ANALYSIS OF TOOTH REPLACEMENT IN ADULT LEOPARD GECKOS

by

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Abstract

Though most dentate vertebrates replace their teeth at least once in the course of their lives, the process of tooth replacement is poorly understood. This is mainly because the major tooth development model is the mouse which only has one generation of teeth. Our previous work suggested that tooth renewal in geckos might involve dental epithelial stem cells and that these putative stem cells become transit- amplifying cells when exposed to canonical WNTs. Here we further investigate this idea using adult leopard geckos (*Eublepharis macularius*). To further previous findings from our lab that the dental apparatus is a WNT responsive tissue we perturbed the WNT pathway by agonist and antagonist organ cultures of oral tissue explants. BIO stimulated proliferation at an intermediate concentration of 20 μM but not at higher or lower concentrations. This suggests that in vivo, cells are responding to gradients of WNT activity. We also looked at associated BMP and FGF pathways via in situ histology and organ culture manipulation respectively and found alternating patterns of gene expression. We then mapped areas of high canonical WNT signaling and found that nuclear staining for phospho beta catenin was principally found in the outer enamel epithelium and successional lamina. We moved to an in vivo strategy to allow for better tissue survival. Palatal injections of LiCl or the control reagent NaCl were delivered to the base of the maxillary teeth. We found that LiCl increased proliferation in the successional lamina and cervical loops, areas that normally have higher proliferation. We conclude that certain regions of the dental epithelium are sensitive to change in canonical WNT signaling and that this signaling is potentially kept to a localized region via BMP inhibition of the WNT pathway. Regions of the dental lamina that contain putative stem cells may require signals in addition to WNTs to stimulate the formation of transit amplifying cells. Future work will further elucidate the many signaling cascades required for tooth succession to occur.

Preface

All *Eublepharis macularius* animals were purchased from Triple R Corns in Aldergrove, BC.

SOX2 immunohistochemistry work was done in collaboration with Dr. Irma Thesleff's lab in Helsinki, Finland. My involvement was fixing, embedding and sectioning leopard gecko, corn snake, and ball python tissues to ship to Finland.

The animal work was carried out under UBC Ethics approval # A11-0352. This protocol was approved Dec. 22, 2011 and is renewed annually.

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List of Abbreviations

ALK3 aka BMPR1A - bone morphogenetic protein receptor 1A
ALK4 aka ACVR1B - activin A receptor, type IB
am - ameloblasts
BMP - bone morphogenetic protein
BrdU - 5-bromo-2'-deoxyuridine
CCD - Cleidocranial dysplasia
cl - cervical loop
d - dentin
DEJ - dentino-enamel junction
DKK3 - dickkopf WNT signaling pathway inhibitor 3
dl - dental lamina
e - enamel
FGF - fibroblast growth factor
FGFR - fibroblast growth factor receptor
GFP - green fluorescence protein
HH - hedgehog
ic - interstitial cells
IF - immunofluorescence
IGFBP5 - insulin-like growth factor binding protein 5
IP - intraperitoneal
je - junctional epithelium
LEF1 - lymphoid enhancer binding factor 1
LGR5 - leucine rich repeat containing G protein coupled receptor 5
LiCl - lithium chloride
LRC - label retaining cell
mxb - maxillary bone
NaCl - sodium chloride
oe - oral epithelium
oee - outer enamel epithelium
pCTNBB1 - phosphorylated ß-catenin
RUNX2 - runt related transcription factor 2
sa - secretory ameloblasts
sl - successional lamina
SOSTDC1 - sclerostin domain containing 1
Sox2/9 - SRY (sex determining region) box 2/9
sr - stellate reticulum
t - tooth
TA - transit amplifying cell
TCF3/4 - transcription factor 3/4
WNT - wingless
ZOI - zone of inhibition

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Finally I would like to thank the Douro Valley wine makers of Portugal. Your delicious goblets of late night encouragement were welcomed and appreciated whole- heartedly.

Dedicated to my mother Doris Holmes

Though you still don't understand what I do,
you've always supported me in doing it

Chapter 1 – Introduction

1.1 Tooth replacement and *Eublepharis macularius*

Most dentate vertebrates, including humans, replace their teeth. Snakes and other lizards continually replace their teeth throughout life (polyphyodonty) whereas humans and other mammals only replace their teeth maximally once (diphyodonty) (Jernvall and Thesleff, 2012). Diphyodont species generate deciduous or milk teeth which are lost and replaced by the permanent teeth. Tooth replacement in mammals happens towards the end of the fetal and into the post- natal period. The mouse dentition is highly specialized, consisting of continually erupting incisors with a set of three non-replacing molars joined by a toothless diastema. Despite the abundance of vertebrate species that replace their teeth, very little is known about the underlying mechanisms. In answering the question of which molecules control the tooth replacement process, this field of research can begin to realize the goal of replacing missing or broken teeth in humans.

Several groups have begun to look at the tooth replacement process in various model and non-model systems including teleost fish such as zebrafish, cichlids (Fraser et al., 2013), sharks (Zerina and Smith, 2005), and mammals such as the ferret and shrew (Handrigan et al., 2010; Huysseune, 2006; Huysseune and Thesleff, 2004; Jarvinen et al., 2009). Various species of fish prevail in publications on tooth replacement research.

Zebrafish form pharyngeal teeth which are generated in a unique way from epithelial crypts deep in the oral tissues (Melfi, 1982). Each fish tooth has a single successional tooth that forms when the erupted tooth is close to exfoliation. The differentiation and proliferation of the successional lamina appears to be differentially temporally controlled. In teleost fish the eruption of the tooth from the gum triggers the

successional lamina to proliferate outwards, starting the generation of a replacement tooth (Huysseune and Witten, 2008).

Reptiles differ from fish in that multiple generations of teeth exist underneath the erupted, functional tooth. To understand tooth replacement in reptiles I will begin by describing the initiation of teeth and how individual teeth form. Then I will explain the process of tooth succession. Teeth arise from the dental lamina, a band of thickening of the oral epithelium that marks out the position of the tooth row. For some animals such as snakes there are two rows of teeth in the upper jaw and these arise from two dental laminas. All dentate animals (reptiles and mammals) have only a single marginal row of teeth in the mandible. The lamina forms as an ingrowth of oral epithelial cells into the underlying mesenchyme of the jaw, and from it, by further cell proliferation, develop localized swellings on the labial side that go on to form tooth germs. Each tooth bud invaginates to form first a cap and then a bell stage enamel organ. The enamel organ is comprised of an outer and inner enamel epithelium connected at the base of the developing tooth by cervical loops as well as stellate reticulum region at the apex of the tooth between these two tissue layers. The enamel organ increases in size, until histodifferentiation begins. The dentin is deposited first, followed by the enamel. Then the cervical loops will either carry on to form the root or will cease growth so that the rootless tooth attaches directly to bone. In lizards and snakes the usual situation is for teeth to be pleurodont, fused to a slanting bone ridge. In alligators, a tooth socket forms similar to mammals and they have a thecodont, or root attachment (Berkovitz, 2000).

Many dentate reptiles have multiple generations of teeth in various states of formation. These additional teeth are all connected to each other by the dental lamina and

are called a tooth family. The dental lamina is always lingual to the teeth and is present throughout life. The extension of the dental lamina past the terminal tooth in the family is the successional lamina. In other words, the successional lamina originates from the outer enamel epithelium of the preceding tooth. Once the functional tooth is nearly ready to exfoliate, the tooth immediately behind it is fully mineralized and ready to erupt and 2-3 more teeth are in various stages of development. The mesenchyme surrounding the successional lamina or free end of the dental lamina begins to condense and the most distal tooth bud begins to form on the labial side. The initiation of generational teeth is slightly different from the first teeth, which originate from the primary dental lamina. (Richman and Handrigan, 2011)

In lizards the successional lamina begins to proliferate well before tooth eruption and then remains temporarily quiescent before commencing the replacement tooth generation process (Handrigan et al., 2010). I hypothesize that in fact there are potentially two discrete signaling events in tooth replacement, the initiation and proliferation of the successional lamina, and the differentiation of the successional lamina to form the enamel organ and replacement tooth.

1.2 Phylogeny of geckos

The leopard gecko belongs to a group of animals known as amniotes, which are any animal that develop with an extra- embryonic membrane. This group of organisms is sub-divided into the mammals (Synapsida) and the reptiles (Sauropsida). These two subgroups underwent parallel evolution, likely sharing a common extinct stem amniote. The reptiles retained the ability to replace their teeth throughout life whereas mammals

may only replace them maximally once. Not all reptiles have teeth however and so the Sauropsida can further be classified into two groupings of reptiles that do develop a dentition; Squamata, and Crocodilia. The leopard gecko is one of the most basal lizards in the squamate group, which includes all snakes and lizards. (Richman, 2013)

1.3 Selection of a reptile model for experimental studies

Though other reptilian models are being used elsewhere for research purposes there are barriers to using these animals for experimental research. The *Anolis carolinensis* (the green anole lizard) has a full genome sequence for example (Alfoldi et al., 2011). The disadvantages are that they are harder to obtain from breeders and harder to maintain in the lab. Their body size is small and this makes it challenging to perform intraoral procedures. There are also several crocodilians whose genomes will soon be published (St John et al., 2012). Obvious animal husbandry and manipulation hazards prevent development of these organisms as a good research model. Tooth development has been described for the African Rock python, Ball python, Corn snake, several venomous fanged snakes, Veiled chameleon, as well as another gecko species, the Pictus gecko (Buchtová et al., 2007; Buchtová et al., 2012; Vonk et al., 2008; Zahradnicek and Horacek, 2008; Zahradnicek et al., 2008). So far a genome is only available for the Burmese python so molecular studies remain to be difficult in most reptile models.

We chose to exclusively study the Leopard gecko (*Eublepharis macularius*) due to its ease of husbandry and safe in vivo manipulation capabilities. The Leopard gecko forms marginal teeth in a manner very similar to mammals. Geckos replace their teeth continually throughout their lives. They also have a set of over 80 teeth on each jaw,

which increases the power of the study. Our lab developed the Leopard gecko as a model organism for tooth replacement research and has published several papers on embryos and post-hatching adult geckos (Handrigan et al., 2010; Handrigan and Richman, 2011; Richman and Handrigan, 2011).

1.4 Stem cell markers in comparative systems

A stem cell is a slowly dividing cell that can give rise to a multitude of different cell types. Stem cells reside in a specific niche of an organ or tissue. The stem cell niche is an environment that promotes asymmetric cell division. One daughter cell renews the stem cell population while the other daughter cell becomes a rapidly dividing transit amplifying cell. The asymmetric cell division was first demonstrated in the hair follicle. Tritiated thymidine was administered to newborn mouse pups over 7 days and the animals were euthanized after an additional month of growth. Here, a population of slow cycling cells was shown to be concentrated in the hair follicle bulge (Cotsarelis et al., 1990). This stem cell population gives rise to rapidly dividing transit- amplifying cells while self- renewing. The transit amplifying cells undergo a limited number of divisions before differentiating into matrix secreting cells at the base of the follicle (Alonso and Fuchs, 2006). These label retaining cells were later confirmed to indeed be stem cells, which give rise to new hair follicles as well as reepithelializing the epidermis for wound repair (Blanpain et al., 2004).

The identification of stem cells relies on the combination of markers present and absent on a cell population. In other words, it is not a single protein that identifies a stem cell but rather a group of proteins that form an expression profile for a particular cell

type. The recent review entitled "25 Years of Epidermal Stem Cells" (Ghadially, 2012) lists hundreds of markers for epithelial and mesenchymal stem cell populations throughout the body. One promising marker that is expressed in many types of epithelial stem cells is Lgr5, a Wnt target gene and cell surface protein (Haegebarth and Clevers, 2009). In the intestinal crypt and hair follicle bulge Lgr5 specifically labels stem cell populations. Lgr5 is extensively expressed throughout embryonic development but ceases global expression at the time of birth. It remains to be expressed in small groups of cells in the eyes, intestine, liver, lungs, and hair follicle and could potentially be a long sought after universal stem cell marker.

A marker of a stem cell niche is the extracellular matrix molecule, tenascin. Tenascin is expressed in the hair bulge (Kloepper et al., 2008) possibly supporting the stem cells. In the bird, the feather follicle contains a stem cell population that gives rise to all components of the feather (Yue et al., 2005). The collar niche is marked by mesenchymal Tenascin C expression surrounding the epithelium (Lin et al., 2013). Thus Tenascin may be a good marker of stem cell niches.

Identifying stem cells solely by marker expression is not sufficient to prove they are multipotent. Many groups have used transplantation and lineage tracing experiments to define their fate (Lin et al., 2013; Yue et al., 2005). An example of a transplantation study is one carried out on the mammary gland (Shackleton et al., 2006). It was found that a marked increase in α6 and β1 integrins existed in a cell population residing against the basement membrane of the mammary gland duct. When these cells close to the basement membrane were transplanted to tissue devoid of glands, a full mammary gland

was regenerated. This experiment conclusively demonstrated the integrin positive cells were indeed multipotent and were likely stem cells.

Lineage tracing has been carried out in detail in the developing hair bulge using the promoter of transcription factor Sox9 to drive expression of LacZ in all Sox-9 expressing stem cells and their progeny (Nowak et al., 2008). The Sox9-Cre/R26R mice not only had blue hair follicles but also had blue sebaceous glands and epidermis. Upon wounding the skin, cells from the hair bulge healed the epidermis and gave rise to new hair follicles and sebaceous glands. A similar experiment has been done in the mouse continuously erupting incisor using a different member of the Sox family and ameloblasts were labeled (Juuri et al., 2012).

1.5 Dental stem cells

The reptilian dentition is renewed throughout life and the formation of the successional lamina is a key factor in this process. The reptilian dental epithelium contains a group of label retaining cells that could be stem cells. In my project I will explore the identity of the putative dental epithelial stem cells based on another system; the hair. Others have speculated that epithelial stem cells might regulate tooth replacement as they do hair renewal (Huysseune and Thesleff, 2004).

The second system that can be used as a comparison for our work is the mouse continuously erupting incisor. It was recently shown that LacZ driven by an inducible *SOX2* promoter in transgenic mice positively identify stem cells in the labial cervical loop of the continually erupting incisors (Juuri et al., 2012). These stem cells give rise to blue ameloblasts on the labial aspect of the tooth, which secrete the enamel necessary for

continual tooth growth. The stem cell population and its niche are controlled in part by Activin, BMP, FGF, and Follistatin signaling events (Wang et al., 2007).

There are also stem cells in the mesenchymal component of teeth. For example two different cell surface markers CD44 and STRO-1 are commonly found in dental pulp stem cells (Huang et al., 2009a; Stevens et al., 2008). It was thought that dental pulp stem cells would show similarities to bone marrow stem cells but this does not appear to be the case. It was found that CD34 specifically marks bone marrow stem cells and that these cells give rise to all of the cellular components of blood (Kiel et al., 2005), however dental pulp stem cells are markedly negative for this stem cell marker (Yan et al., 2010).

1.6 Stem cell studies in gecko

Using the pulse-chase method of identifying putative stem cells that the hair follicle researchers administered (Cotsarelis et al., 1990), our lab was successful in identifying a putative stem cell population on the lingual side of the dental lamina - adjacent to the terminal enamel organ (Handrigan et al., 2010). These are WNT responsive cells that overlap with the expression of stem cell markers such as *LGR5, DKK3,* and *IGFBP5*. This population of cells is presumably self- renewing while giving rise to transit amplifying cells that differentiate into successional laminae of future tooth generations. This population is also potentially controlled by a stationary mesenchymal niche in neighboring tissue (Richman and Handrigan, 2011). Understanding the stem cell origins of tooth replacement is only the first step of many in fully teasing apart what this entire process entails. Tooth replacement is a dynamic process and must be looked at not on an individual tooth basis but on a whole mouth model where signaling cascades and

cross-talk control not only development of one tooth but might actually inhibit or activate development of neighboring teeth also.

I chose a subset of epithelial stem cell markers and one from the mesenchymal component of teeth to define stem cell populations in gecko teeth. My aim was to use the location of these cells to propose their function during continuous tooth replacement.

1.7 Zahnreihen and zones of inhibition theory

Tooth replacement in reptiles appears to be a scheduled event that is not influenced by breakage or wear but simply happens on a temporal basis (Berkovitz, 2000; Edmund, 1960). It is also crucial to understand that this replacement process is not happening at the same time along the jaw but is patterned, with teeth 1,3,5... being most similar in developmental stage and teeth 2,4,6... also being similar but different than the first grouping (Edmund, 1960; Röse, 1893). This alternating pattern of developing teeth in the replacement process begs the question as to what is controlling this phenomenon and why it occurs.

Almost a century ago this wave pattern of development in teeth was observed and the term Zahnreihen was coined (Woerdeman, 1921). In this model there is a staggered development of replacement teeth on a diagonal line lingual and caudal to the erupted tooth. This set up of repeating Zahnreihe units was thought to exist so that there would always be an erupted tooth present in an area of the jaw, even if its neighboring tooth had shed. These diagonal patterns are not precise though, with different species exhibiting different replacement patterns, so it is very hard to generalize (Edmund, 1960).

To explain how the Zahnreihe exist it was proposed that a zone of inhibition (ZOI) is present in the dental lamina preventing teeth from developing within this spherical radius (Osborn, 1970; Osborn, 1977). In this model a developing tooth or mesenchymal region secretes an inhibitory signal to the surrounding tissue. At distances where this signal is still strong enough to act, no tooth will develop. This scheme mainly describes tooth patterning in the developing jaw—as the jaw lengthens during growth more distance is created posterior to the developing tooth emitting inhibitory signals, and out of range of the radius of this signal another tooth can develop. In adult animals a similar inhibitory signaling may still be patterning the jaw but possibly through a different mechanism.

Mouse tooth experiments have successfully supported the ZOI model. When the mesenchyme was trimmed away from mouse incisors grown in organ culture, extra teeth developed, potentially showing that the mesenchyme was inhibiting the dental lamina and subsequent tooth replacement in these monophyodont animals (Munne et al., 2009). In further trying to understand which signaling pathways could contribute to the ZOI, a conditional deletion of *SOSTDC1* (a WNT and BMP antagonist) in the epithelium and mesenchyme lead to double incisor formation (Munne et al., 2010). In the reptile, there may be signaling between the dental lamina and adjacent mesenchyme that is creating an inhibitory environment and thus leading to alternating patterns of dental development. To further elucidate this network of signaling one must first better understand signals present in the dental lamina, successional lamina, enamel organ and adjacent mesenchyme.

1.8 WNT signaling in the tooth replacement process

There are minimal studies on the molecular signaling underlying differentiation and the mechanisms driving the successional lamina to replace teeth. The knowledge of which molecular signals may be involved in tooth replacement has arisen from studies on *Mus muscularis*, the predominant model for studying tooth generation. Mutations that lead to supernumerary teeth, dental agenesis, or tooth fusion can give insight into what signals may be controlling the successional lamina.

There is mounting evidence that the most important signaling events controlling the successional lamina are WNT/β-catenin cascades. The canonical WNT signaling pathway involves stabilization and nuclear accumulation of β-catenin (Willert and Nusse, 2012). The WNT family of secreted proteins consists of 19 members in mammals with the same 19 "WNT-like" genes annotated in the Anole lizard, that can trigger various cellular responses (Willert and Nusse, 2012). In the oral epithelium, WNT is necessary for activation of the odontogenic mesenchyme and resulting tooth initiation. In mouse studies, activation of WNT signaling leads to multiple supernumerary teeth (Järvinen et al., 2006). Numerous ectopic teeth are formed all over the oral cavity. The culture of single molar tooth germs revealed that a single tooth can give rise to up to 40 teeth. WNT pathway signal transducers (AXIN2) and target transcription factors (LEF1) are also strongly and specifically expressed in the proliferating successional lamina and adjacent mesenchyme of the snake (Handrigan and Richman, 2010b). *AXIN2,* a downstream target of WNT signaling (Clevers, 2006), has been seen to cause tooth agenesis when mutated (Lammi et al., 2004). *AXIN2* is an inhibitor of the WNT pathway, thus further revealing the importance of WNT signaling in successional lamina control and tooth replacement.

It has also been identified that *TCF3* and *TCF4* are expressed in the dental lamina and enamel organ respectively (Handrigan et al., 2010). These are both downstream targets of active WNT signaling and show that WNTs are indeed highly involved in proliferation of the successional lamina and development of the dental apparatus.

1.9 Other signaling pathways controlling successional lamina formation

Extensive research has been done exploring the roles that WNTs might play in successional lamina formation. There are however many signaling cascades that are orchestrating the tooth replacement process. It has been shown that WNT activity is regulated by HH (hedgehog) and BMP in the ball python (Handrigan and Richman, 2010b). Here researchers looked at the expression of several WNT and BMP members and found that *WNT10B* and *WNT6* were restricted specifically to the labial side of the early dental lamina. When the BMP pathway was inhibited *LEF1* expression was increased suggesting that BMPs negatively regulate the expression of WNT pathway ligands, but this has not yet been tested experimentally. *BMP2* is subsequently expressed in the labial mesenchyme surrounding the successional lamina, correlating with maintaining WNT activity on the labial side where teeth will form. *BMP2* may also direct proliferation of the successional lamina in a lingual direction. Phosphorylated SMAD was also seen localized to the nucleus in the developing enamel organ and successional lamina of developing teeth in the python, again showing BMP activity is present in these structures (Handrigan and Richman, 2010b). None of the aforementioned work looks directly at the successional lamina or stem cell responsiveness in adult geckos with fully formed tooth families.

Other groups studying the mouse incisor cervical loop have investigated BMP and, FGF signaling. *FGF10* null mice do not develop a cervical loop at the base of the tooth suggesting that this must be a survival factor necessary for maintaining the stem cell population that resides in the cervical loop (Harada et al., 2002). It has also been shown that the mesenchyme surrounding the cervical loop expresses *FGF3* and *FGF10* with their receptors *FGFR1B* and *FGFR2B* present in the dental epithelium, implicating mesenchymal control over epithelial proliferation (Kettunen et al., 1998). Another group (Wang et al., 2007) further unraveled the network of signals controlling the cervical loop stem cell population. They found that *FGF3* was strongly expressed in the mesenchyme surrounding the cervical loop on the labial side where ameloblasts differentiate and produce enamel for the continually erupting tooth. On the lingual side of the cervical loop *BMP4* in the mesenchyme was restricting *FGF3* expression via *ALK3* and *ALK4* in the dental epithelium. This restriction of FGF activity is partly why there is no differentiation of ameloblasts on the lingual side of the incisor and subsequently no enamel deposition.

1.10 Aims of my study

It is apparent that the process of tooth development and replacement is very complex with multiple signaling pathways acting at different times to control the proliferation of structures, direction of growth, differentiation of cells, and maintenance of a stem cell population. Here I chose to look for stem cell markers using pulse-chase combined with immunofluorescence studies, to investigate the roles that WNT, BMP, and FGF signaling may play in the dental epithelial morphogenesis as well as periodicity in

teeth along the dental arch, and to perform proof of principle experiments to show that tooth replacement can be manipulated in vivo in adult geckos.

Chapter 2 - Materials and Methods

2.1 Animal husbandry

Both adult and embryonic Leopard geckos were obtained from a local breeder (Triple R Corns, Aldergrove, BC). Eggs were washed with iodine tincture 1:10,000 to disinfect and incubated in moistened vermiculite at 30°C until needed. Adult geckos were kept in terraria with heat-pads underneath half of the bottom surface area to allow the animals to self-regulate their temperature. "Hide-houses" with damp paper towel were also placed in each terrarium to assist the animals with shedding of skin and to give them a dark place to hide, relieving stress on the animals. Water dishes were cleaned and changed daily and the animals were fed mealworms that had been previously gut-loaded for two days with a blend of dry dog food, skim milk powder, rolled oats, and a prepackaged reptile vitamin powder blend. Mealworms were also provided with a potato as a consumable water source. Daily paperwork tracked the cleaning of the terraria, feeding, and water change schedule. Weekly weighing of the animals took place to ensure good health of the animals.

2.2 Oral BrdU administration

Animals used for the pulse/chase study were fed a solution of 1mg/ml of BrdU in water orally every day for 2 months during the labeling period. A yellow pipette tip was placed onto the end of a plastic transfer pipette, and approximately 300-500 µl of BrdU was loaded. The BrdU pipette was placed into the mouth of the animal, and liquid orally

administered. The throat was also massaged to assist with swallowing the BrdU and to ensure complete ingestion.

2.3 Oral LiCl injections

Preparation for oral injections began 12 hours prior with an oral administration of 0.1 mg/kg body weight Meloxicam (a common veterinary non-steroidal analgesic). On the day of the injections the animal was first placed under general anesthetic using a forced inhalation of 4% isoflurane. The mouth of the animal was then propped open and a palatal nerve block of 0.5% lidocaine with 1:400,000 epinephrine at a maximum dose of 7 mg/kg body weight was injected. This both assured the animal would feel no pain from following injections as well as constricting capillaries to keep experimental solutions localized to injection site. Under a stereoscope, approximately 150 µl of 1M LiCl with 2.5 mg/ml DiI (1,1'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Invitrogen) was injected to the base of the teeth through the palatal tissues along the length of the jaw with 3-4 injection sites. 1M NaCl with 2.5 mg/ml DiI was used on the contralateral side as a control. The animal was then allowed to awake and monitored for responsiveness post-operatively. These injections continued for three consecutive days. On the fourth day a 200 µl intraperitoneal injection of 10 mg/ml BrdU was administered 3 hours prior to euthanasia.

2.4 Tissue explant organ cultures

Animals were anaesthetized using forced inhalation of 4% isoflurane and then euthanized via decapitation. Oral tissues were rough dissected in cold PBS and then transferred to cold dissection media (1X Hanks without Ca^{2+}, Mg^{2+}, and 10% fetal bovine serum) for finer dissections. Dental tissues were then grown via Trowell-type organ culture (Handrigan and Richman, 2010a) in media containing 1:1 DMEM:F12, 10% fetal bovine serum, 1 μg/ml ascorbic acid, 10 mM beta glycerol phosphate disodium salt hydrate, 1X antibiotic/antimycotic (Gibco), 1X L-glutamine, and 10 μg/ml gentamycin. Experimental small molecules were then added to this media at several concentrations (See Table 2.1). Bead implants were placed into some cultures. Affi-gel blue beads 100-200 μm in diameter were soaked in human Fgf2 (1 mg/ml, Peprotech) or Tris (tris (hydroxymethyl) aminomethane) buffer and placed inside the gum tissue through a small incision towards the base of the teeth.

All tissues were cultured in a humidified incubator with 5% CO_2 at 30°C with daily media changes for various amounts of time depending on experiment. Before fixing the tissues, 10 μM BrdU was added for 3 hours.

Table 2.1 Small molecules used for organ culture media

Molecule Name	Activity	Pathway (loss or gain of function)	Company obtained from	Concentrations used
XAV939	Tankyrase PARP activity inhibitor	Wnt(-)	Cayman Chemical	0.1μM 1μM 10μM
BIO	GSK-3β inhibitor	Wnt(+)	EMD BioSciences	2μM 20μM 60μM
SU5402	Competitive inhibitor of the tyrosine kinase activity of FGFR-1	Fgf(-)	Calbiochem	0.1μM 1μM 10μM

2.5 Statistical analysis of BrdU cell proliferation data

BrdU labeled sections were photographed and two channels were separated, one for nuclear staining with Topro3 and the other for BrdU staining with AlexaFluor 488. Regions of interest were identified using morphological features of the teeth and relationship to the oral cavity. Total cell counts for each region and each channel were made using ImageJ Cell counter plugin and the percentage of cells that incorporated BrdU was determined. For organ cultures the biological replicates were independent

cultures (n= 3 per condition). For the LiCl injected animal the biological replicates consisted of 7 different teeth in the same animal. There were two technical replicates for each section and mean percentage BrdU labeled cells were used for statistical analysis. Statistical analysis was carried out with Statistica v.7 (StatSoft) software. A multi-factorial ANOVA was performed followed by Tukey's post hoc testing to determine between group differences. The dependent variable was the proportion of BrdU labeled cells and treatment and region were the two independent variables. P values 0.05 or less were considered statistically significant. Using an ANOVA test assumes the data is normally distributed. Though my "n" values were potentially too low to make this assumption, performing parallel t-tests (data not shown) exhibited the exact same documented significant differences. The factorial ANOVA statistics were used due to the fact that Standard Error is included in the statistical algorithm. Graphical illustration of BrdU data used error bars representing standard deviations around the mean.

2.6 Tissue preparation for IF and histology

Organ culture tissues were fixed overnight in 4% Paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS) at 4°C, decalcified with 15% EDTA for 2 weeks at 4°C and then embedded in 2% agarose gel with water. Agarose blocks with small segments of the jaw were dehydrated to 70% ethanol and processed into wax for sectioning.

For adult jaws, tissues were dissected and placed into Bouin's fixative for 24h, followed by 48 h of decalcification in Morse solution (Nakatomi et al., 2006). Morse's solution consists of 10% w/v sodium citrate and 22.5% v/v formic acid and is used at room temperature until bone is soft when probed with a pin. Bouin's fixed tissues were

then washed in multiple changes of 70% ethanol to remove the fixative before processing into wax.

2.7 Immunofluorescence and TUNEL staining

Tissues processed into paraffin wax were cut into 7 μm coronal sections. Sections were mounted on TESPA (3-triethoxysilylpropylamine, Sigma) coated slides and baked at 65° C overnight, or on Superfrost Plus slides (Thermo-Fisher). Paraffin was removed from slides with two twenty- minute xylene baths and tissues were then rehydrated through a graded alcohol series. Tissues were then washed in two changes of PBS. Antigen retrieval was performed via steaming tissues for 20 minutes submerged in Diva Decloaker (BioCare Medical). After a PBS wash, 5% horse serum was applied for 2 hours at room temperature to block non-specific binding of antibody. Primary antibody (See Table 2.2) was then applied overnight at 4°C. The next day, three 5 minute washes in PBS were performed before applying secondary antibody AlexaFluor 488 goat anti-mouse, or donkey anti-rabbit (Invitrogen) diluted to 1:200 in PBS for 1 hour at room temperature. Alternative sections were used for TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assays as per ApopTag plus in situ apoptosis fluorescein detection kit (Millipore). This is the indirect method of labeling TUNEL positive cells using Digoxygenin tagged oligo dT which is detected with a secondary antibody to Dig that is conjugated to FITC. Tissues were then washed in two changes of PBS before applying nuclear stain TO-PRO-3 Iodide (Life Technologies) diluted to 1:5000 for 20 minutes. After three washes in PBS slides were mounted using ProLong Gold antifade reagent either with or without DAPI (Life Technologies) and imaged with

confocal microscopy. Images were taken with a Leica DM6000 CS confocal microscope. The microscope was focused at the level of the brightest nuclear stain for TO-PRO-3 using either 25X water immersible long working distance objective or 63X oil objective. On average 4-5 image slices were taken through each 7 µm thick section with the confocal. A maximum image projection was created to combine signal from all slices and give a more complete representation of expression in the tissue using ImageJ software.

Table 2.2 Primary antibodies

Antibody	Company obtained from	Antibody type and animal raised in	Dilution used
anti-Sox9 (human)	Aviva Biosystems (P050)	Polyclonal (rabbit)	1:50
anti-CD44 (human)	Hybridoma Bank (HC4C)	Monoclonal (mouse)	1:100
anti-phospho β-catenin (CTNNB1, human)	Millipore (05-665)	Monoclonal (mouse)	1:100
anti-phospho SMAD (mouse)	Cell Signaling (3104)	Polyclonal (rabbit)	1:200
anti-Sox2 (human)	Millipore (AB5603)	Polyclonal (rabbit)	1:500-1:2000
anti-Tenascin (chicken)	Hybridoma Bank (M1-B4)	Monoclonal (mouse)	1:100
anti-Cytokeratin 19 (human)	DAKO (RCK108)	Monoclonal (mouse)	1:150
anti-CD34M (mouse)	LifeTechnologies (MEC14.7)	Monoclonal (rat)	1:100
anti-CD34R (mouse)	LifeTechnologies (RAM34)	Monoclonal (rat)	1:100
anti-Integrin β1 (mouse)	BD Pharmingen (553715)	Monoclonal (rat)	1:100
anti-Integrin β4 (mouse)	Pharmingen (553745)	Monoclonal (rat)	1:100
anti-Integrin α6 (human)	Pharmingen (33771A)	Monoclonal (rat)	1:100

2.8 Non-fluorescent immuno-staining

Sections for Sox2 immunohistology (Millipore) were rehydrated and heated in a microwave in 10 mM sodium-citrate buffer (pH 6.0). Immunostaining was performed using the Ultravision Large Volume Detection System Anti-Rabbit, HRP Kit (Thermo Scientific) and the DAB Peroxidase Substrate Kit (Vector Laboratories, SK4100). This work was done by the Thesleff lab (Juuri et al., 2013).

2.9 Cloning of cDNAs

Jaw segments containing teeth from an adult gecko were snap-frozen in liquid nitrogen and then processed through Qiagen's RNeasy Midi kit (Qiagen) using the QIAShredder (Qiagen) for isolation of total RNA. Random-primed cDNA was created using ABI High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems).

2.10 Degenerate primer design

NCBI GenBank was used to obtain mRNA sequence of *Mus musculus*, *Gallus gallus*, and *Anolis carolinensis*. FASTA formatted sequence was then entered in EBI's ClustalW2 multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the output screened for conserved regions across species that flank a minimum 400 base pair region. Conservative sequence was then entered into the Primer3 online tool (http://frodo.wi.mit.edu) and primers with lowest number of degenerate sites across species were chosen. For *SPRY2* the forward primer 5'-ACACRAATGAGTACACRGARS-3' and reverse primer 5'-GGATAMGTGCACTCCTTRCACT-3' were chosen which would yield a product of 418 base pairs. Sequence has been submitted to Genbank and assigned accession number KC822433. Previously cloned gene fragments were also used including a 772 base pair *WNT7A* clone, GenBank accession number GU080292.1, and a 606 base pair *BMP2* clone, GenBank accession number JF795594.1.

2.11 Degenerate PCR

A PCR master mix was combined with 1X PCR buffer, 0.4mM dNTP mix, 0.4μM forward and reverse primer sets, 1 unit Taq DNA polymerase, and 20 ng of template cDNA. Master mix was aliquoted into 0.5mL PCR tubes. Tubes were placed in a thermocycler for 30 rounds of: 94°C denaturation for 45 seconds, 45 seconds of annealing at temperature 3°C below Tm of specific primer set, and elongation at 72°C for one minute. Following these 30 cycles a final 72°C elongation was performed for 10 minutes and the samples then brought back down to 4°C for storage. One tenth of the completed reaction mix was then mixed with loading dye and run on a 1% agarose gel made with TAE (Tris-acetate-EDTA) along with a 100 b.p. ladder. If a positive band of correct size was identified then the remainder of the reaction mix was run on a new gel for gel extraction and purification.

2.12 Cloning of PCR products

PCR amplicons of predicted sizes were isolated from gel medium, and cloned into pGem-T Easy Vector System kit (Promega). After overnight incubation at 4ºC this ligation mix was then transformed into DH5α competent bacteria for replication. Ampicillin-treated plates were treated with 40μL each of X-Gal (40mg/mL) and IPTG (0.1M) before bacterial plating in order to screen for colonies with inserts. After overnight incubation, white colonies positive for insert were then used to inoculate 5mL of culture media with Ampicillin that were grown overnight at 37°C with shaking. Plasmid DNA was purified using a standard alkaline-lysis DNA mini-prep protocol, and concentrations were read by nano-drop spectrometry. Samples were then sent to

GENEWIZ, Inc. for sequencing. Sequence results from GENEWIZ were BLASTed against the nucleotide database using Blastn to confirm sequence identity. Sprouty2 was successfully cloned.

2.13 Radioactive in situ hybridization

A developed protocol (Rowe et al., 1992) with the following modifications was used. Probes are cleaned up using BioSpin 30 (BioRad) columns instead of LiCl precipitation. DTT was also reapplied to the eluent to prevent oxidation of sulfur due to DTT being stripped through the column. *WNT7A*, GenBank accession number GU080292.1, and *BMP2*, GenBank accession number JF795594.1 were successfully hybridized. Spry4 probe was also used, but no signal was seen. Probe concentration was 10^5 cpm/µl and slides were exposed for between 4 and 6 weeks at 4°C in the dark.

Chapter 3 - Results

3.1 Orally administered BrdU is insufficient for pulse-chase identification of stem cells in developing teeth

Previous work of others had used a pulse-chase technique to try and identify a putative stem cell population in perpetually renewing teeth. By administering BrdU for extended periods of time followed by a long chase period of no feeding, it was assumed that cells retaining the BrdU integration were slow cycling and putative stem cell populations (Handrigan et al., 2010). This work also demonstrated that a pulse period of one week was not sufficient to fully label all cells in the developing tooth. To further try and label all cells, I performed a one-month long pulse of daily oral BrdU administrations. While I expected more comprehensive labeling of all cells I found that there was very little incorporation of BrdU in any of the teeth at zero hours (Fig. 3.1A). There was some incorporation in heart and colon but not 100% (Fig. 3.1B,C). My data is very different than that of the previous publication. My interpretation is that the amount of BrdU ingested per gram of body weight was insufficient to saturate all of the DNA being synthesized. In the previous study a newly hatched animal was used which is about 10 times lighter than the animal used here. A future BrdU concentration as high as 50 mg/kg could be attempted for better cell saturation.

In a second animal that was fed BrdU for one month I chased the pulse for 14 weeks to determine if there were label- retaining cells present (Fig. 3.1D). Not surprisingly based on the zero hour data there were no BrdU-labeled cells detectable in the dental lamina. I would normally not have carried out the chase experiment without determining the zero-hour labeling results however due to a very protracted

decalcification protocol it took the full length of the chase period before the jaw skeleton was soft enough to section. I have since resolved this problem and use a much more rapid decalcification procedure outlined in Chapter 2 which takes a matter of days instead of months. Regrettably, it was not possible to study label- retaining cells under the experimental conditions I used.

In the absence of new data on the position of label retaining cells I extended the work on the stem cell paradigm by examining other phases of dental epithelial renewal.

Figure 3.1 Oral BrdU pulse chase to label slowly proliferating cells in tooth families

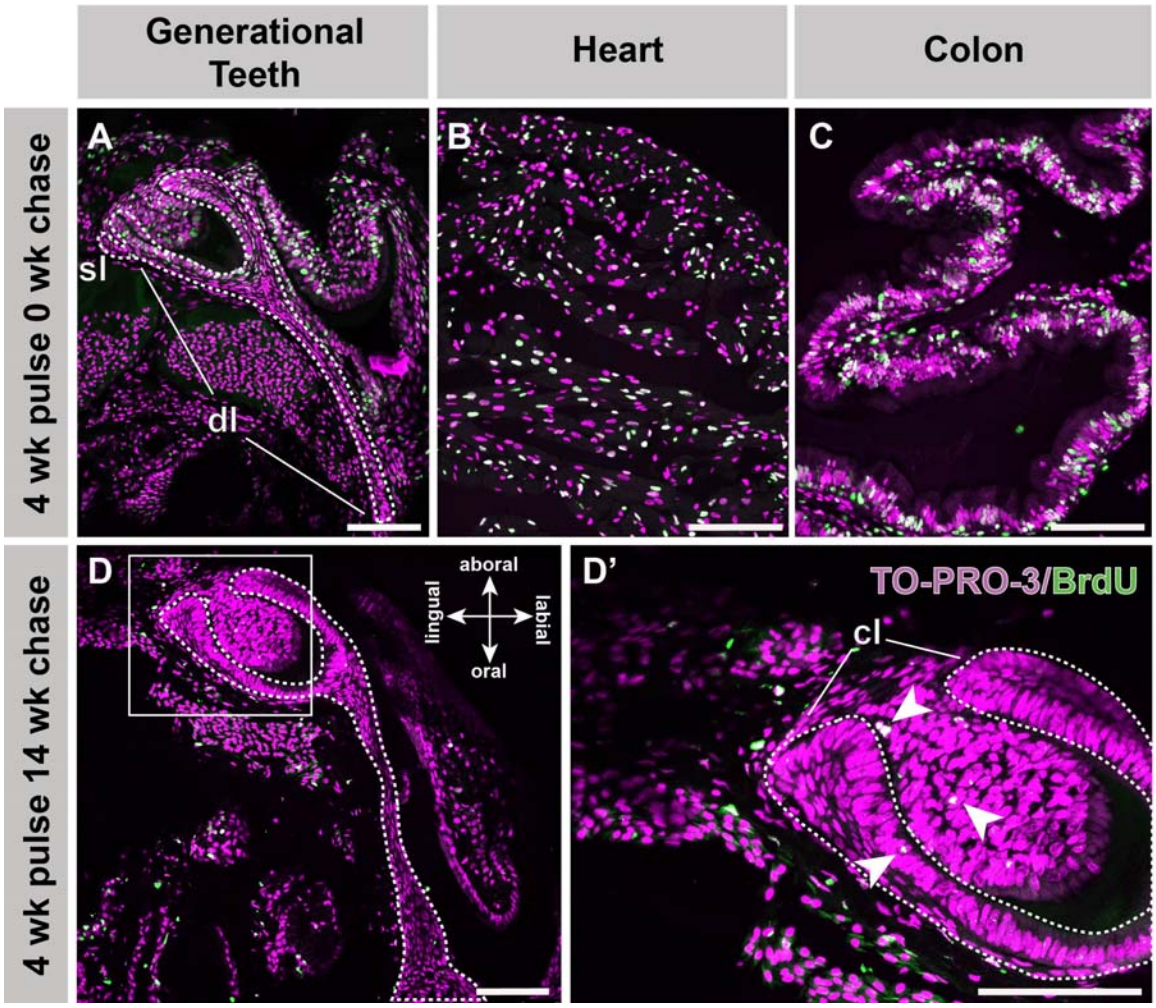

Fig. 3.1 – BrdU antibody staining of adult gecko maxillary teeth in transverse section. Images were taken with a confocal microscope using Alexa 488 conjugated secondary antibody and TO-PRO3 nuclear staining. These are maximum intensity projections of 5 slices. (**A-C**) Proliferating cells that have incorporated BrdU after 4 weeks of oral administration. The heart and colon had less than 50% of the cells labeled (**B,C**). **D**) After a 14 week chase the majority of dental epithelial cells had lost the BrdU signal A small number of labeled cells are indicated in the pulp and inner enamel epithelium (arrowheads in **D'**) Key: cl, cervical loop; dl, dental lamina; sl, successional lamina; Scale bars = 100μm.

3.2 Tooth replacement follows a distinctly different path than other epithelially derived structures including hair

Hair passes through three distinct phases: growth (anagen), regression (catagen) and rest (telogen).. During anagen phase, matrix- secreting cells at the base of the hair follicle continually secrete keratin pushing the hair outward towards the skin surface. Catagen phase is represented by a regression of the lower hair follicle by apoptosis of the epithelial cells. The follicle regresses until it meets the permanently residing bulge structure, the hair falls out of the skin, and the follicle enters telogen phase where the bulge rests so that the stem cells can prepare to receive an activating signal to begin anagen phase (Blanpain and Fuchs, 2009).

Hair passes through several phases including a catagen phase where the erupted hair follicle undergoes apoptosis before the new follicle replaces it (Blanpain and Fuchs, 2009). I hypothesized that since gecko teeth also cycle during replacement, parts of the dental lamina may have increased apoptosis. The regions of dental lamina with higher cell death may be periodic along the jaw, for example, affecting every other tooth. I therefore carefully examined serial sections though 1.5 mm of the jaw from 1 animal. There were approximately 23 tooth families analyzed with the TUNEL assay. No apoptosis was detected in the dental lamina, instead there was signal in the junctional epithelium between the tooth and the oral epithelium (Fig. 3.2A,A') where the erupting tooth was present. Logically cells would need to die and make room for the large tooth pushing into the oral cavity. Therefore teeth are not following the same type of regeneration process as the hair. We do not expect therefore that manipulating pathways that lengthen hair cycling will affect the rate of tooth replacement in geckos. For example

FGF5 (fibroblast growth factor 5) null mice exhibit longer hair follicles than wild-type mice due to a delay in transition to catagen phase (Hebert et al., 1994); WNT (wingless) signaling is necessary for hair follicle anagen phase (Van Mater et al., 2003); and BMPs (bone morphogenetic protein) are needed for differentiation of the transit amplifying cells (Kulessa et al., 2000). These pathways are active during tooth renewal but serve other functions.

Figure 3.2 TUNEL analysis of normal tooth replacement

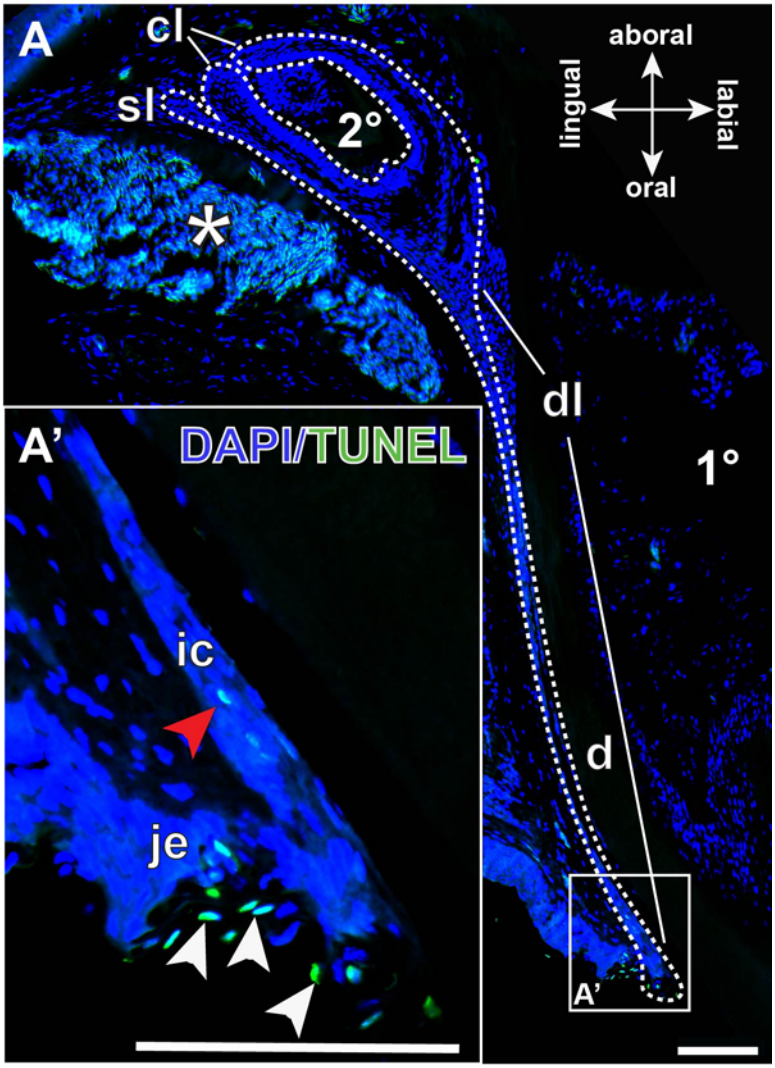

Fig. 3.2 - Fluorescent detection of fragmented DNA with widefield fluorescence microscopy in transverse sections of adult maxillary gecko teeth. **A**) In the low power view, few TUNEL positive cells were identified within the dental lamina and successional lamina. **A'**) There are apoptotic cells at the junctional epithelium, (white arrow heads) at the boundary between the erupted and unerupted portion of the tooth. There are also several apoptotic cells in the interstitum of the dental lamina (red arrowheads). Key: cl, cervical loop; d, dentin; dl, dental lamina; ic, interstitial cells; je, junctional epithelium; sl; successional lamina; 1° and 2° refer to teeth in one family; asterisk denotes auto fluorescence of blood cells. Scale bars = 50µm.

3.3 Stem cell antibodies are not sufficient to label a stem cell population in Leopard gecko teeth

Though pulse-chase experiments and catagen phase analysis did not further the results in trying to identify a putative stem population nor explain the process of tooth replacement, previous results showed that a putative stem population exists (Handrigan et al., 2010). A series of immunofluorescence (IF) experiments were performed to establish a set of antibodies that could positively identify the stem cell niche or specific population and eliminate the need for lengthy pulse-chase experiments. The antibodies tested were chosen from many previous studies loosely grouped according to pertinence to tooth stem cell research or hair follicle bulge stem cell studies. Sox 9, CD34, and integrins α6, ß1 and ß4 have all been used to identify hair follicle bulge stem cells (Blanpain et al., 2004; Trempus et al., 2003; Vidal et al., 2005; Zhu et al., 1999) while Tenascin, and Cytokeratin 19 are involved in hair stem cell niche maintenance (Kloepper et al., 2008). With regards to tooth related stem cell identification, Sox2 expressing cells were identified as mouse cervical loop stem cells (Juuri et al., 2012) and dental pulp stem cells have been identified via Sox9, and CD44 expression (Karaoz et al., 2010). The proteins analyzed therefore included 2 transcription factors, Sox2 and Sox9, cell surface proteins, the integrins and CD44, and extracellular matrix proteins, Cytokeratin 19, Tenascin. I modified the IF conditions to test different pretreatments, frozen versus paraffin sections, different microscopy methods (widefield and confocal) and different methods of detection using peroxidase enzyme as opposed to fluorescently labeled secondary antibodies. Out of all antibodies attempted, (Table 2.2) Sox2, Sox9, Cytokeratin 19 and

CD44 had positive signal (Fig. 3.4A-D). The antibodies that did not give signal looked identical to the preimmune control (data not shown and Fig. 3.4E).

The data on Sox2 antibody expression was obtained in a lab in Finland (Irma Thesleff) using tissue sections that I provided. The antibody generally cross-reacted with oral and dental epithelium in the gecko, corn snake and python (Juuri et al., 2013). There was no special preference for transit amplifying cells. Sox2, did not localize to the dental lamina but instead labeled the newly deposited enamel matrix as seen in a glancing section of a tooth in late bell stage (Fig. 3.4A, B). This staining must be an artifact since Sox2 protein should be restricted to nuclei. Sox9 stained the ameloblasts and the stellate reticulum cells (Fig. 3.4C). Again staining was cytoplasmic which may be an artifact. Positive control sections that contained cartilage had nuclear Sox9 expression (data not shown) therefore in some tissues the antibody worked as expected. The reasons for cytoplasmic staining of the inner enamel epithelium are not entirely clear.

Cytokeratin 19 antibody stained the cytoplasm and extracellular matrix surrounding dental epithelial cells with some stronger staining in the lingual side of the dental lamina (arrow in Fig. 3.4C). There was no regional concentration of signal that would be consistent with presence of a localized stem cell niche (Kloepper et al., 2008). CD44 which is a trans- membrane glycoprotein (hyaluronin receptor) had minimal signal however, it is very interesting to note that there is expression in the extracellular matrix confined to the dentino-enamel junction (DEJ) and in the secretory ameloblasts (Fig. 3.4D). My data is consistent with previous descriptions of CD44 expression in pre-secretory ameloblasts. These authors showed that CD44 is expressed just at the positions where the basal lamina is being degraded and enamel is starting to be deposited

(Felszeghy et al., 2001). CD44 expression in the DEJ or ameloblasts was not seen in preimmune controls (Fig. 3.4E). The antibody to CD44 did cross react with the Gecko protein but did not serve as a marker for stem cells.

The final antibody tested was against Tenascin. Even though this antibody was raised against chicken protein which has a closer evolutionary relationship to geckos, there was no specific staining (data not shown).

The lack of specific markers for stem cells necessitated a change in research direction and that was to study the role of specific signaling pathways in regulating cell proliferation in the tooth.

Figure 3.3 Stem cell and stem cell niche antibody staining of the dental apparatus

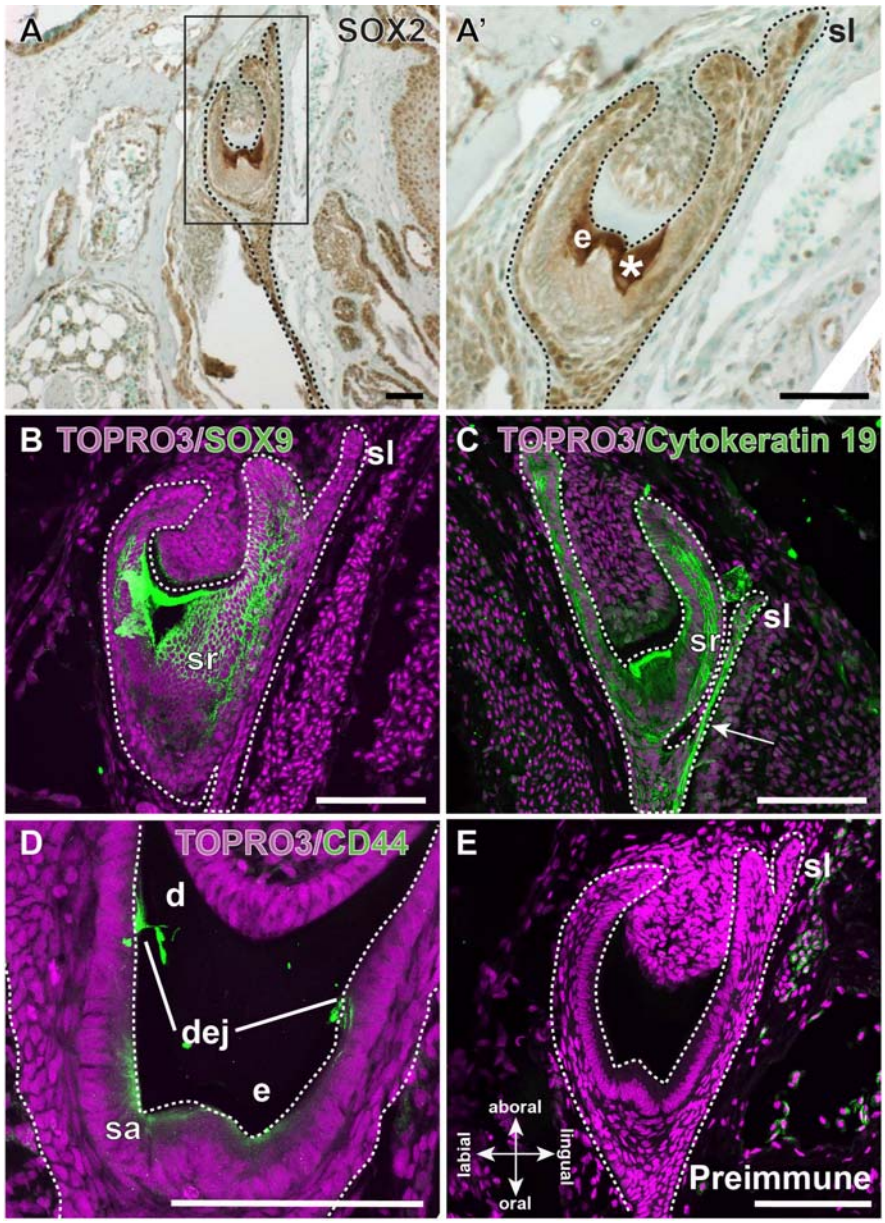

Fig. 3.3 - Immunohistochemistry (**A, A'**) and immunofluorescence staining (**B-E**) of adult gecko teeth in transverse section. Maximum intensity projections were made from a Z stack of 5 slices using a confocal microscope. **A**) Nuclear staining for Sox2 transcription factor is ubiquitous throughout epithelium of the developing tooth and oral cavity. The asterisk in **A'** marks non-specific binding of antibody to enamel matrix. **B**) Sox9 expression in the pulp and stellate reticulum is cytoplasmic and therefore non-specific. There is no Sox9 expression in the dental lamina or successional lamina. **C**) Cytokeratin 19 is highly expressed in the cytoplasm of dental epithelium including the stellate reticulum. There is increased staining on the lingual side of the

dental and successional lamina (arrow in **C**). **D**) CD44 is cell membrane protein and appears to be specifically expressed at the dentino-enamel junction as well as the secretory ameloblasts. **E**) The preimmune control shows no green signal. Key: d, dentin; dej, dentino-enamel junction; e, enamel; sa, secretory ameloblasts; sl, successional lamina; sr, stellate reticulum. Scale bars 100μm.

3.4 Normal expression of *WNT7A* and *BMP2* in developing teeth

Multiple signaling pathways have been shown to influence stem cell populations and promote either formation of transit amplifying cells or renewal of the original stem cells. Two of these pathways are WNT and BMP, again closely following the hair follicle bulge stem cell research where it was shown that WNT activity drives the transitory periods of the hair cycle in an out of phase wave with mesenchymal BMP activity (Greco et al., 2009). When BMP expression is high and WNT activity low the hair maintains in a refractory period of the replacement cycle and when vice versa the hair becomes competent for regeneration. I decided to map expression of representative ligand *BMP2* since it specifically was shown to cycle out of phase with WNT activity in the hair cycle (Plikus et al., 2008) as well as being implicated in our labs previous studies on reptilian tooth crown development (Handrigan and Richman, 2011). I used a slightly different approach to previous gene expression studies from our lab. Though both *BMP2* and *WNT7A* were described in prior work (Handrigan and Richman, 2010a, 2011), these studies only examined transverse tissue sections of very young geckos with one generation of teeth. I examined sagittal sections through multiple tooth families in order to look for differences in neighboring teeth as well as the interdental dental lamina. In the 3D reconstructions of the LRCs in geckos they were clustered at specific level of the dental lamina suggesting they were residing in a niche (Handrigan et al., 2010). The

niche might consist of cells expressing a specific growth factor. The question being addressed is whether there is any evidence of periodic gene expression that might explain why there are clusters of stem cells and why there is an alternating pattern of tooth replacement along the jaw (Edmund, 1960; Osborn, 1970). I also wanted to investigate if there were reciprocal patterns of expression that might indicate some cross regulation between the WNT and BMP pathways that is necessary for homeostasis in hair replacement.

I performed radio in-situ hybridization experiments on adult animals (Fig. 3.4 A-D). The signal for both *WNT7A* and *BMP2* was present in different stages of tooth development in the replacement process. *BMP2* is expressed primarily in the dental pulp of younger tooth generations including the odontoblast layer (Fig. 3.4B) whereas *WNT7A* seems to be expressed in the odontoblast layer of both young tooth generations as well as partially erupted neighboring teeth (Fig. 3.4D). *WNT7A* also showed foci of expression in some maxillary bone as well as the cervical loops of young and further developed teeth (arrows and white arrowheads in Fig. 3.4D). I also noted the periodicity of both *BMP2* and *WNT7A* expression and how this correlated with developmental stages of teeth. *BMP2* seems to be more highly expressed in younger teeth whereas *WNT7A* is expressed in more mature teeth that have formed dentin and enamel and have already erupted. Erupted teeth (teeth 2 and 4 in Fig. 3.4B) are adjacent to partially formed teeth (Teeth 1 and 3 in Fig. 3.4D). The signaling pathways responsible for development appear to shift from BMPs in early development to WNTs in later development and erupting stages.
In my next experiments I perturbed the WNT signaling pathway. BMP2 is downstream of the WNT pathway but quite a lot of work had already been done with manipulations of

BMP signaling and its effect on WNT readouts in our lab (Handrigan and Richman, 2010b). Since less was known about the FGF pathway and no previous manipulations were done on this pathway in our lab, I explored FGF signaling in my gecko system. To elucidate if WNT and FGF pathways regulate the tooth replacement process, I performed in vitro organ culture manipulations.

Figure 3.4 Normal expression of *WNT7A* and *BMP2* in transverse and sagittal sections of adult gecko jaws

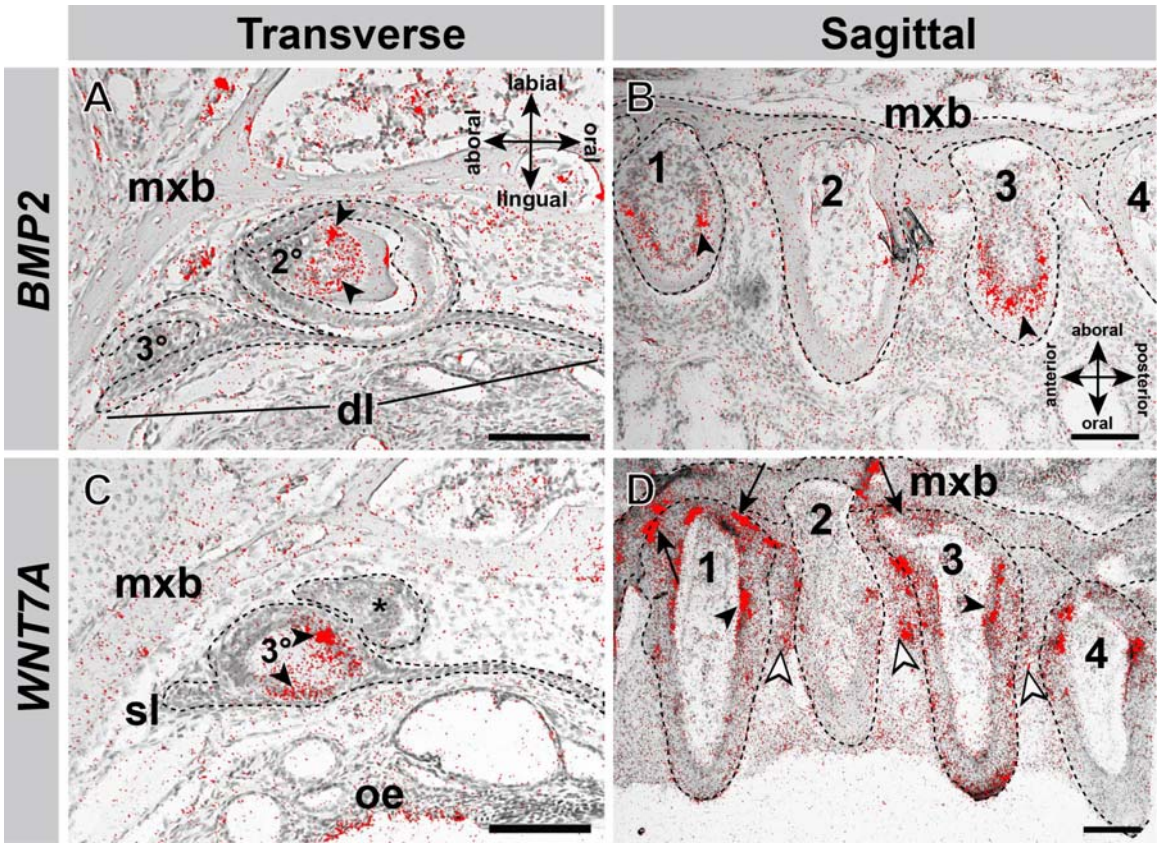

Fig. 3.4 – Radioactive in situ hybridization of paraffin sections from adult gecko maxillas. Sections were photographed in dark field and brightfield. The darkfield signal was superimposed on the brightfield image using Photoshop. Silver grains were selected and pseudo-coloured red to increase the contrast relative to the tissue section. **A**) *BMP2* expression is localized to odontoblast layer of the dental pulp (arrowheads) and in the osteoblast layer on top of the maxillary bone. There is no expression in the enamel organ. **B**) In sagittal sections there appears to be more abundant expression in alternating teeth that are less mature than their neighbours (teeth 1 and 3, arrowheads). **C**) *WNT7A* is primarily localized to odontoblasts (arrowheads) as well as oral epithelium. **D**) In the sagittal section the older teeth (1, 3, arrowheads) have more expression in the odontoblast layer and in the attachment to bone (arrows) than younger teeth (2,4). There is also expression between the teeth possibly in epithelium (white arrowheads). Key: dl, dental lamina; oe, oral epithelium; sl, successional lamina; 2° and 3° refer to teeth in one family; mxb, maxillary bone; asterisk denotes section cut into neighbouring tooth family. Compass in **A** same for **C**, in **B** same for **D.** Scale bars 100µm.

3.5 Signaling pathways that regulate tooth development in vitro

3.5.1 Fibroblast growth factor signaling is not sufficient to increase proliferation in in vitro organ cultures

Fibroblast growth factor (FGF) signaling has been shown to play a key role in the cyclic nature of hair regeneration. It has been show that FGF18 for instance keeps the hair bulge out of its highly proliferative anagen phase (Plikus, 2012). In a dental context, *FGF10* is expressed adjacent to the mouse incisor cervical loop and could be maintaining that stem cell niche or promoting the formation of transit amplifying cells (Harada et al., 2002). I hypothesized that FGFs might play a similar role in tooth replacement, being active to increase proliferation of the dental lamina where known label retaining cells would give rise to transit amplifying cells.

To study the effects of FGF signaling on tooth development and replacement, organ cultures were performed with both an agonist of FGF signaling (insertion of a bead soaked in FGF2 protein) or an FGFR antagonist, SU5402, which was added to organ culture media. FGF2 beads were successfully applied close to teeth in the organ cultures. Surprisingly, FGF2 beads placed adjacent to the successional lamina in a 24 hour culture did not significantly alter the number of BrdU labeled cells of the terminal tooth or dental lamina of the culture (Fig. 3.5B).

The cultures treated with SU5402 did not yield sufficient tissue representation for proliferative counts after a 4-day culture period. This may be in part due to the toxicity of SU5402 as well as experimenter error in dissection and tissue manipulation, which resulted in excision of parts of the dental lamina. Thus FGF manipulations were

inconclusive and changes to the experimental methods are necessary, perhaps using in vivo models instead of organ cultures.

Figure 3.5 Fibroblast growth factor signaling effects in in vitro organ cultures on tooth development

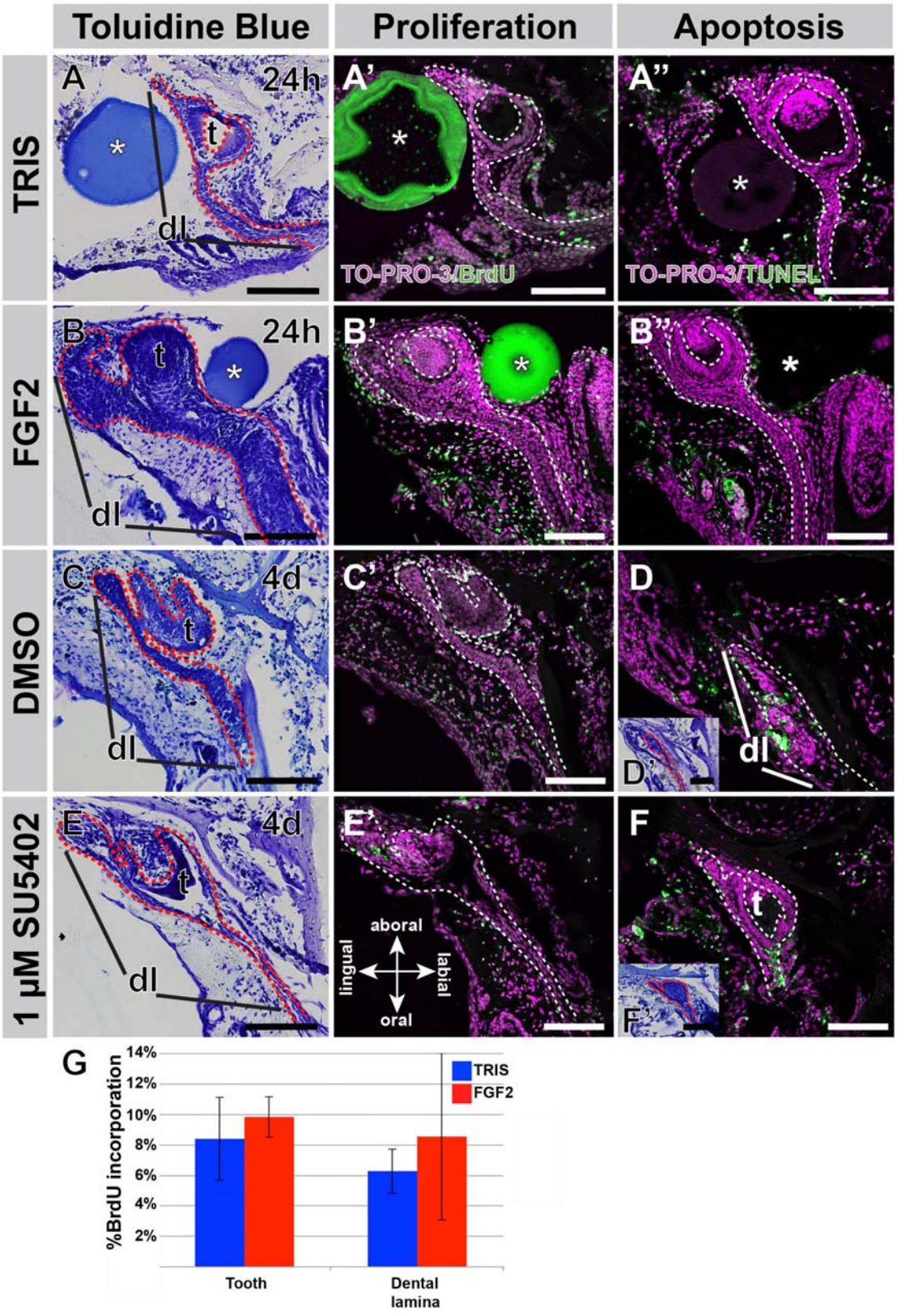

Fig. 3.5 – Transverse sections through maxillary organ cultures treated with FGF2 beads or SU5402 FGFR antagonist. Confocal imaging of gecko teeth, maximum intensity projections of 5 slices. In vitro bead placement was targeted directly to dental lamina of the terminal tooth (Asterisks). **A,A'**) Tris soaked bead and (**B,B'**) FGF2 bead did not affect proliferation or apoptosis (**A'',B''**). The FGF2 bead was placed on the opposite side from the successional lamina in some samples. **C-F**) Four- day SU5402 cultures failed to thrive due to a combination of manipulation of tissues and toxicity of reagent. Note that teeth in panels **D** and **F** are different than in **C** and **E**. **G**) Percentage labeled cells in the tooth and dental lamina are not significantly different between treated and controls, n=3. Error bars are 1 SD around the mean. Key: dl, dental lamina; t, tooth. Scale bars 100μm.

3.5.2 Activation of canonical WNT signaling increases terminal tooth proliferation at specific agonist concentrations whereas inhibition leads to a wearier result

I next used the same agonist/antagonist approach to study the role of WNT signaling in tooth replacement. We had previously used LiCl and BIO which are both GSK3ß inhibitors which result in activation of the canonical WNT pathway (Sato et al., 2004). LiCl was used on snakes and resulted in strong up- regulation of WNT target gene *LEF1* (Handrigan and Richman, 2010b). BIO was used on gecko organ cultures and this compound induced ectopic *TCF4* expression (Handrigan et al., 2010). To antagonize the canonical pathway our previous study on snake teeth used DKK1 protein-soaked beads. This antagonist was able to reduce *LEF1* expression in snake dental lamina (Handrigan and Richman, 2010b). For my studies I continued to use BIO as an activator and a new compound, XAV939 as an antagonist (Huang et al., 2009b). The antagonist works by stabilizing the AXIN component of the ß-catenin destruction complex which then actively degrades ß-catenin and inhibits WNT mediated transcription. The important advantage of using small molecules is that it is simple to add them to culture media and the compounds can be added over several days. Protein soaked beads tend to be exhausted after just 24h so longer term effects on morphogenesis cannot be studied. In

my organ culture experiments I grew the explants for 4 days in order to give the tissues sufficient time to exhibit a phenotype. In order to improve tissue viability, I also made several changes to the tissue culture conditions compared to previous studies (Buchtová et al., 2008; Handrigan et al., 2010; Handrigan and Richman, 2010a, b). First I decreased the incubator temperature to 30°C to improve viability of the tissue. Second, in addition to Penicillin/Streptomycin with amphotericin B (an antifungal) I added gentamycin to have broader spectrum protection against fungal and bacterial growth. In contrast to previous studies done on prehatching animals all of my cultures were made from adult animals in which the oral cavity is non-sterile.

I first conducted dose-response experiments in order to determine the optimal concentration to effect changes in proliferation and morphogenesis without inducing excessive toxicity. I performed proliferation studies by counting BrdU positive cells in the dental lamina and successional lamina as well as in the enamel organ, which included counting the outer enamel epithelium and cervical loops. Stellate reticulum and dental pulp cells were not counted. BIO was tested at 2, 20 and 60 μM (See Table 2.1) and it became apparent that cells survived at all concentrations but proliferation was only induced in response to the 20 μM concentration (Fig. 3.6B,H). Significantly higher proliferation was induced in 20 μM BIO treated enamel organs compared to DMSO controls ($p<0.05$). The lack of effect on the dental lamina in my study differs from previous analysis in the BIO treated gecko organ cultures made from prehatching animals (Handrigan et al., 2010). In the previous work, there were increases specifically in the lingual dental lamina which normally is very quiescent.

In the control cultures, the typical mean values for BrdU labeling for the enamel organ was 8% and for the dental lamina values were lower, on average 4% (Fig. 3.6 G-I). The TUNEL analysis showed minimal signal in the dental lamina or the enamel organs of 4-day DMSO treated cultures showing this time point to be a suitable time point to look for phenotypic genes in cell proliferation, morphology and in the future, gene expression (Fig. 3.6F). The addition of BIO did not qualitatively increase the number of TUNEL positive cells at any of the concentrations used. However there were areas of ectopic apoptosis that were caused by dissection of the tissues. In some cultures apoptosis was also seen in the dental pulp which may indicate poor diffusion of culture media to this cavity (Fig. 3.6F).

Figure 3.6 Activation of canonical WNT signaling in in vitro organ cultures effect on tooth development

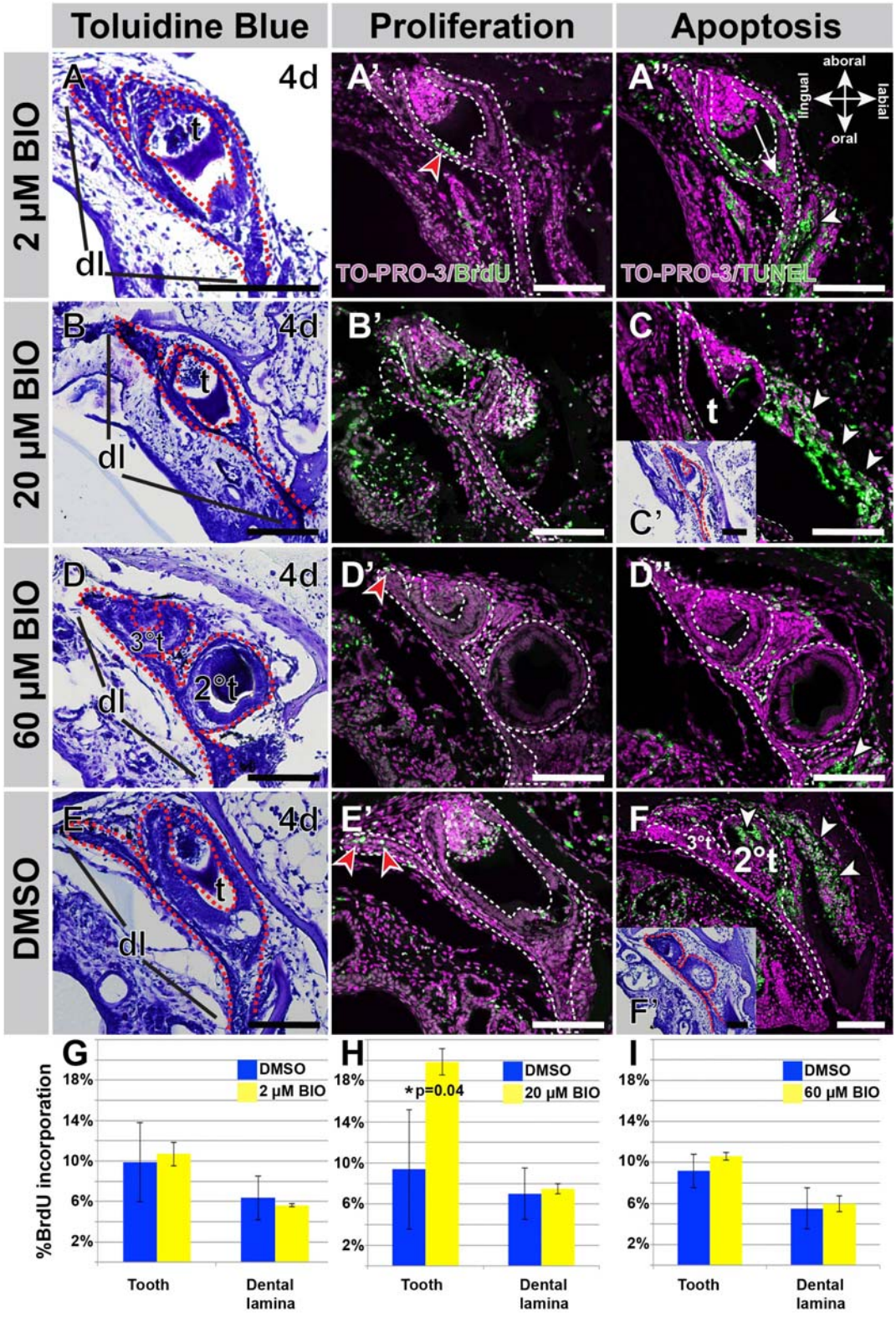

Fig. 3.6 - Transverse sections through maxillary organ cultures treated with graded concentration of BIO canonical WNT agonist. Confocal imaging of gecko teeth, maximum intensity projections of 5 slices. Proliferation counts were performed on dental lamina and enamel organ cells (red arrowheads). 2μM and 60μM (**A** and **D**) BIO treatments had no significant effect on proliferation of teeth and dental lamina of in vitro organ cultures (**G** and **I**) when compared to DMSO controls (**E-F**). 20μM BIO (**B,C**) however significantly increased proliferation in the terminal tooth ($p<0.05$) (**H**). TUNEL results (**A''**, **C**, **D''**, and **F**) also show very little apoptosis in the dental lamina of developing teeth showing these 4 day cultures were amenable to treatment. Ectopic apoptosis was seen (white arrowheads) as well as expected stellate reticulum apoptosis (arrow in **A''**). Note **C** and **F** are different sections than **B** and **E**. Statistics from factorial ANOVA with Tukey's HSD Post Hoc testing n=3 Error bars are 1 SD around the mean. Key: dl, dental lamina; t, tooth; 2°t and 3°t refer to teeth in the same family. Scale bars 100μm.

I tested three concentrations of XAV939, 0.1, 1 and 10 μM but there was insufficient dental tissue to assess proliferation in the 1 μM cultures (data not shown). The 0.1 μM dose increased proliferation in the enamel organ but this was not significant (Fig. 3.7A,A',F). It is unclear why the DMF controls in the 0.1μM proliferation study show a mean labeling index of 6% whereas the dental lamina is much higher, 15%. The large variability in the proliferation counts of the 0.01 μM XAV treated cultures precludes definitive conclusions. A 10 μM concentration decreased proliferation in the enamel organ ($p=0.01$) as compared to DMF controls (Fig. 3.7B,B', G). Here the values for the DMF controls were similar to those for DMSO in the BIO experiments so it appears the culture conditions promoted normal growth of the tissues (10% and 6% values respectively; Fig. 3.7F,G). Although there are some limitations to the data, it is apparent that canonical WNT signaling is required and sufficient for proliferation in the enamel organ but not in the dental lamina.

Figure 3.7 Inhibition of WNT signaling in in vitro organ cultures effect on tooth development

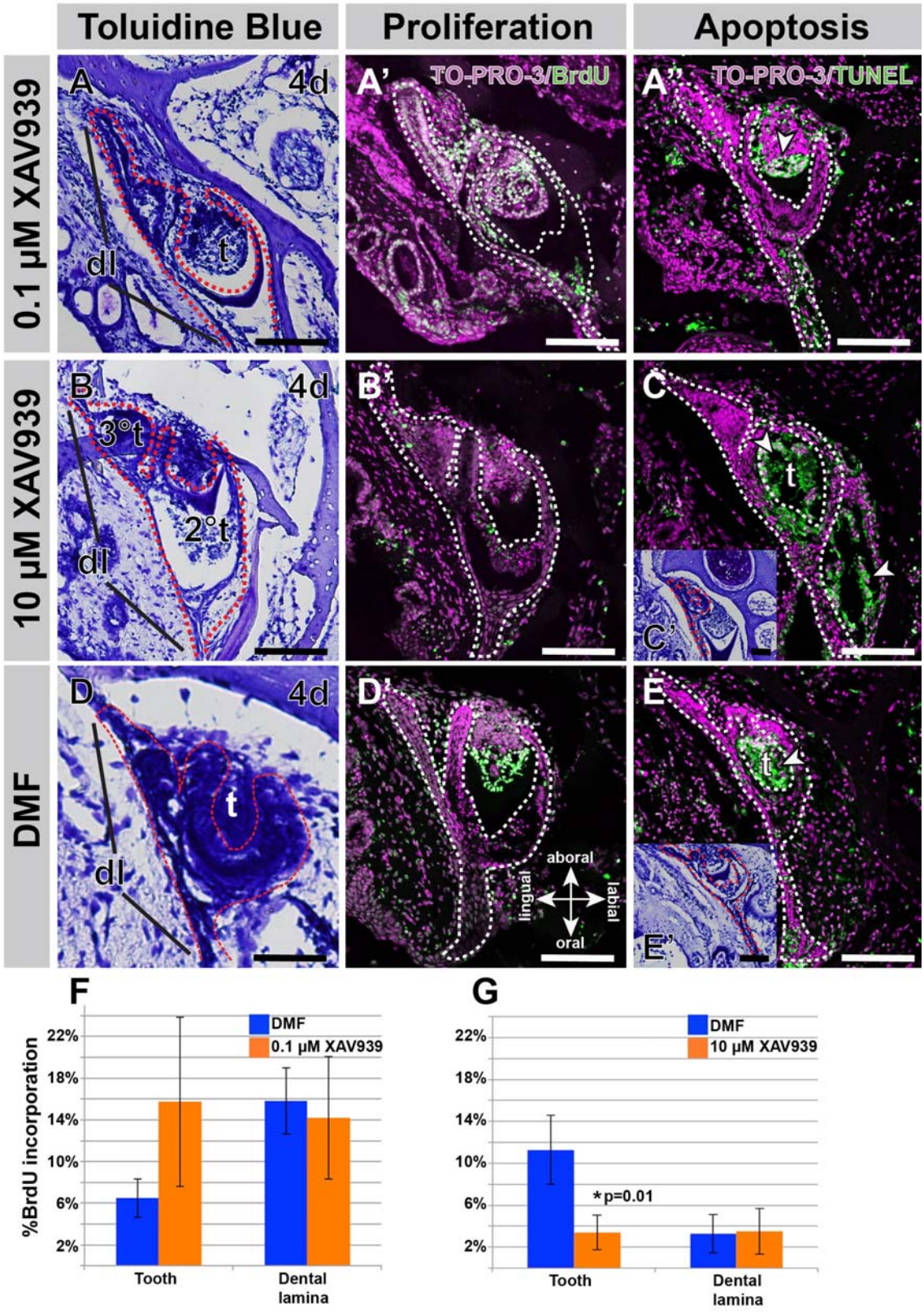

Fig. 3.7 - Transverse sections through maxillary organ cultures treated with graded concentration of XAV939 canonical WNT antagonist. Confocal imaging of gecko teeth, maximum intensity projections of 5 slices. 0.1μM XAV939 had questionable effects on in vitro organ cultures (**A**). Though proliferation seemed to increase in the developing tooth (**F**) as compared to DMF controls (**D-E**) the variability was too large for significant results. Also control proliferation counts in this treatment category seem to be lower than any other control group potentially showing non representative results. 10μM XAV939 (**B-C**) decreased proliferation (p=0.01) in the developing teeth of organ cultures as compared to DMF controls (**D-E**). Negligible TUNEL positive cells show tissues were amenable to 4 day culture treatment. Ectopic apoptosis seen in most cultures (arrowheads) especially in the dental pulp cavity showing growth media penetration might have been insufficient. Note **C** and **E** are different sections than **B** and **D**. Statistics from factorial ANOVA with Tukey's HSD Post Hoc testing n=3 Error bars are 1 SD around the mean. Key: dl, dental lamina; t, tooth; 2°t and 3°t refer to teeth in the same family. Scale bars 100μm.

3.6 Canonical WNT signaling plays a predominant role in terminal tooth development for replacing tooth families

3.6.1 Activated β-catenin marks canonical WNT activity in successional lamina and outer enamel epithelium of terminal tooth development

The previous organ cultures in my work and all previous studies on snakes and geckos did not directly test whether activation of the canonical pathway was present in specific regions of reptile teeth. The previous read outs were expression of the target genes *LEF1, TCF3, TCF4, AXIN2*. Here I used an antibody to phosphorylated beta catenin in adult gecko teeth. In areas of active signaling, β-catenin staining will translocate from the cytoplasm to the nucleus (Wu et al., 2013). Fortunately, the antibody cross- reacted with gecko tissues and using high- resolution confocal microscopy I was able to visualize differences in staining patterns (Fig. 3.8A-D'). Activated β-catenin (and therefore presumable canonical WNT activity) was present in some nuclei of the successional lamina (Fig. 3.8B', arrowheads). Not surprisingly the

outer enamel epithelium shows a predominance of nuclear β-catenin in the developing terminal tooth (Fig. 3.8C', arrowheads). This data fits perfectly with the increase in outer enamel epithelial proliferation in 20μM BIO organ cultures as well as the decrease in proliferation seen in 10μM XAV939 organ cultures. On a side note it is interesting to note activated β-catenin's role in cell-cell communication and scaffolding as becomes apparent as staining is enriched in the ameloblast cell membranes of Fig. 3.8 D-D''.

Figure 3.8 Activated β-catenin immunofluorescence

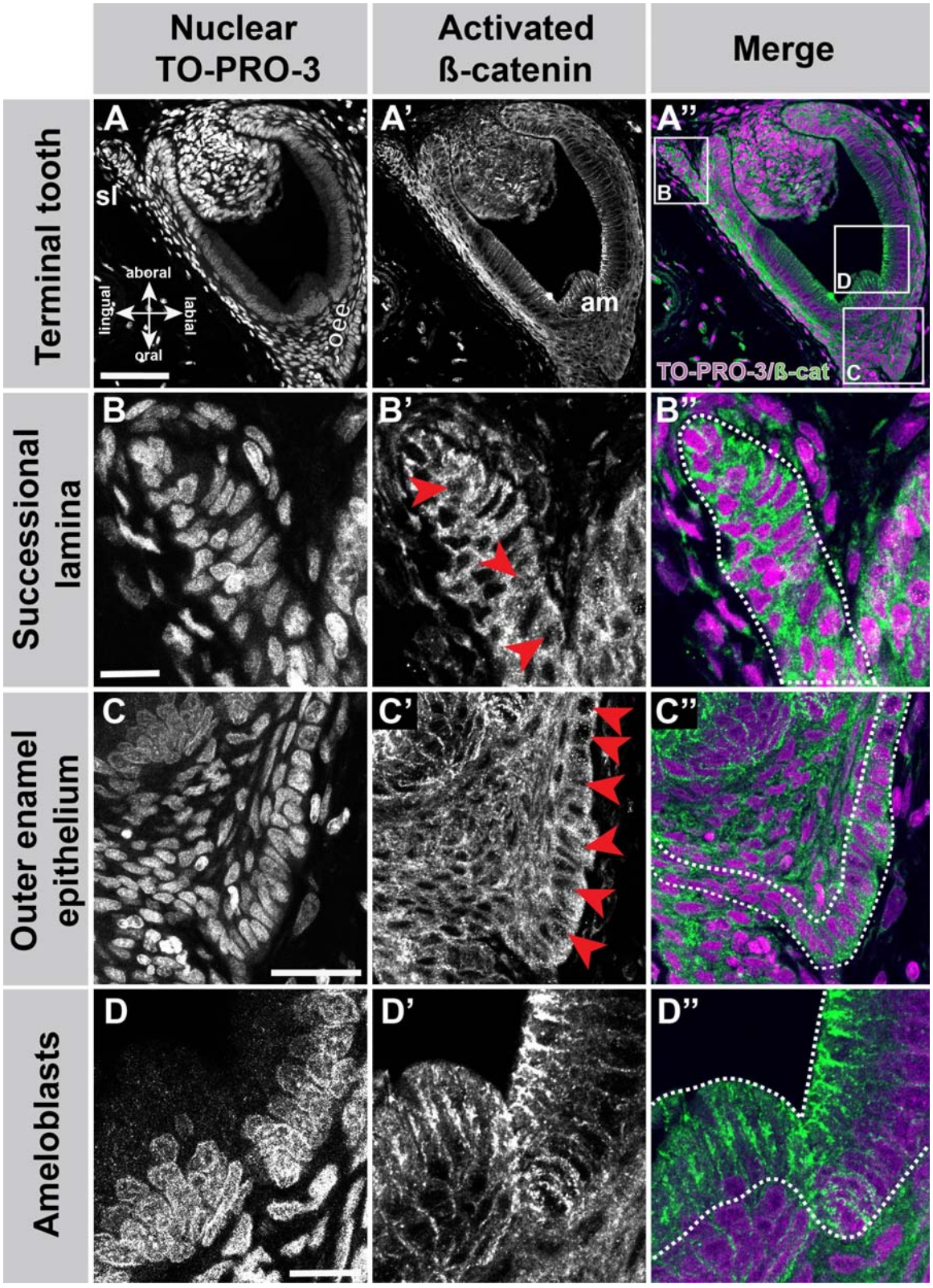

Fig. 3.8 - Transverse sections through maxillary terminal tooth. Immunofluorescence with activated β-catenin photographed at high resolution with a 63X oil immersion lens on a confocal microscope. **A-D'** are maximum intensity projections of Z stack of 8 slices. Canonical WNT signaling occurs in select successional lamina nuclei (arrowheads in **B'**) as well as the majority of outer enamel epithelium nuclei (arrowheads in **C'**). Activated β-catenin is also strongly expressed in the ameloblast cytoplasm (**D''**). Key: am, ameloblasts; oee, outer enamel epithelium; sl, successional lamina. Scale bars **A** 50μm; **B** 10μm; **C**, **D** 25μm.

3.6.2 Injection of LiCl stimulates local and specific increases in cell proliferation of in vivo terminal tooth development

Activation of canonical Wnt signaling has been shown to increase cell proliferation in gecko teeth in vitro both in my experiments and those of others from our lab. The limitations of organ culture studies on adult jaw segments is that tissues are complex and dense which might prevent optimal nutrition of the cells. The values for proliferation were quite low in general and made quantification difficult. I therefore pioneered an in vivo model for studying the effects of WNT signaling on tooth development. Here I used LiCl (a potent canonical WNT activator (Handrigan and Richman, 2010b) and NaCl (control) and injected these compounds locally next to the maxillary teeth. I first determined that I could see the path of the injection using DiI in the solutions being injected (Fig. 3.9A). I also developed inhalant anesthesia protocols with my supervisor to control animal behavior during the injections.

I injected 1M LiCl daily over a 3- day time course into the right palate and 1 M NaCl into the left palate of the same animal. Teeth on the right side that overlapped with DiI staining (Fig. 3.9A) had increased proliferation not only in the successional lamina as seen in Fig. 3.9D ($p<0.05$, as seen in I), but also in the cervical loops and outer enamel

epithelium as seen in Fig. 3.9E ($p<0.001$, as seen in I). The increases were modest but significant. There was no significant difference in proliferation in the dental lamina either beside the terminal tooth or extending up to the oral epithelium. This is different to results obtained in organ culture using BIO (Handrigan et al., 2010). Note that in the NaCl injected teeth the values for enamel organ proliferation were around 12% whereas the dental lamina close to the tooth was 6%. These values are similar to those obtained in 4 day organ cultures treated with DMSO and DMF which validates the in vitro approach. As a follow up, I also stained the teeth that were injected with LiCl and NaCl. Activated ß-catenin immunofluorescence was similar in distribution in the LiCl injected tissues as the control NaCl injected tissues. The proportion of cells with nuclear staining was not possible to quantify therefore differences between LiCl and NaCl injected teeth could not be resolved.

Figure 3.9 In vivo injection of LiCl

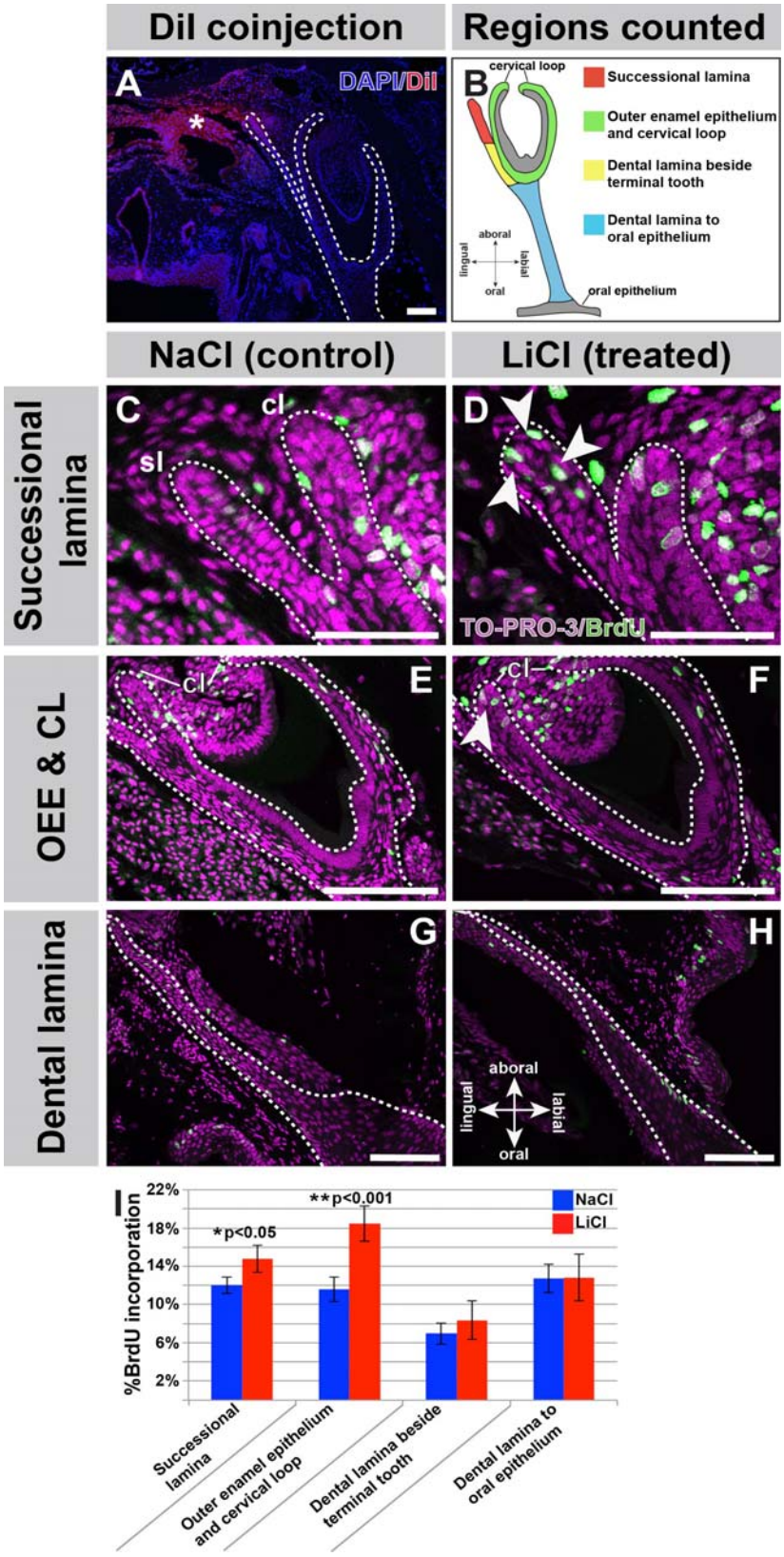

Fig. 3.9 - Transverse sections of maxillary tooth apparatus post in vivo treatment. Confocal microscopy, maximum intensity projections of 5 slice, Z-stack. **A**) Co-injection of DiI with LiCl or NaCl allowed precise identification (asterisk) of injection site for proliferation analysis. Proliferation counts were performed on a region specific basis as outlined in **B**. Visibly noticeable are the increases in LiCl treated proliferation of successional lamina (arrowheads in **D**) and the cervical loop region (arrowheads in **F**) as compared to NaCl injected controls **C** and **E** respectively. LiCl caused a significant increase in proliferation in the successional lamina ($p<0.05$) and outer enamel epithelium and cervical loop ($p<0.001$) (**I**). The dental lamina itself appeared to be unaffected by this treatment (**G**, **H** and right hand bars in **I**) n=8 Error bars are 1 SD around the mean. Key: cl, cervical loops; sl, successional lamina. Scale bars **A** 100µm; **C**, **D** 25µm; **E**-**H** 50µm.

Chapter 4 Discussion

4.1 Limitations of BrdU pulse-chase labeling of putative dental epithelial stem cells juvenile geckos

Feeding juvenile geckos BrdU every day for one month did not result in sufficiently labeled dental apparatus cells. Though integration of the BrdU occurred in the highly proliferative cervical loop and successional lamina tip, the rest of the dental lamina remained unincorporated. Without proper initial labeling of the dental lamina, label- retaining cells (LRCs) were not identified and future planned experiments to further understand this putative stem cell population abandoned. My data differs from that produced from our own lab on newly hatched geckos (Handrigan et al., 2010). With a shorter pulse period of one week these animals had far better cell labeling than my cohort. The number of cells labeled at the zero hour time point was 57%. Followed by a 16-week chase period, LRCs were successfully identified in the lingual side of the dental lamina. One reason for my lower BrdU incorporation even after a longer pulse period may be the size of the animal. Geckos typically weigh around 5 grams at hatching and gain 2-3 grams per week. The animal I used for the zero hour time point weighed 20 grams at the start of the pulse, topping off at 33 grams after one month. The amount of BrdU administered was 0.4 mg per day. I did not increase the amount of BrdU according to body weight. Another reason for the lack of labeling in my experiment might have been that the growth of the animal was more rapid at hatching than at the adult stage. Certainly the weight plateaus once the animal reaches 45-50 grams. One can imagine that at hatching the cell cycle may be shorter, more cells would be entering S phase and thus more cells would incorporate BrdU.

Very recently, another group have used similar techniques to successfully identify a LRC population in the alligator dental lamina (Wu et al., 2013). Using newly hatched alligators and an intraperitoneal (IP) BrdU injection daily for one week some success was achieved in labeling the dental apparatus and after a 4 week chase only presumable slow cycling stem cells remained labeled (Wu et al., 2013). The authors state that 80% of the cells within the teeth are labeled but only 10% of cells in the dental lamina incorporated label. Nevertheless, they were still able to detect slowly dividing cells at 2 and 4 weeks post chase in the dental lamina but only in those laminae that had not moved onto bud stage. The new approach used in this study is the double- label experiment. Initially alligators were pulsed for one week with daily IP Chlorodeoxyuridine (CldU) injections and following a 4 week chase were administered an IP Iododeoxyuridine (IdU) injection 3 hours prior to euthanasia. These two nucleotide analogues can be detected with different antibodies. Consequently LRCs were labeled differentially with CldU and the transit amplifying cells were labeled with IdU. The authors used immunohistochemistry techniques which precluded the detection of dual labeled LRC and transit amplifying (TA) cells. Had fluorescent secondary antibodies been used it would have been possible to show that the LRC cells gave rise to TA cells. This dual label technique has not yet been attempted on geckos but would be an excellent platform for studying effects of in vivo treatments on transit amplifying cell proliferation and is a future direction that could be followed.

The common limitation in all of these pulse-chase experiments is getting all of the cells in the dental lamina labeled with a thymidine analogue. In hair bulge studies the setting aside of stem cells occurs during initial hair development (Myung and Ito, 2012;

Nowak et al., 2008). Along the same lines, I hypothesized that dental epithelial stem cells are specified during embryonic tooth development. I therefore attempted to administer BrdU to pre-hatching geckos. Several methods were attempted including injections of BrdU through the egg wall, desiccation of the egg followed by placement of the egg on top of BrdU-soaked vermiculite, and finally, desiccation of the egg followed by filling the dimple that forms with BrdU solution (Data not shown). No embryos survived these egg manipulations long enough to test for BrdU incorporation. It is apparent that the pulse-chase technique for identifying putative stem cells in post-hatching animals remains the best method.

It is important to recognize that we have no definitive proof of whether the label retaining cells are in fact stem cells. We need to show that these LRCs give rise to the next generation tooth. This was not shown in the alligator work (Wu et al., 2013) since the chase part of the experiment was too brief to allow a new tooth to form (4 weeks chase but each tooth takes about 6 months to form). Future experiments should use the dual label method and chase the animals for a sufficient period of time to find cells in the next generation tooth.

4.2 Markers of stem cells still need to be found in the reptile system

The pulse-chase methods are able to label populations of putative stem cells but the data would be stronger if there was co-localization of other stem cell markers. The previous work from our lab used RNA in situ hybridization to find localized areas of expression of several genes that characterize hair and mouse incisor stem cells (Handrigan et al., 2010). In this study *LGR5, DKK3, LGFBP5* were found to localize in

small regions of the dental lamina in approximately the same region as the LRCs. In the alligator the authors did not show co-localization of any stem cell markers (Wu et al., 2013). Gecko tissue sections proved to be very difficult to achieve positive IF signal. Though a whole host of antibodies were tested only a select few obtained results. Of these, the antibodies tended to label epithelium only and in a non- specific manner. Cytokeratin 19 and Sox9 had been previously shown to mark a stem cell niche in the hair follicle bulge as well as transit amplifying cells, respectively (Kloepper et al., 2008; Vidal et al., 2005). I collaborated with a lab in Helsinki, Finland (Irma Thesleff) to see if Sox2 might identify transit- amplifying cells in the gecko dental lamina however no regionally restricted labeling was observed (Juuri et al., 2013). Instead they showed through lacZ lineage tracing that the Sox2 expressing cells from a first molar contributed specifically to the addition of the second and third molars (Juuri et al., 2013). Also shown was that an epithelial conditional deletion of *SOX2* lead to a hyperplastic dental epithelium but all three molars formed distal addition of molars. Even with the encouraging results in mouse molars, Sox2 is not a marker of only subsets of dental epithelial cells in reptiles. Instead it is a general marker of all epithelia, oral plus dental.

It is possible as noted in Chapter 3 that the protein sequence of the leopard gecko is too dissimilar from other organisms for epitope cross reaction to the antibodies attempted. There is no genome sequence for gecko. Without cloning all the genes myself it is hard to determine whether protein sequences of gecko are divergent or conserved relative to the species of immunogen protein.

4.3 Normal *WNT7A* and *BMP2* expression supports the tooth generation periodicity theories of others

The diagonal replacement pattern observed in the gecko has previously been discussed by others and is termed Zahnreihen (Edmund, 1960; Woerdeman, 1921). The molecular basis for the alternating state of development was proposed to be the result of zones of inhibition (ZOIs) along the dental lamina band within the surrounding mesenchyme (Osborn, 1973). Though the exact molecular mechanism is yet to be understood, it would be fitting that BMPs expressed in the mesenchyme could be exhibiting this ZOI effect on the otherwise proliferative WNTs in the dental lamina. When I investigated *BMP2* expression in sagittal sections of the gecko an interesting finding was that this alternating tooth pattern of expression did exist, however the mesenchymal expression was within the teeth rather than between teeth. *BMP2* was expressed explicitly in the odontoblast layer of younger teeth. The expression pattern did show an alternating pattern, with every other tooth being younger and therefore exhibiting *BMP2* odontoblast expression. Interestingly, *WNT7A* had the opposite expression profile with it being more abundantly expressed in the odontoblast layer of the erupted generation teeth (though still present in the enamel organs of lesser developed teeth) again in an alternating fashion. Both of these genes follow the observed periodicity of reptilian tooth development and replacement. It is possible that the signals do specifically originate from the teeth of the second- generation teeth that are incompletely formed, usually in the cap or bell stage of development. As long as the teeth have not yet attached to the bone crest, growth factors originating in the pulp of teeth could potentially diffuse out and affect neighboring tooth development. I propose that once the signaling

teeth mature and attach to the jaw bone (depending on the species in question) the signals would no longer diffuse out of the teeth. Tooth attachment would extinguish inhibitory signals allowing neighbouring teeth to set up new zones of inhibition. This model could explain why we observe an alternating developmental pattern in adult tooth replacement.

Another idea to explain alternating tooth replacement is that there could be signals in the mesenchyme that affect the stem cells in the dental lamina. To fit with the ZOI model I expected to find *BMP2* expression in the mesenchyme lingual to the dental lamina where it could have an inhibitory effect on the stem cell population. However, my study only included sagittal sections through the center of tooth rows and so the lingual to dental lamina mesenchyme tissues were not represented. In future studies sections through the dental lamina and mesenchyme adjacent to the dental lamina should be examined to look for periodic gene expression.

It was found that crosstalk between WNTs and BMPs exists in the hair replacement process. BMP inhibits WNT activity and keeps the hair in a refractory period before the expression profiles reverse to allow the hair to grow (Greco et al., 2009). I hypothesized that teeth might follow a similar control mechanism. The higher BMP levels may limit WNT activity in the younger teeth and prevent differentiation of odontoblasts and elongation of the cervical loops. It appears that an overlap of BMP and WNT activity in the odontoblasts of lesser-developed teeth might be limiting WNT activity and could potentially keep these teeth at a younger developmental time point than their neighbors. I found an intriguing differential response to the WNT activator BIO that was dependent on the concentration of the reagent. The dose-response data is consistent with the cells sensing a gradient of WNT activity. To address these questions of cross talk

and gradients of activity it would be necessary to look for effects of BMP agonists and antagonists on WNT pathway readouts such as *AXIN2* and *LEF1* or vice versa. Such experiments were carried out previously in the snake (Handrigan and Richman, 2010b). I did perform experiments using BIO and the WNT antagonist XAV so these cultures could be used for in situ hybridization experiments using *BMP2* and *WNT7A* probe.

4.4 WNT plays a key role in generational tooth development and successional lamina outgrowth as examined with in vivo and in vitro systems

Through experimental perturbations of the WNT pathway it is apparent that this is a necessary signaling member in successional lamina and terminal tooth development schemes. Proliferation results showed that activating this pathway with either BIO administered to in vitro organ cultures, or LiCl through in vivo injections caused a significant increase in proliferation of the outer enamel epithelium (OEE), cervical loop, and successional lamina (Fig. 4.1), whereas inhibiting this pathway with XAV939 in in vitro organ cultures caused a significant decrease in proliferation in the developing terminal tooth. It is important to note that all reagents were only effective at a specific concentration. When LiCl was injected into the mouth at a 20mM concentration instead of 1M no proliferation differences were observed (data not shown), BIO was only effective at a 20μM concentration with both 2μM and 60μM concentrations yielding no differences to controls, and XAV939 showing inhibitory effects at 10μM only. In the animal there could be differences in the availability of ligand, the expression of inhibitors or the ability of cells to respond to the signal. All of these could combine to set up a

morphogenetic field where cells closer to the source of the signal would respond differently than those further away.

The main readout of cellular response to changes in the level of WNT signaling I used was proliferation. I also attempted to look for changes in nuclear localization of phosphorylated ß-catenin. I found expression in the nucleus of the majority of OEE cells as well as several successional lamina cells as seen with IF but did not see a difference between LiCl and NaCl. It is possible that the LiCl had diffused into the blood stream and redistributed systemically affecting both sides of the jaw. I do not feel this is likely since I was able to detect statistically significant differences in cell proliferation. The antibody staining results could not be quantified and therefore we may have missed a trend to more nuclear localization in some regions of the dental epithelium. I leave open the possibility that another reagent such as BIO may cause a more clear effect on nuclear localization. Examining ß-catenin localization in the organ cultures could also yield informative results.

Figure 4.1 Two approaches to activating WNT signaling increased proliferation in distinct tissues

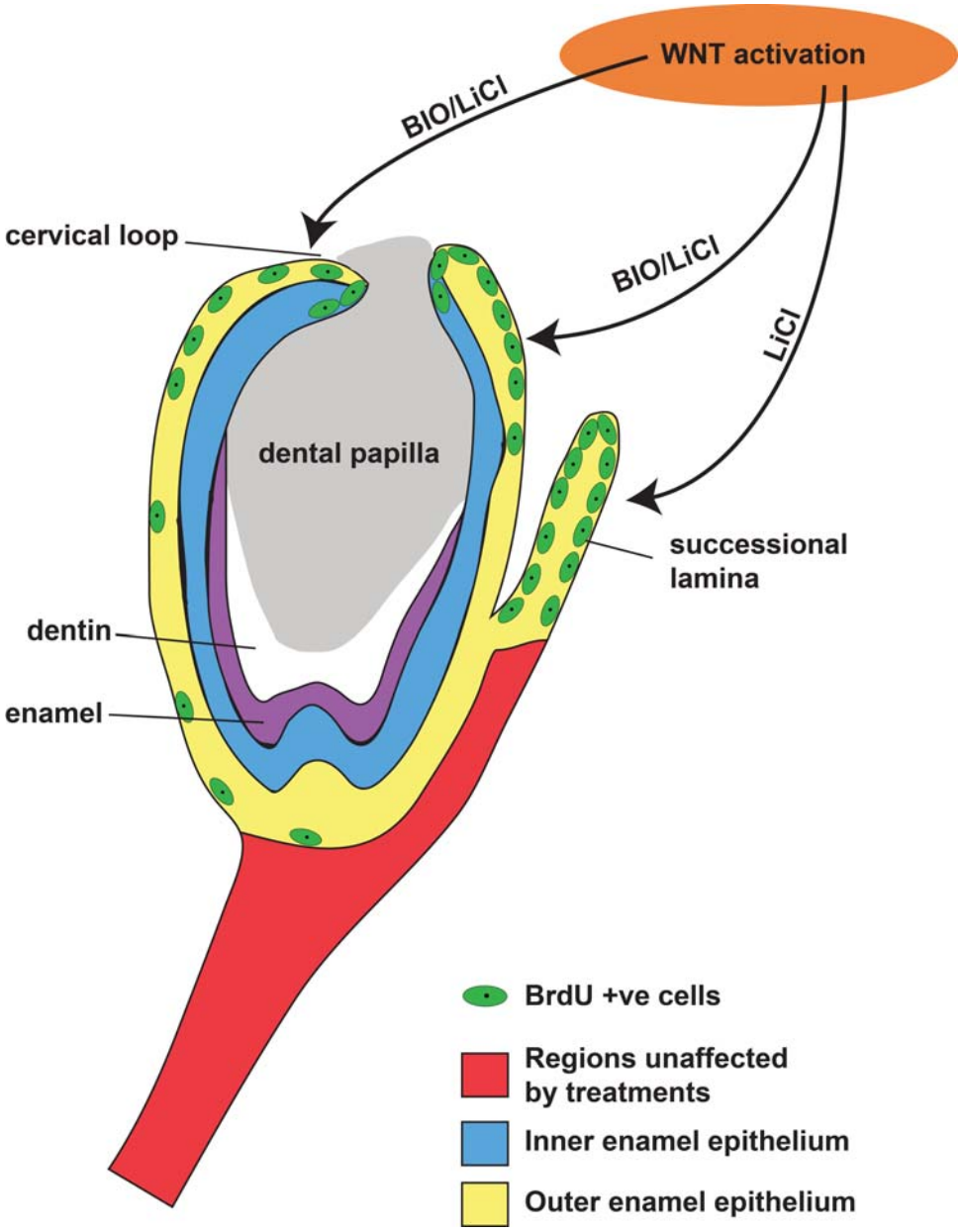

Fig. 4.1 - Transverse schematic of a terminal tooth. Activating WNT signaling with BIO in vitro or LiCl in vivo caused an increase in proliferation in the cervical loop and outer enamel epithelium. XAV inhibited proliferation in the outer enamel epithelium and cervical loop. Only LiCl increased proliferation in the successional lamina however. The dental lamina adjacent to the tooth and out to the oral cavity showed no change in proliferation when subjected to either treatment condition.

Though BIO was previously used in our lab to show early dental lamina to be responsive to WNT activation as shown by increased proliferation and ectopic *TCF4* expression (Handrigan et al., 2010), this is the first time that anyone has shown actively replacing teeth from adult geckos to also be WNT responsive. With regards to inhibiting this pathway, this work is the first time anyone has successfully used XAV939 in an organ culture system to inhibit WNT activity resulting in lowered proliferation of gecko teeth. Our lab has used DKK1 protein soaked beads in the past to inhibit WNT signaling in the early dental lamina which resulted in lower *LEF1* expression and proliferation (Handrigan and Richman, 2010b) but never examined adult replacing teeth until now. The caveat to my study is a lack of WNT signaling readouts post- experimental manipulation. Ideally an expression analysis of experimental cultures with regards to *AXIN2*, *LEF1*, or *pCTNBB1* would ultimately show how the WNT pathway was affected and lead to a clearer picture of what is happening in the replacement process of adult gecko teeth.

It is apparent that canonical WNT signaling is required for proliferation in the enamel organ but not in the dental lamina. This differs somewhat from previous analysis in the BIO treated gecko organ cultures made from prehatching animals (Handrigan et al., 2010) where there were increases specifically in the lingual dental lamina. The significant difference is that the previous study was looking at very early dental lamina in organ cultures where no teeth had developed yet. From this early dental lamina there was a difference in proliferation profile after BIO treatment where increases were seen on specifically the lingual aspect of the dental lamina. In my study there were multiple tooth generations present in the organ cultures and I showed that the dental lamina is very

minimally proliferative. I was unable to study a difference between labial vs. lingual proliferation, as the number of proliferating cells in the dental lamina was insufficient for such differentiation. I believe it is hard to compare the results of these two distinct studies as they are studying different developmental time points and different tissue structure.

4.5 The value of in vitro organ cultures in studying tooth succession

Appropriate DMSO and DMF controls were used in all organ culture proliferation studies and it is interesting to note that normal BrdU incorporation of tissues held constant for control organ cultures as well as NaCl injected control tissues. The trend stands that after a 3-hour BrdU administration at the end of either experiment the tissues of the tooth (cervical loop and outer enamel epithelium) show approximately an 8% BrdU incorporation whereas the dental lamina shows 4%. This has one of two implications. It first shows that the 4-day organ culture was a viable treatment since the tissues were just as proliferative at the end of the study as the in vivo model. In general the organ cultures survived a 4- day culture period as shown by proliferation still happening at the end of the experiment. There was also minimal TUNEL positive cells in the epithelial tissues of interest. However, there was some ectopic TUNEL staining from experimenter manipulations and tearing of tissues as well as dental pulp TUNEL signal. Presumably the dental pulp of teeth in organ cultures are simply not receiving nutrients from the media due to the thickness of the tissue being cultured as well as the presence of hard tissues such as bone and enamel blocking direct diffusion.

4.6 Further experimental conditions must be explored before a definitive role of FGF signaling in the tooth replacement process can be achieved

Though FGF2 bead placements were very successfully placed in close proximity to the successional lamina of in vitro organ cultures, after 24 hours of culture no proliferation differences were observed. This culture time is however notably different then the 4 day BIO and XAV939 cultures where effects were witnessed. It is possible that the time frame was simply too short to cause any changes at the cellular level. Using the FGF inhibitory molecule SU5402, no results were obtained due to several reasons. I found that SU5402 is highly cytotoxic with the majority of my first experimental cultures dying within a couple of days. After repeating this experiment with lower SU5402 concentrations there simply were not enough cultures in the cohort with teeth and dental lamina present to populate a proliferation study. Again this could have been due to the cytotoxicity of the reagent, however TUNEL results show minimal apoptosis in these cultures. Future directions involve in vivo SU5402 administration via oral bead placement or injection since this system is not as sensitive as an organ culture. Due to time constraints I was also not able to study FGF pathway member's expression profiles. I successfully cloned a fragment of leopard gecko *SPRY2* which is currently involved in a radio in situ hybridization with my organ culture tissues as well as normal tissues which will hopefully reveal FGF competent cell populations in the regenerating tooth family apparatus.

4.7 Tooth renewal versus tooth regeneration in the reptile

Previous studies on tooth replacement have relied heavily on established research on the hair follicle bulge stem cell and resultant hair replacement cycle. Here I show that the two processes are quite unique from one another. Most importantly the hair undergoes true regeneration by passing through a catagen phase first before going into anagen. Regeneration is defined as a process that passes through a blastema stage where cells dedifferentiate into an immature, pluripotent state. Regeneration of amphibian limbs and mouse digits are examples where dedifferentiation takes place first and then the new appendage is formed very similarly to its embryonic development, with the same key genes becoming active to pattern the chimeric limb (Bryant et al., 2002; Fernando et al., 2011). The dental lamina never passes through a catagen or blastema type phase. Instead there is survival of the dental lamina into adulthood and the ability to reform a new successional lamina over and over again. It is therefore more accurate to describe tooth replacement as renewal rather than true regeneration. However I maintain that stem cells are present in the gecko dental lamina and that these will eventually be shown to be required for reforming the successional lamina.

What are the signals that may be stimulating the dental lamina and therefore the stem cells? Similar to hair replacement, canonical WNT signaling plays a pivotal role in successional lamina growth as well as proliferation of the developing tooth. Though BMP and FGF pathways have been implicated in hair replacement cycling, further research must be done to determine whether these pathways are actively participating in the tooth replacement process. Supernumerary teeth could be induced in the otherwise monophyodont mouse with constitutive activation of ß-catenin (Järvinen et al., 2006) or

ablation of Apc (adenomatous polyposis coli), a WNT inhibitor (Wang et al., 2009). However these two studies have some limitations, as wild-type mice do not replace their teeth. It has been determined however, that an inherited autosomal dominant mutation in the *APC* gene is the underlying cause of familial adenomatous polyposis (FAP) more commonly known as Gardner's Syndrome (Ballhausen, 2000). This syndrome presents as malignant carcinomas and adenomas throughout the colon and maxillofacial regions, as well as the development of many impacted or unerupted supernumerary teeth. This is a prime example of the importance of WNT signaling effecting tooth development and replacement as truncated APC protein results in degradation of the ß-catenin destruction complex, allowing hyper-activation of this signaling pathway (Ballhausen, 2000).

Supernumerary teeth are also a common phenotype of human Cleidocranial Dysplasia (CCD). This affliction is a result of a number of different mutations in the *RUNX2* (runt related transcription factor 2) gene. This gene helps signaling between the mesenchyme and epithelium and it was shown that it specifically binds the *FGF3* promoter (Ryoo and Wang, 2006). In mutated individuals, *FGF3* is thus down- regulated resulting in the phenotypes seen. *RUNX2* influences several signaling pathways but most interestingly plays a role in FGF and BMP signaling (Zaidi et al., 2006). This begins to explain the importance of FGF and BMP in tooth succession though the networks of signaling pathways have only begun to reveal themselves.

There is evidently a role for WNT, BMP, and FGF signaling in the process of tooth replacement. I have begun to show the importance of these pathways and the outcome of experimentally manipulating each one. Much more work must be done before a clear picture will emerge on the spatiotemporal expression and control of each one of

these pathways and their influences on tooth succession. The leopard gecko has proven to be invaluable in tooth replacement research and will continue to be developed as an excellent model system for such studies.

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