AN ANCESTRAL GENE NETWORK REGULATES CONTINUOUS TOOTH REGENERATION AND DENTICLE DEVELOPMENT IN ELASMOBRANCHS

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"The only way to obtain an explanation of animal diversity in form is by comparative analysis of developmental gene regulatory networks in suitably chosen species. As an experimental enterprise, this is an effort scarcely begun. But the door thus opens on a domain that is equally fruitful for development and evolution, indeed the domain where these subjects merge"

Professor Eric H. Davidson, 2006

THESIS SUMMARY

Sharks and rays (elasmobranchs) regenerate their teeth via a novel system in which multiple teeth develop in advance of function as a dental conveyor belt. Given their ancestral phylogenetic position, extant elasmobranchs therefore provide ideal models to study the ancient state of gnathostome tooth regeneration (polyphyodonty). Despite this potential, they have received little attention, leaving a significant gap in knowledge. The research presented here addresses this by using suitable species of catshark and ray as comparative models to investigate conserved gene expression during elasmobranch tooth development and regeneration.

In both sharks and rays, the conserved expression patterns of the dental stem cell marker *Sox2* first identifies a putative dental stem cell niche (SCN). Using the catshark as a primary model, PCNA is then used to define cell proliferation dynamics, followed by further investigation of the expression of genes representative of the Wnt-*β-catenin,* BMP, FGF and hedgehog signaling pathways. Their expression patterns, and those of additional regulatory genes, imply deeply conserved roles in the elasmobranch dentition. This is particularly apparent in tooth morphogenesis in which the expression of several genes identifies a putative signaling center comparable to the mammalian enamel knot. PCNA and gene expression further define a continuous epithelial connection between the dental and oral epithelium, adding further experimental evidence to support the role of a dental SCN in elasmobranch tooth regeneration. These studies inspire the production of the first hypothetical elasmobranch dental gene regulatory network (GRN) models.

This research further addresses the role of conserved genes in the development of dermal denticles, which in chondrichthyans enhance hydrodynamic efficiency and function as dermal body armour. These gene expression patterns imply similar regulatory roles to those in teeth, suggesting their evolution by a mechanism of gene network co-option. This further inspires the production of a denticle GRN model. When considered in light of existing theories of tooth and denticle evolution, this comparative expression data adds renewed perspective regarding their possible origins, as implied by their respective developmental similarities and differences.

Chapter 1

GENERAL INTRODUCTION

1.1 Teeth as evolutionary-developmental models

Evolutionary-developmental biology (evo-devo) seeks to show how alterations in development can lead to the morphological variation and novelty acted upon by natural selection (Gilbert and Epel, 2009; Wallace, 2011). Nowhere in nature is morphological diversity more apparent than in the dentitions of jawed vertebrates (gnathostomes). Given their crucial functional roles in food acquisition, processing, and defence, teeth have been repeatedly targeted and modified by natural selection during the course of gnathostome evolution. As a result, they have undergone extensive remodeling to produce diverse dental phenotypes, each exquisitely adapted to individual feeding requirements. In conjunction with jaws, teeth have therefore proven a major determinant in the success of gnathostomes, functioning at the forefront of feeding ability and defence (Smith, 2003; Fraser *et al*., 2008; Vonk *et al*., 2008).

One such commonality in several gnathostomes is the ability to renew or completely regenerate teeth (Harada *et al*., 1999; Wang *et al*., 2007; Fraser *et al*., 2013; Wu *et al*., 2013; Gaete and Tucker, 2013). In these respects teeth are remarkably unique, retaining the ability to continuously evolve, while preserving the capacity to develop and regenerate (Fraser *et al*., 2006a; 2006b; 2008; 2012; 2013). This is in part due to the modular nature of teeth, which while derived from the vertebrate exoskeleton, develop with relative autonomy. This modularity has therefore been proposed to be a major driving force in the intrinsic evolvability of teeth (Stock, 2001). From an evo-devo perspective, teeth are therefore ideal models, integrating key elements of development and regeneration with phenotypic plasticity and diversity (Smith *et al*., 2009a; Koussoulakou *et al*., 2009; Fraser *et al*., 2008; Pfennig and Ehrenreich, 2014; Johanson and Smith, 2005).

1.1.1 Current theories regarding the origins and evolution of teeth

Teeth comprise hard-tissue units, incorporating dentine and often a superficial layer of enamel or enameloid, surrounding a central pulp cavity attached to a laminar base of bone or cartilage (Ørvig, 1967; 1977). This is illustrated in the early adult teeth of

Scyliorhinus canicula (small-spotted catshark) (Fig. 1.1A), which incorporate these basic structural elements. As a consequence of their mineralised composition, teeth are highly preserved throughout the fossil record, where they have served as vital diagnostic tools for identifying important vertebrate morphologies and phylogenies (Smith and Hall, 1990; Donoghue and Sansom, 2002; Sire and Huysseune, 2003; Johanson and Smith, 2005; Rücklin *et al*., 2012). However, this has been complicated by the associated preservation of dermal denticles (placoid scales), mineralised structures found in the integument of extant elasmobranchs, which function to enhance hydrodynamic efficiency and act as dermal body armour (Ørvig, 1967; 1977; Schaeffer, 1977; Reif, 1982). This is further illustrated in the denticles of the catshark (Fig. 1.1B), which share considerable compositional and structural homology with teeth (collectively termed odontodes). This shared homology has therefore presented a challenge when attempting to construct an accurate picture of their respective evolutionary origins, leading to several theories which continue to generate considerable debate (Ørvig, 1967; Reif, 1982; reviewed by Huysseune *et al.,* 2009; Smith and Coates, 1998; Fraser *et al*., 2010; Donoghue and Rücklin, 2014).

The classical 'outside-in' model proposes oral teeth to have evolved at around the same time as jaws, by in-folding into the mouth of an external odontode-competent ectodermal epithelium (Reif, 1982; Jolie, 1968; Schaeffer, 1977; Romer, 1936). This model therefore considers oral odontodes to be functional derivatives of modified dermal denticles, co-opted to function as teeth through mixing of ectodermal and endodermal tissues within the oral cavity. The tenets of this model are largely based upon the anatomical resemblance of teeth and dermal denticles, and their respective proximities at the endo-ectodermal boundary of the mouth opening, despite no current evidence of a transition or grading existing between the two (Fig. 1.1C) (Smith and Coates, 2000; Smith, 2003). The recently modified 'outside-in' model builds upon the original by suggesting the developmental potential to develop teeth to have occurred as a consequence of direct cellular mixing of external ecto and endodermal tissues via both the mouth and gill slits (Huysseune *et al*., 2009). These hypotheses are reliant upon phylogenetic events inferred from fossil evidence and in the case of the latter, conclusions drawn from observations of endo-ectodermal tissue

morphodynamics during vertebrate embryological development (Soukup *et al*., 2008; Graveson *et al*., 1997; Linden, 1929; Sellman, 1946; Wilde, 1955).

These theories are challenged by the 'inside-out' model, which proposes teeth to have first evolved in the endodermal pharynx of jawless vertebrates (agnathans), in advance of the advent of jaws. In this scenario, the ability to develop oral teeth is believed to have occurred following anterior transfer of developmental potential to the oral region by recruitment of a gene network deployed to pattern pharyngeal denticles. The 'inside-out' model advocates the view that teeth and dermal denticles evolved independently from separate endo and ectodermal origins, respectively, with the unique patterning potential required to make teeth, restricted to the endodermal tissues of the oro-pharynx (Smith and Coates, 1998; 2000; 2001). This model draws support from existing studies of tooth development, which show similar sets of genes to be expressed in both the oral teeth of mammals and pharyngeal teeth of fish (Wise and Stock, 2006; Borday-Birraux *et al*., 2006; Jackman *et al*., 2004).

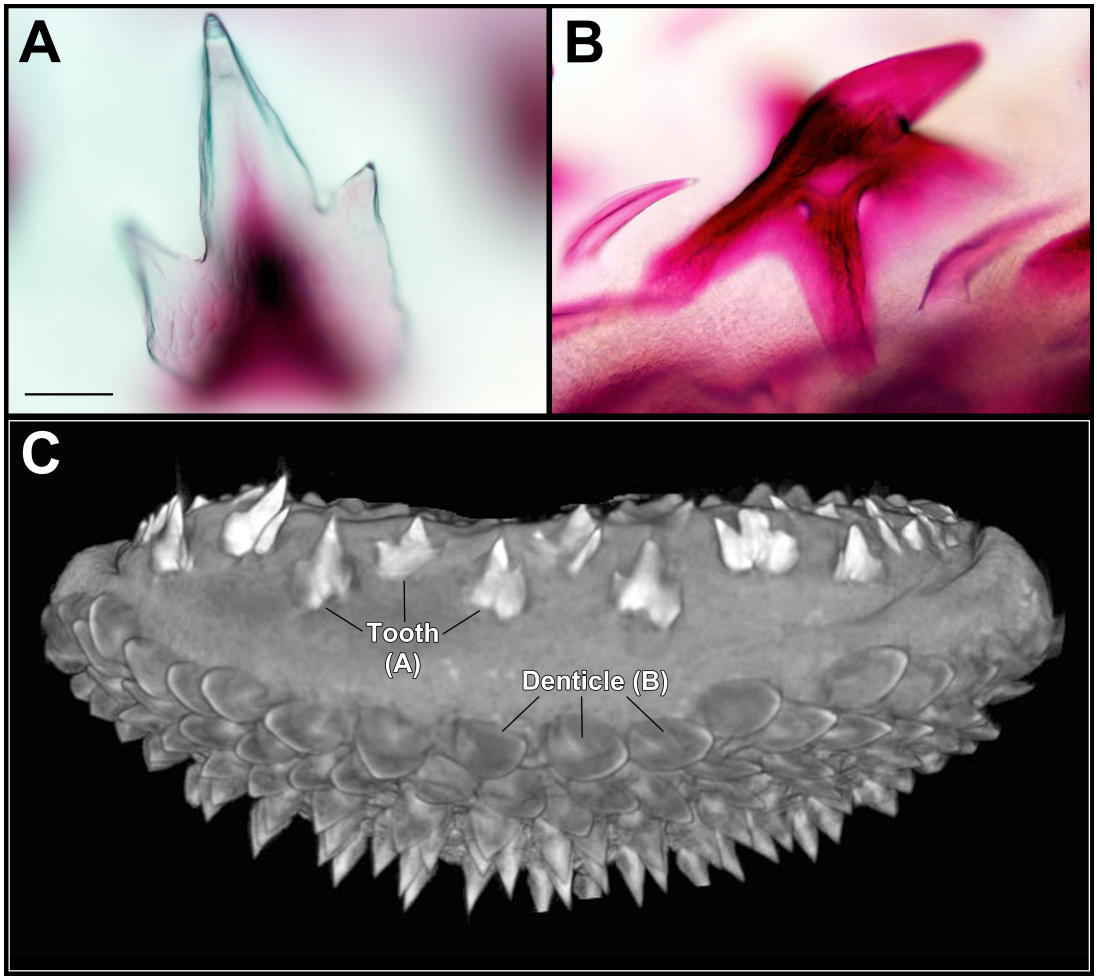


Figure 1.1 Odontogenic diversity in sharks. The teeth (A) and dermal denticles (B) in *Scyliorhinus canicula* (small-spotted catshark) illustrate the basic structural components of odontodes. Both constitute hard-tissue units, comprising an outer layer of enameloid (or enamel) and an inner layer of dentine surrounding a central pulp cavity attached to a laminar base of bone or cartilage (Ørvig, 1967; 1977). Micro-CT imaging of the lower jaw dentition (labial view) of the catshark shows development of teeth (A) within the internal oral cavity and dermal denticles (B) from the external integumentary surface (ectoderm). The significant degree of anatomical and developmental homology shared between both has resulted in several models proposing alternative scenarios of their evolution. One such model, the 'outside-in' theory, advocates the evolution of teeth from in-folding into the oral cavity of an external odontode-competent ectoderm. In this scenario, teeth are therefore viewed as functional derivatives of denticles (Reif, 1982). However, in the absence of any evidence showing teeth and denticles to grade into one another at the oral margin (as shown above), this theory remains largely unsubstantiated and open to challenge. Scale bar: (A-B) 100 μm. Specimen prepared within the University of Sheffield and micro-CT carried out by Brian Metscher, University of Vienna.

This ongoing debate has recently been revitalised by Donoghue and Rücklin (2014), who have challenged the 'inside-out' model, while advocating the 'outside-in' model, following reinterpretation of a combination of developmental and fossil evidence (Donoghue and Rücklin, 2014). Developmental evidence is drawn from studies showing teeth to develop from endoderm, dermis (ectoderm) and a mixture of both epithelia, therefore reducing distinctions between the two (Soukup *et al*., 2008; reviewed by Donoghue and Rücklin, 2014). Supporting fossil evidence is drawn from reanalysis of conodont elements, which are considered to have evolved independently of teeth and denticles, and tooth-like replacement in thelodonts, also considered an independent occurrence of teeth and tooth replacement, which evolved later in stem group gnathostomes. Phylogenetic evidence comes from fossil analysis of several distinct lineages, implying the appearance of odontodes in the dermal skeleton and their inward extension via the oral, nasal and pharyngeal orifices (Donoghue and Rücklin, 2014).

Fraser et al. (2010) also recently proposed the 'inside-and-out' model, which postulates that odontodes can potentially develop from any epithelium (endo or ectoderm), which collaborates with a neural crest-derived mesenchyme. Since existing studies have shown these two interacting tissue layers to contain all the molecules required for teeth to develop, the 'inside and out' model aims to progress current thinking by shifting the emphasis toward the role of gene networks in odontode evolution, rather than inferences drawn from an ambiguous mixture of fossils and extant specimens. In this scenario it is hypothesised that all odontodes share a deep molecular homology, united by their common derivation from an antecedent epithelial sensory receptor, with odontogenic potential acquired only following its collaboration with neural crest-derived mesenchymal tissues (Fraser *et al*., 2010).

1.1.2 The evolutionary origins of teeth in ancient fishes

Despite this on-going debate, current evidence suggests the first teeth to have evolved in conodonts ("cone teeth"), an extinct group of early jawless (agnathan)

eel-like fish prevalent during the Cambrian and Devonian periods. Conodonts lacked scales, but possessed patterned tooth-like protuberances in the oro-pharyngeal cavity, termed conodont elements. These share compositional similarities with the teeth of recently evolved gnathostomes, comprising phosphatic hard tissue containing dentine and lamellar crown tissue similar to enamel (Donoghue and Sansom, 2002; Donoghue *et al.,* 2006; Purnell, 1995). These synapomorphies have therefore led to their proposed classification as early vertebrates (Donoghue *et al*., 2000). However, this remains a contentious issue. While the presence of enamel, bone and dentin is indicative of an affinity with vertebrates, histochemical evidence suggests these hard tissues not to be truly homologous (reviewed by Aldridge and Purnell, 1996. Furthermore, interpretation of Cambrian conodont fossils suggests an affinity with chaetognaths, a group ancestral to euconodonts and therefore also to vertebrates (reviewed by Donoghue *et al*., 2000).

The existence of other agnathan groups often prevalent during similar periods, and presenting evidence of odontodes, has further complicated matters. For example, the heterostracans, agnathans lacking teeth, but possessing bony armoured plates covered in dermal denticles, underwent a major radiation during the Silurian and Devonian periods (Halstead, 1973; Blieck, 1984; Smith and Hall, 1990). Conversely, the agnathan thelodonts, which radiated during the Silurian and Devonian periods, possessed skin denticles and patterned oro-pharyngeal tooth whorls similar to those in extant elasmobranchs (e.g. *Loganellia scotia*) (der Brugghen and Janvier, 1993; Smith and Coates, 1998).

The armour-plated osteostracans also underwent a major radiation during the Silurian and Devonian. These agnathans possessed ordered scales ornamented on their dermal armour, but were lacking in oro-pharyngeal denticles (Reif, 1982; Donoghue and Sansom, 2002; Sire *et al*., 2009). The armour-plated placoderms are the first known jawed vertebrates, phylogenetically positioned at the basal node of the gnathostome lineage, after the osteostracans but before the first sharks, during the Silurian and Devonian periods (Young, 1986; Janvier, 1996; 2001). Many Placoderm taxa possessed both internal and external odontodes, with those positioned in the rear of the gill chamber showing marked signs of organisation compared with those on the

external trunkshield. Crucially, those positioned internally showed evidence of similarities to those of *Loganellia*, implying a deeply conserved tooth patterning mechanism in early vertebrates (Johanson and Smith, 2003; der Brugghen and Janvier, 1993).

1.1.3 The evolution of chondrichthyans and the rise of jaws

The chondrichthyans (sharks, rays and holocephalans) are an enigmatic and diverse group of gnathostomes, characterised by their flexible skeletons predominantly comprising prismatic calcified cartilage and lacking in endochondral bone (Moss, 1977). Today, sharks represent some of the apex marine predators, with an evolutionary history spanning some 450-500 million years. During this time, they have survived four mass extinctions and undergone two major modifications to give rise to the holocephalans (chimaerids and rabbitfishes) and batomorphs (flattened rays). Despite these divergences, the chondrichthyan bodyplan has remained relatively unchanged, making extant forms representative of the ancient state of gnathostomes (Compagno, 1990; Long, 2011; Klimley, 2013).

The fossilised teeth, denticles and in exceptional circumstances, full dermal skeletons, left behind by extinct chondrichthyans have proven instrumental in reconstructing their morphological characteristics and phylogenetic history. Chondrichthyan odontodes are composed of dentinous tissues (typically semidentin and orthodentin), however, remnants of perichondral bone found in primitive sharks, such as *Akmonistion*, implies that they may once have had the potential to develop bone, but lost this during the course of their evolution (Donoghue *et al*., 2006; Coates and Sequeira, 2001). Some fossil evidence suggests early sharks to have diverged from placoderms; however, similarities in denticle morphology with some agnathans also implies a possible relationship with thelodonts (Johanson and Smith, 2005). Despite this, the first articulated sharks are believed to have evolved during the Early Devonian Period, as suggested by fossils with shark-like features, such as the presence of prismatic calcified cartilage (e.g. *Doliodus problematicus*) (Miller *et al.,* 2003). Most groups of Devonian sharks are represented primarily by their various tooth morphologies. For example, *Portalodus bradshawae* possessed large,

bicuspid teeth, whereas *Mcmurdodus whitei*, the earliest known relative of the neoselachians (modern sharks), possessed complex multicuspid teeth capped with an enameloid crown and a root structure diagnostic of modern sharks (Long and Young, 1995; Turner and Young, 1987).

During the Late Devonian, these early sharks further diversified to several new groups, such as the Cladoselachiformes, so named because of their multicuspid cladodont teeth (Gillis and Donoghue, 2007; Klimley, 2013). Cladoselache also presented features common to primitive sharks of the Devonian and Early Carboniferous Periods, such as a terminally orientated mouth (compared to the subterminal mouths of modern sharks). The upper jaw (palatoquadrate) was also firmly attached to the braincase via ligaments, limiting the extent of jaw gape and therefore prey item choice, a characteristic further apparent in members of the Order Xenocanthiformes (Fig. 1.2A-B) (Klimley, 2013). During the Devonian and Permian Periods, sharks continued to diversity, producing some peculiar morphological features. The cladodont, *Stethacanthus altonensis*, developed a dorsal baseplate of enlarged dermal denticles ('brush complex') comprising globular calcified cartilage, supported by a modified dorsal fin (Ørvig, 1951; Coates and Sequeira, 2001). The highly successful hybodontiformes diversified during the Triassic to survive into the Late Cretaceous, alongside the neoselachians. The presence of sharp multicuspid and flat-plated teeth within this order implies a variable diet of fishes and hard-shelled prey, yet as with other primitive sharks, the upper jaw remained attached to the braincase (e.g. *Hybodus*) (Fig. 1.2C) (Reif, 1978; Benton, 2005; Klimley, 2013).

During the Jurassic and Cretaceous Periods, the neoselachians further diversified, giving rise to the major groups of extant sharks and rays (Nelson, 2006). Several innovations conferred significant advantages over the selachians, notably an increasingly mobile set of jaws. The upper jaw, previously fused to the braincase, was now attached to the chondrocranium via a modified ligament system, while the lower jaw became indirectly attached via the hyomandibula, a skeletal element derived from the first gill arch. These modifications allowed the upper jaw the freedom to move up and down relative to the braincase, while enabling the lower jaw to slide back and forward. This combination of increased jaw gape and horizontal

protrusion and retraction conferred the ability to consume larger prey and to gouge pieces of tissue from prey too large to consume whole (Fig. 1.2D). In modern shark lineages, teeth became further modified to enable increasingly efficient sawing of tissue. This is illustrated in the adult teeth (lower jaw) of the bull shark (*Carcharhinus leucas*), which incorporate multiple serrations (Fig. 1.2E) (Kriwet *et al.,* 2009; Klimley, 2013).

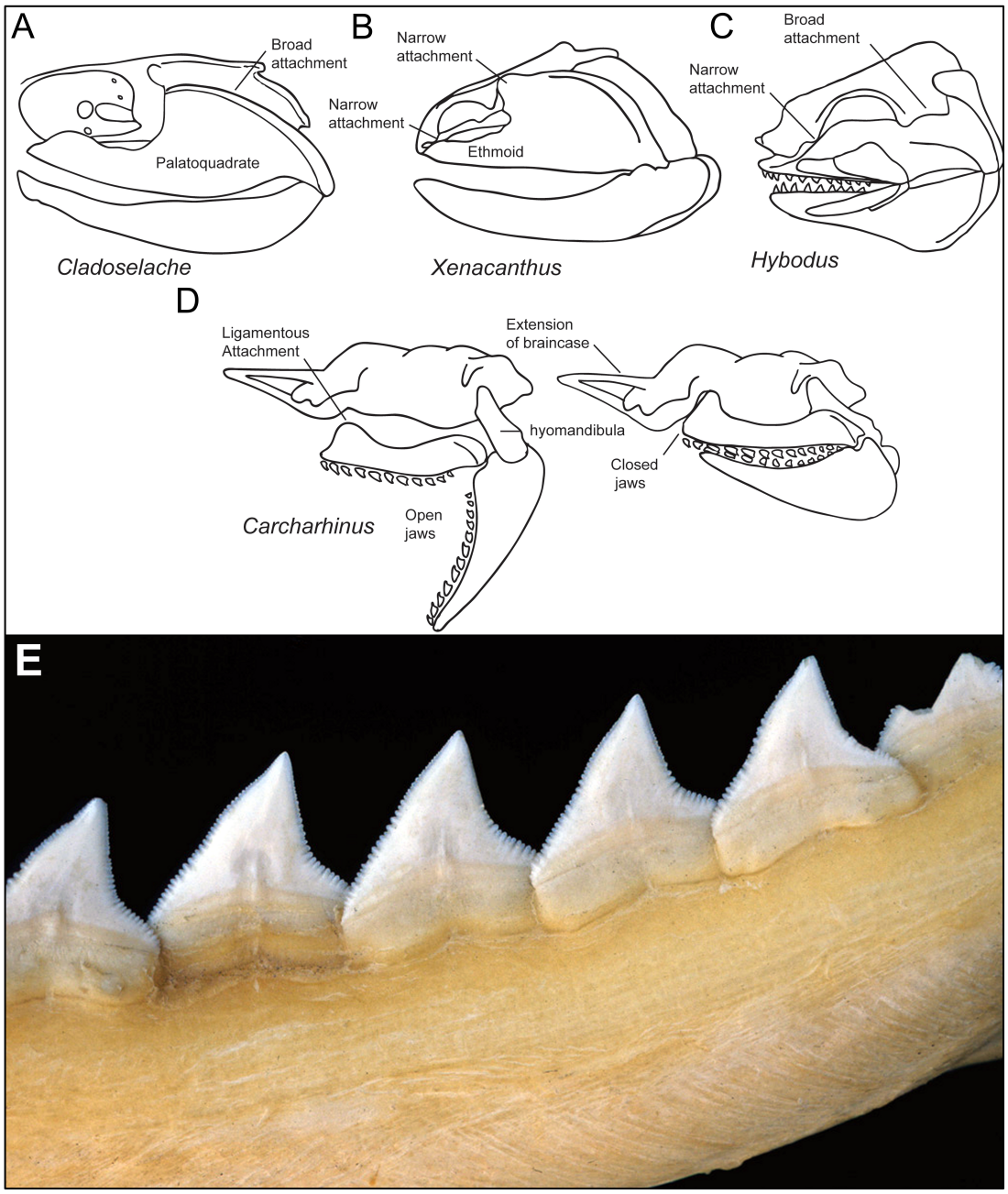


Figure 1.2 The evolution of jaw and tooth morphology in chondrichthyans. Early sharks, such as *Cladoselache* (A), *Xenacanthus* (B) and *Hybodus* (C), exhibit primitive jaw morphology, marked by a terminally-orientated mouth and close attachment of the upper jaw (palatoquadrate) to the braincase, limiting the extent of jaw gape and therefore, prey item choice. In the subsequently evolved neoselachii (modern sharks), several functional innovations conferred considerable predatory advantage. In members of the *Carchariniformes* (ground sharks), this is marked by an increasingly mobile set of subterminal jaws; the upper attached to the braincase by a modified ligament system, and the lower indirectly, via the hyomandibula (D). This detachment of the jaw apparatus from the braincase allowed for greater articulation, increasing vertical gape, while allowing horizontal freedom of movement. In neoselachians, additional modifications in tooth shape further enhanced their ability to saw through tissue. This is apparent in the lower jaw

dentition of the bullshark (*Carcharhinus leucas*), which incorporates multiple serrations (E). These modifications therefore conferred both the ability to consume larger prey items, and gouge out and saw through pieces of tissue too large to consume whole. A-D modified from Klimley, 2013 and E from Norbert Wu Productions (http://www.norbertwu.com).

As a result of these, and additional modifications, the modern sharks continued to radiate and diversify, giving rise to all known extant groups (Fig. 1.3). The largest group, the galeomorphs, comprises 250 extant species, including the heterodontiformes (e.g. bullhead sharks), orectolobiformes (e.g. whale sharks), Laminiformes (e.g. great whites) and carchariniformes (e.g. hammerheads). Remaining groups include the hexaniformes (e.g. frilled and cow sharks), squaliformes (e.g. spiny dogfish), squatiniformes (e.g. angel shark) and the batoids (skates and rays). The suborder batoidea are specifically adapted to bottom feeding, reflected by their compacted bodies, enlarged pectoral fins and ventrally orientated jaws housing flattened, pavement-like teeth specialised for crushing hard-shelled prey (e.g. eagle rays) (Compagno, 1990; Benton, 2005; Klimley, 2013; Summers, 2000). The holocephalomorphs diverged from shark-like ancestors during the Devonian to form their own subclass, which includes the living holocephalans (e.g. chimaerids, elephant sharks and rabbitfish) (Coates and Sequeira, 2001). Extant holocephalans differ to other chondrichthyans in their operculum covering the gill arches, but retain some features common to primitive sharks, notably fusion of the palatoquadrate to the braincase, which combined with their broad tooth plates forms a powerful crushing plate dentition. This specialisation is reflected by their lifestyle as bottom-dwellers, where they feed on hard-shelled prey, such as molluscs and crustaceans (Benton, 2005; Klimley, 2013). The broader extent of their diverse feeding adaptations is further exemplified by recent reclassification and inclusion in this clade, of the extinct yet enigmatic genus *Helicoprion*, characterised by its unique spiral tooth whorl (Tapanila *et al*., 2013).

The evolution of the highly successful osteichthyan (bony) fishes has also generated considerable phenotypic diversity, exemplified by the teleosts, which have undergone four major radiations to constitute 96% of all extant living fish (Bone and Moore, 2008). However, despite this, it remains apparent that primitive chondrichthyans have proven instrumental in establishing the basic bodyplan required for the success of subsequent gnathostomes. This is largely due to the evolution of highly mobile jaws, which combined with numerous modified dental phenotypes, has led to their current status as apex marine predators (Klimley, 2013).

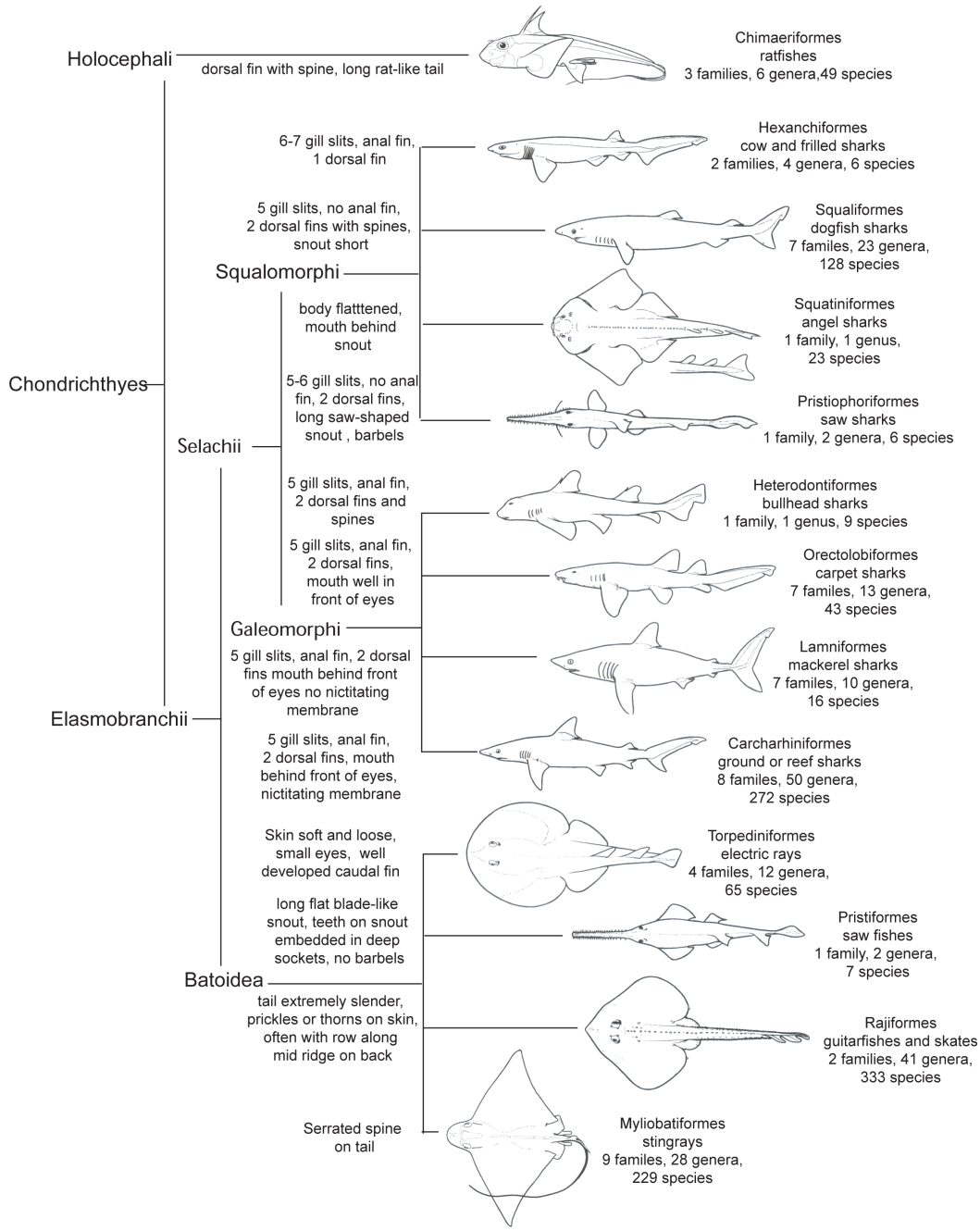


Figure 1.3 Phylogenetic groups and diagnostic features of extant chondrichthyans. These comprise the subclasses elasmobranchii and batoidea (sharks and rays) and holocephali (chimaeriformes). The sharks (selachii) form two major subdivisions (squalomorphi and galeomorphi), with dental phenotypes marked by pointed teeth specialised for piercing soft prey (Klimley, 2013; Compagno, 1990). The highly derived rays and skates (batoidea), form two morphologically distinct orders, defined by their dorso-ventrally flattened body shapes and flattened tooth plates, specialised for crushing hard-shelled prey (durophagy) (Summers, 2000). The subclass holocephali (rat and rabbitfishes) are so named because of their rodent-like tooth plates, also specialised for durophagy (Didier *et al*., 1994). These highly successful dentoskeletal ecomorphotypes, each derived from the same versatile cartilaginous dermal skeleton, highlights chondrichthyans as exemplars of evolutionary plasticity and adaptation. Figure from Klimley, 2013.

1.2 Tooth development and regeneration

1.2.1 Historical perspectives of tooth patterning

Prior observations of the dentition as a well-ordered series of elements led to the early concept of patterning through morphogenetic fields in advance of any knowledge of the molecular regulation of tooth development. Butler (1939) first proposed the putative role of morphogenetic fields in determining the development of teeth as a meristic series. This 'field' theory postulated morphological similarities in neighbouring teeth to result from the local effects of a common developmental field, with regional variations occurring in a field gradient-dependent manner. Butler therefore proposed field gradients to be critical determinants of corresponding morphological gradients, relating these to tooth size, shape and complexity, relative to their position within the gradient itself (Butler, 1939; 1956). Butler further proposed tooth number and shape to result from an imposed signaling mechanism and advocated the evolution of teeth as an integrated system rather than as independent units (Butler, 1963).

Osborn (1978) subsequently proposed his 'clone' theory, in which the patterning of teeth in a particular class resulted from a single clone of pre-programmed cells; for example, a molar clone of cells to initiate molar development. He also introduced the concept of zones of inhibition in which clones of cells developed in accordance with set boundaries, preventing the initiation of new teeth until the existing clone had progressed beyond a set threshold stage of development. Despite Osborn's "fields versus clones" title, both are now considered complementary, with teeth proposed to develop from an integrated reaction and diffusion system in which activators induce placode formation, while inhibitors regulate tooth spacing within the inter-placoidal regions to maintain their development as ordered arrays (Osborn, 1978; Pispa and Thesleff, 2003; Mitsiadis and Smith, 2006).

In the shark dentition, teeth are initiated at specific time differentials to develop in close proximity, while maintaining strict spatial boundaries with neighbouring teeth (Smith *et al*., 2009a). This is apparent in both extinct and extant sharks, such as *Akmonistion* and the frill shark (Chlamydoselachus) in which ordered tooth families

are widely spaced apart (Smith, 2003). In accordance with some of the tenets set by the field and clone theories, investigation of dental pattern morphology in sharks (e.g. Heterodontus) has led to the supposition that the arrangement of teeth as individual families results from positional cues, such as putative fields of inhibition, allowing teeth to develop in certain loci, while preventing their induction in positions already occupied (Reif, 1976; 1984). In light of contemporary understanding of development, positional cues equate to pattern formation, a fundamental process by which cells acquire specific identities to generate increasingly complex levels of organisation (Wolpert, 2007).

1.2.2 The evolutionary significance of the dental lamina

The dental lamina develops as an in-fold of the oral epithelium into the oral mucosa to form the specialised compartment within which teeth develop (Reif, 1980; 1982). In most gnathostomes, the dental lamina is required for tooth development (Fraser *et al*., 2004; 2006a; Smith *et al*., 2009a; 2009b; Buchtová *et al*., 2008; Järvinen *et al*., 2009; Smith, 2003). This is, however, with the exception of some gnathostomes in which embryonic first-generation develop in its the absence. For example, in some alligators, such as *Alligator mississipiensis*, early first generation teeth initiate from the oral epithelium, developing as rudimentary, degenerative structures in advance of dental lamina formation. These are subsequently replaced before or shortly after birth by functional teeth formed within a dental lamina (reviewed by Sire *et al*., 2002; Westergaard, 1986; Westergaard and Ferguson, 1987; 1990).

However, despite such exceptions, the appearance of the dental lamina is likely to have been an essential prerequisite for the evolution and subsequent diversification of teeth (Reif, 1982; Smith *et al*., 2009b). It has previously been proposed that teeth evolved at around the same time the brachial arches become modified to function as dorso-ventrally opposing jaws. In this scenario, dermal denticles lining the oral margin were recruited to function as teeth in collaboration with jaws (the ‘outside-in’ model) (Reif, 1982). However, reconsideration of agnathan fossil taxa containing evidence of oro-pharyngeal teeth (or tooth-like elements), such as those summarised in Section 1.1.2, challenge this view. This therefore suggests an alternative scenario

in which teeth were already present some 50 million years before the advent of jaws ('inside-out' model) (Smith and Coates, 1998). Many of these taxa also show evidence of a common tooth patterning mechanism. In the agnathan *Loganellia*, pharyngeal denticles are arranged as patterned whorls with associated evidence of sequential addition and directionality (der Brugghen and Janvier, 1993; Smith and Coates, 2001). The pharyngeal denticles of some placoderms are also arranged as patterned rows, which differ to the dermal tubercles covering the external surface of the trunk shield (Johanson and Smith, 2003).

Early chondrichthyans also present evidence of ordered tooth patterning; exemplified by the specialised tooth whorls of the Euchondrocephalan *Helicoprion*, a self-evident example of a well-ordered tooth replacement system (Tapanila *et al*., 2013). The primitive shark *Akmonistion zangerli* possesses pharyngeal joined denticles, while the oral teeth of *Doliodus* are positioned to suggest a commonly deployed patterning mechanism, likely the product of an early dental lamina, proposed by Reif to be universal to all gnathostomes (Smith, 2003; Miller *et al.,* 2003; Reif, 1982). Given the fossil evidence of patterned odontodes in the oro-pharynx of several agnathans, it is possible, at least in these groups, that a modular forerunner to the dental lamina is deeply conserved in vertebrates. While at a basal level, this developmental module is likely homologous in its odontogenic potential, it is possible that its derivatives may have evolved independently in different stem and crown gnathostome groups to produce their respective dental patterning mechanisms (Smith, 2003; Smith and Johanson, 2003; Johanson and Smith, 2005).

1.2.3 Teeth develop from a collaborative epithelium and mesenchyme

Teeth, like other epithelial appendages, such as the hair follicle, feather bud and scales, develop from two adjacent tissue layers: the epithelium and underlying neural crest-derived ectomesenchyme. These epithelial derivatives share a common series of early developmental stages, resulting from sequential, reciprocal signaling interactions between both cell layers (Pispa and Thesleff, 2003; Thesleff and Sharpe, 1997; Jervall and Thesleff, 2000; Tucker and Sharpe, 2004). The importance of these collaborating tissue layers in tooth development has been shown in rodents in which

in vitro expansion of combined epithelial-mesenchymal cell populations produces early tooth primordia. These 'toothlets' can then be transplanted into the mouth to form a whole tooth (Yamamoto *et al*., 2003; Duailibi *et al*., 2004; Nakao *et al*., 2007; Ikeda *et al*., 2009).

While this patterning process begins in the epithelium, the role of the neural crest-derived ectomesenchyme is of equal importance. The evolution of teeth is believed to be linked to the appearance in early vertebrates of the neural crest (NC), a transient cell population originating from the neuro-ectoderm of the early neural tube, which undergo an epithelial to mesenchymal transition (Smith and Hall, 1993; Gans and Northcutt, 1983). During early embryogenesis, ectomesenchymal NC cells migrate along the anterior-posterior axis of the neural tube toward the head, where during craniofacial development they colonise the face to produce several patterned elements, including the branchial arches from which the upper (maxillary) and lower (mandibular) jaws develop (Miletich and Sharpe, 2003). As a multipotent mesenchymal cell population, cranial NC cells therefore have the potential to differentiate to several cell types, most notably those that produce hard tissues, such as cartilage, bone and dentin, the principle constituent of teeth. This is evident in higher vertebrates, in which many of the dermal bones of the skull and oro-pharynx are NC-derived. The importance of the NC for vertebrate evolution and development is therefore apparent (Couly *et al.,* 1993; Jiang *et al.,* 2002). While the early emergence of the NC is subject to ongoing debate, its key role as a vertebrate-specific synapomorphy is further apparent, as shown in extant lampreys, where the cranial and pharyngeal cartilages are NC-derived (Miletich and Sharpe, 2003; Langille and Hall, 1988; McCauley and Bronner-Fraser, 2003). In fossil agnathans, dermal armour and teeth containing enameloid, dentine and dermal bone presents further evidence to support the role of a rapidly emerging NC-derived ectomesenchyme in the provision of cell types required to produce early odontogenic innovation (Donoghue and Sansom, 2002; Smith and Hall, 1990).

1.2.4 Common stages of tooth development

Despite ongoing debate regarding the evolutionary origins of teeth, much is known about their development, the majority of which has come from studies of mammalian models (Järvinen *et al*., 2006; Jussila *et al*., 2014). The mouse dentition in particular has provided the principle model for tooth development, from which a common series of stages can be defined (Peters and Balling, 1999). The first morphological sign of tooth development (odontogenesis) is marked by localised thickening of the oral epithelium, which invaginates into the underlying neural crest-derived mesenchyme, forming the primary dental lamina (Fig. 1.4) (reviewed by Tucker and Sharpe, 2004). The epithelium further proliferates into the mesenchyme, which responds by condensing around the epithelium, forming a tooth bud (Pispa and Thesleff, 2003; Tummers and Thesleff, 2008). Cap and bell stages (morphogenesis) are marked by continued inward extension of the epithelium into the mesenchyme and by the activation of a transient signaling center within the epithelial tip, termed the enamel knot. This signaling center determines future tooth cusp morphology by instructing surrounding cells to differentiate to the required cell types before apoptosing, following completion of function (Jernvall and Thesleff, 1994; Vaahtokari *et al*., 1996a; 1996b Jernvall *et al*., 1998). During cap and bell stages, the lateral epithelium of the tooth bud begins to encase the underlying mesenchyme to form the cervical loop. In gnathostomes incapable of tooth renewal or replacement, roots develop from the cervical loop to form Hertwig's epithelial root sheath (HERS), which directs root growth and has limited proliferative potential (Jernvall and Thesleff, 2012). Late bell stage (advanced morphogenesis) is marked by complete enclosure of the dental mesenchyme within the invaginating dental epithelium, accompanied by cytodifferentiation during which epithelial cells differentiate to enamel-secreting enameloblasts and mesenchymal cells to dentin-secreting odontoblasts. Following secretion of these hard tissue matrices and root formation, the tooth erupts into the oral cavity (Tucker and Sharpe, 2004). Tooth initiation, morphogenesis and differentiation are therefore regulated by sustained sequential, reciprocal signaling interactions between this collaborative epithelium and neural crest-derived ectomesenchyme (Tummers and Thesleff, 2009).

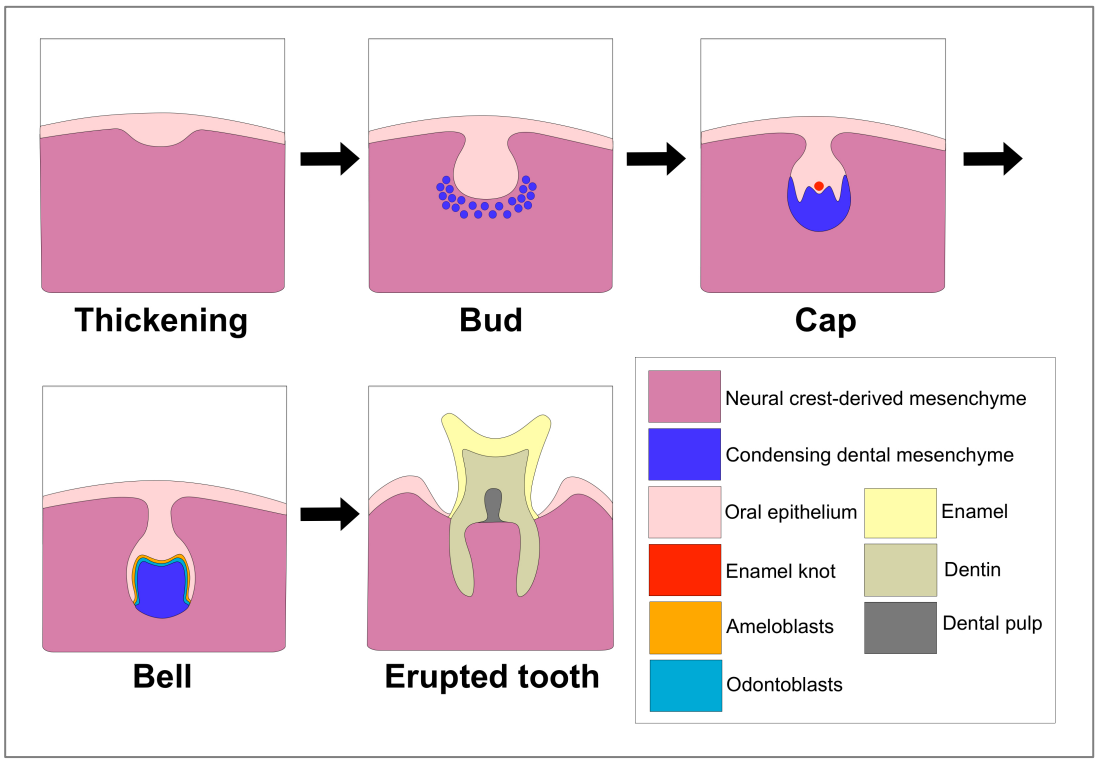


Figure 1.4 Common stages of tooth development. In the mammalian dentition, the first sign of tooth development is marked by thickening of the oral epithelium. This thickening subsequently grows and invaginates into the underlying neural crest-derived mesenchyme to form the primary dental lamina. The mesenchyme responds by condensing around the epithelium, forming a tooth bud. Subsequent cap and bell stages are marked by further inward extension of the epithelium, which wraps itself around the mesenchyme. At this stage, a transient signaling center termed the enamel knot activates within the epithelial tip to regulate tooth morphogenesis by directing surrounding cell activity. Late bell stage is marked by complete enclosure of the mesenchyme within the invaginating epithelium. At this stage, cytodifferentiation occurs, with epithelial cells differentiating to enamel-secreting enameloblasts and adjacent mesenchymal cells to dentin-secreting odontoblasts. These cells secrete hard tissue matrices, followed by root formation and subsequent eruption of the tooth into the oral cavity (Pispa and Thesleff, 2003; Vaahtokari *et al*., 1996a; 1996b; Tucker and Sharpe, 2004; Tummers and Thesleff, 2008). Figure reproduced and modified from Tucker and Sharpe, 2004.

1.2.5 Signaling pathways regulating tooth development and regeneration

Molecular studies of tooth development have shown this process to result from the deployment of numerous signaling molecules, receptors and transcription factors in dental tissues. The genes that produce these molecules are expressed exclusively within the epithelium, the mesenchyme or between the two, and are frequently redeployed during different stages of development (reviewed by Thesleff and Sharpe, 1997). These genes belong to several highly conserved pathways and families, predominantly the BMP (bone morphogenetic protein), FGF (fibroblast growth factor), hedgehog, Wnt and Notch families of signaling molecules (Vainio *et al.,* 1993; Wilkinson *et al*., 1989; Heikinheimo*,* 1994; Jernvall *et al*., 1994; Kettunen and Thesleff, 1998; Bitgood and McMahon, 1995; Vaahtokari *et al.,* 1996a; 1996b; Iseki *et al*., 1996; van Genderen *et al.,* 1994; Zhou *et al.,* 1995; Kratochwil *et al.,* 1996; Behrens *et al.,* 1996; Järvinen *et al.,* 2006; Mitsiadis *et al.,* 1995a; 1997). The organisation of these pathways into larger assemblages forms a genomic control system of considerable complexity; a gene regulatory network (GRN) (Peter and Davidson, 2011). In tooth development, genes from these pathways are expressed in controlled cascades as an odontogenic GRN (oGRN) to regulate cells committed to tooth initiation, budding, morphogenesis and differentiation. To understand the complexity of the oGRN as a whole therefore requires some appreciation of its constituent regulatory components (reviewed by Tummers and Thesleff, 2009).

The Homeobox (Hox) genes are critical determinants of tooth shape and position (Sharpe, 1995; McCollum and Sharpe, 2001). The overlapping ectomesenchymal expression domains of Hox genes regulate canine and molar tooth development, shown by induced remodulation of their expression, resulting in alterations to tooth number, size, shape and differentiation (Tucker *et al*., 1998; Plikus *et al*., 2005). The Distal-less (Dlx) transcription factors constitute a highly conserved family of Hox genes with important roles in mammalian tooth development (Robinson and Mahon, 1994; reviewed Stock *et al.,* 1996). *Dlx1* and *2* are co-expressed in the mesenchyme during mammalian molar tooth development. Here, their critical role in regional specification of tooth pattern is shown in mouse transgenic knockouts, which develop normal incisors and mandibular molars, but fail to develop maxillary molars

(Thomas *et al*., 1997). In humans, *Dlx3* mutations cause Tricho-Dento-Osseous (TDO) Syndrome, which causes several dental defects, including enamel hypodysplacia and taurodontism (Price *et al*., 1998). In mice, deletion of mesenchymal *Dlx3* impairs differentiation of odontoblasts and dentin deposition, showing its critical role in regulating odontoblastic cell activity in advance of matrix deposition (Duverger *et al*., 2012). The broader interactions of *Dlx3* further define its role in biomineralisation, shown during skeletal development in which stimulation by *Bmp2* induces *Runx2*-mediated transcription via *Dlx3* protein-DNA interactions, while direct *Dlx3*-*Runx2* protein-protein interactions have the opposite effect (Hassan *et al.,* 2006).

The bone morphogenetic protein (BMP) family of signaling molecules belong to the TGF-β superfamily, which regulate various developmental processes, including bone formation, as implied by their name (Urist, 1965; Reddi, 1992; Wozney, 1992). During early tooth development, *Bmp2*, *4* and *7* are co-expressed in the thickened presumptive epithelium, with *Bmp4* shifting to the condensing mesenchyme concomitant with the transfer of inductive potential (Vainio *et al*., 1993; Åberg *et al*., 1997). During subsequent tooth morphogenesis, all three are co-expressed within the enamel knot and show marked associations with the differentiation of odontoblasts and ameloblasts, thus demonstrating the importance of BMP signaling in all aspects of tooth development (Åberg *et al*., 1997; Bégue-Kirn *et al*., 1992). This is further apparent in transgenic mice expressing a defective copy of the BMP receptor *Bmpr1*, which causes tooth arrest at bud stage (Andl *et al*., 2004). During tooth development, BMPs also show marked interactions with other genes, shown by *in vitro* addition of *Bmp4*-releasing beads to presumptive dental mesenchymal tissue, which induces expression of the transcription factors *Msx1* and *Msx2* (Vainio *et al.,* 1993).

Several members of the fibroblast growth factor (FGF) family of signaling molecules are also expressed during various stages of tooth development. *Fgf8* is expressed in the presumptive epithelium, *Fgf4* the enamel knot and *Fgf9* in both, and during odontoblastic and ameloblastic cell differentiation (Heikinheimo, 1994; Jernvall *et al*., 1994; Kettunen and Thesleff, 1998). *Fgf10* and *3* are also expressed at various stages of mouse molar tooth development, *Fgf10* in the presumptive dental

epithelium and mesenchyme during tooth initiation, and *Fgf3* in the mesenchyme at late bud stage. *Fgf3* is further expressed in the enamel knot and both *Fgf3*/*10* are co-expressed in the mesenchymal dental papilla during advanced morphogenesis, where they are subsequently down-regulated concomitant with terminal differentiation of odontoblasts (Kettunen *et al*., 2000). FGFs predominantly function as positive regulators of epithelial and mesenchymal cell proliferation during tooth morphogenesis (Jernvall *et al.,* 1994; Kettunen and Thesleff, 1998). This role is further evident in the mouse incisor, where *Fgf3*/*10* stimulate proliferation of epithelial stem cells required for ameloblastic fates. *Fgf3* -/- and *Fgf10* +/- mutants showing reduced tooth growth and severe enamel hypoplasia reflect such a role, while repression by *Bmp4* further demonstrates a direct interaction between FGF and BMP signaling (Harada *et al.,* 1999; 2002; Wang *et al.,* 2007). *In vitro* assays further imply functional similarities to BMPs, shown by the effects of *Fgf4*, *8* and *9* on inducing both cell proliferation and *Msx1* expression in dental tissues (Kettunen and Thesleff, 1998).

The hedgehog family of signaling molecules is highly conserved, with various roles in mammalian tooth development (Kumar *et al*., 1996; Vaahtokari *et al*., 1996a; reviewed Thesleff and Sharpe, 1997). During mouse molar tooth development, the hedgehog ligand sonic hedgehog (*Shh*) is first expressed in the early epithelial thickenings, where it positively regulates tooth initiation through induced cell proliferation (Bitgood and McMahon, 1995; Hardcastle *et al*., 1998). This is shown by *Shh* inhibition, which results in early tooth arrest (Cobourne *et al*., 2001). *Shh* is subsequently co-expressed in the enamel knot with *Bmp2*, *4*, *7* and *Fgf4* to regulate tooth cusp morphogenesis through continued stimulation of surrounding epithelial cell proliferation (Vaahtokari *et al*., 1996b). The critical role of *Shh* in molar tooth morphogenesis is further demonstrated through conditional deletion prior to cap stage, which results in reduced tooth size and severe disruption to molar tooth morphology. However, normal deposition of enamel and dentin suggests that *Shh* signaling is not required for differentiation of ameloblasts and odontoblasts (Dassule *et al*., 2000). However, during mouse incisor renewal, *Shh* is expressed in the differentiating progeny of dental stem cells, where it signals to stimulate differentiation of enameloblasts, therefore demonstrating a conserved requirement

for *Shh* in tooth initiation, morphogenesis and cell differentiation (Seidel *et al*., 2010).

The wingless (Wnt) signaling pathway is also highly conserved in tooth development and is most commonly defined by the *β-catenin* transcription factor. During periods of signaling inactivity, cytosolic *β-catenin* is phosphorylated/ ubiquitylated by the Axin•APC•GSK3 destruction complex, resulting in proteolytic cleavage. However, during signaling, binding of Wnt ligands to the receptor *Frizzled* inhibits phosphorylation by the destruction complex, resulting in cytoplasmic saturation and nuclear translocation, where *β-catenin* forms a transcription complex with T cell-specific transcription factor/ lymphoid-enhancer-1 (*TCF*/*Lef1*) to regulate expression of target genes (Seidensticker and Behrens, 2000; reviewed by Gordon and Nusse, 2006). The crucial role of Wnt signaling in tooth development is demonstrated by constitutive stabilisation of *β-catenin* in the ectoderm, which results in continual formation of enamel knots and supernumerary tooth buds (Järvinen *et al*., 2006). Conversely, inactivation of *β-catenin* in the dental epithelium and mesenchyme arrests tooth development at bud stage (Liu *et al.,* 2008; Chen *et al*., 2009). The important role of *Lef1* in tooth development is shown in deficient mice, which undergo tooth arrest at bud stage, while overexpression leads to increased invagination of the presumptive dental epithelium and formation of ectopic hair follicles and tooth-like structures (Zhou *et al*., 1995).

The *Notch* receptor and its ligand, *Jagged* (*Serrate*), are expressed at several stages of tooth development. *Notch 1*, *2* and *3* are expressed in the epithelium, however, a pronounced Notch-negative region of epithelial cells in close proximity to the mesenchyme implies a role in negative regulation of Notch expression in these cells, which are fated to undergo ameloblastic fates to further suggest an interaction involving regulation of ameloblastic cell fate determination. This is further shown through *in vitro* tissue recombination in experiments, in which epithelial cells adjacent to recombined mesenchyme are also Notch-negative (Mitsiadis *et al*., 1995a; 1997).

The ectodysplasin signaling pathway is commonly defined by the *Eda* ligand, its receptor *Edar* and adaptor protein *Edaradd* (Tucker and Sharpe, 2004). The critical role of ectodysplasin signaling in tooth development is shown in transgenic (*Tabby*) mice, in which *Eda* and *Edar* deficiencies cause missing or abnormally shaped teeth (Pispa *et al*., 1999; Peterková *et al*., 2002). Conversely, upregulation of *Eda* signaling increases the extent of the molar field to produce supernumerary teeth (Tucker and Sharpe, 2004). In humans, hypohidrotic ectodermal dysplasia (HED) is linked to mutations in *Eda* and *Edaradd*, causing similar phenotypic dental abnormalities, including hypodontia and tooth agenesis (Aswegan *et al*., 1997; Chassaing *et al*., 2006). In *Tabby* mice, these adverse dental phenotypes may result from abnormal development of the dental lamina, accompanied by increased apoptosis. Supernumerary phenotypes have therefore been proposed to result from the persistence of tooth germs, which would normally undergo apoptosis (Boran *et al*., 2005; reviewed by Townsend *et al*., 2009).

In addition to these core signaling pathways, several smaller gene families and individual genes are involved in tooth development. The secreted ligand, Sclerostin domain-containing protein 1 (*Sostdc1, Ectodin, Wise, USAG-1*), regulates tooth development through multiple interactions with hedgehog, FGF, BMP and Wnt-*β-catenin* signaling (Laurikkala *et al*., 2003; Munne *et al*., 2009; Cho *et al*., 2011; Ahn *et al*., 2010). *Sostdc1* is principally recognised for its role as a BMP antagonist, functioning as part of a feedback loop with *Shh* and *Fgf4* to define the spatiotemporal expression domains of BMPs surrounding the enamel knot (Laurikkala *et al*., 2003). The critical role of *Sostdc1* in regulating the spatial distribution of enamel knots and cusp morphogenesis is further shown in *Sostdc1* -/- mice, which develop enlarged enamel knots, extra teeth and exhibit altered cusp patterns (Kassai *et al*., 2005). In mouse incisor development, deletion of *Sostdc1* also results in the development of additional incisors, while its expression domains confined to the mesenchyme, suggest a role in Wnt inhibition, which promotes incisor growth. This is shown through *in vitro* cell culture experiments in which addition of mesenchymal tissue results in *de novo* incisor development (Munne *et al*., 2009). *Sostdc1* further interacts with hedgehog and Wnt signaling via controlled negative feedback loops in which Wnt signaling induces expression of *Shh* to in turn inhibit Wnt-*β-catenin* indirectly

via *Sostdc1*. This interaction is proposed to regulate the spatial pattern of developing teeth via a feedback loop in which each functions as activator, mediator and inhibitor, respectively (Cho *et al.,* 2011).

The paired-like homeodomain genes *Pitx1* and *2* are members of the RIEG/ PITX family of transcription factors with critical roles in tooth development. This is apparent in *Pitx2* mutations, which in humans cause Rieger syndrome, characterised by severe tooth and eye abnormalities (Semina *et al*., 1996). Deletion of *Pitx2* in mice also results in several dental abnormalities, including loss of the enamel knot (Lin *et al*., 1999). In the mammalian dentition, *Pitx2* is believed to confer initial odontogenic competence, shown by its expression in the early dental lamina of the ferret (Jussila *et al.,* 2014). In the mouse dentition, *Pitx2* is also expressed in the presumptive dental epithelium and throughout subsequent stages of tooth development, while *Pitx1* is expressed both in the mesenchyme and epithelium, becoming progressively restricted to the latter. Both also interact with FGFs and BMPs, shown by implantation of *Fgf8* and *Bmp4*-releasing beads, which transiently induce and repress *Pitx1*/*2* expression, respectively (St.Amand *et al*., 2000).

Neurite growth-promoting factor 2, or *Midkine* (*MK*), is expressed differentially between the epithelium and mesenchyme during various stages of mouse molar tooth initiation, budding and morphogenesis (Mitsiadis *et al*., 1995b). In the mouse incisor, *MK* is also expressed in the enamel knot, where it is believed to transiently prevent apoptosis and in the cervical loop, where its localisation to regions of high proliferative activity implies a positive regulatory role in stimulating cell proliferation. In the incisor, *MK* is also expressed in odontoblasts in the mesenchymal papilla and pre-ameloblasts in the inner dental epithelium, suggesting a role in regulating cell differentiation (Mitsiadis *et al.,* 2008). The critical role of *MK* in tooth development is further demonstrated through culture of tooth germs with *MK*-neutralising antibody, which in molars inhibits morphogenesis and differentiation and in incisors, growth, differentiation and mineralisation (Mitsiadis *et al*., 1995b; 2008).

Several members of the Forkhead box (Fox) family of transcription factors regulate various aspects of tooth development, including cell proliferation, apoptosis, migration and differentiation (Schmidt *et al*., 2002; Sunters *et al*., 2003; Fosbrink *et al.,* 2006; Myatt and Lam, 2007). In the mouse dentition, *Foxi3* is implied to regulate tooth initiation and morphogenesis, while in the canine dentition heterozygous mutations result in missing teeth (Shirokova *et al*., 2013; Drögemüller *et al.,* 2008). Mutations in several additional Fox genes also produce a broad spectrum of pronounced dental abnormalities, such as *Foxo1*, which in mice results in enamel hypomaturation defects (Póche *et al.,* 2012). In mice, *Foxn3* mutations produce elongated incisors and in humans, *Foxc1* point mutations cause microdontia (small teeth) and hypodontia (numbering few) (Samaan *et al*., 2010; Honkanen *et al*., 2003).

Runx2 belongs to the Runt family of master transcription factors, which play essential roles in bone and tooth development by promoting cell differentiation to osteoblasts and maturation-stage ameloblasts (Zhao *et al*., 2007; D'Souza *et al.,* 1999). The importance of *Runx2* in bone and tooth development is apparent in *Runx2* -/- mice, which lack ossified bone as a result of maturational arrest of osteoblasts and in humans, where mutations cause cleidocranial dysplasia, characterised by delayed tooth eruption and development of supernumerary teeth (Komori*,* 2006; Mundlos *et al.,* 1997). *Runx2* interacts with several signaling pathways, shown in mice where induced activation of *β-catenin* results in enhanced ossification via *Runx2*, while inactivation causes ectopic formation of chondrocytes at the expense of osteoblast differentiation (Day *et al*., 2005). During skeletal development, Wnt-*β-catenin* signaling also targets mesenchymal *Runx2* to stimulate differentiation of osteoblasts (Gaur *et al*., 2005). *Runx2* also interacts with BMP signaling, shown in mouse mandibular explants, in which addition of *Bmp4* protein induces expression of early *Runx2*-dependent genes, suggesting an indirect role for *Bmp4* in stimulating subsequent *Runx2* expression (James *et al*., 2006). *Runx2* is also known to function downstream of epithelial FGF signaling to in turn regulate mesenchymally-expressed *Fgf3*. Over-expression of *Runx2* in *Runx2* -/- cells further induces *Fgf3* expression, implicating *Fgf3* as a downstream target of *Runx2* signaling in the dental mesenchyme (Åberg *et al*., 2004).

Members of the Twist family of basic helix-loop-helix transcription factors also play central roles biomineralisation by regulating pre-osteoblastic and osteoblastic cell activity (Murray *et al.,* 1992; Rice *et al.,* 2000). *Twist* +/- mice exhibit increased bone formation and cranial sutures, and activation increases expression of mesenchymal stem cell (MSC) markers, while decreasing osteogenesis, implying a role in regulating self-renewal of MSCs (El Ghouzzi *et al.,* 1997; Isenmann *et al*., 2009). This *Twist*-mediated self-renewal occurs through transient binding to the DNA-binding domain of *Runx2*, preventing transcription of target genes and inhibiting osteoblast differentiation. When released, *Runx2* is able to trigger osteoblast differentiation, marked by expression of various mineralisation genes (Bialek *et al.,* 2004). While knowledge of the direct function of *Twist* in tooth development remains comparatively limited, a role involving regulation of human dental pulp stem cells (hDPSCs) is implied by *Twist* overexpression assays, which enhance expression of tooth-specific markers. Furthermore, a conserved interaction with *Runx2* is shown by co-transfection assays, in which *Twist* stimulates *Dspp* promoter activity by antagonising *Runx2* (Li *et al*., 2011b).

The Hippo signaling pathway plays an important role in controlling organ size through regulation of cell proliferation, differentiation and apoptosis (Barry and Camargo, 2013; reviewed by Zhao *et al*., 2008; 2010). In particular, the Hippo genes *Yap* (yes-associated protein) and its paralogue, *Taz* (transcriptional co-activator with PDZ-binding motif), or *wwtr1*, are prominent effectors of transcriptional activity through interactions with other DNA-binding proteins (Kanai *et al*., 2000; Hiemer and Varelas, 2013). Recent studies have revealed increasingly prominent roles for *Yap* and *Taz* in regulating various aspects of tooth development. This is shown by constitutive activation of *Yap1* in transgenic (Tg) mice, which produces several dental abnormalities, including widening of the dental lamina and abnormal tooth morphogenesis, characterised by displacement of the enamel knot to the upper region of the enamel organ. Associated abnormalities include the absence of apoptotic activity at the tip of the enamel organ, with apoptosing cells restricted to the mislocated enamel knot, accompanied by a lack of cell proliferation. *Yap1* Tg mice also show altered *Edar* and *E-*/ *P-cadherin* expression, and aberrant *Shh, Fgf3*/*4* and *Wnt10a* expression, restricted to the tip of the enamel organ. Epithelial-mesenchymal

proliferation is also reduced, implying roles in regulating cell proliferation, movement and polarisation during molar tooth morphogenesis (Liu *et al*., 2014). During mouse incisor development, *Yap1* is also expressed in the apical bud and transit amplifying cells, suggesting a role in stem cell division and proliferation during incisor growth (Li *et al*., 2011a). The role of *Taz* in tooth development has been shown by its *in vitro* application to hDPSCs, which induces formation of mineralised extracellular matrices, marked by increased expression of the odontogenic markers *Msx1*/*2*, *DSPP* and *Dlx5* (Suh *et al*., 2012).

1.2.6 Mammalian models of tooth development and regeneration

Most extant mammals differ to other gnathostomes in their limited capacity for tooth regeneration (Jernvall and Thesleff, 2012). This is with the notable exception of manatees (*Trichechus*), which have developed the capacity for molar tooth replacement. In the regenerating manatee dentition, replacement molars initiate in the posterior-most region of each jaw quadrant within continuously developing crypts (Domning and Hayek, 1984). From here, developing molars move anteriorally through constant cycles of reabsorption and deposition of alveolar bone, where at the oral margins worn crowns are shed (Beatty *et al*., 2012).

However, the majority of mammals replace their teeth just once (diphyodonty), during which primary (deciduous) teeth are replaced by a permanent set of adult teeth (van Nievelt and Smith, 2005). This is suggested by evidence found in the fossil record, which shows early mammals to have undergone a progressive reduction in tooth replacement capacity, with a corresponding increase in dental complexity and precise occlusion (Kielan-Jaworowska *et al*., 2004; Jernvall and Thesleff, 2012). This is further reflected in the basal state of the mammalian dentition, which comprises a morphologically distinct set of regionalised incisors, canines, premolars and molars (heterodonty) (Evans *et al*., 2007). It is therefore apparent that mammals have evolved these complex molar cusp shapes to counteract tooth loss and wear, such as the tall, hypsodont teeth found in rodents, horses and cows (von Koenigswald, 2011).

However, some mammals have responded to tooth loss and wear by retaining the ability to continuously grow their teeth throughout life (hypselodonty). This is apparent in the continuously growing incisors of aye-aye lemurs, and molars in voles, sloths and guinea pigs (Mittermeier *et al*., 1994; Tummers and Thesleff, 2003; Hunt, 1959; Tummers and Thesleff, 2008). Shrews develop rudimentary milk teeth, which do not erupt. These are subsequently replaced by adult permanent teeth, with both sets showing conserved *Shh* expression in the enamel knot. The shrew dentition is therefore demonstrative of a derived, yet common patterning mechanism, with primary teeth suppressed as a consequence of development of the secondary replacement dentition (Järvinen *et al*., 2008). In ferrets, replacement teeth initiate from a dental lamina connected with the lingual deciduous tooth enamel organ. Replacement teeth develop as an offshoot of the enamel organ, marked by *Shh* in the enamel knot and associated expression of *Sostdc1* and *Axin2* (Järvinen *et al*., 2009). These mammalian replacement phenotypes therefore demonstrate the conservation of partial tooth regenerative potential among mammals, as further defined by a common set of regulatory genes (Järvinen *et al*., 2009; 2008; Jernvall and Thesleff, 2012).

Due to its general accessibility and experimental amenability as a developmental model, the mouse has become the principle mammalian system for studying molecular regulation of tooth development. This has provided considerable knowledge, such as that summarised in section 1.2.5. Yet despite its prominence as a tooth development model, the mouse possesses an unusual dentition consisting of a single incisor and three molars arranged in a row on both the left and right quadrants of the upper and lower jaws. Mouse incisors and molars are further separated by a large toothless region (diastema) and in the case of the latter, are limited to only one round of growth (monophyodonty) (Tucker and Sharpe, 2004). Despite these disadvantages, the mouse incisor has retained the unique, if reduced, capacity for lifelong renewal, resulting from continual deposition of hard enamel along the labial surface and softer dentin and cementum along the lingual side. This depositional asymmetry results in marked differences in hardness between the lingual and labial surfaces, maintaining the overall sharpness, and therefore functionality, of the incisor (Smith, 1980; Harada *et al*., 1999).

This derived form of hypselodonty results from the deployment of two dental stem cell niches (SCNs), housed within the large and small cervical loops on the labial and lingual sides of the incisor, respectively (Fig. 1.5). This is first implied by BrdU pulse-chase experiments; a method commonly used to identify slow-cycling cells. At the time of administration (pulse), the thymidine analogue BrdU (5-Bromo-2'-deoxyuridine) incorporates into DNA during S-phase of the cell cycle and is retained only by slow-cycling cells. The localisation of BrdU label-retaining cells therefore provides initial indications of their stem or stem-like identities (Shimada *et al*., 2008). Having been further confirmed by lineage tracing, progeny of the stem cells housed within the labial SCN proliferate along the basal epithelium toward the tooth apex as transit amplifying (TA) cells, where they differentiate to enameloblasts to maintain continual enamel deposition (Harada *et al*., 1999; Wang *et al*., 2007; Siedel *et al*., 2010; Juuri *et al*., 2012).

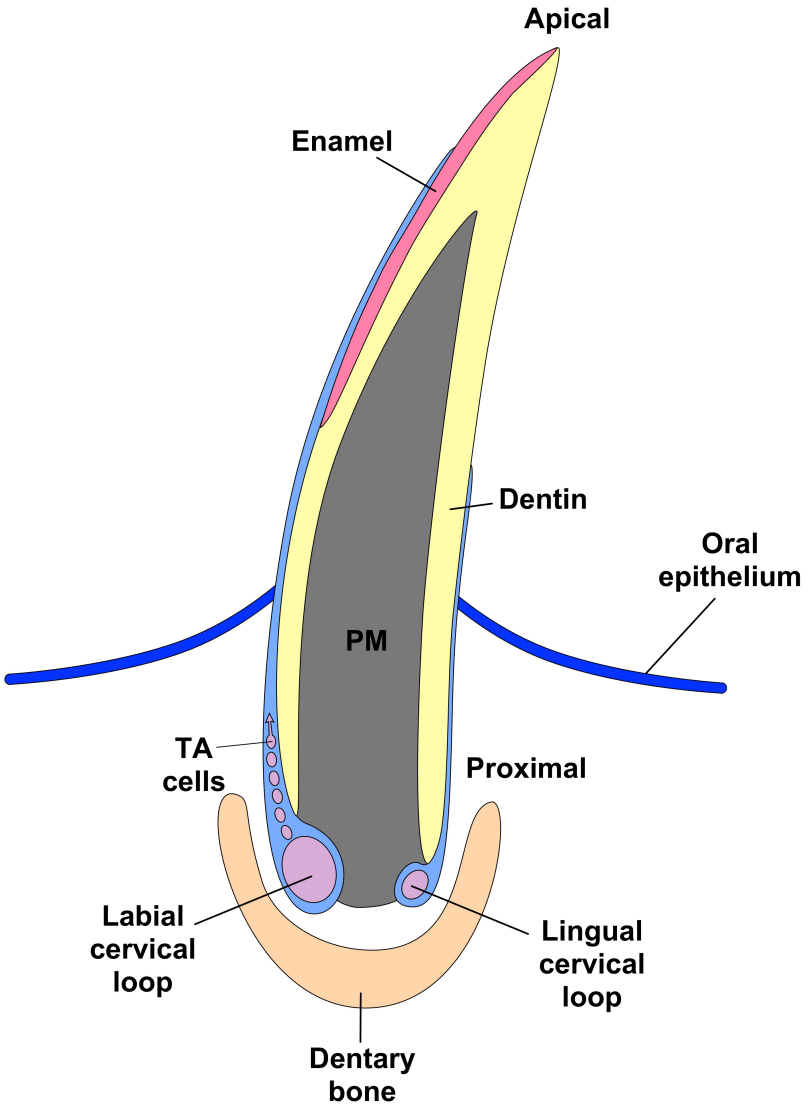


Figure 1.5 Mouse incisor renewal. In the mouse incisor, dental stem cells (SCs) housed within the cervical loops (CLs) at the proximal end maintain sharpness through continual asymmetric deposition of hard enamel and soft dentin along the labial and lingual surfaces, respectively. Stem cell progeny derived from the labial CL proliferate apically as transit amplifying (TA) cells, where they differentiate to enameloblasts to maintain continual enamel deposition (Harada *et al*., 1999; Wang *et al*., 2007; Siedel *et al*., 2010; Juuri *et al*., 2012).

Stem cells are defined by their capacity to self-renew, divide and perpetuate a given lineage by differentiating to the required cell types. Within adult tissues subject to homeostatic control or vulnerable to injury, reservoirs of stem cells are maintained within specialised microenvironments, or 'niches'. Stem cell niches (SCNs) serve to supply the cell types required to replenish these tissues, while regulating their proliferative potential (reviewed by Fuchs *et al*., 2004). Given the crucial role of stem cells in tissue renewal, considerable research has been dedicated to their isolation and characterisation. This has presented a challenge when attempting to isolate specific stem cells from mixed populations in cell culture. While beneficial in establishing specific cell lines, this approach has further limited perspective regarding the underlying molecular mechanisms regulating stem cell function (reviewed by Fuchs and Segre, 2000). Recent research into the regulation of stem cells and their niches has therefore shifted towards characterisation by molecular methods. These efforts have sought to understand stem cell function through systematic dissection of the gene regulatory mechanisms underlying their function. To this end, the mouse incisor has provided an exemplary model system for studying the behaviour of stem cells and the tissue-specific signaling mechanisms regulating their function (Harada *et al*., 1999; 2002; Wang *et al*., 2007; Juuri *et al*., 2012).

During cap and bell stages of incisor development, the lateral epithelium of the bud progressively encases the underlying dental mesenchyme to form the cervical loops, the core of which will house the adult SCNs (Harada *et al*., 1999; Tummers and Thesleff, 2003; 2008). These epithelial compartments are surrounded by mesenchymal cells, which signal to the epithelium to regulate stem cell self-renewal and differentiation. These signals maintain the balance between cell renewal and differentiation (Wang *et al*., 2002; 2007; Klein *et al*., 2008; Parsa *et al*., 2010). Mesenchymally expressed *Fgf3* and *Fgf10* signal to the epithelium to stimulate stem cell proliferation and survival of progeny, while *Bmp4* regulates the inductive effects of *Fgf3* through targeted inhibition. However, *Activin* further inhibits the repressive effects of *Bmp4*, while limiting *Fgf3* to the labial dental mesenchyme, resulting in increased stem cell proliferation within the larger, labial SCN (Wang *et al*., 2007). Mice deficient in *Fgf10* also fail to develop cervical loops and inhibition by neutralising antibody induces apoptosis within the cervical loops, suggesting a

critical role in stem cell maintenance within the developing incisor (Harada *et al*., 2002). In the lingual SCN, *Follistatin* functions to limit the number of stem cells, thus determining the overall characteristic asymmetry of the incisors (Wang *et al*., 2004). In addition to the SCNs contained within the cervical loops, incisor growth is regulated by two associated mesenchymal SCNs (Lapthanasupkul *et al*., 2012). The mesenchymal component of the mouse incisor is marked by the Polycomb repressive complex (PRC1) genes *Ring1a*/*b*, both of which are strongly expressed in the apical mesenchyme of the incisor, coincident with regions of high cell proliferation and TA cells. In mutant mice deficient for *Ring1a*/*b*, cell proliferation within the dental mesenchyme in close proximity to both epithelial cervical loops is greatly reduced and incisor development and is impaired leading to growth arrest. Given these dramatic phenotypic defects, *Ring1a*/*b* are proposed to mark two associated mesenchymal stem cell (MSC) niches with critical signaling roles in regulating the proliferation of cells committed to odontoblastic fates, both within the dental mesenchyme and from the mesenchyme to the cervical loops (Lapthanasupkul *et al*., 2011). Recent cell lineage tracing and expression studies have further identified a MSC niche housed within the neurovascular bundle (NVB) at the base of the incisor (Zhao *et al*., 2014). Secretion of *Shh* from associated NVB sensory nerves activates the hedgehog receptor *Gli* in peri-arterial cells and a subpopulation of pericytes, stimulating their commitment to mesenchymal lineages. Mesenchymal derivatives of peri-arterial cells are defined by the absence of classical MSC marker expression, whereas NG2+ pericytes express classical MSC markers, collectively contributing to incisor homeostasis and injury repair, respectively (Zhao *et al*., 2014).

Continual remodulation of these epithelial and mesenchymal signals regulates the balance between renewal and proliferation to maintain this asymmetry (Harada *et al*., 1999; Wang *et al*., 2007; 2004; Lapthanasupkul *et al*., 2011; Zhao *et al*., 2014). With regard to the epithelial SCN, the importance of these genes in regulating this balance is demonstrated by deletion of *Follistatin*, which causes ectopic enamel formation along the lingual side of the incisor and stimulation of cervical loop growth, resulting in adverse asymmetry (Wang *et al*., 2004; 2007). Inhibition of FGF function in *Fgf3* -/- and *Fgf10* +/- mutants also causes reduced incisor growth and severe hypoplasia, while overexpression of the BMP inhibitor *Noggin* stimulates hyperproliferation of

stem and progenitor cells in the cervical loops, resulting in incisor overgrowth (Wang *et al*., 2007; Plikus *et al*., 2005). Recent integration of expression data pertaining to the stem cell marker *Sox2* in the cervical loop of the incisor has provided further evidence to support its role as a dental stem cell niche (Juuri *et al*., 2012).

1.2.7 Next generation models of tooth regeneration

While the mouse dentition has proven essential for identifying some fundamental components of the dental gene regulatory network (GRN), it remains inherently disadvantaged in several respects. Firstly, in accordance with general mammalian trends, the mouse dentition is both reduced and regionalised (Luo, 2007). This pronounced regionalisation has resulted in both spatial and temporal decoupling of morphogenesis (molars) and renewal (incisors). Secondly, while mouse dental cell lineages are unrestricted in their capacity to form a whole tooth, during renewal, those derived from the incisor SCN lack this differentiation potential and are predominantly restricted to ameloblastic fates only. In mice, these factors combined, essentially nullify the potential for *de novo* tooth replacement and therefore the prospect of fully comprehensive insights into the molecular framework regulating this regenerative process (Harada *et al*., 1999; Wang *et al*., 2007). However, the fact that ectopic tooth formation can be induced in mice through stimulation of canonical Wnt signaling implies the latent conservation of a core dental regenerative network (Järvinen *et al*., 2006). This is also apparent in chick embryos in which *in vivo* activation of *β-catenin* induces ectopic rudimentary tooth germs (Harris *et al*., 2006). In chick mandibular tissue, introduction of exogenous *Bmp4* and *Fgf8*-soaked beads also induces expression of early dental patterning genes and formation of a vestigial dental lamina, further suggesting the retention of latent, yet potent, odontogenic potential (Chen *et al*., 2000).

Studies of ferret tooth replacement support the idea that in mammals retaining this capacity, replacement teeth initiate from a secondary (successional) dental lamina, which forms as a growth extension of the primary tooth (Järvinen *et al*., 2009). Human teeth, which are only replaced once, also contain a remnant of the dental

lamina, which may become activated later in life to form odontogenic tumours, further supporting the conservation of this latent tooth replacement capacity (Philipsen and Reichart, 2004; Ide *et al*., 2007). The successional lamina (SL) may therefore be conserved in polyphyodont gnathostomes to initiate and sustain replacement teeth (Huysseune, 2006; Fraser *et al*., 2006a; 2013). With this existing knowledge in hand, to fully address the cellular and molecular basis of tooth regeneration, research efforts have inevitably refocused upon polyphyodont gnathostomes, those capable of *de novo* tooth replacement. In essence, biologists aim to use these models to understand how nature makes and replaces teeth throughout life. A comprehensive understanding of the molecular pathways regulating this specific type of organ regeneration therefore provides important opportunities to answer questions regarding their putative conserved roles in tooth regeneration, while opening new avenues of research in regenerative dentistry.

1.2.8 Osteichthyan models of tooth development and regeneration

Osteichthyan (teleost) fish predominantly replace their teeth as a 'one-for-one' system, in which individual teeth are cyclically replaced at each position by a single, successive tooth Fraser *et al*., 2006a; 2006b; 2013). Given its experimental accessibility, the zebrafish (*Danio rerio*) has recently been used as a teleost tooth replacement model, revealing some components of the core oGRN. In zebrafish, *Shh* is expressed during tooth initiation and morphogenesis, its critical role shown by exposure to the hedgehog pathway inhibitor cyclopamine, which causes tooth arrest at these stages (Jackman *et al*., 2010). The zebrafish dentition also expresses conserved *Pitx2*, *Pax9* and *Fgf8*, the importance of FGF signaling shown by treatment with the FGF inhibitor SU5402, which prevents dental epithelial morphogenesis (Jackman *et al*., 2004).

However, like other cyprinids, zebrafish lack oral teeth, with those present restricted to the fifth ceratobranchials, the pharyngeal jaws (van der Heyden and Huysseune, 2000; Huysseune *et al*., 1998). This reduced and highly specialised dentition has therefore presented a further obstacle when attempting to elucidate the 'global' tooth replacement oGRN. However, the rainbow trout (*Oncorhynchus mykiss*) possesses

an extensive set of oral and pharyngeal replacement teeth, providing a useful alternative. Molecular studies of trout tooth development have further revealed the conserved expression of *Pitx2* and *Shh*, which in accordance with their known roles are proposed to initiate the odontogenic cascade, resulting in mesenchymal *Bmp4* expression (Fraser *et al*., 2004). In trout tooth replacement, *Pitx2* and *Bmp4* are further expressed at sites of replacement tooth initiation, while restriction of *Shh* to first generation teeth suggests a role confined to tooth initiation. These reiterative expression patterns provide evidence to support the redeployment of conserved genes in tooth replacement, while providing a set of markers to identify cells committed to odontogenic fates (Fraser *et al*., 2006a). However, the rainbow trout dentition develops without a classical dental lamina, with replacement teeth initiated from a set of thickened dental epithelial cells surrounding the developing tooth (Fraser *et al*., 2006a; 2006b).

Cichlids (Family Cichlidae) on the other hand, possess both oral and pharyngeal teeth, and a successional lamina similar to that in zebrafish, with replacement teeth developing from within a bony (intraosseous) cavity underlying each functional tooth position (Fraser *et al*., 2013). Molecular studies of cichlid tooth development and replacement have proven highly informative, revealing the expression of numerous conserved markers common to the hedgehog, BMP, FGF, Notch and Wnt-*β-catenin* pathways. Interestingly, during cichlid tooth replacement an epithelial 'stripe' connects the surface oral epithelium to the dental epithelium, with cells proliferating into small openings in the bony matrix (gubernacular canals) (Fig. 1.6). This epithelial stripe also expresses the stem cell marker *Sox2,* which localises strongly to the labial oral epithelium above the replacement tooth cavity, marking a proposed dental SCN supplying progenitor cells required for tooth replacement (Fraser *et al*., 2008; 2013; Juuri *et al*., 2012). In the cichlid dentition, these genes are proposed to function in accordance with their mammalian counterparts, to confer initial dental competence and regulate tooth development and morphogenesis. Subsequent redeployment of these genes in the replacement cichlid dentition provides compelling evidence to support deeply conserved roles linking tooth regeneration to morphogenesis, a process evolutionarily lost in mammals. The critical importance of these genes in the odontogenic cascade is shown by targeted chemical disruption of

associated pathways, producing altered phenotypes in first and subsequent tooth generations ranging from irrecoverable tooth arrest to disruption of morphogenesis and modified dental morphologies. The outcome of these studies have therefore proven crucial in demonstrating the reiterative deployment of conserved pathways in tooth regeneration, while revealing the considerable level of developmental plasticity intrinsic to teeth, as shown by 'induced evolution' through modification of dental phenotypes by chemical treatment (Fraser *et al*., 2008; 2013).

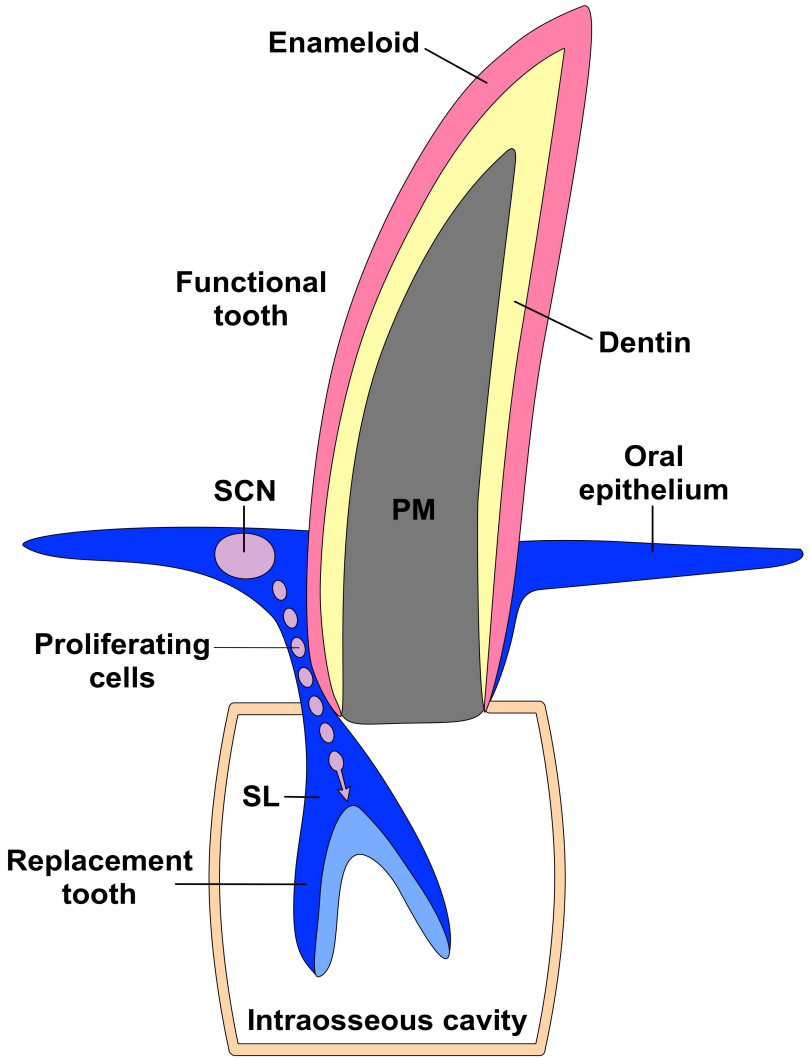


Figure 1.6 Cichlid 'one-for-one' tooth replacement. In the cichlid dentition, replacement teeth develop beneath the functional tooth from a successional lamina (SL) housed within a bony (intraosseous) crypt connected to the oral surface via a continuous epithelial stripe. At the oral surface labial to the functional tooth, this epithelium expresses the stem cell marker *Sox2*, marking a putative dental SCN from which progenitor cells proliferate into the SL to maintain tooth replacement capacity. In collaboration with conserved signaling pathways, this SCN is proposed to recouple cyclical tooth regeneration to morphogenesis, a process lost in mammals. Figure modified from Fraser *et al*., 2013.

Studies of several additional osteichthyan dentitions have further highlighted the recurrent importance of the dental lamina, stem cells and conserved signaling pathways, in maintaining tooth replacement capacity. In the adult upper jaw dentition of the gobiid (*Sicyopterus japonicus*), replacement teeth develop as a semi-circular strand from within a thin, plate-like dental lamina. New tooth buds are connected with the dental lamina via the dental epithelium and develop from the ventro-labial margin. This region coincides with high cell proliferation, suggesting this to be the site of repeated tooth initiation. This unique tooth replacement system is proposed to have evolved to meet the specialised algae-scraping feeding requirements of the gobiid (Moriyama *et al*., 2010).

Putative dental stem cells have recently been localised to the pharyngeal replacement dentition of the medaka (*Oryzias latipes*). Here, BrdU pulse-chase experiments have identified slow-cycling dental epithelial cells expressing *Sox2* at the posterior end of replacement tooth families, where they are proposed to maintain continuous tooth replacement (Abduweli *et al*., 2014). In the African bichir (*Polypterus Senegalus*), BrdU labeling has also identified label-retaining cells within an offshoot of the outer dental epithelium (ODE), which actively proliferate during tooth replacement. While this is proposed to occur in the absence of a classical dental lamina, these cells are also thought to constitute putative dental stem cells (Vandenplas *et al*., 2014).

The considerable potential of the conserved odontogenic genetic toolkit in generating novel dental diversity is further evident in the adult pufferfish *Monotrete suvattii*, which develops an apomorphic beak-like structure originating from four initial medial teeth common to other osteichthyans. During the transition from first generation teeth to adult beak, labial surface epithelial cells proliferate into the underlying bony cavity and successional lamina to form stacked bands of dentine, which maintain the beak throughout life. Investigation of early odontogenic events associated with this remarkable developmental transition show the expression of at least four conserved genes, including *Shh*, *Pitx2*, *Bmp4* and *Pax9* (Fraser *et al*., 2012).

1.2.9 Reptilian models of tooth development and regeneration

While studies of the osteichthyan dentition have made considerable progress in determining key aspects of molecular regulation of tooth regeneration, similar studies of reptilian polyphyodonty have proven enormously insightful. Most reptiles, such as crocodiles, alligators and squamates (lizards and snakes), undergo lifelong tooth replacement (Edmund, 1962; Westergaard and Ferguson, 1987; Richman and Handrigan, 2011). In squamates, such as the corn snake, several replacement teeth are linked together as a 'many-for-one' series in which each develops from a SL formed by an offshoot of the replacement tooth bud. This permanent SL is further connected to the oral surface by a continuous epithelial stripe (Fig. 1.7) (Gaete and Tucker, 2013; Handrigan *et al*., 2010; Buchtová *et al*., 2008; Zahradnicek *et al*., 2008). The early crocodilian dentition is similar, with each family consisting of a single functional and replacement tooth, continuous with the dental lamina and oral epithelium. However, in contrast with squamates, the crocodilian dental lamina loses its connection with the oral surface and teeth during subsequent stages (Wu *et al*., 2013). These differing strategies may be reflected by their contrasting replacement rates, with squamates replacing their teeth four times per year, compared to once in crocodilians (Edmund, 1960; 1962).

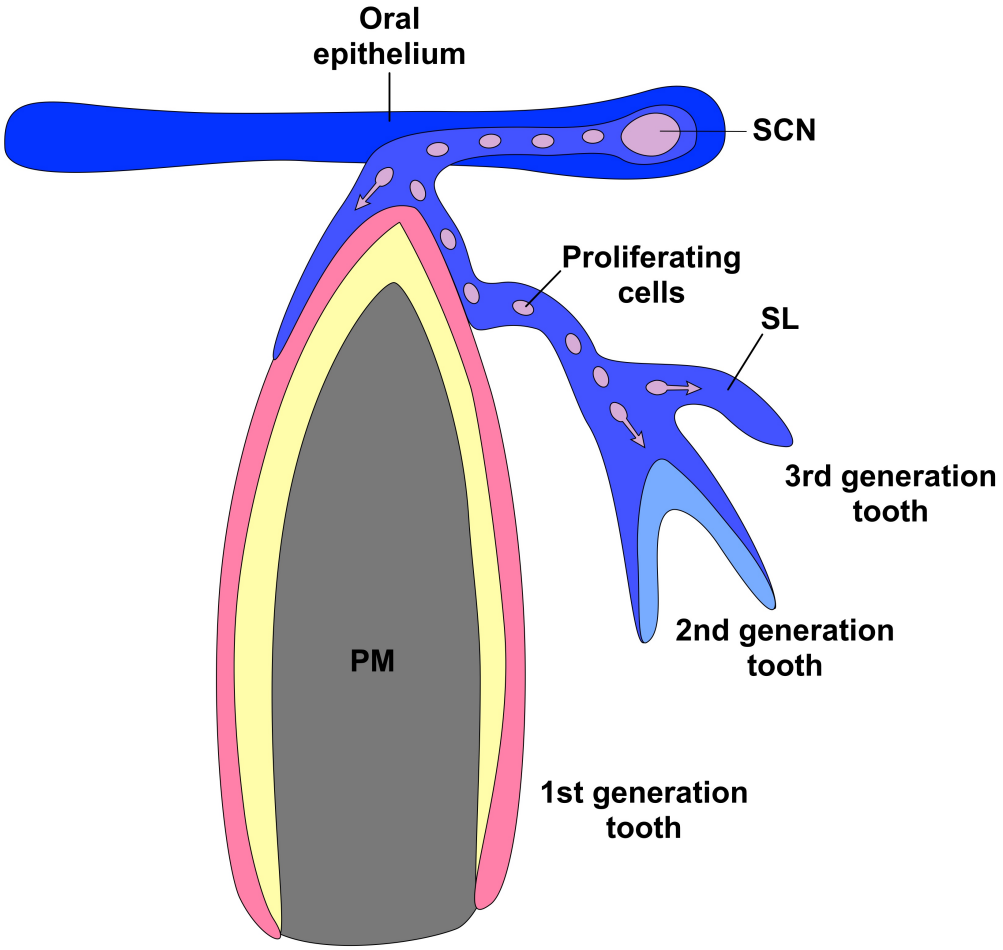


Figure 1.7 Reptile 'many-for-one' tooth replacement. In the snake dentition, successive replacement tooth generations develop as a series from a SL formed by an offshoot from the replacement tooth bud. At the oral surface, *Sox2* marks a putative dental SCN through which progenitors proliferate into the SL via a continuous epithelial stripe to supply cells required to maintain tooth replacement capacity (Gaete and Tucker, 2013).

In gnathostomes, onset of early dental competence is marked by the odontogenic band (OB), a restricted field of gene expression appearing in advance of any epithelial thickenings to define the location of future teeth (Smith *et al*., 2009a). In the teleost dentition this is marked by early expression of *Shh* and *Pitx2* in a continuous arc spanning the width of the jaw (Fraser *et al*., 2004). However, in the mouse dentition, the OB is split into two separate regions of *Shh* expression, resulting from the absence of canines and premolars and the presence of a diastema (Cobourne *et al*., 2004). However, the early snake and alligator dentitions closely resemble those of teleosts, marked by a continuous band of *Shh* expression across the jaw arc to define the position of the future dental lamina (Harris *et al*., 2006; Buchtová *et al*., 2008). During formation of the snake dental lamina, *Shh* is continually expressed on the non-tooth budding side at the junction of the oral and dental epithelium, where it is believed to promote dental epithelial ingrowth into the mesenchyme and establish its characteristically acute asymmetry. *Shh* is subsequently expressed in the developing anlagen during tooth initiation and the enamel organ during cap stage, accompanied by an associated absence of cell proliferation and presence of apoptosis in the stellate reticulum. These critical roles are shown by phenotypic defects resulting from stage-specific exposure to cyclopamine, including prevention of initial epithelial ingrowth, disruption to normal depth and angulation, and disorganised enamel organs lacking in clear cell differentiation (Buchtová *et al*., 2008).

Expanded studies of *Shh* expression in the python, gecko and bearded dragon have also proven informative. In all three, *Shh* is expressed in both the oral epithelium and the acutely angled aspect of the dental lamina, while *Ptc1* is expressed in both the dental epithelium and mesenchyme, suggesting a functional role for *Shh* in both autocrine and paracrine signaling (Handrigan and Richman, 2010a). The critical requirement for *Shh* in normal squamate tooth morphogenesis is further shown by exposure of dental explants to cyclopamine, which at bud stage results in flattened tooth shapes and accompanied loss of *Ptc1* expression. Conversely, placement of *Shh*-releasing beads next to the dental lamina increases cell proliferation in both the dental epithelium and mesenchyme, showing their responsiveness to hedgehog signaling (Handrigan and Richman, 2010a). However, a requirement for *Shh*

signaling during successional lamina induction is ruled out by a lack of *Ptc1* expression in the lamina, which develops normally following cyclopamine treatment. The bearded dragon differs to other squamates in its evolutionary reduction in tooth replacement capacity and vestigial teeth, which are typically aborted. It is therefore of interest that the adverse dental phenotypes of cyclopamine-treated pythons and geckos resemble the superficial teeth of bearded dragons. Reduced responsiveness to *Shh* signaling in bearded dragon tooth morphogenesis, marked by the absence of *Ptc1* expression, may contribute to the premature abortion of their vestigial teeth and prove representative of a characteristic evolutionary trait (Handrigan and Richman, 2010a). Developmental arrest of the successional lamina in bearded dragons is further supported by studies showing an absence of the expression of the Wnt readout genes *Axin2* and *Tcf7*, potentially further marking its transient nature (Richman and Handrigan, 2011).

Studies of reptilian tooth replacement have further integrated hedgehog with Wnt and BMP signaling to reveal their evolutionary conservation and putative interactions. In the python dentition, Wnt signaling is active throughout tooth initiation and replacement (Handrigan and Richman, 2010b). *Lef1* and *Axin2* are consistently expressed in the primary and successional lamina, accompanied by the ligands *Wnt6* and *Wnt10b*, suggesting a role for sustained canonical Wnt signaling in both primary tooth initiation and replacement tooth budding. BrdU labeling of proliferating *Lef1*/*Axin2*+ cells in the dental and successional lamina suggests a role for Wnt signaling in promoting cell proliferation to maintain continuous tooth replacement. This is evident in dental explants treated with LiCl, which stimulates Wnt signaling by inhibiting GSK3*β*, resulting in increased dental epithelial cell proliferation. In the python dentition, hedgehog and Wnt gene expression occupy complementary domains, implying cross talk between the two pathways. Here, hedgehog signaling is proposed to restrict Wnt activity to the dental epithelium during tooth replacement, by negatively regulating mesenchymal *Bmp4*. This is shown by exposure of dental explants to cyclopamine, which downregulates *Ptc1* expression, while upregulating *Lef1*. *Bmp2* and *4*-soaked beads also induce Wnt signaling in dental mesenchymal implants, collectively implying a circuit in which

Shh restricts Wnt activity to the dental lamina, while BMPs induce Wnt activity in the primary and successional lamina (Handrigan and Richman, 2010b).

The reptilian dentition has also provided comprehensive insights into the role of dental stem cells in continuous tooth replacement (Handrigan *et al*., 2010). In the leopard gecko, BrdU labeling of cells housed within the lingual aspect of the dental lamina identifies label-retaining cells (LRCs) exhibiting low rates of cell proliferation. These cells do not contribute to morphogenesis, are organised into high-density pockets in close proximity to the successional lamina and express the stem cell markers *Lgr5*, *Dkk3* and *Igfbp5*. These LRCs are also responsive to induced activation by canonical Wnt signaling, suggesting a population of putative dental epithelial stem cells, the immediate progeny of which contribute to formation of the successional lamina and replacement teeth. 3D reconstructions imply individual clusters of LRCs to be positioned in the inter-dental region, from which progenitors may contribute to tooth replacement by feeding into two adjacent tooth families (Handrigan *et al*., 2010).

The alligator dental lamina also shows complex compartmentalisation, forming a bulge at the distal end, where putative dental stem cells expressing *β-catenin* are thought to contribute to replacement tooth cycling (Wu *et al*., 2013). Here, BrdU labeling also identifies slow-cycling cells (SCCs) localised to the distal tip, where the number of transit amplifying cells increases during replacement tooth initiation and decreases toward the end of replacement growth phases. Their putative role in tooth replacement is further shown by physical extraction of the functional tooth, which activates these SCCs to initiate the tooth cycle. In alligator mandibular explants, overexpression of *Wnt3a* induces cell proliferation in the lamina and causes bulge expansion, while addition of the Wnt inhibitor sFRP1 reduces replacement tooth size. These results suggest that both may regulate cell cycling in the dental lamina by inducing or inhibiting the conversion of dental stem cells to transit amplifying cells during tooth replacement (Wu *et al*., 2013).

In the corn snake dentition, cells expressing *Sox2* localise to the lingual aboral dental lamina and oral epithelium, both linked together by the intervening dental epithelium

(Gaete and Tucker, 2013). At the oral surface, *Sox2* is proposed to mark a putative dental SCN from which progenitor cells proliferate into the SL to maintain tooth replacement capacity (Fig. 1.7). However, *Sox2* is not expressed in the lingual aspect of the dental lamina, where *Lef1* is expressed. The organisation of *Sox2* and *Lef1* into two separate domains in the dental lamina suggests a regulatory mechanism in which Wnt-*β-catenin* signaling inhibits *Sox2* expression to regulate compartmentalisation of immediate progenitor cells marked for odontogenic fates. This is further supported by DiI labeling of cells within the successional lamina, which shows some to contribute to the next tooth generation, while a subset remain in the lamina. The critical role of Wnt-*β-catenin* signaling in this process is made apparent through targeted inactivation of GSK3*β*, resulting in increased numbers of developing tooth germs and associated disruption of normal polarity of tooth initiation and cell differentiation. The putative interaction of *Lef1* and *Sox2* is made further apparent by the expansion of *Lef1* expression domains and corresponding reduction in *Sox2* domains, restricted to the oral epithelium. This study therefore provides further evidence to support the deployment of putative dental stem cells in polyphyodont tooth replacement and a role for Wnt-*β-catenin* signaling in maintaining the balance between pluripotency and induced cell proliferation in the successional lamina (Gaete and Tucker, 2013).

In several reptilian species, including the American alligator, green iguana, leopard gecko and ball python, *Sox2* is also expressed in the dental lamina connecting the tooth to the oral epithelium (Juuri *et al*., 2013). In these species, during first generation tooth development, a continuous stripe of *Sox2*+ cells extends from the oral epithelium into the lingual aspect of the dental lamina via a continuous epithelial connection. In the alligator and iguana, *Sox2* is expressed in the oral surface and outer enamel epithelium terminating lingually to the first generation tooth. These expression domains are maintained during development of the successional lamina, prior to its detachment from early replacement teeth. In the early snake dentition, *Sox2* is expressed symmetrically on both sides of the dental lamina and absent from the free end of the successional lamina, which actively proliferates to produce the next generation tooth. These expression patterns are similar to those in the early ferret dentition, implying a conserved role for *Sox2* in conferring early dental

competence and maintenance of progenitors sequestered for odontogenic fates, possibly through negative regulation of successional tooth formation (Juuri *et al*., 2013). Taken as a strong marker of 'stemness', the conserved expression of *Sox2* within the regenerative component of several gnathostome dentitions is therefore further indicative of a common strategy involving the reutilisation of the same set of core regulatory pathways to perpetuate this extraordinary process (Juuri *et al*., 2012; 2013; Gaete and Tucker, 2013; Fraser *et al*., 2013; Abduweli *et al*., 2014).

1.3 Chondrichthyan tooth development and regeneration

1.3.1 The shark tooth conveyor belt

In contrast with the 'one-for-one' tooth replacement systems found in osteichthyans, elasmobranchs (sharks and rays) have evolved a unique 'many-for-one' system (Reif, 1976; Reif, 1980; Summers, 2000), in which multiple teeth develop in advance of function as a dental conveyor belt. These differ to the compound tooth plates of Chimaeroid (Holocephalan) fishes, which comprise a single mandibular pair in the lower jaw and two pairs in the upper jaw (Didier *et al*., 1994). Rather than being shed or replaced, chimaeroid teeth develop continuously from the oral margin, frequently stacking up at the occlusional surface to form hypermineralised tooth plates, which are continuously worn away (e.g. the extinct genus *Janassa*) (Didier *et al*., 1994). Elasmobranch 'many-for-one' tooth replacement is most commonly recognised in the shark dentition, in which replacement teeth are typically arranged as sequential rows, or 'families', developing outward across the jaw arc (Reif, 1980). As replacement teeth move forward, they are shed at the oral margin independently of wear and replaced by a successive functional tooth (Reif, 1980). This replacement strategy is facilitated by the lyodont nature of chondrichthyan teeth, which are anchored not in bone, but superficially in a continuous sheet of connective tissue in the jaw cartilage. This replacement system therefore ensures an uninterrupted supply of functional teeth to replace those frequently lost or damaged during vigorous feeding activity (Moss, 1967).

Some of the 'many-for-one' phenotypes common to sharks and rays are illustrated in Fig. 1.8. Imaging by x-ray radiography (Fig. 1.8A, horizontal) and dried specimen (Fig. 1.8B, lingual) of the unicuspid replacement dentition of the blacktip reef shark (*Carcharhinus melanopterus*) shows typical shark tooth replacement, in which individual adjacent rows, initially recumbent with the oral surface, develop outward along the jaw, assuming vertical orientation at the oral margin where they become functional. Similarly, in the grey reef shark (*C.amblyrhinchus*) dentition (Fig. 1.8C, horizontal) adjacent tooth rows also develop in close proximity as ordered arrays, therefore further demonstrating a considerable degree of spatiotemporal patterning control. Micro-CT imaging of the catshark (*S.canicula*) dentition (Fig. 1.8D, horizontal) shows some variation in dental phenotype and arrangement, with individual families of tricuspid teeth staggered as alternating rows. Micro-CT of the thornback ray (*Raja clavata*) dentition (Fig. 1.8E) further illustrates the fuller extent of elasmobranch polyphyodonty, with flattened teeth arranged as a continuous interlocking crushing surface, specialised for feeding on hard-shelled prey (durophagy) (Smith, 2003; Summers, 2000).

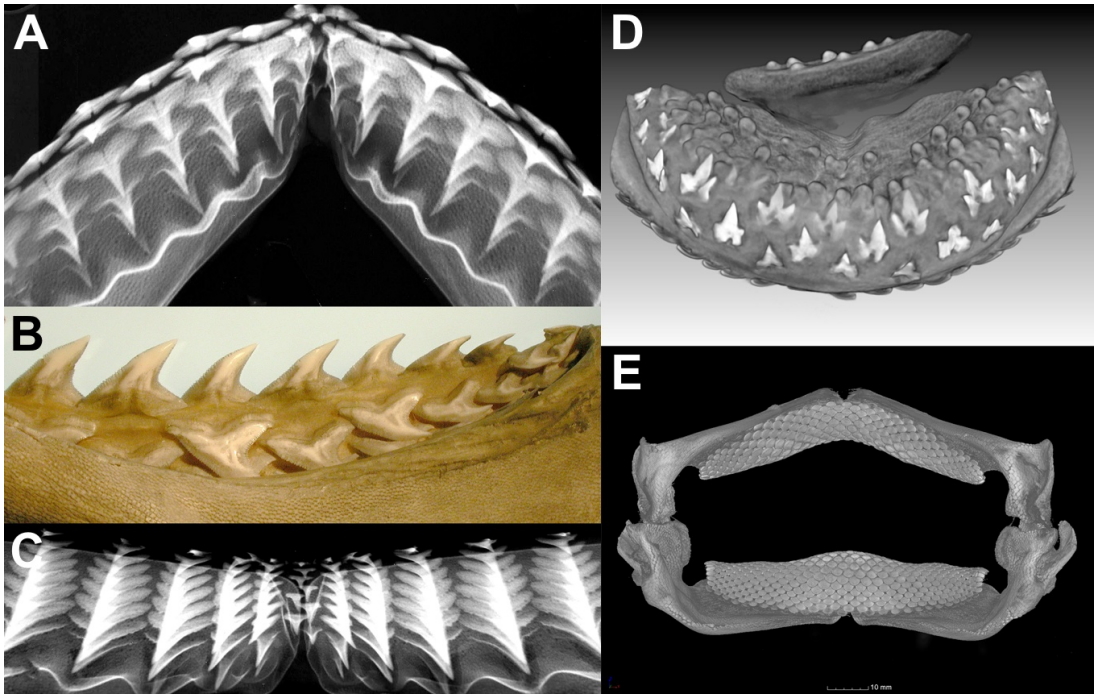


Figure 1.8 Elasmobranch tooth replacement diversity. X-ray radiograph (A, horizontal) and dried specimen (B, lingual) of tooth replacement morphology in the blacktip reef shark (*Carcharhinus melanopterus*). In *C.melanopterus*, replacement tooth rows (families) develop outward across the jaw arc, with functional teeth (vertical) at the oral margin followed by a successive series of closely spaced, replacement teeth. This 'many-for-one' tooth replacement strategy is further illustrated by similar radiographs of the grey reef shark (*C.amblyrhinchus*) dentition, in which adjacent replacement tooth rows develop in close proximity, while retaining developmental pattern through maintenance of precise spatial boundaries (C, lingual). In contrast with the reef shark's unicuspid teeth, Micro-CT imaging of the small-spotted catshark (*Scyliorhinus canicula*) dentition (D, horizontal) shows an alternating series of tricuspid tooth families, therefore showing variation of a conserved developmental mechanism deemed representative of the ancient state of chondrichthyans (Smith, 2003). This is further apparent in the thornback ray (*Raja clavata*) dentition (E) in which replacement teeth form a continuous, interlocking crushing surface specialised for feeding on hard-shelled prey (durophagy). In sharks and rays, individualistic variations in dental phenotype are therefore united by a many-for-one tooth replacement system common to extant and extinct species. Images (A-C, E) kindly provided by Moya Smith, NHM London. Specimen for image (D) prepared within the University of Sheffield and micro-CT carried out by Brian Metscher, University of Vienna.

In extant sharks and rays, these individual specialisations in dental phenotype and replacement strategy are considered derivatives of a many-for-one system representative of ancient chondrichthyans (Smith, 2003). While current evidence suggests the basic biomechanical principles of tooth replacement to be conserved among the majority of elasmobranchs, replacement rates can vary between species. For example, the catshark undergoes a replacement cycle every 18-38 days, while the adult leopard shark (*Triakis semifasciata*) and young lemon shark (*Negaprion brevirostris*), replace their teeth every 9-12 and 10-14 days, respectively (Reif *et al*., 1978; Reif, 1980; Moss, 1967). Tooth replacement rates also show seasonal variations, as seen in juvenile nurse sharks (Order orectolobiformes), which undergo increased replacement during seasonal periods coincident with increased water temperature. This is presumed to result from periods of increased activity and therefore food consumption during summer months. Accelerated growth and changing metabolic requirements are also suggested to contribute to increased replacement rates during these periods (Luer *et al*., 1990).

While elasmobranch tooth morphology has been investigated in several species, the majority of knowledge of shark tooth development has come from the embryonic catshark (Reif, 1976; 1980; Smith *et al*., 2009a; 2009b). Early dental competence is marked by localised thickening of the oral epithelium and condensation of the underlying mesenchyme adjacent to the dorsal-medial aspects of the palatoquadrate (upper) and Meckel's (lower) jaw cartilages, respectively (Fig. 1.9A) (Reif, 1980; Smith *et al*., 2009a). Shown here in the lower jaw (Fig. 1.9A, boxed area), this restricted band of columnar epithelial cells and associated mesenchymal condensates defines the extent of the odontogenic band (OB), as morphologically distinct from the surrounding oral epithelium and mesenchyme (Fig. 1.9B, dotted line) (Buchtová *et al*., 2008; Smith *et al*., 2009a). This thickened epithelium subsequently invaginates into the mesenchyme, forming the primary dental lamina (DL) (Fig. 1.9C). Subsequent inward extension of the dental epithelium into the mesenchyme is accompanied by formation of early first generation tooth germs, marking development of the primary dentition (Fig. 1.9D, T1). Primary teeth (Fig. 1.9E, T1) develop linguo-labially toward the oral margin, accompanied by further extension posteriorally, of the free end of the dental lamina. The developmental transition from

the primary to successional lamina (SL) is defined by initiation of second-generation tooth placodes (Fig. 1.9E, T2). The dental lamina is further partitioned into several cellular domains, which in continuous association with the dental mesenchyme (DM) comprise the inner dental epithelium (IDE) from which teeth develop, intervening outer dental epithelium (ODE) and surrounding middle dental epithelium (MDE) (Fig. 1.9E). The successional lamina and herein named adjoining 'outward-outer dental epithelium' (O-ODE) further define the maintenance of a continuous connection with the overlying oral epithelium (Fig. 1.9E) (Smith *et al*., 2009b). Within the successional lamina, replacement teeth continue to progress through these characteristic stages, shown here at bud stage (Fig. 1.9F, T3) and advanced morphogenesis, as defined by cytodifferentiation and early secretion of mineralised hard tissue matrices (Fig.1.9F, T2). This includes a superficial outer layer of enameloid and inner layer of dentin, surrounding the pulpal mesenchymal papilla. Following formation of the basal plate, the functional tooth erupts into the oral cavity (not shown) (Moss, 1967; Reif *et al*., 1978; Reif, 1976; 1978; 1980; 1982; 1984).

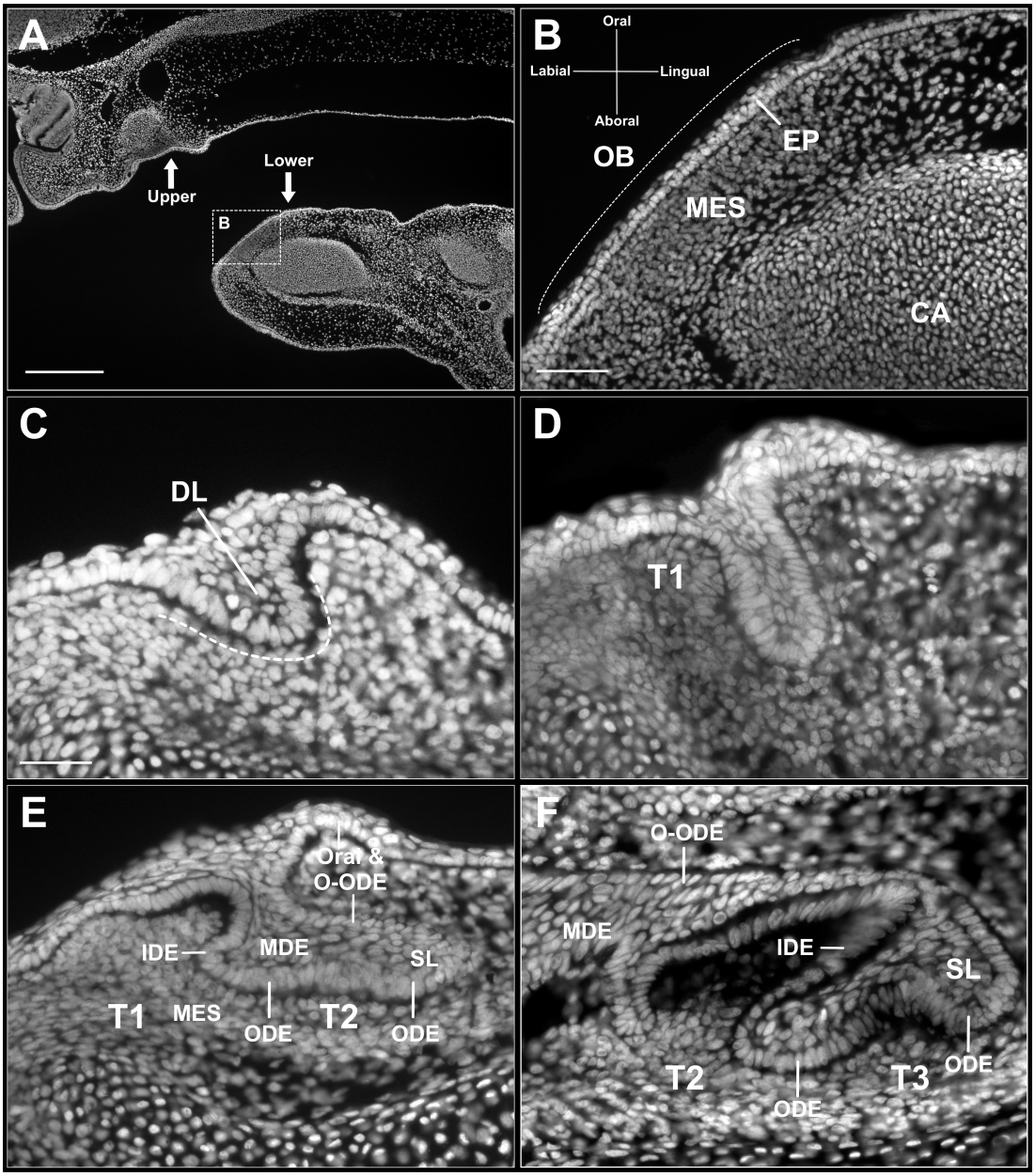


Figure 1.9 Shark tooth development and replacement (DAPI). In the catshark, teeth initiate from a restricted region of thickened oral epithelium (EP) and underlying condensing mesenchyme (MES) positioned adjacently to the upper and lower jaw cartilages (CA) (A) (Reif, 1980; Smith *et al*., 2009a). Shown in the lower jaw (A, boxed area), these epithelial-mesenchymal tissue layers define the extent of early dental competence: the odontogenic band (OB) (B, dotted line) (Buchtová *et al*., 2008; Smith *et al*., 2009a). Initiation of the primary dental lamina (DL) is marked by invagination of the prospective dental epithelium into the mesenchyme (C, dotted line). This invagination continues, followed by initiation of early first generation teeth (D, T1). Within the DL, teeth progress through a characteristic series of stages, shown here at morphogenesis (E, T1) (Reif, 1980). The DL itself consists of several distinct cellular domains, which in continued association with the dental mesenchyme (DM) include the tooth-forming inner dental epithelium (IDE), adjoining outer dental epithelium (ODE) and surrounding middle dental epithelium (MDE) (E-F). The full extent of the DL further defines a continuous epithelial

connection maintained between the dental and overlying oral epithelium: the 'outward-outer dental epithelium' (O-ODE) (E-F). Early second-generation tooth placodes (T2) initiate from the thickened dental epithelium, continuous with the ODE and free end of the DL, which extends posteriorally to form the successional lamina (SL) (E) (Smith *et al*., 2009b). Within the SL, replacement teeth continue to progress through these characteristic stages (F), shown here during bud stage (T3) and advanced morphogenesis (T2). As replacement teeth near the outer jaw margin, mineralised hard-tissue matrices are secreted, accompanied by basal plate formation and eruption of the functional tooth into the oral cavity (not shown) (Moss, 1967; Reif *et al*., 1978; Reif, 1976; 1978; 1980; 1982; 1984). Scale bars: (A) 1 mm, (B) 100 μm, (C-F) 100 μm.

Given the unique polyphyodont dentition and important phylogenetic position of sharks and rays, it is surprising that they have received little attention as tooth replacement models. This could in part be attributed to the relative inaccessibility of the advancing embryo for molecular developmental studies. However, due to the increasing availability of versatile embryological methods and genomic resources, elasmobranchs are progressively entering the mainstream of developmental biology (Takechi *et al.,* 2011; Wang *et al*., 2012). Three particularly promising emerging developmental models include the small-spotted catshark (family Scyliorhinidae), thornback ray and little skate (family Rajidae), which given their small size and experimental amenability, are well suited to studies of elasmobranch embryology. In accordance with their larger cartilaginous kin, both undergo continuous 'many-for-one' tooth replacement and possess contrasting dental phenotypes, providing excellent comparative models to study elasmobranch tooth regeneration. They also possess extensive evolutionary histories, having appeared during the Jurassic and Cretaceous Periods, respectively (Klimley, 2013). These combined attributes therefore provide the means by which to investigate the ancient state of the polyphyodont dentition relative to successive gnathostomes (Fig. 1.10), and so assess the fuller extent of its evolutionary-developmental modification (Reif, 1980; Summers, 2000; Smith, 2003, Smith *et al*., 2009a; 2009b).

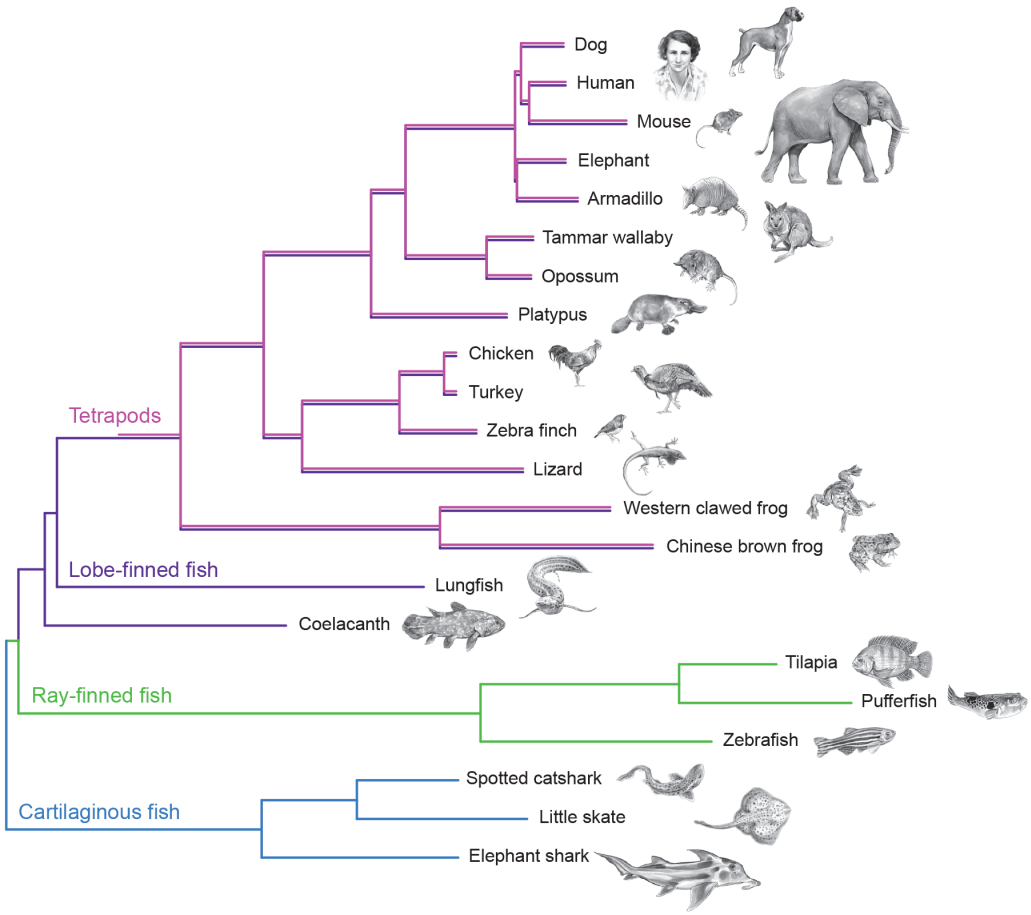


Fig. 1.10 The phylogenetic position of chondrichthyans (cartilaginous fish) relative to other gnathostomes. Given their comparatively basal position, the cartilaginous fishes (sharks, rays and holocephalans) offer a uniquely placed lineage of extant gnathostomes to investigate the ancient state of tooth development (Smith, 2003). As representative elasmobranchs (sharks and rays), the Scyliorhinidae (catsharks) and Rajidae (rays and skates) further provide ideal developmental models to investigate gene regulatory control of 'many for one' tooth regeneration (Smith *et al*., 2009a; Debiais-Thibaud *et al*., 2011). Given their extensive evolutionary histories, both also offer the potential for valuable insights into how the same conserved gene circuits may have been modified during the course of gnathostome evolution, concomitant with its apparent reduction in tooth regenerative capacity (Jernvall and Thesleff, 2012). Figure modified from Amemiya *et al*., 2013.

1.3.2 Molecular patterning of the shark dentition and the involvement of putative dental stem cells

In the catshark dentition, the transition from primary to successional dental lamina is short (Fig. 1.9E) and tooth replacement is frequent. While the first replacement teeth are non-functional and typically aborted, hatchlings possess at least four generations of replacement teeth, with those erupted incorporating extensive matrices of enameloid and dentin (Reif *et al*., 1978; Reif, 1980). In stark contrast with squamates and crocodilians, the frequency of shark tooth replacement is therefore indicative of a comparatively vigorous regenerative system (Edmund, 1960; 1962; Moss, 1967). In this respect, the arrangement of individual tooth families as highly ordered arrays implies shark tooth replacement to be tightly orchestrated, with replacement teeth developing reiteratively in close proximity while maintaining precise spatial boundaries and predictable eruption times (Smith *et al*., 2009a). The sequential addition model (SAM) attempts to account for this by proposing a putative mechanism in which individual tooth families develop from a single tooth primordium, which acts as an initial signaling center to determine subsequent tooth pattern. In this model, restricted bands of gene expression define the primary epithelial competence required to set in motion the linear, unidirectional development and sequential addition of each tooth row within the median, left and right hand regions of the jaw (Smith, 2003). This is shown by recent investigation of *Shh* expression in the catshark dentition, which provides some evidence to support both the SAM and the deployment of conserved odontogenic pathways in ancient gnathostomes. During catshark tooth development, *Shh* is expressed in the early epithelial thickenings coincident with the OB, subsequently localising to the dental lamina to determine the positions of future tooth loci, before reappearing in the developing tooth cusps during morphogenesis (Smith *et al*., 2009a). While less clear in terms of specific roles, the expression of *Shh*, *Epha4*, *Runx1/3* and several members of the *Dlx* family in catshark tooth and denticle development (Table 1.1) provides further evidence to support the deep conservation in elasmobranchs, of odontogenic genes and pathways (Johanson *et al*., 2008; Freitas and Cohn, 2004; Hecht *et al*., 2008; Debiais-Thibaud *et al*., 2011).

Table 1.1 Gene expression in the teeth and dermal denticles of sharks.

Gene	Tooth	Denticle
Shh	✓	✓
Epha4	✓	✓
Runx1-3	✓	✓
Dlx1	✓	✓
Dlx2	✗	✓
Dlx3	✓	✓
Dlx4	✓	✓
Dlx5	✓	✓

Existing developmental studies of embryonic catsharks show the conservation of odontogenic genes in tooth and dermal denticle development. Given the comparatively basal phylogenetic position of extant sharks compared with other gnathostomes, these studies therefore provide provisional evidence to support the deeply conserved odontogenic roles of these genes (Smith *et al*., 2009a; Johanson *et al*., 2008; Freitas and Cohn, 2004; Hecht *et al*., 2008; Debiais-Thibaud *et al*., 2011).

While these provisional studies have proven informative, comprehensive understanding of regulatory control, both in terms of canonical signaling pathways and the regulation of stem cells, in chondrichthyan tooth replacement remains limited. From observations of zebrafish and cichlid tooth replacement it has been proposed that dental stem cells may be contained in the epithelium (successional lamina) from which new tooth buds develop (Huysseune and Thesleff, 2004). This supposition is based upon the fact that in the zebrafish pharyngeal dentition, replacement teeth develop from an epithelial lamina resembling the intestinal crypt of the gut (Huysseune and Sire, 2004). As discussed, from recent molecular studies in the cichlid dentition, Fraser and colleagues (2013) offer an alternative scenario in which the source of stemness originates from the labial oral surface epithelium, a supposition further supported by similar studies of the reptile dentition (Fraser *et al*., 2013; Gaete and Tucker, 2013).

Despite some ongoing disparity over the source of dental stem cells, these studies continue to emphasise their importance in maintaining tooth regenerative capacity. Linkage of conserved signaling pathways to the deployment patterns of dental stem cells therefore offers a crucial vantage point from which to obtain a global perspective of the gene network regulating tooth regeneration. In this respect, routine histological investigation of the catshark dentition has led to some intriguing hypotheses regarding possible sources of progenitor cells and associated sites of regulatory control (Smith *et al*., 2009b). In the catshark dentition, this is further highlighted by subdivision of the dental lamina into several cellular domains (Fig. 1.9E). Of these, the outward-outer dental epithelium (O-ODE) is of particular interest, given its continuous connection with the successional lamina and oral surface (Fig. 1.9E). This oral-dental epithelial connection is established during development of the lamina (Fig. 1.9C-D) and is maintained during early tooth replacement. While this may change during subsequent ontogeny, the formation and maintenance of this epithelial connection strongly resembles those in the ferret and reptilian dentitions, implying a conserved role in establishing early dental competence, and possibly in early tooth replacement (Smith *et al*., 2009b; Juuri *et al*., 2013; Gaete and Tucker, 2013).

During shark tooth initiation, it has been proposed that dental stem cells are first deployed in the OB (marked by *Shh*) to later become set aside as progenitors for tooth production (Smith *et al*., 2009b). The OB is therefore viewed as a dental lamina primordium fated to develop into the epithelial compartment required to retain dental progenitor cells. Given the crucial role of the successional lamina in maintaining tooth replacement capacity, Smith and colleagues (2009b) have proposed putative dental stem cells to reside within the MDE of the lamina, where they are activated by regulatory signals to undergo tooth-specific fates. Integral to this is the associated hypothesis that regulatory control of tooth replacement (and therefore stem cell fates) may be orchestrated either from the junction of the adjoining oral and dental epithelium (hypothesis 1) or at the growth extension; the successional lamina (hypothesis 2) (Fig. 1.11) (Smith *et al*., 2009b).

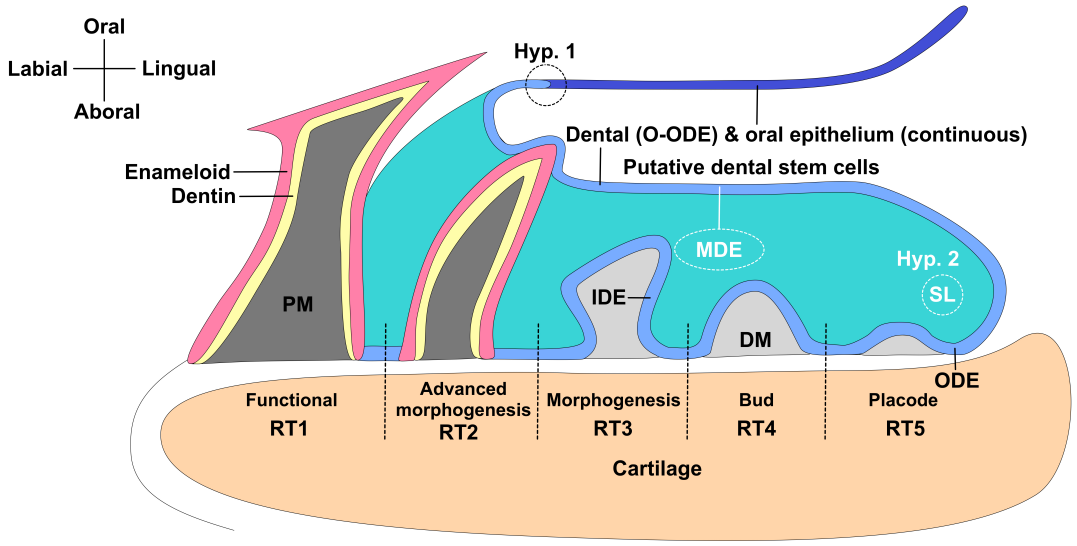


Figure 1.11 Proposed sites of gene regulatory control and dental stem cells in the shark dentition. In hypothesis 1, regulatory control is proposed to occur at the junction between the adjoining oral and dental epithelium ('O-ODE'). Alternatively, in hypothesis 2, this is proposed to occur within the growth extension (successional lamina). Common to both is the source of dental stem cells, distributed throughout the intermediate middle dental epithelium (MDE) of the dental lamina (Smith *et al*., 2009b).

1.4 Aims, objectives and rationale of work presented in this thesis

These hypotheses represent an interesting coalescence of ideas drawn from provisional molecular studies and associated histology (Smith *et al*., 2009a; 2009b). When considered in conjunction with existing developmental studies of the osteichthyan and reptilian dentitions, it can be further hypothesised that in elasmobranchs, such as sharks and rays, a common regenerative strategy couples odontogenic gene expression to stem regulatory control of tooth replacement (Fraser *et al*., 2006a; 2006b; 2013; Abduweli *et al*., 2014; Buchtová *et al*., 2008; Handrigan and Richman, 2010a; 2010b; Wu *et al*., 2013; Gaete and Tucker, 2013; Juuri *et al*., 2013). With this existing knowledge in hand, current hypotheses can be further developed to define the principle aims and objectives of this study. These are:

- To use gene expression to identify putative dental stem cells in the embryonic shark and ray dentitions and to compare and contrast these expression patterns between elasmobranchs and successive gnathostomes, in order to infer their ancestrally conserved roles in 'many-for-one' tooth replacement.

- To use gene expression to identify conserved signaling pathways in the shark and ray dentitions and compare and contrast their expression patterns, so as to infer the extent to which their roles may also be conserved between elasmobranchs and successive gnathostomes.

- To use gene expression to investigate the role of conserved signaling pathways in denticle development and compare and contrast these with teeth, so as to infer how the same core set of genes may have been co-opted during vertebrate evolution to promote the evolution of odontodes.

- To use cell lineage tracing and chemical manipulation techniques to further investigate the roles of putative dental stem cells and conserved signaling pathways in shark tooth regeneration and denticle development.

Chapter 2

MATERIALS AND METHODS

2.1 Common Reagents and Buffers

BBR	Boehringer Blocking Reagent (Roche) 10g in 100ml MAB; autoclave; store at -20°C
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate; 500µl/l solution being treated; leave overnight then autoclave
ddH_2O	Double-distilled Water
DIG	Digoxygenin
dNTPs	Stock of dATP, dCTP, dGTP, dTTP; each 25µmol stocks (Roche)
DTT	Dithiothreatol
EtOH	Ethanol
FGS	Foetal Goat Serum
Gelatin/Albumin	2.2g gelatin in 450ml PBS, heat to dissolve then cool; add 135g chick egg albumin; stir three hours; add 90g sucrose; store at -20°C
H_2O	Water
H_2O_2	Hydrogen peroxide
KOH	Potassium Hydroxide
LB	Luria-Bertani media; 5g tryptone (Difco); 2.5g yeast (Difco); 5g NaCl; made up to 500ml with dH_2O
LB agar	7.5g agar (Difco) in 500ml LB; autoclave.
MAB	100mM maleic acid; 150mM NaCl; 15g NaOH; pH 7.5; autoclave
MABT	MAB + 1% Tween-20
MS 222	Tricaine Methanesulfonate
NTMT	1ml 5M NaCl; 2.5ml 2M Tris-HCl (pH 9.5); 1.25ml 2M $MgCl_2$; 10% Tween-20; make up to 50ml with H_2O; use fresh, do not store.
PBS	10 tablets (Oxoid; 0.16M NaCl; 3mM KCl; 8mM Na_2HPO_4; 1mM KH_2PO_4 (pH 7.3)) in 1 litre H_2O; autoclave
PBST	PBS + 0.1% Tween-20

4% PFA	Paraformaldehyde: 4g PFA in 100ml PBS (pH 7.4); store -20°C
TBS	Tris-buffered saline: 60g Tris; 88g NaCl (pH 6.0); make up to 1 litre with ddH_2O
TST	18ml 5M NaCl; 5ml 2M Tris-HCl (pH 9.5); 1.8ml Tween-20; make up to 1.8 litre ddH_2O
20xSSC	175.3g NaCl; 88.2g trisodium citrate (pH 7 with citric acid); make up to 1 litre ddH_2O; autoclave
5xTBE	54g Tris; 25.5g ortho-boric acid; 20ml 0.5M EDTA (pH 8); make up to 1 litre with ddH_2O

2.2 Embryos

2.2.1 Storage, staging and fixation of embryos

Scyliorhinus canicula embryos were collected from sites along the Menai Strait region, UK and sourced from Station Biologique, Roscoff, France. *Scyliorhinus Stellaris* and *Raja clavata* embryos were sourced from Native Marine Centre Ltd., Portland, UK. *Leucoraja erinacea* embryos were kindly donated by Dr. Andrew Gillis. Embryos were transported in seawater to the University of Sheffield, Department of Animal and Plant Sciences, where they were transferred to a re-circulating aquarium in seawater at 16°C and allowed to develop to the required stages of development. For studies of tooth development, embryos were typically collected at Stages 18-20, coincidental with initiation of the dental lamina and early tooth germs, and Stages 23-35 for tooth replacement. For studies of denticle development, embryos were typically collected at Stages 24-33, coincidental with development of early denticle primordia. Embryos were staged in accordance with those described by Reif (1980). Embryos were collected by removal from egg casings and euthanised (0.17mg/ml MS 222 (tricaine); Sigma-Aldrich, in normal seawater) prior to fixation (Westerfield 1995). Following euthanisation, yolk sacs were removed using a sterile scalpel and embryos fixed overnight in 4% Paraformaldehyde; Sigma-Aldrich, in PBS, and stored at 4°C.

2.2.2 Tissue preparations

Following fixation, embryos were dissected by removal and bisection of the head, depending upon the stage. All dissecting tools and surfaces were first treated with RNaseZAP™ (Sigma-Aldrich) to remove contaminating RNases. Specimens processed for paraffin section *in situ* hybridisation and immunohistochemistry were rinsed twice, and washed three times, for 10 minutes with PBST and dehydrated by washing for 10 minutes each in a graded series of ethanol (25%, 50%, 75% ethanol in DEPC-PBST), followed by two, 5 minute washes in %100 ethanol. Tissue was then bleached with 3% hydrogen peroxide (H_2O_2) in ethanol for three, 5 minute washes to remove residual blood and reduce pigmentation, washed twice with 100% ethanol, and stored in 100% ethanol at least overnight at -28°C.

2.3 Histological Methods

2.3.1 Paraffin embedding and sectioning

Embryos were collected, fixed and processed to 100% ethanol as described. Specimens were transferred to mesh containers and processed for paraffin embedding, first by decalcification of tissue and 3 changes of 70% alcohol over 24 hours, and 3 changes of 90% alcohol over 24 hours. Specimens were then washed 3 times in absolute alcohol, each for 2 hours, followed by 2 washes in chloroform and left in chloroform overnight. To remove traces of the alcohol and chloroform, specimens were transferred through three, 30 minute washes of hot wax, followed by the final wax, orientation and embedding. Wax blocks containing the specimens were embedded in moulds, set on a cooled bench and stored overnight at 4°C. Sections were cut in sagittal and horizontal plane at a thickness of 14μm, using a microtome (Leica RM2145) with a Leica disposable blade. Sections were placed onto a drop of pre-heated (42°C) autoclaved DEPC-H_2O, on Superfrost Plus glass slides (Menzel-Gläser/ Thermo Scientific), and left to dry and adhere to the slides on a heated plate at 42°C overnight.

2.3.2 Whole mount alizarin red staining

Embryos were fixed for 24 hours (4% PFA/PBS) prior to skeletal preparation, washed 3 times, for 5 minutes in PBS and 3 times, for 5 minutes in ddH_2O. Specimens were then neutralised by washing through a graded series of sodium borate in ddH_2O (10%, 20%, 30%) and digested in 0.5% *(w/v)* trypsin in 30% sodium borate in ddH_2O until epidermis and connective tissue were sufficiently cleared. Following initial clearing, specimens were washed three times, for 5 minutes in 30% sodium borate in ddH_2O and placed in a solution of 0.01% (*w/v*) alizarin red (Sigma-Aldrich) in 0.5% KOH in ddH_2O until sufficiently stained (typically 24-48 hours depending upon the stage). After alizarin staining, the surrounding tissue was de-stained by washing three times, for 5 minutes in 0.5% KOH in ddH_2O. If required, specimens were then further cleared by placing in a solution containing 0.5% trypsin (*w/v*) in 30% sodium borate in ddH_2O and then in 0.5% of KOH in ddH_2O, until tissue was sufficiently cleared to observe internal detail. The final

clearing step included washing the specimens through a graded series of glycerol in 0.5% (*w/v*) KOH in ddH_2O (25%, 50%, 75% and 100%); Specimens were left in each grade for 24 hours to equilibrate by sinking and stored at room temperature in 100% glycerol for subsequent imaging. Whole mount skeletal preparations were analysed at low-power using a stereo microscope (Leica M125) and photographed with a Nikon *Coolpix*® 4500 digital camera attachment. High-power microscopy of skeletal preparations was carried out using a high-power compound microscope (Olympus BX51) and photographed with an Olympus DP71 Universal digital camera attachment. Images were viewed using Adobe® Photoshop CS5 Extended (v. 12.0 x 64).

2.4 Molecular Methods

2.4.1 RNA isolation

S.canicula embryos were staged in accordance with Reif (1980), prior to storage at -80°C in 50 ml screw-cap tubes. For isolation of RNA, fresh whole *S.canicula* embryos were homogenised for 5 minutes at 25Hz using a TissueLyser (Qiagen), in 1 ml of TRIzol® Reagent (GibcoBRL) with 1 x 5mm stainless steel bead to effect tissue disruption. Tissue was further homogenised to assist breakup of genomic DNA using a QiaShredder column (Qiagen) according to the manufacturers instructions. Insoluble tissue was removed from the sample by centrifugation at 13,000 x rpm for 2 minutes at RT, leaving a pellet, with the remaining supernatant containing the RNA. The homogenised samples were transferred to a 1.5 ml RNase-free tube and 200µl of chloroform added per 1 ml of TRIzol® Reagent, inverted for 15 seconds and incubated for 2-3 minutes at RT. After centrifugation at 4,000 x rpm at 4°C for 15 minutes the layers separate and RNA remains in the upper colourless aqueous layer. The aqueous phase was transferred to a fresh RNase-free tube, adding 500 µl of isopropanol per 1 ml of TRIzol® originally used, mixed well by inverting 5 times and incubated for 30 minutes at RT, then centrifuged for 15 minutes at 13,000 x rpm, when a gel-like pellet forms. The pellet was washed with 1 ml 75% ethanol (1:1 of original TRIzol® used), re-suspended by flicking the tube and centrifuged at 13,000 x rpm for 5 minutes at 4°C. The ethanol was removed and the pellet was then left to

air-dry for 5-10 minutes. The RNA pellet was re-dissolved in RNase-free H_2O and cleaned using an RNeasy MiniElute Cleanup Kit (Qiagen) according to the manufacturers instructions and stored at -80°C. Approximate concentrations of RNA samples were measured using an Agilent 2100 Bioanalyser.

2.4.2 cDNA synthesis

First strand cDNA was synthesised using an Ambion RETROscript® RT-PCR Kit according to the manufacturers instructions. 4µl of total RNA (~4µg) was added to 2µl of Oligo(DT) and 10µl of Nuclease-free H_2O. The reaction mixture was mixed and spun briefly and incubated at 72°C for 3 minutes. The reaction mixture was placed on ice for 5 minutes and the remaining RT components added, including 2µl of reverse transcriptase 10X reaction buffer, 4µl dNTP mix, 1µl ribonuclease inhibitor and 1µl MMLV-RT to produce a total volume of 24µl. This was mixed gently, spun briefly and incubated for 1 hour at 42°C. The addition of the MMLV Reverse Transcriptase allows cDNA to be generated from a total RNA stock. The reaction was then incubated for 10 minutes at 92°C to inactivate the MMLV. Approximate cDNA concentration was measured using UV spectrophotometry and cDNA stored at -80°C.

2.4.3 Polymerase chain reaction (PCR)

Genes of interest were selected due to their known roles in odontogenesis and stem cell regulation. Primers constructed from previously published chondrichthyan sequences were obtained from the National Centre for Biotechnology (NCBI) website (http://www.ncbi.nlm.nih.gov). Novel chondrichthyan sequences for genes of interest were identified using the Vertebrate Time Capsule Transcriptome Database (VTcap) (http://transcriptome.cdb.riken.go.jp/vtcap) and the North East Bioinformatics Collaborative (NEBC) Transcriptome Database (http://skatebase.org). Novel sequences were identified through alignment of homologous sequences from related vertebrate taxa and putative chondrichthyan sequences viewed and verified by NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1997). Primers for published and novel sequences were

constructed using Primer 3, V.0.4.0. Regions of conserved coding sequence were chosen for prime sites, generally between 18 and 24 bases (6-8 amino acids) in length, either end (5' and 3') of coding sequence attaining the optimum sequence length for isolation and amplification of *S. canicula* cDNA via the polymerase chain reaction (PCR) (see Section 2.4.4). Designed primer sequences were then ordered through Sigma-Aldrich (http://www.sigmaaldrich.com/united-kingdom.html).

2.4.4 PCR procedure

The Polymerase Chain Reaction (PCR) involved the mixture of 6.25µl of one pair of primers (0.4µM concentration of each primer; forward 5' and reverse 3'), with the following: 12.5µl of Qiagen® Multiplex PCR Master Mix (HotStarTaq DNA Polymerase), 1µl of *S.canicula* cDNA (diluted to 1:5 concentration with nuclease-free H_2O) and 5.25µl of nuclease-free H_2O, to produce a final reaction mixture volume of 25µl. The dilution factor of template cDNA required for optimal PCR was established empirically. Positive and negative control reactions were always performed. PCR amplification was carried out using an MJ Research PTC-225 Peltier Thermocycler. Once the tubes were added, a thermal cycling programme of 44 cycles was started:

Activate polymerase	95°C 15 min
Denature	94°C 30 secs
Anneal	60°C 1min 30 secs
Extend	72°C 30 secs

Once the 44 cycles were complete a final step of 72°C for 10 min was added to allow complete extension of the primers. The PCR products were stored at -20°C. Amplified fragments were analysed on a 1.5% agarose TBE gel containing 0.5µg/ml SYBR® Safe (Invitrogen) DNA Gel Stain and correct fragment base pair length was estimated with a 100bp marker ladder (NEB) alongside the PCR products. Gels were viewed under Ultra Violet (UV) light, photographed and bands of correct size excised from the gel using an Invitrogen Safe Imager and DNA recovered using a

Qiagen® gel extraction kit according to the manufacturer's instructions. Approximate DNA concentrations were measured using UV spectrophotometry.

2.4.5 Cloning of PCR fragments (products)

DNA inserts were ligated into the pGEM®-T Easy Vector System I (Promega). The following were added together: 1µl of vector, 5µl of 2X Rapid Ligation Buffer, 3µl of purified insert DNA and 1µl of T4 DNA Ligase. All were mixed and incubated overnight at 4°C. 3µl of the ligation product (plasmid vector plus insert) were transformed into 30µl of competent *Escherichia coli* cells (JM109; Promega), mixed and incubated for 30 minutes on ice, then heat shocked at 42°C for 60 seconds and immediately incubated on ice for a further 2 minutes. 300µl of LB (Luria-Bertani) media was added and the cells were incubated at 37°C for approximately 1 hour. Using a sterile L-shaped glass rod the cells were spread on LB agar plates containing ampicillin (50µg/ml) and incubated overnight at 37°C. Positive transformants were identified by blue-white selection (IPTG-XGAL) and single colonies picked and inoculated into 5 ml LB containing ampicillin (50µg/ml, 1:1000). Liquid cell cultures were then incubated in an orbital shaker at 37°C, 300 rpm overnight. Bacterial cells were harvested by centrifugation at 3440 rpm for 45 minutes at 4°C. Plasmid DNA was recovered using a QIAprep® Spin Plasmid Purification Miniprep Kit according to the manufacturer's instructions. Miniprep plasmid DNA product was analysed on a 1.5% agarose TBE gel containing 0.5µg/ml SYBR® Safe (Invitrogen) DNA Gel Stain and viewed under Ultra Violet (UV) light.

2.4.6 DNA sequencing

Plasmid DNA was sequenced using an Abi 3730 DNA Analyser with BigDye 3.1 cycle sequencing kit, located at the Core Genomics Facility (University of Sheffield Medical School). In order to confirm the degree of homology (percentage identity and coverage) of both published and novel cloned sequences, BLAST searches were carried out using the NBCI website (Altschul *et al.,* 1997). For published sequences, BLAST searches against original sequences were carried out for direct confirmation

of sequence homology. For novel chondrichthyan sequences, the degree of homology was assessed through NCBI BLAST searches against known orthologues conserved amongst vertebrate species.

2.4.7 T7/ SP6 polymerase chain reaction (PCR)

The T7/ SP6 Polymerase Chain Reaction (PCR) of Miniprep plasmid DNA involved the mixture of 2.5µl of T7 and 2.5µl of SP6 Universal Primers (10µM concentration of each primer; forward 5' and reverse 3'), with the following: 12.5µl of GoTaq® Green Master Mix (Promega), 1µl of Miniprep plasmid DNA and 6.5µl of Nuclease-free H_2O, to produce a final reaction mixture volume of 25µl. Positive and negative control reactions were always performed. PCR amplification was carried out using an MJ Research PTC-225 Peltier Thermocycler. Once the tubes were added, a thermal cycling programme of 29 cycles was started:

Activate polymerase	95°C 2 min
Denature	95°C 1 min
Anneal	43°C 1min
Extend	74°C 1 min

Once the 29 cycles were complete, a final step of 74°C for 5 min was added to allow complete extension of the primers. The PCR products were stored at -20°C. Amplified fragments were analysed on a 1.5% agarose TBE gel containing 0.5µg/ml SYBR® Safe (Invitrogen) DNA Gel Stain and correct band sizes estimated with a 100bp marker ladder (NEB) alongside the PCR products. Gels were viewed under Ultra Violet (UV) light and photographed.

2.4.8 Transcription of DIG-labelled RNA probes

The synthesis of RNA probes was performed in RNase-free conditions using DEPC-treated H_2O and RNase-free pipette tips. RNA probes were transcribed from T7/ SP6 PCR product in a total reaction volume of 22µl composed of the following: 10µl DEPC-H_2O, 4µl of 5X transcription buffer, 2µl 10mM DTT, 2µl 10X DIG-RNA

labeling mix (Roche), 1µl (20 units) RNase Inhibitor (Roche), 2µl T7/ SP6 PCR product and 1µl (~20 units) T7/ SP6 polymerase. The reaction was incubated for 2 hours at 37°C; then 2µl (20 units) of DNase I (Roche) added and the reaction vortexed and incubated for a further 15 minutes at 37°C to digest the DNA template. The reaction was then subject to an RNA cleanup procedure using an RNeasy kit (Qiagen) in accordance with the manufacturer's instructions. 5µl of probe was analysed on a 1.5% agarose gel containing 0.5µg/ml SYBR® Safe (Invitrogen) DNA Gel Stain. Gels were viewed under Ultra Violet (UV) light and photographed.

2.4.9 Paraffin section *in situ* hybridisation of RNA probes

In situ hybridisations were all performed on 14µm paraffin-embedded tissue sections mounted on Superfrost Plus glass slides (Menzel-Gläser/ Thermo Scientific). Tissue sections were de-paraffinised by washing once for 10 minutes in Histo-Clear (National Diagnostics) and rehydrated by washing for 5 minutes each, in a graded series of ethanol (100%, 75%, 50% and 25% ethanol/ DEPC-PBST (PBS + 0.1% Tween-20); followed by two, 2 minute washes in DEPC-PBST and two, 2 minute washes in DEPC-H_2O, on a rocking table. High-temperature heating was used to increase the efficiency of detection of target mRNA sequences ('heat-induced nucleic acid-retrieval'). 0.1M citric acid solution (DEPC-H_2O), pH 6.0 was heated within a plastic Tupperware container, using a Panasonic Genius 800W microwave set to full power, for 10 minutes. Tissue sections were placed immediately within the heated citric acid solution using a glass slide rack and microwave heated for an additional 7 minutes at full power. The citric acid was allowed to cool for 15 minutes and tissue sections washed twice, for 5 minutes in DEPC-H_2O, on a rocking table. Tissue sections were then subject to pre-hybridisation treatment (50% formamide, 25% 20X SSC, 0.1% tween-20, DEPC-H_2O, pH adjusted to 6.0 by adding 92µl 1M citric acid/10ml pre-hybridisation buffer: stored at -20°C). Tissue sections were then incubated in pre-hybridisation buffer at 61°C for 1 hour. RNA probes were diluted to 1:50 in hybridisation buffer (50% formamide, 25% 20X SSC, 0.1% tween-20, 5mg/ml torula (yeast) RNA, 50µg/ml heparin, DEPC-H_2O, pH adjusted to 6.0 by adding 92µl 1M citric acid/10ml hybridisation solution: stored at -20°C). Diluted probes were then denatured by heating to 95°C for 15 minutes and applied to tissue

sections by pipetting using RNase-free tips, and sealed with coverslips. Slides were placed within a humidity chamber containing damp tissue (pre-hybridisation buffer), sealed with cling film and incubated overnight at 61°C.

Following overnight incubation, slides were placed in a slide holder within a glass staining dish in fresh pre-hybridisation buffer preheated to 51°C, ensuring immediate removal of coverslips. Tissue sections were then washed once, for 1 hour at 51°C in pre-hybridisation buffer, once, for 5 minutes in 25% pre-hybridisation solution in 75% 2X SSC, once for 10 minutes in 2X SSC and three times, for 30 minutes in 0.2X SSC. Tissue sections were then washed twice, for 5 minutes in TST (500µl NaCl, 250µl Tris-HCl pH 7.5, 50µl tween-20 in DEPC-H_2O) rocking at RT. Tissue sections were incubated in 20% foetal goat serum (FGS)/2% Boehringer Blocking Reagent (BBR) in MABT for 1 hour 30 minutes, each section covered with Parafilm and placed within a sealed incubation chamber containing damp tissue (TST) at RT. This was then replaced with anti-DIG-AP antibody diluted to 1:2000 in MABT, sections covered with Parafilm and incubated at 4°C overnight.

Sections were washed with TST six times, for 1 hour at RT, once overnight rocking, at 4°C, and two times, for 5 minutes in NTMT (1ml NaCl, 2.5ml Tris pH 9.5, 1.25 ml $MgCl_2$, 0.1% tween-20 in DEPC-H_2O). Tissue sections were then placed within an incubation chamber containing damp tissue (NTMT), and BM Purple (Roche) applied to tissue by pipetting and covered with Parafilm. The colour-producing reaction was allowed to proceed at RT, monitoring closely to avoid over-development of signal. Colour reaction times varied between probes, typically taking 1 to 7 days. Following completion of signal development, the reaction was stopped by washing twice, for 5 minutes in NTMT. For Haematoxylin counterstaining, tissue sections were dipped 3 times in 25% Haematoxylin Solution, Gill No. 3 (Sigma-Aldrich), and excess stain removed under gentle running tap water. Tissue sections were then placed in ddH_2O and dehydrated and cleared by washing for 5 minutes each, in a graded series of ethanol/ ddH_2O (25%, 50%, 75%), twice, for 5 minutes in 100% ethanol, and twice, for 10 minutes in Xylenes Histological Grade (Sigma-Aldrich). Tissue sections were then mounted using DePeX mounting medium Gurr® (VWR). For DAPI fluorescent counterstaining and mounting, tissue sections were

washed twice, for 5 minutes in NTMT and three times, for 5 minutes in PBS. Tissue sections were then counterstained in DAPI solution (1:2000 in PBS) for 5 minutes and washed once for 10 minutes, and once for 5 minutes in PBS. Tissue sections were then mounted using Fluoroshield™ histology mounting medium (Sigma-Aldrich). Slides were then placed under an Olympus BX51 Upright Compound Microscope and images taken using an Olympus DP71 Universal digital camera attachment. Bright-field (transcribed RNA) and DAPI (fluorescent histology) images were then viewed and adjusted using Adobe® Photoshop CS5 Extended (v. 12.0 x 64). For pseudo-colour image processing and overlays, bright-field images were inverted, colour adjusted and overlain with DAPI images to produce final composites. Following image processing, tissue sections were stored at 4°C.

2.5.0 Paraffin section immunohistochemistry

Immunohistochemistry was performed on 14μm paraffin-embedded tissue sections mounted on Superfrost Plus glass slides (Menzel-Gläser/ Thermo Scientific). Tissue sections were de-paraffinised by washing once, for 10 minutes in Histo-Clear (National Diagnostics) and rehydrated by washing for 5 minutes each in a graded series of ethanol (100%, 75%, 50% and 25% ethanol in PBST), followed by two, 2 minute washes in PBST and two, 2 minute washes in ddH_2O. 0.1M citric acid solution (ddH_2O), pH 6.0 was heated within a plastic Tupperware container, using a Panasonic Genius 800W microwave set to full power, for 10 minutes. Tissue sections were placed immediately within the heated citric acid solution using a glass slide rack and microwave heated for an additional 7 minutes at full power. The citric acid was allowed to cool for 15 minutes and tissue sections washed twice, for 5 minutes in ddH_2O and twice, for 5 minutes in 10% TBS (ddH_2O), rocking.

The primary antibody solutions were prepared using an Anti-SOX2 antibody ab97959 (Abcam®), diluted to 1:250, and an anti-PCNA antibody ab29 (Abcam®), diluted to 1:800 in 10% TBS buffer containing 1% foetal goat serum (FGS). Antibody solutions were applied to tissue sections by pipetting and covered with Parafilm. Slides were then placed within a humidity chamber containing damp tissue (10% TBS), and incubated overnight at 4°C.

Following primary antibody incubation, tissue sections were washed twice, for 5 minutes in 10% TBS, and once, for 15 minutes with 3% hydrogen peroxide (H_2O_2) in 10% TBS rocking, to block endogenous peroxidases. Secondary antibody solutions for primary anti-SOX2 and anti-PCNA antibodies were prepared using peroxidase-labeled goat anti-rabbit IgG, and anti-mouse IgG (DAKO), respectively, diluted to 1:200 in 10% TBS. Secondary antibodies were applied to tissue sections by pipetting, covered with Parafilm and incubated for 1 hour at RT within a humidified slide box containing damp tissue (10% TBS). Tissue sections were then washed twice, for 5 minutes in 10% TBS, rocking. The colour-producing reaction was carried out using 1 drop active DAB/ml substrate buffer (DAKO) applied to tissue sections, covered with Parafilm and incubated at RT within a humidified box containing damp tissue (10% TBS). Tissue sections were monitored closely until signal fully developed, typically taking 5 to 30 minutes, and colour reaction stopped by washing twice, for 5 minutes in 10% TBS.

For methyl green counterstaining, sections were washed twice, for 5 minutes in ddH_2O and counterstained in 0.05% methyl green for 5 minutes, followed by washing in ddH_2O to remove excess stain. Tissue sections were then dehydrated quickly by dipping twice, 10 times in 100% ethanol (to avoid removal of counterstain), and cleared by washing twice, for 10 minutes in Xylenes Histological Grade (Sigma-Aldrich). Tissue sections were then mounted using DePeX mounting medium Gurr® (VWR). For DAPI fluorescent counterstaining and mounting, tissue sections were washed twice, for 5 minutes in PBS. Tissue sections were then counterstained in DAPI solution (1:2000 in PBS) for 5 minutes and washed once for 10 minutes, and once for 5 minutes in PBS. Tissue sections were then mounted using Fluoroshield™ histology mounting medium (Sigma-Aldrich). Slides were then placed under an Olympus BX51 Upright Compound Microscope and images taken using an Olympus DP71 Universal digital camera attachment. Bright-field (translated protein) and DAPI (fluorescent histology) images were then viewed and adjusted using Adobe® Photoshop CS5 Extended (v. 12.0 x 64). For pseudo-colour image processing and overlays, bright-field images were inverted, colour adjusted and overlain with DAPI images to produce final composites. Following image processing, tissue sections were stored at 4°C.

2.5.1 BrdU pulse-chase experiments

Embryos were collected by removal from egg casings, placed in a petri dish in normal seawater and 5-bromo-2'-deoxyuridine (BrdU; 1 mg/ml) administered twice daily for 7 days by pipetting the solution into the mouth (pulse). Treated embryos were then reinserted into egg casings and allowed to develop for 7 days in normal seawater (chase) prior to euthanisation and fixation as per section 2.2.1. Following fixation, embryos were dissected by removal of the head and tissue prepared as per section 2.2.2, and paraffin embedded and sectioned as per section 2.3.1.

Anti-BrdU immunohistochemistry was performed on 14μm paraffin-embedded tissue sections mounted on Superfrost Plus glass slides (Menzel-Gläser/ Thermo Scientific). Tissue sections were de-paraffinised by washing once, for 10 minutes in Histo-Clear (National Diagnostics) and rehydrated by washing for 5 minutes each in a graded series of ethanol (100%, 75%, 50% and 25% ethanol in PBS/ Triton X-100) (Sigma-Aldrich), followed by two, 2 minute washes in PBS/ Triton X-100. For antigen retrieval, two modified protocols were used. In both methods, 0.1M citric acid solution (ddH_2O) was heated to 95°C in a glass slide dish and the tissue sections placed immediately within the heated citric acid solution using a glass slide rack and heated at 95°C for 10 minutes. In the first method (chapter 6, Fig. 6.2A), tissue sections were transferred to 2N HCl heated to 37°C and incubated for 1 hour. In the second method (chapter 6, Fig. 6.2B-D), tissue sections were transferred to 2N HCl heated to 50°C and incubated for 1.5 hours at 50°C. In both methods, tissue sections were then washed once, for 5 minutes in 0.1M sodium tetraborate (Sigma-Aldrich).

The primary antibody solution was prepared using an anti-BrdU antibody (DSHB, G3G4) diluted to 1:500 in PBS containing 8% FGS and applied to tissue sections, each covered with Parafilm and placed within a sealed humidity chamber containing damp tissue (PBS), and incubated overnight at 4°C. Tissue sections were then incubated in 8% foetal goat serum (FGS) for 1 hour. The secondary antibody solution was prepared using an Alexa Fluor® 488 conjugate goat anti-mouse IgG secondary antibody (Life Technologies) diluted to 1:200 in PBS. The secondary antibody solution was applied to tissue sections, each covered with Parafilm and

placed within a sealed humidity chamber containing damp tissue (PBS), and incubated for one hour at 4°C. Tissue sections were then washed three times, for 5 minutes in 0.3% PBS/ Triton X-100, rocking. For DAPI fluorescent counterstaining and mounting, tissue sections were washed twice, for 5 minutes in PBS. Tissue sections were then counterstained in DAPI solution (1:2000 in PBS) for 5 minutes and washed once for 10 minutes, and once for 5 minutes in PBS. Tissue sections were then mounted using Fluoroshield™ histology mounting medium (Sigma-Aldrich) and placed under an Olympus BX51 Upright Compound Microscope and images taken using an Olympus DP71 Universal digital camera attachment. Images were then viewed and adjusted using Adobe® Photoshop CS5 Extended (v. 12.0 x 64).

2.5.2 DiI fate mapping experiments

Embryos were collected by removal from egg casings and anaesthetised (0.085mg/ml MS 222 (tricaine); Sigma-Aldrich, in normal seawater). DiI (cell tracker CM-DiI, C-7000, Molecular probes) was then resuspended in 100% ethanol and injected by mouth aspirator into upper jaw dental tissues. Post-labeling embryos were then reinserted into egg casings and allowed to develop for 7 days in normal seawater prior to euthanisation and fixation as per section 2.2.1. Following fixation, embryos were dissected by removal of the head and specimens transferred into gelatin/albumin embedding solution for 30 minutes before being transferred into moulds with approximately 4 ml gelatin/albumen embedding solution. 2.5% gluteraldehyde fixative was then placed around the embryos within gelatin/albumen and mixed gently. Moulds containing specimens were then sealed in Ziploc bags containing dampened tissue and left overnight to set at 4°C. The following day, the blocks were cut from the moulds and prepared for sectioning. Blocks were mounted on a specimen block with superglue and left to set. The mounted blocks were then cut in sagittal plane in PBS using an Intracell Vibratome® sectioning machine at a thickness of 40μm. Sections were then placed onto clean glass slides, PBS removed and covered with 100% glycerol and a glass coverslip. Vibratome sections were placed under an Olympus BX51 Upright Compound Microscope and images taken

using an Olympus DP71 Universal digital camera attachment. Images were then viewed using Adobe® Photoshop CS5 Extended (v. 12.0 x 64).

2.5.3 Bead implantation experiments

Preliminary gain- and loss-of-function experiments were carried out by implanting Affi-Gel® Blue, formate-derivitised AG1X2 (Bio-Rad) and Heparin-Acrylic (Sigma-Aldrich) microbeads in dental tissues and the dorsal epithelium associated with early denticle primordia. Beads were soaked in cyclopamine (4 mg/ml in DMSO), LDN (4 mg/ ml in DMSO), SU5402 (2 mg/ml in DMSO) (Sigma-Aldrich), and recombinant N-terminal SHH protein (1 mg/ml in DMSO) (eBioscience). An aliquot of beads were placed in the center of a 100 mm petri dish and several small drops of water placed around the edge to prevent excessive drying of beads during incubation. 5-10µl of chemical was then applied to the beads by pipetting and incubated for 2 hours at room temperature with the lid slightly ajar. Prior to implantation, embryos were collected by removal from egg casings and anaesthetised (0.085mg/ml MS 222 (tricaine); Sigma-Aldrich, in normal seawater). Beads were implanted adjacent to upper jaw dental tissues using watchmakers forceps No. 5 (Dumont) and embryos reinserted into egg casings and allowed to develop overnight in normal seawater, prior to euthanisation and fixation as per section 2.2.1. Embryos were then dissected by removal of the head and tissue prepared and paraffin embedded as per sections 2.2.2 and 2.3.1, respectively. The resulting tissue sections were not examined for changes in morphology and gene expression and cannot therefore be discussed in the current study.

Chapter 3

SOX2 DEFINES A PUTATIVE DENTAL STEM CELL NICHE IN THE REGENERATING ELASMOBRANCH DENTITION

3.1 Summary

To identify putative dental stem-like cells in elasmobranch tooth development, the expression patterns of *Sox2* were compared and contrasted in the shark species *Scyliorhinus canicula* and *S.stellaris* (small and greater-spotted catshark) and ray species *Raja clavata* (thornback ray) and *Leucoraja erinacea* (little skate). To complement gene expression, Sox2 immunohistochemistry was also used to co-localise secreted functional protein.

In the shark dentition, *Sox2* is expressed in the first epithelial thickenings concomitant with initiation of the odontogenic band, and later in the early dental lamina. During tooth development, *Sox2* progressively localises to a cluster of surface oral epithelial cells linked to the dental lamina via a continuous stripe of *Sox2*+ dental epithelium. This cell cluster is proposed to constitute a putative dental SCN. In the ray dentition, *Sox2* expression also marks the odontogenic band and during tooth development further localises to a similar cell cluster, continuous with a stripe of *Sox2*+ oral and dental epithelium. These expression patterns therefore imply, among elasmobranchs, a highly conserved dental developmental and regenerative strategy. Furthermore, in the elasmobranch dentition, the expression domains of *Sox2* and the Wnt gene *Lef1*, show marked similarities to the replacement dentition of the snake (Gaete and Tucker, 2013), implying a conserved interaction in which *Lef1* restricts *Sox2* expression, in order to maintain the epithelial compartment required for continuous tooth regeneration.

3.2 Introduction

3.2.1 The Scyliorhinidae and Rajidae as tooth replacement models

As reviewed in chapter 1: section 1.3, *Scyliorhinus canicula* (the small-spotted catshark) has become a prominent representative elasmobranch tooth replacement model (Smith *et al.,* 2009a; 2009b; Debiais-Thibaud *et al*., 2011). Alizarin red staining of the catshark dentition (upper jaw) shows individual replacement tooth families to develop outward as a continuous 'band' across the jaw margins (Fig. 3.1A). At least two visible tooth generations (boxed area, T1-T2) develop in a staggered pattern toward the oral margin, where they are shed and replaced by the next tooth of the family sequence. Individual teeth constitute multicuspid (typically tricuspid) units of one large cusp, flanked by two accessory cusps specialised for piercing soft-bodied prey (Fig 3.1B). Compositionally, teeth are hypermineralised, comprising dentin interspersed with numerous odontoblast tubules (arrow), capped with a superficial layer of enameloid (Reif, 1978; 1980; Gillis and Donoghue, 2007). In accordance with other polyphyodont gnathostomes, replacement teeth initiate from within an extension of the dental lamina termed the successional lamina (SL) (Huysseune, 2006; Fraser *et al*., 2006a Järvinen *et al.,* 2009).

Routine histology (upper jaw, sagittal plane) reveals the catshark SL (Fig 3.1C-D). Shown at Stage 26-27 (Reif, 1980), two generations of early replacement teeth (T1-T2) develop outward in close succession from the SL, housed within the jaw cartilage (Fig. 3.1C-D). In the absence of gene expression data relating to specific markers, prior histological investigation of the SL in the replacement shark dentition has led to some hypotheses regarding putative sites of regulatory control and stem cells. This is defined by a continuous connection maintained between the oral epithelium and SL via the herein named outward-outer dental epithelium (O-ODE) (Fig. 3.1D, arrow) (Smith *et al*., 2009b). In this scenario, it is hypothesised that regulatory control of the dentition may localise either to the junction of the oral and dental epithelium (hypothesis 1) or the SL itself (hypothesis 2), with the source of dental SCs localised to within the middle dental epithelium (MDE) (Smith *et al.*, 2009b) (reviewed chapter 1: section 1.3.2, Fig. 1.11).

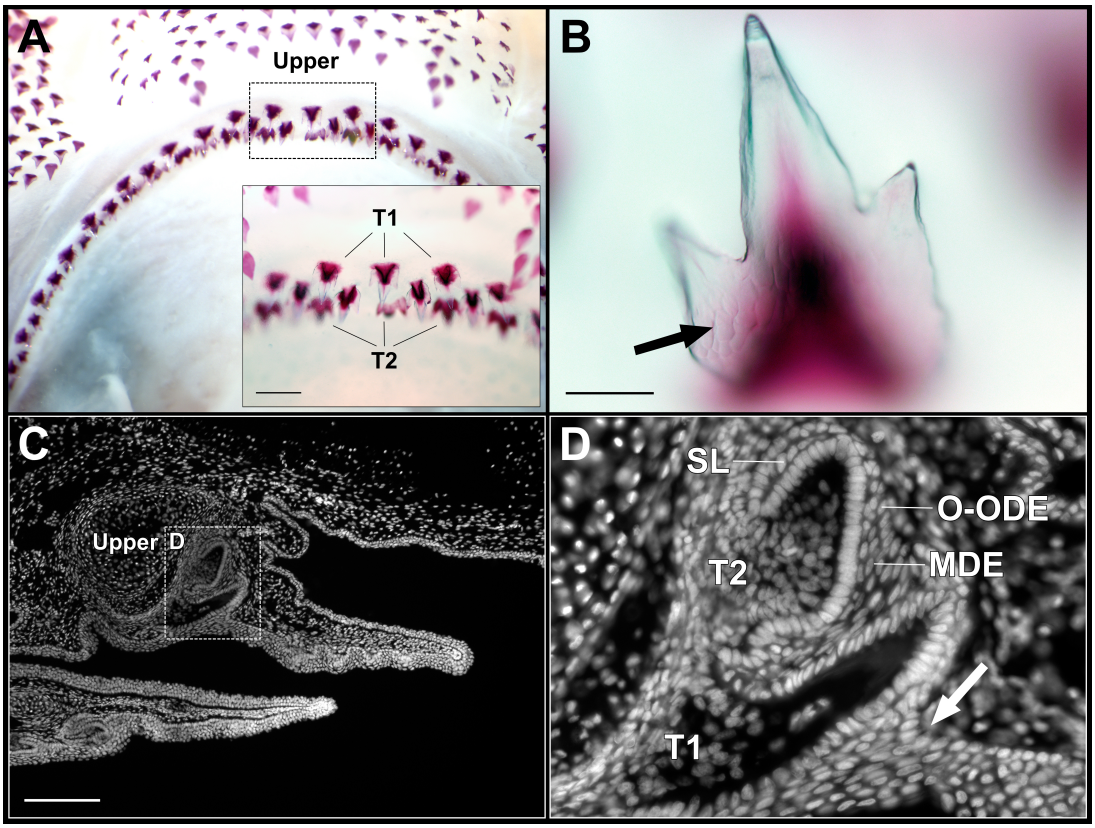


Figure 3.1 'Many-for-one' tooth replacement in sharks (*S.canicula*). Alizarin red staining of the early catshark dentition shows characteristic shark tooth replacement morphology. Shown in the upper jaw, first and second generation teeth (boxed area, T1-2) develop outward (linguo-labially) in a typical odd-even pattern toward the jaw margin, where they are shed and systematically replaced (A). Morphologically and compositionally, individual teeth are characteristically tricuspid, comprising hypermineralised tissues capped by a thin layer of enameloid. Visible also, are numerous odontoblast tubules (B, arrow). Viewed in thin section (sagittal plane, DAPI), replacement teeth (T1-2) develop outward from the successional lamina (SL), an epithelial compartment proposed to supply the regenerative potential to maintain continuous tooth replacement in polyphyodont gnathostomes (C, boxed area, D) (Huysseune, 2006; Fraser *et al*., 2006a Järvinen *et al.,* 2009). Histological investigation of the catshark dentition has led to additional hypotheses regarding the possible deployment of dental stem cells in the middle dental epithelium (MDE) and associated regulatory loci inclusive of the SL itself (hypothesis 1) and the junction between the oral and dental epithelium (hypothesis 2) (D, arrow), also continuous with the SL via the outward-outer dental epithelium (O-ODE) (Smith *et al*., 2009b). Scale bars: Large image (A) unscaled, small image (A) 1 mm, (B, D) 100 μm, (C) 200 μm.

Skates and rays of the Family Rajidae evolved during the Cretaceous Period, approximately 146 mya, therefore offering a suitably positioned phylogenetic group to investigate their unique tooth replacement phenotype (Klimley, 2013). Alizarin red staining of the embryonic teeth in the little skate (*Leucoraja erinacea*) highlights the ray dentition (Fig. 3.2A), comprising a characteristic set of dorso-ventrally opposing flattened tooth plates. Two early tooth generations (T1-2) are present, with first generation teeth (T1) visible at the oral margin. Individual first generation teeth are broad and circular, containing lightly calcified hard tissues (Fig. 3.2B). Viewed in thin section (Fig. 3.2C) (sagittal plane), the embryonic jaws of the thornback ray (*Raja clavata*) show the histological component of the ray dentition (boxed area), defined by a narrow dental lamina connected to the oral epithelium via the adjoining O-ODE. Within the DL (Fig. 3.2D), the O-ODE is further continuous with the SL from which two early tooth generations (T1-2) develop, each undergoing morphogenesis and bud stages, respectively (Fig. 3.2D).

The embryonic ray dentition presents some comparatively unique morphological characteristics, with early tooth shape and level of mineralisation (defined by alizarin staining) reflective of the adult dentition (Fig. 3.2A-B). In the shark dentition, the developmental transition from embryonic to adult tooth morphology occurs over many early rounds of replacement, with initial rudimentary (degenerative) teeth aborted in advance of function or developing as non-functional 'shards' (Reif, 1980; Reif, 1976). This early developmental trait is reflective of other polyphyodont gnathostomes, which show similar patterns of vestigial tooth development in advance of the adult functional dentition (reviewed by Sire *et al*., 2002; Järvinen *et al*., 2008). In rays, this therefore implies the comparatively rapid transition from the non-functional to functional adult dentition, a process likely dependent upon the availability of dental progenitor cells and the gene regulatory mechanisms, which determine their function.

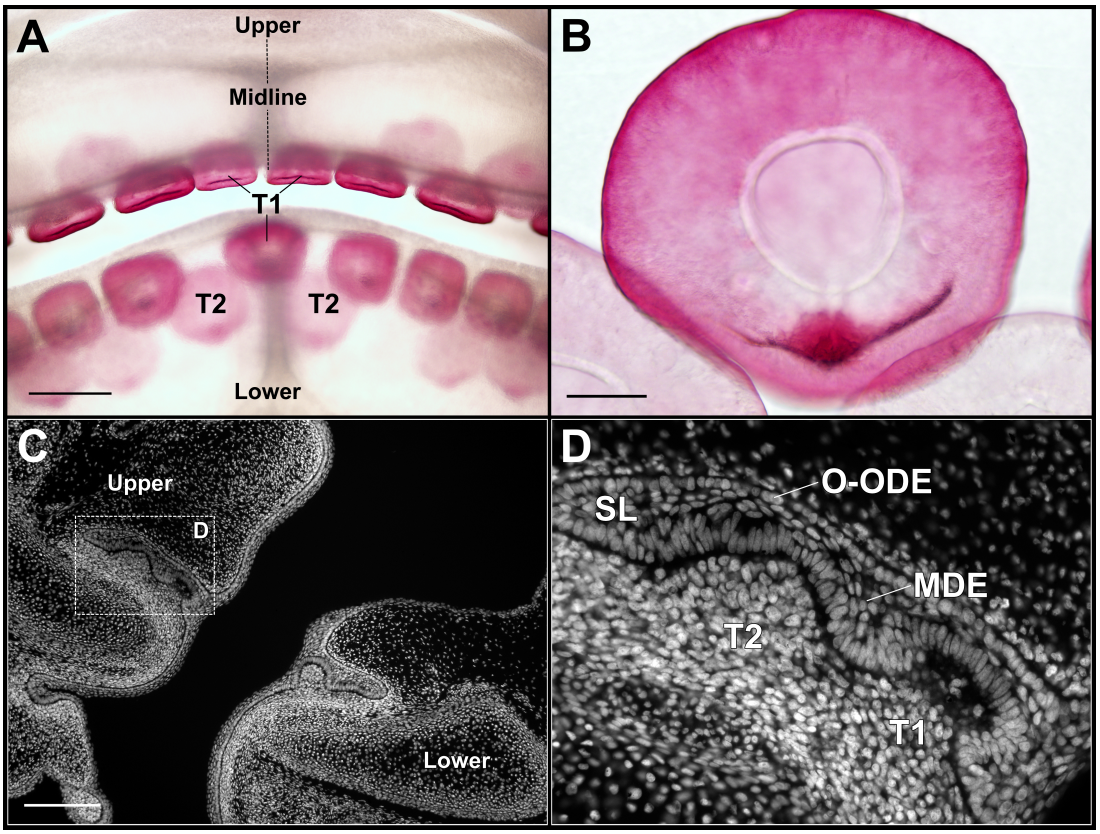


Figure 3.2 'Many-for-one' tooth replacement in rays. Alizarin red staining of the skate dentition (*L.erinacea*) shows characteristic ray tooth morphology. The upper and lower jaws house a set of compact, dorsoventrally opposing tooth plates, in adults forming an interlocking crushing surface specialised for feeding on hard-shelled prey (Summers, 2000; Underwood *et al*., 2015) (A). In the embryonic skate dentition, two early tooth generations are present (T1-2), with early first generation teeth visible at the oral margin. The specialised nature of the ray dentition is further defined by individual replacement teeth, which constitute broad, circular units with lightly mineralised crowns (B). Thin sections (sagittal plane, DAPI) of the embryonic thornback ray dentition (*R.clavata*) define a comparatively narrow DL (boxed area) also continuous with the oral epithelium (C). In further accordance with the shark dentition, the O-ODE is also continuous with the SL (D) from which two characteristically compact tooth generations (T1-T2) develop, interspersed by the surrounding MDE. Scale bars: (A) 1 mm, (C), 200 μm, (B, D) 100 μm.

3.2.2 *Sox2* marks putative dental stem cells

Given their contrasting dental morphologies and comparatively ancestral phylogenetic positions, the catshark and little skate/ thornback ray provide ideal models to investigate stem-regulatory control of elasmobranch tooth regeneration. However, the identification of dental stem cells is dependent upon detection of undifferentiated progenitors and daughter cells committed to odontogenic fates (Harada *et al*., 1999). While lone markers are insufficient to definitively confirm stem cell identities within a heterogeneous population, one strong marker of stemness is the SRY (sex determining region Y)-box 2 (*Sox2*) transcription factor (reviewed by Driessens and Blanpain, 2011). In particular, *Sox2* has become a prominent dental stem cell marker. During mouse incisor development, *Sox2* is expressed in the epithelial SCN in the labial cervical loop region and adjacent enamel epithelium. Combined with fate mapping of transit amplifying cells, this suggests a homeostatic role in the maintenance of progenitor cells (Juuri *et al*., 2012; Zhang *et al*., 2012). During deciduous tooth development in the ferret, *Sox2* is also expressed in the lingual dental epithelium, progressively extending from the oral epithelium to the SL during early cap stage (Juuri *et al*., 2013). In the initiation of human deciduous premolars, *Sox2* is expressed in the lingual DL and high levels of expression have been reported in human ameloblastomas, high recurrence odontogenic tumours resulting in serious craniofacial abnormalities (Morgan, 2000). Interestingly, multiple supernumerary teeth and the persistence of deciduous teeth have been reported in patients with *Sox2* anophthalmia syndrome, therefore implying, in humans, a highly conserved role in regulating tooth number (Numakura *et al*., 2010).

Sox2 is further conserved in polyphyodont gnathostomes, such as reptiles and osteichthyan fish, which have become increasingly used for molecular studies of tooth regeneration accordingly (Juuri *et al*., 2013; Gaete and Tucker, 2013; Fraser *et al*., 2013; Abduweli *et al*., 2014). In the American alligator (Wu *et al*., 2013) and leopard gecko (Handrigan *et al*., 2010), putative stem and slow cycling cells have been localised to the DL. Both these and additional reptiles, including the green iguana, ball python and corn snake, express *Sox2* in domains indicative of

associations with SC-mediated tooth regeneration (reviewed chapter 1: section 1.2.9, Fig. 1.7) (Juuri *et al*., 2013; Gaete and Tucker, 2013). In Lake Malawi cichlids, *Sox2* is expressed in delineated regions of the oral and dental epithelium associated with replacement teeth (Fraser *et al*., 2013) and in the medaka (*Oryzias latipes*), BrdU pulse-chase experiments have identified slow-cycling dental epithelial cells expressing *Sox2* at the posterior end of replacement tooth families, where they are proposed to maintain continuous tooth replacement capacity (Abduweli *et al*., 2014).

3.2.3 Aims and objectives

Smith et al. (2009b) recently highlighted the current lack of gene expression data for relevant markers to be a limiting factor in identifying the dental stem cells responsible for elasmobranch tooth regeneration (Smith *et al*., 2009b). To address this gap in knowledge, the aims and objectives are as follows:

- To use the catshark and ray as comparative models to investigate the expression of the stem cell marker *Sox2* (mRNA and protein), in order to identify putative dental stem cells in the elasmobranch dentition.

- To compare and contrast the expression patterns of *Sox2* during shark and ray tooth development and further compare these with successive gnathostomes, so as to infer the broader extent of evolutionary conservation of this dental regenerative strategy.

- To use this comparative expression data to develop a hypothetical elasmobranch *Sox2* dental stem cell niche (SCN) model.

3.3 Results

3.3.1 *Sox2* is expressed during early dental competence and in the dental lamina in elasmobranchs

Early dental competence is defined by the odontogenic band (OB), a localised region of thickened oral epithelium and condensing mesenchyme, which expresses the dental patterning genes *Pitx2* and *Shh* (Fraser *et al*., 2004; Smith *et al*., 2009a; Buchtová *et al*., 2008). To investigate the possible role of *Sox2* in defining early dental competence in elasmobranchs, its expression patterns were investigated prior to development of the dental lamina. Descriptions of tooth stages and associated cellular histology are in accordance with those previously described by Reif, 1980; Smith *et al*., 2009b, with the exception of the herein named 'outward-outer dental epithelium' (O-ODE).

In the shark dentition, *Sox2* mRNA was first expressed in the upper and lower jaw epithelium in restricted patterns, prior to development of the primary dental lamina (DL) (Fig. 3.3A, boxed areas). Both *Sox2*+ epithelia show marked signs of localised thickening, coincident with the onset of dental competence defined by the OB (Fig. 3.3B-C). During development of the primary DL (lower jaw), epithelial *Sox2* expression was maintained, extending into the in-folded DL via an epithelial stripe and terminating lingually to the first tooth placode (T1) (Fig. 3.3D, DAPI counterstain) and (E, haematoxylin counterstain). Sox2 protein was also detected at the same stage, restricted to the proximal aspect of the DL, terminating lingually to the first tooth placode (T1) (Fig. 3.3F). Throughout, no *Sox2* expression was detected in the underlying mesenchyme.

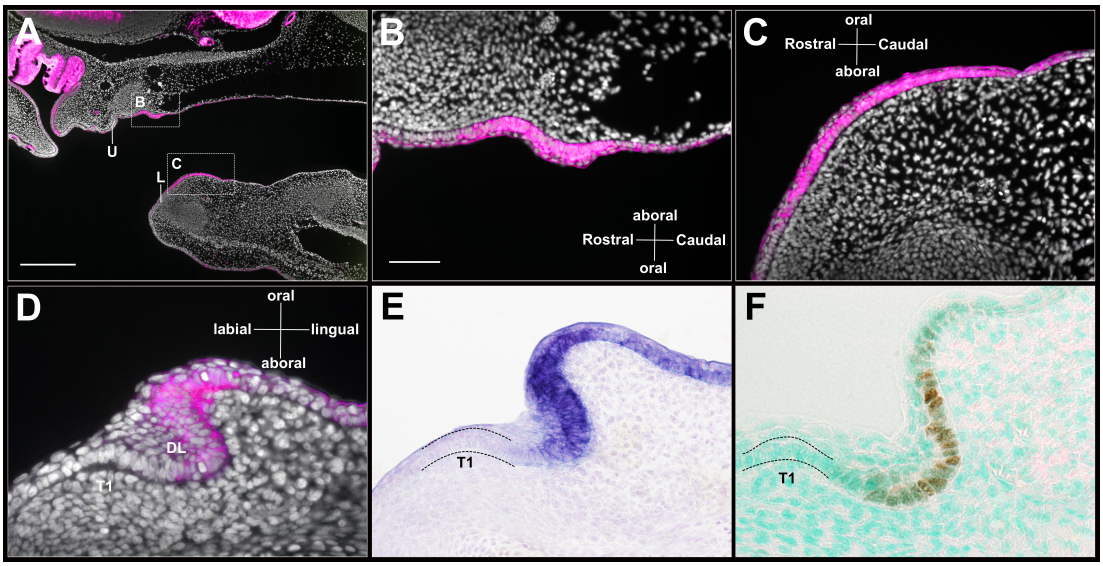


Figure 3.3 *Sox2* expression during initiation of the shark dentition (A-C, *S.stellaris*; D-F, *S.canicula*). Shark tooth induction is marked by *Sox2* expression in the oral epithelium of the upper (U) and lower (L) jaws (boxed areas) (A, DAPI). Both *Sox2*+ epithelia show signs of localised thickening, coincident with formation of the OB (B, upper and C, lower jaw, DAPI). Subsequent development of the primary DL is marked by in-folding of this oral epithelium into the underlying condensing mesenchyme (D-F), which shows no corresponding expression. During DL development (lower jaw), *Sox2* mRNA is continually expressed in a stripe of in-folding epithelium, lingual to the first tooth (T1) placode (D, DAPI and E, haematoxylin). Sox2 protein is also expressed in the same proximal DL, lingually to the first tooth placode (T1) (F). Scale bars: (A) 1 mm, (B-F) 100 μm.

In order to compare *Sox2* expression in the early shark dentition with other elasmobranchs, this was further investigated in two species of ray, *Raja clavata* (thornback ray) and *Leucoraja erinacea* (little skate). In the thornback ray, *Sox2* was first expressed in restricted regions the upper and lower jaws (Fig. 3.4A, boxed areas, DAPI) in approximately overlapping patterns. In the upper jaw, (Fig. 3.4B), *Sox2* expression marked a delineated stripe of thickened oral epithelium. This pattern was further apparent in the lower jaws (Fig. 3.4C), with *Sox2* marking a similar region of thickened columnar epithelial cells. In both cases, *Sox2* showed no signs of expression in the underlying mesenchyme. Sox2 IHC produced similar patterns, with protein localised to the same dorso-ventrally opposing domains of the upper and lower jaws (Fig. 3.4D, boxed areas). In the upper and lower jaw epithelium (Fig. 3.4E-F, respectively), Sox2 protein marked a similar set of columnar epithelial cells, with no further indications of localisation to the mesenchymal. These expression patterns therefore closely follow those of the early shark dentition, indicating a role for *Sox2* in conferring early dental competence.

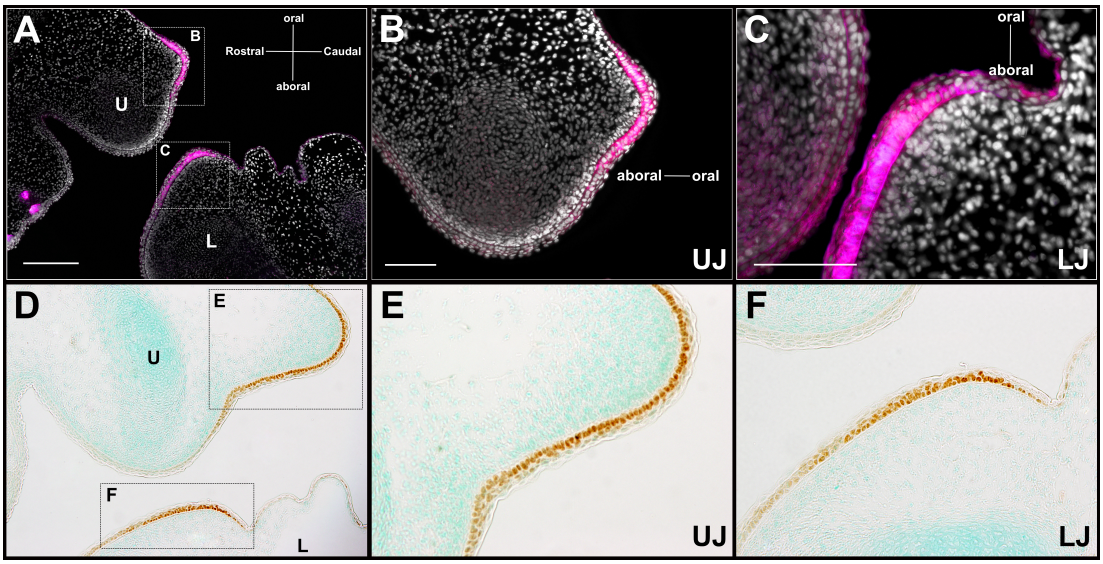


Figure 3.4 *Sox2* expression during initiation of the ray dentition (*R.clavata*). In the upper and lower jaws (A, boxed areas, DAPI), *Sox2* expression marks delineated regions of oral epithelium in approximately overlapping domains. These restricted expression patterns define a thickened stripe of epithelium in both the upper (B) and lower (C) jaws, with no corresponding expression in the underlying mesenchyme. Localisation of Sox2 protein produces similar patterns (D, boxed areas), marking a similar population of columnar epithelial cells in the upper (E) and lower (F) jaws, with no further localisation to the mesenchyme. Scale bars: (A) 1 mm, (B, D) 100 µm, (C, E, F) 100 µm.

3.3.2 *Sox2* is maintained in the oral-dental epithelium during development of first generation teeth

During development of first-generation teeth in the catshark (lower jaw, LJ), Sox2 protein was detected in the surface oral epithelium continuous with the dental epithelium (Fig. 3.5A-B). This was specifically localised to the taste buds (TB, arrows) and a defined cluster of epithelial cells positioned at junction between the oral and dental epithelium (Fig. 3.5A, boxed area). These Sox2+ cells were strongly marked at the junction (Fig. 3.5B, arrow) and extended into the lamina via the continuous epithelial stripe of O-ODE, progressively reducing in signal intensity to terminate lingually to the first tooth bud (T1). *Sox2* mRNA was expressed in a similar pattern, extending from the localised oral epithelium into the DL via the intervening O-ODE, terminating lingually to the first tooth undergoing morphogenesis (Fig. 3.5C). In the upper jaw (UJ), Sox2 protein (Fig. 3.5D-E) and *Sox2* mRNA (Fig. 3.5F) localised to a similar stripe of oral epithelial cells in the MV, continuous with the O-ODE and adjoining DL. These further terminated in the DL, lingually to the first generation tooth (T1). In horizontal plane, a continuous band of Sox2 protein was also detected lingually to the DL and individual first generation tooth germs (Fig. 3.5G, arrows). In horizontal plane, Sox2+ cells showed some superficial evidence of localisation to within the epithelium of individual tooth germs (Fig. 3.5G, boxed area and 3.5H, arrows); however, this is inconsistent with sagittal plane expression patterns, which relative to the position of teeth in section, may exclude corresponding cellular expression.

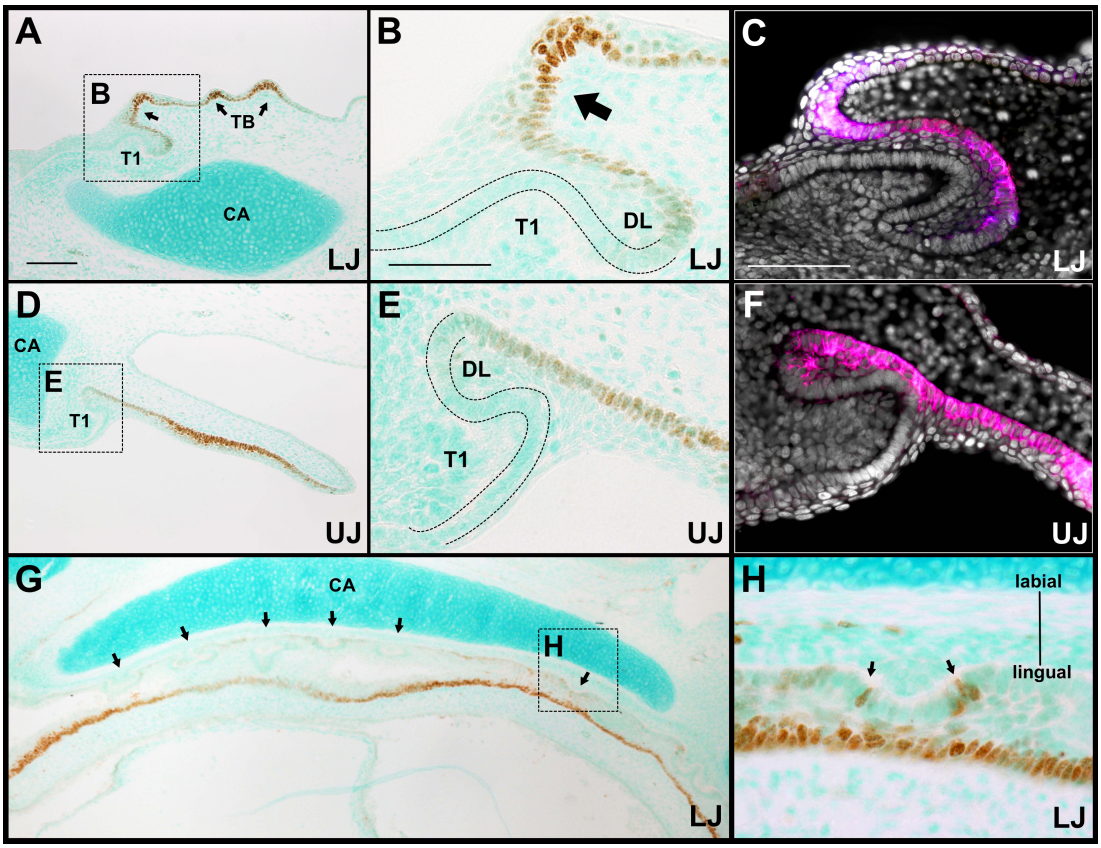


Figure 3.5 *Sox2* expression during shark tooth development (*S.canicula*). During first-generation tooth development (lower jaw, LJ), Sox2 protein is expressed as a continuous epithelial stripe, extending into the lamina via the O-ODE and terminating lingually to the first tooth bud (T1) (A, boxed area). Sox2 protein further localises to developing TBs (arrows). In addition, Sox2 localises to a cell cluster positioned at the oral and dental epithelial junction (B, arrow), extending into the DL via the intervening O-ODE to terminate lingually to the first generation tooth. *Sox2* mRNA (lower jaw) shows a similar pattern, expressing strongly at the oral-dental epithelial junction and extending into the DL via the O-ODE to terminate lingually to the first tooth (C, DAPI). In the upper jaw (UJ), Sox2 protein is expressed in similar patterns, localising to the epithelium of the MV, continuous with the O-ODE and DL (D, boxed area). Similarly, this continuous Sox2+ epithelial stripe extends into the DL, terminating lingually to the first tooth (T1) (E). *Sox2* mRNA shows an identical pattern (F, DAPI), expressed in the epithelium of the MV, continuous with the O-ODE and DL, terminating lingually to the first generation tooth. Horizontal sections show a continuous band of Sox2+ cells spanning the entire jaw lingually to first-generation tooth germs (G, arrows). Some Sox2+ cells also show evidence of localisation to within individual tooth germs (G, boxed area and H, arrows); however, in the absence of corresponding sagittal expression patterns, this remains inconclusive. Scale bars: (A, D, G) 200 µm, (B, E, H) 100 µm, (C, F) 100 µm.

In the current study it was not possible to investigate the expression of *Sox2* during development of the DL in rays, due a lack of the required stages; however, expression was profiled during the development of first and second-generation teeth. During development of the primary dentition, both the upper and lower jaws showed a well-developed DL housing first generation teeth undergoing morphogenesis. In the upper and lower jaws (U/LJ), *Sox2* was expressed in the oral epithelium continuous with the O-ODE and DL (Fig. 3.6A). The inward restriction of this *Sox2*+ oral-dental epithelium was defined by its delineated expression domains, ending a short distance within the buccal cavity (data not shown). In the upper jaw (UJ), *Sox2* mRNA localised strongly to the O-ODE (Fig. 3.6B), ending within the SL lingually to the early second-generation tooth placode (T2). Lower jaw (LJ) expression was similar, in accordance with the shark dentition showing further localisation to the oral-dental epithelial junction to the DL (Fig. 3.6C, arrow). The distribution of Sox2 protein was similar, localising to the same oral epithelium and the O-ODE, terminating in the proximal aspect of the DL lingual to first generation teeth (Fig. 3.6D-F). In line with the expression patterns shown by mRNA, Sox2 protein showed further evidence of localisation to the same defined cell cluster at the oral-dental epithelial junction in the lower jaws (Fig. 3.6F, arrow). IHC (methyl green) also showed Sox2 protein in the oral and dental epithelium to follow this pattern of distribution, ending in the SL lingually to early replacement teeth (T2) (Fig. 3.6G). Within the DL (upper jaw), Sox2 protein showed continued localisation to the O-ODE, continuous with the SL, ending lingually to the epithelial thickenings of second-generation teeth (T2) (Fig. 3.6H). In the lower jaw (Fig. 3.6I, arrow), Sox2 protein further localised to the same epithelial cell cluster positioned at the oral-dental epithelial junction.

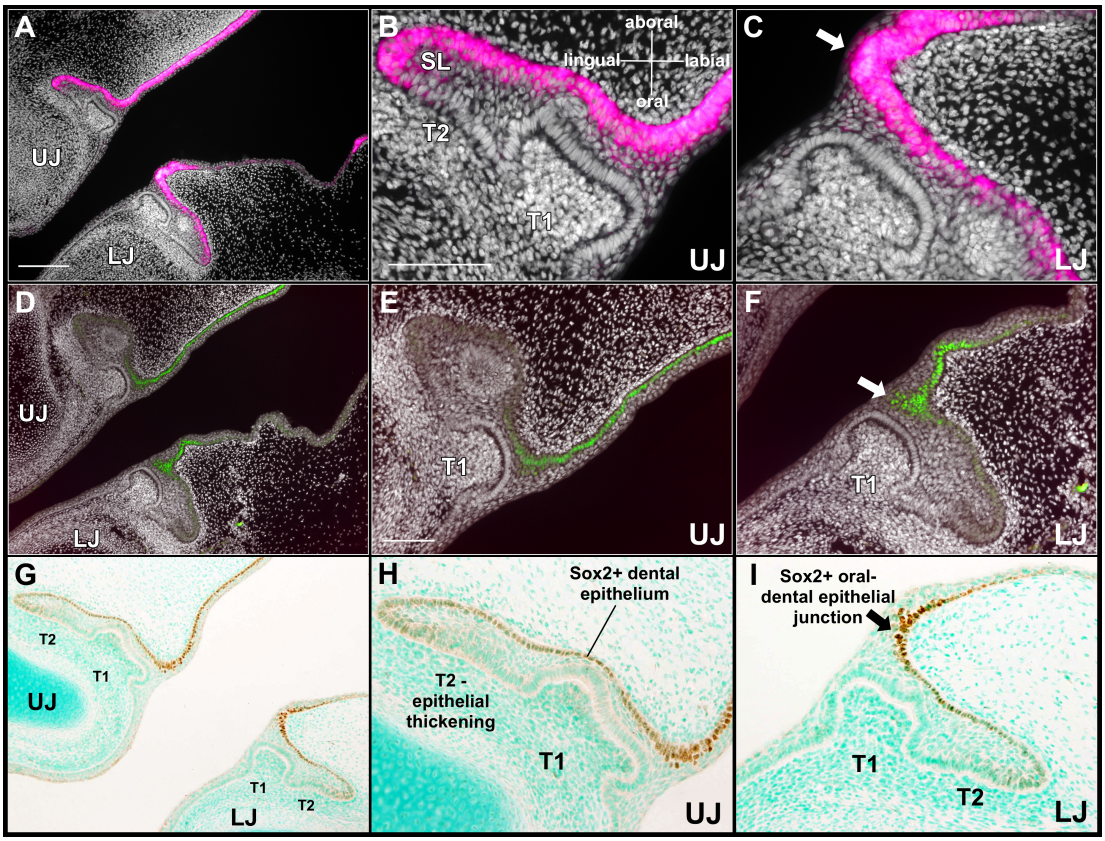


Figure 3.6 *Sox2* expression during ray tooth development (*R.clavata*). During development of first generation and early second-generation teeth, *Sox2* mRNA localises strongly to the oral and dental epithelium continuous with the DL in both the upper and lower jaws (U/LJ) (A, DAPI). In the upper jaws (UJ), *Sox2* mRNA is expressed in the O-ODE, terminating lingually to the first early replacement tooth placode (T2) in the SL (B, DAPI). In the lower jaws (LJ), this expression pattern is similar, while showing further localisation to a defined cell cluster within the oral-dental epithelial junction (C, arrow, DAPI). Sox2 IHC shows similar patterns in the upper and lower jaws, with protein expressed in the oral and dental epithelium continuous with the DL (D-F, DAPI). In further accordance with mRNA expression patterns, Sox2 protein (lower jaws) localises to a defined cell cluster at the junction of the oral the dental epithelium (F, arrow, DAPI). Additional Sox2 IHC (methyl green) further defines these patterns (G), with expression localising to the O-ODE (H, UJ), continuous with the SL, ending lingually to early thickened replacement tooth placodes (T2). These patterns of protein distribution further localise *Sox2* to the same oral-dental epithelial junction as sharks (I, LJ, arrow), identifying commonalities between both. Scale bars: (A, D, G) 200 µm, (B, C, H) 100 µm, (E, F, I) 100 µm.

3.3.3 *Sox2* expression during tooth replacement defines a Sox2+ cell cluster

During subsequent shark tooth replacement (upper jaw, UJ), *Sox2* mRNA was continually expressed in the oral epithelium of the MV (Fig. 3.7A) and taste buds (arrows) continuous with the O-ODE. *Sox2* showed sustained localisation to the dental epithelial stripe (Fig. 3.7B, dotted line) ending in the SL, lingually to first generation teeth (advanced morphogenesis, T1), second and third generation teeth (morphogenesis, T2 and placode, T3 stages, respectively). In line with lower jaw expression patterns, *Sox2* mRNA further localised to a defined cluster of taste bud-like epithelial cells proximal to the SL (Fig. 3.7C, boxed area). This cell cluster (Fig. 3.7D, arrow) is positioned sub-epithelially at the oral-dental epithelial junction continuous with the O-ODE, continuous with the SL. The distribution of Sox2 protein followed this pattern (Fig. 3.7E), localising to the O-ODE extending into the SL (boxed area) and taste buds. Sox2 protein also showed marked localisation to within the lingual aspect of the SL (Fig. 3.7F, arrow 1), while further defining a similar pocket of positive cells positioned closely to the oral-dental epithelial junction (Fig. 3.7F, arrow 2). Additional Sox2 IHC (methyl green counterstain) further defined the continuous Sox2+ epithelial connection maintained between the oral epithelium and SL, and associated taste buds (arrow) (Fig. 3.7G, boxed area). These expression patterns define one possible source of epithelial dental 'stem-like' cells (Fig. 3.7H, arrow), potentially originating from a putative dental stem cell niche (SCN) at the oral-dental epithelial junction.

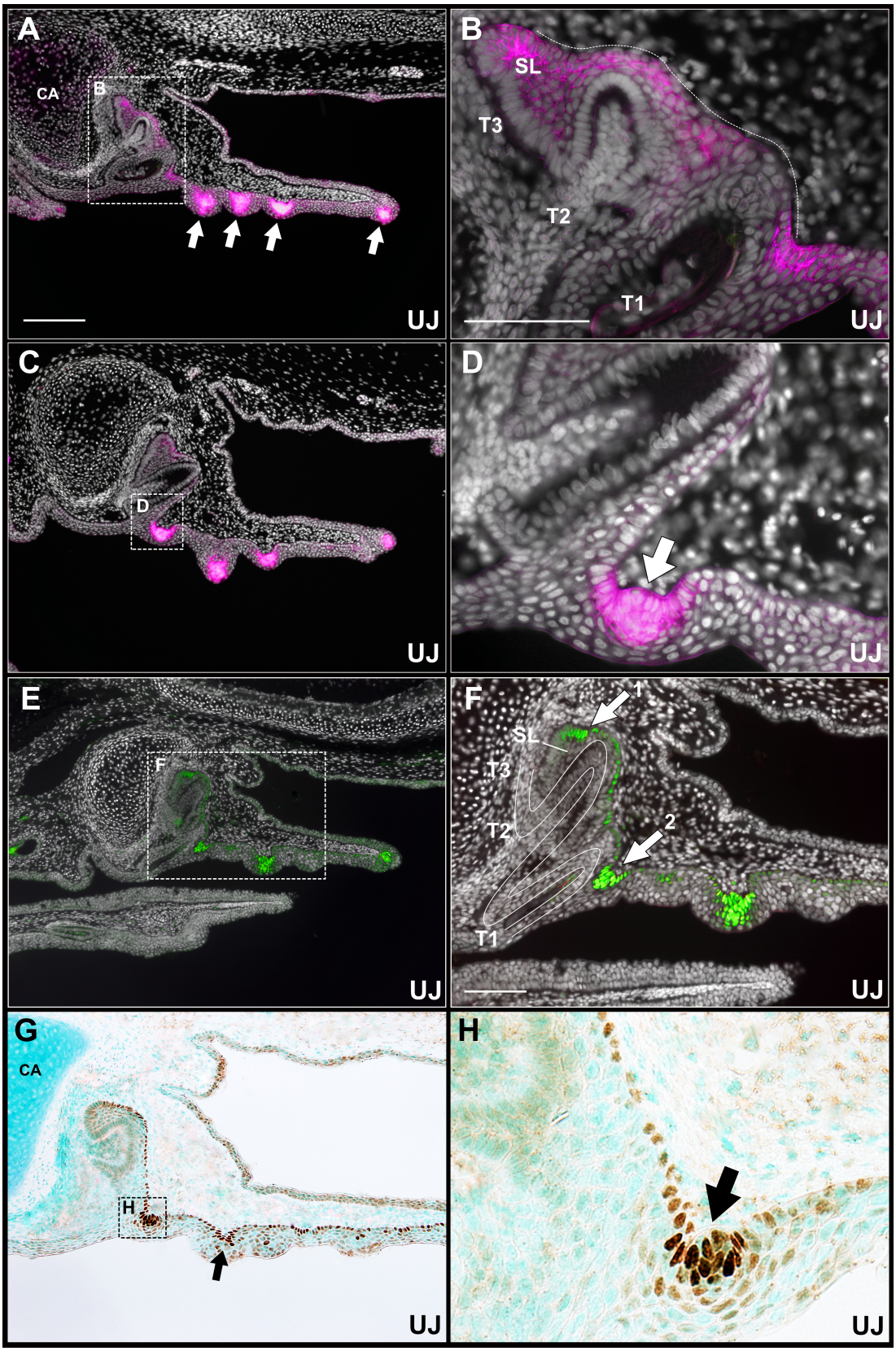


Figure 3.7 *Sox2* expression during shark tooth replacement (*S.canicula*). During early tooth replacement (upper jaw, UJ, T1-T3 stage) *Sox2* mRNA expression is maintained in the oral epithelium (MV) and TBs (arrows), continuous with the dental epithelium (A, DAPI). Sustained *Sox2* expression further localises to the O-ODE and

SL (dotted line), terminating lingually to the third generation tooth placode (T3) (B, DAPI). *Sox2* mRNA further localises to a taste bud-like, sub-epithelial cell pocket continuous with the O-ODE (C, boxed area, DAPI). This epithelial cell cluster (D, arrow, DAPI) shows marked association with the SL, as defined by its connection with the adjoining O-ODE. Sox2 IHC shows a similar pattern, with protein localised to the oral-dental epithelium, continuous with the SL (boxed area) (E, DAPI). Sox2 protein localises to the O-ODE and lingual aspect of the SL (F, arrow 1, DAPI), while further defining a similar pocket of cells in the distal aspect (F, arrow 2). Additional IHC (methyl green) further defines the Sox2+ oral-dental epithelium (G), marking a distinct cell cluster (G, boxed area and H, arrow) continuous with taste buds (G, arrow) and the SL. Scale bars: (A, C, E, G) 200 μm, (F) 100 μm, (B, D, H) 100 μm.

In embryonic rays (*R.clavata*), subsequent stages of early tooth replacement were defined by the development of two generations of teeth; the first undergoing morphogenesis (T1) and second, bud stage (T2) (Fig. 3.8). During these stages, *Sox2* was continually expressed in similar patterns to those in first generation teeth, with mRNA localised to the oral epithelium continuous with the adjoining dental epithelium (Fig. 3.8A). *Sox2* expression continued to extend into the SL via the O-ODE (Fig. 3.8B), terminating lingually to the second-generation tooth (upper jaw, UJ, T2). The distribution of Sox2 protein followed this pattern, extending from the oral and dental epithelium into the SL, terminating lingually to second-generation teeth (Fig 3.8C-D). These expression patterns were further complemented by additional Sox2 IHC (methyl green), showing a sustained band of Sox2+ epithelium connecting the SL with the oral epithelium via a continuous stripe of O-ODE (Fig. 3.8E-F).

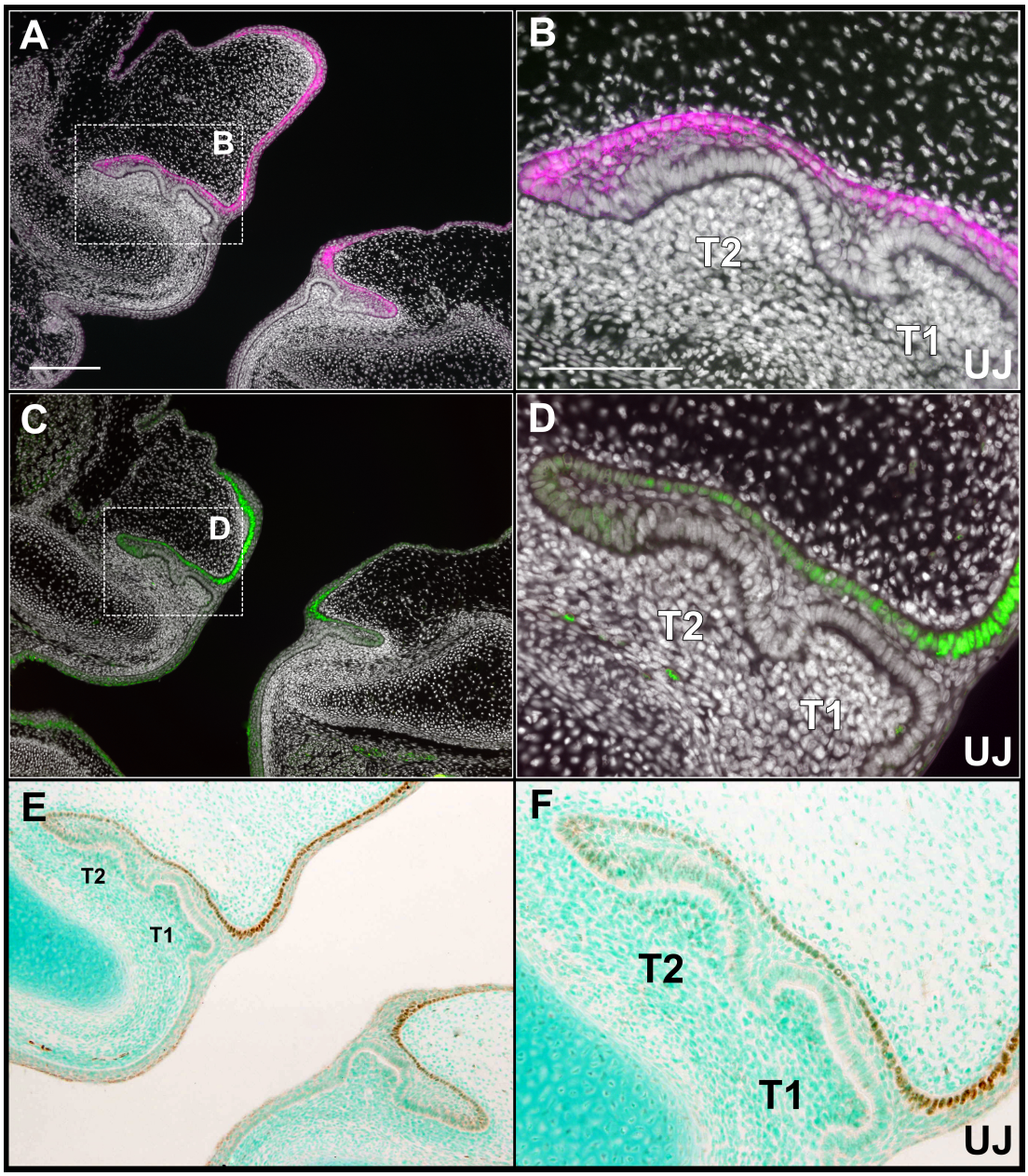


Figure 3.8 *Sox2* expression during early ray tooth replacement (*R.clavata*). During development of second-generation teeth (upper jaw, UJ), *Sox2* expression patterns remain largely unchanged, with mRNA expressed in the oral epithelium continuous with the dental epithelium (A, DAPI). Expression continues to extend into the SL via the O-ODE, terminating lingually to the second-generation tooth bud (T2) (B, DAPI). Sox2 IHC produces a similar pattern, with protein expressed in the oral epithelium and O-ODE, continuous with the SL and terminating lingually to early second-generation teeth (T2) (C-D, DAPI) and (E-F, methyl green). Scale bars: (A, C, E) 200 μm, (B, D, F) 100 μm.

3.3.4 Lingual *Sox2* marks a highly proliferative successional lamina

In this study, the availability of thornback ray (*R.clavata*) embryos was limited. Little skate (*L.erinacea*) embryos were therefore subsequently used to investigate *Sox2* expression in horizontal plane. In addition, PCNA was used to define cell proliferation dynamics in dental tissues. PCNA (proliferative cell nuclear antigen) expression is maximal during mitosis (S Phase) of the cell cycle, labeling highly proliferative zones of predominantly dividing progenitors and identifying regions of early differentiation, each characterised by strong and weak immunoreactivity, respectively (Bravo and MacDonald-Bravo, 1987; Ferreiro-Galve *et al.,* 2010).

At this stage of development, at least two generations of teeth were observed, marked by PCNA immunoreactivity in dental tissues within the parasymphysis of the lower jaw (LJ) (Fig. 3.9A, boxed area). Within the SL, early replacement teeth (G1-2) were present, arranged in an odd-even configuration with proliferating dental epithelial cells surrounded by a compartment of condensing mesenchymal cells (Fig. 3.9B). It is of interest that in the intervening MDE, cells associated with individual tooth germs showed some indications of movement. While in the absence of cell fate mapping data this remains unconfirmed, cell movement is implied by dynamic distribution patterns and polarity, compared with cells distributed labially to first generation teeth. In the upper jaw (UJ), PCNA showed strong immunoreactivity within the SL at equivalent stages, marking two generations of teeth undergoing bud stage (T2) and morphogenesis (T1) (Fig. 3.9C).

In symphyseal dental tissues, *Sox2* was expressed in restricted regions of the SL in both the upper and lower jaws (Fig. 3.9D, boxed areas). In the lower jaw, *Sox2* mRNA showed strong bias to the lingual aspect of the lamina, extending in between individual tooth germs in the intervening MDE (Fig. 3.9E). In the upper jaw at equivalent stage, expression patterns were similar to those shown in sagittal plane, with mRNA restricted to the oral epithelium and O-ODE, continuous with the SL and terminating lingually to second-generation teeth (T2) (Fig. 3.9F). The distribution of Sox2 protein followed similar patterns, restricted to the lingual SL (Fig. 3.9G, boxed area). The level of expressed protein was also comparatively

reduced, compared with mRNA, restricted to a defined cell population aligned to the lingual-most extent of the SL and showing marked association with developing tooth germs in a pattern of distribution similar to that shown by PCNA (Fig. 3.9H). In the upper jaw, the distribution of Sox2 protein was similar, expressed in a comparatively well-defined cell population focal to the adjoining oral epithelium, O-ODE and SL (Fig. 3.9I).

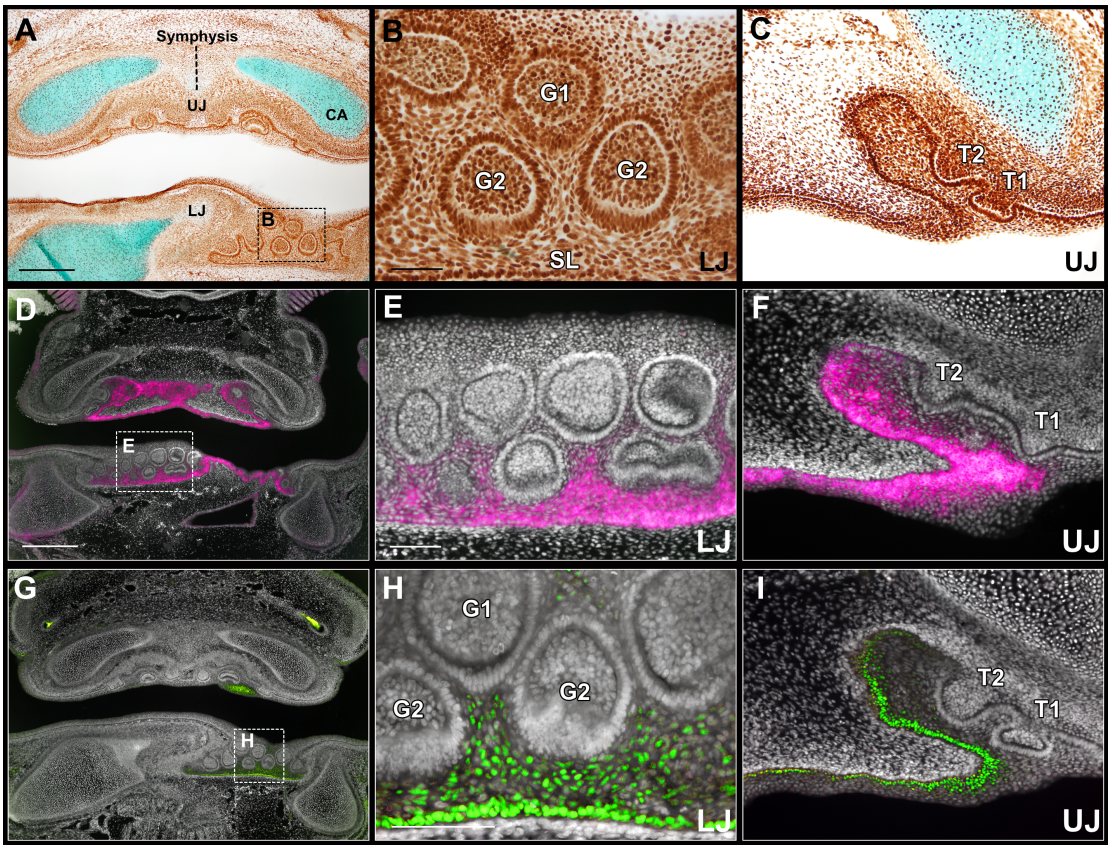


Figure 3.9 Cell proliferation dynamics and *Sox2* expression patterns during ray tooth replacement as shown in horizontal plane (*L.erinacea*). In lower jaw (LJ) parasymphyseal dental tissues, PCNA immunoreactivity shows cell proliferation within the SL (A, boxed area). PCNA defines two replacement tooth generations (G1-G2) (B), while tentatively indicating cell movement. While in the current study this remains unconfirmed, this is implied by the distribution patterns of cells in the inter-tooth region (MDE), their polar morphology and associations with individual developing tooth germs, compared with cells distributed labially. In the upper jaw (UJ), PCNA further defines two generations of teeth within the SL (C) (T2, bud and T1, morphogenesis). At equivalent stage, *Sox2* mRNA is strongly expressed within the SL in both the upper and lower jaws (D, boxed areas, DAPI). *Sox2* mRNA shows marked association with individual tooth germs, biased to the lingual extent of the SL and extending to within the MDE (E, DAPI). *Sox2* mRNA in the upper jaw (parasymphysis) shows similar expression patterns to those shown in sagittal plane, restricted to the oral epithelium and O-ODE, terminating lingually to second generation (T2) tooth buds (F, DAPI). Sox2 IHC produces a similar pattern, with expressed protein localised to within the SL (lower jaw, parasymphysis) (G, DAPI). Here, Sox2 protein forms a comparatively well-defined band aligned with the lingual-most aspect of the SL, with positive cells extending into the MDE between individual tooth germs in a pattern similar to that defined by PCNA (H, DAPI). Upper jaw Sox2 IHC expression follows a similar pattern to mRNA, with protein restricted to a well-defined band of cells spanning the oral epithelium and O-ODE, continuous with the lingual aspect of the SL (I, DAPI). Scale bars: (A, D, G) 1mm, (B, C, E, F, I) 100 μm, (H) 100 μm.

3.3.5 *Sox2* and *Lef1* are expressed in delineated domains during elasmobranch tooth development

In order to further investigate the putative interactions of *Sox2* with other molecular markers of tooth development, these were compared with the expression of the Wnt marker, lymphoid enhancing factor 1 (*Lef1*), which during activation of this pathway associates with *β-catenin* to trigger transcription of target genes (reviewed by Gordon and Nusse, 2006). A developmental series for *Lef1* were produced for the shark (chapter 4); however, in the absence of a similar series for the ray, these were compared during early tooth replacement only. In the shark, *Sox2* was characteristically expressed in the O-ODE, terminating within the successional lamina (Fig. 3.10A). *Lef1* was expressed exclusively in a delineated region of the dental epithelium, coincident with the thickened epithelium of the replacement tooth placode (Fig. 3.10B, arrow). The expression patterns of *Sox2* during ray tooth replacement were as previously described (Fig. 3.8), with expression also terminating in a sharply delineated region of the successional lamina (Fig. 3.10C, arrow). In the ray, *Lef1* expression was further restricted to the IDE of both first and second generation teeth (Fig. 3.10D), also terminating in the successional lamina coincident with the delineated region set by *Sox2* (arrow).

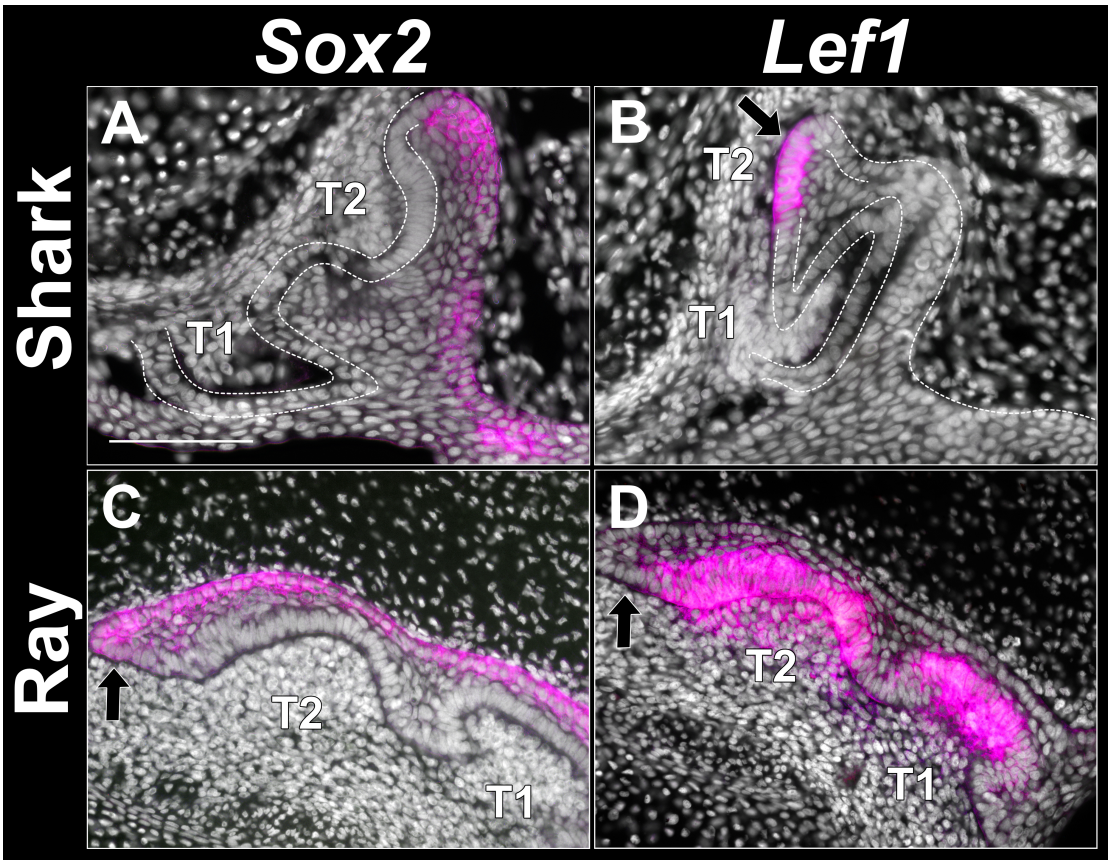


Figure 3.10 Comparative *Sox2*/ *Lef1* expression in the shark and ray replacement dentition (*S.canicula* and *R.clavata*). In shark tooth replacement (upper jaw), *Sox2* is consistently expressed in the O-ODE and SL (A) bordering with *Lef1*, expressed in the dental epithelium coincident with RT placodes (B, arrow). In the ray dentition (upper jaw), these patterns are further conserved, with *Sox2* also restricted to the O-ODE/ SL (C), bordered by *Lef1*, expressed in the most proximal replacement tooth (D, arrow). Scale bar: 100 μm.

3.4 Discussion

3.4.1 Ancestrally conserved *Sox2* marks putative dental stem cells in the elasmobranch dentition

In several gnathostomes, teeth are only able to develop following formation of the DL (Fraser *et al*., 2008; Buchtová *et al*., 2008; Juuri *et al*., 2013) and common to many, replacement teeth in sharks develop lingually to existing tooth generations (Reif, 1980). This has led to the supposition that regulatory control of elasmobranch tooth replacement may originate within, or close to, the growth extension of the dental lamina (successional lamina), lingual to developing teeth (Smith *et al*., 2009b). When considering the crucial role of the mouse incisor SCN in tooth renewal, it is reasonable to speculate that a similar SCN regulated by a commonly deployed network of regulatory circuits are deeply conserved in ancient gnathostomes to maintain this regenerative capacity (Harada *et al.,* 1999; Wang *et al*., 2007). In sharks, the DL/ SL has been suggested to be ideally positioned to house the SCN required for continuous tooth replacement (Smith *et al*., 2009b). In line with this idea, local signals regulating the activity of dental progenitors are postulated to originate either from the oral and dental epithelial junction proximal to the lamina (hypothesis 1) or within the lingual aspect of the DL itself (hypothesis 2). Common to both, dental SCs regulated by these signals are suggested to be broadly distributed within an expanded region of the DL (MDE) intermediate to these two regions (reviewed chapter 1: section 1.3.2, Fig. 1.11) (Smith *et al*., 2009b).

Studies of cichlid tooth development show that replacement teeth initiate from an epithelial SL housed within an intraosseous (bony) crypt underlying each functional tooth (Fraser *et al*., 2013) and incorporation of the DNA synthesis marker ^{3}H-Thymidine into dividing cells within the SL epithelium have indicated this to be the location of a putative dental SCN (Huysseune and Thesleff, 2004). In zebrafish, replacement pharyngeal teeth also initiate from an epithelial SL, collectively implying these tissues to constitute one possible source of dental stem cells (van der Heyden and Huysseune, 2000; Huysseune *et al*., 1989; Huysseune and Thesleff, 2004). More recently, putative dental stem cells have also been localised to the pharyngeal replacement dentition of the medaka (*Oryzias latipes*). BrdU pulse-chase

experiments have identified slow-cycling dental epithelial cells expressing *Sox2* at the posterior end of replacement tooth families, where they are proposed to maintain continuous tooth replacement capacity (Abduweli *et al*., 2014). In the shark and osteichthyan dentitions, comparisons drawn between these tissue loci and SCNs known to continually replenish epithelial organs such as the incisor, intestinal epithelium and hair follicle, have further implied the source of putative dental SCs to originate from within the basal epithelium underlying, or adjacent to functional teeth (Harada *et al*., 1999; Wang *et al*., 2007; Potten *et al.,* 2003; Plikus *et al*., 2008; 2011; Huysseune and Sire, 2004).

However, when considering *Sox2* as a dental SC marker, recent molecular evidence offers a possible alternative scenario. For example, in the cichlid dentition, *Sox2* strongly localises to the labial surface oral epithelium connected to the SL via a continuous stripe (gubernacular cord) of *Sox2*+ cells. In cichlids, this surface oral epithelium is proposed to house a putative dental SCN from which progenitors proliferate into the SL to maintain tooth replacement capacity (Fraser *et al*., 2013). Patterns of *Sox2* gene expression in the elasmobranch dentition also imply the source of dental stem-like cells to originate from a restricted region of surface oral epithelium extending into the DL as a continuous stripe. In the elasmobranch dentition, the DL itself constitutes an in-folding of an initial *Sox2*+ stripe of oral epithelium, the odontogenic band. This restricted presumptive dental epithelium further expresses the dental patterning gene *Shh* (Smith *et al*., 2009a). This co-expression data supports the existing idea that the OB may constitute a DL primordium, which in continued association with the oral epithelium, retains stem-like properties during subsequent ontogeny (Smith *et al*., 2009b).

Further supporting evidence for this is found in reptiles. In the early python dentition, similar expression of *Shh* in the competent oral epithelium of the OB defines the future position of the prospective DL, which fails to develop following inhibition by cyclopamine treatment (Buchtová *et al*., 2008). The python and several additional squamate reptile species also express *Sox2* in the DL connected with the oral epithelium (Juuri *et al*., 2013). In the corn snake dentition, cells expressing *Sox2* localise to the lingual aboral dental lamina and oral epithelium, both linked together

by the intervening dental epithelium. At the oral epithelial surface, *Sox2* is proposed to mark a putative dental SCN from which progenitor cells proliferate into the SL to maintain tooth replacement capacity, in much the same way as the cichlid (Gaete and Tucker, 2013; Fraser *et al*., 2013). This is further supported by DiI lineage tracing, which shows the retention of labeled cells in the SL and incorporation into replacement tooth generations, implying the lamina to house putative dental SCs (Gaete and Tucker, 2013). In the gecko, similar cell lineage tracing by incorporation of BrdU into cells of the DL also indicates the presence of a localised dental SC population. In the alligator dentition, localised asymmetric *Sox2* expression lingual to first generation teeth shows marked resemblance to the expression patterns observed in the shark and ray dentitions (Handrigan *et al*., 2010; Juuri *et al*., 2013; Wu *et al*., 2013). These studies provide compelling evidence to support a highly conserved role for *Sox2* in regulating tooth regeneration (Juuri *et al*., 2013; Gaete and Tucker, 2013). It can therefore be inferred that in the elasmobranch dentition, the *Sox2*-expressing OB may constitute an initial pool of putative dental epithelial SCs, fated to form the primary dental lamina. During subsequent tooth development and early replacement, sustained *Sox2* expression at the junction of the oral epithelium and O-ODE defines a putative dental SCN supplying progenitor cells required for continuous tooth replacement. This is most apparent in the early developing shark dentition, which shows a distinctive *Sox2*+ taste bud-like cell cluster at the oral-dental epithelial junction, connected with an adjoining stripe of *Sox2*+ O-ODE extending into the lamina and terminating lingually to the first tooth placodes (section 3.3.2).

These remarkable similarities to the reptilian dentition are made further apparent by integration of *Lef1* expression data. In the ball python (*Python regius*) dentition, a direct role for Wnt signaling in tooth development and replacement is implied by the sustained expression of *Lef1* and *Axin2* in the dental epithelium and mesenchyme of the growth extension of the lamina tip (Handrigan and Richman, 2010b). In python dental explant tissue cultures, induced upregulation of Wnt-*β-catenin* signaling through targeted inactivation of GSK3*β* further increases dental epithelial cell proliferation. These experiments collectively demonstrate a direct role for canonical

Wnt signaling in the regulation of ordered tooth replacement through coordinated cell proliferation and extension of the lamina (Handrigan and Richman, 2010b).

In the corn snake dentition, *Lef1* is also expressed in the SL, while cells expressing *Sox2* localise to the lingual aboral dental lamina and oral epithelium, which houses a proposed dental SCN (Gaete and Tucker, 2013). The organisation of *Sox2* and *Lef1* into these two opposing domains within the epithelial compartment suggests a regulatory mechanism in which Wnt-*β-catenin* signaling inhibits *Sox2* expression to regulate compartmentalisation of progenitor cells marked for immediate odontogenic fates (Gaete and Tucker, 2013). In the corn snake dentition, similar up-regulation of Wnt-*β-catenin* signaling through GSK3*β* inhibition results in development of supernumerary tooth germs and associated disruption of normal polarity of tooth initiation and cell differentiation (Gaete and Tucker, 2013). This is further marked by the expansion of *Lef1* expression domains and a corresponding reduction in *Sox2* domains, restricted to the oral epithelium. In the corn snake dentition, this therefore supports a direct role for Wnt signaling in negatively regulating the expression domains of *Sox2*, so as to orchestrate the development and sequential replacement of teeth (Gaete and Tucker, 2013).

In both the shark and ray dental epithelium, *Sox2* and *Lef1* occupy similar opposing domains, with *Sox2* restricted to the O-ODE continuous with the SL, where *Lef1* is consistently expressed in the dental epithelium focal to developing teeth. This is further apparent in horizontal plane (Fig. 3.9), with *Sox2* predominantly expressed in a defined band aligned to the lingual face of the lamina, with a gradient of *Sox2*+ cells extending linguo-labially into the MDE and developing tooth germs. Given these striking similarities between the reptilian and elasmobranch dentitions, it is proposed here that *Lef1* may be acting in a conserved role by restricting *Sox2* to the lingual aspect of the lamina to maintain the controlled compartmentalisation of cells committed to tooth-specific fates (Gaete and Tucker, 2013). Future studies to manipulate Wnt-*β-catenin* signaling in the elasmobranch dentition will be required to further investigate these putative roles and interactions.

3.4.2 *Sox2* dental stem cell niche model

Inferences drawn from these gene expression patterns suggest a highly conserved role for *Sox2* in elasmobranch tooth replacement; however, a conclusive function remains unclear. *Sox2* gene function has been extensively studied, with recent findings suggesting an essential role in stabilising the pluripotent state of embryonic SCs (Masui *et al*., 2007; Niwa, 2007). Expression of *Sox2* in the prospective dental epithelium of the mouse incisor, and its progressive restriction to the dental SCN, suggests an early role in the specification of dental epithelial SCs, while fate mapping of *Sox2*+ cells during incisor renewal also implies a subsequent role in replenishing dental epithelial tissues (Juuri *et al*., 2012). In both the mammalian and reptilian dentitions, *Sox2* is further proposed to confer initial dental competence. Conditional deletion further results in hyperplastic dental epithelium, suggesting a role in the maintenance of progenitor dental epithelial cells (Juuri *et al*., 2013).

While in the elasmobranch dentition, definitive conclusions cannot yet be drawn, based upon its known function, *Sox2* is proposed to play a central role in regulating the undifferentiated state of dental progenitor SCs, reflected by its expression in the OB and a putative dental SCN. This supposition culminates in the production of the first hypothetical elasmobranch dental regenerative model (Fig. 4.11). In this model, *Sox2* is first expressed in the thickened oral surface epithelium in advance of the DL, marking onset of initial dental competence (OB) (Fig. 3.11A). During subsequent DL development, *Sox2* is expressed in the in-folding dental epithelium lingual to the first tooth bud, extending a short distance posteriorly from the lamina into the surface oral epithelium. Expression at this stage is proposed to coincide with the supply of undifferentiated progenitor cells required for sustained development of the DL itself and first generation teeth (Fig. 3.11B). Following this, *Sox2* expression is maintained in a stripe of dental epithelial cells (O-ODE) extending into the DL from the SCN positioned at the oral-dental junction and proximal to the taste buds (TB) (Fig. 3.11C). The SCN is taken to be the primary site of undifferentiated dental progenitors, while cells emanating into the DL/ SL via the O-ODE are postulated to be putative proliferating TA cells, committed to odontogenic fates. Throughout early first tooth induction (shown in chapter 4) and replacement, *Lef1* expression in the tooth position proximal to the DL/ SL is proposed to restrict *Sox2* expression to

within the lingual aspect of the epithelial compartment, in order to maintain a supply of undifferentiated dental progenitors (Fig. 3.11B-C). In both the shark and ray, the primary source of stemness therefore appears to derive from the OB, as marked by *Sox2*, which forms a persistent connection between the DL/ SL and the surface oral epithelium. The maintenance of this connection appears to be required for the continued supply of dental progenitors for continuous tooth regeneration.

The distribution of *Sox2*+ cells within the lamina itself and their putative interaction with *Lef1* is further illustrated schematically in horizontal plane, with *Lef1* creating a putative zone of inhibition to maintain dental progenitors in a 'holding pattern' of quiescence against the lingual face, until sequestered by the appropriate signals to commit to odontogenic fates (Fig. 3.11D). In the gecko dentition, the distribution of putative dental SCs follows a similar pattern, with clonal cells aligned against the lingual face of the SL, where they are proposed to periodically feed into individual tooth families as 'waves' in synchronisation with temporal timing of tooth replacement phases (Handrigan *et al*., 2010). 3D reconstructions of the gecko SL imply further alignment of dental SCs within the inter-dental region, where one cluster may feed into two adjacent tooth families (Richman and Handrigan, 2011). In the elasmobranch dentition, the distribution of *Sox2*+ cells in the SL is not indicative of a similar pattern of clustering. However, in light of expression patterns, which show a gradient of positive cells to spread linguo-labially into the MDE in between families of tooth germs, it is tempting to speculate that this may prove indicative of a conserved mechanism in which waves of clonal cells are periodically stimulated to proliferate into adjacent tooth families during active replacement phases (Fig. 3.11D, arrows).

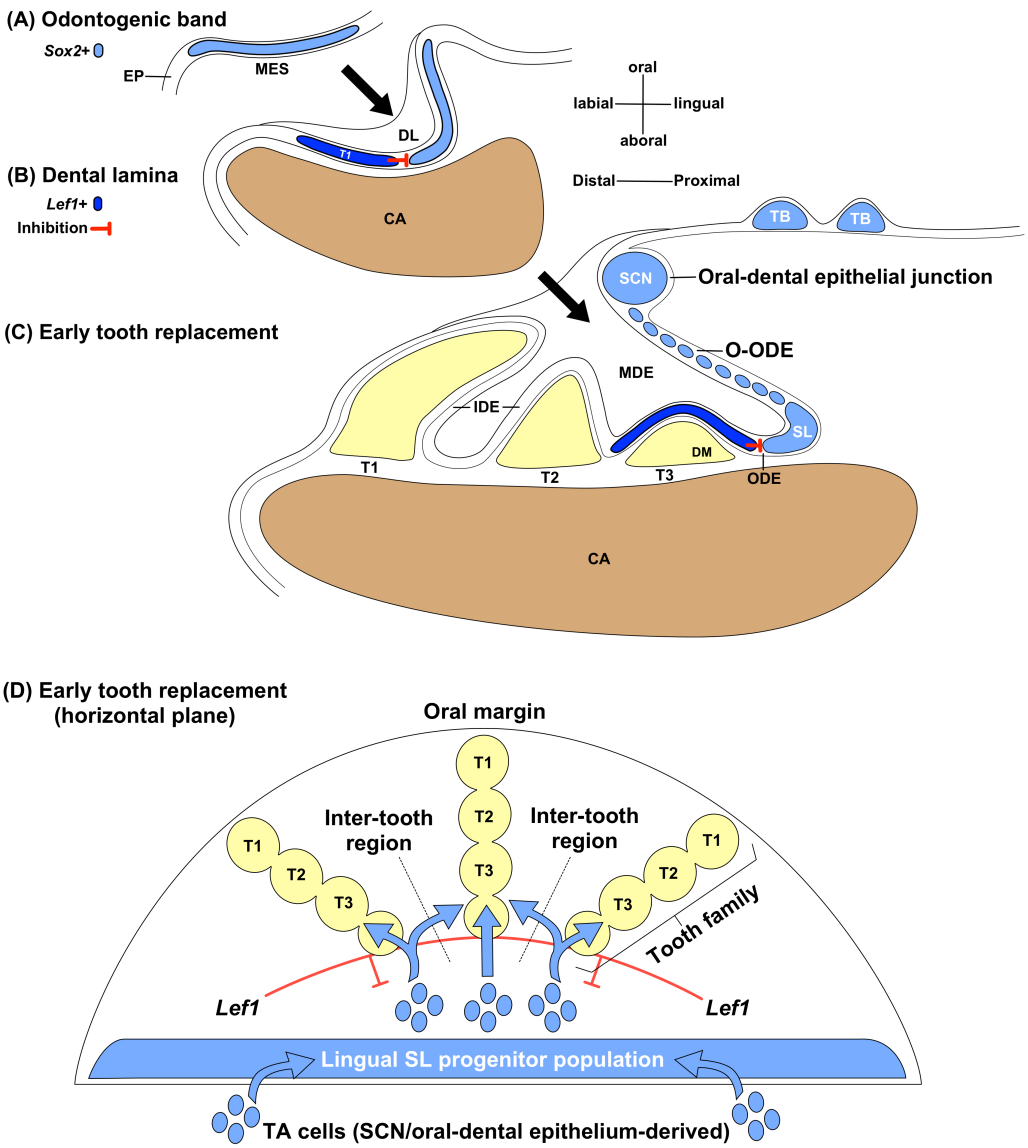


Figure 3.11 Elasmobranch *Sox2* dental SCN model. Based upon the expression patterns of *Sox2* in the shark and ray dentitions, a hypothetical model can be proposed (represented here in the lower jaw). In this model *Sox2* is first expressed in the thickened oral epithelium, marking initial dental competence (OB) (A). During subsequent development of the DL, *Sox2* expression becomes restricted to the O-ODE and proximal aspect of the lamina, terminating lingually to the first tooth placode (T1), as a result of inhibition by *Lef1*. This expression extends from the base of the lamina towards the oral surface as a continuous epithelial stripe, where it ends (B). During development of the first tooth (T1) and early replacements (T2-T3), *Sox2* remains restricted to the lingual DL/ SL resulting from continued *Lef1* inhibition, and is maintained in the epithelial stripe (O-ODE), connected with a defined cluster of *Sox2*+ epithelial cells positioned at the dental-oral epithelial junction (C). This cell cluster is proposed to constitute a putative dental stem cell niche (SCN) from which transit-amplifying (TA) cells proliferate into the lamina to sustain continual tooth replacement capacity. *Sox2* also strongly localises to taste buds developing in the oral cavity. Depicted in horizontal plane (D), within the SL, *Sox2*+ dental progenitors

align against the lingual face, where they are transiently held within a putative zone of inhibition created by *Lef1*. During active phases of tooth regeneration, progenitors (clonal cells) are stimulated by the appropriate signals to proliferate into adjacent tooth families (arrows) to undergo odontogenic fates. These expression patterns show marked similarities to the reptilian dentition, suggesting a highly conserved mechanism of tooth regeneration common to polyphyodont gnathostomes (Gaete and Tucker, 2013; Juuri *et al*., 2013). EP, epithelium; MES, mesenchyme; ODE, outer dental epithelium; O-ODE, outward-outer dental epithelium; T, tooth; CA, cartilage; SL, successional lamina; MDE, middle dental epithelium; IDE, inner dental epithelium; SCN, stem cell niche; TB, taste bud.

3.4.3 Additional putative interactions of *Sox2* in the elasmobranch dentition

The expression of *Lef1* provides some evidence to support direct regulation of *Sox2* from within the DL/ SL. However, given that *Sox2* is also expressed in the SCN and as putative TA cells in the intervening O-ODE, it is probable that further levels of regulatory control are orchestrated from these cell loci. In this respect, while the amounts of expressed *Sox2* mRNA and protein have not yet been quantified, it is of interest that visually, the level of expressed RNA appears to consistently exceed that of protein, implying inhibition of translated Sox2. In the snake dentition, it is proposed that Sox2 may intrinsically inhibit its own expression in the DL and in mouse ES cells, increased *Sox2* and associated *Oct4*, *Klf4* and *c-Myc*, repress expression of endogenous *Sox2* via a negative feedback loop involving the *Sox2* activator *Foxo1* (Gaete and Tucker, 2013; Ormsbee Golden *et al*., 2013). Given this apparent difference in *Sox2* mRNA and Sox2 protein, it therefore remains highly likely that additional factors, inclusive of *Sox2* itself, may exercise further levels of regulatory control in dental tissues to maintain the required balance between cell pluripotency, proliferation and differentiation. In this respect, gene expression data pertaining to markers commonly expressed in dental stem cells, such as *ABCG2, Bmi-1, Oct-3*/*4*, *Nanog*, *Dkk3* and *Igfbp5*, are likely to provide further insights into the role of putative dental stem cells in this process (Li *et al.,* 2011a; Handrigan *et al*., 2010).

3.4.4 Future studies of mesenchymal stem cells in the elasmobranch dentition

In addition to epithelial dental SCs, the isolation and characterisation of mesenchymal dental stem cells (MDSCs) has also generated considerable research interest, due to their accessibility, multipotent properties and high proliferation capacity (reviewed by Rodríguez-Lozano *et al*., 2012; Huang *et al*., 2009). Of these, dental pulp stem cells (DPSCs) constitute a highly plastic cell population with the ability to differentiate to several cell types, including odontoblast-like lineages (Gronthos *et al*., 2000; Zhang *et al*., 2006). *In vitro* treatment of human DPSC cultures with the small molecules Pluripotin (SC1), 6-bromoindirubin-3-oxime and rapamycin, decreases cell proliferation and differentiation capacity, while increasing expression of *Sox2*, *Oct4*, *Nanog* and *Stro-1*, showing conserved sets of pluripotency

factors to regulate stemness (Al-Habib *et al*., 2013). The regenerative potential of DPSCs has also been shown by *in vitro* studies, in which combined DPSCs and SCs derived from the apical papilla (SCAP) induce *de novo* regeneration of dental pulp tissues (Huang *et al*., 2010). In mouse incisor renewal, the importance of the mesenchymal component has also been shown through the identification of mesenchymal SCNs, which in collaboration with their epithelial counterparts, maintain incisor growth and renewal through similar signaling cascades (reviewed chapter 1: section 1.2.6) (Lapthanasupkul *et al*., 2011; Zhao *et al*., 2014). In the absence of specific MSC markers, their putative role in the shark dentition remains unresolved. However, in both sharks and rays, the conserved expression of several genes in the dental mesenchyme (to be shown in chapter 4) highlights the important role of mesenchymal cells and the signaling cascades regulating their behaviour. While it remains highly likely that many of these mesenchymal markers are directly involved in MSC regulation, this remains speculative, warranting future studies.

3.5 Conclusions

This study identifies *Sox2* as the first putative dental stem cell marker expressed in the regenerating shark and ray dentitions. The expression patterns presented here provide initial evidence to define both the source of initial odontogenic potential required for elasmobranch teeth to form (OB) and one possible superficial source of dental 'stem-like' cells required for continuous tooth regeneration. Localisation of this putative dental stem cell niche (SCN) to the oral epithelium and a *Sox2*+ epithelial stripe connected with the dental lamina is in partial agreement with existing hypotheses regarding regulatory control of tooth regeneration in sharks (Smith *et al.,* 2009b). This study therefore proposes a new hypothetical model in which *Sox2* marks quiescent progenitor cells maintained in the SCN. From here, dental progenitors are stimulated to proliferate into the lamina as putative transit amplifying cells during active phases of tooth replacement to maintain this regenerative cycle. In accordance with similar studies of the snake dentition, *Lef1* is proposed to restrict the expression of *Sox2* to the lingual aspect of the dental lamina to maintain transient control of undifferentiated dental precursors, while further controlling the extension of the successional lamina during the sequential addition of new replacement teeth (Gaete and Tucker, 2013). Evolutionarily, this collaboration therefore suggests a regenerative strategy conserved amongst polyphyodont gnathostomes for 450-500 million years of vertebrate evolution (Juuri *et al*., 2013; Gaete and Tucker, 2013).

Chapter 4

AN ANCESTRAL GENE REGULATORY NETWORK PERPETUATES TOOTH REGENERATION IN ELASMOBRANCHS

4.1 Summary

In chapter 3, the stem cell marker *Sox2* was used to investigate the role of putative dental stem cells in the elasmobranch dentition, implying a tooth regenerative strategy common to sharks, rays and other polyphyodont gnathostomes. However, tooth renewal and replacement is known to further result from the deployment of a core set of genes expressed in the Wnt-*β-catenin*, hedgehog, FGF and BMP pathways (Harada *et al*., 1999; 2002; Järvinen *et al*., 2009; Fraser *et al*., 2006a; 2008; 2013; Handrigan and Richman, 2010a; 2010b; Gaete and Tucker, 2013). In this study, the primary focus was on the catshark as a polyphyodont model to investigate the conserved roles of genes expressed in these pathways during tooth development. The ray was further used as a secondary model to draw additional comparisons.

In the shark dentition, PCNA further defines patterns of cell proliferation during various stages of tooth development. *In situ* hybridisation then defines the expression of *β-catenin, Shh, Ptc2, Pitx1/2* and *Bmp4* during early dental competence. These, and the Wnt gene *Lef1* are subsequently expressed in the early dental lamina and first tooth placodes. During tooth development and replacement, these genes, and an expanded set of markers, including *Sostdc1, Taz, Fgf3*/*10, Midkine, Meis2, Foxq1, Twist, Runx2, Dlx3* and *Sparc*, are further expressed in patterns indicative of conserved roles. Similar expression patterns in the ray dentition further imply a significant degree of conservation among elasmobranchs.

This study further demonstrates two key findings. Firstly, during morphogenesis, reduced cell proliferation in the epithelial tooth tip concomitant with nested gene co-expression implies the conservation in the shark dentition, of a signaling center comparable to the mammalian enamel knot (Vaahtokari *et al.,* 1996a). Secondly, PCNA and gene expression combined, further define the oral-dental epithelial connection previously identified using *Sox2*, adding additional evidence to support the deployment of a dental stem cell niche in the regenerating shark dentition. These collective expression patterns offer new perspective regarding the deep conservation in gnathostomes, of a core dental gene regulatory network, and inspire production of the first hypothetical shark tooth GRN model.

4.2 Introduction

4.2.1 A core dental GRN is conserved in polyphyodont gnathostomes

Recent studies addressing gene regulatory control of tooth development have progressively shifted from mammals to polyphyodont gnathostomes capable of *de novo* tooth regeneration dentition (reviewed by Tucker and Fraser, 2014). As previously highlighted, these have predominantly focused upon reptiles and osteichthyan fish, in both cases identifying the deployment of highly conserved molecules belonging to the hedgehog, Wnt and BMP signaling pathways (Buchtová *et al.,* 2010; Handrigan and Richman 2010a; 2010b; Handrigan *et al.,* 2010; Fraser *et al*., 2004; 2006a; 2008; 2013; 2012; Abduweli *et al*., 2014). However, as previously highlighted, knowledge of gene regulatory control of shark tooth development remains limited to just a few genes and in relatively superficial detail (reviewed chapter 1: section 1.3.2).

To date, expression in the catshark dentition of the hedgehog pathway ligand sonic hedgehog (*Shh*) provides the strongest evidence to support the ancestral conservation of core odontogenic signaling pathways in ancient gnathostomes (Smith *et al*., 2009a). In the embryonic shark dentition, *Shh* is first expressed in the early epithelial thickenings of the odontogenic band and dental lamina to determine the positions of future tooth loci. Following its initial expression, *Shh* reappears in the developing tooth cusps during morphogenesis, implying a deeply conserved odontogenic role (Smith et *al*., 2009a; Dassule and McMahon, 1998; Cobourne *et al*., 2004; Fraser *et al*., 2004; Harris *et al*., 2006; Buchtová *et al*., 2008). In addition, the expression of *Epha4*, *Runx1/3* and several members of the *Dlx* family of Homeobox genes in the shark dentition further supports the deep conservation of a core dental GRN (Freitas and Cohn, 2004; Hecht *et al*., 2008; Debiais-Thibaud *et al*., 2011).

4.2.2 Aims and objectives

Given the limited extent of current knowledge of gene regulatory control of elasmobranch tooth development, a more comprehensive survey of epithelial-

mesenchymal gene expression remains outstanding. To address this gap in knowledge, the aims and objectives are as follows:

- To use the catshark as a primary model to investigate cell proliferation dynamics and the expression of genes representative of conserved signaling pathways (Table 4.1) in the embryonic shark dentition.

- To profile their expression patterns during shark tooth initiation, development and replacement and compare and contrast these with a subset of genes expressed in the embryonic ray dentition.

- To further compare and contrast these with successive gnathostomes, so as to infer the extent of their ancestrally conserved roles in maintaining elasmobranch tooth regeneration capacity and develop a hypothetical shark tooth gene regulatory network (GRN) model.

Table 4.1 Candidate genes cloned for expression analysis. Unannotated sequences (no accession) obtained from raw transcriptomic databases SkateBase (www.skatebase.org) and Vertebrate TimeCapsule, VTcap (http://transcriptome.cdb.riken.go.jp/vtcap). Cloned sequence identities confirmed by 3730 Sequencing and BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene name, abbreviation	Role	Pathway/ family/ interaction	Database/ accession
Sonic hedgehog, *Shh*	Lig	Hh	HM991336.1
Patched-2, *Ptc2*	Rec		EU814484.1
β-catenin, *β-cat*	TF	Wnt	AF393833.1
Lymphoid enhancer-binding factor 1, *Lef1*	TCR		VTcap
Paired-like homeodomain transcription factor 1/2, *Pitx1/2*	TF	Pitx	SkateBase, AB625610.1
Sclerostin domain-containing protein 1, *Sostdc1*	Lig	Bmp/ Wnt/ Hh	VTcap
Runt-related transcription factor 2, *Runx2*	TF	Runx	EU241884.1
Fibroblast growth factor 3/10, *Fgf3/10*	Lig	Fgf	SkateBase
Neurite growth promoting factor 2, Midkine, *MK*	Lig	Midkine	VTcap
Bone morphogenetic protein 4, *Bmp4*	Lig	TGF-β	SkateBase
Twist-related protein, *Twist*	TF	Twist	EU196400.1
Transcriptional co-activator with PDZ-binding motif, *Taz* (*wwtr1*)	TCR	Hippo	SkateBase
Forkhead-box q1, *Foxq1*	TF	Fox	EU438755.1
Meis protein-2, *Meis2*	TCR	Hox	SkateBase
Distal-less homeobox transcription factor 3, *Dlx3*	TF	Hox	JX270826.1
Secreted protein acidic and rich in cysteine, *Sparc*	GP	Sparc	EU241888.1

Abbreviations: GP, glycoprotein; LIG, ligand; REC, receptor; TF, transcription factor; TCR, transcriptional co-regulator.

4.3 Results

4.3.1 PCNA analysis defines the proliferating shark dentition

In chapter 3, PCNA was used to investigate cell proliferation in the ray successional lamina. To investigate cell proliferation dynamics during catshark tooth development, PCNA was further applied, using a combination of stages in the upper and lower jaws. At the lower jaw oral margin, PCNA first labeled a distinct population of thickened, columnar epithelial cells overlying an associated condensing mesenchyme (Fig. 4.1A, boxed area). This localised epithelial-mesenchymal cell population is presumed to mark the onset of early dental competence, commonly defined by the odontogenic band (OB) (Fig. 4.1B, dotted line) (Fraser *et al*., 2004; Smith *et al*., 2009a). During subsequent development of the primary dental lamina, the dental epithelium (DE) formed an in-fold into the underlying dental mesenchyme (DM), marked by PCNA immunoreactivity, predominantly biased to the lingual aspect (Fig. 4.1C, arrow). During placode formation, PCNA continued to label the thickened epithelium of the first tooth (Fig. 4.1D, arrow) and at bud stage, the corresponding epithelium-mesenchyme (Fig. 4.1E, arrow).

During morphogenesis, PCNA immunoreactivity remained strong in the DL and reduced in the apical inner dental epithelium (IDE) and mesenchyme of the tooth (Fig. 4.1F, arrow 1), marking early differentiation of odontoblasts and ameloblasts. Sustained proliferation in the early successional lamina (SL) accompanied development of second-generation tooth placodes (Fig. 4.1G, arrow) lingual to advancing first generation teeth (T1). During tooth replacement, PCNA continued to label proliferating cells in the SL, coincident here with third generation teeth (T3) undergoing early morphogenesis, with a progressive reduction in PCNA immunoreactivity in second (T2) and first (T1) generation teeth, undergoing advancing morphogenesis (Fig. 4.1H). Throughout tooth development, PCNA further defined a continuous epithelial connection maintained between the DL/ SL and the surface oral epithelium (O-ODE), terminating in a distinct cluster of non-proliferative epithelial cells (Fig. 4.1F, arrow 2) and (Fig. 4.1H, dotted line).

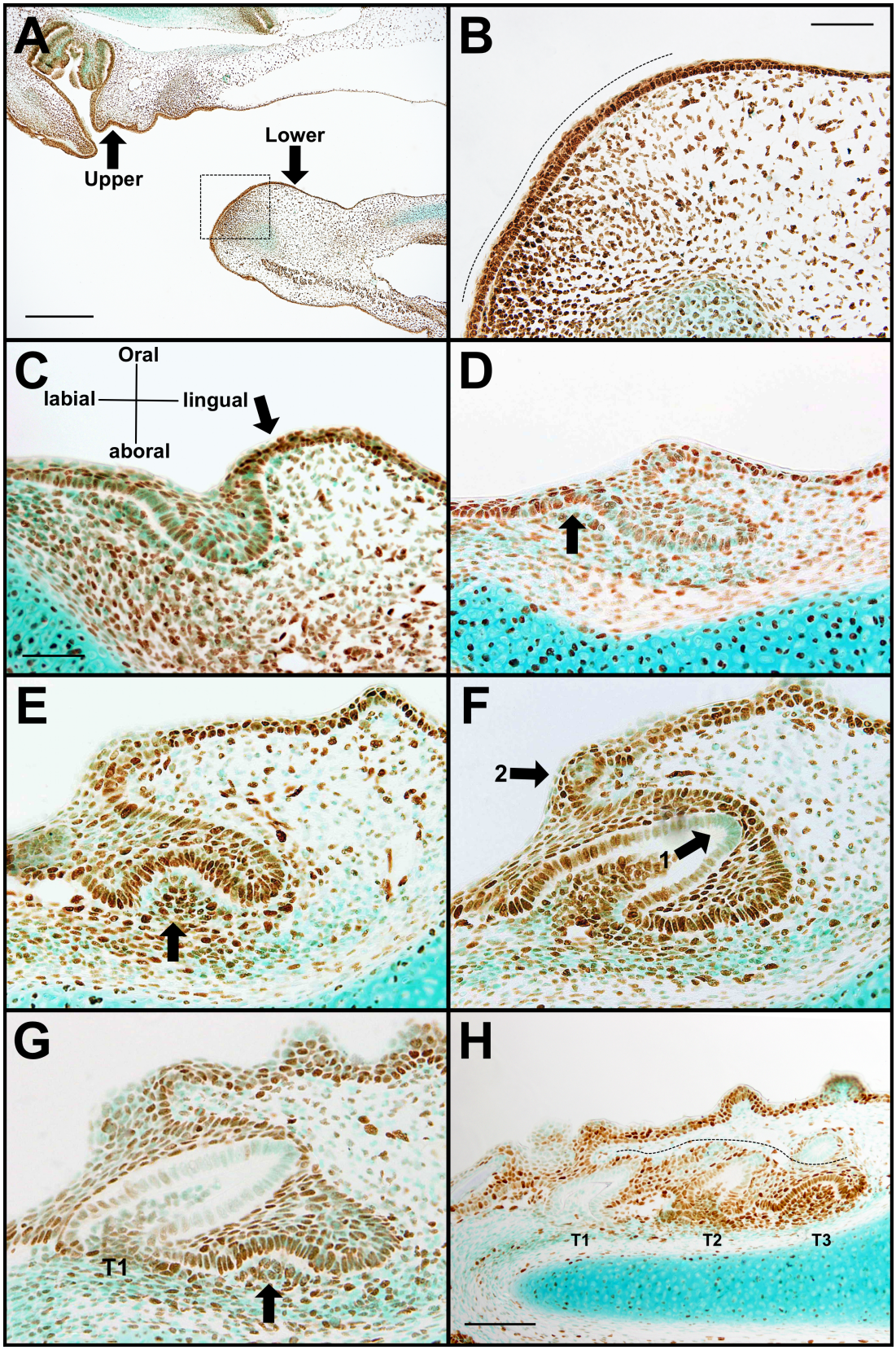


Figure 4.1 PCNA IHC of catshark lower jaw tooth development (A-B, *S.stellaris*; C-H, *S.canicula*). In the lower jaw oral margin (A, boxed area), PCNA immunoreactivity marks initial epithelial thickening and associated mesenchymal condensation to define early dental competence (odontogenic band) (B, dotted line).

During development of the primary DL, PCNA continues to mark epithelial in-folding into the DM, biased to the lingual aspect (C, arrow). During development of first generation teeth, PCNA marks the first tooth placode, accompanied by further extension of the DL (D, arrow). Epithelial cells budding into the surrounding DE, define the first tooth bud overlying the condensing DM (E, arrow). During morphogenesis, epithelial-mesenchymal cells differentiate to ameloblasts and odontoblasts, marked by reduced PCNA immunoreactivity in the apical tooth (F, arrow 1). The first replacement tooth placodes (G, arrow) develop in the continuously proliferating SL, lingual to advancing first generation teeth (T1). During tooth replacement, PCNA immunoreactivity in the SL marks third generation teeth (T3, early morphogenesis), progressively reducing in preceding generations undergoing advancing morphogenesis (T2-1). Throughout tooth development and replacement, a continuous connection is maintained between the DL/ SL and the surface oral epithelium via the O-ODE, terminating in a distinct spherical cluster of non-proliferative cells (F, arrow 2) and (H, dotted line). Scale bars: (A) 1 mm, (B) 100 μm (C-G) 100 μm, (H) 200 μm.

In the upper jaw, PCNA labeling of the thickened epithelium and condensing mesenchyme also defines the onset of early dental competence (Fig. 4.2A, boxed area). In accordance with the lower jaw, this restricted band of thickened columnar epithelial cells is presumed to mark the OB, and the future site of the prospective DL (Fig. 4.2B, dotted line) (Fraser *et al.,* 2004; Smith *et al.,* 2009a). During development of the early DL, PCNA strongly labeled invaginating oral epithelial cells (Fig. 4.2C, dotted line). These thickened epithelial cells were continuous with the ventral axis of the maxillary valve (MV), a broad free-fold of oral tissue spanning the jaw arc and functioning to regulate water intake (Hamlett, 1999). During first generation tooth development, PCNA strongly labeled the first thickened epithelial placodes and condensing mesenchymal cells in the DL (Fig. 4.2D, arrow 1), forming a continuous connection with the oral epithelium of the MV and merging with developing taste buds (Fig. 4.2D, arrow 2). During the transition from bud to early morphogenesis, PCNA immunoreactivity remained constant in the IDE, while increasing in the mesenchymal compartment to mark associated cell proliferation. At this stage, early tooth shape also became apparent, shown by PCNA labeling of polarised cells in the epithelial tip (Fig. 4.2E). By advanced morphogenesis, reduced PCNA immunoreactivity in the tip (Fig. 4.2F, arrow) implied reduced cell proliferation, concomitant with onset of early cell differentiation to odontogenic fates.

During tooth replacement, continued PCNA labeling of epithelial-mesenchymal cells in the SL indicated sustained cell proliferation (coincident here with morphogenesis, T2) (Fig. 4.2G, boxed area). A lack of PCNA immunoreactivity during advanced morphogenesis (T1) further indicated terminal differentiation of dentin-secreting odontoblasts and enameloid-secreting ameloblasts. Sustained PCNA immunoreactivity at the base of the tooth implied continued cell proliferation associated with early differentiation of odontoblasts for basal plate formation (Fig 4.4G, arrow 1). PCNA labeling also defined several well-developed taste buds in the ventral epithelial surface of the MV (Fig 4.2G, arrows). During replacement tooth (RT) morphogenesis, a small cluster of medial epithelial cells nested within the tip lacked PCNA immunoreactivity (Fig. 4.2H, arrow). While morphologically indistinct from neighbouring cells, the absence of proliferative activity in this cell cluster implied withdrawal from the cell cycle and terminal differentiation (Bravo and

MacDonald-Bravo, 1987). In the upper jaws, PCNA labeling also defined a continuous epithelial connection maintained between the DL and the surface oral epithelium via the O-ODE. This connection appears to be formed during development of the dental lamina (Fig. 4.2C) and maintained during early tooth replacement (Fig. 4.2G, arrow 2).

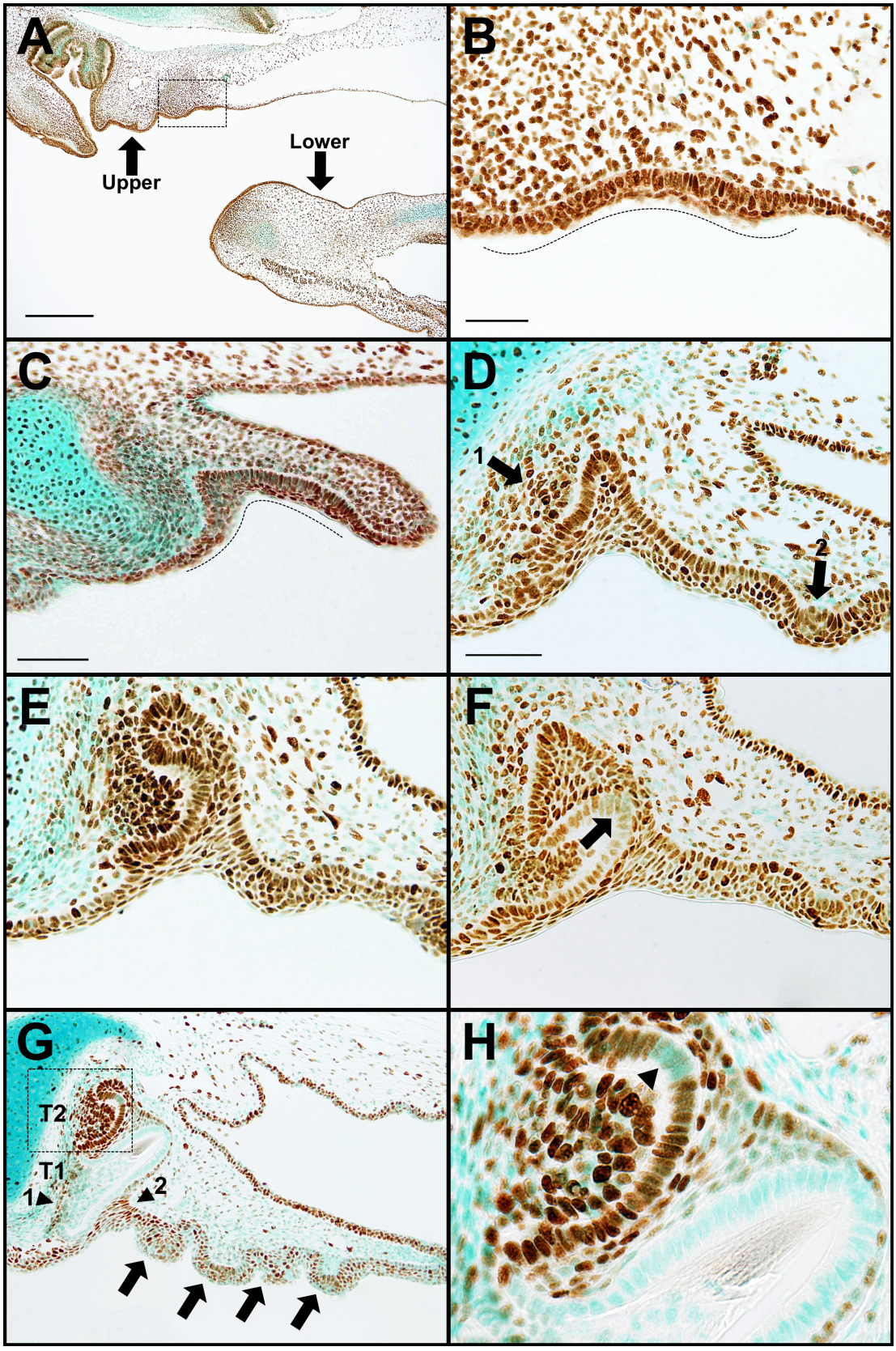


Figure 4.2 PCNA IHC of catshark upper jaw tooth development (A-B, *S.stellaris*; C-H, *S.canicula*). In the upper jaw, PCNA immunoreactivity further defines localised thickening of the oral epithelium and condensing mesenchyme to mark onset of

dental competence (A, boxed area). A restricted band of thickened columnar epithelial cells mark the OB, and future position of the prospective DL (B, dotted line). Sustained PCNA immunoreactivity marks formation of the early DL as this epithelium, continuous with the MV, in-folds into the underlying DM (C, dotted line). Development of the first tooth bud is defined by outgrowth of thickened dental epithelial cells, associated with underlying mesenchymal condensates (D, arrow 1). The O-ODE is also continuous with the basal epithelium of the MV, defined by early taste bud papillae (D, arrow 2). During first tooth morphogenesis, PCNA immunoreactivity continues to show sustained condensation of the underlying DM and early epithelial growth polarity (E). During advanced morphogenesis, reduced PCNA immunoreactivity in the apical IDE and DM of the tooth further implies cell differentiation in advance of matrix deposition (F, arrow). PCNA continues to label the IDE and DL during RT morphogenesis (G, boxed area T2), with a corresponding reduction in immunoreactivity during advanced morphogenesis (T1), accompanied by associated deposition of mineralised tissue. In the basal DM of the papilla, sustained PCNA immunoreactivity marks continued cell proliferation concomitant with basal plate formation (G, arrow 1), while continuing to define a connection between the dental and oral epithelium (G, arrow 2). During RT morphogenesis, a nested cluster of cells in the medial epithelial tip (H, arrow) lack PCNA immunoreactivity, compared with the surrounding IDE and DM. Their implied withdrawal from the cell cycle suggests a functional requirement distinct from surrounding epithelial cells. Scale bars: (A) 1 mm, (B, D, E, F) 100 μm (C, G) 200 μm.

4.3.2 Conserved gene expression domains indicate early dental competence

Very little is known about gene regulatory control of shark tooth development. While analysis of several genes has implied roles in the shark dentition, a detailed study of those representative of conserved pathways remains largely unaddressed (Freitas and Cohn, 2004; Hecht *et al*., 2008; Smith *et al.,* 2009a; Debiais-Thibaud *et al*., 2011). To address this gap in knowledge, candidate odontogenic genes representative of major signaling pathways were cloned for *in situ* hybridisation, to investigate their epithelial-mesenchymal expression during shark tooth initiation, development and replacement. With respect to descriptions of gene expression relating to ligands and growth factors, it is noted that short-range autocrine and intra-epithelial signaling are considered synonymous, while expression domains encompassing the epithelium and mesenchyme are considered representative of long-range paracrine signaling (Handrigan and Richman, 2010a). As previously shown by PCNA analysis, the odontogenic band (OB) is an initial field of competence characterised by early thickening of the prospective dental epithelium to mark the position of the future DL and teeth (Fraser *et al*., 2004; Smith *et al.,* 2009a). To investigate gene regulatory control of early dental competence, the expression patterns of conserved genes were investigated.

The *Wnt-β-catenin* signaling pathway is highly conserved in vertebrates, regulating multiple stages of tooth development and other epithelial organs, such as the hair follicle (Gordon and Nusse, 2006; Järvinen *et al*., 2006; Suomalainen and Thesleff, 2010; Richman and Handrigan, 2011; Fraser *et al*., 2013; Zhang *et al.,* 2008). During OB formation, *β-catenin* was extensively expressed throughout the epithelium, showing some preferential expression in the marginal oral epithelium in both the upper (U) and lower (L) jaws (Fig. 4.3A, boxed areas). These expression domains coincide with localised thickening of the oral epithelium and are therefore presumed to mark the OB in the upper and lower jaws (Fig. 4.3B and C, respectively). The hedgehog signaling pathway is also highly conserved in osteichthyan, reptilian and shark tooth development, with *Shh* being one of the earliest expressed markers of dental competence (Jackman *et al.,* 2010; Fraser *et al.,* 2004; 2008; 2012; Harris *et al*., 2006; Buchtová *et al*., 2008; Smith *et al.*, 2009a). During development of the

OB, *Shh* was also expressed in a restricted region of the oral epithelium in both the upper and lower jaws (Fig. 4.3D, boxed areas). Expression was further restricted, compared with the extensive expression domains of *β-catenin,* defining the focal extent of early dental competence in the upper (Fig. 4.3E), and lower jaws (Fig. 4.3F, arrow). During tooth development, *Shh* signals to target cells via its receptors *Ptc1/2* (Cobourne *et al*., 2004). In several gnathostomes, the hedgehog signaling pathway operates through *Shh* and *Ptc* to regulate early dental competence, tooth initiation and morphogenesis. This therefore provides an additional readout gene to assess the broader extent of hedgehog signaling (Hardcastle *et al*., 1998; Cobourne *et al*., 2004; Jackman *et al.,* 2010; Fraser *et al*., 2013; Handrigan and Richman, 2010a). During OB formation, *Ptc2* was co-expressed with *Shh* in the same thickened oral epithelium (Fig. 4.3G, boxed areas) in both the upper and lower jaws (Fig. 4.3H & I, respectively), to further suggest the role of hedgehog signaling during early dental competence.

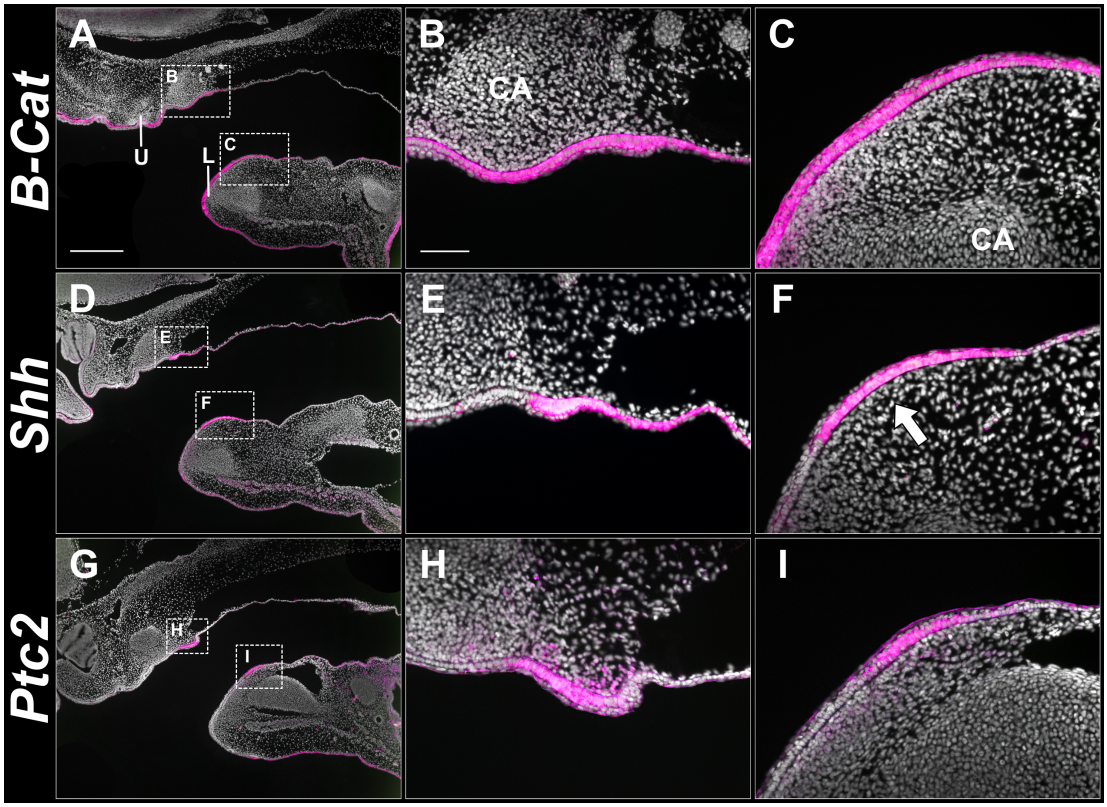


Figure 4.3 *β-catenin, Shh* and *Ptc2* expression patterns in the odontogenic band (OB) (*S.stellaris*). *β-catenin* is expressed throughout the epithelium of the upper (U) and lower (L) jaws (A), however, restriction to regions of thickened oral epithelium presumed to coincide with the OB, (boxed areas), implies a role of establishing early dental competence. This is prominent in the thickened oral epithelium adjacent to the cartilage in the upper (B) and lower jaws (C). *Shh* further defines the OB, shown by its expression domains in the presumptive dental epithelium of the upper and lower jaws (boxed areas) (D). Expression is further restricted compared to *β-catenin,* delineating focal regions of thickened odontogenic epithelium in the upper (E) and lower (F, arrow) jaws. *Ptc2* is co-expressed with *Shh* in the upper and lower jaw epithelium (G, boxed areas). Both genes are expressed in the same restricted region of thickened epithelium in the upper (H) and lower (I) jaws, further implying a role for hedgehog signaling in the early priming of prospective dental tissues. Scale bars: (A, D, G) 1 mm, (B, C, E, F, H, I) 100 μm.

Pitx1 and *Pitx2* both regulate tooth and limb development, *Pitx2* being one of the earliest expressed markers of dental competence (Marcil *et al*., 2003; Semina *et al*., 1996; Mitsiadis and Drouin, 2008; Fraser *et al.,* 2004; 2008; 2012; Klopocki *et al*., 2012; Duboc and Logan, 2011). *Pitx1* was expressed in the thickened oral epithelium and mesenchyme in the upper and lower jaws, coincident with formation of the OB (Fig. 4.4A, boxed areas). *Pitx1* was strongly expressed in the thickened epithelium of the upper jaw, further spreading into the underlying mesenchyme (Fig. 4.4B). This was also evident in the lower jaw, with expression strongest in the epithelium, while spreading comparatively further into the underlying mesenchyme (Fig. 4.4C). *Pitx2* was expressed in a similar pattern, in the upper and lower jaws localising to a restricted region of epithelium and mesenchyme at the oral margins (Fig. 4.4D, boxed areas). Expression was also stronger in the thickened epithelium in both the upper and lower jaws, while also spreading into the underlying mesenchyme (Fig. 4.4E and F, respectively).

The bone morphogenetic proteins (BMPs) are some of the earliest expressed markers of dental competence, achieved through reciprocal signaling between the epithelium and mesenchyme (reviewed by Thesleff and Sharpe, 1997). During mammalian tooth induction, *Bmp4* is expressed first in the epithelium, before shifting to the mesenchyme to transfer this inductive potential (Vainio *et al*., 1993). *Bmp4* was first expressed in the prospective odontogenic mesenchyme (Fig. 4.4G, boxed areas). In both the upper and lower jaws (Fig. 4.4H & I, respectively), *Bmp4* localised primarily to the condensing mesenchyme underlying the thickened epithelium, showing marked association with the early OB. *Bmp4* showed no signs of epithelial expression, however, due to its characteristic tendency to shift between the epithelium and mesenchyme, a similar stage-specific pattern cannot be ruled out.

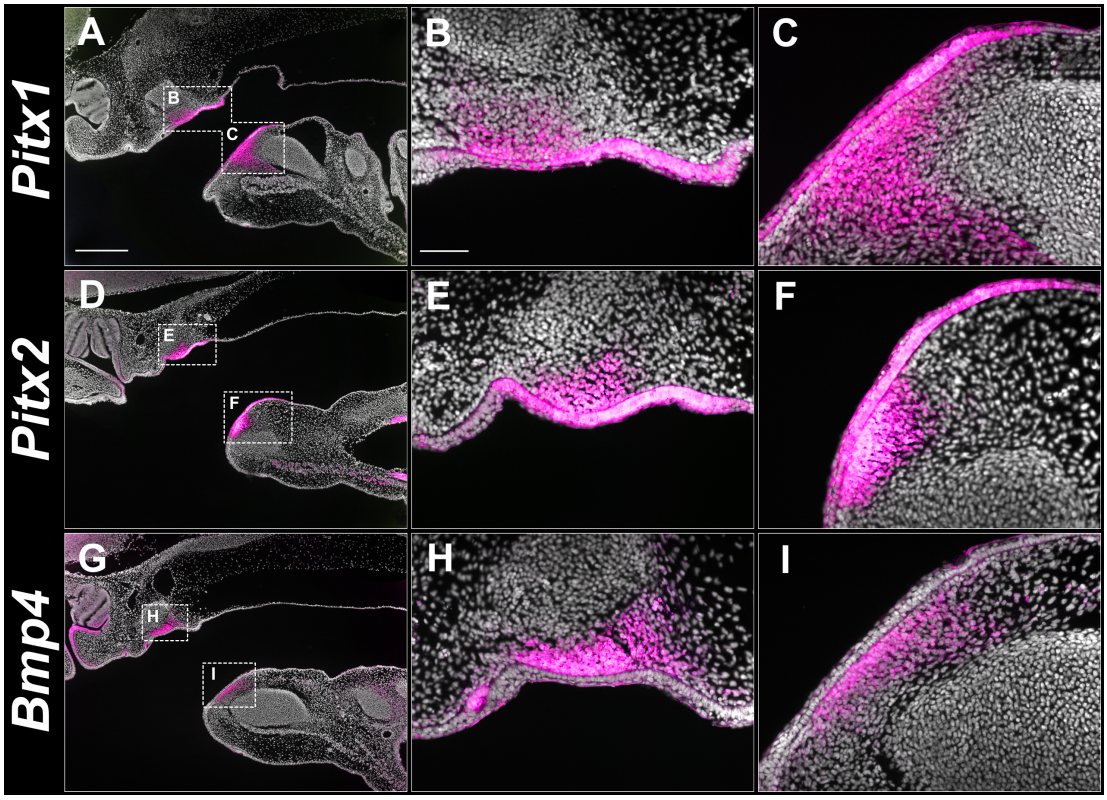


Figure 4.4 *Pitx1/2* and *Bmp4* expression patterns in the odontogenic band (*S.stellaris*). During early priming of odontogenic tissues, *Pitx1* is expressed in both the upper and lower jaw epithelium and mesenchyme (A, boxed areas). Expression is comparatively stronger in the thickened epithelium, spreading inward into the underlying mesenchyme in both the upper (B) and lower (C) jaws. *Pitx2* is expressed in a similar pattern, localised to the odontogenic epithelium, while spreading inward into the underlying mesenchyme (D, boxed areas). Expression is also comparatively stronger in the thickened epithelium in both the upper (E) and lower (F) jaws, implying both epithelial induction and concomitant transfer of odontogenic potential to the underlying prospective DM, possibly to define the future position of the DL. This is most apparent in the comparatively deepened expression domains of *Pitx1* in the lower jaw (C). *Bmp4* is expressed primarily in the condensing mesenchyme, both in the upper and lower jaws (G, boxed areas). Localisation of *Bmp4* to this mesenchyme, directly underlying the thickened epithelium, in both the upper (H) and lower (I) jaws, implies a role in regulating early odontogenic mesenchymal induction. Scale bars: (A, D, G) 1 mm, (B, C, E, F, H, I) 100 µm.

4.3.3 Conserved gene expression domains mark development of the dental lamina and first generation teeth

During subsequent formation of the DL and first tooth placodes, expression of both *β-catenin* and its binding partner, *lymphoid enhancing factor 1* (*Lef1*), were investigated (Seidensticker and Behrens, 2000). In the lower jaw (LJ) DL, *β-catenin* was first expressed in the first tooth placode (Fig. 4.5A, arrow 1) and the O-ODE continuous with the surface oral epithelium. At this lamina-oral surface interface, expression extended to, and terminated within, a small cluster of epithelial cells (Fig. 4.5A, arrow 2). In the upper jaw (UJ) of the same individual, *β-catenin* was expressed in a similar pattern, in the early tooth placode (Fig. 4.5B, arrow), and weakly in the epithelium of the early MV (Fig. 4.5B, dotted line). Given the difference in timing of first tooth initiation between the lower and upper jaw, it is implied that in sharks, *β-catenin* is one of the earliest expressed genes, marking individual tooth placodes at an early stage. *Lef1* expression was comparatively more restricted, localised to the epithelial thickenings of the first tooth placodes within the DL in both the lower and upper jaws (Figs. 4.5C-D, arrows), respectively.

During tooth initiation, *Pitx1* was strongly expressed in the dental epithelium, coincident with the first tooth placode (Fig. 4.5E, arrow), and in the lingual DE extending to the oral epithelium via the O-ODE (Fig. 4.5E, dotted line). In the upper jaw, *Pitx1* was expressed in a similar pattern, coincident with the early forming DL (Fig. 4.5F, arrow), extending outward along the ventral DE continuous with the MV (Fig. 4.5F, dotted line). *Pitx2* was also expressed strongly in the lingual epithelium of the DL and in a restricted region of underlying DM (Fig. 4.5G, dotted line). This pattern was matched in the upper jaw, with expression in the early forming DL extending both inward into the DM, and outward along the ventral axis of the MV (Fig. 4.5H, dotted line).

Though previously expressed in the OB, at this stage *Shh* was not detected in first generation tooth placodes in the lower jaw (Fig. 4.5I, arrow 1) or the early DL of the upper jaw (Fig. 4.5J, dotted line). Expression was instead localised only to a cluster of epithelial cells positioned at the lower jaw oral surface-DL interface (Fig. 4.5I,

arrow 2) and in the upper jaw to the distal tip of the MV (dotted line) (Fig. 4.5J). While in partial agreement with prior observations by other experimenters, these expression patterns are inconsistent with those anticipated during primary tooth induction. In the current study, this disparity has been attributed to the absence of a crucial embryonic stage in which *Shh* is expressed in the first placodes (Smith *et al*., 2009a). Future expression studies will therefore be required to address this discrepancy.

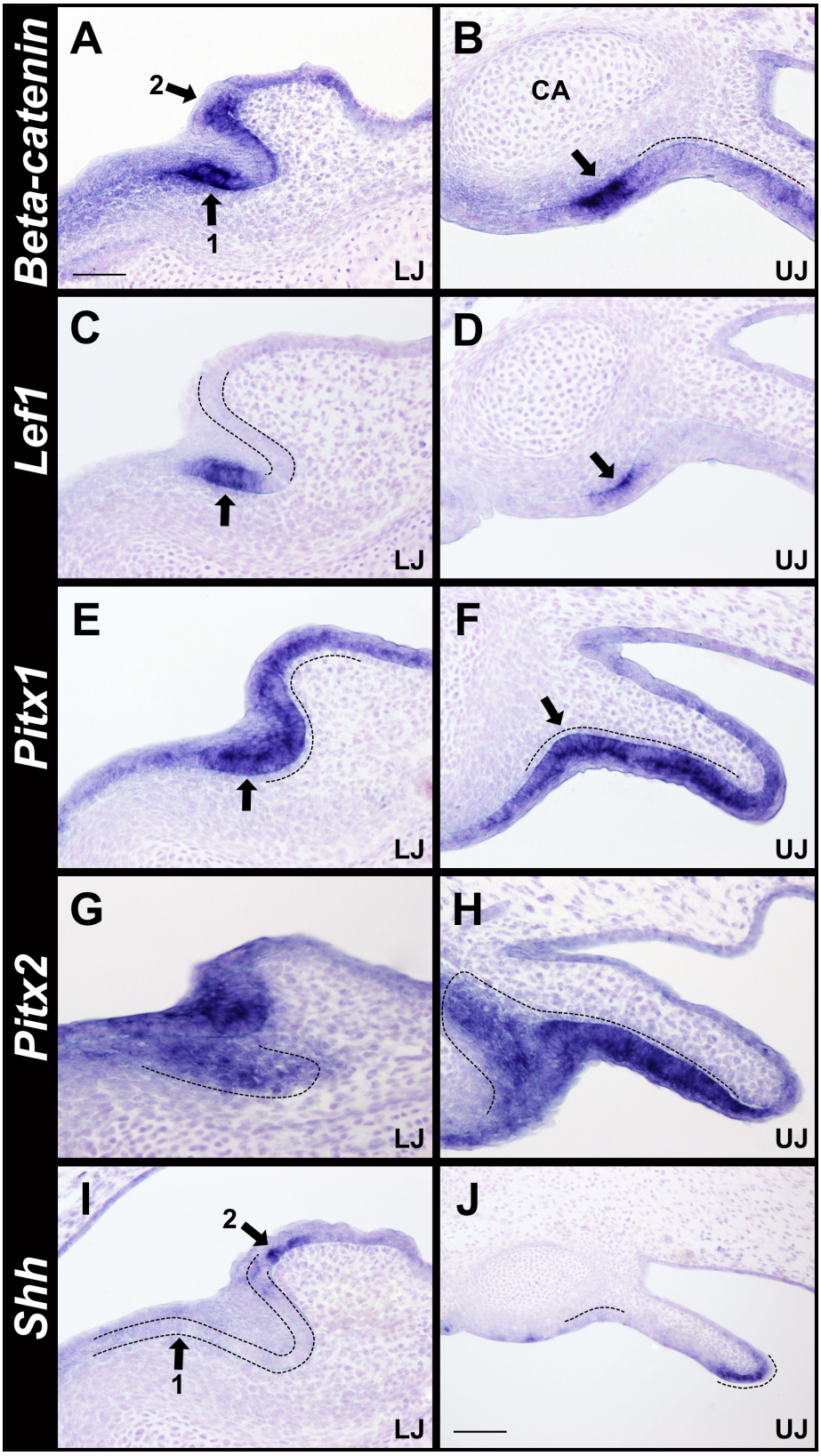


Figure 4.5 Gene expression patterns during shark tooth initiation (*S.canicula*). During development of the DL (lower jaw, LJ), *β-catenin* is strongly expressed in the epithelium of the first tooth placode (A, arrow 1) and a cluster of epithelial cells at the DL-oral surface interface via the O-ODE (A, arrow 2). In the upper jaw (UJ), *β-*

catenin is also strongly expressed in the early first tooth placode (B, arrow) and weakly in the epithelium continuous with the MV (B, dotted line). In the lower jaw, *Lef1* is expressed primarily in the epithelium of the first tooth placode and in the upper jaw, the early first tooth placode (C-D, arrows). *Pitx1* is expressed throughout the DL, inclusive of the first tooth placode (E, arrow), extending into the lingual DL and adjoining dental epithelium (E, dotted line). Expression is similar in the upper jaw, localised to the early DL (F, arrow) and extending to within the MV via the epithelium (F, dotted line). *Pitx2* is expressed in a similar pattern, however; in addition, expression spreads to within the underlying DM in both the upper and lower jaw (G-H, dotted lines). In the lower jaw DL, *Shh* expression is not detected in the first tooth placode (I, arrow 1); localised only to a restricted region of the oral epithelium at the interface with the distal DL (I, arrow 2). In the upper jaw, expression is also absent in the early DL (J, dotted line), localised only to the distal tip of the MV (J, dotted line). Scale bars: (A-I) 100 μm, (J) 200 μm.

4.3.4 Gene expression during tooth development and early replacement

To expand upon the gene expression patterns identified during shark tooth initiation, both these and an additional set of markers were used to analyse for expression during subsequent tooth development and early replacement. Due to practical limitations regarding consistent stage matching of individuals with respective markers, some variability in developmental stages is presented here. This dataset is therefore reflective of a combination of stages representative of first generation teeth and where available, early second generation replacements.

4.3.4.1 Sustained *β-catenin* and *Lef1* are delineated by *Sostdc1* expression in first/ second-generation teeth

The expression of *β-catenin* and *Lef1* were first analysed, along with the secreted ligand Sclerostin domain-containing protein 1 (*Sostdc1*), which regulates tooth development through controlled regulation of Wnt, Bmp, Fgf and Hh signaling (Ahn *et al*., 2010; Laurikkala *et al*., 2003; Cho *et al*., 2011; Kassai *et al.*, 2005; Munne *et al*., 2009). During tooth development and early replacement (lower jaw, LJ) *β-catenin* was expressed in the early RT placodes (Fig. 4.6A, T2) and in the epithelium and underlying DM of first generation tooth buds (Fig. 4.6A, T1). At this stage, expression was also maintained in the O-ODE connecting the SL and surface oral epithelium (Fig. 4.6A, dotted line). During advanced morphogenesis (lower jaw), expression was absent from the tooth (Fig. 4.6B, T1), limited to the epithelium and mesenchyme of the developing RT bud (Fig. 4.6B, T2). At advanced bud-early morphogenesis (upper jaw, UJ), *β-catenin* was strongly expressed in the IDE and DM (Fig. 4.6C), with some superficial expression extending outward via the basal layer of the O-ODE within the MV (Fig. 4.6C, dotted line).

In accordance with first tooth initiation patterns, *Lef1* expression was restricted to the epithelium of the second-generation tooth placode (Fig. 4.6D, T2) (lower jaw) and absent during advanced morphogenesis (Fig. 4.6D, T1). During bud stage (upper jaw), expression was also restricted to the epithelium of the tooth bud (Fig. 4.6E, T2), and absent during advanced morphogenesis (Fig. 4.6E, T1). During upper jaw second-generation tooth development, *Lef1* was also expressed in the epithelial

placode (Fig. 4.6F, T2), however, during onset of advanced morphogenesis, expression localised to defined regions of the IDE, (Fig. 4.6F, T1, arrow 1), including the medial aspect of the tooth tip (Fig. 4.6F, arrow 2).

At these stages, *Sostdc1* expression was less clearly defined, during bud formation (lower jaw) localising to the epithelium of the tooth bud in an apparent demarcation of the medial region of the tooth unit itself (Fig. 4.6G, arrows). This pattern was repeated in the upper jaw, with expression bordering the lingual and labial epithelium of the developing tooth bud (T2) (Fig. 4.6H, arrows). During the onset of advanced morphogenesis (upper jaw), this flanking pattern was better defined, with expression domains in two regions of IDE (Fig. 4.6I, arrows), bordering a *Sostdc1*-negative medial tooth region.

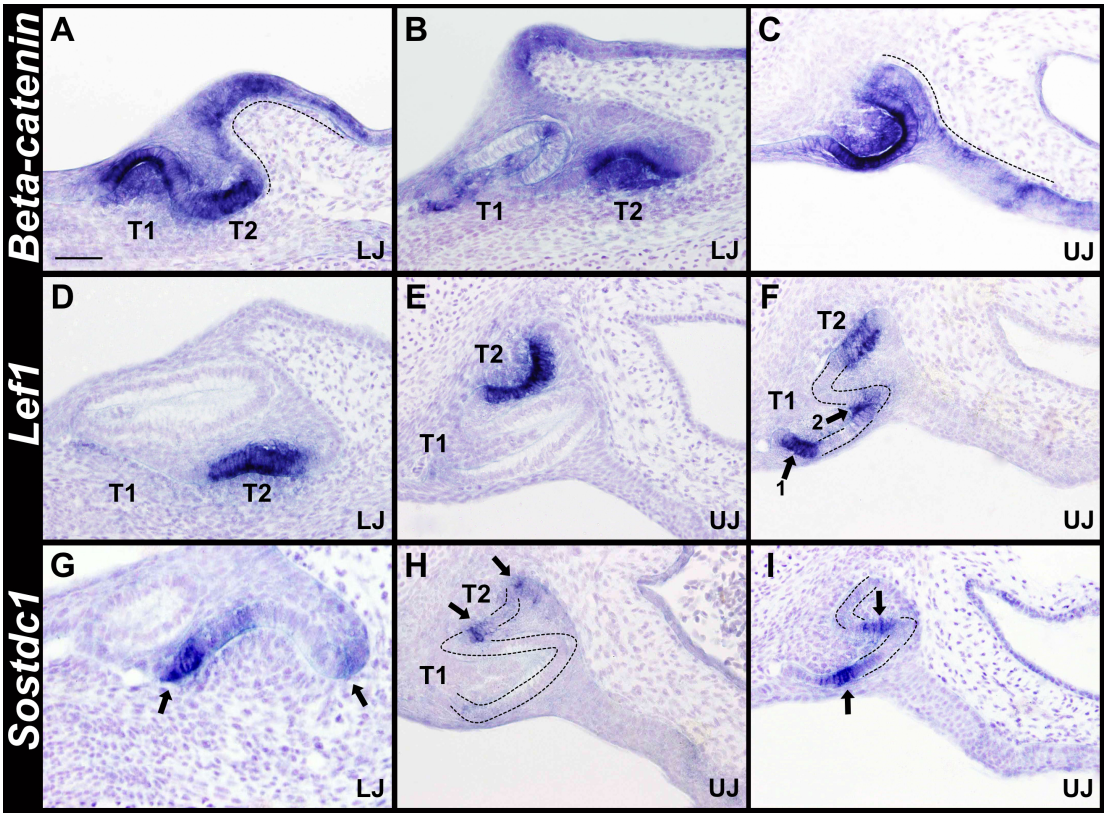


Figure 4.6 Expression patterns of *β-catenin, Lef1* and *Sostdc1* during shark tooth development and early replacement (*S.canicula*). In the lower jaw (LJ), *β-catenin* is expressed both within the dental epithelium and mesenchyme during bud stage (A, T1) and in the epithelial placode (A, T2) connected with the oral surface by a stripe of *β-catenin*+ O-ODE (A, dotted line). During morphogenesis, expression is largely absent (B, T1) confined to the epithelium and mesenchyme of the second-generation tooth bud (B, T2). During late bud stage-early morphogenesis (upper jaw, UJ), *β-catenin* is also strongly expressed in the IDE and underlying DM (C), with expression further extending to within the basal epithelium of the MV via the O-ODE (C, dotted line). *Lef1* is expressed primarily in the tooth placode (lower jaw) (D, T2) and absent during advanced morphogenesis (D, T1). During bud stage (upper jaw) (E, T2), *Lef1* is expressed in the dental epithelium and absent during advanced morphogenesis (E, T1). At placode and advanced morphogenesis stages (also upper jaw), *Lef1* is expressed in the dental epithelium (F, T2) and a defined region of IDE (F, T1, arrow 1), including the tooth tip (F, arrow 2). During lower jaw bud stage, *Sostdc1* is expressed in the lingual and labial dental epithelium bordering the medial region of the tooth bud (G, arrows), a pattern repeated in the upper jaw (H, T2, arrows). This pattern is further defined during morphogenesis (upper jaw), with expression localised to the lingual and labial IDE of the tooth (I, arrows). Scale bar: 100 μm.

4.3.4.2 Epithelial *Pitx1* and mesenchymal *Pix2* are redeployed in first/ second-generation teeth

During early morphogenesis (upper jaw, UJ), *Pitx1* was strongly expressed in the DL (Fig. 4.7A, arrow) and the basal DE extending to within the MV (Fig. 4.7A, dotted line). Expression was absent in the DM and generally absent in the IDE of the tooth unit itself. During early tooth replacement (lower jaw, LJ), *Pitx1* was also expressed in the SL lingual to developing teeth (Fig. 4.7B, arrow) and in the IDE surrounding the tooth during advanced morphogenesis (Fig. 4.7B, T1). Consistent *Pitx1* expression, both within the DL and IDE during advanced morphogenesis, suggests a role in tooth induction and in the regulation of cell activity states during subsequent stages. *Pitx2* was strongly expressed in the DM during morphogenesis (upper jaw), with some outspread into the underlying mesenchyme (Fig. 4.7C, dotted line). During early tooth replacement (also upper jaw), *Pitx2* was also expressed in the DM during bud stage (Fig. 4.7D, T2) and reduced during advanced morphogenesis, confined to a restricted region of the mesenchymal papilla (Fig. 4.7D, T1, arrow). Contrary to expression patterns during DL development and early tooth initiation, *Pitx2* was not expressed in the DL or SL, raising the possibility of a cell-type specific role during tooth replacement. Furthermore, in stark contrast to other vertebrates previously studied, the expression patterns of *Pitx2* are suggestive of an alterative role in tooth development, perhaps representative of the ancestral state of polyphyodont gnathostomes (St.Amand *et al.,* 2000; Jussila *et al.,* 2014).

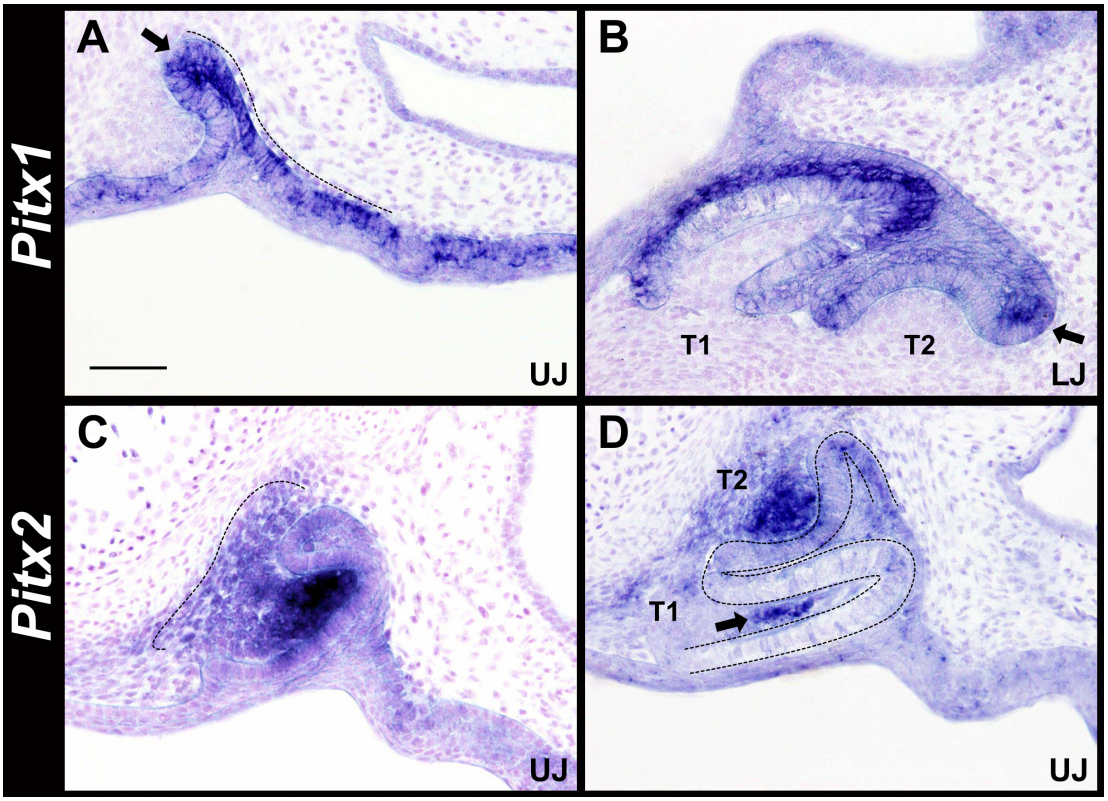


Figure 4.7 Expression patterns of *Pitx1/2* during shark tooth development and early replacement (*S.canicula*). In the upper jaw (UJ) during early morphogenesis *Pitx1* is expressed in the DL (A, arrow) and DE continuous with the MV, and absent in the DM and IDE of the tooth itself. During early replacement (lower jaw, LJ), *Pitx1* is also expressed in the DL (B, arrow) and during advanced morphogenesis (T1), in the IDE surrounding the tooth. In the upper jaw, also during morphogenesis, *Pitx2* is strongly expressed in the DM, with some outspread into the underlying mesenchyme (C, dotted line). During early tooth replacement (also upper jaw), *Pitx2* is also expressed in the DM during bud stage (D, T2), reducing significantly during advanced morphogenesis (D, T1, arrow). Scale bar: 100 μm.

4.3.4.3 *Shh*/ *Ptc2* and *Taz* mark activation of the hedgehog and hippo pathways in first/ second-generation teeth

The expression patterns of *Shh* during tooth initiation were ambiguous compared to existing studies of the shark dentition, largely due to its unexpected absence in first generation tooth placodes (Smith *et al*., 2009a). However, in several gnathostomes, *Shh* is redeployed to regulate subsequent stages of tooth development (Vaahtokari *et al*., 1996a; Fraser *et al*., 2006a; 2013; Handrigan and Richman, 2010a). This is shown in mammalian tooth morphogenesis, during which *Shh* marks activation of the enamel knot (Vaahtokari *et al*., 1996a). To clarify the role of hedgehog signaling during shark tooth development, expression of *Shh* and its receptor *Ptc2* were therefore further investigated during subsequent tooth development and early replacement. *Shh* was first expressed during early first generation tooth morphogenesis (upper jaw, UJ) in a population of cells nested within the polarised epithelial tip (Fig. 4.8A, arrow) and (Fig. 4.8B, arrow, DAPI). During early tooth replacement (lower jaw, LJ) this pattern was repeated, with expression restricted to the same cells, strongly during morphogenesis (Fig. 4.8C, T1), and initially in the early second-generation tooth bud (Fig. 4.8C, T2, arrow). At similar early stages of replacement (lower jaw), expression was also confined to the epithelial tip (Fig. 4.8D, T1, arrow, DAPI) and absent in the thickened epithelium of the first replacement tooth placode (Fig. 4.8D, T2, DAPI) and SL.

During development of the first tooth bud (upper jaw), *Ptc2* was expressed in the dental epithelium (Fig. 4.8E, arrow 1), DL (Fig. 4.8E, arrow 2) and the underlying DM (Fig. 4.8E, arrow 3) (DAPI). During subsequent bud to early morphogenesis, (also upper jaw) expression spread throughout the full extent of the IDE and DM (Fig. 4.8F, DAPI). The proximal expression domains of ligand (*Shh*) and receptor (*Ptc2*) imply, at least at the RNA level, evidence of a short-range, intra-epithelial (autocrine) and epithelial-mesenchymal (paracrine) requirement for hedgehog signaling during tooth morphogenesis. The expression of *Ptc2* in domains negative for *Shh*, further implies hedgehog signaling to operate through other as yet unidentified secreted ligands.

The transcriptional co-activator with PDZ-binding motif (*Taz, wwtr1*) and its paralogue, yes-associated protein (*Yap*), serve as major downstream effectors of the Hippo signaling cascade to regulate organ size and tumour suppression by transactivating target gene expression (Kanai *et al*., 2000; Wang *et al*., 2014; Camargo *et al*., 2007; Zhang *et al*., 2011). Knowledge of the role of *Taz* and *Yap* in tooth development has largely been limited to studies of *Yap1*-deficient mice, which undergo abnormal tooth morphogenesis, marked by mislocation of the enamel knot, aberrant apoptosis and ablated cell proliferation (Liu *et al*., 2014). The conserved odontogenic role of *Yap1* has further been shown by its expression in the apical bud of the mouse incisor (Li *et al*., 2011a). Understanding of the role of *Taz* in tooth development is comparatively limited, so far implied only by its ability to induce formation of mineralised extracellular matrices and associated gene expression when applied to human dental pulp stem cells (hDPSCs) (Suh *et al*., 2012). *Taz* expression was first detected during morphogenesis (upper jaw) in the medial epithelial basal membrane of the tooth tip (Fig. 4.8G, arrow). This pattern was identical in the lower jaw during preceding stages of morphogenesis, with expression maintained in the same cell population of the IDE (Fig. 4.8H, arrow). Analysis of *Taz* expression during subsequent stages of RT development revealed signs of variable regionalisation, marked by its expression in the extracellular matrix (ECM) in between the dental epithelium and mesenchyme (section 4.3.5).

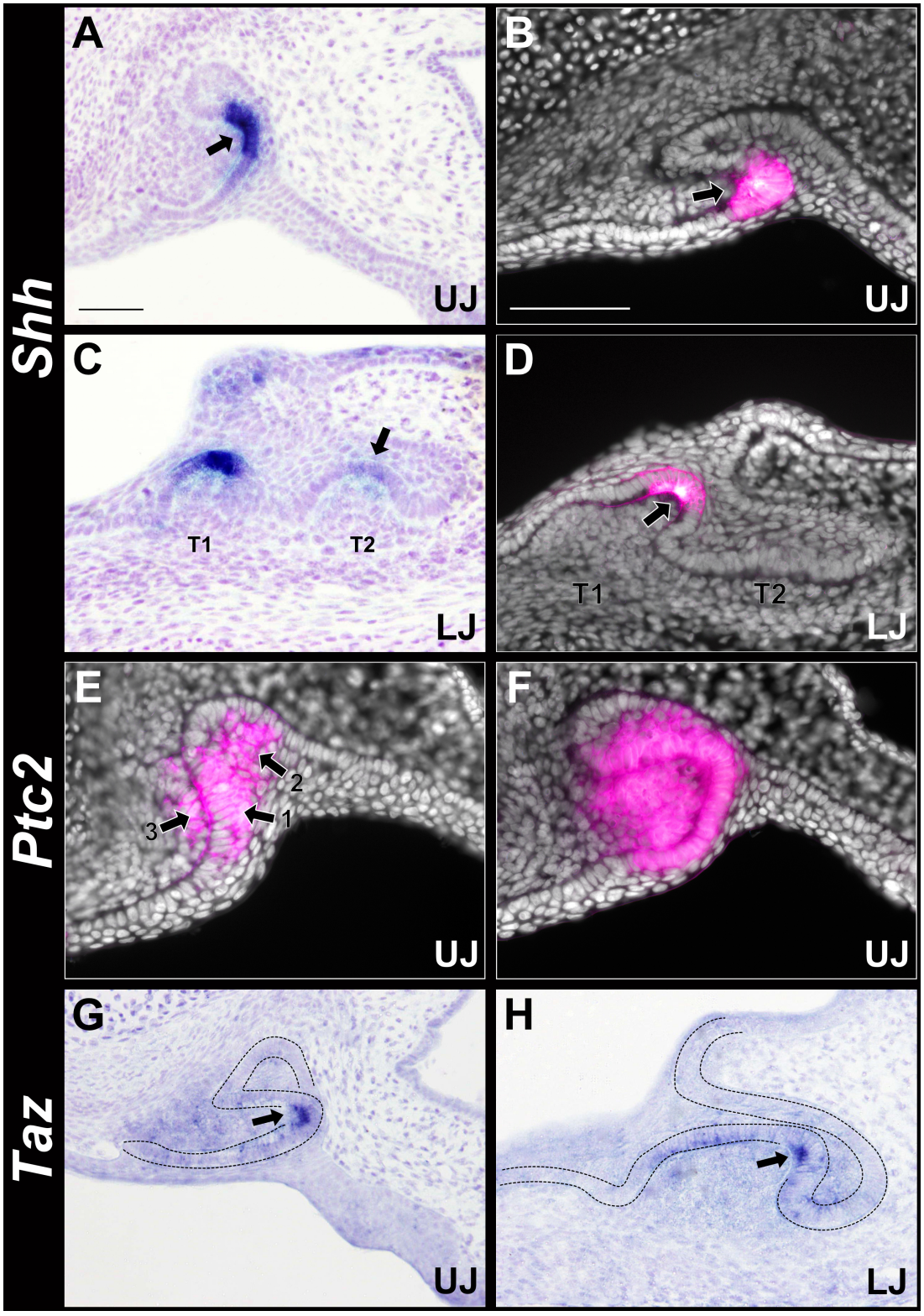


Figure 4.8 Expression patterns of *Shh, Ptc2* and *Taz* during shark tooth development (*S.canicula*). In the upper jaw (UJ) during morphogenesis, *Shh* is expressed in a nested population of epithelial cells in the tooth tip (A, arrow), also shown in (B, arrow) (DAPI). This pattern repeats during lower jaw (LJ) tooth replacement in the same set of epithelial cells, expressing *Shh* during morphogenesis (T1) and weakly during bud stage (T2) (C, arrow). This is further shown at similar stages of

replacement, with *Shh* expressed at T1 stage in the lower jaw (D, arrow), while absent in the first replacement placode (D, T2) and SL (DAPI). During upper jaw first tooth bud development, *Ptc2* is expressed in the dental epithelium (E, arrow 1), DL (E, arrow 2), and superficially in the DM (E, arrow 3) (DAPI). During subsequent morphogenesis, *Ptc2* is extensively expressed both throughout the IDE and the underlying DM, implying both short and long-range hedgehog signaling functions (F, DAPI). Throughout morphogenesis, *Taz* is expressed in a population of epithelial cells nested within medial IDE of the tooth tip (upper jaw) in a similar pattern to *Shh* (G, arrow). This pattern repeats in the lower jaw, with expression localised to the same nested cell population (H, arrow). Scale bars: (A, C, G, H) 100 μm, (B, D, E, F) 100 μm.

4.3.4.4 FGF signaling is marked by *Fgf3*/*10* in developing first-generation teeth

The fibroblast growth factors (FGFs) regulate tooth growth, morphogenesis and apoptosis through controlled epithelial-mesenchymal stimulation of cell proliferation and division (Jernvall *et al*., 1994; Kettunen and Thesleff, 1998; Heikinheimo, 1994; Vaahtokari *et al*., 1996b). As representative members of the FGF family, *Fgf3* and *Fgf10* were cloned for expression analysis. *Fgf3* was first detected during first generation tooth morphogenesis (upper jaw, UJ) in the DM and medial IDE of the tooth bud (Fig. 4.9A, arrow 1) and taste buds (Fig. 4.9A, arrow 2). During advanced morphogenesis (also upper jaw), mesenchymal expression progressively reduced, while epithelial expression continued to localise to the tooth tip, marking a spherical cluster of polarised *Fgf3*+ IDE cells (Fig. 4.9B, arrow). *Fgf10* was primarily expressed during bud stage in the same cluster of medial epithelial cells, with no corresponding mesenchymal expression (Fig. 4.9C, arrow) (DAPI) and (4.9D, arrow) (haematoxylin). While the epithelial-mesenchymal expression patterns of *Fgf3* imply both short and long-range signaling functions, restriction of *Fgf10* to the epithelial tip implies a short-range autocrine signaling function only. Future expression studies targeting corresponding receptors will be required to assess the fuller extent of FGF signaling in the shark dentition.

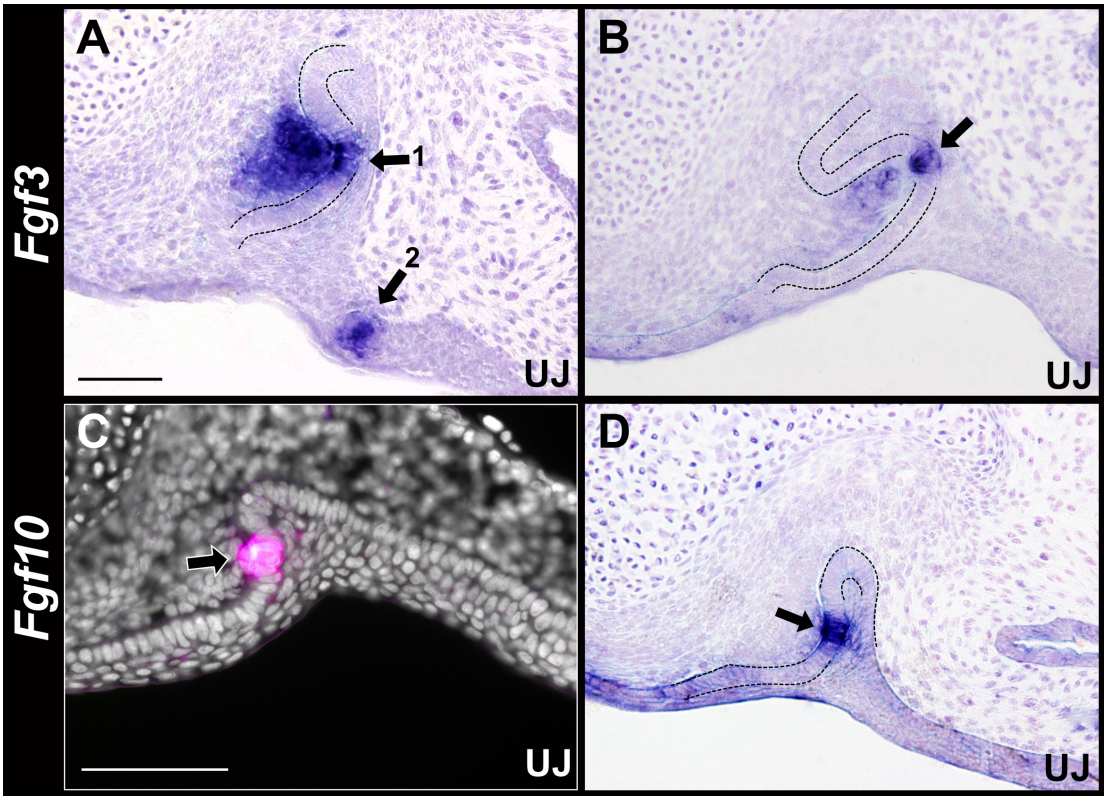


Figure 4.9 Expression patterns of *Fgf3/10* during shark tooth development (*S.canicula*). In the upper jaw (UJ) during morphogenesis, *Fgf3* is expressed in the medial IDE of the tooth tip (A, arrow 1) and underlying DM. *Fgf3* is also expressed in the taste buds (A, arrow 2). During advanced morphogenesis, expression reduces in the DM, further localising to a spherical cluster of IDE cells in the tooth tip (B, arrow). During bud stage (upper jaw), *Fgf10* is expressed in the medial epithelium of the tooth tip only (C, arrow) (DAPI), with no expression in the DM, also shown in (D, arrow) (haematoxylin). Scale bars: (A, B, D) 100 μm, (C) 100 μm.

4.3.4.5 Epithelial-mesenchymal *Bmp4* is expressed in first/ second-generation teeth

In addition to its early role in tooth induction, *Bmp4* is also expressed in the enamel knot during tooth morphogenesis and in the IDE and DM during advanced morphogenesis. During incisor renewal, *Bmp4* also interacts with FGFs, *Activin* and *Follistatin* in the cervical loop SCNs to regulate stem cell proliferation, showing roles in tooth induction, morphogenesis, cytodifferentiation and the regulation of dental SCs (Jernvall *et al*., 1994; Vaahtokari *et al.,* 1996a; Åberg *et al*., 1997; Zhang *et al*., 2000; Handrigan *et al.,* 2010; Vainio *et al*., 1993; Wang *et al.,* 2007). *Bmp4* expression during first generation tooth initiation was not recorded in the current study and cannot therefore be discussed, however, during advanced bud stage, *Bmp4* was strongly expressed in the DM (upper jaw, UJ) and basement membrane of the IDE. At this stage, expression was conspicuously absent in the medial epithelial tip of the bud (Fig. 4.10A, arrow). However, during morphogenesis *Bmp4* was strongly expressed both in the DM and medial IDE of the tip, coincident with other markers (Fig. 4.10B, arrow). During early tooth replacement, epithelial expression in the first generation tooth, now undergoing advanced morphogenesis, ceased (Fig. 4.10C, T1), localising strongly to the basal DM of the papilla. Weak mesenchymal expression underlying the first replacement tooth placode (Fig. 4.10C, T2) was also visible, though no corresponding epithelial expression was detected. During lower jaw (LJ) early tooth replacement, *Bmp4* was expressed both in the epithelium and mesenchyme of the early tooth bud (Fig. 4.10D, T2), and in accordance with the upper jaw, strongly in the basal DM of the papilla during advanced morphogenesis (Fig. 4.10D, T1). These transient epithelial-mesenchymal expression patterns are generally consistent with those in the dentitions of other gnathostomes, implying a highly conserved role in tooth induction, morphogenesis and cytodifferentiation in advance of matrix deposition (Vainio *et al*., 1993; Åberg *et al*., 1997; Fraser *et al*., 2004; 2008; 2013).

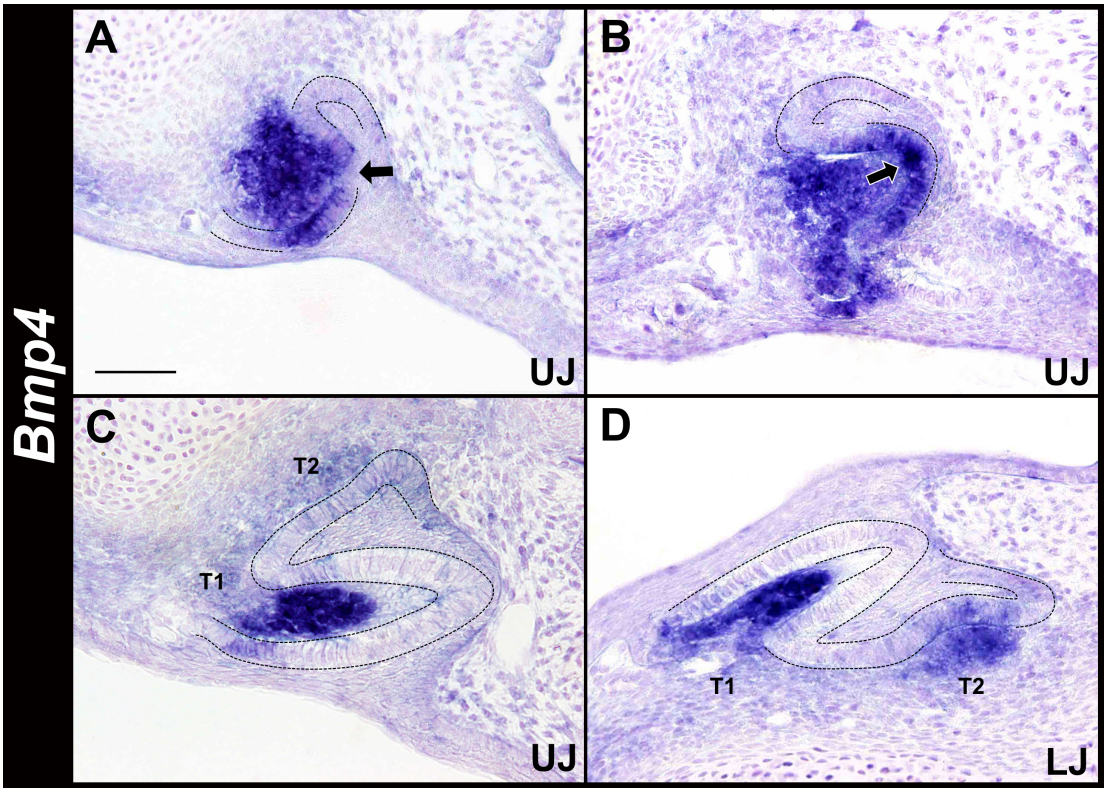


Figure 4.10 Expression patterns of *Bmp4* during shark tooth development and early replacement (*S.canicula*). In the upper jaw (UJ) during advanced bud stage, *Bmp4* is strongly expressed in the DM and lateral IDE, with a gap in expression in the medial epithelial tooth tip (A, arrow). During subsequent morphogenesis, mesenchymal expression is maintained, while epithelial expression spreads within the IDE of the tooth tip (B, arrow). In the upper jaw during advanced morphogenesis, expression localises to the basal DM of the papilla (C, T1), with some weak mesenchymal expression underlying the second-generation tooth placode (C, T2). In the lower jaw (LJ), *Bmp4* is expressed in the epithelium and mesenchyme of the early second generation tooth bud (D, T2) and in line with the upper jaw, strongly in the basal DM of the papilla during advanced morphogenesis (D, T1). Scale bar: 100 μm.

4.3.4.6 Epithelial-mesenchymal *Midkine* is further expressed in first-generation teeth

Neurite growth-promoting factor 2, or *Midkine* (*MK*), is transiently expressed between the epithelium and mesenchyme during mouse molar and incisor initiation, morphogenesis and cytodifferentiation, including in the incisor enamel knot and cervical loop (CL) SCN (Mitsiadis *et al.,* 1995b; 2008). In the shark dentition, *MK* expression was first detected at first generation bud stage (upper jaw, UJ) in the mesenchyme and weakly in the overlying medial epithelium (arrow), though it is likely that *MK* is expressed in advance of this stage (Fig. 4.11A). During subsequent morphogenesis (also upper jaw), expression in the mesenchymal compartment increased, accompanied by a corresponding increase in the medial epithelial basement membrane of the tooth tip (arrow) (Fig. 4.11B). During advanced morphogenesis (upper jaw), *MK* expression was maintained in the DM of the papilla and moderately reduced in the tooth tip IDE (Fig. 4.11C). This expression pattern was identical in the lower jaw (LJ) during advanced tooth morphogenesis (Fig. 4.11D).

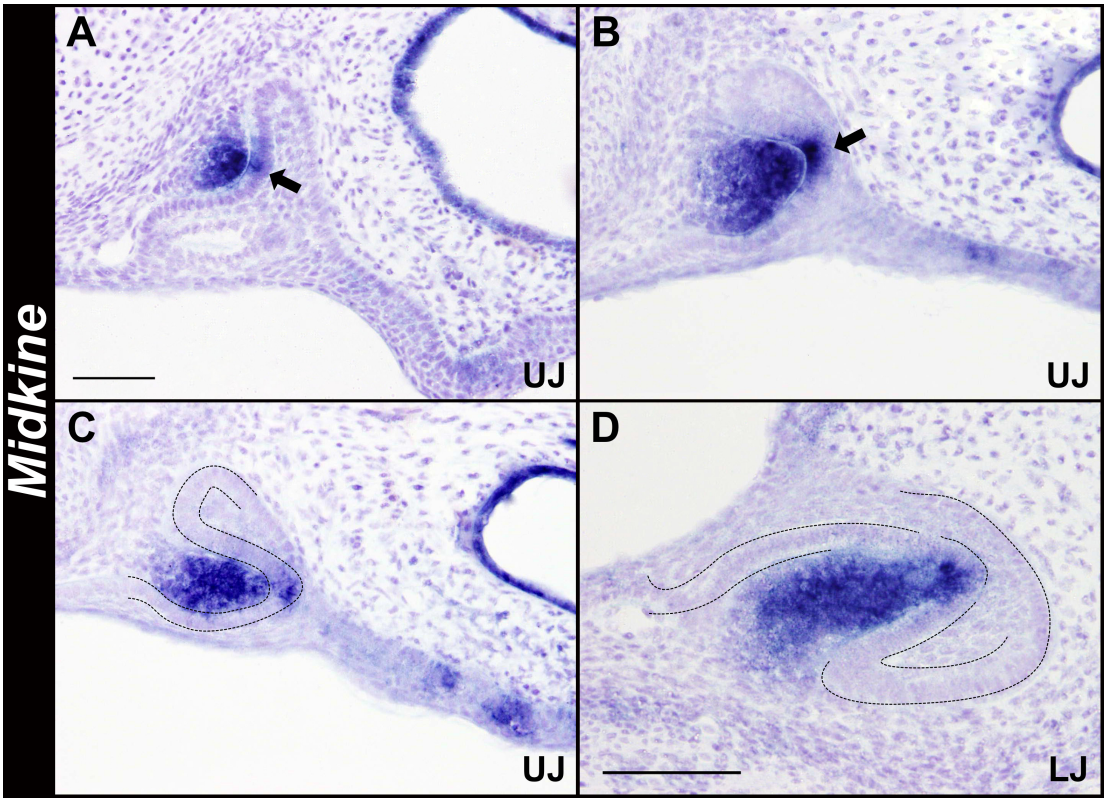


Figure 4.11 Expression patterns of *Midkine* (*MK*) during shark tooth development (*S.canicula*). In the upper jaw (UJ) (bud stage), *MK* is strongly expressed in the mesenchyme and weakly in the overlying epithelium of the tooth tip (arrow) (A). During subsequent morphogenesis, both mesenchymal and epithelial expression increase, the latter restricted to the medial epithelial basement membrane of the tooth tip (arrow) (B). During advanced morphogenesis (upper jaw), expression is maintained in the DM of the papilla and moderately reduced in the IDE of the tooth tip (C). This expression pattern is repeated in the lower jaw (LJ) (D). Scale bars: (A-C) 100 µm, (D) 100 µm.

4.3.4.7 *Meis2* is expressed in the dental lamina, while *Foxq1* marks the oral-dental epithelial junction in first-generation teeth

The *Meis2* transcription factor regulates limb outgrowth through antagonistic interactions with BMPs, also interacting with *Pitx2* during development (Capdevila *et al.,* 1999; Paylakhi *et al.,* 2011). *Meis1/2* also control retinal cell cycling to maintain early cells in a rapidly proliferating state (Heine *et al*., 2008). The developmental repertoire of *Meis2* therefore suggests conserved roles in epithelial appendage development and regulation of progenitors. During upper jaw (UJ) first tooth development (bud stage), *Meis2* expression was detected only in the DL (Fig. 4.12A, arrow). Expression was also detected in the developing taste buds, eye and brain (not shown), the latter two consistent with known expression patterns. While limited to this single stage, the expression of *Meis2* in the shark DL implies a putative role in tooth initiation and/ or regulation of progenitors marked for odontogenic fates. This novel expression further provides the scope for future investigation in the catshark dentition and those of other gnathostomes.

Members of the Forkhead box (Fox) family of transcription factors regulate various aspects of tooth development (Shirokova *et al*., 2013; Poché *et al.,* 2012). Recent expression studies of *Foxq1* have further shown its conservation in the embryonic catshark dentition, in the current study promoting further investigation of its putative odontogenic role (Wotton and Shimeld, 2011). During development of first generation teeth, *Foxq1* was expressed in taste buds throughout the oral epithelium (Figs. 4.12B-C, arrows). Though not directly apparent, *Foxq1* was further expressed in taste bud-like structures within the oral epithelium proximal to the junction with the O-ODE and DL (Fig. 4.12B, arrow*) (upper jaw). This expression pattern was further apparent in the lower jaw (LJ), with *Foxq1* localised to cell loci continuous with the O-ODE (Figs. 4.12C-D, arrows*).

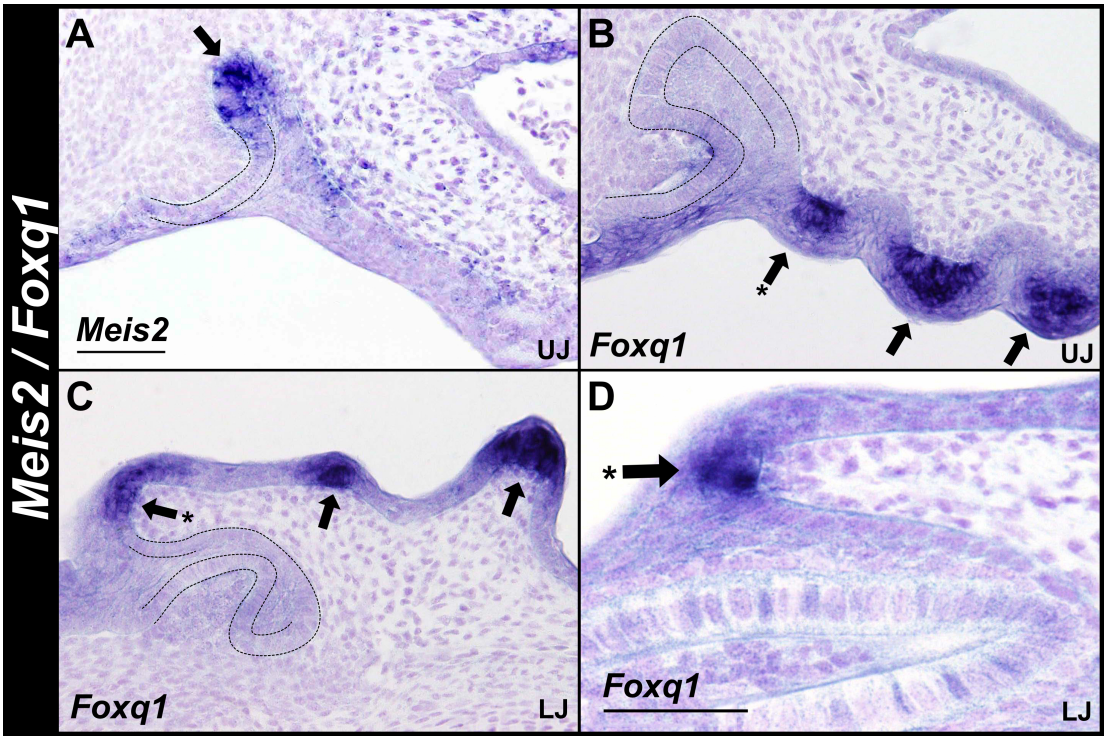


Figure 4.12 Expression patterns of *Meis2* and *Foxq1* during shark tooth development (*S.canicula*). During first generation tooth development (upper jaw, UJ), *Meis2* is expressed primarily in the DL (A, arrow), developing brain, eye and taste buds (not shown). In addition to taste buds (B-C, arrows), *Foxq1* is expressed in taste bud-like structures at the junction of the oral epithelium and O-ODE (B, arrow*) (upper jaw). This is further apparent in the lower jaw (LJ) in which *Foxq1*+ cell clusters localise to oral epithelial loci continuous with the adjoining O-ODE (C-D, arrows*). Scale bars: (A-C) 100 µm, (D) 100 µm.

4.3.4.8 Mesenchymal markers expressed during tooth development

The *Twist* transcription factor regulates skeletal development by specifying cell fates through negative regulation of *Runx2* (Bialek *et al*., 2004). During tooth development, *Twist* regulates odontoblastic cell fates, interacts with FGFs and BMPs, and regulates mesenchymal stem cells (D'Souza *et al.,* 1999; Bourgeois *et al.,* 1998; Rice *et al*., 2005; Russell *et al.,* 2010). During bud stage (upper jaw, UJ), *Twist* was expressed in the DM (Fig. 4.13A, arrow). During early tooth replacement (lower jaw, LJ), *Twist* was expressed in the same pattern, localising to the mesenchyme during tooth bud formation (T1) and initiation of the second-generation tooth placode (T2) (Fig. 4.13B, arrow). The conserved expression of *Runx2* in osteichthyan teeth, and catshark teeth and denticles, implies conserved roles involving regulation of cells marked for odontoblastic fates (Fraser *et al*., 2008; Hecht *et al*., 2008). To further characterise the putative role of *Runx2* in the catshark dentition, its expression patterns were investigated. While limited here to first-generation bud/ early morphogenesis stages (upper jaw), *Runx2* was expressed in the DM (Fig. 4.13C, arrow 1) and DL (arrow 2). Future studies of *Runx2* will be required to expand upon this initial expression pattern.

Distal-less Homeobox (Dlx) transcription factors regulate various aspects of tooth development and are expressed in the teeth and denticles in the catshark (Thomas *et al*., 1997; Debiais-Thibaud *et al*., 2011). During development of the first tooth bud (upper jaw), *Dlx3* was strongly expressed in the DM, which is in partial agreement with results produced by previous experimenters (Fig. 4.13D, DAPI). As with *Runx2*, future studies will be required to clarify the role of *Dlx3* in the catshark dentition, however, the availability additional *S.canicula* Dlx probes provides such an opportunity. The mineralised matrix protein *Sparc*/ *Osteonectin* is expressed during the late differentiation of preodontoblasts to odontoblasts, implying a role in regulating cell lineages committed to hard tissue formation (Papagerakis *et al*., 2002; Kim *et al*., 2012). In the catshark dentition, *Sparc* was primarily expressed in the DM of the papilla during advanced morphogenesis (upper jaw), with no corresponding epithelial expression (Fig. 4.13E). In the lower jaw this pattern was repeated, with strong *Sparc* expression in the DM during advanced morphogenesis (Fig. 4.13F).

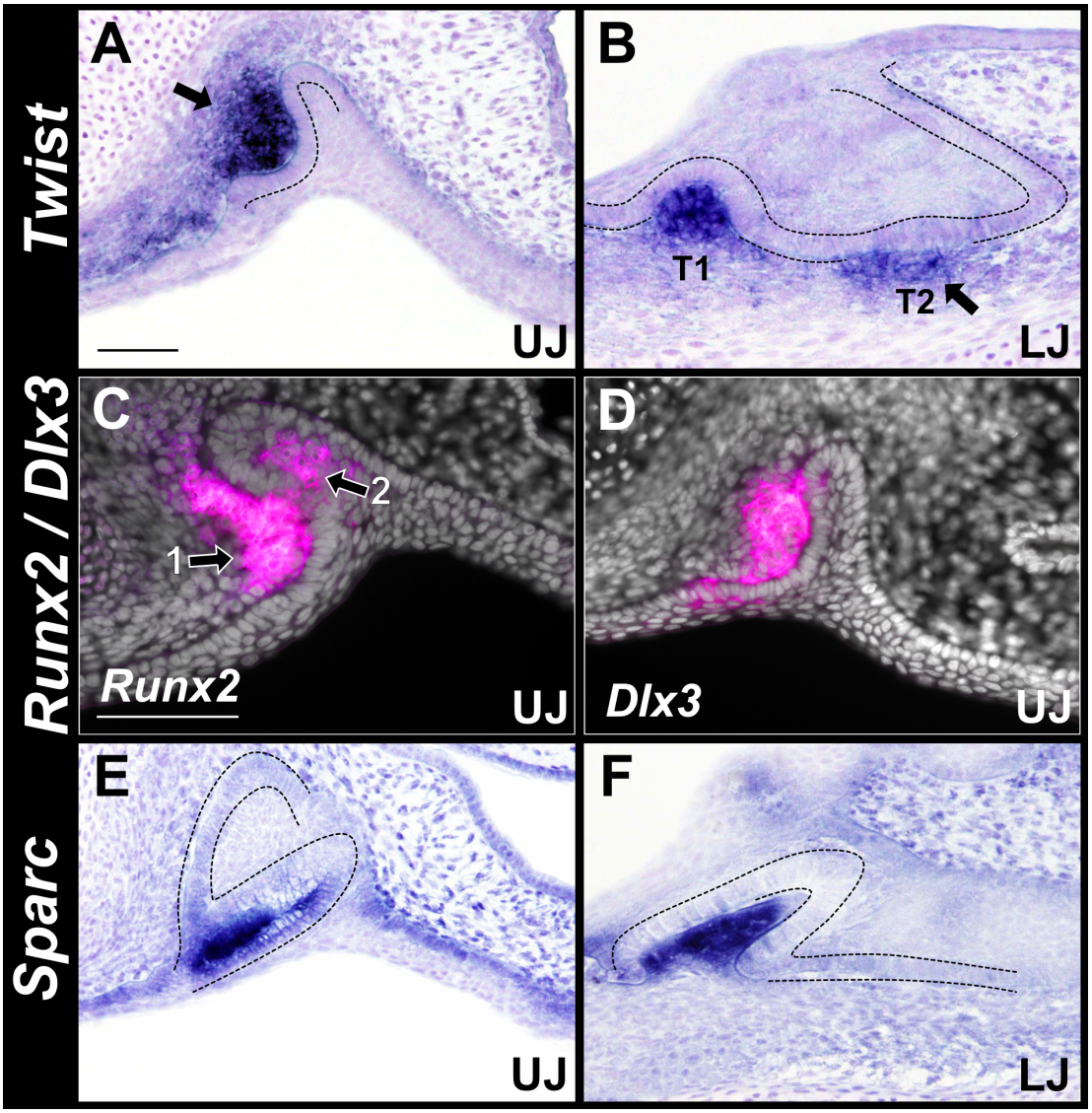


Figure 4.13 Expression patterns of *Twist, Runx2, Dlx3* and *Sparc* during shark tooth development (*S.canicula*). During bud stage (upper jaw, UJ), *Twist* was strongly expressed in the DM (A, arrow) and during early tooth replacement (lower jaw, LJ), in the same pattern at bud (T1) and placode (T2) stages, the latter within the basal DM underlying the replacement tooth placode (B, arrow). At late bud-morphogenesis stage (upper jaw), *Runx2* was expressed predominantly in the DM (C, arrow 1), with some evidence of expression in the DL (C, arrow 2), while *Dlx3* was only expressed mesenchymally (D). *Sparc* was expressed primarily in the DM during advanced morphogenesis in both the upper and lower jaws (E-F). Scale bars: (A, B, E, F) 100 μm, (C, D) 100 μm.

4.3.5 Gene expression during advanced tooth replacement

The gene expression patterns described thus far have proven informative in terms of understanding the initial patterning and early replacement potential of the elasmobranch dentition. However, it remains to be seen whether the same gene circuits are reiteratively activated in the same way during subsequent rounds of tooth regeneration. Identification of the expression domains of the same genes during advanced tooth replacement is vital to understanding their putative individual roles, interactions and therefore the overall regenerative potential of the elasmobranch dental GRN. This is of particular importance in light of the fact that first generation teeth often develop superficially at the oral surface as vestigial appendages in advance of successive functional teeth. These rudimentary teeth are typically aborted by subsequent shedding or resorption, as the dental patterning programme assumes its role in producing functional replacement teeth (Sire *et al*., 2002; Järvinen *et al*., 2008). To expand upon these existing gene expression patterns, in the current study a similar set of markers were used to analyse for expression during advanced tooth replacement, generally limited to T1-T4 stage. As previously highlighted, some variation in developmental stages is presented here and where necessary, a combination of upper and lower jaw expression are used to illustrate a given developmental series. As opposed to the combination of haematoxylin and DAPI previously used, here DAPI was used as a primarily histological counterstain to better visualise surrounding dental histology.

4.3.5.1 *β-catenin, Lef1* and *Sostdc1* are redeployed during tooth replacement

During advanced stages of tooth replacement, *β-catenin* was strongly expressed in the dental epithelium and weakly in the mesenchyme in early replacement tooth (RT) buds (Fig. 4.14A, T3) (lower jaw, LJ). During subsequent advanced budding stages, (upper jaw, UJ) expression progressively increased in the DM, while remaining constant in the IDE (Fig. 4.14B, T3). At this stage, *β-catenin* expression also appeared to extend to within the SL (Fig. 4.14B, arrow). During RT morphogenesis (upper jaw), *β-catenin* was expressed in the IDE and DM (Fig. 4.14C, T2), and in the epithelium of RT placodes (Fig. 4.14C, T3) and adjoining SL. Though generally absent during advanced morphogenesis, there were some indications of residual *β-*

catenin expression in the medial IDE of the tooth tip (Fig. 4.14A, arrow). In accordance with its expression patterns during early tooth development, *Lef1* was expressed primarily in the epithelium of RT placodes (Fig. 4.14D, T4) (lower jaw) and the medial IDE of the tooth tip during morphogenesis (Fig. 4.14D, T3, arrow). Throughout intervening RT bud stages (lower jaw), expression remained constant in the epithelium of both early (Fig. 4.14E, T4) and subsequent RT buds (Fig. 4.14F, T3) (upper jaw). *Sostdc1* expression was detected primarily in the epithelium during RT placode stage (Fig. 4.14G, T2) (lower jaw), a pattern repeated in the upper jaw (Fig. 4.14H, T2). Due to an absence of RT bud stage expression, *Sostdc1* could not be fully assessed in the context of its flanking expression domains in the dental epithelium. However, some expression in the lateral IDE during morphogenesis (Fig. 4.14I, T2, arrow) (upper jaw) corresponds with preceding stages, implying a reiterative role during tooth replacement. Both *β-catenin* and *Lef1* RT expression domains also shared strong similarities with those during tooth development, further suggesting reiterative roles during RT initiation, development and morphogenesis.

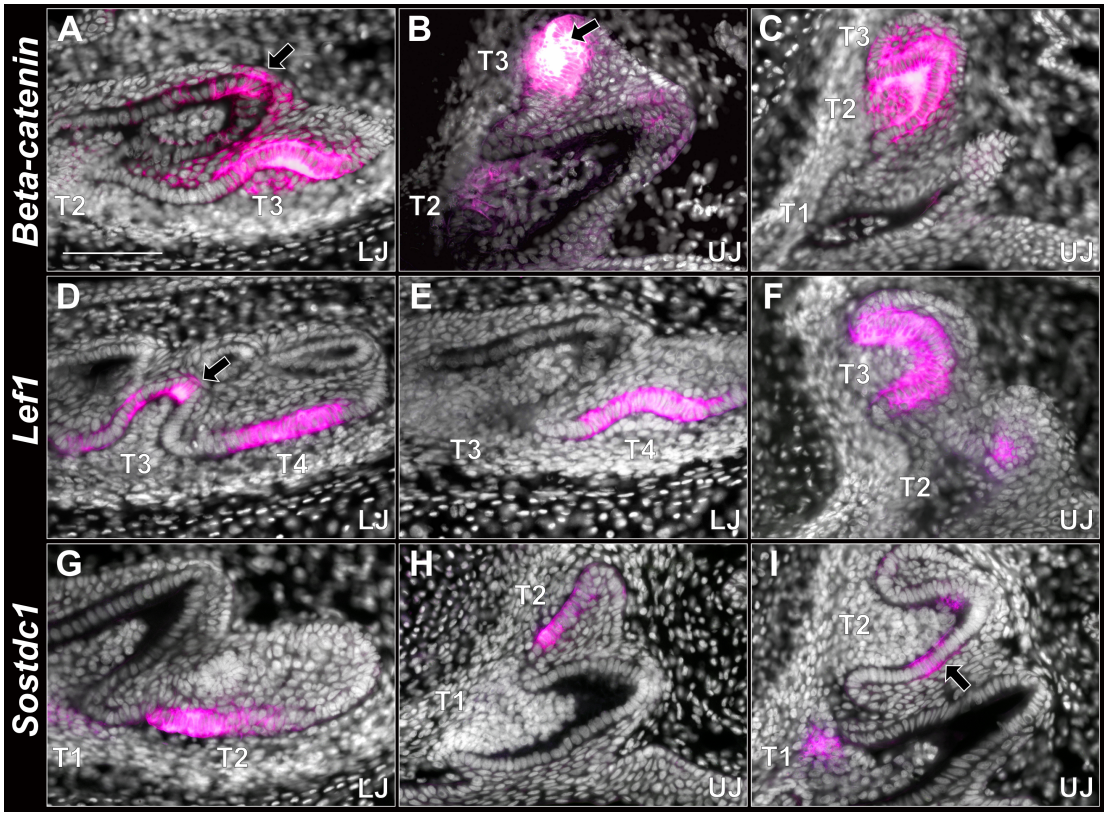


Figure 4.14 Expression patterns of *β-catenin, Lef1* and *Sostdc1* during advanced shark tooth replacement (*S.canicula*). In the lower jaw (LJ), *β-catenin* is strongly expressed in the dental epithelium during early RT bud formation (A, T3), with some indications of expression in the epithelial tip during advanced morphogenesis (A, T2, arrow). In the upper jaw (UJ) RT bud, *β-catenin* is intensely expressed in the dental epithelium and mesenchyme, continuous with the SL (B, T3, arrow). During upper jaw RT morphogenesis, expression in the IDE/ DM continues (C, T2), extending into the epithelium of the RT placode and SL (C, T3). In the lower jaw, *Lef1* is strongly expressed in the epithelium of the RT placode (D, T4) and tooth tip IDE during morphogenesis (D, T3, arrow). In both the intervening early RT bud (E, T4) (lower jaw) and advancing bud (F, T3) (upper jaw) epithelial *Lef1* expression is maintained. In both the lower and upper jaw, *Sostdc1* is expressed in the RT epithelial placode (G-H, T2) and in the upper jaw in discrete regions of the IDE during RT morphogenesis (I, T2, arrow). Scale bar: (A-I) 100 μm.

4.3.5.2 Epithelial-mesenchymal *Pitx1*/*2* and *Bmp4* are further redeployed during tooth replacement

During advanced tooth replacement, *Pitx1* was expressed in the dental epithelium and mesenchyme during placode stage (Fig. 4.15A, T3) (upper jaw, UJ). Though expression during mid-stage budding was not profiled here, *Pitx1* was subsequently expressed in the IDE and DM during morphogenesis (Fig. 4.15B, T2) (upper jaw), the epithelium during early placode stage (Fig. 4.15B, T3), and in the SL. During advanced morphogenesis (lower jaw, LJ), *Pitx1* was further expressed in the SL and IDE surrounding the tooth (Fig. 4.15C, T3 and T2, respectively). Though these expression patterns largely follow those of preceding stages, some differences were apparent, notably expression in the DM and an increase in the epithelium, both absent during preceding stages. This marked shift in expression domains in the advancing dentition implies a possible change in role during advanced tooth replacement. At bud stage (Fig. 4.15D, T2), *Pitx2* was expressed in both the dental epithelium and mesenchyme, contrasting with preceding equivalent stages in which expression localised primarily to the mesenchyme. However, during subsequent morphogenesis, *Pitx2* assumed its more characteristic expression patterns in the DM of the advancing tooth (Fig. 4.15E, T2) (upper jaw) and in the adjoining DM of the RT placode (Fig. 4.15E, T3). During advanced morphogenesis (upper jaw), *Pitx2* continued to localise to the DM of the papilla (Fig. 4.15F, T2) with some evidence of continued expression in the SL adjacent to the RT placode (Fig. 4.15F, T3).

Bmp4 produced a characteristic pattern during advancing tooth replacement, first expressing in the dental epithelium and mesenchyme during early bud stage (Fig. 4.15G, T3) (upper jaw). During advanced morphogenesis, *Bmp4* was expressed in the DM of the papilla (Fig. 4.15H, T2) (lower jaw), preceded by the third generation tooth bud, expressing in the epithelium and mesenchyme (Fig. 4.15H, T3). In the upper jaw, *Bmp4* was also expressed in the DM during advanced morphogenesis (Fig. 4.15I, T2) and markedly absent during placode stage (Fig. 4.15I, T3). These expression patterns are largely in line with preceding stages, implying reiterative roles in regulating epithelial-mesenchymal interactions and mesenchymal cell activity states during budding and morphogenesis, respectively.

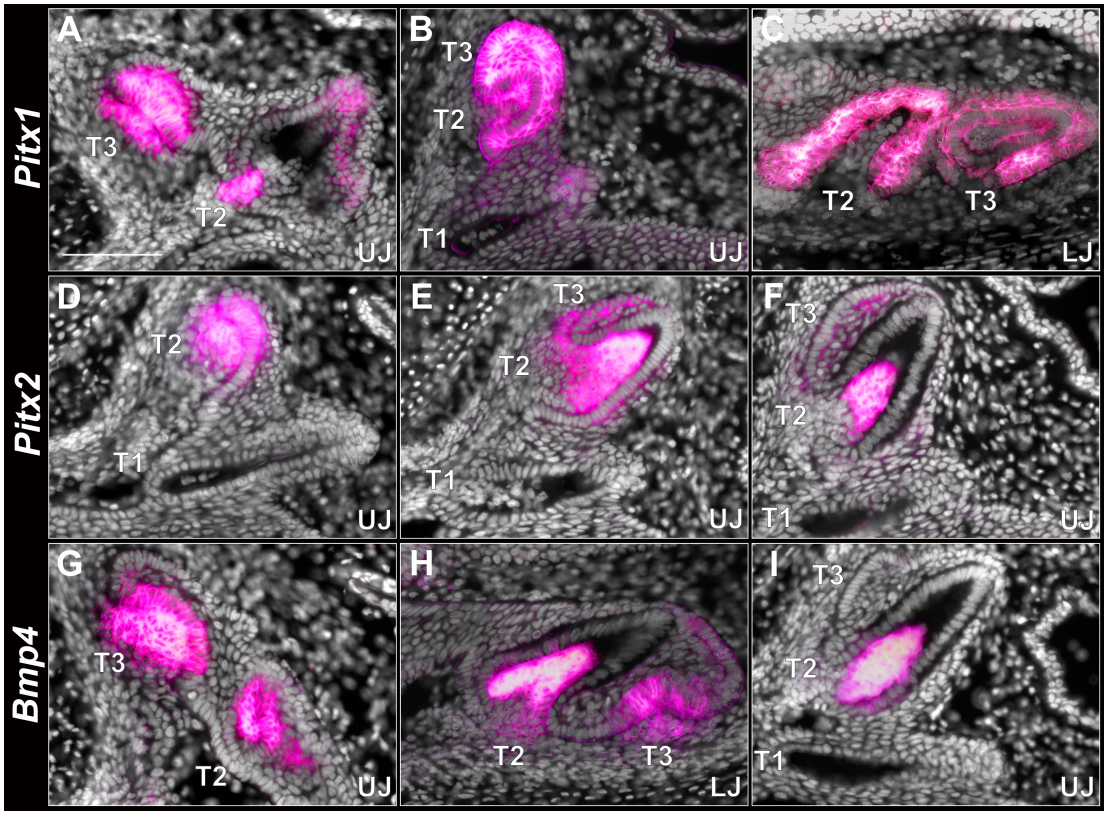


Figure 4.15 Expression patterns of *Pitx1*/*2* and *Bmp4* during advanced shark tooth replacement (*S.canicula*). In the upper jaw (UJ), *Pitx1* is expressed in the dental epithelium and mesenchyme of the RT placode (A, T3), IDE/ DM during morphogenesis (B, T2) and epithelial placode (B, T3) and SL. In the lower jaw (LJ), *Pitx1* is also expressed in the IDE during advanced RT morphogenesis (C, T2) and SL. In the upper jaw, *Pitx2* is expressed in the dental epithelium and mesenchyme during bud stage (D, T2) and DM during RT morphogenesis (E, T2) and placode formation (E, T3). During advanced morphogenesis (upper jaw), *Pitx2* is expressed in the basal DM of the papilla (F, T2) and weakly in the SL coincident with the RT placode (F, T3). In the upper jaw, *Bmp4* is expressed in the dental epithelium and mesenchyme during early RT bud formation (G, T3). In the lower jaw, this pattern repeats (H, T3), subsequently localising strongly to the DM of the papilla during morphogenesis (H, T2). This pattern also repeats in the upper jaw (I, T2). Scale bar: (A-I) 100 μm.

4.3.5.3 *Shh*/ *Ptc2* and *Taz* mark sustained hedgehog and hippo signaling during tooth replacement

In line with its expression patterns in first generation teeth, *Shh* was expressed in the medial IDE of the tooth tip during early RT morphogenesis (Fig. 4.16A, T2, arrow) (upper jaw, UJ), but was absent during advanced morphogenesis (Fig. 4.16A, T1). This was further evident in the lower jaw (LJ) dentition, in which *Shh* was expressed in the IDE of successive replacement tooth tips (Fig. 4.16B, T2-T3, arrows), but was absent within the SL, further implying a role specific to regulation of morphogenesis. *Ptc2*, however, was first expressed in both the dental epithelium and mesenchyme of early RT buds (Fig. 4.16C, T3) (upper jaw). During early bud stage (Fig. 4.16D, T2) (lower jaw), sustained expression in the dental epithelium and mesenchyme showed an apparent bias toward the posterior region of the tooth bud (Fig. 4.16D, T2). In the advancing tooth bud (Fig. 4.16E, T2) (upper jaw), *Ptc2* expression continued to spread throughout the IDE/ DM and was markedly absent during subsequent advanced morphogenesis (Fig. 4.16E, T1). However, during preceding tooth morphogenesis, *Ptc2* was strongly expressed in the IDE and DM of the tooth (Fig. 4.16F, T2) (upper jaw), collectively implying both short and long-range roles for hedgehog signaling in the epithelium and mesenchyme during corresponding stages of advanced RT development.

Compared with its expression in the epithelial tip during first generation tooth morphogenesis (section 4.3.4), the expression of *Taz* in subsequent stages of RT development showed some regional variability. This was first evident during late bud-early morphogenesis stage (Fig. 4.16G, T3), in which expression localised specifically to the extracellular matrix (ECM), situated in between the dental epithelium and mesenchyme. This expression pattern was essentially the same at equivalent stages in the upper jaw (Fig. 4.16H, T2). During subsequent RT morphogenesis, expression shifted to the anticipated domains focal to the medial IDE of the tooth tip (Fig. 4.16I, T2, arrow), while showing marked signs of upregulation in the directly underlying ECM.

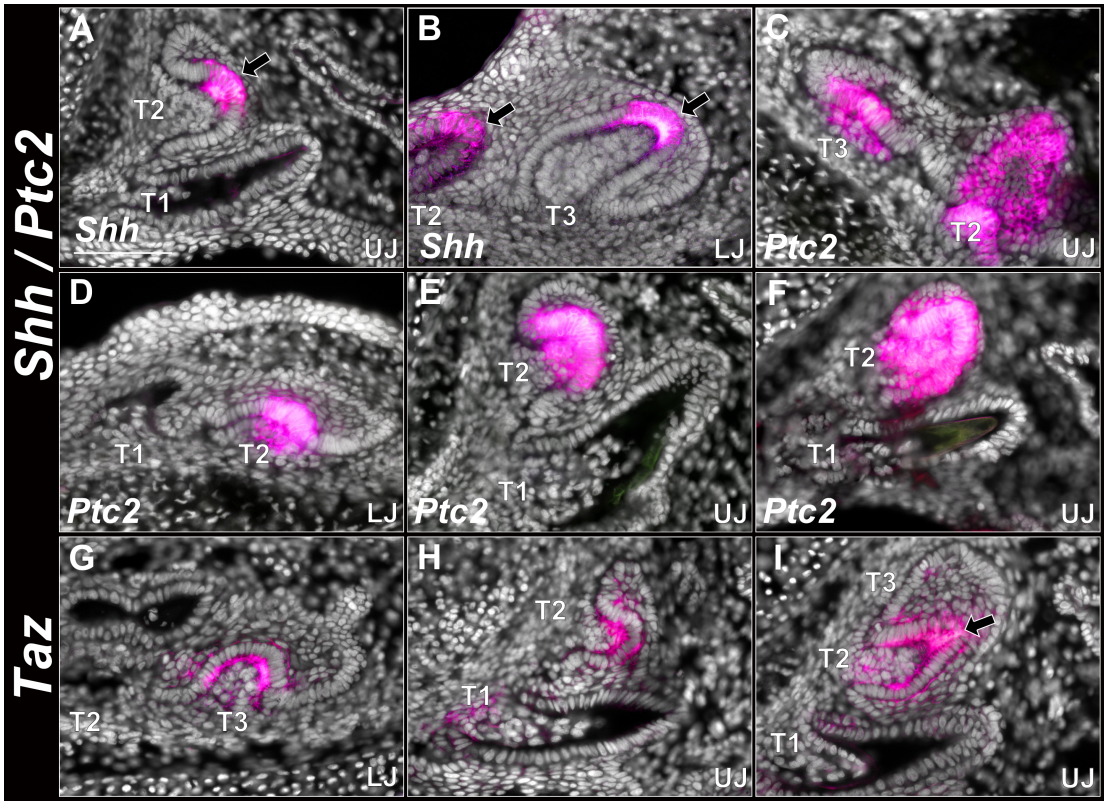


Figure 4.16 Expression patterns of *Shh, Ptc2* and *Taz* during advanced shark tooth replacement (*S.canicula*). In the upper jaw (UJ), *Shh* is expressed in the medial IDE of the tooth tip during early RT morphogenesis (A, T2, arrow), but is absent during advanced morphogenesis (A, T1). In the lower jaw (LJ), this pattern repeats, with expression restricted to the replacement tooth tip IDE (B, T2-T3, arrows) and absent in the SL. In the upper jaw, *Ptc2* is first expressed in the dental epithelium and mesenchyme during RT bud formation (C, T3). In the lower jaw during subsequent budding, this expression pattern continues, biased to the posterior region of the tooth bud (D, T2). In the upper jaw during advancing tooth bud formation (E, T2), expression in the dental epithelium and mesenchyme is maintained, subsequently spreading throughout the IDE/ DM during RT morphogenesis (F, T2). In partial contrast with its expression patterns in first generation teeth, during late budding-early morphogenesis, *Taz* is expressed specifically in the extracellular matrix (ECM) in between the dental epithelium and mesenchyme (G, T3) (lower jaw), a pattern repeated in the upper jaw (H, T2). In the upper jaw during morphogenesis, *Taz* shows characteristic localisation to the medial IDE of the polarised tip (I, T2, arrow), while showing signs of upregulation in the directly underlying ECM. Expression remains consistent throughout the adjoining ECM and is absent during advanced morphogenesis (I, T1). Scale bar: (A-I) 100 μm.

4.3.5.4 *Fgf3*/*10* show reiterative FGF signaling during tooth replacement, while *Meis2 and Foxq1* further mark the successional lamina and IDE

In the lower jaw (LJ), *Fgf3* was strongly expressed in the medial epithelium of the RT bud and underlying DM (Fig. 4.17A, T4), but absent in both tissues during advanced RT morphogenesis (Fig. 4.17A, T3). However in the upper jaw (UJ), during preceding stages of RT morphogenesis *Fgf3* was continually expressed in the IDE and DM of the tooth (Fig. 4.17B, T2). In partial contrast to the expression patterns shown during first generation tooth morphogenesis, expression the epithelial tip alone was not observed during advanced tooth replacement. This may be attributed to some variation in staging, or prove indicative of a more fundamental shift in role during subsequent stages of tooth replacement. During advanced tooth replacement (upper jaw), *Fgf10* was expressed reiteratively in the medial epithelial tip (Fig. 4.17C, T2-3, arrows), though this remains only partially clear due to the position of the tooth (T2) in section. *Fgf10* was, however, continually expressed in the same cells during RT morphogenesis (Fig. 4.17D, T2, arrow) and absent during subsequent advanced morphogenesis (Fig. 4.17D, T1) (upper jaw). Collectively, these expression patterns strongly imply a sustained role for FGF signaling in regulation of epithelial-mesenchymal interactions (*Fgf3*), and tooth cusp morphogenesis (*Fgf3*/*10*). The extent of *Meis2* expression during advanced tooth replacement was limited, however, in accordance with preceding stages, expression localised primarily to the SL, with some evidence of outward expression into the ODE continuous with RT placodes (Fig. 4.17E, T3) (upper jaw). These expression patterns imply a role for *Meis2* in regulating RT initiation. The expression patterns of *Foxq1* during advanced tooth replacement were indicative of a potential shift in role compared with preceding stages, with expression restricted to the IDE of the tooth during advanced RT morphogenesis (Fig. 4.17F, T2). While unclear, restriction of *Foxq1* to the IDE during this advanced stage is indicative of a cell context-specific role in regulating epithelial cell activity.

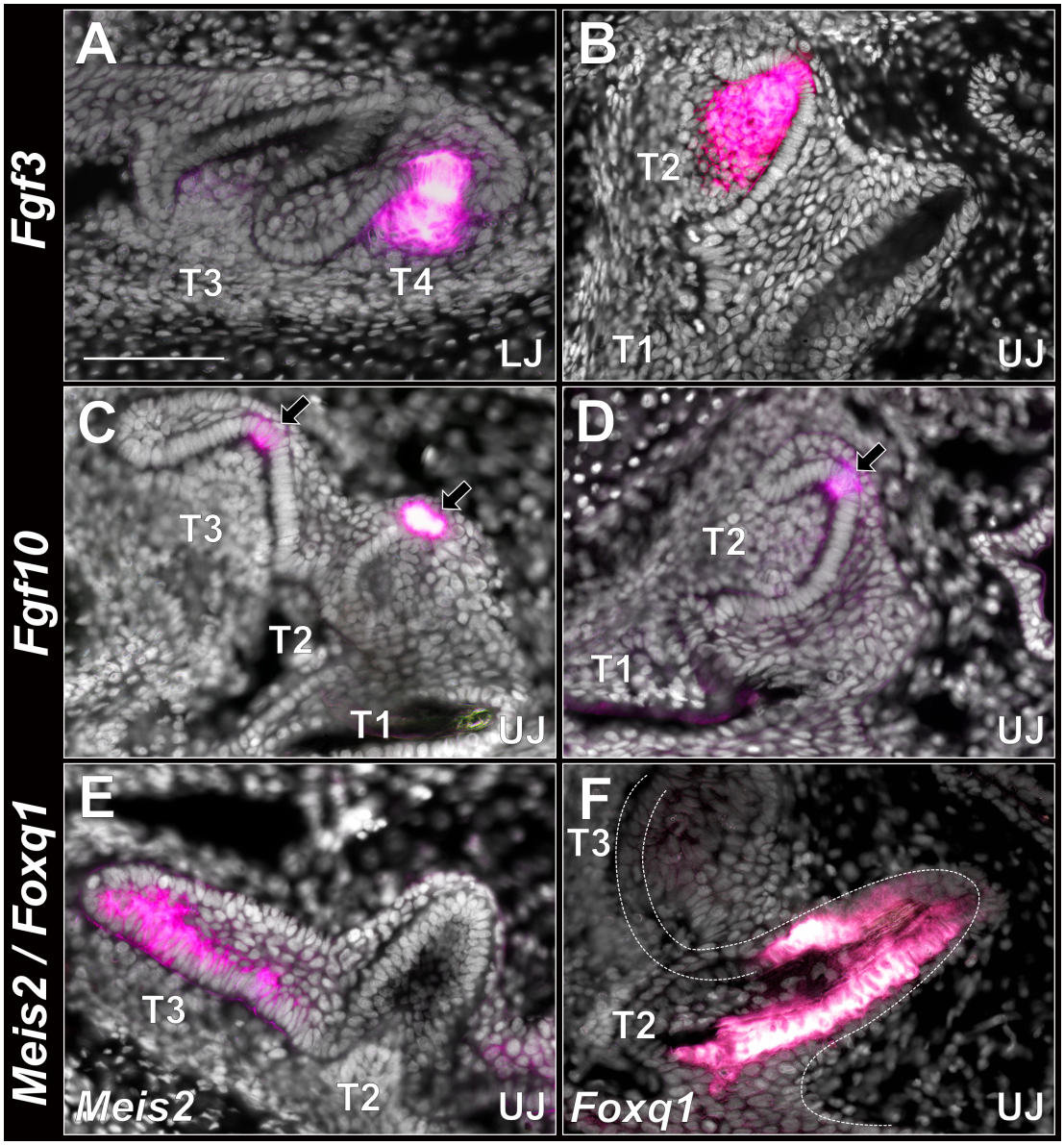


Figure 4.17 Expression patterns of *Fgf3*/*10, Meis2* and *Foxq1* during advanced shark tooth replacement (*S.canicula*). In the lower jaw (LJ) early RT buds (A, T4), *Fgf3* is strongly expressed in the medial epithelium of the tooth tip and underlying DM, and absent during advanced morphogenesis (A, T3). In the upper jaw (UJ) during intervening morphogenesis (B, T2), this expression in the tooth tip IDE and DM is maintained. In the upper jaw during bud stage, *Fgf10* is reiteratively expressed in a restricted population of cells in the medial epithelium of the tooth tip (C, T2-3, arrows), though due to the position of teeth in these sections, this remains only partially clear. During morphogenesis (D, T2, arrow), *Fgf10* is expressed in the same cells in the tooth tip IDE, but is absent during subsequent advanced morphogenesis (D, T1). In the upper jaw, *Meis2* is expressed in the SL, continuous with the adjoining ODE and RT placode (E, T3), while *Foxq1* is expressed throughout the IDE of the RT during advanced morphogenesis (F, T2). Scale bar: (A-F) 100 μm.

4.3.5.5 Mesenchymal markers are further redeployed during tooth replacement

During RT morphogenesis (upper jaw, UJ), *MK* was strongly expressed throughout the DM and medial IDE of the tooth tip (Fig. 4.18A, T2), and during advanced morphogenesis (Fig. 4.18A, T1), in the DM of the papilla. In the lower jaw (LJ) during RT morphogenesis (Fig. 4.18B, T2), *MK* was also expressed in the DM, but was absent in the RT placode (Fig. 4.18B, T3). These expression patterns are generally in accordance with those in first generation teeth, implying reiterative roles in regulating mesenchymal cell activity and tooth morphogenesis during advanced tooth replacement. In the upper jaw, *Twist* was strongly expressed in the DM of the early RT bud (Fig. 4.18C, T3) and in the DM bilaterally to the basal papilla during advanced morphogenesis (Fig. 4.18C, T2). In accordance with preceding stages, during advanced bud stage (Fig. 4.18D, T3), mesenchymal expression was maintained (upper jaw). During advanced tooth replacement (upper jaw), *Runx2* was first expressed in the DM of RT buds (Fig. 4.18E, T2), extending caudally into the DM of the adjoining RT placode (Fig. 4.18E, T3). *Runx2* was further expressed in the DM and lingual IDE during RT morphogenesis (Fig. 4.18F, T3, arrow), but was absent in both the IDE and DM during advanced morphogenesis (Fig. 4.18F, T2) (upper jaw). *Dlx3* was expressed in the DM and IDE, during RT morphogenesis (Fig. 4.18G, T3), and in the basal DM of the papilla during advanced RT morphogenesis (Fig. 4.18G, T2) (upper jaw). In accordance with preceding patterns, during advanced RT morphogenesis, *Sparc* expression was restricted to the DM of the papilla (Fig. 4.18H, T2) and absent in the SL (upper jaw).

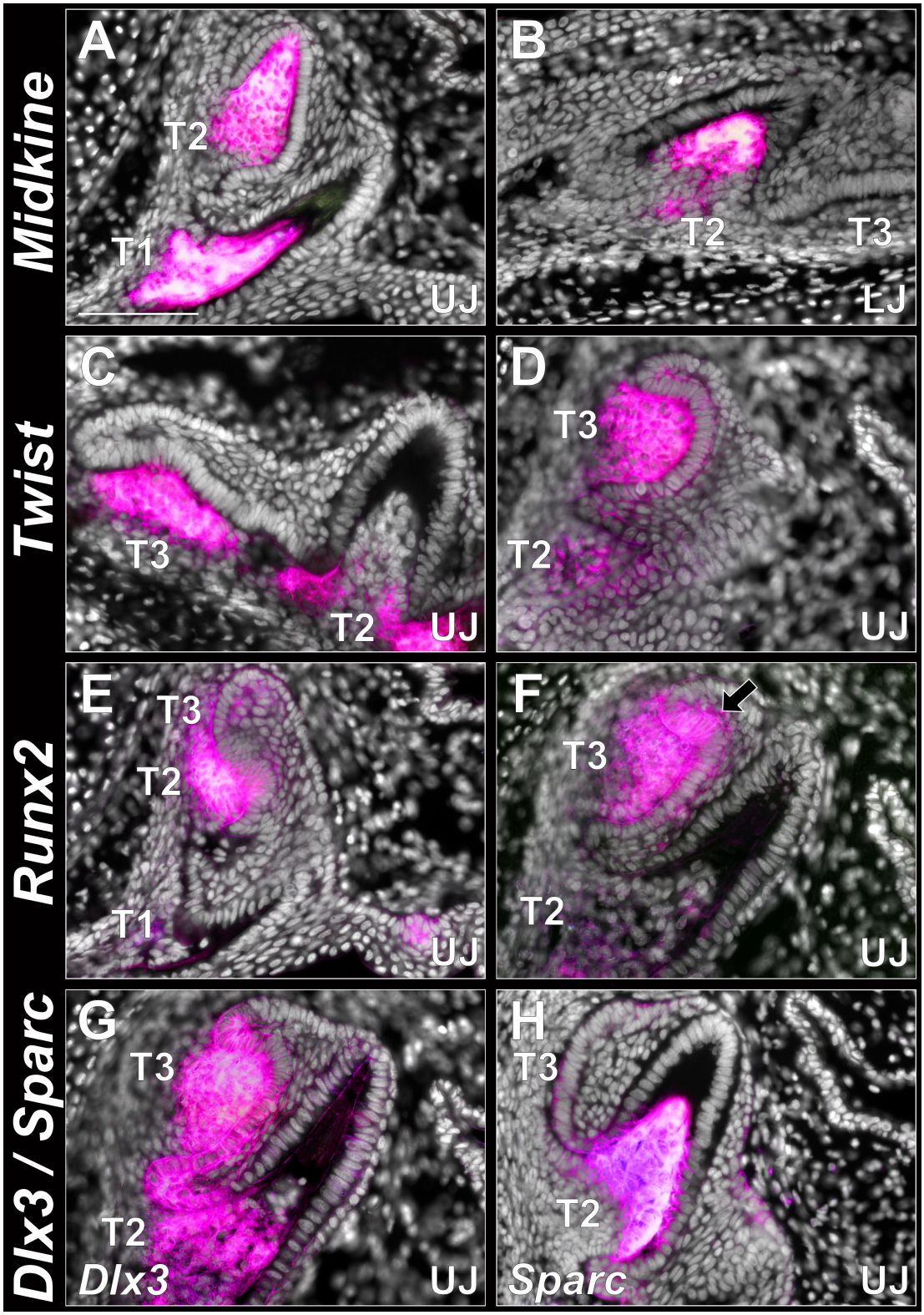


Figure 4.18 Expression patterns of *Midkine, Twist, Runx2, Dlx3* and *Sparc* during advanced shark tooth replacement (*S.canicula*). In the upper jaw, during RT morphogenesis, *Midkine* (*MK*) is expressed in the DM and medial IDE of the tooth tip (A, T2), and the basal DM of the papilla during subsequent advanced RT morphogenesis (A, T1). This expression pattern is further repeated in the lower jaw during RT morphogenesis (B, T2). In the upper jaw, *Twist* is expressed in the DM of

the early RT bud (C, T3) and bilaterally in the basal DM during advanced RT morphogenesis (C, T2). During intervening bud stage (D, T3), *Twist* is also expressed primarily in the DM. In the upper jaw, *Runx2* is expressed in the DM during RT bud (E, T2) and placode formation (E, T3). During subsequent RT morphogenesis, *Runx2* is continually expressed in the DM and IDE (F, T3, arrow), but absent during advanced RT morphogenesis (F, T2). During RT morphogenesis, *Dlx3* is expressed in the DM and IDE (G, T3), but is restricted to the basal DM of the papilla during advanced morphogenesis (G, T2). *Sparc* expression is restricted to the DM of the papilla during advanced RT morphogenesis (H, T2) and absent during preceding stages. Scale bar: (A-H) 100 μm.

4.3.6 Gene expression and cell proliferation during ray tooth development

In chapter 3, the shark and ray were used as comparative models to investigate the role of putative dental stem cells in the elasmobranch dentition, as implied by the common expression patterns of the stem cell marker *Sox2*. In the current study, the thornback ray (*Raja clavata*) was further used to investigate the extent to which commonly deployed signaling pathways are conserved in ray tooth development, thus adding further evidence to support the activation of a common GRN in polyphyodont gnathostomes with contrasting dental phenotypes.

In the ray dentition, this is first implied by the conserved expression of *β-catenin* and *Lef1* (Fig. 4.19, A-F). During early tooth replacement, *β-catenin* was expressed in the upper and lower jaws (Fig. 4.19A). This was predominantly restricted to the IDE (Fig. 4.19B-C) during early development of second-generation teeth (T2), therefore showing partial contrast with the shark dentition in which *β-catenin* is expressed in both the epithelium and mesenchyme. As previously shown (chapter 3), the conservation of canonical Wnt signaling in the ray dentition is further implied by the expression of *Lef1* (Fig. 4.19D). *Lef1* was co-expressed with *β-catenin* at similar stages in near identical domains focal to the IDE in the upper and lower jaws (Fig. 4.19E-F, respectively). In further accordance with the shark, *Pitx1*/*2* were also expressed in the upper and lower jaws during ray tooth development and early replacement (Fig. 4.19G and J, respectively). In the upper jaw (Fig. 4.19H), *Pitx1* was expressed throughout the dental/ successional lamina and thickened epithelium of the RT placode (T2) and absent in mesenchyme. In the lower jaw (Fig. 4.19I), expression was restricted to the successional lamina and adjoining dental epithelium, with no corresponding expression in the mesenchyme. In the upper jaws, *Pitx2* was expressed in the epithelial RT placode (Fig. 4.19K, T2), successional lamina and adjoining dental epithelium. *Pitx2* was also expressed throughout the directly underlying dental mesenchyme and absent from the mesenchymal papilla of the first generation tooth (T1) during morphogenesis. In the lower jaw (Fig. 4.19L), these expression patterns were similar, however, during bud stage (T1) *Pitx2* was expressed both in the dental mesenchyme of the tooth unit and underlying dental mesenchyme.

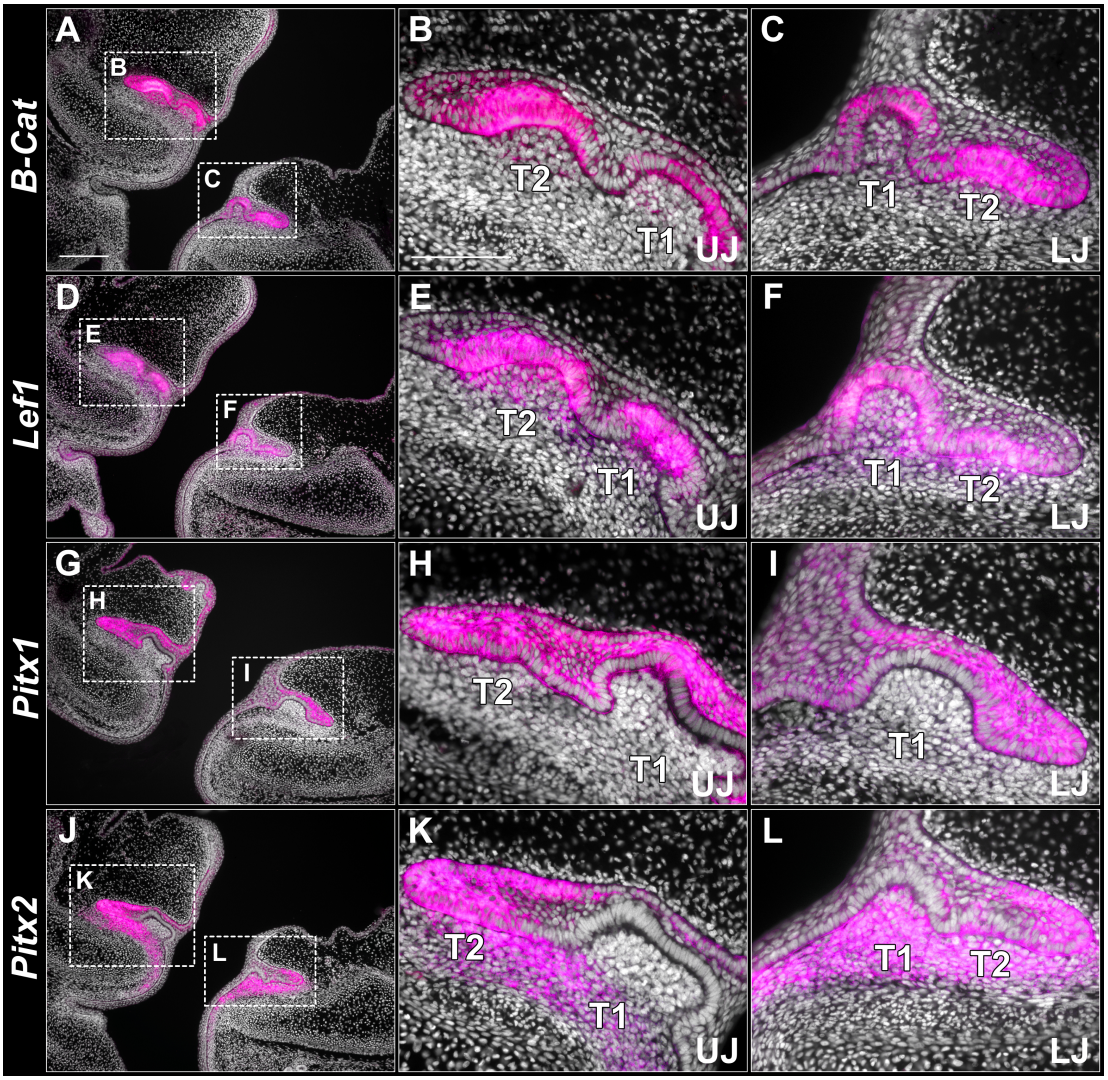


Figure 4.19 Conserved expression of *β-catenin*, *Lef1* and *Pitx1/2* in the ray dentition (*R.clavata*). In the ray, *β-catenin* (A-C) and *Lef1* (D-F) expression within the dental epithelium imply the common deployment of canonical Wnt signaling both within and amongst elasmobranchs. In both shark and ray tooth development, the conservation of these core developmental pathways is further implied by the common expression of epithelial *Pitx1* (G-I) and epithelial-mesenchymal *Pitx2* (J-L). Scale bars: (A, D, G, J) 200 μm, (B, C, E, F, H, I, K, L) 100 μm.

To define additional components of the ray tooth GRN, particularly those deployed during tooth morphogenesis, the expression patterns of *Bmp4*, *Shh* and *Midkine* were investigated, along with associated cell proliferation dynamics as defined by PCNA. *Bmp4* was expressed in both the epithelium and mesenchyme in the upper and lower jaws (Fig. 4.20A). In the upper jaws (Fig. 4.20B), *Bmp4* was expressed in the successional lamina and mesenchyme in association with the second-generation RT placode (T2). In the lower jaw (Fig. 4.20C), this expression pattern was essentially identical. The expression patterns of *Shh* were less clear, with expression visible in the lower jaw tooth only (Fig. 4.20D). The absence of teeth in the upper jaw dental lamina (Fig. 4.20E) was presumably due its inter-dental position in section. Despite this, a stripe of *Shh*-expressing oral epithelium was visible (arrow), showing some potential similarities with expression patterns in the early upper jaw of the catshark (Fig. 4.5J). In the lower jaw, a single bud-stage tooth was visible (Fig. 4.20F, T1); its position at the oral margin suggestive of a non-functional vestigial anlagen to be shed in advance of subsequent functional replacements. Despite this, *Shh* expression was observed in the medial epithelium in a pattern similar to that in the shark, though in the absence of additional stage-specific expression profiles, this remains circumstantial. The expression patterns of *Midkine* (*MK*) proved more informative, as clearly defined in the upper and lower jaws (Fig. 4.20G). In the upper jaw (Fig. 4.20H), *MK* was expressed in the medial IDE of the tooth at bud stage (T1), with some further signs of expression in the successional lamina. In the lower jaw (Fig. 4.20I), expression was also apparent, focal to the medial IDE and extending bilaterally to encompass the flattened aspect of the tooth. These expression patterns therefore provide further evidence to support the activation of similar gene circuits in two elasmobranchs with contrasting dentitions. In line with the early shark dentition, PCNA showed the ray dental lamina to be highly proliferative. This was particularly apparent in the upper jaw (Fig. 4.20J-K), in which both the O-ODE and adjoining IDE of first and second-generation teeth (Fig. 4.20K, T1-2) showed strong immunoreactivity. In the lower jaw first generation tooth (Fig. 4.20L, T1), this was further apparent; however, at these stages no reduction in cell proliferation in the medial IDE was apparent, therefore showing some notable differences with the shark dentition.

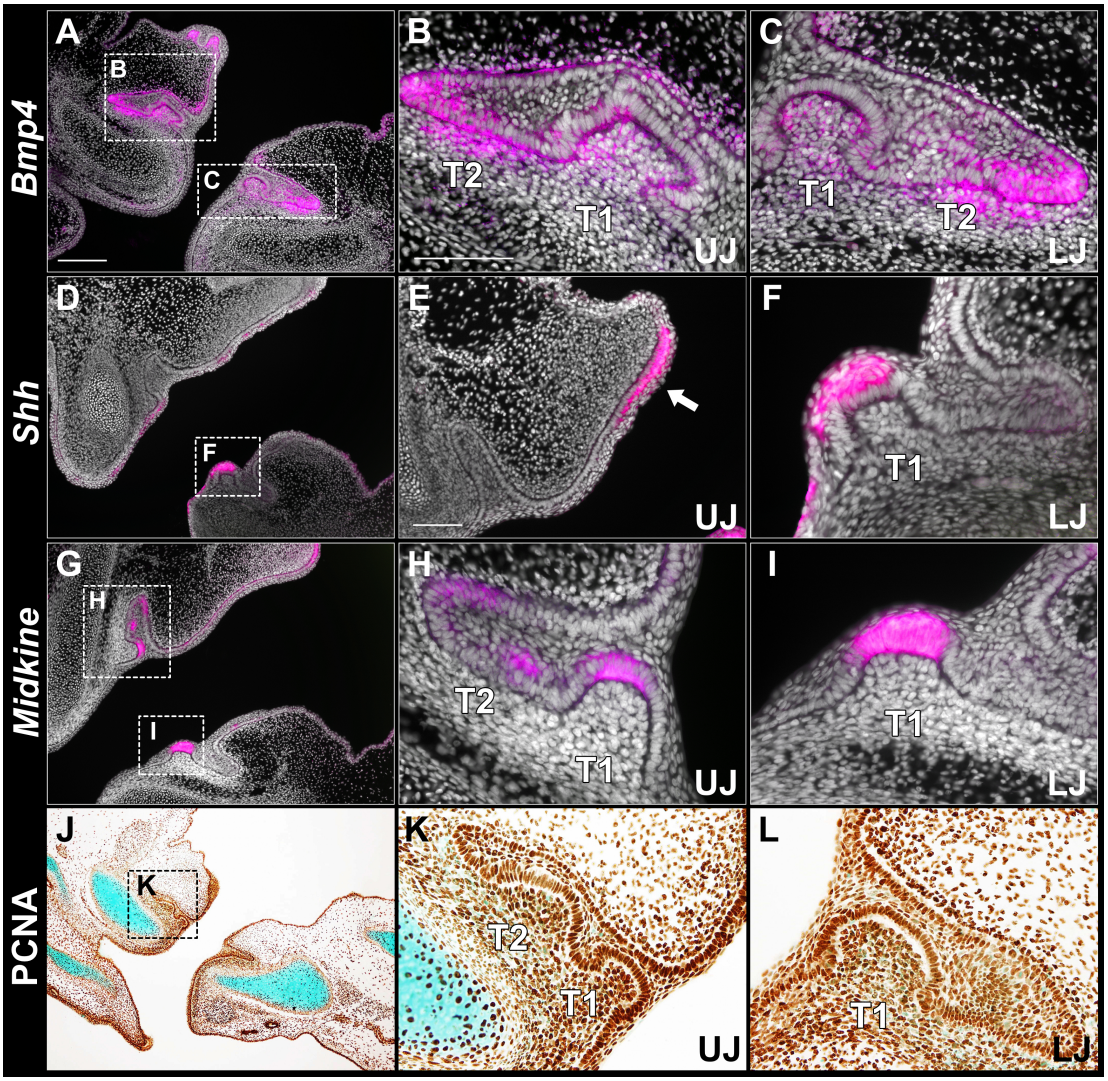


Figure 4.20 Conserved expression of *Bmp4, Shh*, *Midkine* and PCNA cell proliferation in the ray dentition (*R.clavata*). During ray tooth development, epithelial-mesenchymal *Bmp4* (A-C) defines the broader extent of gene regulatory conservation common to the shark dentition. This is made further apparent by the expression patterns of *Shh* (D-F) and *Midkine* (G-I). Both *Shh* (F) and *MK* (H-I) localise to regions of the IDE indicative of conserved roles during elasmobranch tooth morphogenesis. PCNA (J-L) defines the proliferative extent of the ray dental/ successional lamina. In both upper (K, T1-2) and lower (L, T1) jaw tooth development, PCNA immunoreactivity in the O-ODE and adjoining IDE of the tooth marks actively proliferating cells. However, this partially contrasts with the shark dentition, which at similar stages shows a marked reduction in cell proliferation in the medial aspect of the IDE. These patterns of cell proliferation and gene expression therefore highlight key similarities and differences between the shark and ray dentitions. Scale bars: (A, D, G, J) 200 μm, (B, C, E, F, H, I, K, L) 100 μm.

4.3.7 Investigation of *in situ* cell death during shark tooth morphogenesis

The detection of PCNA-negative cells in the medial epithelial tip of the tooth during morphogenesis (Fig. 4.2H), combined with subsequent gene expression data (*Shh*, *Fgf3*/*10*, *MK* and *Taz*), tentatively implies a signaling center comparable to the mammalian enamel knot to be ancestrally conserved in the shark dentition. Localisation of apoptotic activity in the same nested population of cells in the mammalian enamel knot has led to the proposition that apoptosis may be the principle mechanism by which its signaling function is terminated (Vaahtokari *et al*., 1996b). Subsequent studies have provided additional evidence to support this apoptotic potential, which appears to be further dependent upon *Caspase3*-mediated cell death (Jernvall *et al.,* 1998; Setkova *et al.,* 2007; Shigemura *et al.,* 2001; Matalová *et al*., 2006). In this study, Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (DeadEnd^{TM} Colorimetric TUNEL System, Promega), was first used to investigate DNA breaks indicative of apoptosis. To further identify putative apoptotic activity, Caspase3 immunohistochemistry (ab13847, Abcam) and *in situ* hybridisations were also carried out.

The TUNEL assay was carried out under standard and modified experimental conditions; however, these produced negative results in both dental and surrounding embryonic tissues. Casepase3 immunohistochemistry and *in situ* hybridisation experiments produced similar negative results, identifying no apparent signs of protein or mRNA in embryonic tissues. In all cases, appropriate controls were also run, producing similar results. This therefore leaves the possible role of apoptosis in shark tooth morphogenesis currently unresolved. Despite these negative results, it can still be hypothesised that apoptosis may yet prove to be one of the causal mechanisms by which the growth transition involved in shark tooth morphogenesis is effected. In the following discussion, interpretation of some of the gene expression patterns presented in this study are in line with this concept, however, it is acknowledged that these putative roles remain strictly hypothetical. This therefore warrants future experiments to target apoptotic activity in the embryonic shark dentition, in order to further substantiate the ancestral conservation of the enamel knot, or a similar signaling module.

4.4 Discussion

4.4.1 A conserved gene regulatory network patterns an ancient dentition

The study of tooth regeneration in ancient gnathostomes, such as elasmobranchs, provides the potential for crucial insights into evolution and development (Smith *et al*., 2009a; 2009b; Debiais-Thibaud *et al*., 2011). As comparatively basal extant gnathostomes, sharks and rays are representative of a seemingly ancient 'many-for-one' polyphyodont tooth replacement phenotype (Reif, 1976; 1980; 1982; Reif *et al*., 1978). Given their extensive evolutionary history, sharks in particular provide a crucial opportunity to further explore gene regulatory control of tooth replacement at its near-most basal origins. Evolutionary-developmental biology (evo-devo) is a comparative discipline, which frequently aims to ascertain putative gene function by cross-comparison in different developmental systems (Carroll, 2005). In this comparative context, the genes discussed here have, wherever possible, been assigned putative roles based upon critical evaluation of their known functions in other systems. It is acknowledged, however, that these inferences are further drawn from their stage-specific expression patterns (for example, during early epithelial thickening or tooth morphogenesis). In the absence of functional assays to manipulate gene function, these conclusions therefore remain hypothetical.

4.4.2 Gene co-expression domains infer regulation of early dental competence

In the osteichthyan dentition, the initial patterning potential required for dental competence is defined by the odontogenic band (OB); a continuous band of thickened oral epithelium expressing early tooth patterning genes, such as *Shh, Pitx2* and *Bmp4* (Fraser *et al*., 2004; 2008). This initial patterning mechanism is further conserved in the snake dentition, where *Shh* is expressed in a continuous OB to determine the position of the future dental lamina (Buchtová *et al*., 2008). Similarly, in the prospective shark dentition, PCNA and associated gene co-expression domains appear to define early dental competence, as inferred from this thickened oral epithelium and condensing mesenchyme (Smith *et al*., 2009a).

Of those implied to confer early dental competence, the Wingless (Wnt) signaling pathway is one of critical importance, most commonly defined by the *β-catenin* transcription factor (reviewed chapter 1: section 1.2.5). In both the mouse and snake dentitions, Wnt-*β-catenin* signaling is highly conserved, regulating various stages of tooth development (Järvinen *et al*., 2006; Handrigan and Richman, 2010b; Gaete and Tucker, 2013). In this study, expression of *β-catenin* in the early epithelial thickenings of the shark dentition suggests a deeper level of ancestral conservation and a role for Wnt signaling in defining initial dental competence. In the mouse dentition, Wnt-*β-catenin* signaling in the early dental epithelium induces dental competence by activating the underlying presumptive dental mesenchyme (Chen *et al*., 2009). In the adult mouse dentition, constitutive activation of *β-catenin* results in uncontrolled tooth budding, while deletion inhibits tooth development and reduces tooth number (Järvinen *et al*., 2006; Liu *et al.,* 2006). In the corn snake dentition, activation of *β-catenin* induces enhanced cell proliferation and budding of ectopic tooth germs along the DL (Gaete and Tucker, 2013).

Viewed in the context of its conserved role, it is therefore implied that *β-catenin* may positively regulate early odontogenic events by inducing cell proliferation in the prospective dental epithelium and/ or activating the underlying dental mesenchyme. Such a conserved role is further inferred from related studies showing, in the early epithelial thickenings (as implied by PCNA) of the shark and ray dentitions, co-expression of the stem cell marker *Sox2* (chapter 3). In the reptile dentition, *Sox2* is expressed in the first epithelial thickenings to define initial dental competence (Juuri *et al*., 2013). In the prospective shark dentition, the overlapping expression of *β-catenin* and *Sox2* at these early stages suggests a conserved interaction involving induced epithelial cell proliferation from a *Sox2*+ progenitor population (chapter 3). However, future functional studies to perturb Wnt-*β-catenin* signaling will be required to further substantiate this.

The co-expression of *Shh* and *Ptc2* at these early stages further adds to current evidence regarding a role for hedgehog signaling in shark tooth development (Smith *et al.*, 2009a). However, in contrast to the extensive expression domains of *β-catenin,* those of *Shh* and *Ptc2* are comparatively restricted, marking the focal extent of the

prospective dentition. The hedgehog signaling pathway is highly conserved, in vertebrates regulating the development of several epithelial appendages, including teeth, feathers, hair and scales (Hausmann *et al.,* 2009; Matus *et al*., 2008; Cobourne *et al*., 2004; Dassule *et al*., 2000; Siedel *et al.,* 2010; Harris *et al*., 2005; Sire and Akimenko, 2004). The critical role of hedgehog signaling in the mouse dentition is shown by *Shh* inhibition, which causes tooth arrest at initiation stage (Cobourne *et al*., 2001). Hedgehog signaling is further conserved in the osteichthyan dentition, in several species regulating various stages of tooth induction and morphogenesis (Jackman *et al.,* 2010; Fraser *et al.,* 2004; 2006a; 2008; 2012; 2013). In the reptilian dentition, *Shh* is also conserved, in snakes delineating the extent of the prospective odontogenic epithelium and specifying the position of the future DL. This is shown by *Shh* inhibition, which prevents initiation of the DL and associated ingrowth into the mesenchyme (Buchtová *et al.,* 2008). In the shark dentition, co-expression of both ligand and receptor in a same restricted epithelium therefore implies a short range (autocrine) signaling function for *Shh* and a conserved role in defining early dental competence by marking the position of the future DL. Once again, this supposition is drawn from early *Shh*/ *Ptc2* expression domains in this thickened epithelium, warranting future functional studies targeting hedgehog signaling during formation of the OB.

However, the role of hedgehog signaling in early dental competence is further supported by associated co-expression of the transcription factors *Pitx1* and *Pitx2*, both of which regulate various aspects of tooth and limb development (Semina *et al*., 1996; Mitsiadis and Drouin, 2008; Marcil *et al.,* 2003; Duboc and Logan, 2011; Klopocki *et al.,* 2012). *Pitx2* in particular is strongly linked to tooth initiation, shown by its conserved expression in the OB of the trout and cichlid (Fraser *et al.,* 2004; 2008, respectively). In the shark, co-expression of *Pitx1*/*2* in the odontogenic epithelium therefore suggests a conserved role in tooth initiation; however, both show further marked associations with the underlying mesenchyme. In the mouse dentition, *Pitx1* is expressed in both the epithelium and mesenchyme, while *Pitx2* is restricted to the epithelium (St.Amand *et al*., 2000). In the osteichthyan dentition, *Pitx2* is also only expressed in the epithelium, where it is proposed to confer early dental competence with mesenchymally expressed *Bmp4* (Fraser *et al*., 2004). These

epithelial-mesenchymal co-expression patterns may therefore prove indicative of an ancestral role in which both confer early dental competence through expression in both cell layers. The expression patterns of *Pitx1* in particular are indicative of a role in determining the position of the future DL, shown by comparatively deepened mesenchymal expression domains, compared with the upper jaw. This is further consistent with previous observations suggesting the lower jaw dentition to develop in advance of the upper (Reif, 1980).

The bone morphogenetic protein (BMP) family of secreted signaling molecules are so named due to their ability to induce ectopic bone formation (Urist, 1965; Reddi, 1992; Wozney, 1992). In mouse tooth induction, *Bmp4* is expressed in the presumptive dental epithelium of the early tooth placode, sequentially inducing its own expression in the mesenchyme during budding. During tooth morphogenesis *Bmp4* is subsequently expressed in the enamel knot and during differentiation of odontoblasts and ameloblasts. *Bmp4* is therefore implied to mediate epithelial-mesenchymal interactions during tooth initiation and regulate cusp morphogenesis and subsequent differentiation of cells involved in matrix deposition (Vainio *et al.,* 1993; Åberg *et al.,* 1997). *Bmp4* is also expressed in similar patterns in both the reptilian and osteichthyan dentitions, implying conserved roles in regulating epithelial-mesenchymal induction (Handrigan and Richman, 2010b; Fraser *et al*., 2004; 2008; 2012; 2013).

In the prospective shark dentition, *Bmp4* shows no apparent association with the epithelium, expressing primarily in the mesenchyme. These expression domains therefore present some differences compared with other gnathostomes, making it difficult to define a precise role during these early odontogenic inductive events. Given the comparatively ancient phylogenetic position of sharks, this may therefore prove indicative of an ancestral mesenchymal function for *Bmp4*, subsequently modified in successive gnathostomes. In the mouse dentition, the expression domains of *Pitx1*/*2* and *Bmp4* are maintained by antagonistic interactions; however, this is further inconsistent with the overlapping expression patterns in the early shark dentition (St.Amand *et al*., 2000). In the osteichthyan dentition, *Pitx2* is proposed to activate the odontogenic mesenchyme by inducing expression of *Bmp4* (Fraser *et al.,*

2004). In the shark, co-expression of mesenchymal *Pitx1/2* with *Bmp4* provides some evidence to support a conserved interaction to activate the prospective dental mesenchyme, possibly through induced expression of *Bmp4*. Given the characteristically transient epithelial-mesenchymal expression patterns of *Bmp4*, future expression and functional studies will be required to further determine the nature of these putative interactions.

4.4.3 Gene co-expression domains mark first generation tooth initiation

Following onset of early dental competence, sustained epithelial-mesenchymal gene expression is proposed to enable development of the primary dental lamina and first generation tooth placodes. This early patterning process is further defined by associated *Sox2* (chapter 3), expressed at the oral surface continuous with the primary DL via an intervening 'stripe' of O-ODE. Furthermore, *Sox2* shows progressive localisation to a defined cell cluster at the oral and dental epithelial junction, marking a putative dental stem cell niche (SCN). This is further inferred from the snake and cichlid dentitions, in which *Sox2* marks a similar putative dental SCN connected with the successional lamina via a continuous epithelial stripe (Gaete and Tucker, 2013; Fraser *et al*., 2013). In the shark dentition at this stage of development, *β-catenin* is co-expressed with *Sox2* in the SCN and the adjoining *Sox2*+ O-ODE, progressively weakening before reappearing in the first tooth placodes. Recent studies of ball python tooth development have implied a role for Wnt signaling in stimulating cell proliferation during inward growth of the primary DL (Handrigan and Richman, 2010b). Given the overlapping cell proliferation shown by PCNA, it can be inferred here that *β-catenin* may hold a conserved role in stimulating the proliferation of *Sox2*+ progenitor cells to maintain development of the DL. This is further supported by addition studies of *β-catenin* function, which imply an additional role in regulating changes in cell adhesion, a cellular remodeling process anticipated during ingrowth and expansion of the DL (Behrans *et al*., 1996).

The co-expression of *β-catenin* and *Sox2* in the surface SCN and O-ODE presents further interesting possibilities regarding early regulation of tooth progenitor cell maintenance, proliferation and differentiation. During alligator tooth development, *β-catenin* expression in the stem cell bulge implies a role either in promoting or

inhibiting commitment of quiescent SCs to tooth-specific fates (Wu *et al*., 2013). In the hair follicle (HF) SCN, stabilised *β-catenin* expression is low, subsequently increasing in TA cells and so implying cell-context specific roles in SC maintenance and regulation of the transition between progenitors and proliferating TA progeny (Lowry *et al*., 2005). This apparent functional disparity in maintenance of pluripotency versus proliferation and lineage commitment is not uncommon and has generated considerable debate. One proposed mechanism to account for this is a critical cell context-dependent switching mechanism and in this respect, the early shark dentition may present such an example (reviewed by Miki *et al*., 2011). It is feasible that in this context, *β-catenin* may function as a molecular rheostat, to transiently maintain undifferentiated progenitors in the SCN, followed by a switch in regulatory function to stimulate proliferation of TA cells in the intervening O-ODE leading to the DL. Some supporting evidence for this can be found in time-course treatment studies of induced pluripotent stem cells (iPSCs), in which Wnt-*β-catenin* signaling modulates cellular reprogramming by enhancing expression of endogenous pluripotency factors, such as *Oct4, Sox2, Klf4 and Sall4*. This regulatory role is, however, transient, occurring only at the initial reprogramming stage to establish the undifferentiated state, followed by a switch in role once no longer required (Zhang *et al*., 2014). In the early shark dentition, this interaction correlates with the overlapping co-expression of *Sox2* and *β-catenin*, both strongly expressed in the SCN and O-ODE, progressively reducing in the DL. In the shark dentition, it is therefore possible that *β-catenin* may further stimulate the expression of *Sox2* (and probably additional pluripotency factors) in the SCN to maintain cells in an undifferentiated state, prior to a switch in regulatory function in the O-ODE to induce cell proliferation of undifferentiated progeny (here implied to be putative TA cells) into the DL. While hypothetical, this is generally consistent with other systems in which Wnt-*β-catenin* modulates cellular reprogramming of progenitors in a cell context-dependent manner (Sato *et al*., 2004; Anton *et al*., 2007). As previously highlighted, functional studies to manipulate Wnt-*β-catenin* signaling will be required to better understand its putative roles and interactions.

In the shark dentition, *β-catenin* is further expressed in the first epithelial tooth placodes, showing an associated role in tooth induction. In the mammalian and

reptilian dentitions, *β-catenin* is also strongly associated with tooth induction, shown by induced activation and inhibition, resulting in supernumerary teeth or impairing their development, respectively (Järvinen *et al*., 2006; Gaete and Tucker, 2013; Liu *et al.,* 2008). In further accordance with its known role, *β-catenin* is therefore implied to induce development of tooth placodes in the shark dentition. However, associated expression of *Lef1* in the tooth placode epithelium further implies a cell context-specific role for Wnt-*β-catenin* signaling. Stabilised *β-catenin* binds *Lef1* in the nucleus, forming a bipartite transactivation complex to regulate gene transcription (Seidensticker and Behrens, 2000). In the mammalian dentition, *Lef1* expression in the presumptive epithelium gives rise to tooth placodes, suggesting a role in early tooth induction, while targeted inactivation arrests tooth development after formation of the epithelial bud. Subsequent development of teeth and whiskers also requires transient *Bmp4*-mediated activation of *Lef1*, further suggesting a role in mediating inductive tissue interactions (Kratochwil *et al.,* 1996). During primary shark tooth induction, *Lef1* is co-expressed with *β-catenin* in the epithelial tooth placodes, which when taken in the context of their conserved roles, suggests a positive regulatory role in tooth initiation, possibly through induced cell proliferation, as implied by PCNA.

However, when considering the expression of *Sox2*, an inhibitory role can further be considered. As previously discussed in chapter 3, in the snake dentition, organisation of the dental epithelium into *Lef1*+ and *Sox2*+ compartments, in which *Lef1* is expressed in the SL and *Sox2* is not, implies an inhibitory role. Overactivation of Wnt-*β-catenin* signaling results in expansion of these *Lef1* expression domains, with associated restriction of *Sox2* to the oral epithelium (Gaete and Tucker, 2013). A similar phenotype is produced in the mouse lung epithelium in which activation of Wnt-*β-catenin* signaling induces expanded *Lef1* expression domains, while reducing those for *Sox2* (Hashimoto *et al*., 2012). In the shark dentition, sustained *Sox2* expression in the O-ODE and lingual aspect of the DL implies a conserved interaction in which *Lef1* spatially restricts *Sox2* to maintain a pool of quiescent dental progenitors periodically sequestered to commit to odontogenic fates when induced by appropriate signaling (chapter 3).

The expression domains of *Pitx1/2* in the DL and first tooth placodes show some variation compared to their expression in the OB, with *Pitx1* restricted to the epithelium. This shift in expression implies a corresponding shift in role. Induced Wnt signaling through addition of *Wnt3a* to pancreatic *β* cells stimulates *Pitx2* to promote *cyclin D2* expression, promoting increased cell proliferation. Conditional activation of *β-catenin* produces similar phenotypes (Rulifson *et al*., 2007). This is further consistent with Wnt-induced *Pitx2* expression, mediated by *Lef1,* to stimulate cardiac development and pituitary proliferation (Kioussi *et al*., 2002). *Pitx1* is also expressed in the epithelial mouse tooth anlagen and -/- mutants show defective cell proliferation, implying a direct regulatory interaction involving Wnt-*Pitx1/2*-mediated cell proliferation (Mitsiadis and Drouin, 2008; Lanctôt *et al*., 1999; Szeto *et al*, 1999). In the shark DL, *Pitx1/2* are strongly co-expressed with *β-catenin,* and during tooth induction, with *β-catenin/Lef1* in the tooth placodes. Given the known interactions and overlap with *β-catenin/Lef1* in a highly proliferative dental epithelium (shown by PCNA), *Pitx1/2* may constitute downstream Wnt-*β-catenin* targets to regulate development of the DL and tooth placodes through sustained stimulation of cell proliferation. The continued expression of *Pitx2* in the mesenchyme remains unclear, however, this may be to stimulate mesenchymally-expression genes further involved in tooth development. Furthermore, in the shark dentition, mesenchymal *Pitx2* expression may prove representative of an ancestral function, subsequently modified in the dentitions of successive gnathostomes (St.Amand *et al*., 2000; Fraser *et al*., 2004; 2006a). This is further implied by related expression studies of first generation tooth development in the thornback ray (*Raja clavata*) in which *Pitx2* is expressed in similar epithelial-mesenchymal domains. Given the known proliferative effects of *Pix2*-induced *cyclin D2* expression (Rulifson *et al*., 2007), future studies targeting the expression of cyclins in the shark dentition may shed further light upon these putative roles and interactions.

While the initial expression of *Shh* in the shark OB is consistent with other gnathostomes, its expression domains in the primary DL present some inconsistencies. In the cichlid dentition, early *Shh* expression defines the extent of the OB, before localising to individual tooth germs, each formed from a separate DL (Fraser *et al.*, 2008). In contrast, elasmobranch teeth develop within a continuous

DL, shown previously to express *Shh* during first tooth induction (Smith *et al*., 2009a). Similar expression patterns in the early snake dentition further imply a conserved role for *Shh* in regulating first generation tooth induction (Buchtová *et al*., 2008). In this study, the absence of *Shh* expression during first generation tooth induction has been attributed to a gap in staging, highlighting the requirement for future additional expression studies to resolve this discrepancy. Despite this, the early expression of *Shh* at the junction between the oral and dental epithelium is of interest. In the early snake dentition, *Shh* is expressed in similar domains, localised to the acutely angled side of the dental lamina, where it is proposed to delineate the position of the odontogenic epithelium and promote dental epithelial ingrowth. This is supported by functional studies using cyclopamine treatment, which prevents ingrowth and disrupts normal depth and angulation (Buchtová *et al*., 2008). These common expression patterns therefore provide some evidence to support a deeply conserved regulatory function for *Shh* in determining both the position and future inward growth of the primary dental lamina. This is supported by initial expression of *Shh* in the OB and it is anticipated that similar future studies to ablate normal *Shh* gene expression at these stages will shed further light upon these suppositions.

In the current study, it is of further interest that this initial site of *Shh* expression defines the future position of the elasmobranch dental SCN, marked by co-expressed *β-catenin* and *Sox2* (chapter 3). These expression domains are therefore potentially indicative of an important function for this restricted oral epithelium in determining both epithelial ingrowth and establishing the pool of dental progenitors required for tooth development and continuous replacement. In the hair follicle SCN, *Shh* is required to maintain bulge cells capable of becoming epidermal SCs (Brownell *et al.,* 2011). Wnt-*β-catenin* signaling relayed through *Shh* and *Bmp* signals is the principle mechanism underlying HF cell fate change, while overexpression of *β-catenin* causes HF tumours, accompanied by upregulation of *Shh* and *Ptc*, suggesting synergistic interactions to promote proliferation of progeny in hair cell lineages (Suzuki *et al*., 2009; Niemann *et al.,* 2003). During tumour progression in the tongue epithelium, dormant activation of *β-catenin* leads to *Shh/ Ptc1* expression, in turn diminishing nuclear *β-catenin* through feedback loops. *Shh* also provides proliferative cues in chronic myeloid leukemia progenitor cells through downstream *β-catenin* signaling

(Schneider *et al*., 2010; Su et *al.,* 2012). *Sox2* is also highly expressed in medulloblastomas associated with pathological activation of *Shh* signaling. Deletion of *Sox2* from cell cultures with constitutive *Shh* signaling results in decreased proliferation, while overexpression causes enhanced proliferation, implying a co-regulatory role in tumour cell proliferation (Ahlfeld *et al.,* 2013). Given the known interactions between *Shh*, *β-catenin* and *Sox2* in regulating cell proliferation, a similar putative role in the early shark SCN, in which these genes act in concert to regulate the inward proliferation of dental progenitors into the early dental lamina, can be inferred. It may then follow that *β-catenin*-*Pitx1/2*-mediated cell proliferation further promotes cell proliferation into the dental lamina to maintain the supply of progenitors committed to tooth-specific fates.

4.4.4 Gene co-expression domains regulate elasmobranch tooth development and regeneration

Formation of the successional lamina (SL) is marked by further inward growth extension of the free end of the primary dental lamina and initiation of first replacement tooth placodes, which progress through a series of common stages as a result of continued reciprocal signaling interactions between the epithelium and mesenchyme (Smith *et al.,* 2009a; 2009b). The early expression of *β-catenin, Lef1, Shh, Ptc2*, *Pitx1/*2, *Bmp4* and *Sox2* determine preceding odontogenic events, however, little is known about the subsequent roles and interactions of these, and addition genes, during shark tooth development and replacement. In this section of the study, the expression patterns of an expanded set of dental patterning genes were therefore investigated during elasmobranch tooth development and replacement.

4.4.4.1 *β-catenin*/ *Lef1* mark sustained Wnt signaling during tooth development

During development of first and second-generation teeth in the catshark, *β-catenin* and *Lef1* are expressed in similar domains to those in tooth initiation. *β-catenin* is initially expressed epithelially in the SCN continuous with the O-ODE, and subsequently down-regulated in the lingual DL before reappearing in the tooth placode epithelium with *Lef1*. In the shark, *β-catenin* is strongly expressed in both the dental epithelium and mesenchyme with epithelially-expressed *Lef1,* whereas in

the ray *β-catenin* appears to be restricted primarily to the dental epithelium. While this shows some tissue-specific variation, these co-expression patterns imply reactivation of the same set of regulatory circuits to induce RT placode and bud formation through sustained stimulation of cell proliferation. In the shark and ray, sustained *Lef1* expression in the tooth placode epithelium is indicative of an inductive role, while corresponding restriction of *Sox2* to the lingual aspect of the DL (chapter 3) is further consistent with a continued inhibitory role, as shown in the snake dentition (Gaete and Tucker, 2013). Furthermore, in the corn snake, *Lef1* expression in the free end of the successional lamina is proposed to regulate its continual extension, in order to maintain the correct spacing of teeth (Gaete and Tucker, 2013). Given the sustained expression of *Lef1* in the dental/ successional lamina of the shark and ray, and the common requirement for its controlled extension during 'many-for-one' tooth replacement, this role may be conserved, so as to regulate the sequential addition of new replacement teeth (Smith *et al*., 2009b; Gaete and Tucker, 2013). It is, however, acknowledged that in all cases, current interpretation of *β-catenin* mRNA expression as a readout for canonical Wnt signaling provides only partial evidence to support its various proposed roles in the embryonic shark and ray dentitions. Future immunohistochemistry experiments targeting nuclear (activated) protein at similar stages, will therefore be required to further elucidate the role of Wnt signaling in the elasmobranch dentition.

Despite this, the expression patterns of Wnt genes and additional markers continue to prove insightful, in terms of their possible roles in the shark dentition. During shark tooth morphogenesis, *β-catenin* and *Lef1* become progressively restricted to specific regions of the IDE and DM, including the epithelial tooth tip. In mouse molar tooth morphogenesis, signaling molecules belonging to the hedgehog, FGF, BMP and Wnt families are expressed in a knot-shaped cluster of non-proliferative epithelial cells in the tooth tip, appropriately termed the enamel knot. Originally identified in histological sections, the enamel knot is now known to constitute a highly conserved organising center, which determines the size and shape of teeth by signaling to surrounding cells to trigger the transcriptional responses required to induce their appropriate growth activity (Ahrens, 1913; Butler, 1956; Vaahtokari *et al*., 1996a; 1996b; Keränen *et al.,* 1998; Jernvall *et al*., 1994; Jernvall and Thesleff, 2000;

Kratochwil *et al.,* 1996; Järvinen *et al*., 2006). Similar conserved patterns of gene expression in vole, shrew and ferret tooth morphogenesis imply the enamel knot to be highly conserved in the mammalian dentition (Keranen *et al.,* 1998; Yamanaka *et al*., 2007; Järvinen *et al*., 2009). In the cichlid dentition, *Shh, Fgf3*, *Bmp2/4* and several additional genes are expressed in the tooth tip in similar domains, supporting the assumption that the enamel knot, or a similar forerunner, may be conserved in all gnathostomes presenting some complexity in cusp morphology (Fraser *et al*., 2013; Vaahtokari *et al*., 1996a; Jernvall *et al*., 1994).

In the mammalian dentition, *β-catenin* plays a key regulatory role in tooth morphogenesis, shown by its constitutive stabilisation, which results in continual formation of enamel knots and supernumerary tooth buds (Järvinen *et al*., 2006). Conversely, inactivation of *β-catenin* in the dental epithelium and mesenchyme arrests tooth development at bud stage (Liu *et al.,* 2008; Chen *et al*., 2009). *Lef1*-deficient mice also show signs of defective tooth development at bud stage and fail to form the enamel knot (van Genderen *et al*., 1994; Kratochwil *et al*., 1996; Chen *et al*., 2009). While in the current study, a definitive role for *β-catenin* and *Lef1* in shark tooth morphogenesis remains unclear, their co-expression in the epithelial tip implies a conserved ancestral function to determine future tooth cusp morphology through transcriptional control of associated regulatory genes.

In the shark dentition, during bud and morphogenesis stages, *Lef1* also shows marked signs of demarcation to defined regions of the dental epithelium. These restricted expression domains could be partially accounted for by effects of the secreted ligand, Sclerostin domain-containing protein 1 (*Sostdc1*), which regulates tooth development through multiple interactions with hedgehog, FGF, BMP and Wnt-*β-catenin* signaling (Ahn *et al*., 2010; Cho *et al*., 2011; Kassai *et al*., 2005; Laurikkala *et al.,* 2003). Existing studies of *Sostdc1* function imply a role in BMP inhibition; however, *Sostdc1* further interacts with Hh and Wnt signaling in controlled feedback loops in which Wnt signaling induces expression of *Shh*, which in turn inhibits Wnt-*β-catenin* indirectly via *Sostdc1*. This feedback loop is proposed to regulate critical phases of mammalian tooth development through spatial delineation of expression domains,

including that of the enamel knot (Laurikkala *et al*., 2003; Cho *et al.,* 2011; Kassai *et al.,* 2005).

In the shark dentition, during development of first and second-generation teeth, *Sostdc1* also delineates the *β-catenin* /*Lef1*+ dental epithelium in the first tooth placode and bud, containing a medial *Sostdc1*-negative region. During advanced tooth morphogenesis, *Sostdc1* is expressed in a similar pattern, further implying a role involving demarcation of Wnt signaling to impose spatial boundaries bordering the developing tooth. In addition to its proposed antagonistic effects, *Sostdc1* may further contribute to tooth induction, shown by its expression in the epithelium of RT placodes. How *Sostdc1* expression is induced and whether this involves a similar feedback loop to that of the mouse dentition remains unclear, however, given the deployment of these pathways in the shark dentition, a deeply conserved ancestral interaction is likely. Functional studies to manipulate these signaling pathways will be required to better characterise their putative roles and interactions.

4.4.4.2 Sustained *Pitx1*/*2* expression patterns suggest continued roles in tooth development

Following their initial expression at stages proposed to coincide with early dental competence and formation of the primary dental lamina, *Pitx1*/*2* are continually expressed during tooth development and early replacement. In the primary and secondary dentitions of the shark, *Pitx1* is restricted to epithelial domains encompassing the O-ODE, IDE and DL, and in first-generation teeth in the ray, *Pitx1* expression is further restricted to the dental epithelium. However, during subsequent shark tooth replacement, this extends to the dental mesenchyme, as initially observed in the OB. In the mouse dentition, *Pitx1* regulates epithelial cell proliferation, emphasised its absence in the enamel knot through inhibition by *Bmp4* (Mitsiadis and Drouin, 2008). In the early shark and ray dentition this role may be partially conserved. In both, *Pitx1* is strongly expressed in the proximal aspect of the DL, implying a role in positively regulating tooth induction, possibly by stimulating cell proliferation. Throughout bud to early morphogenesis there is also a marked absence of *Pitx1* expression in the IDE, including the epithelial tooth tip, which is consistent

with the mammalian enamel knot (Mitsiadis and Drouin, 2008). During advanced shark tooth morphogenesis, *Pitx1* is also strongly expressed in the marginal IDE of the tooth, implying a role in regulating pre and post-ameloblast cell activity. However, during subsequent shark tooth replacement, *Pitx1* is expressed both in the dental epithelium, including the epithelial tooth tip, and mesenchyme during RT placode development and morphogenesis. In the shark dentition, reactivation of *Pitx1* in the dental mesenchyme therefore implies a role in regulating mesenchymal cell activity specific to tooth replacement stages. This may involve regulation of epithelial-mesenchymal interactions and during morphogenesis, mesenchymal cell proliferation; however, in both cases this currently remains unclear.

It is interesting to note that in *Pitx1* -/- mutant mice, incisors and maxillary (upper jaw) molars develop normally, however, during advanced stages of mandibular (lower jaw) development, teeth exhibit several phenotypic abnormalities, including small size, fused first and second molars, and reduced numbers of cusps. In the mouse dentition, these independent patterning mechanisms are assumed to account for development of correct molar tooth morphology and therefore the mandibular-maxillary processes as functional apparatus (Mitsiadis and Drouin, 2008) (Mitsiadis and Drouin, 2008). In both the shark and ray, *Pitx1* and additional genes shown here are expressed in developing teeth within both the maxillary and mandibular jaws, with no evidence of preferential expression in either. Since elasmobranch teeth lack the occlusional properties of mammalian teeth, the gene circuits reflective of this 'dual patterning' mechanism may therefore prove indicative of an ancestral phenotypic state subsequently modified in successive gnathostomes, such as mammals, to meet their changing feeding requirements.

In the mouse and ferret dentitions, *Pitx2* is restricted to the presumptive dental epithelium by the inhibitory effects of *Bmp4* (St.Amand *et al.,* 2000; Jussila *et al.,* 2014). It has therefore been proposed that in mammals, *Pitx2* is critical in specifying the odontogenic epithelium and regulating cell proliferation and growth of teeth. This is also reflected by an absence of *Pitx2* expression in the enamel knot and an apparent down-regulation during advanced morphogenesis, consistent with differentiation of ameloblasts and odontoblasts (St.Amand *et al.,* 2000). Expression

of *Pitx2* in the odontogenic epithelium of the zebrafish, trout, pufferfish and cichlid further implies a deeply conserved role in osteichthyan tooth induction (Stock *et al.,* 2006; Fraser *et al.,* 2004; 2012; 2008). However, as previously discussed, in the early shark and ray dentitions, this remains only partially conserved, with *Pitx2* expressed in both the epithelium and mesenchyme during various stages of tooth development. This therefore implies a putative ancestral role specific to the elasmobranch dentition, raising further interesting questions regarding the remodulation of the core dental GRN during gnathostome evolution. Viewed in the context of its known role, in the shark dentition, *Pitx2* may therefore further function to regulate tooth development by mediating epithelial-mesenchymal interactions and/ or stimulating the proliferation of mesenchymal cells marked for tooth-specific fates. It has been proposed that in mammals, *Pitx1* function may be redundant to that of *Pitx2* (Lanctôt *et al*., 1999). Given the comparatively basal phylogenetic position of elasmobranchs, it is therefore tempting to speculate that the apparent overlap in *Pitx1*/*2* epithelial-mesenchymal expression domains may prove indicative of an ancestral interaction in regulating early odontogenic events. It is further tempting to speculate that through remodulation of the dental GRN, this partnership may have been modified during the course of gnathostome evolution, rendering one gene redundant to the other. This supposition is further drawn from studies showing the expression of *Pitx3* in the developing shark dentition (data not presented here), therefore implying significant functional overlap for several members of the PITX family of genes in regulating tooth development in ancient gnathostomes.

4.4.4.3 *Meis2* and *Foxq1* further imply the deployment of dental stem cells

Recent characterisation of the expression patterns of *Sox2* in elasmobranch tooth regeneration (chapter 3) has proven important in identifying a putative epithelial dental stem cell niche. In the reptilian and osteichthyan dentitions, *Sox2* marks a similar SCN and adjoining epithelial stripe, suggesting this regenerative strategy to be highly conserved amongst polyphyodont gnathostomes (Gaete and Tucker, 2013; Fraser *et al*., 2013). In the shark dentition, *Foxq1* further marks the same morphologically distinct cell cluster, while *Meis2* is expressed in the dental/ successional lamina, suggesting sustained roles in dental progenitor cell regulation.

Meis genes encode transcription factors belonging to the TALE (three-amino-acid loop extension) class of homeodomain proteins, with implied roles in cardiac development, limb outgrowth and regeneration, and tumourigenesis (Burglin, 1997; Paige *et al.,* 2012; Capdevila *et al.,* 1999; Mercader *et al*., 2005; Dekel *et al.,* 2006). Meis proteins also play critical roles in cell cycle regulation, fate specification, maintenance of neural SCs and cell proliferation (Bessa *et al.,* 2008; Agoston *et al*., 2014; Kaslin *et al.,* 2009; Heine *et al*., 2008). Given the apparent absence of any pre-existing tooth expression data relating to Meis genes, the detection of *Meis2* transcripts in the primary and successional DL of the shark presents both a novel finding and an associated challenge when considering its putative function. In the adult zebrafish neural SCN, *Meis2* and *Sox2* are co-expressed during progenitor cell proliferation (Kaslin *et al.,* 2009). *Meis1/2* are also expressed in retinal progenitor cells (RPCs) during onset of differentiation. *Meis2*-inactivation reduces *Cyclin D1* expression, while *Cyclin D1* transfection partially rescues RPC proliferation, suggesting positive roles for *Meis1/2* in regulating RPC cell proliferation (Heine *et al.,* 2008). In the shark dentition, one such putative role for *Meis2* may therefore be to induce proliferation of *Sox2*+ progenitor cells (chapter 3) housed with the lingual dental lamina. In human trabecular cells, *Pitx2* knockdown results in a corresponding change in *Meis2* expression (Payalakhi *et al.,* 2011). *Pitx2* and *Meis2* are also co-expressed in overlapping domains during avian craniofacial development (Buchtová *et al*., 2010). The known interaction of *Pitx2* and *Meis2* with cyclins in stimulating cell proliferation further supports a conserved ancestral function in promoting dental progenitor cell proliferation during active phases of tooth development and regeneration (Heine *et al*., 2008; Bessa *et al.,* 2008). In addition to the shark dentition, *Meis2* is further expressed in the developing taste buds, eye and brain, which in the case of the latter two, further supports a deeply conserved ancestral role in regulating the development of these structures (Heine *et al*., 2008; Bessa *et al.,* 2008; Agoston *et al*., 2014).

Members of the Forkhead box (Fox) family of transcription factors carry out diverse functions involving regulation of cell proliferation, apoptosis, migration and differentiation (Schmidt *et al*., 2002; Sunters *et al*., 2003; Fosbrink *et al.,* 2006; Myatt and Lam, 2007). Several Fox genes are also strongly associated with tooth

development and linked to dental abnormalities resulting from their dysfunction (Shirokova *et al*., 2013; Poché *et al.,* 2012; Samaan *et al*., 2010; Honkaken *et al*., 2003). *Foxq1* transcripts have previously been identified in the ampullary organs, denticles and teeth of the embryonic catshark, prompting further investigation in the current study (Wotton *et al*., 2008).

Existing arguments to support the proposed involvement of dental stem cells in tooth replacement have previously drawn evidence from comparisons of the mammalian intestinal crypt and zebrafish dentition in which replacement teeth develop from a morphologically similar pharyngeal epithelium underlying the functional tooth (Huysseune and Thesleff, 2004; van der Heyden and Huysseune, 2000). The regenerative capacity of the intestinal tract is a well-characterised system, comprising a series of invaginations (crypts) housing stem cell niches from which proliferating cells migrate upward to give rise to the various lineages required to maintain the absorptive function of the epithelium (Potten, 1995). Within the crypt, the critical role of conserved signaling pathways in maintaining the balance between cell pluripotency and proliferation is shown by their dysregulation, resulting in malignancy (reviewed by Leedham *et al*., 2005; Marshman *et al*., 2002).

The role of Fox genes in crypt development and maintenance has previously been shown by studies of *Fox-1* (*Fkh6*), with knockout mice showing phenotypic abnormalities in intestinal epithelial architecture and hyper-proliferative crypts. These mice also show upregulation of genes, which increase the efficacy of Wnt signaling, highlighting an indirect role for *Fox-1* in regulating the balance between cell pluripotency and proliferation in the crypt (Kaestner *et al*., 1997). Consideration of the implied role of *Foxq1* in several known cancers provides further relevant insights into its putative conserved role in the elasmobranch dental SCN. In colorectal cancer (CRC) cells, decreased *Foxq1* expression leads to increased cell proliferation (Kaneda *et al.,* 2010). However, studies have also shown *Foxq1* repression to impair cell proliferation, implying cell-context specific roles (Feuerborn *et al.,* 2011). In CRC cells, *Foxq1* further constitutes a direct target for *β-catenin* signaling, which increases its expression (Christensen *et al.* 2013). Given current evidence of the conserved interaction between Wnt-*β-catenin* signaling and

Fox gene function in modulating the balance between cell maintenance and proliferation, a possible ancestral partnership in the dental SCN during early stages of tooth development remains possible.

An ancestral role for *Foxq1* in stimulating cell proliferation in the dental SCN is further inferred from consideration of its putative interactions with *Sox2*, which maintains cell pluripotency by partnering with *Oct4*, *c-Myc* and *Klf4*, as shown by reprogramming of human dermal fibroblasts, which generates induced pluripotent stem cells (Takahashi *et al*., 2007). In cultured adipose tissue stromal cells, overexpression of *Oct4* negatively regulates *Foxq1* and *Foxo1*, further suggesting their conserved roles as positive regulators of cell proliferation (Kim *et al*., 2009). Given the conserved partnership of *Oct4* and *Sox2* in maintaining the pluripotent state of stem cells, this provides further supporting evidence of a role for *Foxq1* in stimulating the proliferation of quiescent progenitors into the dental epithelium to differentiate to odontogenic fates (Nichols *et al*., 1998; Avilion *et al*., 2003). Some further supporting evidence for this is found in studies of Fox gene expression in hair follicle development. At the base of the developing hair follicle, mesenchymal cells of the dermal papilla (DP) become enveloped by epithelial matrix (Mx) cells; a transiently proliferative cell population maintained in an undifferentiated state in advance of their upward migration and differentiation (Hardy, 1992; Schmidt-Ullrich and Paus, 2005). One characteristic feature of Mx cells is their responsiveness to inductive cues from the surrounding microenvironment, which prompts their upward proliferation and subsequent differentiation. Transcriptional profiling of Mx cells has revealed, amongst others, the differential expression of *Foxn1* and *Foxq1*, which taken in the context of their immediate cellular environment, implies a role involving positive regulation of cell proliferation (Rendl *et al*., 2005).

Inferences drawn from comparison of these gene expression profiles, as superimposed upon this defined cell cluster, support its proposed role as a dental SCN housing the progenitors required for continuous shark tooth regeneration. Given that during early rounds of tooth replacement, these expression domains in the SCN tend to reduce while *Sox2* expression is consistently maintained, this suggests remodulation of existing signals or an alternative scenario perhaps involving partial

transferal of this regenerative potential to the successional lamina itself. Despite this, *β-catenin* and *Foxq1* constitute the first identified markers of a putative molecular rheostat dedicated to modulating the balance between cell pluripotency and proliferation, as further defined by the co-expression of *Sox2* (chapter 3).

Given the insights provided here by the intestinal crypt, it is likely that future dissection of further regenerative components of the shark tooth gene regulatory program will prove increasingly insightful when considering their conserved roles in other stem cell niches of similar function. This agenda for future research is immediately highlighted by ongoing studies of the shark dentition, which have identified the expression of the stem cell markers *Bmi1* and *Lgr5* (Martin and Fraser, unpublished). Both genes are expressed in the cervical loop of the rodent incisor and in the intestinal crypt, highlighting their conservation as core components of a commonly deployed regenerative genetic toolkit (Biehs *et al*., 2013; Suomalainen *et al*., 2010; Yan *et al*., 2012). It is noteworthy, that in addition to its expression in the dental SCN, *Foxq1* is strongly expressed in the IDE during advanced tooth replacement morphogenesis, implying a further function. Given the known role of Fox genes in stimulating odontogenic cell differentiation and the expression of target genes required for enamel maturation (inclusive of *Runx2*), it can be inferred that *Foxq1* may also function to stimulate the differentiation of cells marked for ameloblastic fates (Poché *et al.,* 2012).

4.4.5 PCNA and gene co-expression domains define the enameloid knot

The discovery of the enamel knot as a conserved signaling center represents a milestone in understanding the intrinsic complexities of mammalian tooth morphogenesis, and of the wider role of organising centers in epithelial organogenesis (Ahrens, 1913; Vaahtokari *et al*., 1996a; 1996b; Jervall *et al*., 1994; Thesleff and Jernvall, 1997). One characteristic of the enamel knot is its lack of cell proliferation, marked by the absence of BrdU incorporation compared with the surrounding dental epithelium and mesenchyme (Vaahtokari *et al*., 1996a). During shark tooth morphogenesis, PCNA analysis distinguishes a delineated set of non-proliferative cells in the medial IDE, as distinct from the surrounding proliferating

dental epithelium. These cells lack PCNA immunoreactivity, indicating their withdrawal from the cell cycle. This leads to the supposition that such a signaling center analogous to the enamel knot may be conserved in gnathostomes displaying some degree of tooth cusp complexity and that this enameloid knot may therefore retain a similar ancestral function in the elasmobranch dentition. In the current study, this hypothesis is therefore proposed. Unlike mammalian teeth, those in chondrichthyans do not incorporate enamel, capped instead with a superficial layer of hyper-mineralised enameloid. Given these compositional differences, the 'enameloid knot' is so named here accordingly (Shellis and Miles, 1974; 1976; Smith and Hall, 1990; Sasagawa, 1993; Gillis and Donoghue, 2007). While PCNA analysis provides initial support for the existence of the enameloid knot, gene co-expression domains focal to the same delineated set of dental epithelial cells further add to this.

4.4.5.1 *Shh* expression patterns define tooth morphogenesis but not replacement initiation

In the shark dentition, following its initial expression during tooth initiation, *Shh* is expressed symmetrically in the medial IDE at advanced bud stage, subsequently upregulating in the medial epithelial tip during tooth morphogenesis and progressively spreading bilaterally. This pattern reiterates during subsequent rounds of tooth replacement, implying cyclical reactivation of the same hedgehog circuits to regulate tooth morphogenesis. While these patterns therefore consolidate existing expression studies of *Shh* in the shark dentition, a precise role remains unclear (Smith *et al*., 2009a). In the mammalian dentition, the critical role of *Shh* in tooth morphogenesis is demonstrated through conditional deletion prior to cap stage, which results in reduced tooth size and severe disruption to molar tooth morphology. However, normal deposition of enamel and dentin suggests that *Shh* signaling is not required for differentiation of ameloblasts and odontoblasts (Dassule *et al*., 2000). This is further shown in the reptilian dentition, in which exposure of dental explants to cyclopamine at bud stage results in flattened tooth shapes and accompanied loss of *Ptc1* expression (Handrigan and Richman, 2010a). In natural and bioengineered tooth germs, patterns of spatiotemporal *Shh* expression further imply a role in

determining tooth size and shape (Ishida *et al*., 2011). Studies of *Shh* in hair follicle and feather morphogenesis also suggest a conserved role in regulating cell proliferation and growth polarity (St-Jacques *et al.,* 1998; Nanba *et al*., 2003; McKinnell *et al*., 2004). In hair follicle development this is shown in *Shh* null mutant mice, which undergo growth arrest of the dermal anlage, and by cyclopamine inhibition, resulting in impaired formation of the dermal papilla (St-Jacques *et al.,* 1998; Nanba *et al*., 2003). In feather development, cyclopamine inhibition induces ectopic feather domains and dramatic phenotypic changes in feather bud morphology (McKinnell *et al*., 2004)

In the context of these known roles, *Shh* may therefore function to regulate shark tooth morphogenesis and growth polarity through short-range autocrine signaling to the surrounding epithelium of the tooth tip. In the early developing ray dentition, provisional expression studies suggest a conserved role for *Shh* in elasmobranch tooth morphogenesis; however, in comparison with the shark, its epithelial expression domains within the tooth are far less restricted. Given the stark differences in tooth shape between sharks and rays, these differential expression patterns therefore indicate the modification of the same core gene circuits to produce these contrasting dental phenotypes. A conserved role for hedgehog signaling in the shark dentition is further supported by the overlapping epithelial co-expression of *Ptc2* at similar stages of development, implying its responsiveness to hedgehog ligands. However, *Ptc2* is also expressed throughout the DM at various stages, indicating a mesenchymal role. In the leopard gecko, bearded dragon and python, *Ptc1* is also consistently expressed in the dental epithelium and mesenchyme at various stages of tooth development, indicating both autocrine and short-range paracrine roles for hedgehog signaling (Handrigan and Richman, 2010a). In the shark dentition, this role may therefore be ancestrally conserved, with *Ptc2* expressed in the dental mesenchyme directly underlying the epithelium expressing *Shh*. It is therefore possible that *Shh* may be acting as a diffusible short-range paracrine factor to regulate mesenchymal cell activity during tooth budding and morphogenesis. *Ptc2* expression in mesenchymal domains negative for *Shh*, however, further imply hedgehog signaling to operate through other as yet unidentified ligands, warranting future expression studies.

The evolutionary implications of these expression patterns are of further significance. Though expressed during first tooth induction, it has been proposed that *Shh* is not required for initiation of replacement teeth (Fraser *et al*., 2006a; 2013). This is evident in the trout and cichlid dentitions in which *Shh* is expressed in the primary OB and ODE associated with first generation tooth germs, but absent during successive tooth replacement (Fraser *et al*., 2004; 2006a; 2006b; 2008; 2013). In the replacement squamate dentition, *Shh* signaling is also absent during replacement tooth formation and treatment of dental explant tissue with cyclopamine does not affect outgrowth of the SL, which is also negative for *Ptc1* (Buchtová *et al.,* 2008; Handrigan and Richman, 2010a). In the current study, restriction of *Shh* to the epithelial tip during replacement tooth morphogenesis and its marked absence in the thickened epithelium of RT placodes and the successional lamina therefore supports these studies to suggest a conserved ancestral role (Smith *et al*., 2009a). However, in partial contrast with these polyphyodont gnathostomes, a continued requirement for hedgehog signaling during tooth replacement is implied by the sustained expression of *Ptc2* in spatial and temporal epithelial-mesenchymal domains outlying those of *Shh*. This may therefore prove indicative of an ancestral condition and while currently unresolved, it is likely that future studies to perturb hedgehog signaling and target the expression of additional related markers and Shh at the secreted protein level will prove increasingly informative in characterising the fuller extent of hedgehog signaling in the developing shark dentition.

4.4.5.2 FGF expression patterns further define the enameloid knot

In the catshark dentition, expression of *Shh* in the epithelial tip provides initial supporting evidence for the conservation of an enamel knot-type signaling center in the elasmobranch dentition (Vaahtokari *et al*., 1996a). Additional evidence for the existence of such an enameloid knot, and partially conserved ancestral roles for *Fgf3*/*10* in the shark dentition, is found in the expression patterns of FGFs in mammals. During mouse molar development, FGFs expressed in the enamel knot stimulate cell division in the enamel epithelium and dental papilla (Jernvall *et al.,* 1994). *Fgf10*/*3* are expressed at various stages of molar tooth development, *Fgf10* in the presumptive dental epithelium and mesenchyme during initiation, and *Fgf3* in the

mesenchyme at late bud stage. During subsequent morphogenesis, *Fgf3* is expressed in the enamel knot, whereas *Fgf10* is not, and during cap and bell stages both are expressed in the mesenchymal dental papilla. Subsequent down-regulation in post-mitotic odontoblasts correlates with their terminal differentiation and those of neighbouring ameloblasts (Kettunen *et al*., 2000). Though developmentally decoupled, in the mouse incisor, mesenchymal *Fgf3*/*10* also cooperate to stimulate epithelial SC proliferation to maintain a continuous supply of progenitors for ameloblasts. This critical role is reflected in *Fgf3* -/- and *Fgf10* +/- mutants exhibiting reduced tooth growth and severe enamel hypoplasia (Harada *et al.,* 1999; 2002; Wang *et al.,* 2007).

In the catshark dentition, the expression patterns of *Fgf3*/*10* therefore present some interesting similarities and differences with that of mammals. In the catshark, *Fgf3* transcripts are first detected during early first generation tooth morphogenesis in the DM and basal membrane of the central IDE of the tooth tip. *Fgf3* progressively localises to the medial tooth tip IDE, with a corresponding reduction in the DM. These patterns are essentially repeated during subsequent tooth replacement. However, during development of first generation and replacement teeth, *Fgf10* is expressed primarily in the tooth-tip IDE throughout tooth bud and morphogenesis stages. In mouse dental explants, *Fgf10* is sufficient to stimulate dental epithelial cell proliferation in culture, and expression of *Fgf3* in the DM at late bud stage suggests a role in mesenchymal signaling to the epithelium (Kettunen *et al*., 2000). In the catshark dentition, progressive restriction of *Fgf3* expression from the DM to the tooth tip epithelium therefore implies a conserved ancestral role in mediating epithelial-mesenchymal interactions, while *Fgf10* may hold a unique ancestral function as an autocrine factor to stimulate surrounding epithelial cell proliferation during tooth morphogenesis (Kettunen *et al*., 2000). In the mouse dentition, *Lef1* indirectly regulates mesenchymal *Fgf3* and epithelial *Shh* through *Fgf4* (Kratochwil *et al.,* 1996; 2002). In the shark dentition, co-expression of these genes therefore further implies a conserved ancestral interaction, which given their collective localisation to the medial epithelium of the tooth tip, provides further evidence to support the deep conservation in shark tooth morphogenesis of an enameloid knot comparable in function to the mammalian enamel knot.

Despite these apparent similarities, the most striking difference with respect to the mammalian enamel knot is notably expression of *Fgf10* in the dental epithelium of the tooth tip. During mouse tooth morphogenesis, *Fgf10* is expressed both in the epithelium and mesenchyme (Kettunen *et al*., 2000) and in the incisor stem cell niche mesenchymal *Fgf10* stimulates epithelial cell proliferation via Notch signaling (Harada *et al*., 1999). In the shark dentition, restriction of *Fgf10* to the dental epithelium of the tooth tip, coincident with the enameloid knot, may therefore prove indicative of an ancestral circuit modified during subsequent gnathostome evolution.

The assumed status of *S.canicula Fgf10* (*ScFgf10*) as an ancestrally conserved homologue is drawn from NCBI BLAST analysis, showing considerable conserved sequence identity amongst vertebrates, and with no apparent sequence homology shared with other FGF genes. However, the complexity of the FGF gene family is shown by recent molecular phylogenetic analysis, which implies the occurrence of several duplications and losses to account for its current diversity (Oulion *et al*., 2012). Within this phylogenetic framework, *Fgf10* is grouped within a subfamily of closely related FGFs, inclusive of *Fgf7*/*22* (Oulion *et al*., 2012). Due to the considerable complexity of the FGF family of genes, it therefore remains possible that in basal gnathostomes, such as elasmobranchs, *ScFgf10* may yet prove homologous with other mammalian FGFs (e.g. *Fgf8*). This could therefore further account for the differential expression patterns highlighted between the elasmobranch and mammalian dentitions. Interestingly, the BLAST search carried out in this study shows *ScFgf10* to share most significant sequence homology with that of the African coelacanth (*Latimeria chalumnae*). Given the comparatively basal phylogenetic position of elasmobranchs and sarcopterygians (Amemiya *et al*., 2013), this is perhaps unsurprising, therefore emphasising the importance of exercising caution when arbitrarily assigning gene names and function based upon superficial sequence analysis alone. In addition, future expression studies targeting corresponding FGF receptors will be required to investigate the broader signaling range of FGF ligands, such as *Fgf3*/*10*. While the receptors *Fgfr1*/*2* were cloned for this study, both produced inconclusive *in situ* hybridisation results, warranting future expression studies to ascertain the wider extent of FGF signaling in the shark dentition.

4.4.5.3 *Midkine* and *Bmp4* are expressed in the enameloid knot, while marking epithelial-mesenchymal interactions

Neurite growth-promoting factor 2, or *Midkine* (*MK*), is transiently expressed between the epithelium and mesenchyme during mouse molar initiation, budding and morphogenesis, and also in the enamel knot and cervical loop (CL) of the mouse incisor. Tooth growth, differentiation and mineralisation are all inhibited in the presence of *MK*-neutralising antibodies, implying critical roles in tooth induction, proliferation, cytodifferentiation and SC maintenance (Mitsiadis *et al.,* 1995b*;* 2008). In the shark dentition, transcripts are first detected at bud stage, during which *MK* is strongly expressed in the dental mesenchyme and weakly in the overlying epithelium. Expression subsequently spreads throughout the mesenchymal compartment, while increasing in the medial enameloid knot-region of the IDE focal to the epithelial basement membrane. During both first generation and advanced RT morphogenesis, persisted *MK* expression in the tooth tip IDE and mesenchyme of the papilla implies conserved roles in regulating tooth budding, morphogenesis and cytodifferentiation, prior to the secretion of hard tissue matrices.

In the mouse incisor CL, *MK* expression in the dental epithelium and mesenchyme coincides with regions of high cell proliferation, implying a positive regulatory role in promoting cell proliferation (Mitsiadis *et al.*, 2008). During tooth budding in the shark dentition, overlapping *MK* expression and PCNA immunoreactivity in the dental epithelium and mesenchyme further implies a conserved ancestral role. During subsequent morphogenesis, *MK* expression in the enameloid knot-region of the shark dentition further suggests a role in regulating early tooth cusp morphogenesis. *MK* expression in similar domains in the ray dentition further implies this role to be conserved amongst elasmobranchs. In the mouse enamel knot, *MK* is implied to prevent apoptosis until completion of function (Mitsiadis *et al*., 2008). Some supporting evidence for this comes from the wider role of *MK* in inhibiting the apoptotic effects of *Caspase3* during neuronal development and a similar cytoprotective role in mediating tumour cell survival (Owada *et al.,* 1999; Qi *et al.,* 2000). While in the current study, experimental evidence to support apoptosis during shark tooth morphogenesis remains unresolved, *MK* may further be acting in

accordance with its conserved role to inhibit apoptosis of cells in the enameloid knot until its signaling function is complete. Furthermore, in the mouse incisor CL, overlapping localisation of MK and Fgf10 protein to within ameloblast progenitors has suggested a synergistic interaction in maintaining SCs in an undifferentiated state until induced to commit to a specific cell fate (Mitsiadis *et al*., 2008). Since *MK* and *Fgf10* are both co-expressed in approximately overlapping patterns in the enameloid knot-region, a further interaction in regulating undifferentiated progenitors fated to contribute to cusp development cannot be ruled out. In the mouse incisor, *MK* is strongly expressed in odontoblasts in the DM of the papilla and moderately in pre-ameloblasts in the IDE, while culture with *MK*-neutralising antibody also prevents mineralised tissue formation (Mitsiadis *et al*., 2008). *MK* is also transiently expressed during bone formation and repair, implying a conserved role in positively regulating cell activity in advance of matrix deposition (Ohta *et al*., 1999). In the shark dentition during advanced morphogenesis, *MK* is strongly expressed throughout the DM of the papilla, but absent in the IDE lateral to the tooth tip, suggesting a similarly conserved ancestral role in regulating odontoblastic cell activity in advance of matrix deposition. In addition to those previously described, *MK* therefore provides a further marker to support the deep conservation in ancient gnathostomes, of an enameloid knot signaling center dedicated to regulating tooth cusp morphogenesis.

The expression patterns of *Bmp4* are further characteristic of a conserved ancestral function. In the shark dentition, though expression during first tooth initiation was not profiled here, *Bmp4* is strongly expressed in both the dental epithelium and mesenchyme during advanced bud stage, in a pattern indicative of a conserved role involving transmission of odontogenic signals between the epithelium and mesenchyme (Vainio *et al*., 1993). This is further apparent during early ray tooth replacement, in which *Bmp4* is expressed epithelially in the successional lamina and in the underlying dental mesenchyme in association with RT placodes. During shark tooth morphogenesis, a delineated *Bmp4*-negative zone in the medial epithelial tooth tip defines a putative zone of inhibition concomitant with activation of the enameloid knot. In the mammalian dentition, *Bmp4* is implied both to activate the enamel knot and terminate its function through induced apoptosis (Jernvall *et al*., 1998). In the

shark dentition, subsequent in-spread of *Bmp4* into the medial IDE during morphologically distinguishable stages of tooth morphogenesis, provides additional evidence both to support the ancestral conservation of the enameloid knot, and of the involvement of *Bmp4* in its function. The causal factors responsible for the putative initial inhibition of *Bmp4* from the medial epithelium remain unclear. In the python dentition, *Shh* signals emanating from the tooth tip are implied to spatially restrict BMP expression to the DM (Handrigan and Richman, 2010b). In the shark dentition this interaction may be further conserved, marked by initial restriction of *Bmp4* to the DM and IDE lateral to *Shh,* implying such a role for *Shh* in excluding *Bmp4* from the enameloid knot, in advance of its subsequent apoptotic requirement.

Following its expression during morphogenesis, *Bmp4* localises strongly to the basal DM of the papilla, implying a shift in function to regulate mesenchymal cell activity in relation to differentiation of odontoblasts. At this stage, an absence of *Bmp4* expression in the IDE suggests a lack of involvement in regulating pre and post-ameloblastic cell activity. This supposition is at least partially supported by previous studies of *Bmp4* expression in the mammalian dentition, which show a corresponding shift in *Bmp2*/*4* expression to the DM of the papilla. At this stage, both are expressed in odontoblastic and ameloblastic progenitors until their terminal differentiation, suggesting a sustained role in mediating epithelial-mesenchymal interactions required for their terminal differentiation. At this stage, the reliance of *Bmp2*/*4* upon epithelial-mesenchymal interactions is further demonstrated in tissue recombination experiments in which both are absent in mesenchymal tissue cultured in the absence of epithelium, whereas in recombined explants both show preferential expression in regions of odontoblastic and ameloblastic differentiation (Vainio *et al*., 1993). While in the current study, it is not possible to investigate the conserved aspects of this interdependence, similar expression patterns of *Bmp4* imply an ancestral role in regulating pre and/ or post odontoblastic progenitors in the mesenchymal papilla. Future functional studies to perturb BMP signaling, accompanied by further expression studies of additional BMPs and their receptors, will be required to better define these putative roles and interactions. Despite this, the expression patterns of *Bmp4* in the shark dentition strongly support a conserved ancestral function in

conferring initial dental competence, tooth morphogenesis and cell differentiation, through stage-specific epithelial-mesenchymal interactions.

4.4.5.4 *Taz* expression patterns infer multiple functions in the shark dentition

The transcriptional co-activator with PDZ-binding motif, *Taz*, or *wwtr1*, along with its paralogue *Yap* (yes-associated protein), function as major downstream effectors of the Hippo pathway to regulate organ size and tumour suppression by mediating transactivation of target genes (reviewed by Barry and Camargo, 2013; Hiemer and Varelas, 2013). While commonly linked with regulation of cell proliferation and apoptosis, recent studies have revealed an increasingly apparent role for *Taz* and *Yap* in various aspects of mammalian tooth development (Wang *et al*., 2014; Camargo *et al*., 2007; Zhang *et al*., 2011). This has largely been demonstrated by overexpression of the splice variant *Yap1* in transgenic (Tg) mice, which show several dental abnormalities, including widening of the dental lamina and displacement of the enamel knot. *Yap1* Tg mice also show signs of defective apoptosis, restricted to the enamel knot, and absent at the tip of the enamel organ. Associated defects include ablated cell proliferation, altered *Edar* and *E-*/*P-cadherin* expression, and abnormal restriction of *Shh, Fgf3*/*4* and *Wnt10a* to the tip of the enamel organ. Epithelial-mesenchymal cell proliferation is also reduced, implying roles in regulating cell proliferation, movement and polarisation during molar tooth morphogenesis (Liu *et al*., 2014). During mouse incisor development, *Yap1* is also expressed in the apical bud and transit amplifying cells, suggesting regulatory roles in incisor morphogenesis and dental stem cell proliferation (Li *et al*., 2011a).

The role of *Taz* in tooth development is comparatively less clear, limited to *in vitro* studies in which its application to hDPSCs induces formation of mineralised extracellular matrices, accompanied by the expression of *Msx1/2*, *DSPP* and *Dlx5* (Suh *et al*., 2012). While in the current study, this therefore renders any clear conclusions of its putative odontogenic role difficult, some informed speculation can be drawn from the known relationship between both genes. The functional overlap of *Yap* and *Taz* is implied by existing studies, which have highlighted their considerable degree of shared sequence and structural homology (Kanai *et al*., 2000). This is

apparent in lung cancer cells, in which knockdown of *Taz* inhibits cell proliferation and increases apoptosis and the expression of cleaved (activated) *Caspase3*, while overexpression inhibits apoptosis and alleviates *Caspase3* cleavage (Wang *et al*., 2014). Similarly, in colorectal cancer cells, *Taz* knockdown represses cell proliferation and increases apoptosis (Pan *et al*., 2012). A conserved role common to *Taz* and *Yap* is further implied by studies of skin and hair follicle development, in which upregulation of *Yap* promotes cell proliferation and inhibits apoptosis, marked by a decrease in activated *Caspase3*, while downregulation reverses these effects (Zhang *et al*., 2011). In zebrafish development, *Yap1* inhibition also leads to decreased cell proliferation and increased apoptosis, therefore implying this function to be highly conserved between mammals and osteichthyans (Hu *et al*., 2013). A key feature of the mammalian enamel knot is its transient nature, marked by programmed cell death following completion of function, resulting from the proposed apoptotic effects of *Bmp4* (Vaahtokari *et al*., 1996a; Jernvall *et al*., 1998). Given the conserved anti-apoptotic roles of *Yap* and *Taz*, it is therefore tempting to speculate that in the shark dentition, *Taz* may further function in line with its conserved role to confer transient cytoprotection to cells in the enameloid knot, in advance of subsequent apoptosis. This initial conclusion is, however, based upon current consideration of *Yap* and *Taz* as evolutionarily related genes with overlapping functions (Kanai *et al*., 2000). As previously stated, future studies to identify apoptotic activity in the epithelial tip will also be required to further support this proposed role.

However, one additional or perhaps entirely alternative role for *Taz* may be to relay mechanosensory signals to surrounding cells in response to dynamic changes in cell adhesion, movement and rigidity experienced during morphogenesis. Both *Yap* and *Taz* are fundamentally linked to regulatory roles involving cellular responses to mechanical stress (reviewed by Guo and Zhao, 2013). In human mesenchymal stem cells (MSCs), *Yap* and *Taz* have been identified as key signal transducers of mechanosensory responses to changes in surrounding extracellular matrix (ECM) rigidity (Dupont *et al*., 2011). This is shown by their induced nuclear translocation, and therefore activation, within MSCs grown on stiffened substrates. When depleted under these conditions, osteogenic differentiation in inhibited, which is also in line with the conserved role of *Yap* and *Taz* in promoting differentiation of cell lineages

responsible for hard tissue formation (Dupont *et al*., 2011; Cui *et al*., 2003; Suh *et al*., 2012).

Throughout tooth morphogenesis, the dental epithelium and mesenchyme undergo extensive tissue remodeling during the transition from bud to cap and bell stages (reviewed by Peters and Balling, 1999). In the shark dentition, *Taz* shows marked associations with the epithelial-mesenchymal ECM throughout late tooth budding and morphogenesis. This is most apparent during RT morphogenesis, in which *Taz* expression is apparently upregulated in the ECM underlying the medial aspect of the polarised tip, followed by an absence during subsequent stages. This therefore implies a further role in regulating physical changes in cell adhesion, shape and movement, in order to effect this growth transition. When considering the overlapping roles of *Yap* and *Taz*, this supposition is therefore further consistent with the dramatic adverse phenotypes observed in *Yap1* Tg mice, notably deformed tooth morphogenesis marked by mislocation of the enamel knot and associated changes in *E*- and *P-cadherin* expression (Liu *et al*., 2014). Cadherins play critical roles in establishing tissue architecture through regulation of cell-cell adhesion, junction formation and cytoskeletal remodelling (Harris and Tepass, 2010). In the mammalian dentition, the effects of *Yap1* overexpression on tooth morphogenesis and cadherin expression support its hypothesised role as a critical determinant of odontogenic cell movement and polarisation (Liu *et al*., 2014). This supposition is therefore consistent with the expression patterns presented in the current study, which imply a similar ancestral role for *Taz* in regulating dynamic changes in cell-cell adhesion and movement to determine the overall architecture of future replacement teeth. However, given these contrasting proposed roles, future studies targeting the expression of *Yap*, cadherins, and additional up and downstream effectors of the Hippo pathway, will be required to further elucidate their odontogenic functions in the shark dentition.

4.4.5.5 Enameloid knot model and its role as a cusp-making module

In the shark dentition, conserved enamel-knot markers, notably *Shh*, *Fgf3*/*10*, *Bmp4* and *MK,* show marked associations with the non-proliferative epithelial tooth tip and

associated mesenchyme during advanced stages of tooth budding and morphogenesis (Fig. 4.21). While hypothetical, these genes are proposed to operate in controlled autocrine and paracrine signaling cascades to trigger transcription of target genes in order to direct shark tooth cusp morphogenesis. The conservation of the enameloid knot in ancient gnathostomes, such as elasmobranchs, raises interesting questions regarding the evolutionary trajectory of the vertebrate dentition. It has been suggested that the evolvability of the dentition results from its organisation into a meristic series of modules capable of phenotypic adaptation through dissociation, while retaining independent genetic control (Wagner, 1996; Stock, 2001). In the mouse dentition, genetic control of incisor renewal and molar morphogenesis are regionally decoupled, while in polyphyodont gnathostomes, these developmental processes remain unified (Tummers and Thesleff, 2009; Handrigan and Richman, 2010a; 2010b; Handrigan *et al*., 2010; Fraser *et al*., 2013). In the mammalian dentition, the most apparent level of modular organisation is found in individual molar tooth cusps, shown by the enamel knot as a cusp-making module (Jernvall and Thesleff, 2000).

In the current study, the deployment of such a cusp-making module is further implied during shark tooth morphogenesis, therefore forging a putative link between two functionally homologous signaling centers separated by considerable evolutionary timespan. When considering how remodulation of the same cusp-making signals might generate different tooth shapes, some differences identified between the shark and ray provides further insights. In the shark dentition, a lack of PCNA immunoreactivity marks the enameloid knot, whereas in the ray this appears not to be the case. Furthermore, in the ray, *Shh* and *MK* both occupy comparatively expanded expression domains within the IDE, compared to the shark. Given the ray's characteristically flattened tooth cusps, this therefore provides further evidence to support remodulation of the same core signals to generate two contrasting dental phenotypes using the same cusp-making genetic toolkit. The deep conservation in elasmobranchs of the same modular GRN, and its persistence in the mammalian dentition, therefore provides a strong example to support the ability of teeth to undergo significant phenotypic modification and regional dissociation, while retaining the independent gene circuits required for their development (Stock, 2001).

When considering signaling centers as determinants of future tooth shape, it is acknowledged that the paraffin section (sagittal plane) method used here presents certain disadvantages, when compared to whole-mount methods. During mammalian molar tooth morphogenesis, the activation of secondary enamel knots is proposed to account for correct cusp shape and size, thus determining precise occlusion and overall tooth function (Jernvall and Thesleff, 2000). The shark dentition presents no such occlusion, however, elasmobranch teeth still present some degree of complexity, as defined by the tricuspid teeth of the catshark. Given the apparent activation of the enameloid knot in the prospective medial 'primary cusp', it is probable that secondary enameloid knots may then activate in the lateral 'secondary cusps', to define the overall shape of each tooth. In this respect, this approach is likely to provide limited perspective regarding the role of signaling centers in determining overall tooth shape in sharks. To further define the role of cusp-making modules in elasmobranch teeth, future studies targeting expression in additional planes, or whole-mount methods, will be required to develop a fuller picture of the gene circuits controlling their development and regeneration.

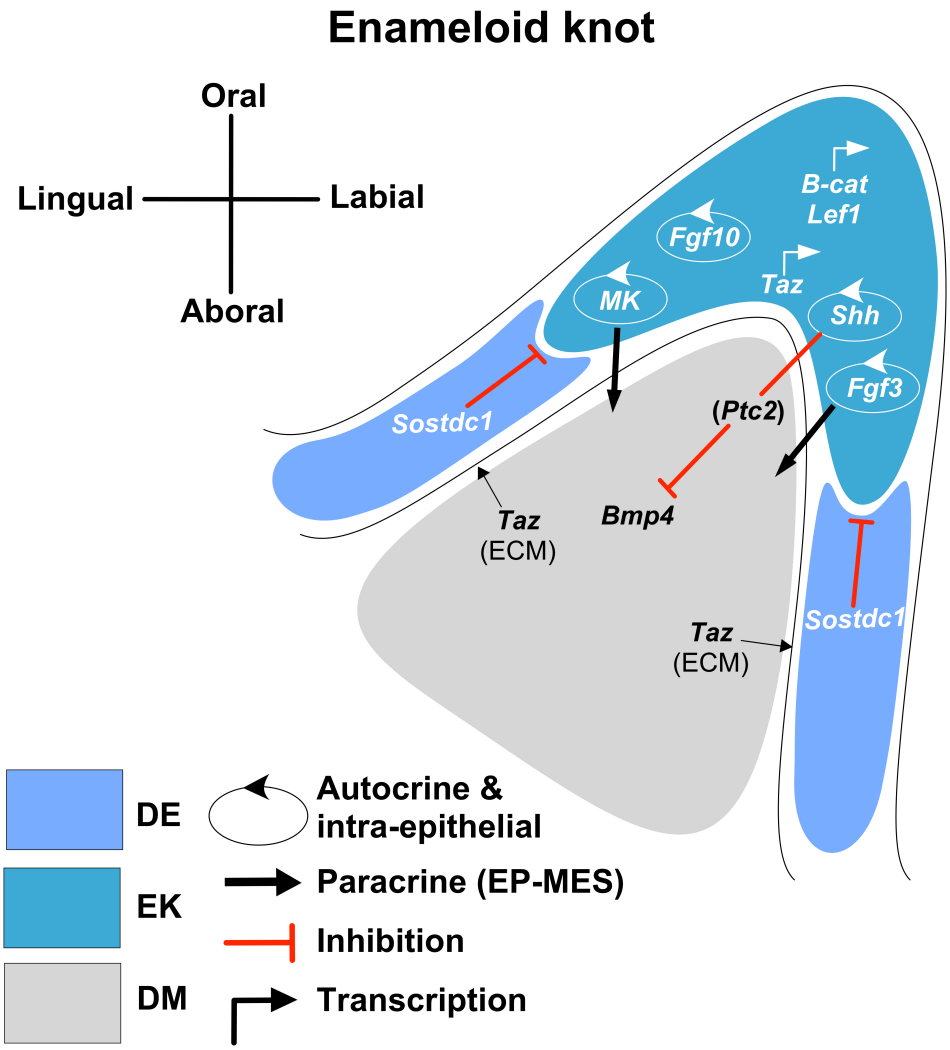


Figure 4.21 Enameloid knot model: Conserved gene expression in the epithelial tip and mesenchyme support its conservation in the elasmobranch dentition. Notable putative roles include *Shh* signaling to regulate tooth cusp shape, mesenchymal cell activity and transiently restrict *Bmp4* to the mesenchyme in advance of its apoptotic role (Vaahtokari *et al*., 1996a; Jernvall *et al.*, 1998). *Fgf3*/*Fgf10* are also proposed to hold conserved ancestral functions (Kettunen *et al*., 2000), while canonical Wnt signaling is inferred from expression of *β-catenin* and *Lef1* (Kratochwil *et al*., 1996; 2002). *MK* is proposed to regulate cell survival and proliferation (Mitsiadis *et al*., 1995b; 2008). *Taz*/ *wwtr1* is also suggested to confer a transient anti-apoptotic role, and/ or to transduce signals to surrounding cells during changes in cell-cell adhesion and movement (Wang *et al*., 2014; Liu *et al*., 2014; Dupont *et al*., 2011). The distinctive expression domains of *Sostdc1* suggest an inhibitory function to delineate the expression domains of activatory genes (Kassai *et al*., 2005). Given their similarities to the mammalian enamel knot, these expression patterns provide the first evidence to support the deployment in the elasmobranch dentition, of a signaling center conserved throughout 500 million years of gnathostome evolution. EK, enamel knot; DM, dental mesenchyme; ECM, extracellular matrix; EP-MES, epithelial-mesenchymal.

4.4.6 Gene co-expression domains regulate odontogenic cell differentiation

The gene expression patterns presented here have largely focused upon those expressed during shark tooth initiation, budding and morphogenesis. However, these processes inevitably culminate in cell differentiation and secretion of hard tissue matrices to complete the tooth in advance of function. The cellular events leading to the deposition of dental hard tissues is therefore a critical process, reliant upon controlled proliferation and differentiation of progenitors to enamel-secreting ameloblasts in the IDE and dentin-secreting odontoblasts in the DM. In the human dentition, DPSCs are of considerable interest, due to their potential to differentiate to odontoblasts, and so facilitate the development of therapeutic tissue-engineering strategies targeting dentin regeneration (Li *et al*., 2011c). Since the evidence presented here suggests tooth replacement to be driven by reiterative reactivation of the same developmental circuits, identification of associated genes regulating hard tissue formation is essential to understanding dental regeneration as a whole.

One such gene is the *Runx2* transcription factor. *Runx2* belongs to the Runt family of master transcription factors, which play essential roles in bone and tooth development (Zhao *et al*., 2007; D'Souza *et al.,* 1999). The critical developmental role of *Runx2* is shown in *Runx2* -/- mice, which lack ossified bone due to maturational arrest of osteoblasts, and in humans, where mutations cause cleidocranial dysplasia, resulting in delayed eruption of teeth, supernumerary teeth and other skeletal abnormalities (Komori*,* 2006; Mundlos *et al.,* 1997). Existing studies show *Runx2* to be expressed in both the teeth and dermal denticles of the catshark, implying an ancestral function in gnathostomes (Hecht *et al*., 2008). The expression analysis presented here further confirms this, showing *Runx2* expression in the DM during development of first generation and subsequent replacement teeth, with some evidence of associated expression in the epithelium and DL.

Given its known role, in the shark dentition *Runx2* is presumed to stimulate the differentiation of mesenchymal cells to odontoblasts during advancing morphogenesis. However, its expression with *β-catenin, fgf3, Bmp4* and *Twist* in advance of these stages provides some evidence of broader putative interactions. In mice, induced activation of *β-catenin* results in enhanced ossification, while

inactivation causes ectopic formation of chondrocytes at the expense of osteoblast differentiation (Day *et al*., 2005). During skeletal development, Wnt-*β-catenin* signaling targets mesenchymal *Runx2* to stimulate subsequent osteoblast differentiation (Gaur *et al*., 2005). In the shark dentition, *β-catenin* and *Runx2* are co-expressed in the DM during tooth budding. It is therefore possible that *β-catenin* may stimulate *Runx2* expression in advance of subsequent *Runx2*-induced cell differentiation. In the mouse dentition, *Runx2* expression in the DM is also stimulated by epithelial signals (Åberg *et al.,* 2004; D'Souza *et al.,* 1999). For example, *Runx2* functions downstream of epithelial FGF signaling, in turn regulating mesenchymally-expressed *Fgf3*. Over-expression of *Runx2* in *Runx2* -/- cells further induces *Fgf3* expression, indicating *Fgf3* to be a downstream target of *Runx2* signaling in the DM (Åberg *et al*., 2004). Since in the shark dentition, *Fgf3* is expressed both in the IDE and DM concomitant with *Runx2*, a similar putative interaction cannot be ruled out. During advanced shark tooth morphogenesis, *Bmp4* is also strongly co-expressed in the DM with *Runx2*. In mice, addition of *Bmp4* protein to mandibular tissue induces expression of early *Runx2*-dependent genes, suggesting an indirect role for *Bmp4* in stimulating subsequent *Runx2* expression (James *et al*., 2006). In the catshark dentition, a putative role involving *Bmp4*-dependent *Runx2* expression is therefore also possible.

The Distal-less (Dlx) family of homeobox transcription factors are a group of highly conserved genes with important roles in mammalian tooth development (Robinson and Mahon, 1994; reviewed by Stock *et al.,* 1996). *Dlx3* in particular is of considerable interest, due to associated mutations causing Tricho-Dento-Osseous (TDO) Syndrome in humans, which results in several dental defects, including enamel hypodysplacia (enamel deficiency) and taurodontism (enlarged pulp space) (Price *et al*., 1998). In mice, deletion of mesenchymal *Dlx3* causes major impairment of odontoblast differentiation and dentin deposition, demonstrating its essential role in regulating odontoblastic cell activity leading to matrix deposition (Duverger *et al*., 2012). The expression of several Dlx genes (including *Dlx3*) in the early catshark dentition and caudal primary denticles has also provided provisional evidence to support an ancestral function in ancient gnathostomes (Debiais-Thibaud *et al.,* 2011). Though in this study, several Dlx genes were cloned for expression analysis, due to

practical reasons it was only possible to investigate the expression of *Dlx3*. During bud stage, *Dlx3* was expressed primarily in the DM, presenting some differences with the mouse dentition in which at this stage, *Dlx3* is expressed in the enamel epithelium (Zhao *et al.,* 2000). However, during advanced tooth replacement, subsequent expansion of *Dlx3* expression domains to encompass both the DM and IDE, are more consistent with those of the mammalian dentition, further suggesting a conserved ancestral role. In terms of possible functional interactions, the overlapping expression domains of *Dlx3* and *Runx2* imply a putative relationship. In the mouse dentition, *Dlx3* positively regulates *Runx2*-mediated transcription through *Dlx3* protein-DNA interactions, while direct *Dlx3*-*Runx2* protein-protein interactions have the opposite effect (Hassan *et al.,* 2006). Given their known interactions, a similar putative conserved relationship in the catshark dentition involving *Dlx3*-mediated modulation of *Runx2*-induced odontoblast differentiation remains possible. Future studies are required to further characterise the expression patterns of Dlx genes in the shark dentition. The availability of several Dlx probes presents such an opportunity.

Twist proteins constitute an evolutionarily conserved family of basic helix-loop-helix transcription factors, which play various roles in embryonic development and disease (reviewed by Barnes and Firulli, 2009). *Twist* also plays a central role in bone development by regulating pre-osteoblastic and osteoblastic cell activity (Murray *et al.,* 1992; Rice *et al.,* 2000). *Twist* +/- mice exhibit increased bone formation and cranial sutures, and activation increases expression of mesenchymal stem cell (MSC) markers while decreasing osteogenesis, implying a role in regulating self-renewal of MSCs (El Ghouzzi *et al.,* 1997; Isenmann *et al*., 2009). This *Twist*-mediated self-renewal occurs through transient binding to the DNA-binding domain of *Runx2*, preventing transcription of target genes and inhibiting osteoblast differentiation. When released, *Runx2* is able to trigger osteoblast differentiation, marked by expression of genes associated with mineralisation (Bialek *et al.,* 2004). In hDPSCs, overexpression of *Twist* produces similar effects, suggesting a conserved partnership between *Runx2* and *Twist* in determining the differentiation of odontoblasts (Li *et al*., 2011c). In the shark dentition, mesenchymal *Twist* expression is first detected at bud stages, though it is likely that *Twist* is expressed in advance of this stage. This is evident during development of early second-generation teeth, in which *Twist* is

expressed in the basal DM underlying the tooth placode. During subsequent tooth replacement, *Twist* is expressed in the DM underlying the RT bud, becoming progressively restricted to basal, bilateral domains of the papilla, separated by the intervening, medial DM. While the specific role of *Twist* in the shark dentition is unclear, given its known role in maintaining undifferentiated SCs and its co-expression here with *Runx2*, an ancestral function in which *Twist* regulates *Runx2* in the DM through transient antagonistic interactions can be inferred.

Secreted protein acidic and rich in cysteine (*Sparc*/ *Osteonectin*) is a multifunctional mineralisation matrix protein with cell-type specific roles in proliferation, survival, morphogenesis and differentiation (Delaney and Hankenson, 2009; Bradshaw, 2009; Mason *et al.,* 1986; Motamed *et al*., 2003; Lane and Sage, 1994). *Sparc* was first described in skeletal tissue and is the most abundant non-collagenous extracellular matrix protein in bone (Kuwata *et al.,* 1985; Termine *et al*., 1984; Robey *et al*., 2006). *Sparc*-deficient mice produce a decreased number osteoblasts and osteoclasts, resulting in profound osteopenia (bone loss), including decreased bone volume and mineral density. This severe phenotype implies a critical role in positive regulation of osteoblastic cell lineage progression (Delany and Hankenson, 2009). In human teeth, *Sparc* is expressed during initial differentiation of odontoblasts and in the extracellular matrix. Due to its prominent expression in the non-mineralised pre-dentin compartment, *Sparc* is, by virtue of its own anti-adhesive properties, implied to regulate cell movement inside the extracellular compartment through macromolecular remodeling (Papagerakis *et al.,* 2002; Thomas, 1984). Furthermore, in rat molars, *Sparc* is expressed in pre-odontoblasts rather than the dentin matrix, suggesting a regulatory role during initial dentin matrix formation (Kim *et al.,* 2012).

In the shark dentition, *Sparc* is not expressed during bud stage, but strongly in the DM of the papilla during both first generation and subsequent RT advanced morphogenesis. This apparent sharp increase in stage-specific expression strongly implies a highly conserved role in stimulating odontoblastic cell activity in the DM in advance of matrix deposition. In terms of the wider *Sparc* interactions in the elasmobranch GRN, there is currently little evidence available from which to draw informed conclusions. However, recent comparison of cDNA libraries generated

from *Runx2* -/- and +/+ mouse molar organs have revealed several differentially expressed *Runx2*-specific downstream targets, including *Sparc* (Gaikwad *et al.,* 2001). Given this known relationship, the overlapping expression domains of *Runx2* and *Sparc* during advanced shark tooth morphogenesis, implies a conserved interaction involving induced dental mesenchymal odontoblastic cell differentiation. The ancestral role of *Sparc* implied by these expression patterns is further consistent with phylogenetic studies implicating *Sparc* as a core component of an ancient subcircuit of enamel/ enameloid matrix proteins involved in the early evolution of mineralised tissue (Sire *et al*., 2005; Kawasaki *et al*., 2004).

4.5 Conclusions

This study uses the catshark and ray as models to identify the deployment of conserved signaling pathways in elasmobranch tooth regeneration. These constitute members of the Wnt-*β-catenin*, hedgehog, FGF and BMP families, and additional regulatory genes with conserved roles. This is inferred through comparison of their respective expression patterns with the mammalian, reptilian and osteichthyan dentitions, thus speculating upon putative gene function. Furthermore, this study demonstrates two additional novel findings. Firstly, in association with *Sox2* expression (chapter 3), PCNA cell proliferation data and additional gene expression patterns provide further evidence to support the deployment of a dental stem cell niche in the elasmobranch dentition. Secondly, using PCNA and gene expression patterns as further comparative tools, this study identifies a putative signaling center analogous to the mammalian enamel knot. This enameloid knot is proposed to regulate shark tooth morphogenesis, as defined by the co-expression of several genes, notably *Shh*, *Fgf3*/*10*, *Bmp4* and *Midkine*, in the non-proliferative tip, therefore demonstrating its ancestral conservation as a cusp-making module. This study therefore identifies the core circuitry of a gene regulatory network conserved for 500 million years of vertebrate evolution. The identification of this dental GRN in a comparatively ancient lineage of polyphyodont gnathostomes therefore recouples tooth morphogenesis with regeneration, a capacity lost in mammals.

Chapter 5

AN ANCESTRAL GENE REGULATORY NETWORK PATTERNS DERMAL DENTICLES IN SHARKS

5.1 Summary

Teeth and dermal denticles (odontodes) are prime examples of morphological novelty (Ørvig, 1967; 1977). In elasmobranchs, teeth enable food acquisition, while denticles act as dermal body armour and enhance hydrodynamic efficiency (Reif, 1980; 1982; Oeffner and Lauder, 2012). Several theories aiming to explain their respective evolutionary origins continue to generate considerable debate (Donoghue, 2002; Huysseune *et al*., 2009), however, recent molecular data has shifted focus toward the role of GRN co-option in their evolution and development (Fraser *et al.,* 2010).

The catshark is a prominent model for investigating denticle patterning, however, current knowledge remains limited (Johanson *et al*., 2008; Debiais-Thibaud *et al*., 2011; Freitas and Cohn, 2004; Hecht *et al*., 2008; Johanson *et al*., 2007). This study addresses this gap in knowledge by studying cell proliferation and conserved gene expression in developing dermal denticles in the catshark. PCNA first shows denticles to develop through a series of stages common to teeth and other epithelial appendages. Gene expression analysis then shows the deployment of the Wnt-*β-catenin,* hedgehog, FGF, BMP pathways of signaling molecules and additional regulatory genes. During morphogenesis, co-expression of *Shh, Fgf3* and *Midkine* in the epithelial tip further supports the ancestral conservation of a signaling module analogous to the enamel knot in mammals (Vaahtokari *et al*., 1996a) and enameloid knot in sharks (chapter 4). Additional expression patterns further imply highly conserved roles, collectively inspiring the production of the first denticle gene regulatory network model.

Comparison of denticle gene expression patterns with teeth (chapter 4) and sensory receptors reveals considerable serial patterning homology. However, one key difference is marked by restriction of the stem cell marker *Sox2* (chapter 3) to the dental lamina and its absence in dermal denticle laminae. This differential expression pattern is therefore indicative of some key differences between the oral and dermal epithelia in their respective capacities to produce replacement teeth and non-replacing denticles. When considered in light of current theories of odontode evolution, these findings therefore offer partial support the ‘inside-out’ and ‘inside and out’ models (Smith and Coates, 1998; Fraser *et al*., 2010).

5.2 Introduction

5.2.1 Denticles as evolutionary-developmental models

Animals frequently find creative solutions to respond to the relentless selective pressures of survival in a competitive natural environment. This is exemplified by the periodic appearance of morphological novelty: structural elements with new adaptive function. Evolutionary novelties may prove critical in conferring the advantage required for an individual to survive, ultimately propagating throughout the population to facilitate the adaptive radiation of a given lineage (Carroll, 2005). Underpinning this phenotypic novelty are the gene regulatory networks (GRNs), which determine their development. The co-option and remodulation of conserved pathways from existing GRNs provides the driving force to generate evolutionary novelty (Peter and Davidson, 2011).

Teeth and denticles (odontodes) are prime examples of evolutionary novelty (Fraser *et al*., 2010). Both derive from the same basic hard-tissue unit, therefore sharing considerable morphological character (Ørvig, 1967; 1977). However, as a consequence of this anatomical homology, an accurate and unifying picture of the events concerning their evolution remains unclear (de Beer, 1971; Donoghue, 2002; Wagner, 1989). Several competing theories attempting to explain the evolution of oral versus dermal odontodes therefore continue to generate considerable debate (reviewed chapter 1: section 1.1.1). Denticles in particular serve several novel functions by acting as dermal body armour and enhancing swimming ability by reducing frictional drag, and optimising water flow dynamics and energy efficiency (Reif, 1978; Reif and Dinkelacker, 1982; Motta *et al*., 2012; Oeffner and Lauder, 2012). The contrasting functional roles of odontodes are illustrated in the teeth (Fig. 5.1A-B) and dermal denticles (Fig. 5.1A, C-E) of the catshark. Morphological studies of catshark scalation (squamation) have revealed at least three apparently distinct fields: the primary denticles arranged as ordered rows in the tail fin (axial lobe) subsequently lost during ontogeny (not shown here), ordered bilateral rows positioned along the body associated with the sensory canals (Fig. 5.1C, E) and those scattered across the integument with seeming irregularity (Fig. 5.1C-D) (Reif, 1980; Ballard *et al*., 1993; Johanson *et al*., 2007).

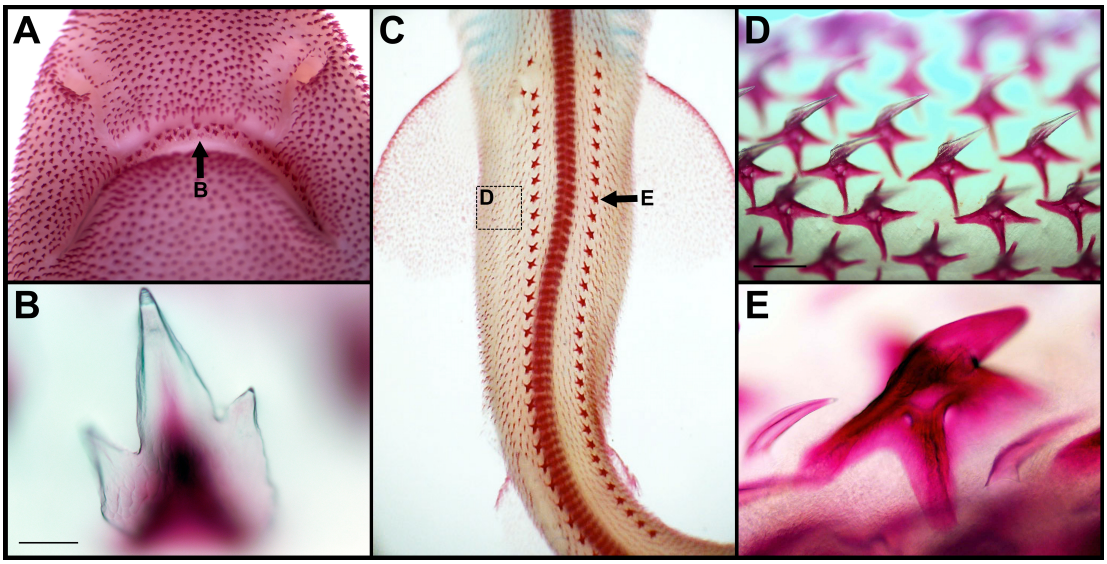


Figure 5.1 Odontogenic diversity in *Scyliorhinus canicula* (small-spotted catshark). Alizarin red staining visualises odontodes as teeth in the oral cavity (A-B) and dermal denticles throughout the integument (A, C-E). Despite their respective functional roles, teeth and denticles are structurally homologous, constructed of mineralised tissue surrounding a central pulp cavity with bone of attachment (B, E). Denticle coverage shows a degree of organisation into spatially distinct populations, such as the bilateral rows positioned along the dorsal surface (C, E), surrounded by a seemingly random scattering (C-D). Denticles further exhibit distinct polarity, orientated to enhance water flow dynamics and therefore optimising hydrodynamic efficiency (D-E). Orientation of specimens: A-B, ventral; C, dorsal; D-E, lateral. Scale bars: (A, C) unscaled, (D) 200 μm, (B, E) 100 μm.

5.2.2 The ambiguous evo-devo repertoire of denticles

Despite the presence of regularly arranged rows of denticles (Fig. 5.1C), it has previously been purported that in contrast with teeth, no common patterning mechanism controls their development (Reif, 1985). In catshark squamation, the distribution and spacing of scattered skin denticles appear to show no evidence to suggest their conversion into a patterned replacement dentition (Fraser and Smith, 2010). Denticles are not added sequentially in rows and their irregular initiation and sizing further appear to follow no distinct pattern to indicate a patterning mechanism comparable with teeth (Fraser and Smith, 2010). Developmental evidence from other chondrichthyan taxa and vertebrates, however, speaks partially to the contrary. Squamation in two species of *Heterodontus* (Port Jackson shark), and the skate (*Leucoraja erinacea*), are reported to occur in a strictly regulated caudo-rostral pattern, implying distinctly regulated patterning mechanisms (Johanson *et al*., 2007; Miyake *et al*., 1999). In the skate, while the timing of denticle initiation varies at different regions of the embryo, eruption timing is almost simultaneous, further suggesting a regulated patterning mechanism (Miyake *et al*., 1999).

Such an ancestral patterning mechanism may also be inferred from successive vertebrates. Analysis of avian feather bud development has shown primordia to originate from a primary row of feather buds along the midline, expressing molecules common to tooth development (Jung *et al*., 1998). The generation of such periodic pattern is hypothesised to result from a Turing-like mechanism in which the diffusion and reaction of chemical morphogens (activators, mediators and inhibitors) over both short and long distances, provides the inductive potential to initiate and sustain stable periodic pattern (Turing, 1952; Meinhardt, 1982; Koch and Meinhardt, 1994). On a conceptual level, this has recently been extended to denticle development (reviewed by Fraser and Smith, 2010). This "nearest neighbour" hypothesis proposes pre-existing denticles to initiate development of neighbouring primordia from localised stem-like cell populations, inducing their development through concentration gradients of activators and inhibitors (Johanson *et al*., 2008). Skin wound-healing experiments carried out on the nurse shark (*Ginglymostoma cirratum*) and the leopard shark (*Triakis semifasciata*), show denticles to develop around wound sites,

implying the requirement for neighbouring skin denticles to promote growth of those lost (Reif, 1978).

In the skate and catshark, denticles develop from individual laminae. Though some may be shed and replaced by adult denticles, others may not (Miyake *et al*., 1999; Reif, 1980). This lack of serial repetition therefore further contrasts sharply with the shark dentition, which contains multiple developing teeth initiated from within a single dental lamina (Reif, 1982; Smith *et al*., 2009a; 2009b). However, as epithelial appendages, denticles develop in much the same way as teeth, a process dependent upon sequential, reciprocal signaling interactions between the epithelium and neural crest-derived mesenchyme (Reif, 1980; Pispa and Thesleff, 2003; Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004). Studies of mammalian, reptilian and osteichthyan tooth development have shown this patterning process to be regulated by a gene regulatory network (GRN) comprising several families of conserved signaling molecules (reviewed by Thesleff and Sharpe, 1997; Kettunen and Thesleff, 1998; Järvinen *et al.,* 2006; Buchtová *et al*., 2008; Handrigan and Richman, 2010a; 2010b; Fraser *et al*., 2004; 2008; 2012; 2013; Smith *et al*., 2009a).

In contrast with osteichthyan scales, which have been modified into various types, dermal denticles have remained largely unchanged throughout their evolution and are therefore considered representative of a plesiomorphic (ancestral) state (Sire and Huysseune, 2003). Denticles therefore provide important developmental tools to gain crucial insights into the evolution of odontodes and to this end, molecular developmental studies using the catshark have proven informative. In the catshark, studies of tooth and denticle development have revealed the conserved expression of the dental patterning genes *Shh*, *Epha4*, *Runx1*/*3* and several members of the *Dlx* family of genes (Smith *et al*., 2009a; Johanson *et al*., 2008; Freitas and Cohn, 2004; Hecht *et al*., 2008; Debiais-Thibaud *et al*., 2011). These studies have therefore provided initial evidence to support the conservation of an ancestral GRN in odontode development; however, a detailed investigation of conserved gene expression in denticle development remains outstanding.

5.2.3 Aims and objectives

Given the current level of ambiguity regarding the origins of teeth and denticles and their respective patterning mechanisms, gene expression analysis continues to provide a vital investigative method to progress current understanding of their evolution and development. To address this gap in knowledge, the aims and objectives are therefore as follows:

- To use the catshark as a model to investigate the expression of genes representative of conserved signaling pathways during denticle development.

- To profile their expression patterns and compare and contrast them with those shown during shark tooth development, in order to ascertain whether both develop using a common genetic toolkit.

- To use these expression profiles both to develop a hypothetical denticle GRN model and input novel molecular developmental data into current models of odontode evolution.

5.3 Results

5.3.1 Denticles develop from a proliferating epithelium and mesenchyme

Shark skin constitutes an outer epithelium (epidermis), neural crest-derived mesenchyme (dermis) and an intermediate acellular layer (basement membrane) (Hamlett, 1999). Denticles develop as placodes from a thickened epithelium and condensing mesenchyme, bulging outward to form a bud. During morphogenesis, this extends, in-folds and polarises to form a papilla, followed by the secretion of hard tissue matrices. These common stages have previously been described using standard histological methods (Reif, 1980). However, in this study a PCNA antibody was used to investigate epithelial-mesenchymal cell proliferation during development of denticle primordia. All tissue sections shown are in sagittal plane and axes of orientation are in accordance with those shown in Fig. 5.2A.

Initiation of denticle placode primordia was marked by localised thickening of columnar epithelial cells proliferating from the basal lamina and associated condensation of the underlying mesenchyme (Fig. 5.2A). During bud stage, the thickened epithelium evaginated to form a bulge, accompanied by continued mesenchymal condensation (Fig. 5.2B). Early morphogenesis was marked by onset of growth polarity and a reduction in cell proliferation in the distal epithelial tip, shown by reduced PCNA immunoreactivity (Fig. 5.2C, dotted line). Subsequent morphogenesis was further accompanied by progressive enclosure of the proliferating mesenchymal compartment (Fig. 5.2D, dotted line). During advancing morphogenesis, growth polarisation continued, accompanied by reduced PCNA immunoreactivity in the epithelial tip, biased to the lingual aspect (Fig. 5.2E, dotted line). By advanced morphogenesis, a total reduction in PCNA immunoreactivity in both the epithelium and mesenchyme implied terminal differentiation of cells to ameloblasts and odontoblasts, respectively. Corresponding matrix deposition in the papilla was also apparent (Fig. 5.2F, arrow 1). Continued PCNA immunoreactivity at the basal lamina of the papilla (Fig. 5.2F, arrows 2-3) showed sustained cell proliferation, possibly concomitant with formation of the basal attachment plate.

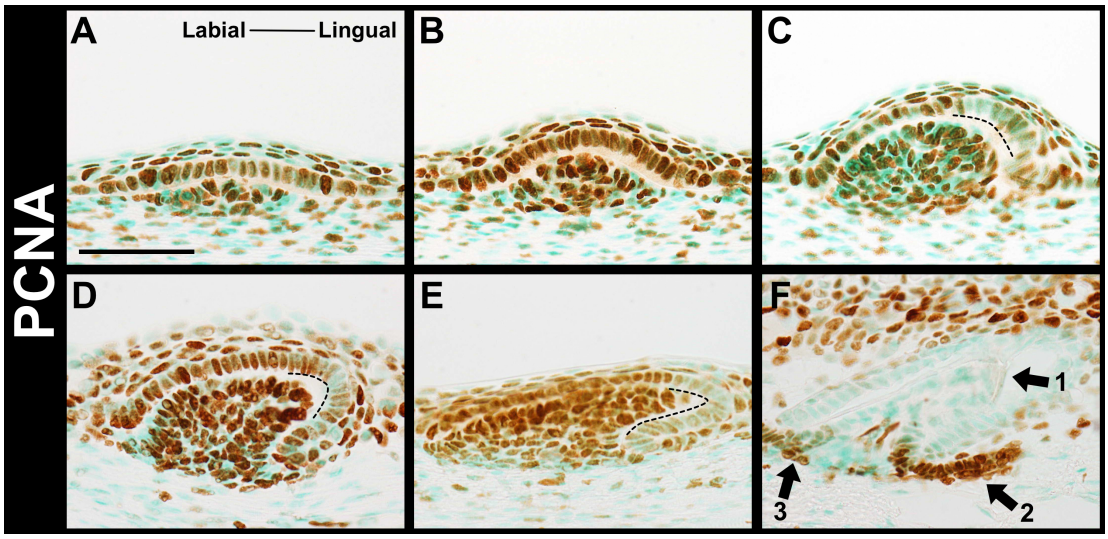


Figure 5.2 PCNA analysis of cell proliferation during denticle development. Denticle placode initiation is marked by localised epithelial-mesenchymal cell proliferation. Basal cells become columnar, thickening to form an epithelial placode, accompanied by associated condensation of the underlying mesenchyme (A). During subsequent budding, continued cell proliferation marks the evaginating epithelium and condensing mesenchyme (B). Onset of morphogenesis is marked by asymmetric growth polarity, accompanied by reduced cell proliferation in the distal epithelial tip, in contrast to the adjoining epithelium and underlying mesenchyme (C, dotted line). During mid-advanced stages of morphogenesis (D-E, respectively), this reduction in epithelial cell proliferation continues, focal to the lingual epithelium of the tip (D-E, dotted line). Following advanced morphogenesis (E), cell proliferation in both the epithelium and mesenchyme is negligible, marked by a total lack of PCNA immunoreactivity (F). Signs of secreted mineralised tissue in the papilla (F, arrow 1), implies terminal differentiation to ameloblasts and odontoblasts. However, residual PCNA immunoreactivity, restricted to the basal lamina, further implies continued proliferation, possibly in connection with formation of the basal attachment plate (F, arrows 2-3). Scale bar: (A-F) 100 μm.

5.3.2 Conserved genes and pathways are deployed in denticle development

To explore the potential deployment of a genetic toolkit common to teeth and denticles, the expression patterns of genes representative of several conserved signaling pathways and families were investigated during denticle development using *in situ* hybridisation. The Wnt gene *β-catenin* is required for initiation and morphogenesis of the hair follicle, feather bud and for the normal development of teeth (Huelsken *et al.,* 2001; Noramly *et al.,* 1999; Järvinen *et al.,* 2006). During denticle development, intense *β-catenin* expression marked individual placodes, restricted to the basal epithelium of each placode-forming unit, often in advance of any obvious signs of corresponding cellular morphological change (Fig. 5.3A). Throughout subsequent stages of bud formation, this epithelial expression was sustained, further spreading to the underlying mesenchyme (Fig. 5.3B-C). During morphogenesis, mesenchymal expression was maintained, while in the epithelium, showing signs of progressive restriction to the labial aspect, becoming absent in both the distal tip and lingual aspect (Fig. 5.3D, arrow, DAPI). During advanced morphogenesis, *β-catenin* expression was completely absent throughout the epithelium, restricted to the basal mesenchyme of the papilla (Fig. 5.3E, arrow, DAPI).

During activation of Wnt signaling, nuclear *β-catenin* activates target genes by binding with *lymphoid enhancing factor 1* (*Lef1*), which is also expressed during tooth and feather development (reviewed by Seidensticker and Behrens, 2000; van Genderen *et al*., 1994; Kratochwil *et al*., 1996). During development of denticle primordia, *Lef1* was initially expressed in a similar pattern to *β-catenin*, marking individual placodes by expression in the basal epithelium (Fig. 5.3F). However, unlike *β-catenin,* during subsequent budding, *Lef1* remained restricted to the epithelium, showing no signs of mesenchymal expression (Fig. 5.3G-H). During morphogenesis, however, *Lef1* was differentially expressed in the epithelium, restricted to the labial aspect and distal tip, while absent in the lingual aspect (Fig. 5.3I, arrow, DAPI). By advanced morphogenesis, expression was restricted primarily to two bilateral regions of the basal mesenchyme adjacent to the papilla (Fig. 5.3J, arrows, DAPI).

The secreted sclerostin domain-containing protein 1 (*Sostdc1*, *Ectodin, Wise*) interacts with BMP, Wnt, FGF and hedgehog signaling to regulate the spatial patterning and morphogenesis of teeth and development of other epithelial appendages, such as the feather bud and hair vibrissae (Munne *et al*., 2009; Ahn *et al.,* 2010; Cho *et al*., 2011; Kassai *et al.,* 2005; Mou *et al*., 2011; Närhi *et al*., 2008; 2012). During denticle placode formation, *Sostdc1* was expressed in the basal epithelium of the placode-forming unit (Fig. 5.3K). However, during early budding, expression appeared to shift bilaterally to the peripheral epithelium, leaving a negative medial region (Fig. 5.3L, arrow). Throughout subsequent bud formation, this central negative region expanded, with expression increasingly restricted to the outer periphery of the denticle bud (Fig. 5.3M). During morphogenesis, *Sostdc1* expression was predominantly restricted to the lingual in-fold of the epithelium directly underlying the polarised tip (Fig. 5.3N, arrow), following which, during advanced morphogenesis, expression shifted to the opposing labial epithelium (Fig. 5.3O, arrow, DAPI).

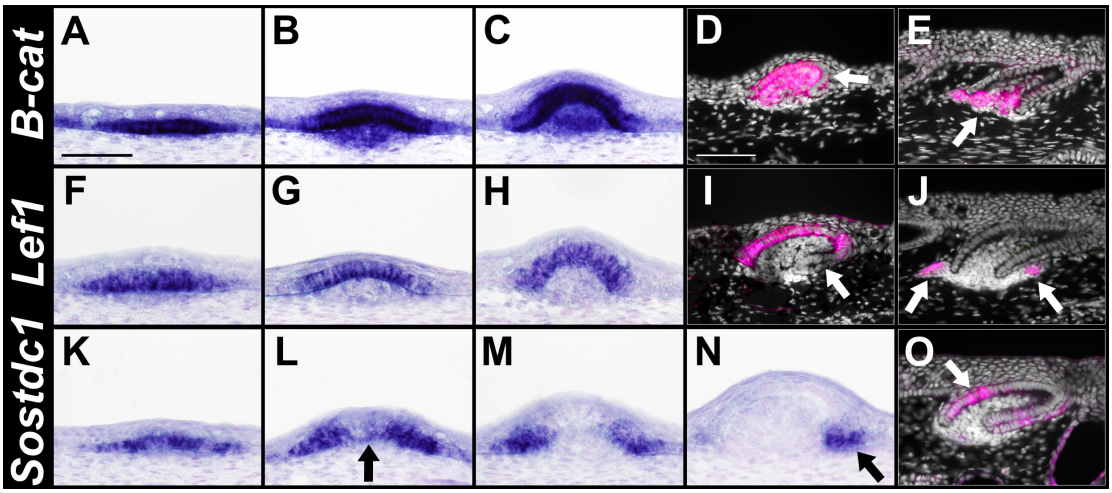


Figure 5.3 Expression of *β-catenin, Lef1* and *Sostdc1* during denticle development. Intense *β-catenin* expression in restricted regions of the basal epithelium marks denticle placode initiation (A), continuing throughout subsequent stages of budding, while further spreading to the underlying condensing mesenchyme (B-C). During morphogenesis, *β-catenin* expression is maintained in the mesenchymal compartment, while becoming progressively restricted to the labial epithelium and absent in tip and lingual aspects (D, arrow, DAPI). By advanced morphogenesis, expression is absent in the epithelium, restricted the basal mesenchyme of the papilla (E, arrow, DAPI). During placode initiation, *Lef1* is co-expressed in the basal epithelium (F) and maintained throughout subsequent budding (G-H). However, during morphogenesis, epithelial expression becomes restricted to the labial epithelium and tip, and absent in the lingual aspect, in a similar pattern to *β-catenin* (I, arrow DAPI). During advanced morphogenesis, epithelial expression is absent, restricted to bilateral regions of the basal mesenchyme adjacent to the papilla (J, arrows, DAPI). Initiation of denticle placodes is further marked by, *Sostdc1*, also co-expressed in the basal epithelium of each placode-forming unit (K). However, during subsequent budding, expression becomes progressively restricted to the peripheral epithelium of the bud, leaving a *Sostdc1*-negative medial region (L, arrow, M). During morphogenesis, expression is predominantly restricted to the lingual in-fold of the epithelium, directly underlying the tip (N, arrow), while during advanced morphogenesis (O, arrow, DAPI) this shifts to the opposing labial epithelium. Scale bars: (A-O) 100 μm.

Shh is expressed during tooth development and other epithelial appendages, such as scales and the feather bud (Bitgood and McMahon, 1995; Vaahtokari e*t al.*, 1996a; Buchtová *et al*., 2008; Fraser *et al*., 2004; 2008; Smith *et al*., 2009a; Jackman *et al*., 2010; Sire and Akimenko, 2004; Ting-Berreth and Chuong, 1996; St-Jacques *et al*., 1998). *Shh* expression has also been localised to the early developing tail denticles of the catshark, albeit it in superficial detail (Johanson *et al*., 2008). During denticle development, *Shh* was not detected the first epithelial placodes, subsequently expressing weakly in the medial epithelium of the early bud (Fig. 5.4A, arrow). During advanced budding, expression was up-regulated in the same restricted epithelium, also showing initial asymmetric bias toward to lingual aspect (Fig. 5.4B). By morphogenesis, expression continued to up-regulate, localising to the polarised distal epithelial tip and spreading bilaterally (Fig. 5.4C-D). During subsequent advanced morphogenesis, expression spread to further encompass the epithelial tip and lateral aspects (Fig. 5.4E-F, DAPI). *Shh* signals to target cells via its receptor, *Ptc2* (reviewed by Ingham and McMahon, 2001). In this study, it was not possible to investigate the full extent of *Ptc2* expression, limited here to advanced morphogenesis showing expression within the basal mesenchyme of the papilla (Fig. 5.4G-H, DAPI). It will therefore be necessary to further investigate *Ptc2* expression in greater depth to assess the broader extent of hedgehog signaling in denticle patterning.

Of the FGF family of signaling molecules, *Fgf3* is highly conserved and expressed during tooth, hair follicle and feather bud development (Bei and Maas, 1998; Kettunen *et al*., 2000; Jackman *et al.,* 2004; Fraser *et al*., 2013; Rosenquist and Martin, 1996; Mandler and Neubüser, 2004). During denticle development, *Fgf3* expression was first detected weakly at late placode stage in the medial mesenchyme underlying the epithelial basement membrane (Fig. 5.4I, arrow). During the early transition to bud stage, mesenchymal expression spread outward, accompanied by a marked increase focal to an asymmetric region of the epithelial basement membrane (Fig. 5.4J). Throughout subsequent morphogenesis, this asymmetric epithelial-mesenchymal expression pattern progressively increased, biased to the polarised region of the tip (Fig. 5.4K-L). However, by advanced morphogenesis, *Fgf3* was predominantly localised to the distal epithelial tip (Fig. 5.4M, arrow), with some

residual mesenchymal expression. The apparent dynamic shift in *Fgf3* expression domains between polarised regions of the epithelium and mesenchyme is further illustrated in Fig. 5.4N and 5.4O (DAPI), during bud stage and morphogenesis, respectively. During subsequent advanced morphogenesis, *Fgf3* showed a further shift in expression, localising primarily to the mesenchymal compartment of the papilla (Fig. 5.4P, DAPI).

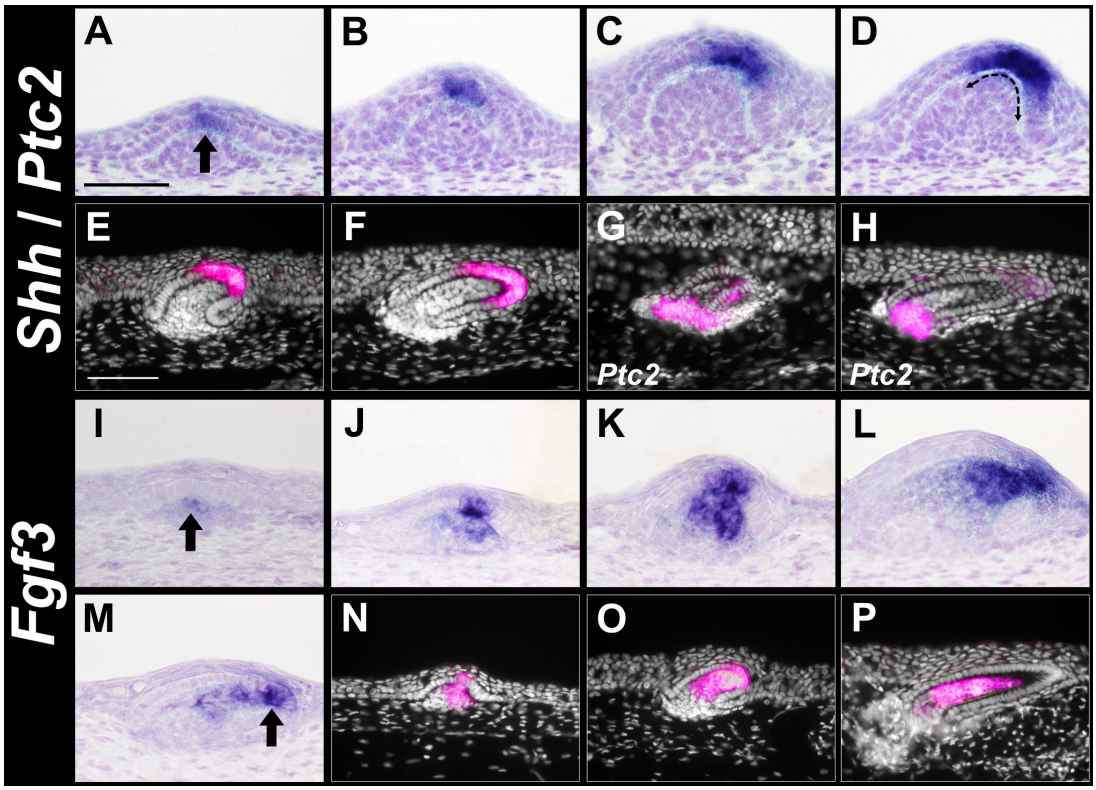


Figure 5.4 Expression of *Shh, Ptc2* and *Fgf3* during denticle development. *Shh* is not expressed during denticle placode initiation. Expression is first detected in the medial epithelium of the early bud (A, arrow), progressively increasing during subsequent budding and showing some lingual bias (B). During early-mid morphogenesis (C-D) and advanced morphogenesis (E-F, DAPI), *Shh* expression intensifies in the distal aspect of the polarised epithelial tip, progressively spreading bilaterally (D, dotted line). In this study, it was not possible to investigate the full extent of *Ptc2* expression, however, during mid-advanced morphogenesis, *Ptc2* is predominantly expressed in the basal mesenchyme directly underlying the papilla (G-H, DAPI). During early budding, *Fgf3* is first weakly expressed in the medial mesenchyme directly underlying the epithelial basal membrane (I, arrow), progressively spreading into the mesenchyme, while becoming restricted to an asymmetric region of the epithelium (J). During subsequent stages of morphogenesis, expression continues to spread, further localising to the polarised distal epithelial tip and associated mesenchyme (K-L). By advanced morphogenesis, expression is almost entirely focal to the epithelial tip (M, arrow). These dynamic epithelial-mesenchymal expression patterns are further shown using DAPI at bud stage (N), morphogenesis (O) and later stages of advanced morphogenesis, during which expression localises to the mesenchymal compartment of the papilla (P). Scale bars: (A-P) 100 μm.

The dynamic expression of *Fgf3* provides evidence to support a conserved role in denticle patterning through epithelial-mesenchymal interactions. However, there are likely to be additional genes expressed to regulate these early inductive events. The heparin-binding growth factor *Midkine* (*MK*) regulates various aspects of cell growth and differentiation and is expressed throughout tooth initiation, morphogenesis, differentiation and renewal (Muramatsu, 1993; Mitsiadis *et al.,* 1995b; 2008). During denticle development, *MK* was first expressed in the thickened epithelium of denticle placodes, with some initial expression in the underlying mesenchyme (Fig. 5.5A). During bud formation, mesenchymal expression was up-regulated (Fig. 5.5B). However, during morphogenesis, mesenchymal expression was further accompanied by an apparent up-regulation in the distal epithelial tip (Fig. 5.5C, arrow). Subsequent morphogenesis was marked by continuance of mesenchymal expression and associated up-regulation in the polarised region of the epithelial tip (Fig. 5.5D). By advanced morphogenesis, expression became restricted to the mesenchyme of the papilla, and absent in the epithelium (Fig. 5.5E, DAPI).

Bone morphogenetic proteins (BMPs) also regulate various aspects of tooth, feather and hair follicle development by mediating epithelial-mesenchymal interactions (Vainio *et al.,* 1993; Åberg *et al.*, 1997; Noramly and Morgan, 1998; Mou *et al*., 2006). *Bmp4* in particular is highly conserved in the formation of epithelial appendages, expressed in tooth, feather and limb bud development (Åberg *et al.,* 1997; Vainio *et al.,* 1993; Jung *et al*., 1998; Niswander and Martin, 1993). During denticle development, *Bmp4* was first expressed weakly during placode formation, restricted to the condensing mesenchyme and absent in the epithelium (Fig. 5.5F). During early bud development, expression in the mesenchymal compartment was up-regulated (Fig. 5.5G). In subsequently developing buds, this was accompanied by superficial signs of expression associated with the epithelium (Fig. 5.5H, arrow). *Bmp4* expression during initial morphogenesis was not profiled here, however, during advanced morphogenesis, expression was further restricted to the mesenchymal compartment of the papilla (Fig. 5.5I-J). In the absence of a complete developmental series, the full extent and therefore putative role of *Bmp4* in denticle development cannot be assessed here warranting further expression studies.

Of the Runt-related (Runx) family of transcription factors, *Runx2* regulates bone calcification by controlling the proliferation and differentiation of cells committed to osteoblastic lineages (reviewed by Camilleri and McDonald, 2006). The deeply conserved odontogenic role of *Runx2* has previously been shown by expression in the teeth and denticles of the catshark, implying its co-option from a common developmental module to allow the evolution of odontodes (Hecht *et al.,* 2008). During denticle development, the role of *Runx2* was further investigated, with expression first detected in the mesenchyme underlying the early denticle placode in advance of any apparent signs of epithelial thickening or mesenchymal condensation (Fig. 5.5K). During subsequent placode formation, this mesenchymal expression was maintained (Fig. 5.5L), however, during bud formation, expression spread from the mesenchymal compartment into the directly overlying medial epithelium (Figs. 5.5M-N, arrows). *Runx2* expression during early stages of morphogenesis was not profiled here, however, during advanced morphogenesis, expression became further restricted to the basal mesenchyme of the advancing papilla (Fig. 5.5O). As with *Bmp4*, future studies of *Runx2* gene expression will be required to produce a full developmental series. During bone development, the *Twist* transcription factor also regulates osteoblastic cell activity (Murray *et al*., 1992; Rice *et al*., 2000). Here, *Twist* co-expression was also detected in the mesenchyme in advance of any apparent signs of placode formation (Fig. 5.5P), subsequently further co-localising with *Runx2* throughout placode and bud stages (Fig. 5.5Q-R, respectively). During morphogenesis, however, *Twist* showed signs of down-regulation and restriction to the labial aspect of the mesenchyme, leaving an apparent negative region of lingual mesenchyme (Fig. 5.5S, arrow). By advanced morphogenesis, *Twist* became progressively restricted to the bilateral periphery of the basal mesenchyme of the papilla (Fig. 5.5T, arrow).

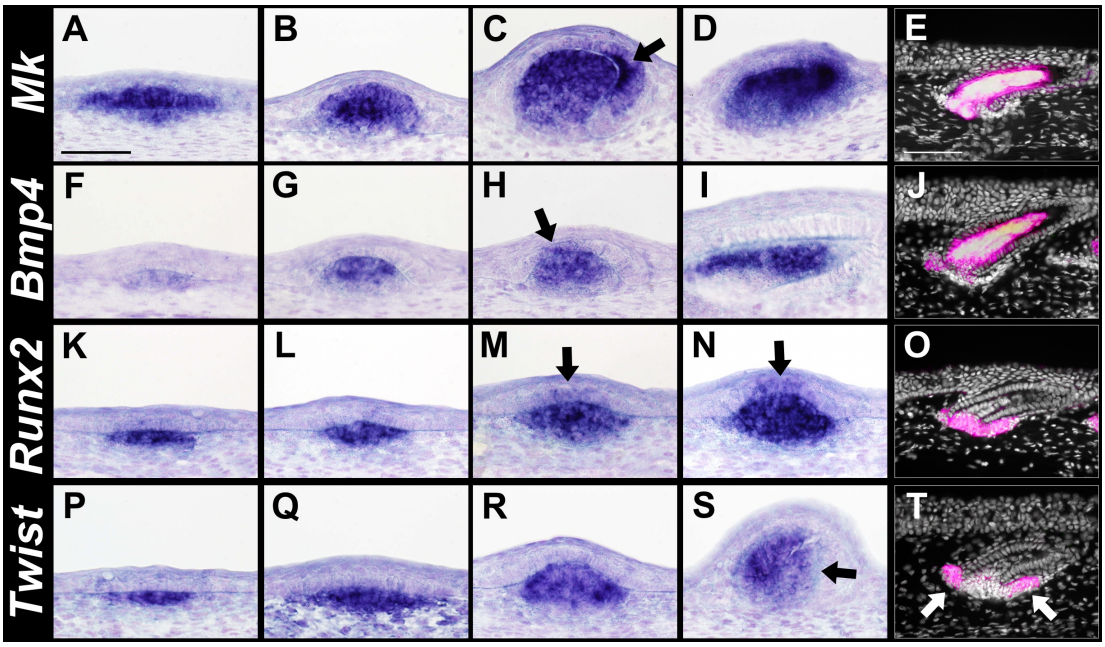


Figure 5.5 Expression of *Midkine, Bmp4, Runx2* and *Twist* during denticle development. *Midkine* (*MK*), is expressed in the epithelium and underlying mesenchyme, during both placode (A) and bud stages (B). During morphogenesis, mesenchymal expression is accompanied by a shift in epithelial expression to a restricted region of the polarised tip (C, arrow). Expression becomes further polarised during advancing morphogenesis, increasingly localised to the lingual mesenchyme, while continuing to spread throughout the epithelial tip (D). However, during advanced morphogenesis, epithelial expression is absent, restricted to the mesenchyme of the papilla (E, DAPI). During placode stage, *Bmp4* is first weakly expressed in the mesenchyme (F), increasing during subsequent budding (G), accompanied by some weak expression in the epithelium (H, arrow). Due to a lack of staging, expression during onset of morphogenesis is not shown here, however, during advanced morphogenesis, *Bmp4* is continually expressed in the mesenchyme of the papilla, and absent in the epithelium (I) and (J, DAPI). *Runx2* is expressed in the mesenchyme during early placode stages (K-L). During subsequent budding, expression remains predominantly restricted to the mesenchyme, with some signs of weak expression in the medial epithelium (M-N, arrows). Due to a lack of staging, expression during onset of morphogenesis is also not shown here, however, during advanced morphogenesis, *Runx2* is expressed in the basal mesenchyme directly underlying the papilla (O, DAPI). *Twis*t is expressed in the mesenchyme at early placode stages (P-Q), remaining restricted to the mesenchyme during subsequent budding (R). However, during morphogenesis, expression becomes restricted to the labial mesenchyme (S, arrow), and during advanced morphogenesis, to the lingual and labial basal mesenchyme, adjacent to the papilla (T, arrows, DAPI). Scale bars: (A-T) 100 μm.

5.3.3 cell proliferation and gene expression patterns during sensory receptor development

To draw comparisons between the development of denticles and other epithelial elements, such as sensory receptors, cell proliferation and gene expression patterns during their development were recorded using a representative set of stages. In accordance with teeth and dermal denticles, elasmobranch taste buds also develop from a proliferating epithelium, shown by PCNA (Fig. 5.6A-C). During initiation of taste bud primordia, thickened columnar cells marked the competent epithelium, forming an early bud (Fig. 5.6A). During subsequent bud formation, the proliferating epithelium evaginated outward with comparatively limited condensation of the underlying mesenchyme (Fig. 5.6B). During subsequent morphogenesis, a marked reduction in cell proliferation was observed in the basal lamina, as implied by reduced PCNA immunoreactivity (Fig. 5.6C, arrow). At this stage, *Shh* expression was observed in an asymmetric pattern in similar cell loci (Fig. 5.6D, arrow). During preceding stages, *Sostdc1* was further expressed, notably in bilateral domains in the periphery of the basal epithelium in similar patterns to denticles (Fig. 5.6E, arrows). During various stages, additional genes, including *Sox2*, *β-catenin, Runx2* and *Foxq1* were expressed, predominantly in the epithelium (Fig. 5.6F-I, respectively). *β-catenin* and *Midkine* were also expressed during the development of pit organs (Fig. 5.6J-K), while *Sox2* in sensory receptor cells within developing Ampullae of Lorenzini (Fig. 5.6L).

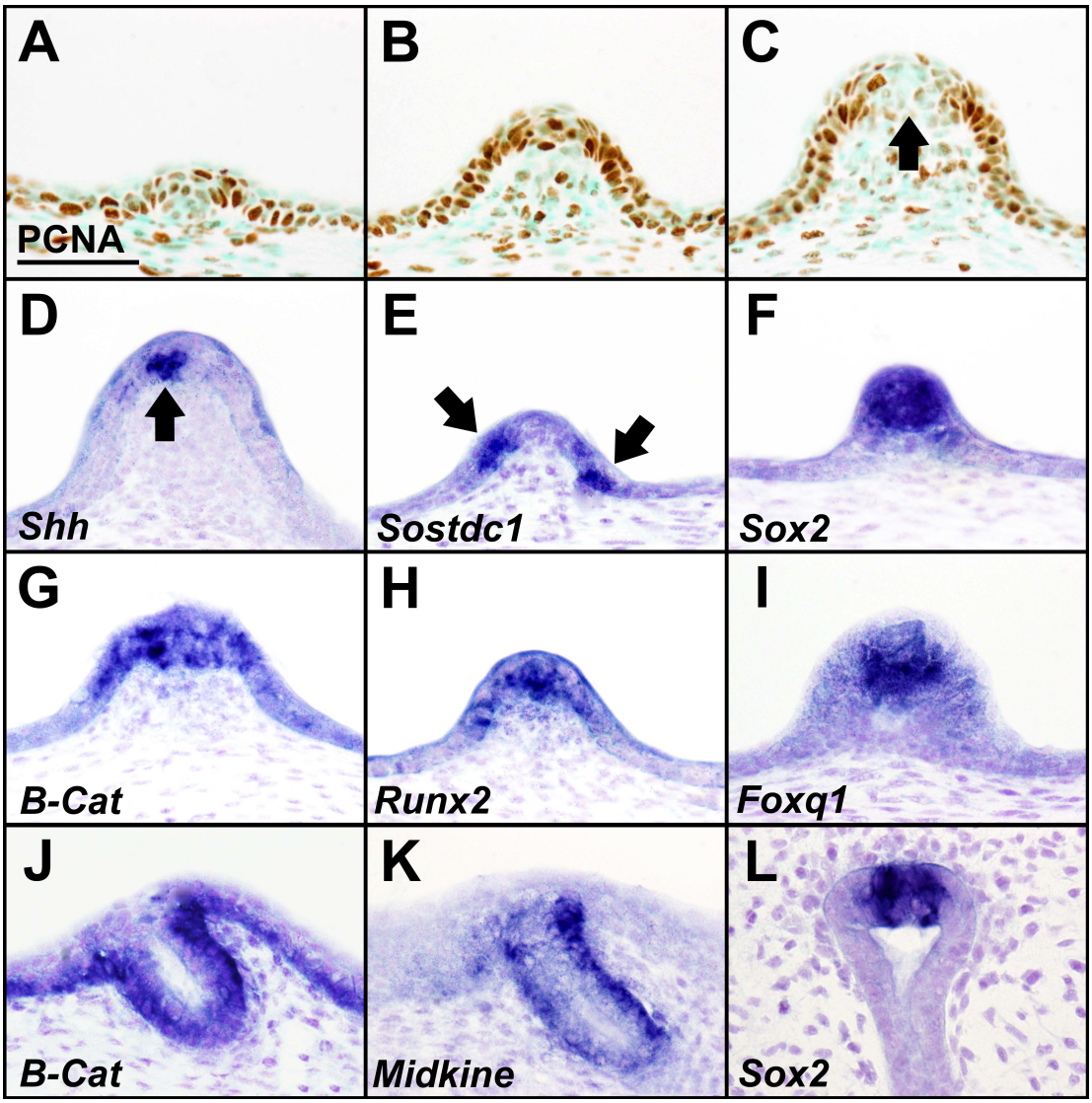


Figure 5.6 Cell proliferation and conserved gene expression in sensory receptors in the catshark. PCNA shows taste bud papillae arise through controlled proliferation of the epithelium, proceeding through early (A) to mid (B) bud stages, followed by morphogenesis, marked by cessation of cell proliferation in the basal lamina of the epithelial compartment (C, arrow). At similar stages, *Shh* is expressed asymmetrically at the basal lamina (D, arrow). At preceding stages, *Sostdc1* is further expressed bilaterally in the periphery of early taste bud papillae (E, arrows). Additional genes, including *Sox2*, *β-catenin Runx2* and *Foxq1* (F-I, respectively) are epithelially expressed at various stages of taste bud development, while *β-catenin* (J) and *Midkine*, (K) are expressed in developing pit organs, and *Sox2* (L) in the sensory cells of Ampullae of Lorenzini. It is noted that *Foxq1* expression during denticle development has been omitted, due to limitations in staging. Scale bar: (A-L) 100 μm.

5.4 Discussion

5.4.1 Comparative gene expression reveals a deeply conserved core GRN

Denticles develop through a series of initial stages common to other epithelial appendages, as illustrated here by PCNA, while gene expression identifies the activation of common developmental pathways, most notably Wnt-*β-catenin,* hedgehog, FGF and BMP signaling, to regulate development. While hypothetical, the expression domains of these genes imply a deeply conserved serial patterning mechanism common to odontodes and other epithelial appendages (reviewed by Biggs and Mikkola, 2014). Here, comparison of their respective expression patterns with those of other gnathostomes provides the basis for some informed speculation regarding their putative developmental roles.

In accordance with their known roles, *β-catenin* and *Lef1* are presumed to function as activators, inducing placode formation through induced cell proliferation as shown by the overlapping patterns of PCNA. This is supported by existing studies of *β-catenin,* which when stabilised produces supernumerary teeth in mice and when forcibly expressed, ectopic rudimentary tooth germs in chicks (Järvinen *et al.,* 2006; Harris *et al*., 2006). Similarly, forced activation of *β-catenin* in chicks, induces ectopic feather bud formation, further suggesting a conserved role in the initiation and development of epithelial placodes (Noramly *et al*., 1999). *β-catenin*-mediated tooth formation is further reliant upon interaction with *Lef1*, shown by inhibition of tooth and hair morphogenesis in *Lef1*-deficient mice (van Genderen *et al.,* 1994). In snake dental explant cultures, overactive *β-catenin* also induces ectopic tooth formation, accompanied by the expansion of *Lef1* expression domains (Gaete and Tucker, 2013).

PCNA and Wnt gene expression patterns present further evidence to support a regulatory role in promoting growth polarisation. During initial morphogenesis, as defined by onset of growth polarity, sustained PCNA immunoreactivity in the labial/dorsal epithelium overlaps with *β-catenin* and *Lef1* expression. Conversely, all show a corresponding reduction in the lingual aspect of the epithelium, implying a role in promoting local changes in growth. In the developing feather bud, highly

localised changes in gene expression are proposed to account the cellular rearrangements and cell polarity required to induce its self-guidance and growth extension (Li *et al*., 2012). During denticle morphogenesis, *β-catenin* is expressed in similar domains to the feather bud, therefore highlighting one possible conserved mechanism by which locally-induced cell proliferation promotes extension of the denticle primordium to confer growth polarity and the acquisition of correct physical form (Li *et al*., 2012). During subsequent advanced morphogenesis, PCNA further marks sustained cell proliferation in the basal papilla accompanied by *β-catenin* and *Lef1* expression, presumably in advance of cell differentiation and secretion of mineralised tissue to complete the basal plate.

However, the periodic patterning of integumentary elements is reliant upon a controlled balance of activators and inhibitors. In avians, the organisation of feathers into ordered arrays results from such a balance, with positive activatory signals inducing placode formation and inhibitory signals responding to delineate placodes as a reaction-diffusion mechanism. These positional cues culminate in well-defined feather tracts, termed macropattern, with the small-scale spacing between individual feathers termed micropattern (Mou *et al.,* 2011). In the catshark, two discrete denticle arrays arranged as the dorsal bilateral rows (Fig. 5.1C) and those of the tail fin (axial lobe), provide some evidence to support the deep conservation in ancestral vertebrates of a macropatterning mechanism, potentially similar in character to that of avians. This is especially apparent when comparing these well-ordered arrays with the seemingly random coverage of denticles developed during subsequent stages of ontogeny (Johanson *et al.,* 2007; Fraser and Smith, 2010).

Part of Reif's odontode regulation theory proposes the ordered distribution of skeletal elements to result from spatial and temporal mechanisms to prevent new elements from developing in excessive proximity to existing ones. Implementation of these zones of inhibition is therefore proposed to account for the observed spacing of developing teeth and denticles (Reif, 1982). In the context of denticle squamation, consideration of *β-catenin* and *Lef1* as positive activators leads to the assumption that inhibitory genes, such as *Sostdc1*, may be responding, so as to regulate stable micropattern. *Sostdc1* spatially restricts the inductive capacity of teeth through

inhibition of BMP and Wnt signaling, and regulates *Shh* through *Wnt-Shh-Sostdc1* feedback loops in which each acts as activator, mediator and inhibitor, respectively (Munne *et al.,* 2009; Cho *et al.,* 2011). During development of the mammary gland and hair follicle, *Sostdc1* also restricts the size and number of developing mammary buds and hair placodes through attenuation of Wnt-*β-catenin* signaling (Närhi *et al.,* 2012). Given the known role of *Sostdc1* in regulating the development of epithelial appendages through antagonistic interactions, the expression patterns shown here are indicative of a zone of inhibition, established to restrict the outward expansion of localised territories established by activatory genes, such as *β-catenin* and *Lef1*. In avians, induced *β-catenin* results in formation of ectopic feather buds and interestingly, during feather placode development, *Sostdc1* is expressed at the periphery and absent from the medial region in a similar pattern to that observed in developing denticles (Noramly *et al.,* 1999; Mou *et al.,* 2011). This is further evident during morphogenesis, where in the lingual aspect of the epithelium *Sostdc1* shows some signs of demarcating *β-catenin* and *Lef1* to the labial epithelium, potentially to inhibit cell proliferation, as shown by a lack of associated PCNA immunoreactivity. While the respective boundaries set by *β-catenin/ Lef1* and *Sostdc1* are not precisely defined, it can be inferred here that in accordance with its known role, *Sostdc1* may be acting as an inhibitor to restrict the expression of these activatory genes to maintain stable micropattern.

In the early developing mouse mandible, Wnt-induced *Shh* subsequently inhibits Wnt-*β-catenin* signaling through controlled feedback loops (Dassule and McMahon, 1998). *Shh* is further expressed in the epithelial tip during morphogenesis, while conditional deletion disrupts molar tooth morphology (Vaahtokari *et al*., 1996a; Dassule and McMahon, 1998). A highly conserved role for *Shh* in determining the growth polarity of epithelial appendages is made further apparent by related studies of feather and hair follicle development (Ting-Berreth and Chuong, 1996; Bitgood and McMahon, 1995; St-Jacques *et al*., 1998). During normal feather development, *Shh* is first expressed at placode and bud stage, followed by its progressive localisation to the posterior epithelium of the lengthening feather bud. However, exogenous overexpression of *Shh* produces abnormally large feather buds lacking in anterior-posterior growth polarity or showing reverse orientation (Ting-Berreth and

Chuong, 1996). During mouse hair follicle development, *Shh* is further expressed in the polarised epithelial tip (Bitgood and McMahon, 1995), while *Shh* -/- mutants undergo abnormal morphogenesis, marked by reduced numbers of anlagens and growth arrest prior to formation of the dermal papilla and epidermal ingrowth (St-Jacques *et al*., 1998). This conserved role is further consolidated by expression studies of zebrafish squamation in which *Shh* is expressed in developing scales, progressively localising to the posterior region of the epithelial fold (Sire and Akimenko, 2004). In denticle development, the near identical expression patterns of *Shh* therefore suggests an even deeper level of conservation. Given its conserved role as a polarising factor, it is inferred that *Shh* may be acting in accordance with this through short-range autocrine signaling to cells nested within the distal epithelial tip, so as to ensure correct orientation in advance of function (Ting-Berreth and Chuong, 1996; Bitgood and McMahon, 1995; St-Jacques *et al*., 1998; Oeffner and Lauder, 2012). Interestingly, an ancestral role for *Shh* specific to denticle morphogenesis is further implied by its apparent lack of expression during placode stage, which correlates with similar observations made during zebrafish scale and mouse hair follicle development, both of which suggest no corresponding inductive requirement (Sire and Akimenko, 2004; St-Jacques *et al*., 1998).

In the absence of a complete developmental series, the broader role of hedgehog signaling in denticle development, as implied by mesenchymally-expressed *Ptc2*, cannot be assessed. However, its expression in the basal papilla during advanced morphogenesis implies a further role for hedgehog signaling in regulating mesenchymal cell activity. Given a corresponding lack of *Shh* expression, it therefore remains to be seen whether hedgehog signaling may be further operating through other as yet unidentified ligands and receptors. This therefore warrants future expression studies targeting additional hedgehog ligands, such as indian hedgehog, which plays an important role in skeletal morphogenesis (St-Jacques *et al*., 1999).

In the mouse dentition, *Fgf3* is dependent upon Wnt-*β-catenin* signaling to in turn induce expression of *Shh* (Kratochwil *et al*., 2002). In the incisor, mesenchymally-expressed *Fgf3* also inhibits *β-catenin* to regulate odontogenic cell fates (Liu *et al*., 2013a). In denticle development, the expression of *β-catenin* upstream of *Shh* and

Fgf3 implies a similar inductive sequence for Wnt-dependent expression of these markers. During early denticle budding, *Fgf3* is first expressed in the medial region of the mesenchyme directly underlying the epithelial basement membrane. Shortly thereafter, expression spreads asymmetrically throughout the epithelium and mesenchyme from this basement membrane, concomitant with the appearance of epithelially co-expressed *Shh*. While it is not possible to confirm the sequential inductive events underlying these expression patterns, it can be inferred that *Fgf3* may be acting in accordance with its known role in inducing *Shh* expression through transfer of this odontogenic potential to the epithelium. Furthermore, given the known role of *Fgf3* in inhibiting *β-catenin,* expression in the distal epithelial tip may be to restrict *β-catenin* during tooth morphogenesis, as implied by associated restriction of *β-catenin* expression to the labial epithelium. The dynamic expression of *Fgf*3 between the epithelial and mesenchyme is also indicative of a role in mediating reciprocal signaling interactions, while subsequent restriction to the mesenchyme of the papilla during advanced morphogenesis implies a role in regulating activity of mesenchymal cells fated to differentiate to odontoblasts. Here, *Fgf*3 may be further acting to restrict *β-catenin*-induced cell proliferation in the mesenchymal papilla during differentiation of odontoblasts, as suggested by expression of *β-catenin* in the basal mesenchyme and associated absence of *Fgf3* at the same stage (Liu *et al*., 2013a).

MK is expressed dynamically between the thickened epithelium and mesenchyme of early teeth and hair follicles to control early inductive events by regulating epithelial-mesenchymal interactions (Mitsiadis *et al.,* 1995b). In accordance with its known role, the expression patterns here present some tentative evidence of deep odontogenic conservation. In the mouse dentition, *MK* is initially expressed in the presumptive epithelium during early tooth induction, subsequently signaling to the mesenchyme, which in turn signals back to the epithelium, suggesting sequential reciprocal signaling interactions. Progressive restriction to the dental mesenchyme and subsequent down-regulation concomitant with timing of odontoblast terminal differentiation further suggests a role in regulating cell differentiation (Kadomatsu *et al.,* 1990; Mitsiadis *et al.,* 1995b). These expression patterns closely follow those observed in denticle development, in which early *MK* expression in the presumptive

epithelium during placode initiation implies an inductive role, followed by expression in the mesenchyme, which apparently signals back to the epithelium shown by expression in the distal epithelial tip. These sequential expression patterns imply a deeply conserved role in mediating epithelial-mesenchymal interactions and positive regulation of cell proliferation as shown by PCNA. In the mouse dentition, *MK* inhibition results in disrupted morphogenesis and differentiation, implying a role in positive regulation of tooth morphogenesis and differentiation of cells associated with matrix deposition (Mitsiadis *et al.,* 1995b; 2008). Given these similarities in expression domains and the known odontogenic role of *MK*, a deeply conserved role in denticle development is inferred.

The role of *Bmp4* in denticle development is far less clear, however, its co-expression with *Bmp2* and *7* in the epithelium and mesenchyme during tooth induction, morphogenesis and cell differentiation is indicative of multiple odontogenic roles (Åberg *et al.,* 1997; Bégue-Kirn *et al.,* 1992; Vainio *et al*., 1993). In the osteichthyan dentition, the role of *Bmp4* in mediating epithelial-mesenchyme interactions through transfer of inductive potential appears to be conserved, shown by characteristically transient expression domains shifting between the epithelium and mesenchyme (Fraser *et al*., 2004). *Bmp4* is also expressed in developing feather buds, induced by *Shh* and *Fgf4,* and subsequently acting to suppress local expression of both to maintain micro and macropattern stability (Jung *et al*., 1998). The conserved inhibitory role of BMPs is also apparent during formation of the limb bud, where they regulate growth through antagonistic interactions with FGFs (Niswander and Martin, 1993). In denticle development, the absence of a complete developmental series for *Bmp4* presents a challenge when drawing conclusions regarding a putative role. However, in its available context, restriction of *Bmp4* to the mesenchyme during placode formation implies an ancestral role in regulation of mesenchymal induction, subsequently modified in succeeding gnathostomes. However, provisional signs of expression in the epithelium during budding are more in line with its conserved role in mediating epithelial-mesenchymal interactions. During advanced mouse tooth morphogenesis, *Bmp4* is expressed in pre-odontoblasts, followed by down-regulation upon terminal differentiation (Vainio *et al*., 1993; Åberg *et al.,* 1997). Culture of mouse induced pluripotent stem cells (iPSCs) in *Bmp4*-containing ameloblasts

serum-free conditioned medium (ASF-CM) also promotes odontogenic differentiation, marked by up-regulation of ameloblast and odontoblast-specific genes, while culture with the *Bmp4* inhibitor noggin abrogates this effect (Liu *et al*., 2013b). Viewed in the context of this role, sustained *Bmp4* expression in the mesenchymal papilla during advanced denticle morphogenesis suggests a role in regulating odontoblastic cell activity. Future studies of stage-specific *Bmp4* expression in denticle development will be required to better assess the full extent of its putative conserved odontogenic role.

Previous studies of Runx gene expression in the catshark have shown their deployment in tooth and denticle development, presenting an opportunity to further explore their putative odontogenic roles and interactions (Hecht *et al.,* 2008). Given its characteristic role in positively regulating mesenchymal cell differentiation to osteoblastic cell lineages (Bialek *et al*., 2004), in the present study *Runx2* was selected for expression analysis, in order to further investigate this conserved role. It was therefore unfortunate that the absence of *Runx2* expression data coinciding with stages of morphogenesis limited an assessment of its putative role. Despite this, a putative role in the positive regulation of cell differentiation is partially supported by sustained *Runx2* expression in the basal mesenchyme of the papilla during advanced morphogenesis, associated with persisted cell proliferation shown by PCNA. As with several of the markers presented here, this prolonged expression is likely associated with ongoing proliferation and differentiation of mesenchymal cells during basal plate formation. However, *Runx2* expression well in advance of morphogenesis implies further roles and interactions. In skeletal development, *Runx2* constitutes a target of Wnt-*β-catenin* signaling in the mesenchyme to induce osteoblast differentiation (Gaur *et al*., 2005). Furthermore, in the mouse dentition, *Runx2* interacts with FGF and BMP signaling, shown by induced *Fgf3* expression by *Runx2* over-expression in -/- mutants, and of early *Runx2*-dependent genes by addition of *Bmp4* protein to mandibular explants (Åberg *et al*., 2004; James *et al*., 2006). The considerable overlap here in expression domains suggest multiple pathway interactions for *Runx2* with Wnt-*β-catenin,* BMP and FGF signaling in advance of cell differentiation. In the case of *β-catenin* and *Bmp4*, this may be to stimulate

Runx2 expression in advance of its role to induce cell differentiation, though its putative interactions with *Fgf3* are less clear.

Despite the absence of a full developmental series, a putative role here for *Runx2* in regulating mesenchymal cell activity in advance of odontoblastic cell differentiation may be further supported by the associated co-expression of *Twist*, which acts to inhibit differentiation of osteoblasts through positive regulation of mesenchymal stem cell (MSC) renewal. During skeletogenesis, activation of *Twist* increases the expression of MSC markers and decreases osteogenesis, suggesting such a role in MSC self-renewal (El Ghouzzi *et al*., 1997; Isenmann *et al*., 2009). This is carried out by negative regulation of *Runx2* gene function through direct interaction with the *Runx2* DNA-binding domain during early skeletogenesis, preventing transcription of genes to inhibit osteoblastic differentiation. However, this interaction is transient and upon release, *Runx2* is free to stimulate osteoblast differentiation by activating target genes. This antagonistic interaction occurs without affecting *Runx2* expression, but with a subsequent reduction in *Twist* expression to trigger osteoblastic differentiation (Bialek *et al.,* 2004). Viewed in the context of their known roles, this relationship may be deeply conserved in denticle development, with both *Runx2* and *Twist* apparently expressed in equal measure throughout initial stages, followed by a subsequent down-regulation of *Twist* during morphogenesis, coincident with the requirement for *Runx2*-induced differentiation of odontoblasts for matrix secretion. This conserved relationship is further implied during advanced morphogenesis, with *Runx2* expressed in the medial basal mesenchyme and *Twist* restricted to the bilateral periphery, to allow terminal differentiation of odontoblasts during basal plate formation.

5.4.2 Denticle GRN model

The co-expression of *Shh*, *Fgf3* and *Midkine* in the epithelial tip, concomitant with its reduced cell proliferation shown by PCNA, suggests significant ancestral conservation. During mammalian tooth development, *Shh, Fgf3* and *MK* are co-expressed with other genes in the non-proliferative enamel knot (Vaahtokari *et al.,* 1996a; Kettunen *et al*., 2000; Mitsiadis *et al.,* 2008; Vaahtokari *et al.,* 1996b). In the

shark dentition, these common expression domains are further apparent (chapter 4), potentially identifying a conserved signaling module; the enameloid knot, proposed to be ancestrally conserved in gnathostomes exhibiting some degree of cusp complexity. The identical expression patterns of these genes during denticle morphogenesis therefore provides further compelling evidence to support the deployment of such a signaling module in odontogenesis. While hypothetical, this module is considered representative of a subset of developmental circuits with the capacity to periodically pattern morphologically similar, yet functionally distinct epithelial appendages, such as teeth and denticles. However, given current evidence to suggest the deployment of similar signaling pathways in other periodically patterned epithelial appendages with polarising properties, this may prove representative of a broader trend deeply embedded within the genome of gnathostomes. In the feather bud, the concept of developmental modules is well-recognised, with similar gene circuits transforming simple, dome-like epithelial primordia to complex, conical organs (Li *et al*., 2012).

As previously highlighted, this developmental transition is proposed to occur through localised physical processes involving changes in cellular reorganisation and polarity, controlled proliferation and apoptosis, and differentiation (Li *et al*., 2012; Närhi *et al*., 2012; Mou *et al*., 2011; Ting-Berreth and Chuong, 1996). The expression patterns presented here in denticle development both expand upon, and extend into deep evolutionary time, this developmental repertoire. This study therefore highlights denticles as important future models, when considering the role of developmental modules as determinants of form and function. However, it its current format, this study provides the initial framework for a hypothetical denticle gene regulatory network (GRN) model (Fig. 5.7). In this model, conserved genes are proposed to function as activators, inhibitors, polarising and differentiating factors, to guide denticle primordia from simple, epithelial placodes to complex, functional appendages. By consideration of the shark model as a comparatively ancient gnathostome, these novel insights highlight a deep molecular homology shared between epithelial appendages and so extend current understanding of epithelial organogenesis to its distant origins.

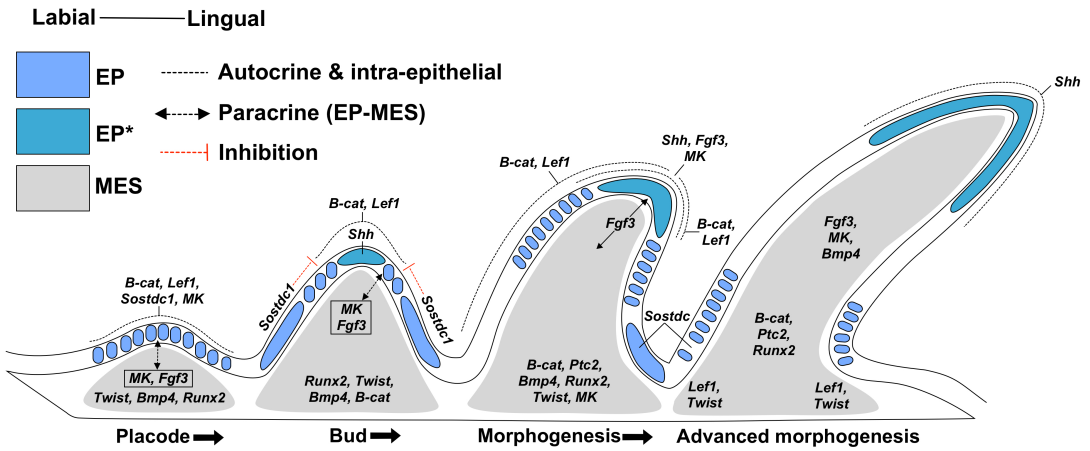


Figure 5.7 Denticle GRN model. Gene cascades regulate denticle development (defined by four common stages) through expression of activators, inhibitors, polarising and differentiation factors. *β-catenin* and *Lef1* are proposed to positively regulate development through induced cell proliferation (Järvinen *et al.,* 2006; Harris *et al*., 2006), while inhibitors, such as *Sostdc1*, are implied to respond by delineating these expression domains, in order to maintain stable micropattern (Cho *et al*., 2011; Närhi *et al.,* 2012; Noramly *et al.,* 1999; Mou *et al.,* 2011). Dynamic expression of *Fgf3* and *MK* between the epithelium and mesenchyme suggests roles in mediating inductive tissue interactions (Kettunen *et al*., 2000; Mitsiadis *et al.,* 2008). In association with *β-catenin,* progressive localisation of *Shh*, *Fgf3* and *MK* to the non-proliferative epithelial tip* infers roles as polarising factors to guide unidirectional growth and regulate subsequent cusp-formation. The co-expression of these genes in this restricted epithelium is proposed to mark the activation of an ancestrally conserved cusp-making module analogous to the mammalian enamel knot (Vaahtokari *et al.,* 1996a), and in the shark dentition, enameloid knot (chapter 4). *Twist* is also proposed to act in accordance with its conserved role to negatively regulate *Runx2*, in advance of its function to induce cell differentiation for matrix deposition (Bialek *et al.,* 2004). EP, epithelium; EP*, epithelial tip; MES, mesenchyme; EP-MES, epithelial-mesenchymal.

It also remains uncertain to what extent the gene expression patterns shown here may translate into micro and macropatterns, as speculated upon in relation to feather development (Jung *et al*., 1998; Mou *et al.,* 2011). The deep conservation of such a periodic patterning mechanism in basal vertebrates remains an intriguing concept and one worthy of further study. The organisation of dermal denticles into at least two distinctly ordered arrays implies some periodically patterned squamation (Reif, 1980; Ballard *et al*., 1993; Johanson *et al.,* 2007). Whether the two bilateral rows provide the localised source of initial patterning potential from which surrounding squamation is derived remains uncertain. Similarities drawn between the expression patterns of *β-catenin, Sostdc1* and *Shh* in denticle and feather patterning (section 5.5.1), tentatively imply a conserved developmental mechanism, however, whether this putative 'micropattern' translates into the stable, well-ordered periodic macropattern intrinsic to avians, remains unknown (Ting-Berreth and Chuong, 1996; Noramly *et al.,* 1999; Mou *et al.,* 2011). It is anticipated that future gene expression studies targeting specific stages of squamation initiation and progression will prove informative. In this respect, the identified involvement here of Wnt, FGF, hedgehog and BMP signaling, further provides the necessary prerequisite for future functional studies to perturb gene expression through local application of pathway-specific chemicals (e.g. the *Shh* inhibitor cyclopamine). Induced alteration of squamation resulting from targeted disruption of localised gene expression is likely to prove immensely informative in further defining the roles and interactions of specific pathways in regulating denticle initiation and patterning.

5.4.3 Comparative tooth-denticle expression patterns provide renewed insights into their evolutionary origins

In chapter 1, the various theories of odontode evolution were reviewed (section 1.1.1.). When reconsidering the 'inside-out' model, which proposes teeth to have evolved in advance of denticles in the oro-pharyngeal cavity (Smith and Coates, 1998; 2001; 2000), some corresponding evidence is found in extinct agnathan taxa. For example, conodonts possessed no dermal armour, but developed patterned tooth-like elements in the oro-pharyngeal cavity (Donoghue *et al.,* 2000; Turner *et al.,* 2010). Furthermore, the extinct thelodont *Loganellia scotia* developed patterned tooth whorls and dermal denticles similar to extant elasmobranchs, suggesting clear distinctions in patterning mechanisms (der Brugghen and Janvier, 1993; Smith and Coates, 1998; 2001; Smith, 2003). The 'inside-out' model also considers commonalities in gene deployment in oral and pharyngeal teeth, with similar genes frequently expressed in the pharyngeal teeth of fish and oral teeth of mice (Wise and Stock, 2006; Borday-Birraux *et al.,* 2006; Jackman *et al.,* 2004; Tucker and Sharpe, 2004). In extant fish possessing oral and pharyngeal teeth, both express a similar set of genes, providing further supporting evidence for the activation of a common GRN at different developmental loci in the oro-pharynx (Wise and Stock, 2006; Fraser *et al.,* 2004; Debiais-Thibaud *et al*., 2007; 2008).

The recently proposed 'inside and out' model has attempted to input renewed perspective into existing debate by further shifting the emphasis toward the role of a common GRN initiated at multiple locations to independently generate odontogenic diversity (Fraser *et al*., 2010). This model proposes teeth and skin denticles to have evolved convergently from any epithelium (oral or dermis) coming into contact with a neural crest-derived mesenchyme. Underpinning this are the gene networks intrinsic to these tissue layers, termed co-expression groups (CEGs). This model therefore postulates that epithelial and mesenchymal co-expression groups, termed epCEGs and mesCEGs, respectively, collaborate to generate odontodes, either as a patterned dentition within the oral epithelium or as dermal denticles in the external ectoderm (Fraser *et al.,* 2010). The 'inside and out' model therefore aims to draw the emphasis away from inferences reliant upon an often ambiguous mixture of comparative fossil and developmental data, toward the contemporaneous deployment

of a common GRN. A crucial aspect of this model is that it is testable in extant organisms and to this end, some support comes from recent investigation of Dlx gene expression in catshark tooth and denticle development, showing the common expression of *Dlx1, 3-5*, and differential expression of *Dlx2* (Debiais-Thibaud *et al*., 2011).

Leading on from this, current investigation of conserved gene expression in tooth and denticle development provides an ideal opportunity to compare the two and make informed judgments regarding their respective evolutionary origins. Given the extensive evolutionary history of elasmobranchs, the initiation of a single GRN to pattern ectodermal denticles and oral teeth via a serial patterning mechanism, would provide strong evidence to support elements of the 'inside-out', and 'inside and out' models (Smith and Coates, 1998; 2001; 2000; Fraser *et al.*, 2010). This is shown in Fig. 5.8, with conserved dental patterning genes expressed in near identical domains to denticles, implying the activation of a common GRN to produce patterned odontodes. The extent of this serial patterning homology is apparent, in both denticles and teeth first showing common epithelial-mesenchymal *β-catenin* expression during morphogenesis (A-B). The shared expression of *Shh* in the epithelial tip (C-D), and *Fgf3* (E-F), in both the tip and underlying epithelium and mesenchyme, adds further support this model. During advanced denticle morphogenesis, *Twist* is expressed in bilateral regions of the basal mesenchyme of the papilla (G, arrows), matching those in tooth development (H, arrows). This comparative expression data therefore offers support for the 'inside and out' model, showing that any collaborative epithelium and neural crest-derived mesenchyme are sufficient to supply the patterning potential required to generate teeth and denticles (Fraser *et al.*, 2010).

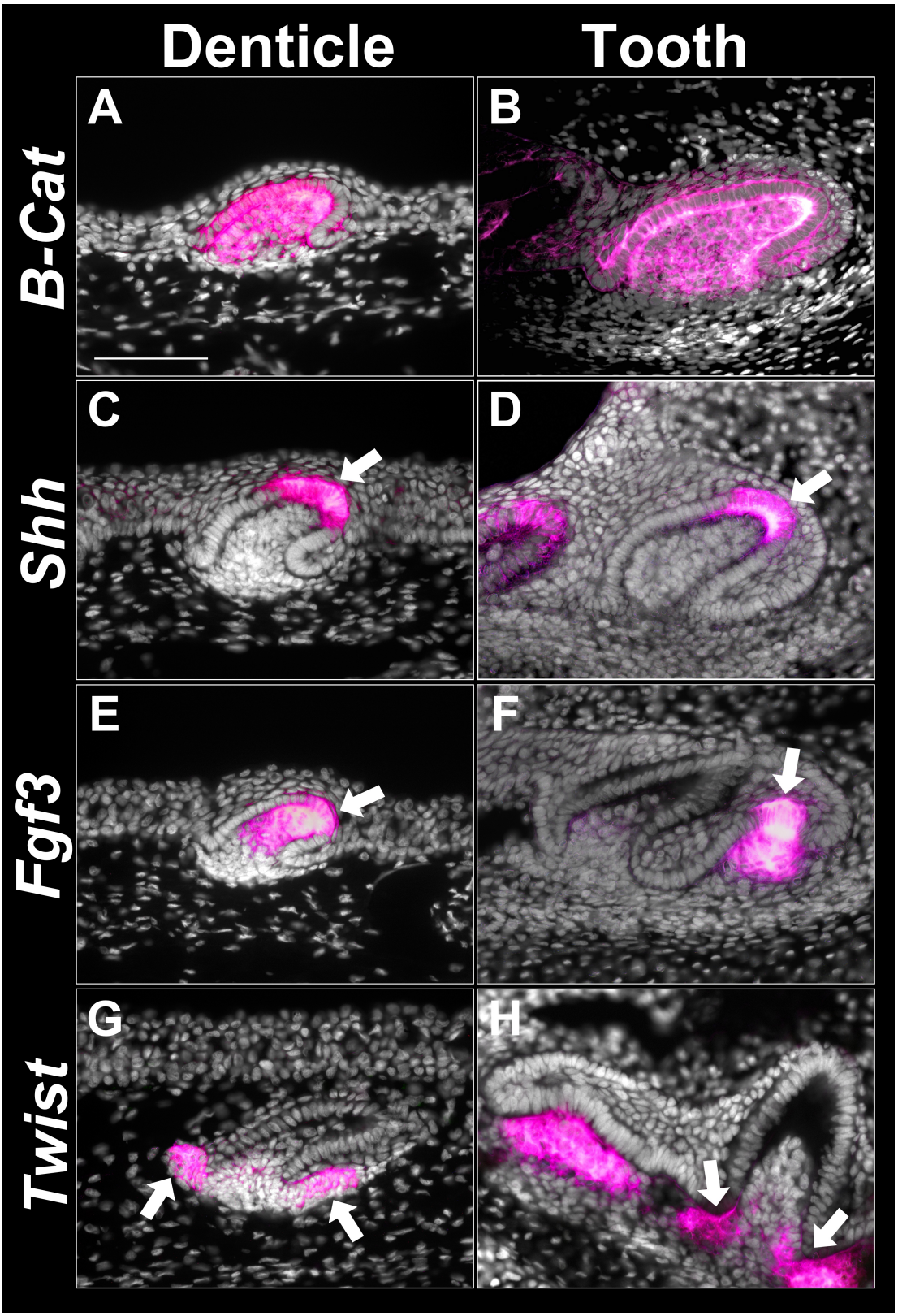


Figure 5.8 Comparative tooth-denticle expression in sharks (1). In dermal denticles and teeth, serially expressed genes imply the deployment of a GRN common to both. The epithelial-mesenchymal expression domains of *β-catenin* (A-B, lower jaw) supports a common odontogenic role, while those of *Shh* and *Fgf3* imply an even deeper level of ancestral conservation in regulating both morphogenesis (C-D, lower

jaw) and epithelial mesenchymal interactions (E-F, lower jaw). The common expression of *Twist* in the periphery of the basal mesenchyme during advanced morphogenesis (G-H, arrows) (upper jaw) further supports a conserved role, collectively implying the simultaneous activation of the same GRN to produce patterned odontodes as ectodermal skin denticles or oral teeth. Scale bar: (A-H) 100 μm.

Despite the morphological similarities common to teeth and denticles, their contrasting mechanisms of development have highlighted some important distinctions regarding their possible evolutionary origins. In elasmobranchs, this is principally that internal teeth develop and continuously regenerate in advance of function within a dental lamina, whereas external denticles exhibit no such pattern, predominantly developing at random by a comparatively simple space-on-demand growth mechanism (Ørvig, 1977; Reif, 1982). The crucial difference between dermal denticles and teeth is therefore the dental lamina, which is proposed to contain the regulatory potential to control tooth shape, position and in gnathostomes capable of tooth replacement, regeneration (Smith *et al*., 2009b).

Fossil agnathans, such as *Loganellia*, provide a compelling argument to challenge Reif's conclusion that teeth evolved concomitantly with jaws as the result of an invading odontode-competent ectoderm. They further provide an argument to support the evolution of oral teeth through transfer anteriorally of odontogenic potential from patterned odontodes in the pharyngeal cavity (Smith and Coates, 1998). In *Loganellia*, the sequential addition of pharyngeal denticles as patterned, joined sets of whorls with clear directionality, shows marked differences to their oral counterparts (der Brugghen and Janvier, 1993; Smith and Coates, 2001). In several placoderms, denticles developing in the rear of the gill chamber also show clear signs of pattern, arranged as rows to differ from the dermal tubercles covering the external surface of the trunk shield (Johanson and Smith, 2003). The pre-patterning potential of early pharyngeal denticles can also be inferred from the primitive chondrichthyan stethacanthid *Akmonistion zangerli*, which possesses pharyngeal joined denticles (Smith, 2003), while the oral teeth of the primitive shark *Doliodus problematicus* are further positioned to suggest the presence of a dental lamina (Miller *et al.,* 2003).

The emphasis here is that while pharyngeal denticles may share some similarities with external dermal denticles by expression of a similar set of developmental genes, they are distinct in their intrinsic pre-patterning potential. In this respect, the positional information required to produce patterned sets of replacement teeth is potentially restricted to the dental lamina (Smith, 2003). The comparative gene expression patterns presented here provide some further insight into this with several

commonly deployed markers expressed in teeth within the dental lamina (oral), and denticles developing from individual laminae (external). This is further shown in Fig. 5.9, by *β-catenin*, *Lef1*, *Fgf3*, *Bmp4*, *Sostdc1* and *Midkine* expression in oral teeth (T) and external denticles (D). Teeth and denticles are further separated by a hypothetical boundary zone at the oral margin (dotted line), previously proposed to restrict this unique patterning potential to the oro-pharynx (Smith and Coates, 1998; 2000; 2001). The physical separation of these two laminar developmental fields therefore provides some measure of support for the 'inside-out' and 'inside and out' models' (Smith and Coates, 1998; Fraser *et al*., 2010).

However, the differential expression of *Sox2* (chapter 3) also suggests some important differences. In the elasmobranch dentition, *Sox2* shows marked associations with the dental lamina, identifying a putative dental stem cell niche (Fig. 5.9G-H, arrow 1) linked to the successional lamina via a continuous epithelial stripe (arrow 2). *Sox2* is also expressed in taste buds (Fig. 5.9H, arrows), which further correlates with those in Lake Malawi cichlids (Fraser *et al*., 2010). In the current study, *Sox2* expression was not detected in developing denticles, which show no corresponding signs of regenerative capacity (Reif, 1980; Miyake *et al*., 1999). In elasmobranchs, the differential expression of *Sox2* therefore provides important molecular data to support the presumed restriction of serial regenerative capacity to the oral dentition. If, in line with the 'inside-out' and 'inside and out' models, the dental lamina is itself to be considered the developmental product of the oro-pharyngeal epithelium, this would suggest some important differences compared with the external dermal epithelium (Smith and Coates, 1998; 2000; 2001; Fraser *et al*., 2010). In partial accordance with the 'inside-out' model, it is therefore tempting to speculate upon the recruitment in deep evolutionary time, of a set of taste receptors by an anteriorally-advancing oro-pharyngeal dentition. This collaboration could conceivably have supplied the regenerative potential required for the evolution of a serial replacement dental patterning system (Smith and Coates, 1998; 2000; 2001).

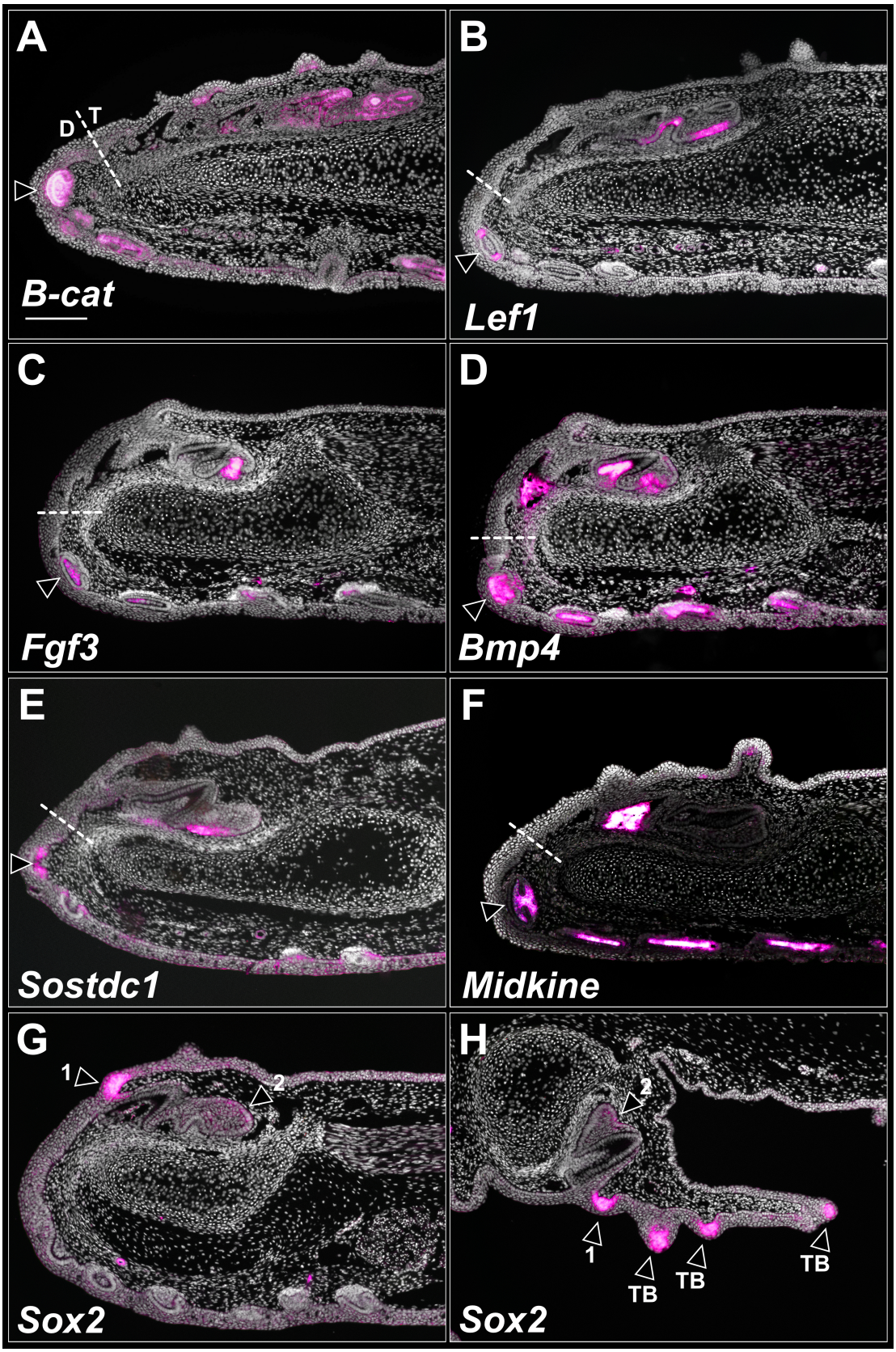


Figure 5.9 Comparative tooth-denticle expression in sharks (2). The expression of a common set of genes in teeth and denticles suggests a significant degree of serial molecular homology. In tooth and denticle development, commonly expressed *β-catenin*, *Lef1*, *Fgf3*, *Bmp4*, *Sostdc1* and *Midkine* (A-F, respectively) define these key

similarities, shown by their expression in oral teeth and dermal denticles. Teeth (T) and denticles (D) are further demarcated by a putative boundary zone (dotted line) demarcating the oral and dermal epithelia. These combined features offer partial support for both the 'inside-out' and 'inside and out' models of odontode evolution (Smith and Coates, 1998; Fraser *et al*., 2010). However, the differential expression of *Sox2*, confined to the dental lamina (G-H), partially contrasts with this to define some key differences. In the oral epithelium, *Sox2* shows clear associations with developing teeth, where its expression marks a putative dental stem cell niche (G, arrow 1) linked with the successional lamina via a continuous epithelial stripe (H, arrow 2). *Sox2* further marks developing taste buds (H, TB, arrows). In partial contrast with these models, this therefore suggests some key differences between the inner (oral) and outer (dermal) epithelia, as defined by a *Sox2*+ dental lamina, which produces replacement teeth via a precisely regulated serial patterning mechanism, in contrast to *Sox2*-negative denticles, which show no corresponding signs of regenerative organisation. Scale bar: (A-H) 200 μm.

5.4.4 Did odontodes evolve from an ancient sensory receptor?

When further considering the 'inside and out' model, the observed expression of *Sox2* in association with teeth and taste buds raises further interesting questions regarding their respective evolutionary origins and whether these might on a different level be interwoven. As an extension of this model, Fraser and colleagues (2010) hypothesised that the odontogenic GRN in its present-day form might conceivably have derived from a pre-established epCEG deployed in an ancestral sensory receptor similar to the taste buds and electroreceptors common to extant chondrichthyans. In subsequent collaboration with a newly acquired neural crest-derived mesCEG, this enhanced GRN could have greatly expanded the number of potential cell fate decisions available to such a receptor. Through remodulation of a common set of signals, this could conceivably have enabled its evolutionary diversification and the eventual appearance of odontodes (Fraser *et al*., 2010).

However, the proposed neural crest-derived origins of denticles remain uncertain. Since denticles develop as exoskeletal elements in the head and trunk, and because they contain dentin matrix, it has been assumed that denticle mesenchymal cells originate from the neural crest (NC) and that both cranial and trunk NC cells may contribute to denticle formation (Smith and Hall, 1990). However, developmental studies using mice and chicks suggest that the trunk NC has no skeletal potential (Hall and Horstadius, 1988; Hall, 1999). Despite this, mouse tissue recombination experiments using cranial NC and ectodermal tissues shows this to confer odontogenic potential, which extends to the most rostral trunk (Lumsden, 1988). Similarly, in axolotls, the odontogenic potential of the NC extends rostro-caudally beyond the cells that make teeth (Graveson *et al*., 1997), while in the skate, the distribution of denticles beyond the head implies both cranial and trunk NC to contribute to their development (Miyake *et al*., 1999).

Despite the uncertain origins and role of such a NC-derived mesCEG in odontode evolution, this study provides some measure of evidence of support that of an epCEG. When proposing the 'inside and out' model, Fraser and colleagues emphasised the important requirement to test this in extant chondrichthyans and in

the current study this has been partially fulfilled (Fraser *et al.,* 2010). In the catshark, several genes expressed during tooth and denticle development are also expressed during development of sensory receptors, such as taste buds, pit organs and Ampullae of Lorenzini (Fig. 5.6). Furthermore, these are expressed in strikingly similar patterns. In common with teeth and denticles, PCNA shows taste buds to develop from a proliferating epithelium, progressing through a common series of stages followed by a marked reduction cell proliferation, implying onset of cellular morphogenesis (Kapsimali and Barlow, 2013). This corresponds with concomitant *Shh*, expressed asymmetrically in similar cell loci, implying the possible conservation of a modular signaling center similar in function to that proposed to regulate elasmobranch tooth (chapter 4) and denticle development (Liu *et al*., 2013c). The expression patterns of *Sostdc1* are of further interest, restricted to bilateral domains in the periphery of the basal epithelium, perhaps to restrict outward growth expansion through a mechanism of lateral inhibition, as also proposed in tooth and denticle development. In addition to *Sox2*, *β-catenin, Runx2* and *Foxq1* are expressed in taste buds, *β-catenin* and *Midkine* in pit organs, and *Sox2* in Ampullae of Lorenzini. With the aforementioned exception of *Sox2*, this universal geneset may therefore prove representative of a larger GRN dedicated to patterning both odontodes and sensory receptors. When considering the proposed role of a collaborative set of ep-mesCEGs in triggering the early evolution of odontodes, their common expression in elasmobranch sensory receptors, especially taste buds, may provide some measure of supporting evidence (Fraser *et al*., 2010).

Despite current inferences from these gene expression patterns favouring a regenerative odontogenic oral epithelium over the dermis, it remains apparent that *Sox2* is expressed in dermal Ampullae of Lorenzini. Should future studies show the oral and dermal epithelia to be entirely homologous in their respective odontogenic regenerative capacities, the intimate developmental connection between the elasmobranch polyphyodont dentition and taste buds remains apparent. Given the seemingly plastic nature of taste buds (Castillo *et al*., 2014), coupled with the important role a regenerating dentition might play in the evolutionary success of early polyphyodont gnathostomes, this would still appear to favour the occurrence of such a co-option event in the oro-pharyngeal cavity. In either case, it remains highly

likely that future research targeting genes expressed both commonly and differentially in teeth and denticles will prove highly informative in determining both their origins and respective modes of development. The hypothesised roles of these genes in establishing periodic pattern, however, remains outstanding. While their expression patterns imply an ancestrally conserved micropatterning role, it remains to be seen how this might translate into macropattern. Once more, comparative gene expression analysis in conjunction with functional assays is likely to play a central role in establishing the fuller extent of these levels of developmental organisation.

5.5 Conclusions

This study uses the catshark as a model to investigate evolutionary novelty by gene co-option through analysis of conserved gene expression in dermal denticle development. PCNA first shows denticles to develop from a proliferating epithelium, while subsequent gene expression patterns reveal the deployment of conserved genes representative of the Wnt-*β-catenin*, hedgehog, FGF and BMP families of signaling molecules. During morphogenesis, reduced cell proliferation in the epithelial tip, combined with the co-expression of *Shh*, *Fgf3* and *Midkine*, implies the activation of a modular signaling center comparable to the enamel knot in mammals (Vaahtokari *et al*., 1996a) and enameloid knot in sharks (chapter 3). Integration of these gene expression patterns with additional markers inspires the production of the first denticle GRN model. In this model, these genes are proposed to function in controlled cascades as activators, inhibitors, polarising and differentiation factors, to regulate denticle development.

Further comparison of these gene expression patterns with those in the shark dentition reveals evidence of significant serial patterning homology, implying the deep conservation in gnathostomes, of a core GRN to produce odontogenic diversity through gene network co-option. This is generally in agreement with elements of the 'inside-out' and 'inside and out' models, which view teeth and denticles as convergent structures (Smith and Coates, 1998; Fraser *et al*., 2010). However, the differential expression of the dental stem cell marker *Sox2*, restricted to the dental lamina, implies some key differences in patterning potential between both epithelia. In partial accordance with the 'inside-out' model, this favours the transfer anteriorally of odontogenic potential, which in collaboration with taste buds may have led to the subsequent evolution of a serially patterned replacement dentition (Smith and Coates, 1998). The expression of odontogenic genes in sensory receptors, such as taste buds reveals additional key similarities. In line with the 'inside and out' model, this therefore also supports concepts relating to the proposed evolution of odontodes from an ancestral epithelial sensory receptor, which in collaboration with newly acquired neural crest-derived fates, may have developed the patterning potential to trigger the evolution of odontodes (Fraser *et al*., 2010).

Chapter 6

FATE MAPPING AND FUNCTIONAL STUDIES OF CONSERVED SIGNALING PATHWAYS

6.1 Summary

Studies of incisor renewal in mammals and tooth replacement in polyphyodont gnathostomes have demonstrated the combined importance of chemical manipulation of key signaling pathways and cell lineage tracing in understanding these regenerative processes. Modified dental phenotypes induced by chemical treatment and localisation of slow-cycling cells and their proliferating progeny provide far greater evidence to support gene function and the role of dental stem cells than expression analysis alone (Järvinen *et al*., 2006; Harada *et al*., 2002; Shimada *et al*., 2008; Juuri *et al*., 2012; Fraser *et al*., 2013; Abduweli *et al*., 2014; Handrigan and Richman, 2010a; 2010b; Wu *et al*., 2013; Gaete and Tucker, 2013).

Following the identification of putative dental stem cells (chapter 3) and conserved signaling pathways (chapter 4), similar experiments were therefore carried out during shark tooth and denticle development. To further characterise the role of putative dental stem cells and associated cell proliferation dynamics implied by *Sox2* and PCNA, respectively, preliminary cell fate mapping studies were used. BrdU pulse-chase experiments were first used to localise slow-cycling and transit amplifying cells in dental tissues. During first generation tooth development, BrdU label-retaining cells were localised to the oral-dental epithelial junction and epithelial-mesenchymal tissues within the dental lamina in patterns indicative of their proposed roles as dental stem cells. DiI lineage tracing was further used to provide supporting evidence for dynamic cell movement in tissues associated with the dental lamina.

To investigate the effects of targeted manipulation of conserved pathways on tooth and denticle development, preliminary gain- and loss-of-function experiments were carried out. This involved the implantation of several bead types soaked in chemicals known to affect hedgehog, FGF and BMP signaling. While it was not possible to analyse the results of these initial experiments, this method demonstrates significant potential, therefore laying the foundations for future related efforts to further understand the roles of these pathways in elasmobranch tooth and denticle development.

6.2 Introduction

The gene expression data presented in chapters 3 and 4 suggests elasmobranch tooth development to result from the deployment of a conserved set of core signaling pathways. This regenerative process is further proposed to be dependent upon a putative integrated dental stem cell niche regulated by genes expressed within these pathways. In other gnathostomes, while gene expression analysis of tooth development has proven insightful, the effects of chemical manipulation of these pathways offers far greater evidence to support of their proposed developmental roles. In mammalian tooth development, such experimental approaches have proven essential in understanding the precise role of developmental genes through gain- and loss-of-function experiments (Järvinen *et al*., 2006; Tucker *et al*., 1998; Cobourne *et al*., 2001; Harada *et al*., 2002). When considering the associated role of dental stem cells in mammalian tooth development and renewal, this has further been shown through cell fate mapping. During mouse incisor renewal, use of cell labeling methods, such as BrdU and DiI lineage tracing to identify putative dental stem cells and their proliferating progeny have provided associated insights into the role of dental stem cells (Shimada *et al*., 2008; Juuri *et al*., 2012).

In polyphyodont gnathostomes, similar methods have proven to be of equal importance when understanding gene function and the role of putative dental stem cells in tooth replacement (Fraser *et al*., 2008; Handrigan and Richman, 2010a; 2010b; Wu *et al*., 2013; Gaete and Tucker, 2013; Abduweli *et al*., 2014). This has previously been shown in functional experiments applied to the reptilian and cichlid dentitions, in which exposure of dental tissues to small molecules targeting common pathways significantly perturbs various stages of tooth development (Buchtová *et al*., 2008; Handrigan and Richman, 2010a; 2010b; Gaete and Tucker, 2013; Fraser *et al*., 2008; 2013). Given the importance of dental stem cells in sustaining the regenerative capacity of the polyphyodont dentition, similar cell fate mapping studies have also provided comprehensive insights. This has been shown in the cichlid, medaka and reptilian dentitions, in which similar lineage tracing studies have also identified putative dental stem cells and progenitors committed to tooth-specific fates (Fraser *et*

al., 2013; Abduweli *et al*., 2014; Handrigan and Richman, 2010b; Handrigan *et al*., 2010; Wu *et al*., 2013; Gaete and Tucker, 2013).

6.2.1 Aims and objectives

Given that similar genes representative of conserved signaling pathways are temporally expressed in reiterative patterns throughout various stages of elasmobranch tooth development, it can be deduced that manipulation of these pathways may impact their expression, inducing similar phenotypic aberrations. In the current study, the hypothesised roles of genes inferred from their respective expression patterns highlights the essential requirement for functional and cell lineage tracing experiments. To address this, the aims and objectives are as follows:

- To use the catshark as a model to further investigate the role of putative dental stem cells and their proliferating progeny through the application of BrdU and DiI cell lineage tracing techniques.

- To further investigate the functional role of conserved genes in tooth regeneration by targeted chemical manipulation of signaling pathways using bead implantation techniques.

- To further investigate the functional role of conserved genes in denticle development by targeted chemical manipulation of signaling pathways using bead implantation techniques.

6.3 Results

The fluorescent lipophilic molecule DiI, when injected into cells, diffuses into the membrane where it is retained. Given its intense fluorescent properties and photostability, DiI label-retaining cells (LRCs) therefore remain strongly marked, while showing a progressive reduction in signal intensity over many divisions, allowing for the localisation of quiescent stem cells and fate mapping of their proliferating progeny. Similarly, the thymidine analogue BrdU (5-Bromo-2'-deoxyuridine) incorporates into DNA during S-phase of the cell cycle and is retained only by slow-cycling cells (SCCs) and their progeny, therefore providing further indications of their stem-like properties (reviewed by Kretzschmar and Watt, 2012). In the context of tooth development, DiI and BrdU have therefore become widely used tools for identifying putative dental stem cell populations and their transit amplifying progeny (Wang *et al*., 2007; Juuri *et al*., 2012; Handrigan and Richman, 2010a; 2010b; Gaete and Tucker, 2013; Wu *et al*., 2013; Fraser *et al*., 2013; Abduweli *et al*., 2014). To further identify the putative dental stem and transit amplifying cells indicated by *Sox2* and PCNA (chapters 3 and 4), BrdU and DiI lineage-tracing experiments were carried out on the catshark.

6.3.1 BrdU lineage-tracing during shark tooth development

This first consisted of a BrdU label pulse-chase experiment, with a pulse administered twice daily for one week, followed by a one-week chase period. Two slightly varying methods of detection were used (described chapter 2, section 2.5.1), resulting in some variation in results. Fig. 6.1A pertains to method 1 and Fig. 6.1B-D, to method 2. During initiation of primary teeth (Fig. 6.1), BrdU+ label-retaining cells (LRCs) localised strongly to the junction of the oral-dental epithelium in both the upper and lower jaws (Fig. 6.1A-B, respectively). This was most apparent in the lower jaw (Fig. 6.1A) during early first generation tooth development, in which BrdU+ cells localised to a cluster of epithelial cells (arrow) positioned at the oral-dental epithelial junction, with some evidence of in-spread into the adjoining O-ODE (dotted line). No LRCs were detected in the thickened epithelium of the first generation tooth placode (T1). In the upper jaw at similar stages (Fig. 6.1B), BrdU+

cells localised to the oral epithelium (arrow) continuous with the dental lamina. In addition, BrdU+ cells were further detected in both the dental epithelium and mesenchyme of the same individual (Fig. 6.1C-D). In the lower jaw (Fig. 6.1C), LRCs were detected throughout the epithelium of the dental lamina, spreading outwards to the O-ODE into the adjoining oral epithelium (1, dotted line). BrdU+ cells were also distributed throughout the directly underlying condensing dental mesenchyme (2, dotted line) in a pattern of demarcation, with no evidence of further outspread into the surrounding mesenchyme. In the upper jaw (Fig. 6.1D), LRCs (arrow) were detected in the dental mesenchyme directly underlying the first generation tooth (T1) during early morphogenesis. BrdU+ cells further localised to both the lingual and labial IDE (arrows) of the tooth, though in partial contrast to the lower jaw, no positive cells were detected within the dental lamina (DL) or the O-ODE and oral epithelium.

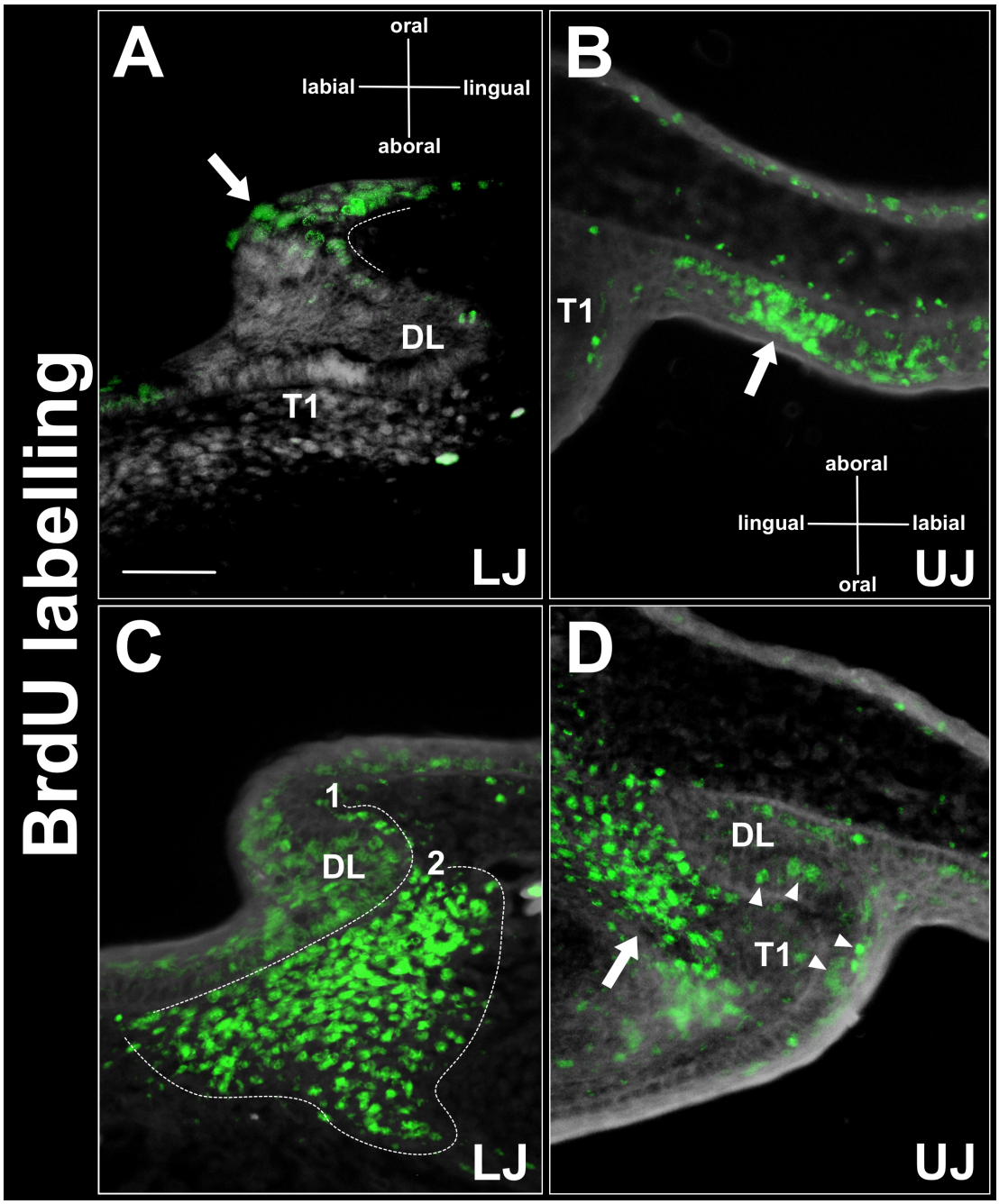


Figure 6.1 BrdU lineage-tracing in the shark dentition. Preliminary experiments show putative BrdU LRCs in the early dentition (7-day pulse plus 7-day chase period). LRCs are first detected in the lower jaw (A) at the oral-dental epithelial junction (arrow) with some evidence of influx into the O-ODE (dotted line). No LRCs are detected in the IDE of the early developing tooth placode (T1). Immunodetection of BrdU LRCs under modified conditions (B-D) produces partially contrasting results. In the upper jaw (B), patterns of BrdU incorporation (arrow) mark putative LRCs at the oral-dental epithelial junction in similar patterns to (A). However, in the same individual (C, lower jaw) LRCs are further detected in the dental lamina, adjoining O-ODE (dotted line 1) and the underlying dental mesenchyme (dotted line 2). In the upper jaw (D), patterns of incorporation in the dental mesenchyme are similar (arrow), while also showing evidence of LRCs within the IDE of the first generation tooth (arrows) during early morphogenesis.

6.3.2 DiI lineage-tracing during shark tooth development

To further investigate the cell fate dynamics of *Sox2*/ BrdU+ cells derived from the oral-dental epithelium, this tissue was injected with DiI to trace these cells in relation to the successional lamina. Out of several individuals treated, one showed indications of having been injected in the required region of upper jaw epithelium concomitant with the dental lamina. This is shown immediately post-treatment with point of injection circled (Fig. 6.2A, bright field) and (Fig. 6.2B, fluorescence). This individual was allowed to develop for 7 days, followed by euthanisation and examination of cell labeling. When viewed in sagittal section (bright field), replacement teeth are not visible; these tissues are therefore presumed to be inter-tooth region. However, oral-dental tissues within the maxillary valve showed indications of label retention (Fig. 6.2C, circle), with the dental lamina (DL) clearly visible as an embayment in the maxillary cartilage (CA). The presence of LRCs was further indicated when viewed under fluorescence (Fig. 6.2D) with the DiI label strongly maintained within a specific region of valve tissue (circle) continuous with the dental lamina. This showed a similar population of LRCs (arrow) focal to the proximal aspect of the successional lamina. LRCs positioned within the intervening O-ODE and distal epithelium also presented some evidence out-spread, indicated by the respective patterns of diffusion shown by the DiI label. Viewed at high magnification under bright field (Fig. 6.2E), the tissue-specific point of injection (circle) is unclear, however, under fluorescence (Fig. 6.2F), LRCs with increased signal strength (circle) showed some bias toward the epithelium. At high magnification, the DiI signal further defined an apparent influx of LRCs into the dental lamina via the O-ODE (arrow 1), near continuous with a secondary population of LRCs marking the successional lamina (arrow 2). In common with results shown by *Sox2*/ BrdU, those presented here therefore tentatively indicate the presence of two distinct cell populations; slow-cycling cells (putative dental SCN) maintained in a defined region of the oral-dental epithelium and another transient group (dental progenitors) in the successional lamina, interspersed by a population of LRCs (transit amplifying cells) in the adjoining O-ODE.

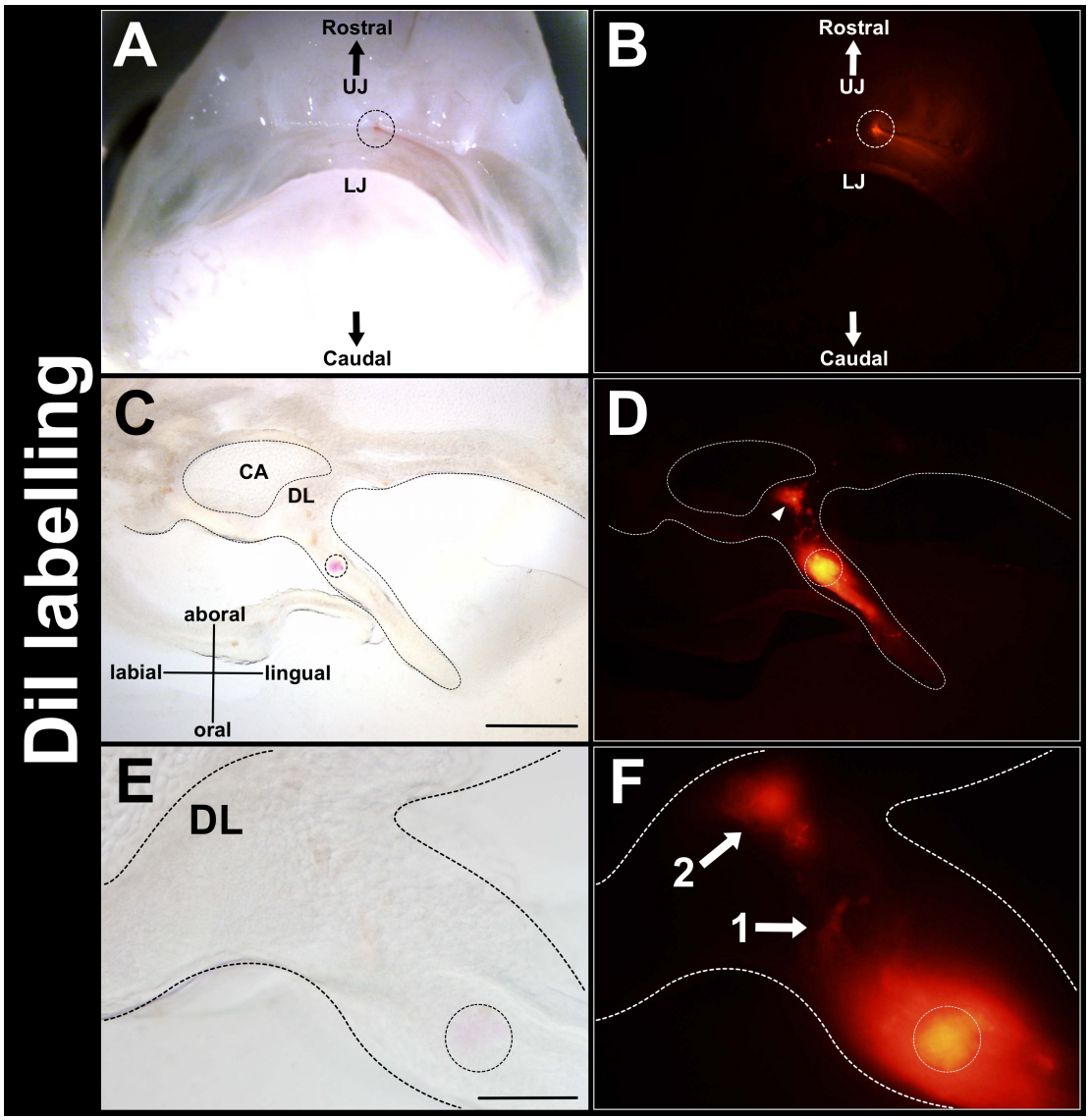


Figure 6.2 DiI lineage-tracing in the shark dentition. DiI label was injected into upper jaw (maxillary) dental tissues, followed by 7 days of development. This is shown post-treatment: bright field (A) and fluorescence (B), with point of injection circled. In section, the label remains in the region of the maxillary valve (C). No teeth are visible in section, however, an embayment in the maxillary cartilage (CA) is presumed to define the extent of the dental lamina (DL). Viewed under fluorescence (D), a population of LRCs clearly marks the point of injection (circle). These cells extend outwards, both distally and proximally toward the lamina, where a second population of LRCs (arrow) marks the successional lamina. It remains unclear as to whether these cells are epithelium or mesenchyme (E, circle), however, under fluorescence (F) these appear biased towards the epithelium. From here, inward diffusion of the DiI signal (arrow 1) toward the dental lamina is presumed to label putative transit amplifying cells within the O-ODE, while retention of the DiI label in the successional lamina (arrow 2) appears to mark a population of LRCs, which may prove representative of progenitor cells immediately marked for tooth-specific fates.

6.3.3 Bead implantation experiments to manipulate conserved signaling pathways

In order to demonstrate the functional roles of conserved signaling pathways deployed in shark tooth and denticle development (chapters 4 and 5), some preliminary chemical manipulation experiments were applied to the catshark. In accordance with similar methodology used with other animal models (Gillis *et al*., 2009; Buchtová *et al*., 2008; Handrigan and Richman, 2010a), these involved implantation of a combination of microbead-types into the shark dentition and outer epithelium associated with early dorsal row denticle primordia. Bead-types used consisted of Affi-Gel® Blue, formate-derivitised AG1X2 (Bio-Rad) and Heparin-Acrylic beads (Sigma-Aldrich). In accordance with their respective physico-chemical properties, beads were soaked in chemicals known to inhibit a given pathway, e.g. cyclopamine (hedgehog), LDN (BMP) and SU5402 (FGF). Conversely, to induce altered phenotypes through targeted up-regulation of gene expression, beads soaked in recombinant protein (e.g. Shh) were also applied using similar methodology.

This is first shown in Fig. 6.3A, in which approximately 13 Heparin-Acrylic beads (arrows) loaded with cyclopamine were successfully implanted into the upper jaw of a prehatchling embryo. These were effectively inserted adjacent to dental tissues beneath a thin strip of transparent oral tissue, which forms an elastic-like band across the maxillary jaw arc. Similar implantations using Affi-Gel Blue beads were carried out on individuals at preceding stages (Fig. 6.3B), with several beads (arrows) also successfully implanted at similar tissue loci. Following implantation, individuals were reinserted into egg capsules and returned to their tanks overnight to allow the chemical to diffuse into dental tissues. The following day, treated individuals were visually inspected and it was found that in all, the beads were absent, presumably dislodged and washed away through jaw movement and/ or re-immersion in seawater. The results of this line of research therefore remain unresolved, warranting further study.

To further investigate the effects of manipulating conserved gene expression during development of dorsal row denticles (chapter 5), similar bead implantation experiments were applied to staged individuals concomitant with induction of early

primordia. This involved the creation of 'pilot holes' at defined locations in the anterior dorsal epithelium, those presumed to coincide with early developing denticles. This is shown in Fig. 6.3C (boxed area) in which several Affi-Gel Blue beads loaded with cyclopamine were successfully implanted into the dorsal epithelium of the integument. Treated individuals were once again reinserted into egg capsules in seawater and following examination the following day, allowed to develop for between 1-2 weeks prior to euthanisation and tissue processing. While several beads were successfully retained (Fig. 6.3D, circles) subsequent examination of surrounding squamation (arrows) showed no apparent signs of induced phenotypes. This line of research therefore also remains unresolved, warranting further functional investigation. In this respect it is further anticipated that these preliminary experiments will facilitate future related efforts to manipulate key signaling pathways and demonstrate their functional roles in denticle development.

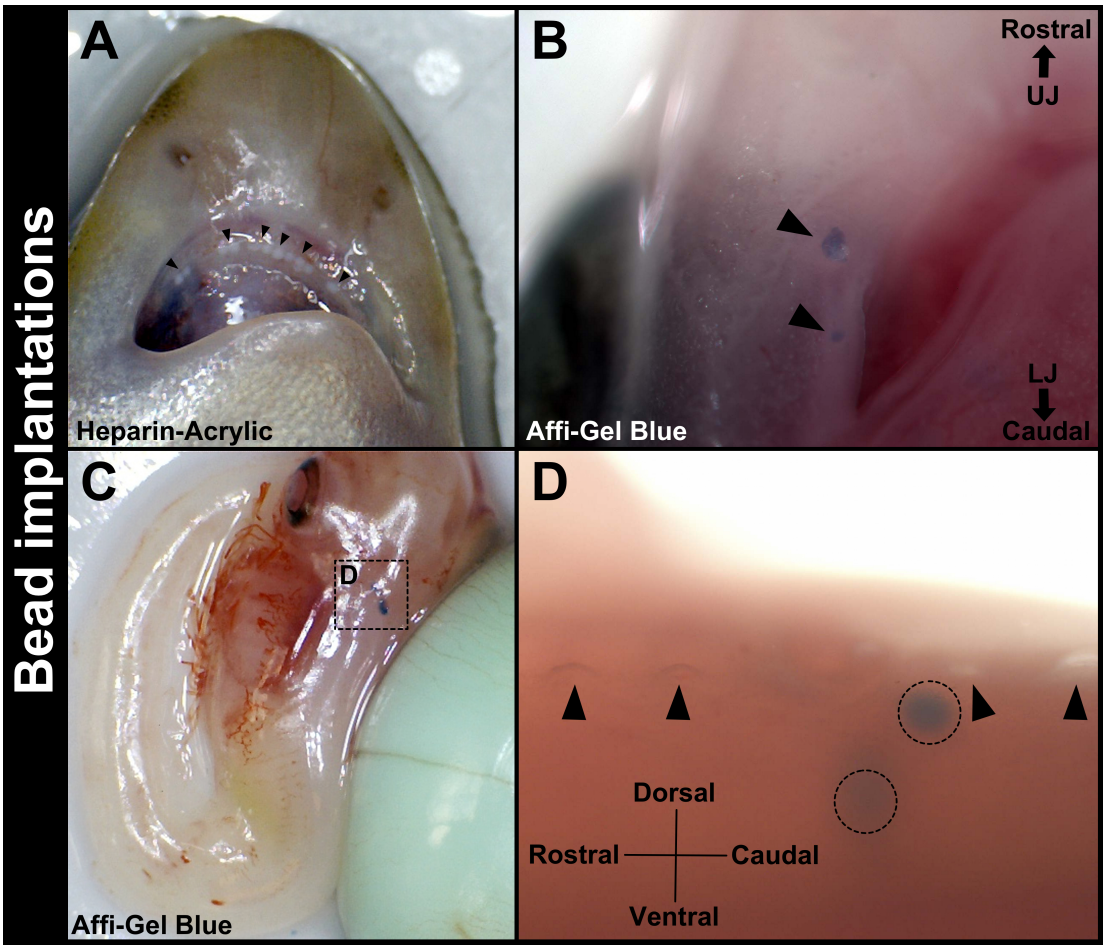


Figure 6.3 Preliminary bead implantation experiments. In the catshark, several microbead types loaded either with chemicals or protein were implanted into dental tissues to induce altered phenotypes. For example, Heparin-Acrylic (A) and Affi-Gel Blue (B) beads (arrows) soaked in the hedgehog antagonist cyclopamine were implanted superficially beneath maxillary jaw tissues adjacent to developing teeth. Embryos were left overnight in seawater and then examined to check for bead retention. These were found to be absent, presumably displaced through jaw and water movement. Using similar methodology, Affi-Gel Blue beads (cyclopamine) were implanted into the integumental epithelium (C) coincident with early dorsal row denticle primordia (boxed area). These were successfully implanted and following development for 1-2 weeks, individuals checked for adverse phenotypes (D). Despite the beads having been retained (circles), no phenotypic abnormalities were apparent with early denticles developing normally (arrows). In the case of both implantation experiments, it was not possible to examine treated tissue for signs of histological phenotypic abnormalities; however, the methodology established during these preliminary implantation experiments are anticipated to be of value when carrying out similar functional experiments in the future. Having been taken with a USB digital microscope, reference images shown are unscaled.

6.4 Discussion

In this chapter, cell lineage tracing (BrdU and DiI) and functional studies (bead implantations) have been used to add further experimental evidence to support the deployment of dental stem cells (chapter 3) and conserved signaling pathways (chapter 4) in elasmobranch tooth development. Given the conservation of these core pathways in denticle development (chapter 5), similar functional studies have been used to further investigate their patterning in sharks.

6.4.1 Functional investigation of tooth and denticle development

Manipulation of reptilian tooth development using a combination of these experimental approaches has proven successful in the past. In the corn snake, inhibition of hedgehog signaling by treatment of dental explant tissue with cyclopamine prevents dental lamina ingrowth, while in the python this produces a shortened dental lamina, lacking in acute angulation (Buchtová *et al*., 2008). In the leopard gecko, exposure of dental explant tissue to cyclopamine induces dental phenotypic defects in teeth and the dental lamina; while in the bearded dragon this reduces dental epithelial-mesenchymal cell proliferation and disrupts dental morphology (Handrigan and Richman, 2010a). Conversely, in the bearded dragon, gain-of-function using Shh-loaded beads increases cell proliferation in these tissues as shown by BrdU (Handrigan and Richman, 2010a).

When considering similar functional experiments in the embryonic shark dentition, this approach therefore holds significant promise. The amenability of elasmobranchs to gain- and loss-of-function experiments by chemical treatment has previously been demonstrated in the little skate (Gillis *et al*., 2009). In skate branchial ray development, treatment with retinoic acid produces mirror-image duplications of branchial rays and ectopic *Shh* expression. Implantation of beads loaded with Shh protein induces a single ectopic branchial ray, while cyclopamine and the FGF antagonist SU5402 prevents neighbouring branchial ray development (Gillis *et al*., 2009). In the current study, the application of these techniques to the shark dentition was therefore tested and proved to be technically challenging, most notably when

attempting to implant beads in soft dental tissues without risking excessive damage. Given the relative inaccessibility of the lower jaw to implantation in ventral orientation, efforts focused on delivering beads to the upper jaw. As previously shown here, several bead-types loaded with chemicals were superficially implanted adjacent to dental tissues within the natural aperture formed by the thin band of soft tissue spanning the jaw margins. Despite these efforts, the beads were lost shortly after implantation, casting some doubt over the suitability of this technique. While these individuals were euthanised and processed for analysis of histology and gene expression, due to practical considerations it was not possible to achieve this in the current study. It therefore remains unknown whether the relatively short exposure periods (12 hours or less, depending upon the point of loss) induced any detectable changes in gene expression and/ or dental phenotype. Should this prove not to be the case, a whole embryonic immersion strategy may provide a suitable alternative. However, given the comparatively indiscriminate exposure of the embryo to chemicals compared with the use of beads, this comes at the risk of affecting the broader expression of other genes. On a GRN level this may therefore prove less informative regarding the function of individual genes and associated pathways on a tissue-specific basis. Furthermore, this level of exposure to chemicals may result in lethality, making this approach wholly impractical. A dental explant tissue culture system, such as those previously used during reptile tooth development, might therefore provide a suitable alternative (Buchtová *et al*., 2008; Handrigan and Richman, 2010a; Gaete and Tucker, 2013).

In chapter 5, a core set of conserved signaling pathways were identified in denticle development. Given their similar expression patterns in teeth, a serial patterning mechanism evolved through gene network co-option was proposed. Within this conceptual framework, a further hypothesis involving a patterning mechanism similar feather development was discussed (Jung *et al*., 1998; Mou *et al.,* 2011). In order to further investigate this idea, some preliminary bead implantation experiments similar to those targeting the dentition were carried out during early development of the two dorsal denticle rows. The aim of these experiments was to ascertain the impact (if any) of manipulating their early development on surrounding patterns of squamation. As previously shown, these preliminary implantations were

successful, with several cyclopamine-loaded beads implanted beneath the external ectoderm proximal to the early dorsal row denticle primordia. However, upon subsequent examination of these tissue loci, no phenotypic aberrations were apparent, leaving this hypothesis unresolved. Despite the limited success of these functional experiments, they demonstrate future potential in terms of their practical applicability to elasmobranch tooth and denticle development. These lines of research therefore remain under continued practical review, particularly in the context of denticle development as indicated by the initial results of recent chemical manipulation of tail denticle patterning in the catshark (Gareth Fraser and Kyle Martin, personal communication, January 2015).

6.4.2 Initial fate mapping experiments identify putative label-retaining cells

In mammals and polyphyodont gnathostomes, the use of fate mapping to trace dental stem cells and their progeny has previously been demonstrated with considerable success (Harada *et al*., 1999; Wang *et al*., 2007; Juuri *et al*., 2012; Handrigan and Richman, 2010a; 2010b; Gaete and Tucker, 2013; Wu *et al*., 2013; Abduweli *et al*., 2014; Vandenplas *et al*., 2014). In the current study, the expression patterns of *Sox2* (chapter 3) and cell proliferation dynamics shown by PCNA (chapter 4) have proven insightful in terms of localising putative dental stem cells and their transit amplifying progeny. However, to further progress these initial conclusions beyond this starting hypothesis requires appropriate cell fate mapping experiments to spatially and temporally trace cell movement in dental tissues.

To this end, the results shown by these experiments have proven informative when considered in light of similar experimental approaches in mammals, reptiles and fish. In the mouse incisor, BrdU-pulse chase experiments and DiI labeling indicate the presence of dental stem cells in the cervical loop epithelium (Harada *et al*., 1999). Similar BrdU labeling in wild-type and mutant mice also localise SCCs to the incisor cervical loop, indicating their stem-like properties (Wang *et al*., 2007). In the American alligator, BrdU labeling localises SCCs to the cervical loop, which becomes enriched with TA cells during tooth initiation and growth phases (Wu *et al*., 2013). Similarly, in the leopard gecko, BrdU labeling identifies LRCs in the lingual

dental lamina and in the bearded dragon, the cervical loop, enamel epithelium and papilla (Handrigan and Richman, 2010b; 2010a). In the corn snake dentition, DiI labeling also identifies LRCs in the successional lamina and replacement tooth germs (Gaete and Tucker, 2013). In the Japanese medaka, BrdU+ cells further mark the posterior end of each tooth family, coincident with the expression of *Sox2* (Abduweli *et al*., 2014), while in the African bichir, BrdU marks epithelial cell proliferation coincident with initiation of new replacement teeth (Vandenplas *et al*., 2014).

The commonalities shown by these fate-mapping experiments and those presented here add further evidence to support a similar regenerative strategy. In further accordance with existing conclusions, a dental SCN is therefore proposed to sustain continuous tooth regeneration in elasmobranchs through the supply of quiescent progenitors from a cluster of 'stem-like' cells housed within the oral-dental epithelial junction. Within this progenitor population and the adjoining outward-outer dental epithelium (O-ODE), molecular signals are proposed to induce the proliferation of putative transit amplifying (TA) cells into the dental lamina, where they contribute to the continuous formation of replacement teeth. These initial short-term BrdU experiments provide some support for this, identifying LRCs in several dental tissues including the oral-dental epithelial junction, O-ODE, dental lamina, tooth IDE and underlying dental mesenchyme. The localisation of LRCs in the oral-dental junction and adjoining O-ODE corroborates with the expression patterns of *Sox2* (chapter 3) and additional markers (chapter 4). This is also in general agreement with the patterns of cell proliferation shown by PCNA (chapter 4), adding further evidence to support the migration of TA cells from tissue loci into the dental lamina. The incorporation of BrdU into cells within the IDE of the tooth during morphogenesis is in further agreement with epithelial gene expression patterns in these domains at similar stages (chapter 4). Similarly, BrdU+ cells in the dental mesenchyme are further indicative of cell proliferation induced by various markers expressed within these tissue loci (chapter 4). While these initial fate-mapping experiments have proven promising, it must be considered that the two varying methods of BrdU detection used (chapter 2, section 2.5.1) may have influenced these partially contrasting results, requiring future immunodetection experiments inclusive of strict controls.

Despite this, some additional supporting evidence for these initial conclusions is further found in preliminary DiI experiments. The point of injection, in the upper jaw maxillary valve, appears to coincide with the position of putative dental stem cells, as implied by *Sox2* (chapter 3). Following a relatively short chase period, the diffusive staining patterns of the DiI would appear to mark cells proliferating both distally toward the aboral aspect of the valve and importantly, those moving into the dental lamina. The strong retention of the DiI signal in the original LRC population and reduced pattern of staining in those distributed throughout the adjoining O-ODE provides further preliminary evidence to support their slow-cycling and transit amplifying identities. This is further apparent in the dental lamina, where a diffusely labeled secondary population marks the successional lamina; those cells immediately marked for tooth-specific fates. Future DiI experiments, inclusive of staged individuals and appropriate controls, will be further required to substantiate this.

6.5 Conclusions

In this chapter, cell fate mapping (BrdU and DiI) and chemical manipulation of signaling pathways (bead implantations) were used to further investigate tooth and denticle development in the catshark. Initial bead implantations into dental tissues proved to be of limited success, largely due to the relative inaccessibility of the shark dentition using this approach, while those targeting denticle development were more successful. In both cases, the results of these initial functional experiments remain outstanding, warranting future study. Initial results shown by BrdU and DiI labeling of early teeth show significant promise, highlighting the distribution of putative label-retaining cells in the dental epithelium and mesenchyme in patterns conducive to those previously shown by gene expression and cell proliferation. These preliminary fate mapping experiments therefore add further experimental evidence to support the deployment of dental stem cells and conserved signaling pathways in the elasmobranch dentition, while further providing the initial practical framework for future related efforts to investigate the molecular basis of tooth regeneration and denticle development in elasmobranchs.

Chapter 7

GENERAL DISCUSSION

7.1 Overview

In Chapter 1, the requirement to enhance current understanding of all aspects of tooth regeneration highlighted the importance of studying these processes in the elasmobranch replacement dentition. The issues highlighted were:

1. A limited understanding of the role of dental stem cells in elasmobranch tooth regeneration and the degree to which their functional roles and interactions are conserved, both within and among ancestral gnathostomes, such as sharks and rays.

2. A limited understanding of the role of gene regulatory networks in elasmobranch tooth regeneration and the degree to which they also may be conserved amongst gnathostomes.

3. A limited understanding of the role of gene network co-option in denticle evolution and development and how the deployment of a common genetic toolkit might reflect their respective developmental similarities and differences with teeth.

4. A limited understanding of putative dental stem cells and gene networks in tooth and denticle development as identified in this study, therefore warranting cell-fate mapping and manipulation of signaling pathways to better understand their respective roles.

The work presented in this thesis has addressed these issues using four approaches: Firstly, comparative investigation of the dental stem cell marker *Sox2* in catshark and ray tooth development and regeneration (Chapter 3). Secondly, comparative investigation of cell proliferation and conserved gene expression during catshark and ray tooth development and regeneration (Chapter 4). Thirdly, investigation of conserved gene expression in catshark dermal denticle development, with further comparisons to the catshark dentition (Chapter 5). Finally, cell-fate mapping experiments in the catshark to further characterise the role of putative dental stem cells, and functional manipulation of signaling pathways to further characterise their role in tooth and denticle development (chapter 6). Consequently, these four studies

address the substantial knowledge gaps detailed above of understanding the role of conserved gene networks in elasmobranch tooth regeneration, and in denticle development and evolution. In this section, the key findings for each study are summarised and discussed, with particular emphasis placed upon their implications in defining the direction of future related studies, especially with regard to the results presented in chapter 6.

7.2 *Sox2* defines a putative stem cell niche in the regenerating elasmobranch dentition

In chapter 4, the role of putative dental stem cells in elasmobranch tooth regeneration was investigated. Here, the catshark and ray were used as comparative models with contrasting dental phenotypes to investigate the expression patterns of the dental stem cell marker *Sox2*. A combination of *in situ* hybridisation and immunohistochemistry were used to investigate the distribution of *Sox2* mRNA and protein during tooth initiation, development and early replacement. In both the shark and ray, *Sox2* was expressed in the early epithelial thickenings characteristic of the odontogenic band. During development of the dental lamina, *Sox2* was subsequently expressed in a continuous stripe of O-ODE linked to the oral surface. During tooth development and early replacement, this expression pattern was initially maintained, with progressive localisation to a restricted cell cluster at the junction of the oral and dental epithelium. In the replacement dentition, this cell cluster is proposed to house a dental SCN required for continuous tooth regeneration. This is potentially demonstrative of a conserved ancestral role for *Sox2* in regulating elasmobranch tooth development. This study has therefore demonstrated two particularly novel findings.

Firstly, that in the elasmobranch dentition, *Sox2* potentially defines early dental competence, as implied by its expression in the thickened epithelium in advance of the dental lamina. This finding further supports recent consideration of the odontogenic band as a dental lamina primordium (Smith *et al*., 2009a). Given its conserved role in cell progenitor pluripotency and maintenance, *Sox2* is proposed to play an important role in the transient maintenance of progenitor cells required for development of the dental lamina. During tooth development and replacement, progressive localisation of *Sox2* to an epithelial cell cluster at the oral-dental epithelial junction marks a putative dental SCN linked to the dental lamina by a continuous *Sox2*+ epithelial connection, terminating lingually to developing tooth placodes. This expression pattern shows marked similarities to the reptilian dentition, in which *Sox2* also defines a similar oral-dental epithelial connection, suggesting a deeply conserved regenerative strategy common to gnathostomes with similar tooth

replacement systems (Gaete and Tucker, 2013). During the transition to early tooth replacement, *Sox2* expression within the adjoining O-ODE is maintained, suggesting the continued requirement for this connection with the oral surface. These findings therefore provide partial support for current hypotheses identifying putative sites of gene regulatory control and dental stem cells in the elasmobranch dentition (Smith *et al*., 2009b).

The second important finding here is shown by integration of the canonical Wnt pathway gene *Lef1*, providing further evidence of conserved interactions in the elasmobranch and reptilian dentitions. In the snake dental lamina, *Sox2* and *Lef1* are expressed in opposing domains, suggestive of a direct interaction. This is shown through induced activation of Wnt-*β-catenin* signaling, which increases the expression domains of *Lef1*, while restricting those of *Sox2* to the oral epithelium. In the snake dentition, *Lef1* is therefore presumed to regulate *Sox2* to maintain the balance between cell pluripotency and proliferation. In the elasmobranch dentition, *Lef1* is persistently expressed in the epithelium of replacement tooth placodes in similar opposing domains to *Sox2*, which is restricted to the lingual aspect of the dental lamina. These expression patterns therefore provide provisional evidence to support a conserved interaction involving spatial restriction of *Sox2* by *Lef1* to modulate the balance between progenitor maintenance and induction to tooth-specific fates (Gaete and Tucker, 2013). These initial conclusions culminate in the first hypothetical elasmobranch *Sox2* dental GRN model (Fig. 3.11).

7.3 An ancestral gene regulatory network perpetuates tooth regeneration in elasmobranchs

In chapter 4, the role of a conserved gene regulatory network (GRN) in elasmobranch tooth development and regeneration was investigated, with particular emphasis on the shark dentition. PCNA immunohistochemistry was first used to define cell proliferation during several stages of shark tooth development and the expression patterns of conserved dental patterning genes were investigated by *in situ* hybridisation. PCNA analysis identified the first epithelial thickenings and mesenchymal condensates characteristic of the odontogenic band, while concomitant

expression of *β-catenin, Shh, Ptc2, Pitx1/2* and *Bmp4* in the same tissues implied the onset of early dental competence. During subsequent development of the dental lamina and first generation teeth, *β-catenin, Lef1, Shh* and *Pitx1/2* were continually expressed in dental tissues, therefore implying roles in tooth initiation. During tooth development and replacement, an expanded set of markers further inclusive of *Sostdc1, Taz, Fgf3*/*10, Midkine, Meis2, Foxq1, Twist, Runx2, Dlx3* and *Sparc*, were reiteratively expressed in the dental epithelium and mesenchyme in patterns suggestive of conserved roles as activators, inhibitors, polarising and differentiating factors to maintain serial tooth regenerative capacity. In the ray, the expression patterns of a subset of these genes were investigated, further showing their deployment during tooth development and early replacement. The expression of these genes in near identical domains to the shark dentition further implies their conservation as an ancestral GRN, both within and amongst elasmobranchs.

In addition to providing much-needed data on the deployment of conserved signaling pathways in the replacement shark dentition, this study demonstrated two particularly important novel findings. Firstly, that the co-expression of several dental patterning genes, notably *Shh*, *Bmp4*, *Fgf3*/*10*, *Midkine* and *Taz*, in the distal tip of the tooth during morphogenesis, is indicative of the ancestral conservation of a modular signaling center analogous to the mammalian enamel knot. This enameloid knot is proposed to regulate the development of future tooth cusps in much the same way as its mammalian equivalent, as further implied by a corresponding lack of PCNA immunoreactivity in the same set of epithelial cells. These inferences are drawn from existing studies of the mammalian enamel knot, which is non-proliferative and undergoes apoptosis following completion of function (Jernvall *et al*., 1998). In partial contrast, at similar stages no corresponding lack of cell proliferation was detected in developing ray teeth, through the expression domains of *Shh* and *Midkine* remained focal to the medial IDE. This therefore presents partial evidence for and against the enameloid knot's conservation in the ray dentition. Despite this disparity, these findings are potentially indicative of the conservation of a cusp-making module in gnathostomes displaying some degree of cusp complexity, therefore highlighting its crucial role in defining future tooth shape.

The second important novel finding here is first shown by PCNA, which in common with *Sox2*, defines a continuous connection linking the oral epithelium with the dental lamina. During formation of the dental lamina, *Shh* and *β-catenin* are expressed at the junction between the oral and dental epithelium, followed by expression of *Foxq1*. At these early stages, *β-catenin* and *Pitx1*/*2* are also expressed in the adjoining outward-outer dental epithelium (O-ODE). When considered in light of existing studies of the reptilian dentition, which exhibits similar properties, these tissue loci are proposed to fulfill important roles. These are most notably to position the early dental lamina (*Shh*) and regulate the activity of early *Sox2*+ progenitor cells (*β-catenin, Shh* and *Foxq1*) as a putative epithelial SCN linked to the dental lamina by the adjoining O-ODE. During these early stages, *β-catenin* and *Pitx1*/*2* are further presumed to act in a cell context-specific capacity by regulating the activity of *Sox2* dental progenitors distributed between the SCN and dental lamina.

7.3.1 Elasmobranch dental GRN model

Collectively, these expression patterns inspire production of the first elasmobranch dental gene regulatory network model. Given the principle prominence of the catshark in this study, schematically this model is designed accordingly, however, given the significant serial molecular homology shared with the ray, this model is considered representative of both models. While hypothetical, this model provides novel insights into an ancient dental regenerative network, reflective of the ancestral state of gnathostomes, thus providing dual insights into the development and evolution of the early dentition. This further integrates the expression patterns of *Sox2* to provide a comprehensive picture of the dental regenerative GRN. In this model, gene co-expression domains in the odontogenic band establish initial dental competence, leading to formation of the primary dental lamina (Fig. 7.1A). Here, the same gene circuits remain active to shape the lamina and produce first generation teeth. Throughout this process, a continuous connection is maintained with the oral surface via the O-ODE, terminating in a putative dental stem cell niche (SCN) defined by expression of *Sox2* and additional genes. From here, putative transit amplifying (TA) cells proliferate into the dental lamina to supply the progenitors required for teeth to develop (Fig 7.1B).

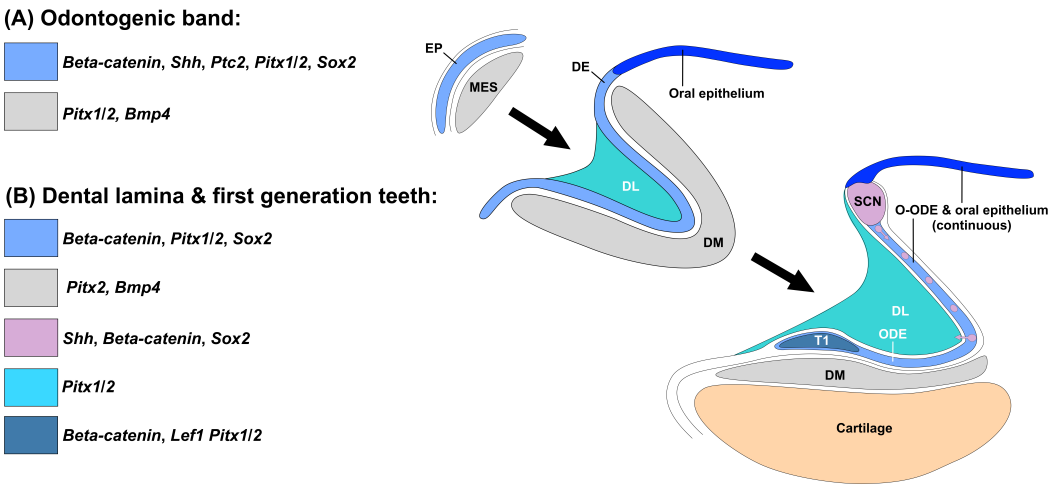


Figure 7.1 Model: Gene regulatory control of shark tooth initiation and development. In the early catshark dentition, early dental competence is marked by conserved gene expression within restricted regions of the oral epithelium (EP) and mesenchyme (MES), defining the extent of the odontogenic band (A). Sustained expression of these genes within the in-folding dental epithelium (DE) and underlying mesenchyme (MES) produces the primary dental lamina (DL) (B), and first tooth placodes. During development of first generation teeth, gene expression domains further define a continuous epithelial connection maintained between the dental and oral epithelium, which houses a putative dental stem cell niche (SCN) (B). This SCN is proposed to supply progenitor cells, stimulated to proliferate into the DL via the intervening outward-outer dental epithelium (O-ODE). Early signaling cascades within the lingual aspect of the DL, dental epithelium and mesenchyme stimulate these cells to commit to tooth-specific fates.

During development of first and subsequent tooth generations, this oral-dental epithelial connection is maintained, marked by sustained *Sox2* expression, while within the DL/ SL, gene circuits are reactivated in the dental epithelium and mesenchyme to regulate tooth initiation, bud formation and morphogenesis (Fig. 7.2). The expression patterns shown by these genes imply their reiterative deployment as putative activators, inhibitors, polarising and differentiating factors, to maintain continuous 'many-for-one' tooth regeneration. This process is proposed to occur as a result of sustained inward proliferation of TA cells from the surface dental SCN to provide a continuous supply of tooth progenitors for life-long tooth replacement. In this respect, the shark dentition shares considerable similarities with the reptilian and osteichthyan (cichlid) dentitions, thus highlighting a dental regenerative strategy common to polyphyodont gnathostomes (Gaete and Tucker, 2013; Fraser *et al*., 2013; Tucker and Fraser, 2014). Given current evidence regarding the dormant capacity for continuous tooth regeneration in mammals, this study adds renewed insights by further identifying, in ancestral gnathostomes, a common set of regulatory genes linked with several dental disorders (Juuri *et al*., 2013; Järvinen *et al*., 2006; Semina *et al*., 1996). It is anticipated that these, and future related efforts, will further enhance work focused upon tooth bioengineering and the design of novel dental regenerative therapies (reviewed by Townsend *et al*., 2009; Ikeda and Tsuji 2008; Volponi *et al*., 2010).

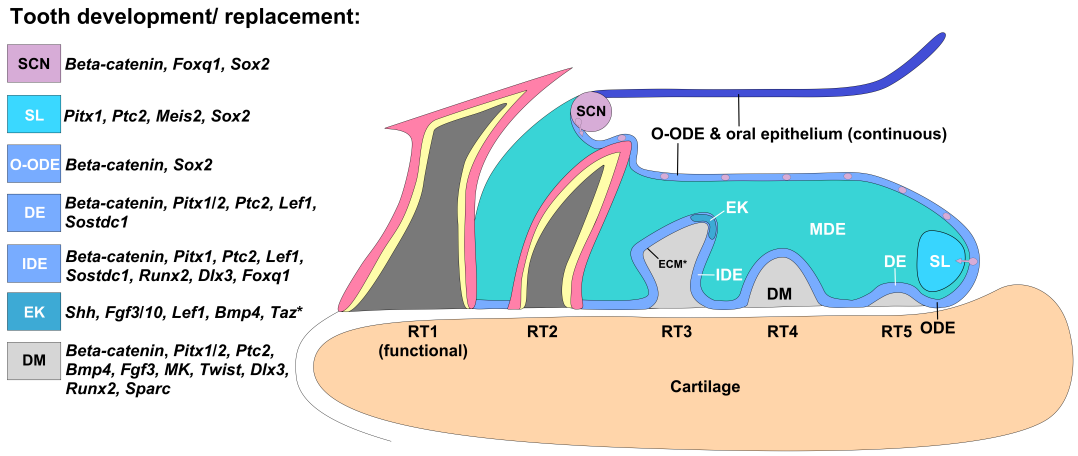


Figure 7.2 Model: Gene regulatory control of shark tooth development and replacement. During subsequent stages of tooth development, the same gene circuits remain active to produce the first tooth buds, followed by activation of a conserved signaling center (enameloid knot, EK) in the tooth tip to control cusp morphogenesis. Subsequent signaling cascades stimulate continued proliferation and differentiation of odontoblasts (DM) and enameloblasts (IDE) in advance of matrix deposition to complete the tooth in advance of function. The transition from primary to successional lamina (SL) is marked by reiterative activation of the same regulatory circuits to produce replacement tooth sets through maintenance of the required balance between cell maintenance, proliferation and differentiation. SCN, stem cell niche; O-ODE, outward-outer dental epithelium; ODE, outer dental epithelium; IDE, inner dental epithelium; ECM, extracellular matrix (*denotes *Taz* expression domains, both in the EK and ECM).

7.3.2 Elasmobranch 'many-for-one' tooth replacement recouples tooth regeneration and morphogenesis

Studies of the cichlid dentition further highlight the important role of polyphyodont gnathostomes in developing a comprehensive understanding of gene regulatory control of tooth regeneration (Fraser *et al*., 2013). In the current study, integration of conserved signaling pathways with *Sox2* adds to existing knowledge by further recoupling gene regulatory control of tooth development and regeneration in ancestral gnathostomes. While phenotypically modified in the osteichthyan and reptilian dentitions, the deep conservation in elasmobranchs of this core dental GRN, identifies a regenerative strategy common to these polyphyodont gnathostomes (Fig. 7.3) (Fraser *et al*., 2008; 2013; Handrigan and Richman, 2010a; 2010b; Handrigan *et al*., 2010; Juuri *et al*., 2013; Gaete and Tucker, 2013). In the mammalian dentition, these gene circuits are greatly modified, decoupling incisor renewal from molar morphogenesis to drastically reduce tooth replacement capacity (Harada *et al*., 1999; Wang *et al*., 2007; Juuri *et al*., 2013). This study provides further evidence to support the organisation of the vertebrate dentition into dissociable modules with intrinsic genetic control, marked by identification in elasmobranchs of the enameloid knot conserved for over 450 million years of gnathostome evolution (Stock, 2001; Vaahtokari *et al*., 1996a). These studies provide novel insights into gene regulatory control of tooth regeneration in an extant group representative of the ancient state of polyphyodont gnathostomes, while shedding new light upon the role of this dental GRN in determining the evolutionary trajectory of the vertebrate dentition.

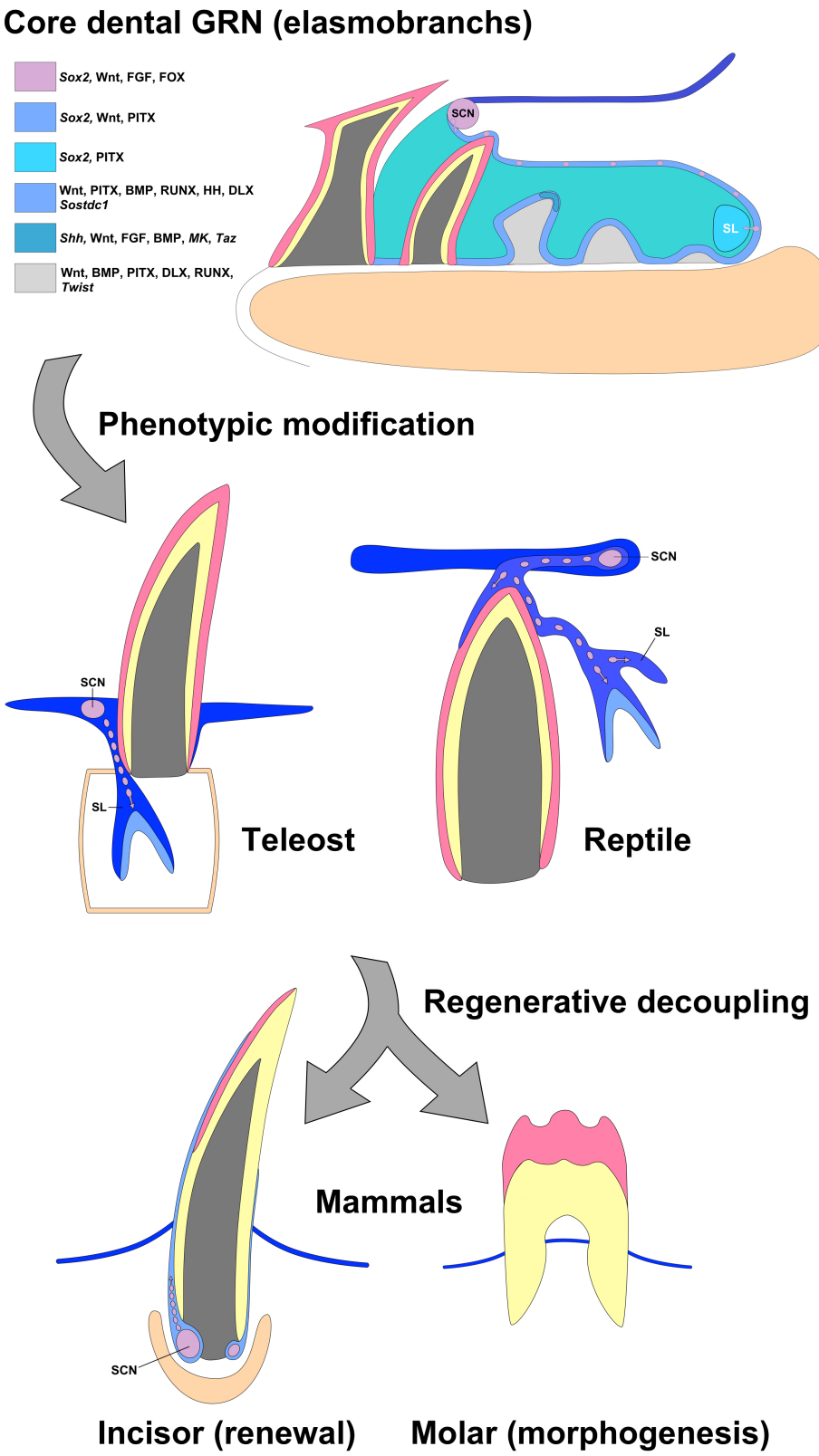


Figure 7.3 An elasmobranch core dental GRN is modified in successive gnathostomes. Recent studies of the cichlid dentition highlight the important role to be played of polyphyodont model systems in elucidating the global regenerative dental gene regulatory network (GRN) (Fraser *et al*., 2013). In the current study, these insights are further extended into deep evolutionary time by identifying in

elasmobranchs, core components of an ancient dental GRN. In polyphyodont gnathostomes, this dental GRN links conserved pathways with stemness to sustain tooth replacement capacity. While phenotypically diverse, in the elasmobranch, osteichthyan (cichlid) and reptilian dentitions, this is shown by the expression of a common set of odontogenic genes and *Sox2*, which marks a putative dental stem cell niche (SCN) connected to the successional lamina (SL) via a continuous epithelial connection (Fraser *et al*., 2008; 2013; Handrigan and Richman, 2010a; 2010b; Handrigan *et al*., 2010; Juuri *et al*., 2013; Gaete and Tucker, 2013). Significant modification of these gene circuits in mammals decouples incisor renewal with molar morphogenesis, drastically reducing tooth replacement capacity (Harada *et al*., 1999; Wang *et al*., 2007; Juuri *et al*., 2013). Despite this, in the shark dentition, a commonly expressed set of genes identifies a putative signaling center (enameloid knot) analogous to the mammalian enamel knot, thus demonstrating its conservation for over 450 million years of vertebrate dental evolution (Vaahtokari *et al*., 1996a).

7.4 An ancestral gene regulatory network patterns dermal denticles in sharks

In the study presented in chapter 5, the catshark was further used as a model to study conserved gene expression in denticle development. PCNA was first used to investigate epithelial-mesenchymal cell proliferation during denticle development, while *in situ* hybridisation revealed the conserved expression of *β-catenin, Lef1, Sostdc1, Midkine, Bmp4, Runx2* and *Twist*, showing the deployment of major signaling pathways in denticle development. This study has therefore demonstrated three particularly novel findings.

Firstly, that in denticle development these genes are expressed in domains comparable with teeth, suggesting the same deeply conserved core GRN to regulate denticle development. This is particularly apparent in the epithelial tip, which in common with the shark dentition shows reduced cell proliferation, accompanied by expression of *Shh, Fgf3* and *Midkine*. This therefore provides further evidence to support the ancestral conservation of a modular cusp-making signaling center analogous to the mammalian enamel knot. This study therefore reveals a significant degree of serial patterning homology between denticles and teeth, implying both to constitute the end products of an ancestral GRN periodically redeployed to generate functional morphological novelty through gene co-option. This study further culminates in the production of a hypothetical denticle GRN model (Fig. 5.7).

The second important finding here is shown through comparison of the spatial expression domains of oral teeth dermal and denticles, highlighting some commonalities and differences, which potentially impact existing models of odontode evolution. One such commonality is the expression of identical genes in internal teeth, which develop within a dental lamina, and external skin denticles in individual laminae, both separated by a hypothetical border at the oral margin (Smith and Coates, 1998). The simultaneous deployment of a single GRN in internal teeth and external denticles provides some supporting evidence for the ‘inside-out’ and ‘inside and out’ models, both of which propose teeth and denticles to have evolved

convergently with no required ectodermal input (Smith and Coates, 1998; 2000; 2001; Fraser *et al*., 2010).

However, one notable difference between the two is the differential expression of *Sox2*, restricted to the dental lamina in cell loci proposed to constitute the elasmobranch dental stem cell niche. The differential expression of *Sox2* is therefore presumed to reflect some important differences in patterning potential between the oral and dermal epithelia. This is most notably the capacity of oral teeth to regenerate sequentially via a serial patterning mechanism, in contrast to dermal denticles, which show no corresponding evidence of developmental organisation. While in general agreement with the 'inside-out' and 'inside and out' models, both of which propose odontogenic potential to be universal to the oral and dermal epithelia, this study therefore highlights some potential key differences (Smith and Coates, 1998; 2000; 2001; Fraser *et al*., 2010). *Sox2*+ taste buds, which show marked associations with the dental SCN, further open up the interesting possibility of an ancestral collaboration, following anterior transfer of odontogenic potential from the oro-pharynx in ancient fishes. In partial accordance with the 'inside-out' model, it could therefore follow that their recruitment by the advancing dentition provided the regenerative potential required for a serially patterned replacement dentition to evolve (Smith and Coates, 1998).

Finally, the serial expression of tooth and denticle patterning genes in elasmobranch sensory receptors highlights some additional key similarities to further suggest a common evolutionary origin. The 'inside and out' model' postulates that teeth and denticles may constitute the evolutionary derivatives of an antecedent epithelial sensory receptor, which in collaboration with newly acquired neural crest-derived cell fates, developed odontogenic potential. With the exception of *Sox2*, restricted to the regenerating dentition, this study therefore offers some supporting evidence for this, shown by the common deployment of these pathways in odontodes and sensory receptors. In line with this scenario, it could therefore follow that progressive remodulation of these common signals led the appearance and subsequent diversification of teeth and denticles (Fraser *et al*., 2010).

7.5 Fate mapping and functional studies of conserved signaling pathways

When considering the role of putative dental stem cells and conserved gene networks in tooth and denticle development, a recurrent theme has been the requirement for cell fate mapping and gain- and loss-of-function experiments to support initial conclusions. In chapter 6, work therefore focused upon consolidating existing data by using a combination of cell lineage tracing experiments (BrdU and DiI) and chemical manipulation experiments (bead implantations).

In order to further localise dental stem cells and their proliferating progeny, preliminary BrdU pulse-chase chase experiments were first carried out. This involved the direct application of the thymidine analogue BrdU (pulse) to the oral cavity, followed by several days development (chase) to label slow-cycling cells (SCCs) and transit amplifying (TA) cells, such as those speculated upon previously (chapters 3 and 4). Similarly, the lipophilic label DiI was injected into dental soft tissues to attempt to label similar cell types. Two varying methods of BrdU immunodetection marked putative label-retaining cells (LRCs) at the oral-dental epithelial junction, dental lamina and underlying mesenchyme. DiI further labelled putative SCCs at the oral-dental epithelial junction and a similar population in the successional lamina, interspersed by diffusely labelled population taken to be putative TA cells. These provisional results are in general agreement with those shown by investigation of *Sox2* expression, supporting the deployment of a dental stem cell niche in the elasmobranch dentition. Future cell lineage tracing studies using similar methods will be required to further validate these results.

Having identified the deployment of conserved signaling pathways in tooth and denticle development (chapters 4 and 5), chemical manipulation experiments were carried out to perturb these pathways and induce altered phenotypes. Loss-of-function experiments involved the implantation of several different bead-types loaded with the pathway inhibitors cyclopamine (hedgehog), SU5402 (FGF) and LDN (BMP). Gain-of-function experiments using Shh recombinant protein were carried out using the same methodology. In the case of tooth development, bead

implantations proved technically challenging. While several beads were superficially implanted adjacent to dental soft tissues, these were subsequently lost, calling into question the suitability of this method. Several beads were successfully implanted into the dorsal epithelium of the trunk adjacent to the early developing denticle rows. Following several days development, embryos were examined for signs of induced phenotypes, however, these were not apparent. In both cases, due to practical considerations it was not possible to examine the histology of treated tissues, leaving the overall results of these experiments unresolved. Despite this, it is anticipated that these initial efforts will facilitate the design and successful application of future experiments to investigate elasmobranch tooth and denticle development.

7.6 Conclusions and future directions

7.6.1 General conclusions

In conclusion, the findings presented in this thesis have contributed to current understanding of gene regulatory control of elasmobranch tooth regeneration in several ways. They have i) provided evidence to support the deployment of dental stem cells in the regenerating elasmobranch dentition, ii) widened knowledge of the role of conserved pathways in elasmobranch tooth regeneration, and iii) shown the role of conserved pathways in regulating denticle development, while inputting renewed perspective into current debate regarding the evolutionary origins of odontodes.

7.6.2 Agenda for future studies of gene expression in elasmobranch tooth development

This work has also highlighted several interesting directions for future research. In Chapter 4, work focused on identifying in the elasmobranch dentition, core signaling pathways involved in mammalian, osteichthyan and reptilian tooth development. While this study has identified several key pathways, these expression profiles present the limited output of a larger gene cloning effort aimed at widening the picture of the elasmobranch dental GRN. These are summarised in Table 7.1, inclusive of those expressed in the Notch and EDA signaling pathways, which are critical in tooth development (Mitsiadis *et al*., 1995a; Pispa *et al*., 1999; Peterková *et al*., 2002; Tucker and Sharpe, 2004). Despite their apparent conservation in the elasmobranch genome, evidence to support their expression in the dentition remains inconclusive. These current conclusions are drawn from *in situ* hybridisation studies carried out under standard and modified experimental conditions, yielding either negative or circumstantial expression data.

The important requirement for additional expression data to demonstrate the deployment of these pathways in the elasmobranch dentition is highlighted by existing studies showing their roles in other gnathostomes (reviewed in chapter 1:

sections 1.2.5-1.2.9). This persistent gap in knowledge therefore sets forth a specific future experimental agenda to broaden understanding of conserved gene expression in the elasmobranch dentition. To this end, it is anticipated that the availability of an expanded set of RNA probes as a further product of this study, will facilitate future expression studies of elasmobranch tooth development and replacement. The success of nucleic acid hybridisations is reliant upon careful modification of experimental conditions, such as salt concentration, annealing and wash temperatures, and probe length. When combined, these parameters form a complex set of variables, which frequently define the critical balance between stable annealment of hybrids to produce positive expression (signal) and removal of excess probe through washes to reduce background (stringency) (Wilkinson, 1993; Polak and McGee, 1998). It is therefore also anticipated that the experimental methods developed as a result of this study (sections 2.4.9-2.5.0) will provide the basis for future refinements to achieve these experimental objectives. The successful cross-hybridisation of catshark RNA probes with rays further exemplifies the versatility of this experimental approach, providing future opportunities to study the molecular basis of tooth regeneration in other elasmobranch species.

Table 7.1 Additional candidate genes cloned for expression analysis. Unannotated sequences (no accession) obtained from raw transcriptomic databases SkateBase (www.skatebase.org) and Vertebrate TimeCapsule, VTcap (http://transcriptome.cdb.riken.go.jp/vtcap). Cloned sequence identities confirmed by 3730 Sequencing and BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene name, abbreviation	Role	Pathway/ family/ interaction	Database/ accession
Activin, *Actv*	Rec	TGF-β	SkateBase
ATP-binding cassette sub-family G member 2, *ABCG2*	MAP	ABC	SkateBase
Axin inhibition protein 2, *Axin2*	STP	Wnt	SkateBase
B-cell lymphoma/leukemia 11B, *Bcl11b*	TF	Bcl11	SkateBase
Bone morphogenetic protein 6, *Bmp6*	Lig	TGF-β	SkateBase
Cysteine-aspartic acid protease 3, *Caspase3*	IST	Caspase	SkateBase
Distal-less homeobox 1/2/4/5, *Dlx1/2/4/5*	TF	Hox	JX270824.1-5.1, 7.1-8.1
Ectodysplasin A, *Eda* Ectodysplasin A receptor, *Edar* Ectodysplasin A receptor-associated adapter protein, *Edar-add*	Lig Rec Rec	Eda	SkateBase
Fibroblast growth factor receptor 1/2, *Fgfr1/2*	Rec	Fgf	SkateBase
Follistatin, *Fst*	Lig	TGF-β	SkateBase
Forkhead box A2, *FoxA2*	TF	Fox	SkateBase

Abbreviations: IST, intracellular signal transducer; LIG, ligand; MAP, membrane associated protein; REC, receptor; STP, signal transducer protein; TF, transcription factor; TCR, transcriptional co-regulator

Table 7.1 contd.

Gene name, abbreviation	Role	Pathway/ family/ interaction	Database/ accession
Frizzled, *Fz*	Rec	Wnt	SkateBase
GLI family zinc finger 2, *Gli2*	TF	Hh	EU196410.1
Heart and neural crest derivatives expressed 2, *Dhand2*	TF	Hand	VTcap
Iroquois homeobox 2, *Irx2*	TF	Hox	SkateBase
ISL LIM homeobox 1, *Islet1*	TF	Hox	VTcap
Jagged 2, *Jag2*	Rec	Notch	SkateBase
Kruppel-like factor 4, *Klf4*	TCR	Wnt	SkateBase
Leucine-rich repeat containing G protein-coupled receptor 5, *Lgr5*	Rec	Wnt	SkateBase
LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase, *Lfng*	Rec	Notch	SkateBase
Low density lipoprotein receptor-related protein 4/5/6, *Lrp4/5/6*	Rec	Wnt	SkateBase
Neurogenic locus notch homolog protein 2, *Notch2*	Rec	Notch	SkateBase
Noggin, *Nog*	Lig	TGF-β/ Wnt	SkateBase
Odd-skipped related 2, *Osr2*	TF	Osr	SkateBase
Paired box 9, *Pax9*	TF	Pax	KC507188.1

Table 7.1 contd.

Gene name, abbreviation	Role	Pathway/ family/ interaction	Database/ accession
Paired-like homeodomain transcription factor 3, *Pitx3*	TF	Pitx	VTcap
Patched 1, *Ptc1*	Rec	Hh	AB647262.1
Polycomb complex protein BMI-1, *Bmi-1*	TF	Wnt/ Hh/ Notch	SkateBase
POU class 3 homeobox 1, *Pou3f1*	TF	Hox	VTcap
Serrate, *Srrt*	Lig	Notch	VTcap
Smoothened, *Smo*	Rec	Hh	SkateBase
Programmed cell death 2, *PDCD2*	Lig	TNF-α/NF-kB	FP885828.1
Ring finger protein 1, *Ring1*	TF	HMG-box	SkateBase
Runt-related transcription factor 1/3, *Runx1/3*	TF	Runt	EU241883.1, DQ990015.1
Semaphorin 3C, *Sema3c*	Lig	Wnt	SkateBase
Sprouty, *Spry*	Lig	Fgf	SkateBase
SRY (sex determining region Y)-box 9, *Sox9*	TF	HMG-box	EU241880.1
Tumour protein P63, *TP63*	TF	P53	VTcap
Wingless-type MMTV integration site family member 5a/7b, *Wnt5a/7b*	Lig	Wnt	SkateBase

7.6.3 Agenda for future studies of dental stem cells in elasmobranch tooth development

When considering *Sox2* as a dental stem cell marker, further emphasis is placed upon the importance of identifying additional markers of 'stemness' in the elasmobranch dentition. In the present study, *in situ* hybridisation of several additional genes strongly linked with stem cells, such as *ABCG2*, *Bmi-1*, *Lgr5* and *Klf4* (Table 7.1), has proven inconclusive. Integration of the expression patterns of these and additional stem cell markers (e.g. *Oct4*/ *Pou5f1*, *Nanog* and *Stat-3*) therefore provides important future opportunities to enhance current knowledge of stem-regulatory control of elasmobranch tooth dentition (Rizzino, 2009; Li *et al*., 2011a; da Cunha *et al*., 2013). In association with this, future cell fate mapping/ lineage tracing experiments using the methodology developed in this study are likely to be of further value, as indicated by the preliminary results shown in chapter 6. In this respect, identification here of key expression domains in the elasmobranch dentition have proven important in fulfilling the initial requirements for future transcriptomic profiling. This is likely to be further achieved through laser capture microdissection (LCM), in which physical isolation of specific cell populations allows identification of differentially expressed genes by qRT-PCR. These transcriptional profiles are likely to prove informative when studying the differential expression of odontogenic and stem-regulatory genes expressed in the putative SCN, dental lamina and intervening dental epithelium.

7.6.4 Agenda for future studies of denticle development

In Chapter 5, work focused on the role of conserved gene regulatory networks in denticle patterning and discussed the putative generation of odontogenic diversity by gene co-option. Here, integration of tooth and denticle expression data provides evidence to support this hypothesis, inputting renewed perspective into existing models of odontode evolution. In both respects, this offers future opportunities to expand upon the gene expression profiles shown in the present study, while in conjunction with future studies of tooth development, identifying commonly and differentially expressed genes. It is anticipated that these future studies will shed

further light upon conclusions drawn from this work, especially with respect to genes expressed at the boundary of the oral margin, which may identify a zone of inhibition demarcating the oral epithelium and outer dermis.

7.6.5 Agenda for future functional studies of tooth and denticle development

Finally, the initial gain- and loss-of-function experiments carried out here further highlight an important future research agenda to understand the role of genes and associated pathways in elasmobranch tooth and denticle development. The results of the initial bead implantations currently remain largely unresolved. While future examination of the resulting histology may prove indicative of their successful application, the technical difficulties experienced along the way indicate that other experimental approaches may be more suitable. With respect to tooth development, a dental explant tissue culture system similar to those successfully used in reptile tooth development might therefore provide a suitable alternative (Buchtová *et al*., 2008; Handrigan and Richman, 2010a; Gaete and Tucker, 2013). Alternatively, whole embryonic chemical immersions, such as those previously used to study cichlid tooth development, may prove equally useful (Fraser *et al*., 2008; 2013). However, in comparison to the targeted approach shown by bead implantations, these may prove disadvantageous with respect to the ubiquitous treatment of tissues with chemicals. The drawbacks of this approach are therefore the potential for embryonic lethality and perturbation of the broader network of signaling pathway interactions, which may make interpretation of changes in gene expression and development difficult. Furthermore, whole-embryonic immersions may create additional problems regarding effective penetrance of chemicals into dental tissues. In this respect, an explant tissue culture system is further advantageous in that dental epithelial and mesenchymal tissues are immediately exposed to the effects of chemicals applied. A live-slice tissue culture system is therefore likely to be the most effective method to achieve this and may be of further benefit when labeling putative dental stem cells, allowing fate-mapping of dental progenitors using live-cell imaging.

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