeper within the pharynx (inside-out) before their eventual cooption and elaboration in jaws of early gnathostomes (1, 3). However, neither the developmental genetic basis of this cooption event nor the evolutionary mechanisms underlying the elaboration and diversification of this peculiar odontode module are known. To resolve these outstanding questions, it is necessary to clearly differentiate true teeth from other odontode modules using reliable homology criteria. Successional regeneration, the iterative emergence of replacement odontodes within organized families (e.g., tooth-whorls) on the jaw margins characterizes the dentition of crown gnathostomes (Chondrichthyes (including acanthodians) + Osteichthyes). Polarized rows of sequentially added oral odontodes occur earlier in the fossil record, including on pharyngeal arches of the jawless thelodont *Loganellia* (2, 8, 9) and in dentitions of stem gnathostomes such as arthrodiran placoderms (10, 11). However, whether these patterned odontodes are ancestral to or convergent with true successional tooth whorls of crown gnathostomes is still unclear.

Due to the secondary loss of polyphyodonty in most established vertebrate model organisms (e.g., mouse, chick, frog, zebrafish), developmental mechanisms regulating successional regeneration of oral teeth remain poorly understood. Correspondingly, how the evolutionary transformation between simple unidimensional addition of new odontodes into an expanding growth zone as seen in skin denticles and the complex successional regeneration of odontodes in a one-for-one or many-for-one family-structured pattern occurred is unknown. To resolve the evolutionary relationship between oral teeth and other odontodes and to develop a model describing the emergence of successional regeneration from simple nonsuccessional odontode addition, we compared the developmental genetic basis for successional regeneration of oral teeth with the development of other odontodes (skin denticles) using the emerging chondrichthyan model, the small-spotted catshark (*Scyliorhinus canicula*) (12, 13) (Fig. 1 *A* and *B*).

Results

Successional Regeneration is a Property Unique to Teeth Among Odontodes. We first compared developmental patterning of oral teeth with denticles in catsharks with a range of light microscopy and computed tomographic methods (Fig. 1 and Movie S1). The shark dentition, emerging from tightly constrained developmental fields on the anterior jaw margins (6, 14), includes adjoining tooth families embedded within a permanent epithelial dental lamina (DL) (Fig. 1 *C*–*G*). Each family is comprised of a developmental sequence of regenerating teeth, with the youngest tooth primordia

Significance

Oral teeth and skin denticles share a deep, 450-million-year-old, evolutionary relationship among vertebrates. We investigate how teeth evolved from simpler denticles using sharks as an evolutionary developmental model. The striking ability of shark teeth to regenerate rapidly and continuously is not shared by denticles, and we suggest that this difference is related to the tooth-specific expression of the stem cell factor sox2. We show that epithelial progenitors of the shark dentition share a remarkable developmental relationship with taste buds. We suggest that teeth arose by combining the gene regulatory network for producing simple denticles with that for producing the highly regenerative taste buds in the oral cavity.

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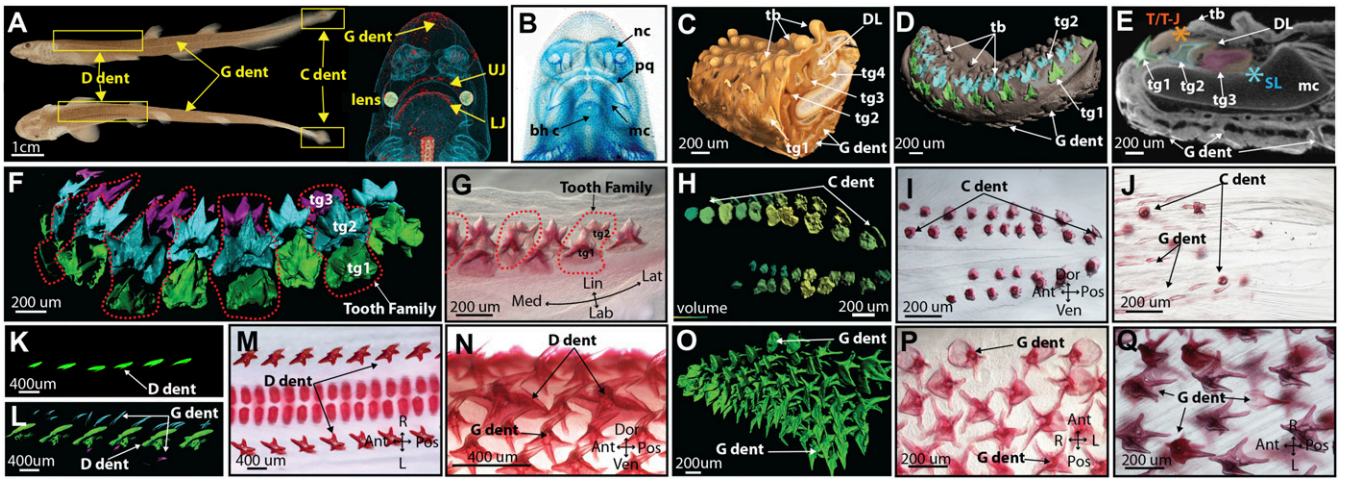

Fig. 1. Developmental diversity of teeth and denticles in *S. canicula*. MicroCT reconstruction (*A*) demonstrating specialized dorsal denticle (D dent), caudal denticle (C dent), and general denticle (G dent) positions relative to teeth on the upper (UJ) and lower (LJ) jaws in a hatchling (St 34;103 mm) and corresponding ventral view of an alcian-blue (cartilage) and alizarin-red (calcium) stained specimen (*B*). Same-stage LJ high-resolution soft-tissue–stained microCT reconstructions (*C* and *D*) and slice (*E*), revealing successional tooth generations (tg1–tg4) within a family embedded in the dental lamina (DL). Taste buds (tb) grade into the DL at the taste/tooth junction (T/TJ), and new teeth are formed adjacent to the successional lamina (SL). Light-sheet fluorescence microscopy (LSFM) isosurface renderings of alizarin-red–stained samples revealing successional tooth families in a hatchling (*F*), sequentially adding embryonic (St 31) caudal denticle rows (*H*) and dorsal denticle rows at St 31 (*K*) and St 33 (*L*), general denticles at St 34 (*O*), and corresponding bright-field images (*G*, *I*, *M*, and *P*). In juveniles (~3 mo post-hatching; 155 mm) the processes of caudal denticle shedding (*J*), dorsal denticle incorporation (*N*), and new general denticle addition (*Q*) can be observed.

emerging from the successional lamina (SL) at the distal end of the DL and the oldest, functional teeth erupting synchronously into the oral cavity (Fig. 1 *C*–*G* and Movie S2). This conveyor-belt mechanism of successional regeneration appears to be a synapomorphy of oral teeth in crown gnathostomes (15, 16). Although both the precocious embryonic caudal tail (Fig. 1 *H*–*J*) and dorsal trunk (Fig. 1 *K*–*N*) denticle rows initially also bear features of organized, sequential development within well-bounded developmental fields, there is no successional family-level organization to their emergence. Moreover, caudal denticles are eventually shed, and dorsal denticles are subsumed into general scalation shortly after hatching; and neither type regenerates into adulthood (Fig. 1 *J* and *N*). Only general adult-type denticles, which are the last to develop during embryogenesis and which radiate around the entire body surface, unbounded by the tight developmental fields which constrain more specialized precocious denticle modules and teeth, continue to form beyond embryonic stages as gaps in the scalation appear with growth, yet without any sign of successional family-level organization (Fig. 1 *O*–*Q*). Overall, these observations suggest that in addition to the oral dentition, the external exoskeleton of the shark also consists of several highly regulated developmental modules of odontodes, but that the property of continuous successional regeneration with family-level organization sets teeth apart from all other odontodes.

Odontode *sox2* Expression Is Exclusive to the Development and Regeneration of Teeth. To determine the degree of genetic similarity between teeth and denticles and search for markers uniquely associated with successional regeneration of teeth, we examined expression patterns of several key regulators of odontogenesis previously identified in bony vertebrates, including β-*catenin*, *bmp4*, *fgf3*, *fgf10*, *lef1*, and *shh*, as well as the secreted calcium-binding glycoprotein *sparc* (*osteonectin*) (Fig. 2). Overall, we found remarkable conservation of gene expression patterns during development of both teeth and denticles in the shark. These results suggest that a shared core odontogenic gene regulatory network (oGRN) is redeployed to make odontodes, whether in the oral or external modules of the exoskeleton, and that all odontodes share a deep molecular homology. Strikingly, whereas most oGRN components examined were conserved, we found that strong *sox2*/sox2 expression characterizes development and regeneration of teeth in both sharks and rays (Fig. 3 and Fig. S1), but is absent from all external denticle types (Fig. 3 and Fig. S2). Sharks, like other polyphyodont vertebrates, must use multipotent odontogenic stem cells to enable the lifelong successional regeneration of teeth (17–22). Previous studies demonstrated the role of Sox2 in regulating adult epithelial stem cells, including those required for both tooth ameloblast and taste-bud regeneration in mouse (23–26). Polyphyodont osteichthyans (17, 20, 21, 26) also express *sox2* in the developing DL during tooth regeneration within putative epithelial stem cell populations. However, despite previous attempts (27), this important stem marker has not been characterized in a chondrichthyan. Our results indicate that expression of *sox2* in the DL during tooth development and regeneration has indeed been conserved at least since the origin of successionally regenerating teeth in crown gnathostomes and therefore serves as a reliable marker of epithelial progenitor/stem cell populations across all vertebrate dentitions. Considered together with its absence during the development of denticles, this suggests that *sox2* represents a molecular marker, expression of which can reliably differentiate between teeth and other odontode types. As the differential expression of the known stem cell marker *sox2* between teeth and denticles is correlated with their vastly different regenerative capacities, we suggest it is functionally significant and contributed to the evolution of continuous successional regeneration in teeth from nonregenerating denticles.

Teeth and Taste Buds Emerge from a Common sox2+ Progenitor Field. To investigate how sox2 could have become incorporated into an otherwise identical oGRN and facilitated the evolution of the permanent epithelial stem cell population necessary for successional tooth regeneration, we examined early expression of *sox2* in developing shark jaws alongside the odontogenic epithelial/mesenchymal marker *pitx2*. Intriguingly, we found that both teeth and anteriormost taste-bud rows emerge from a shared *sox2*+/*pitx2*+ progenitor field, which we term the odontogustatory band (OGB) to reflect its contribution to the DL and anterior taste-bud field (Fig. 3 *A*–*C*). We found that initially overlapping taste/tooth territories within the OGB at St 28/29 (Fig. 3 *A* and *B*) narrowed and shifted progressively from St 31 (Fig. 3 *E* and *F*) to St 32 (Fig. 3 *I* and *J*) such that *pitx2*-expressing odontogenic progenitors became confined to the DL and its nascent tooth buds, whereas *sox2*-expressing progenitors became restricted to the lingual DL and adjacent taste buds. During first tooth regeneration (St 32), shared

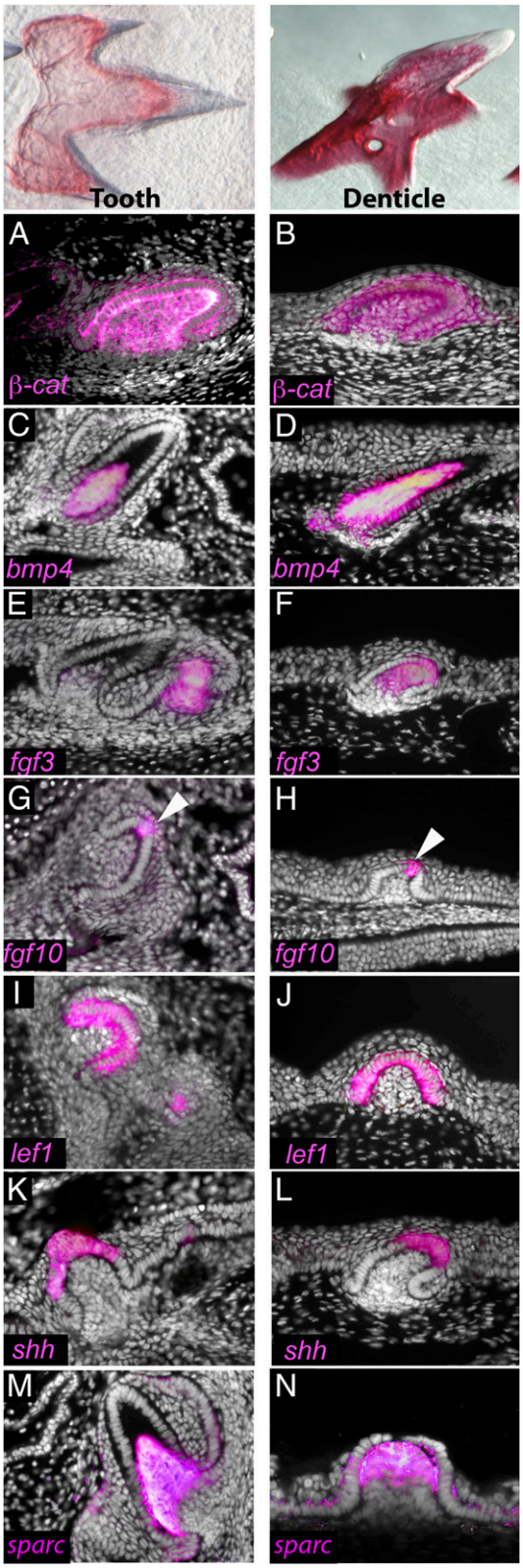

Fig. 2. Conserved gene expression profiles in shark teeth and skin denticles. Teeth (*A, C, E, G, I, K,* and *M*) and denticles (*B, D, F, H, J, L,* and *N*) exhibit similar expression patterns of many genes belonging to a core odontode gene network during representative stages of development. *β-catenin* labels epithelium and mesenchyme of morphogenesis-stage teeth (*A*) and denticles (*B*). *bmp4* is restricted to the mesenchyme in mineralizing teeth (*C*) and denticles (*D*). *fgf3* is expressed in both the epithelial enameloid knot and mesenchyme during early papilla stages of teeth (*E*) and denticles (*F*), whereas expression of *fgf10* at the same stage is restricted to the enameloid knot (white arrowheads) in both teeth (*G*) and denticles (*H*). *Lef1* is expressed throughout the epithelium of papilla-stage teeth (*I*) and denticles (*J*), whereas *shh* expression at the same stage is restricted to a more apical epithelial region in both teeth (*K*) and denticles (*L*). The downstream calcium-binding effector gene *sparc* is expressed throughout the mesenchyme in both teeth (*M*) and denticles (*N*). Signal from in situ hybridization shown in magenta (false colored); nuclear counter stain with DAPI shown in grayscale.

expression domains of *sox2* and *pitx2* were restricted to just two adjoining niches: (*i*) the superficial taste/tooth junction (T/TJ) where the oral epithelium grades into the DL; and (*ii*) the deep successional lamina (SL) (Fig. 3 *M* and *N*), which is immediately adjacent to the site of new tooth formation marked by *lef1* expression (Fig. 3*O*). Arrays of smaller taste buds overlying the basihyal and palate were also associated with clusters of *sox2*-expressing progenitors (Fig. 3*J*), but never exhibit *pitx2* expression (Fig. 3*I*), nor are they organized in distinctive patterned rows like the DL-associated taste-bud rows descended from the OGB. Taste buds are ancient, primitively endodermal epithelial sensory appendages, which predate the origins of teeth and are present in extant jawed (osteichthyans and chondrichthyans) and jawless (lampreys) vertebrates (28). Taste buds regenerate constantly throughout life and in mammals are renewed from populations of Sox2+ stem cells within the papillae or adjacent basal keratinous epithelium underlying the definitive taste bud (24, 29, 30). Emergence of both the dentition and anteriormost taste buds from a confluent field of *sox2*-expressing epithelium in the shark suggests that developmental programs of teeth and taste buds are linked and the evolutionary precursors of epithelial progenitors responsible for tooth regeneration may have been derived from those responsible for the development and regeneration of taste buds.

To track the development of early embryonic progenitors within the OGB until their eventual establishment within putative stem cell niches of the dentition, we performed double immunofluorescent labeling of sox2 and activated β-catenin on semithin sections of a series of *S. canicula* teeth and, for comparison, denticles at equivalent developmental stages (Fig. 3 *C*, *D*, *G, H, K,* and *L*). β-catenin is a key component of the canonical Wnt-signaling pathway known to interact with Sox2 (31, 32) and to regulate both tooth and taste-bud development in osteichthyans (17, 33–35). Throughout early development, we found continuous colocalization of sox2 and β-catenin, first within the basal epithelium in the early OGB (Fig. 3*C*), then extending in an uninterrupted band of highly columnar cells along the lingual edge of the invaginating DL connecting the T/TJ to the SL (Fig. 3*G*). This continuous sox2+ band in the DL remains intact through the first rounds of tooth regeneration at St 32 (Fig. 3*K*), until finally breaking and establishing within the disjointed niches of the T/TJ (Fig. 3*Q* and Movie S3) and the SL (Fig. 3*Q* and Movie S4), in the functional dentition of St 34 hatchlings (Fig. 3*Q*). Interestingly, in hatchlings, intermittent streams of sox2+ cells, usually coexpressing β-catenin, are also detected along the lingual margin of the squamous middle dental epithelia (MDE), a stellate reticulum-like collection of cells within the DL between the two established niches. This suggests that even in feeding-stage sharks with a functional dentition, the superficial T/TJ maintains intermittent connectivity to the deep SL niche (Fig. 3*Q*). These results are also consistent in upper jaws (Fig. S3). Although all taste buds, including those which emerge from the OGB and overlie the T/TJ (Fig. 3*P*) and those which develop deeper within the pharynx unassociated with the DL (Fig. S4), also coexpress sox2 and β-catenin, sox2 was not detected in the basal epithelia during development of denticles, despite comparable expression of β-catenin (Fig. 3 *D*, *H*, and *L*). The use of the epithelial stem cell marker sox2 during early development and regeneration therefore unites teeth with taste buds to the exclusion of other odontode types.

Proliferation of sox2+ Progenitors and Putative Stem Cells. To assess when the sox2+ embryonic progenitors of the OGB become fated as slow-cycling putative adult odontogenic stem cells, we analyzed

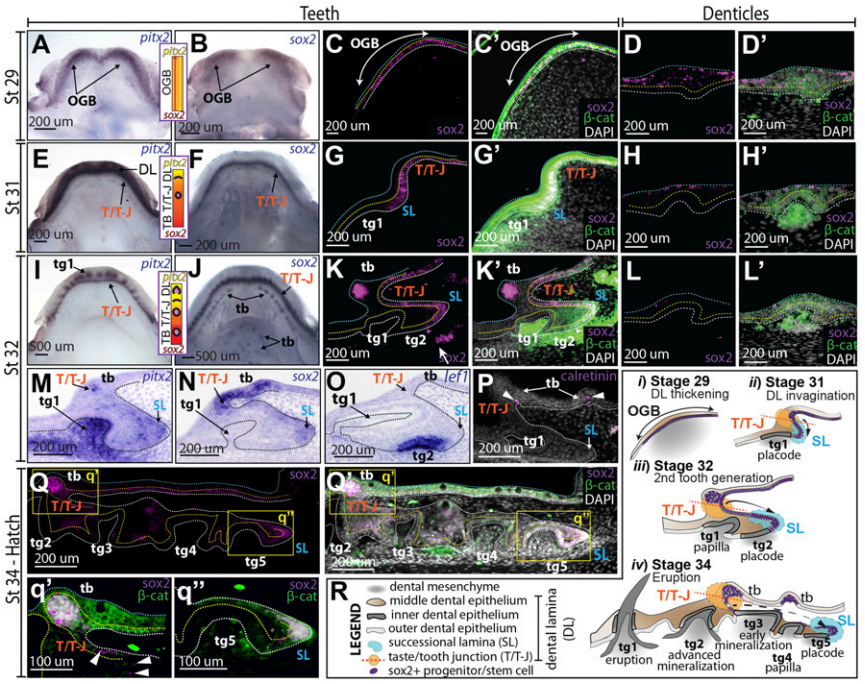

Fig. 3. Sox2+ putative stem cells in the shark dentition emerge from progenitors of the odontogustatory band (OGB). Whole-mount in situ hybridization for *pitx2* and *sox2* in the lower jaw during early OGB establishment (St 29; *A* and *B*), DL invagination (St 31; *E* and *F*), and tooth regeneration (St 32; *I* and *J*) reveals progressive restriction of initially overlapping expression. Immunofluorescence for sox2 (magenta) and β-catenin (green) in sections through the lower jaw dentition (*C, G,* and *K*) and denticles (*D, H, and L*) reveals colocalization of sox2 and β-catenin in a contiguous band of basal epithelium shared between the tooth and taste-bud field. The taste/tooth junction (T/TJ) and successional lamina (SL) retain overlapping *sox2* and *pitx2* expression during regeneration at St 32 (*M, N*), where *lef1* marks the second tooth generation (tg2) (*O*) and calretinin marks prospective gustatory cells (white arrowheads) in taste buds (tb) at both the T/TJ and adjacently (*P*). At hatching (St 34), sox2+ cells of the DL (*Q–Q′*) are concentrated in the T/TJ (*q′*) and the SL (*q″*) where they coexpress β-catenin, but are also found in the middle dental epithelium between the niches (white arrowheads). Schematic of sox2+ progenitor/stem cell distribution (purple) during development of the dentition and taste buds (*R*).

cell-cycle kinetics of the sox2+ cells in the DL through coimmunostaining for proliferating cell nuclear antigen (PCNA), a G1-S phase marker of actively mitotic cells. Beginning in the early OGB (Fig. S5*A*) and continuing from early DL invagination (Fig. S5*B*) until the initiation of second tooth generation (Fig. S5 *C* and *D*), we found that most sox2+ progenitors in the DL coexpress PCNA at consistently high levels. By the third tooth generation at St 33, leading into segregation of the separate T/TJ and SL niches, PCNA expression in sox2+ cells drops markedly (Fig. S5 *E* and *F*), reflecting the slowdown of cell-cycle kinetics expected for long-term maintenance of adult stem cell populations sustained in the discrete T/TJ and SL niches of hatched animals (Fig. S5 *G* and *H*).

Periodicity in Proliferation and Wnt Signal Transduction in the Shark SL. Interestingly, within the SL of juvenile sharks, PCNA expression in sox2+ cells is not permanently attenuated, and reactivation of cell proliferation in this slow-cycling niche correlates with the relative timing of new tooth initiation. In *S. canicula*, development of adjacent tooth families is staggered such that immediately neighboring families initiate regeneration at slightly different times, and this can be visualized via histological stains of serial sections (Fig. S6 *A*, *B*, and *E*). During preinitiation, sox2+ cells of the SL lack PCNA expression (Fig. S6*D*), whereas during initiation of new tooth placodes in the immediately adjacent tooth family, sox2+ stem cells become mitotically active (Fig. S6*E*). Expression of β-catenin in the SL also covaries with timing of tooth initiation. During preinitiation, β-catenin is mostly confined to sox2+ cells (Fig. S6*F*), whereas during initiation, β-catenin is expressed widely throughout the SL and appears to translocate to the nucleus specifically of sox2+ cells (Fig. S6*G*). This implicates the canonical wnt-signaling pathway in the regulating odontogenesis in chondrichthyans, as previously shown in polyphyodont osteichthyans (17, 19, 35, 36).

Label-Retaining Cells Reveal Slow-Cycling Putative Stem Niches in the Shark Dentition. We performed two successive 5-bromo-2′-deoxyuridine (BrdU) pulse–chase experiments to localize slow-cycling stem cells in the shark dentition and further characterize the timing of mitotic deceleration and niche establishment. Two separate 2-wk-long pulses of BrdU confirmed to saturate the epithelium (Fig. S7) during (*i*) the first tooth stage before niche sequestration and (*ii*) the third tooth stage after sequestration of the T/TJ and SL niches of sox2+ cells (Fig. 4 and Fig. S7). Following the first tooth pulse and an 8-wk chase, epithelial BrdU+ label-retaining cells (LRCs) of the DL were largely restricted to sox2+ regions of (*i*) the T/TJ, and (*ii*) the lingual margin of the middle dental epithelium (MDE) overlying tooth cusps (Fig. 4 *A–D*). Notably, at this stage few LRCs were found in the SL (Fig. 4*D*). This is likely a consequence of rapid rate of tooth regeneration at this early stage, necessitating a constant turnover of sox2+ cells within the SL. As with teeth (Fig. 4*D*), epithelial LRCs within denticles were found within the cervical loops (Fig. 4*E*), whereas in taste buds, LRCs were found within the sox2+ domains of the taste-bud core and basal epithelium underlying and adjacent to the taste bud within the papilla (Fig. 4*F*). In the second experiment, a 2-wk BrdU pulse at St 33 was followed by a chase of 12–13 wk until hatching at St 34 (Fig. 4 *G–L*). In this case, sharks accumulated large numbers of LRCs within all sox2+ domains of the DL previously characterized, including (*i*) the basal epithelium of the T/TJ (Fig. 4*H*); (*ii*) the lingual margin of the squamous MDE, especially around tooth cusps (Fig. 4*I*); and (*iii*) the SL (Fig. 4*J*). These results were consistent in upper (Fig. 4) and lower jaw dentitions (Fig. S7). In denticles a few LRCs were found within the cervical loops, but mostly in the overlying keratinizing epithelium (Fig. 4*K*), whereas LRCs associated with taste buds were found in the basal epithelium underlying or adjacent to each taste bud within the papillae (Fig. 4*L*). The deferred accumulation of LRCs within the SL niche compared with the T/TJ coincides with the timing of reduction of PCNA expression in sox2+ cells (Fig. S5) and suggests that kinetic slow-down of the superficial T/TJ and deep SL niches (and the transition from proliferating progenitor to slow-cycling putative stem cell populations) is regulated independently. In denticles, LRCs were largely restricted to cervical loops at the base of each unit and within cusp enameloid knots, regions where LRCs were also found in teeth, but which are decoupled in space and time from the site of tooth regeneration and therefore unlikely to play a role (Fig. 4 *E* and *K*). A recent study examined juvenile sharks for the presence of BrdU LRCs in the dentition following short (2 d) pulse treatments and concluded that a few slow-cycling putative stem cells were present in the MDE, but did not detect LRCs within either the T/TJ or SL (27). We suspect that failure to detect LRCs within the T/TJ or SL in that case was due to lack of saturation and failure to incorporate BrdU into slow-cycling cells whose division cycle may exceed 2 d.

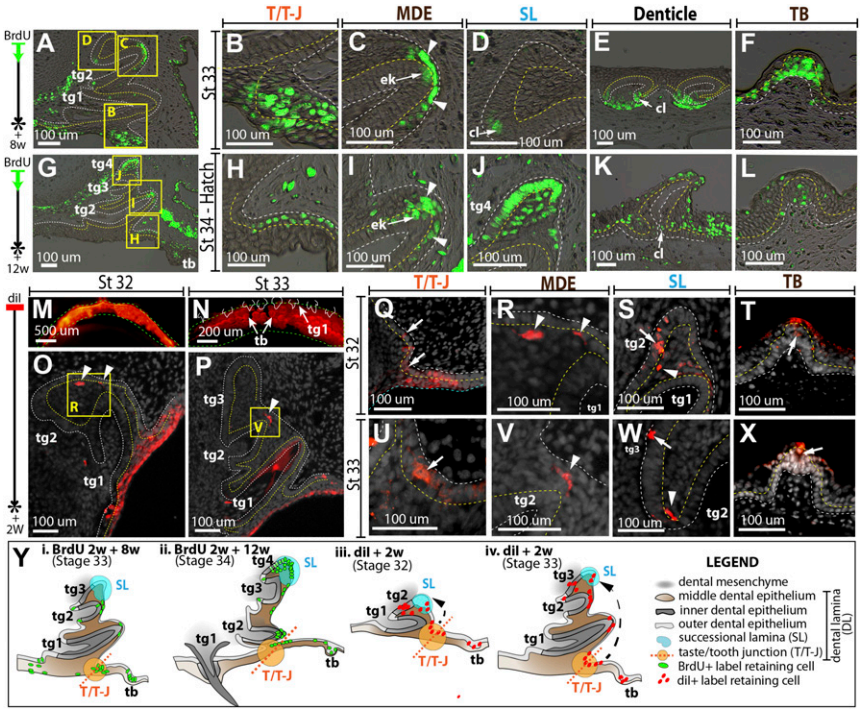

Fig. 4. Label-retaining cells reveal slow-cycling niches and migratory cells in the shark DL. Slow-cycling BrdU+ label-retaining cells (LRCs) (green) in the UJ dentition of St 33 embryos after a 2-wk saturation pulse and 8-wk chase (*A–D*) and St 34 after a 2-wk saturation pulse and 12-wk chase (*G–J*). Corresponding distribution of LRCs in denticles (*E* and *K*) and taste buds (*F* and *L*). High magnification of *Inset* areas from *A* and *G* revealing accumulation of LRCs in the taste/tooth junction (T/TJ) (*B* and *H*), the middle dental epithelium (MDE) concentrated around the enameloid knots (ek) (*C* and *I*), and the successional lamina (SL) (*D* and *J*), which is initially devoid of LRCs in the early rapidly cycling dentition (*D*) but becomes slow-cycling in later stages (*J*). DiI applied superficially to the UJ buccal valve spanning the odontogenic band (OGB)-derived presumptive taste-field of St 32 (*M*) or St 33 (*N*) embryos reveals undiluted dil label-retaining cells static within the T/TJ (*Q* and *U*) (white arrows) migrating through the MDE (*O, P, R,* and *V*) (white arrowheads) and contributing to new tooth placodes within the SL (*S* and *W*) (white arrows). DiI-carrying cells are also incorporated within nascent taste buds formed after labeling at St 32 (*T*) and within taste buds after labeling at St 33 (*X*). Schematic summarizing distribution of labeled cells (*Y*).

The presence of definitive sox2+/PCNA− niches rich in LRCs in the dentition but not the scalation of sharks likely plays a key part in the different regenerative potentials of teeth compared with other odontode types.

Lineage Tracing Reveals Migration of Taste-Associated Cells into the SL. Lipophilic dye (DiI) lineage tracing was performed to uncover the relationship between the T/TJ and the SL before and following their sequestration (Fig. 4 *M–X*). In both cases, DiI labeling was performed across the whole OGB, spanning the length and width of the buccal valve up to and including the T/TJ (Fig. 4 *M* and *N*). Early labeling of the OGB was carried out before taste-bud development (Fig. 4*M*), whereas the late labeling was performed after several taste buds had formed within the OGB (Fig. 4*N*). Strikingly, this revealed that within 2 wk, labeled cells from the T/TJ migrated through the squamous epithelia of the MDE into the SL and directly contributed to new tooth placodes both before (Fig. 4 *M*, *Q*, *R*, and *S*) and after (Fig. 4 *N* and *U–W*) breakdown of the initially continuous band of sox2+ columnar epithelium connecting the niches (Fig. S3). Distribution of DiI labeled cells in the MDE is highly concordant with the observed distribution of both BrdU LRCs and sox2+/PCNA− cells, strongly suggesting this migratory pathway directly connects cells of the superficial T/TJ to the SL and to new teeth, uniting the apparently separate niches of odontogenic progenitors in the shark DL as a single dynamic system. DiI labeled cells were also found within nascent taste buds, which emerged from the OGB following the early treatment supporting the idea that labeled cells contributed to both tooth and taste-bud development (Fig. 4*T*). Although DiI was also found in taste buds following the late-stage labeling (Fig. 4*X*) it is not possible to determine whether these are nascent taste buds or whether they were directly labeled. Therefore, although our results indicate that progenitors within the early OGB are indeed multipotent and contribute to both tooth and taste-bud development, it is still unclear whether the T/TJ maintains this dual function later in development or adopts a role specific to tooth regeneration. Regardless, the presence of DiI-labeled cells migrating from the surface through the DL and contributing to new tooth placodes strongly suggests that both the initial emergence and successional regeneration of teeth in shark rely on superficial taste-associated progenitors.

Discussion

Collaboration Between the Gene Regulatory Network for Odontodes and Taste Buds During Tooth Evolution. Overall our data suggest that evolutionarily conserved sox2+ progenitors, which have their origin in superficial taste-competent oral epithelium, are the source of adult odontogenic stem cells responsible for the successional regeneration of teeth in the shark. This property sets teeth apart from all other odontode types and therefore serves as a reliable homology criterion for differentiating teeth from other odontodes. Although teeth and denticles are homologous as odontodes and share expression of most oGRN components, teeth are more similar to taste buds in both their ontogenetic tissue of origin and use of the sox2/β-catenin pathway during development and regeneration. These data therefore suggest that successionally regenerating teeth evolved as a result of a cooption of the gene regulatory network for odontodes (oGRN) into the oral cavity and its integration with the local sox2-modulated gene regulatory network for regenerating taste buds (tGRN) in early vertebrates before the origin of crown gnathostomes. This study does not resolve the question of whether the first odontodes were external (outside-in) or deeper within the pharynx (inside-out) before their heterotopic shift to the jaws. Although an interesting problem in itself, even a definitive answer from new fossil data would not address the deeper mechanistic question of how teeth came to be so different from all other odontodes. We believe that focus on the developmental genetic mechanisms underlying fundamental biological differences between teeth and other odontode types can complement paleontological data and together offer a richer and more detailed account of tooth evolution.

Materials and Methods

Shark Husbandry. The University of Sheffield is a licensed establishment under the Animals (Scientific Procedures) Act 1986. All animals were culled by approved methods cited under Schedule 1 to the Act. Small-spotted catshark (*Scyliorhinus canicula*) embryos were obtained from Station Biologique de Roscoff, Roscoff, France. Little-skate (*Leucoraja erinacea*) embryos were obtained from Woods Hole Marine Biological Laboratory, Woods Hole, MA. Embryos were raised in an artificial saltwater aquarium at 12 °C at the University of Sheffield, Sheffield, United Kingdom. Embryos were killed with MS-222 (tricaine) at 300 mg/L and fixed in 4% (wt/vol) paraformaldehyde overnight at 4 °C.

X-ray microCT. Scans of *S. canicula* specimen (Fig. 1*A*) were performed using Metris X-Tek HMX ST 225 CT scanner at the Imaging and Analysis Centre, Natural History Museum, London. For high-resolution soft-tissue X-ray microCT scans (Xradia MicroXCT, [www.zeiss.com/microscopy/int/x-ray], Fig. 1 *C–E*), specimens were dehydrated to ethanol and stained with Phosphotungstic Acid (PTA) (0.1% wt/vol in abs. ethanol) for 3 d (37). Three-dimensional reconstructions were made using Drishti (github.com/nci/drishti).

Clearing and Staining. Fixed samples were stained with Alcian blue (cartilage) and/or Alizarin red S as previously described (14).

Light-Sheet Fluorescence Microscopy. Alizain red S stained animals were imaged using a Zeiss Z1 light-sheet microscope under a 5× objective using the 561-nm laser, LP560 dichroic beam splitter, and LP585 filter. Isosurfaces were rendered with the software Imaris 8.1.

Cloning and in Situ Hybridization. Probes for *β-catenin*, *bmp4*, *fgf3*, *fgf10*, *lef1*, *shh*, *sox2*, and *pitx2* were cloned as described previously (14). A 1-kb probe for *sparc* was cloned using the primer sequences (Forward-GCCTGGTGCTCTCTCACTG, Reverse-TAGCGATGGGCAGCAATAG). Both section and whole-mount in situ staining was performed as described in 14.

Immunofluorescence. Dewaxed paraffin slides were subjected to heat-mediated antigen retrieval in hot 0.01M sodium citrate pH = 6.0 for 20 min before blocking and subsequent antibody labeling. Primary antibody labeling with rabbit anti-Sox2 (Abcam ab97959) 1:500, together with mouse anti-PCNA (Abcam ab29) 1:2,000, or mouse anti-active β-catenin (ABC; Merck 05–665) 1:500 was carried out overnight at 4 °C. Secondary antibody incubation with goat anti-rabbit AlexaFluor-647 (Thermo A-21245) 1:250 and goat anti-mouse AlexaFluor-488 (Thermo A-11-001) 1:250 was carried out for 1 h at room temperature. Slides were counterstained with 1 μg/mL DAPI (Sigma D9542) and mounted in Fluoromount (Sigma F4680). Calretinin (calb2) immunofluorescence was performed with rabbit anti-calretinin (abcam ab702) at 1:1,000. Imaging was carried out on an Olympus BX51 upright epifluorescent microscope and visualized with the software Volocity 6.3.

Label-Retaining Assay. Embryos at stage 31 ($n = 15$) or stage 33 ($n = 14$) were reared in 5-L artificial seawater tanks containing 100 μg/mL BrdU (5-bromo-2′-deoxyuridine) for 2 wk with biweekly 50% water changes and daily injection of 100 μL of a 10 mg/mL BrdU into the egg cases. Two animals were killed immediately after each 2-wk pulse to verify epithelial saturation of BrdU. Remaining animals were transferred to fresh artificial seawater and killed periodically following the early (St 31) treatment after chase periods of 1 wk ($n = 2$), 2 wk ($n = 2$), 4 wk ($n = 3$), 6 wk ($n = 3$), and 8 wk ($n = 3$) or following the late (St 33) treatment after chase periods of 2 wk ($n = 2$), 4 wk ($n = 3$), 8 wk ($n = 3$), 12 wk ($n = 3$), and 13 wk ($n = 1$). Animals were fixed in 4% (wt/vol) PFA at 4 °C overnight and vacuum-embedded in paraffin. BrdU immunofluorescence was performed on dewaxed slides following an antigen recovery in 2N HCl for 30 min at 50 °C and staining with mouse anti-BrdU (DSHB G3G4) at 1:250 and goat anti-mouse AlexaFluor-488 (Thermo A-11-001) as described above.

Lineage Tracing Assay. Liquid DiI solution (Thermo V22885) was applied superficially to the buccal valve (bv) at St 32 ($n = 10$) or St 33 ($n = 14$) using a pulled microinjection capillary and aspirator tube. Embryos were anesthetized in MS222 at 100 mg/L and treated with DiI under a Leica (MZ FL III) fluorescent stereomicroscope. Embryos were allowed to recover in aerated fresh artificial seawater until pharyngeal respiration resumed, then returned to their egg cases to develop for 2 wk before processing.

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Supporting Information

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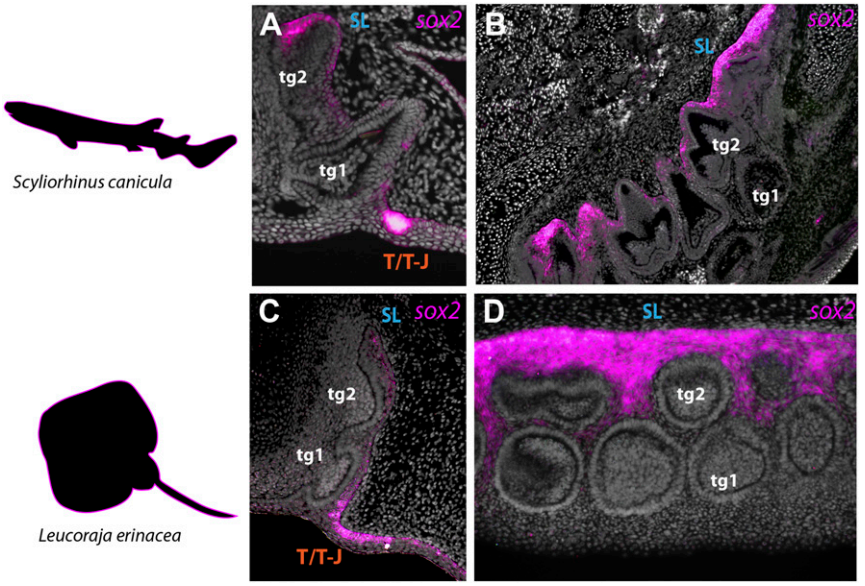


Fig. S1. *Sox2* expression profile in teeth is conserved among sharks and rays (elasmobranchs). *Sox2* expression characterizes restricted populations' epithelial cells in the dental lamina in both the catshark (*A* and *B*, *Scyliorhinus canicula*) and the little skate (*C* and *D, Leucoraja erinacea*). Sagittal section of the upper jaw dentition in St 33 animals (*A* and *C*) reveals strong *sox2* expression in the superficial taste/tooth junction (T/TJ) where the oral epithelium grades into the dental lamina and in the successional lamina (SL) at the most distal invaginated tip of the DL. Tooth generations (tg1, tg2), which develop within the DL between these two *sox2*+ regions populations, show no *sox2* expression after initiation. Horizontal sections (*B* and *D*) of the lower jaw in both species show continuous jaw-wide expression of *sox2* in the successional lamina (SL) surrounding the developing teeth despite vastly different shape, molariform-type teeth in the ray (*D*), and pointed multicuspid teeth in the shark (*B*). Signal from in situ hybridization shown in magenta (false-colored); nuclear counter stain with DAPI shown in grayscale.

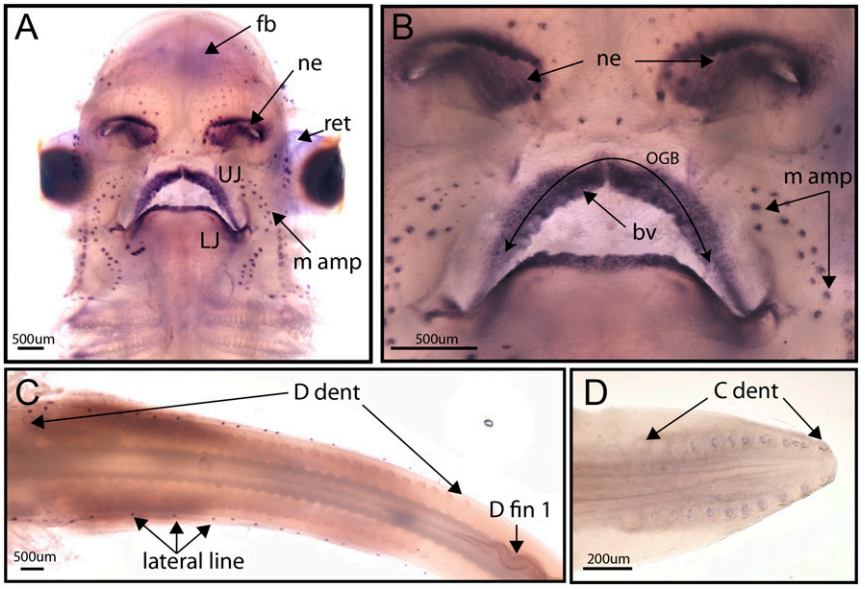


Fig. S2. Whole-mount s*ox2* expression profile in St 29 shark embryos. Ventral view (*A* and close-up *B*) showing s*ox2* is expressed in the upper (UJ) and lower (LJ) odontogustatory band (OGB), the nasal epithelium (ne), the forebrain (fb), retina (ret), and the ampullae of Lorenzini, including the maxillary ampullae (m amp). Along the trunk, *sox2* labels the developing placodes of the lateral line (*C*, dorsal view). Importantly, *sox2* is not expressed in or around any other developing odontodes in the skin; dorsal denticles (D dent) visible as parallel placodal condensations in a dorsal view (*C*) and caudal denticles shown in lateral view with the oldest mineralized denticles on the *Right* and youngest placodal condensations to the *Left* (C dent) (*D*) develop within *sox2* negative epithelia. bv, buccal valve; D fin, dorsal fin.

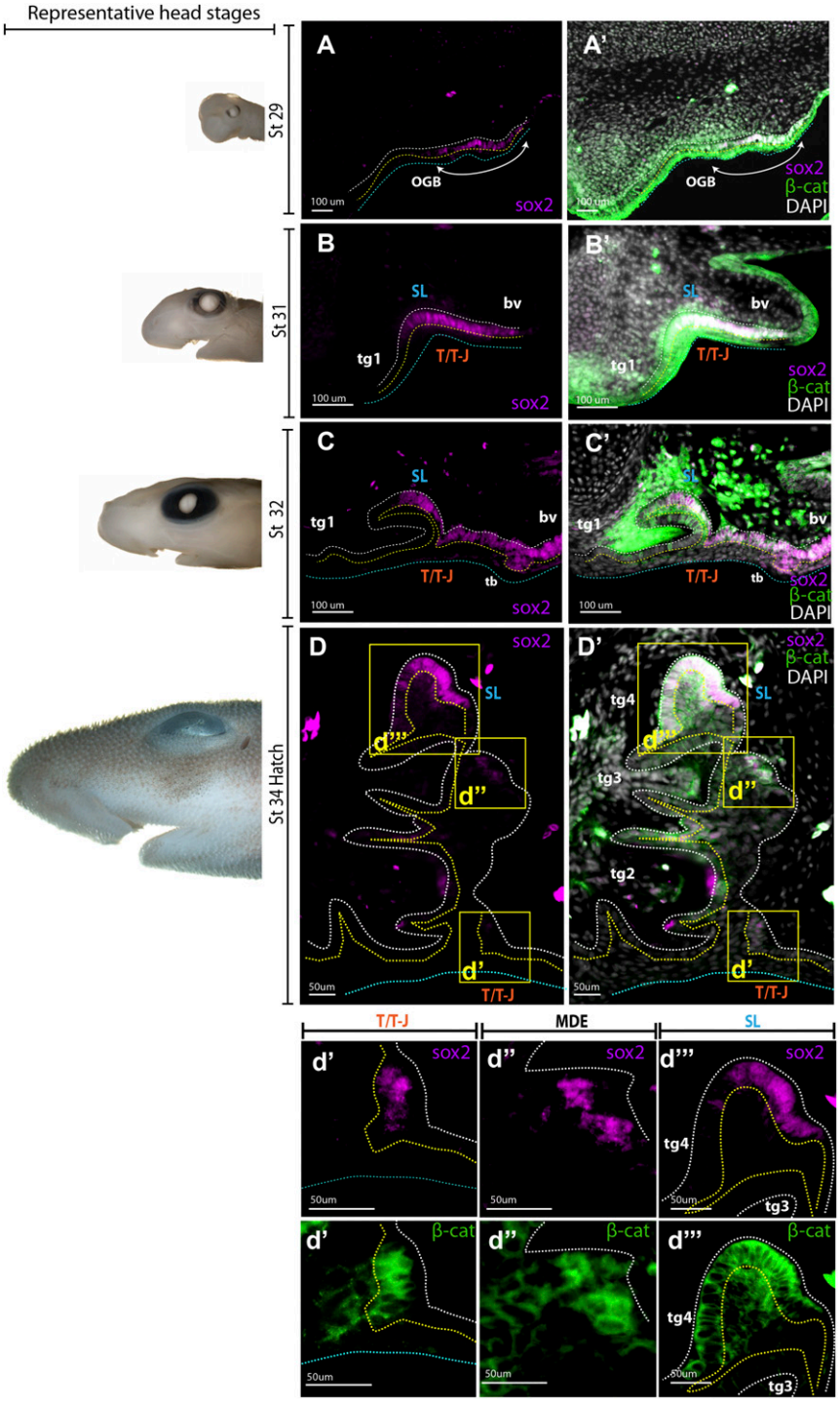


Fig. S3. Sox2 and β-catenin protein expression during development and regeneration of the shark upper jaw dentition. Representative developmental staging series of catshark heads (*Left*) and corresponding sox2 and β-catenin immunofluorescence in sagittal sections of the upper jaw dentition (*Right*; *A*–*D*). The development of the dentition in the upper jaw is slightly delayed compared with the lower jaw. Nevertheless, the dental lamina still progresses through OGB stage (*A*; stage 29), invagination/first tooth stage (*B*, stage 31), and tooth regeneration/second tooth stage (*C*, stage 32) and contains up to four distinct tooth generations in families at the symphysis at hatching (*D*, stage 34). Sox2 (magenta) and β-catenin (green) are expressed in the basal layer of the oral epithelium connecting the taste-bud field emerging from the OGB on the buccal valve (bv) with the taste/tooth junction (T/TJ) and successional lamina (SL) regions of the dental lamina during early development (*A*–*C*). By hatching at St 34, sox2 expression is restricted to the sites of the putative stem cell niches at the T/TJ (*D* and *d*′) and deep in the SL (*D* and *d*‴) in addition to within putative migratory cells of the squamous middle-dental epithelium (d′′, MDE) connecting the two permanent niches and is always associated with β-catenin expression.

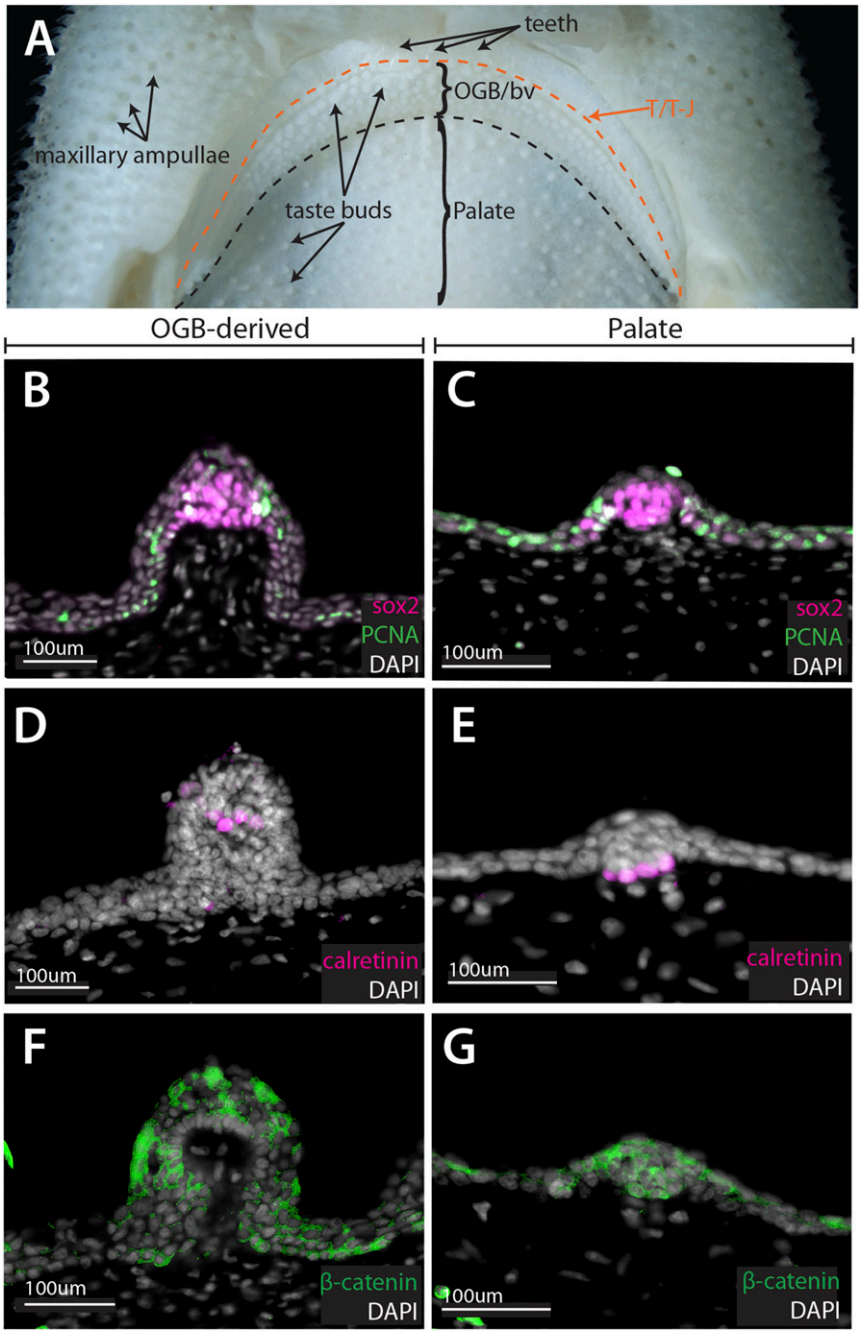


Fig. S4. Equivalent expression of immunofluorescent markers between OGB-derived and palatal taste buds. Distribution of taste buds in the upper jaw of a stage 34 (hatchling) catshark (*A*), revealing teeth erupting labial to the taste/tooth junction (T/TJ), OGB-derived taste-bud rows on the buccal valve (bv), and an array of smaller palatal taste buds. Expression of sox2 (magenta) and PCNA (green) is similar between OGB-derived (*B*) and palatal (*C*) taste buds, which both exhibit a central epithelial core of sox2+/PCNA− cells with a small population of sox2+/PCNA+ cells at its margins where the taste bud sits within the papilla. Calretinin (magenta) marks a relatively small subset of sox2+ cells at the basal epithelial boundary in both OGB-derived (*D*) and palatal (*E*) taste buds. β-catenin (green) is expressed throughout much of the epithelium of the papillae of both OGB-derived (*F*) and palatal (*G*) taste buds. Nuclear counterstain with DAPI shown in grayscale.

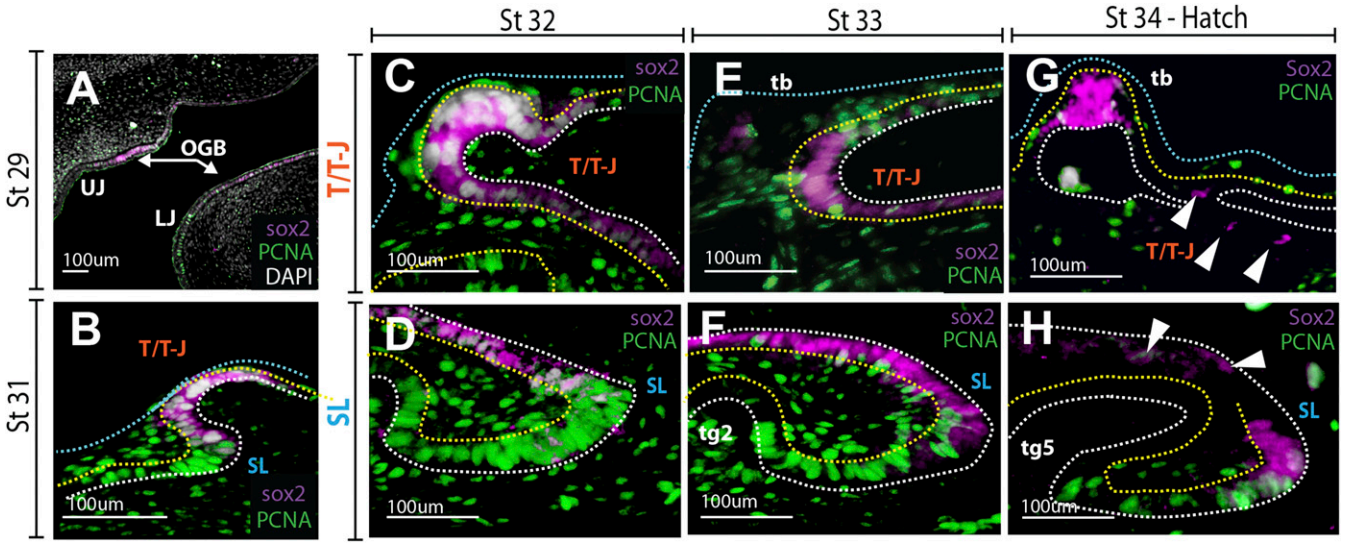


Fig. S5. Differential proliferation of sox2+ progenitors and putative stem cells during dental lamina (DL) development and niche establishment. Sox2 (magenta) and PCNA (green) immunofluorescence showing proliferating sox2+ progenitors coexpressing PCNA (white) during the early OGB (St 29; *A*), DL invagination (St 31; *B*), and early tooth regeneration (St 32; *C* and *D*). During advanced tooth regeneration (St 33; *E* and *F*) and in hatchlings (St 34; *G* and *H*) most sox2+ are devoid of PCNA, including apparently migratory cells in the middle dental epithelium (*H*, white arrowheads), suggesting a transition from proliferating progenitor to quiescent stem cell states.

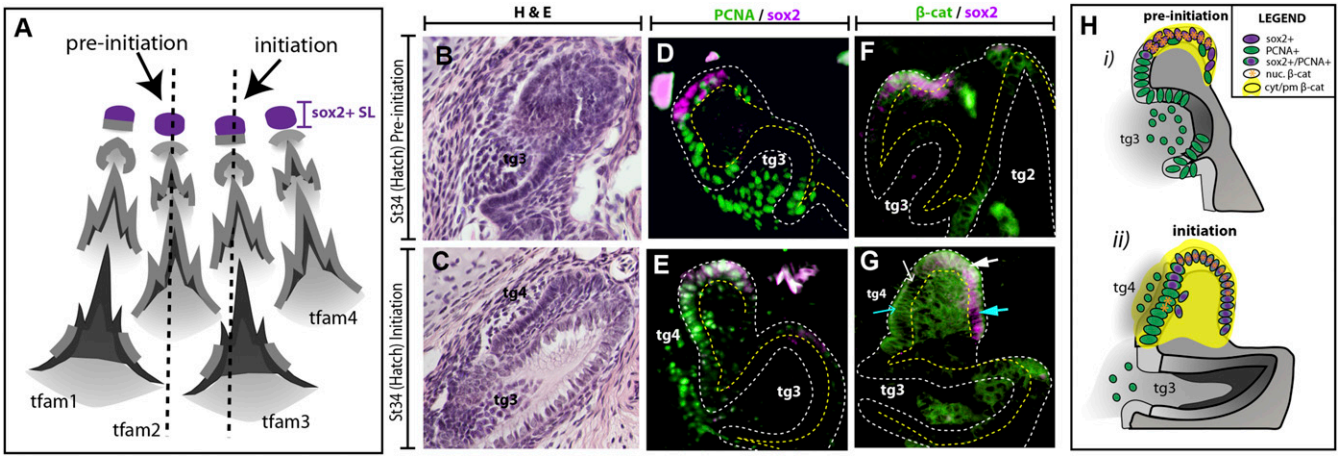


Fig. S6. Cyclical proliferation and expansion of β-catenin expression within the successional lamina. Schematic of adjacent tooth families in *S. canicula* (*A*). H & E histology from adjacent tooth families in the upper jaw of a hatchling catshark showing the SL during preinitiation (*B*) and initiation (*C*) stages of regeneration. PCNA (green) is not expressed in sox2+ cells (magenta) during preinitiation (*D*) but is coexpressed with sox2 during initiation (white cells) (*E*). β-catenin (green) is limited to sox2+ cells (magenta) during preinitiation (*F*) but expands throughout the SL and new tooth placode during initiation (*G*). Schematic representation of sox2, PCNA, and β-catenin expression during regeneration of adjacent tooth families at different stages (*H*).

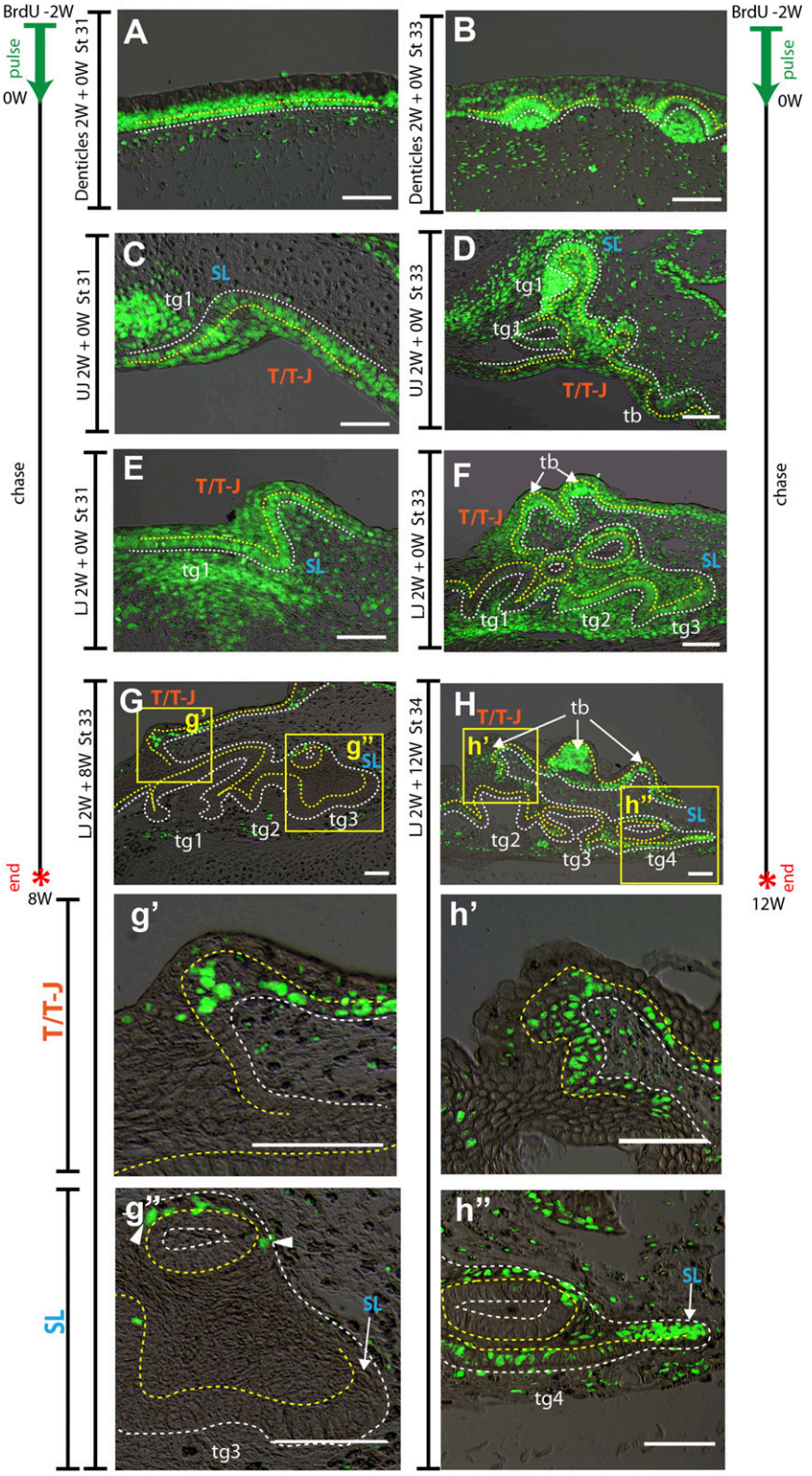
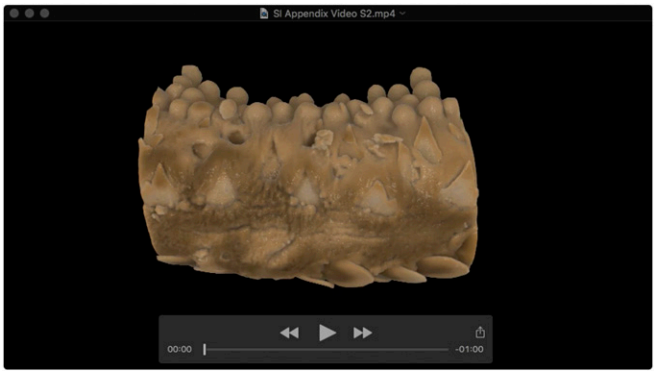


Fig. S7. Label-retaining cell assay (BrdU) in the developing dentition and skin denticles of the shark. Two pulse–chase BrdU experiments were initiated via saturation of tissues at stage 31 (*A*, *C*, and *E*) and stage 33 (*B*, *D*, and *F*) with the main data after the chase period presented in Fig. 4 of the main text. Skin saturation of BrdU (*A* and *B*) led to the results seen in Fig. 4, main text. Later-stage chase periods (8 wk in *G*, *g*′, and *g*′′, and 12 wk in *H*, *h*′, and *h*′′) diluted the label retention to cells with a slower cell cycle, and label was retained in the taste/tooth junction niche (T/TJ) in both the 8-wk (stage 31, *g*′) and 12-wk (stage 33, *h*′) experiments. However, LRCs were only detected in the successional lamina niche in the 12-wk chase experiment from the stage 33 (three-tooth–stage embryo) saturation pulse, suggesting that there is a shift in the pooling of slow-cycling cells from the T/TJ to the both the T/TJ and SL in later stage individuals and that during later stages of tooth regeneration the dentition in sharks might rely on a more localized (SL) niche for immediate and rapid regeneration, while still being fed from the T/TJ surface niche.

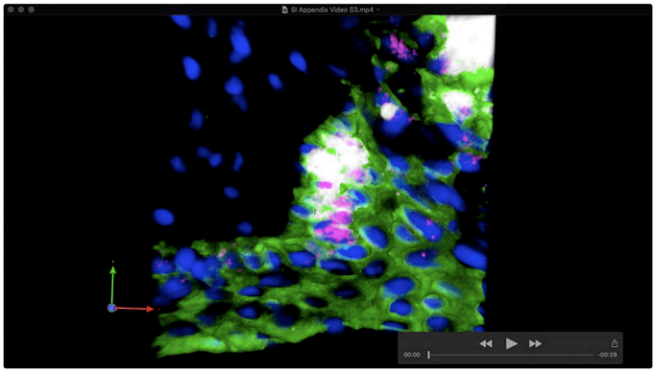
Movie S1. Small-spotted catshark, hatchling (103 mm) whole XCT scan rotation, showing the skin denticles covering the entire body.

Movie S1



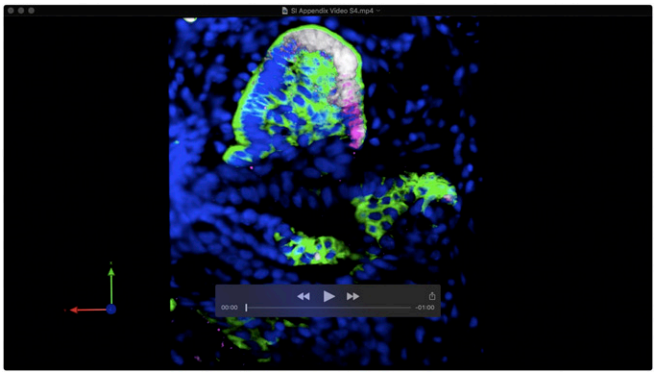
Movie S2. Soft-tissue contrast (PTA-stained) microCT scan of the hatchling catshark lower jaw segment. Large taste buds are present at the junction with the emerging teeth at the jaw margins. Skin denticles are also present on the outer surface of the segment.

Movie S2



Movie S3. Thin-section 3D epifluorescent opacity-rendering of z-stacked image of sox2 (magenta) and β-catenin (green) double immunofluorescence labeling the superficial (taste/tooth junction) dental stem cell niche in the catshark (*Scyliorhinus canicula*).

Movie S3



Movie S4. Thin-section 3D epifluorescent opacity-rendering of z-stacked image of sox2 (magenta) and β-catenin (green) double immunofluorescence labeling the deep successional lamina (SL) stem cell niche for de novo tooth regeneration in the catshark (*Scyliorhinus canicula*)

Movie S4