Chemical manipulation of dental patterning in Malawi cichlids

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Undergraduate Thesis
4/22/2008

Abstract

A dynamic model for odontogenesis is lacking. Cichlids serve as superior organisms for studies in tooth development because of diversity in tooth shape, size, number, number of rows, all of which undergo continuous replacement, as well as the possession of a second set of toothed jaws, within the posterior pharynx. Through *in situ* hybridization we have characterized basic patterns of odontogenesis in cichlids that are conserved throughout vertebrates. We have used the teratogens cyclopamine and SU5402 to reduce the function of Shh and Fgf protein, respectively with a time and dose dependence. Cyclopamine trials indicate that *Shh* is necessary to pattern the dentition and that its expression in the first tooth is essential, as is its interaction with tooth spacing genes in the initiation of a periodic pattern mechanism. Members of the Fgf family are required for proper replacement cycles, possibly through stem cell interactions.

Introduction

The dentition is essential to the survival of nearly all vertebrates. Burdened with prey capture, mastication, defense, and a plethora of specialized roles, defects in the development of teeth are often drastic enough to drive the death of an individual or the evolution of a new species. For their obligation, the body rewards teeth with a great deal of time and energy cultivating them as an intricate yet durable structure. Society recognizes this obligation and spends millions on dental care and education. But for all

our endeavors in maintaining the dentition and treating its diseases, we know and invest very little in understanding the genetic causes of its development. By uncovering the pathways responsible for growing, spacing, replacing and shaping the tooth throughout ontogenesis, we can embark on learning to heal from the root up instead of from the crown down.

With most of the studies involving the molecular causes of tooth development only being presented in the past decade, a suitable organism for research has yet to be chosen. Because the mouse serves as a mammalian relative to humans, most studies involving teeth thus far have been performed in murine laboratories. However, mus musculis is particularly lacking in the mouth. Humans have a dentition comprised of incisors, canines, premolars and molars. Relatively late in human development the primary dentition of the juvenile is replaced by a permanent secondary adult dentition. In murine odontogenesis, there is no replacement and only one set of teeth serves an entire lifespan. This single set of teeth is limited to three molars and one continuously growing rodent incisor in each quadrant of the mouth. Zebrafish are a useful alternative because they have large broods and a quick gestation. While the zebrafish lacks an oral dentition, it has a set of pharyngeal jaws laced with teeth derived from the fifth pharyngeal gill arch that is shared by many osteichthyans. While development of teeth in the pharynx is very similar to that of the mouse, it differs in some fundamental aspects (Stock 2006). An apparent need for a generalized vertebrate model encompassing all aspects of tooth development, from shape to replacement, has emerged and has been cited in the discussions of numerous manuscripts involving the aforementioned odontogenesis models. The Malawi cichlid is well-suited to this role and offers a multitude of diverse, species specific dentitions.

The East African cichlid has several advantages for tooth development studies to both of these models (Streelman 2003). Unlike zebrafish, cichlids have maintained their oral teeth, yet they still poses toothed pharyngeal jaws characteristic of teleosts. These traits lend two dentitions to study with similar but different genetic modulators (Fraser 2006) of odontogenesis in one organism. Intense competition in Lake Malawi has led to the functional phenotypic diversification of both sets of jaws and teeth (Albertson

2005). This divergence has given rise to a range of tens to thousands of teeth uniquely arranged in one to several rows. In addition, this species dependent arrangement and number of teeth can be comprised of a mixed or uniform dentition of unicuspid, bicuspid or tricuspid shape. But perhaps the most interesting aspect of the cichlid dentition is a continuous oral replacement of teeth that other model organisms lack. While these factors accumulate complexity when taken together, the human dentition is no less complex. By comparing genetic expression between select species, we can piece together the individual aspects such as patterning and replacement of odontogenesis.

Studies performed on cichlids can be difficult but very rewarding. Because the cichlid is a relatively young model organism, not much is known about cichlid genetics. By using partial genome sequences across a variety of Lake Malawi cichlid species, we can piece together enough information to perform genetic studies such as *in situ* hybridization. These types of studies can be enhanced by utilizing databases and manuscripts detailing odontogenesis in other vertebrates, in particular the mouse and the zebrafish. Although knockout systems such as lox-pe-cre in the mouse do not exist for cichlids, we can use chemical modulators to manipulate gene expression.

One such chemical modulator is the natural compound cyclopamine, found in the corn lilly *Veratrum californicum*. Cyclopamine was discovered with the strange appearance of cycloptic sheep that consumed the lilly (Keeler 1978). It was later discovered that the cyclopamine alkaloid that was found in Veratrum californicum inhibits SHH signaling, which is essential for inhibiting eye formation in the most medial portion of the presumptive eye field by inhibiting Pax signaling (Macdonald 1995). More importantly for this study, SHH is essential for the development of the mouse and zebrafish dentition. Cyclopamine mimics the actions of the trans-membrane protein Patched (which is inhibited by SHH ligand) by inhibiting another trans-membrane protein smoothened and thus preventing the transcription of SHH regulated genes (Chen et. al. 2003). Therefore, the extracellular application of cyclopamine can act to inhibit the expression of gene transcripts up-regulated by SHH through direct inhibition of the trans-membrane protein smoothened.

The compound synthetic compound SU5402 is a more general chemical modulator (Mohammadi 1997). SU5402 acts by binding specifically to the entire family of trans-membrane fibroblast growth factor (Fgf) receptors. This biniding prevents the function of all Fgf ligands from activating their respective signal transduction pathways. Several Fgf proteins have been implicated in the developing mouse (Pispa 1999) and zebrafish (Jackman 2004) dentition, including Fgf3, Fgf6, Fgf8, Fgf9, and Fgf10. Fgf10 and Fgf3 have also recently been implicated in the maintenance of the stem cell niche of the developing mouse (Harada 2002, Wang 2007) and may serve a role in the development of the cichlids continuously replacing dentition.

We have applied several techniques to cichlids to characterize odontogenesis from initiation to replacement. Clearing and staining techniques were used to analyze the morphometrics of both the adult as well as the developing cichlid for many species. *In situ* hybridization was performed to visualize the expression of genes suspected by previous literature to be involved in the development of the cichlid tooth. Microscopy and histology were used to further discern expression. Finally, chemical modulation by known inhibitors of certain gene transcripts was used to deduce mechanisms of odontogenesis.

Literature Review

Morphogenesis initiation

The first tissues competent to form a tooth in the mouse are demarcated by a dental lamina in the mandible and maxilla. Known as the odontogenic band in osteichthyans (Fraser 2004), no teeth will grow outside of this region and cases of hyperdontia are usually confined here. The interactions in this region of epithelium overlying neural crest derived mesenchyme, are very similar to the milk-line of mammary glands and ectodermal limb development (Thesleff 1995). Cranial neural crest cells migrate from the lateral ridges of the neural plate and participate in the formation of everything from the dental mesenchyme, dental papilla, odontoblasts, dentine, pulp, cementum, and even the chondrocytes of the Meckel's cartilidge (Chai 2000). However, the germ layer origin of the overlying epithelium is still unknown. While the pharyngeal teeth of teleosts have an endodermally derived epithelium, agnathans (jawless fish) like the lamprey show hox gene expression throughout their branchial arches (Cohn 2002). With the loss of hox gene expression in the first branchial arch, gnathastomes were able to develop the meckel's cartilage that gave rise to opposing jaws and their ensuing teeth. Instead of an expansion of the teeth lining the pharynx as in some osteichthyans, it is possible that oral teeth were an invagination of ectodermaly derived structures such as dermal denticles, tooth-like structures that cover the outer surface of ancient jawless fish, similar to those modern elasmobranchs (sharks and rays) (Reif 1982). Zebrafish, the teleost developmental model, lack a dental lamina and teeth in the oral cavity, they still express genes characteristic of tooth development such as sonic hedgehog (shh) and pitx2 in the oral epithelium (Stock 2006). Because of the burdens that teeth carry, they are surrounded by enamel, the hardest substance in the vertebrate body. Enamels durability allows paleontologists

to make inferences based on fossil records of teeth. A more telling story might unfold when the gene expression of pharyngeal teeth and scales to oral teeth of the cichlid are compared.

The knowledge of tooth development and the genes that are involved have been characterized from the mammalian developmental model, the mouse. Inside of the lateral dental lamina of mice, fibroblast growth factor-8(*Fgf8*) is expressed expression during the thickening of the epithelium of presumptive molar tooth sites around day 9-10 of embryonic development. Similarly, bone morphogenic protein 4 (Bmp4) is expressed at day 9-10 in the thickened medial epithelium that patterns the incisors of mice. Bmp4 and Fgf8 are mutually antagonistic in the dental lamina, regulated through feedback loops with paired related homeobox gene 2 (PITX2). Knockouts of PITX2 lead to the expansion of BMP4 and the repression of Fgf8, as well as arrestment of tooth development in the bud stage characteristic of the genetic disease Rieger syndrome (Lu 1999). The thickened epithelium signals the underlying neural crest derived mesenchyme to express paired box transcription factor 9 (*Pax9*). Once the underlying mesenchyme is activated by the epithelium, it is competent to form teeth; however, it cannot form tooth derivatives otherwise. Tissue explant experiments have shown that superimposing mouse epithelium to chicken mesenchyme gives rise to teeth, showing that chickens lost the inducers for teeth in the epithelium, most likely due to flight (Harris 2006). At day 12.5 of mouse embryonic development the mesenchyme gains odontogenic potential and can instruct non-dental epithelium to form teeth (Lumsden 1988). *Fgf8* and *Fgf9* are expressed at the same time and place in the dentary and can be inhibited simultaneously using chemicals such as SU5402 that inhibit all fgf receptors (Mohammadi 1997). While inhibiting just fgf8 through gene targeting results in mice with incisors but no molars (Trumpp 1999), blocking both Fgf9 and Fgf8 through SU5402 results in adontia (Mandler 2001). In contrast, *fgf8* and *pax9* are not

expressed in the tooth buds of pharyngeal zebrafish teeth. Embryos treated with antisense

morpholino oligonucleotides for *fgf3*, *fgf4*, and *fgf8* expression is only mildly affected in tooth

development. However, tooth morphogenesis is blocked in SU5402 exposed zebrafish (Jackman

2004). Interestingly, *pitx2* expression is unaffected by SU5402 treatment in either organism,

suggesting a conserved upstream role of Pitx2 to Fgf8 in both the oral cavity of the mouse and

pharynx of zebrafish.

Molar regions of the mouse express BarH-like homeobox 1 (*Barx1*) and distal-less

homeobox 1 and 2 (*Dlx 1* and *Dlx2*) in the mesenchyme upregulated by overlying Fgf8 and Fgf9

(Thomas 1997). Signals from Bapx1 derived from the developing ear and temporomandibular

joint overlap the molar region (Miller, 2003). BMP4 inhibits the expression of *Barx1* while

upregulating homeobox msh-like 1 and 2 (Msx1 and Msx2) (Bei 1998). Although the murine

pharynx does not express *Dlx2*, the zebrafish expresses both orthologs *dlx2a* and *dlx2b* localized

in tooth sites. The requirement of dlx2 in odontogenesis of zebrafish pharynx and in the oral

cavity of the mouth is likely due to direct downstream regulation by pitx2 (Green 2001). While

loss of either Dlx1 or Dlx2 alone is not sufficient to inhibit tooth formation in the mouse, loss of

both genes arrests maxillary molar development while leaving incisors unaffected. To further

complicate things, simultaneous loss of Dlx1 and Dlx2 does not affect the molars of the

mandible. Dlx5 and Dlx6 are not competent to form teeth alone, but in the absence of both Dlx1

and Dlx2, they can rescue dentary molar formation (Zhao 2000). This pattern demonstrates that

differences in the development of teeth can be seen on not only between branchial arches and the

proximal-distal axis, but between the mandibular and maxillary arches.

Spatial patterning

The expression of *Pax9* by signals from the thickened epithelium leads to a condensation of the epithelium into a dental placode. This placode invaginates into the mesenchyme marking the transition to the bud stage (Thesleff 2003). During the bud stage, *Pitx2* is continuously expressed in the epithelium while other initiation genes like *Fgf8* are inhibited. Shh marks the placodes for all developing teeth (Hardcastle 1998) and upregulate patched 1 (Ptc1) and Gli1 in the mesenchyme (Cobourne 2004), creating a feedback loop. Wingless integration site homologue 7b (Wnt7b) helps space the teeth by creating a zone of inhibition (ZOI) (Sarkar 2000). *Wnt7b* is expressed in regions surrounding Shh expressing dental placodes. The downstream transcription factor of the canonical Wnt pathway *β-catenin* is strongly expressed in developing hair and feathers, but had been difficult to isolate in the teeth. Dental epithelial expressed *Wnt6* induced by epithelial *Fgf8* is co-expressed with the tumor necrosis factor ligand ectodysplasin (*Eda*) in the epithelium. Eda is inhibited in the dental placodes where its receptor, Edar is upregulated by Activin. Activin is a mesenchymaly derived member of the wnt pathway present in all teeth except maxillary molars (Ferguson 1998). Eda's restriction to non-dental epithelium and Edar's restriction to odontogenic placodes is a distinct interaction because of its restriction to one germ layer, as opposed to most other mesenchyme-epithelium signaling pathways of odontogenesis (Laurikkala 2001). This signalling suggests an important role for the ectodysplasia ligand in spacing and organization of teeth within the lamina or odontogenic band. Shh and Wnt7b are two of the only other genes that share a single germ layer interface and have been implemented as regulators of the Eda pathway. Mutant strains of mice for the Wnt mediating Lef1 protein show a downregulation of Eda, suggesting Eda is downstream of Wnt signaling. Tabby and downless mutant mice are knockouts of Eda and Edar, respectively. These

mutants share identical phenotypes to ectodermal dysplasia patients, exhibiting severe deformities in teeth and ectodermally derived structures, including peg shaped molars and severe oligodontia (Miletich 2003).

Shaping the tooth

At day 14 of mouse embryonic development the placode of epithelium in the bell stage begins to fold around a cluster of mesenchymal cells that becomes the dental papilla (Thesleff 2003). At the apex of this newly formed papilla, the overlying epithelium condenses to a new signaling center called the enamel knot. This knot activates a slew of new genes and reenlists earlier acting genes from thickening and the bell stage, such as Shh and BMP4, for new roles. Around day 16 of murine development, the papilla can further fold and develop secondary enamel knots, depending on the shape of the final tooth. During this last stage of tooth development, the mesenchymal tissue juxtaposed to the epithelium will differentiate into dentin secreting odontoblasts, while the immediate overlying epithelium contacting the enamel knots differentiates into enamel secreting amealoblast. Because enamel knots are late acting signaling centers, they provide information about the shape of the presumptive tooth. While certain genes like BMP2, Wnt10a, and Shh are strong markers for the enamel knots, fairly little is known how these and other genes interact in shaping the tooth. The role of BMP's can be found by targeting them specifically or indirectly through one of their inhibitors. Ectodin, one such BMP inhibitor, is found in the epithelium surrounding the enamel knots and confining them to that space. Null mutants of Ectodin in the mouse suffer from an expansion of enamel knots into once delineated epithelium. The resulting phenotype has supernumerary molars, a more complex accelerated

cusp patterning, and a more robust overall dentition (Kassai 2005). Interestingly, the downregulation of BMP activity by implanting BMP inhibitor Noggin soaked beads in the incisor region leads to an all molar phenotype (Tucker 1998). This suggests that BMP's have an early role in the tooth specific interaction with genes like Msx1/2 and Barx1 that are proximal-distal domain specific, and a later more general role in shaping all teeth at the level of the enamel knot. Members of the delta-notch family of extracellular ligands and receptors have also been indicted with shaping the tooth.

Genetic Modulation

In order to identify the exact roles of a gene, many studies will target a specific gene for knockout or upregulation. These knockouts are difficult with certain co-opted homologous genes, because of pleitoropic roles they play elsewhere in the body essential for the viability of the organism. Knockout strains of the entire fgf or shh pathway results in embryos that abort before tooth initiation. Chemical inhibitors, however, have the ability to alter expression of proteins dose dependently. Furthermore, synthetic chemicals like aforementioned SU5402 can be used to target entire gene families at any stage of development through a delayed exposure. The teratogen cyclopamine is one such inhibitor. A natural product of the lily *Veratum californicum*, cyclopamine acts by inhibiting cholesterol-independent downstream transcription activating proteins of the shh signaling pathway. Studies in the cavefish have shown dose dependent phenotypic effects of cyclopamine in the eye (Yamamoto 2004), but none are reported for the teeth. Lithium chloride can also act in a dose dependent manner by upregulating *wnt* family

expression. By inhibiting the inhibitor Glycogen synthase kinase-3β (gsk-3β), canonaical wnt transcription products are upregulated. By treating embryos dose and temporal specific manor, downstream products of major signaling pathways should be altered in a dose dependent manor as well. It may even be possible to manipulate and reproduce species specific phenotypes in the cichlid by slight adjustments of one or more of these genes.

Methods

1.0 Fish husbandry

Different adult species of Lake Malawi cichlids were raised in a re-circulating aquarium system kept at 28ºC on a 12-hour day 12-hour night cycle (Georgia Tech). Species maintained include Copadichromis Eucinistomas, Dimidichromis Compressiceps, Metriaclima Zebra, Cynotilapia Afra, Melanochromis labrosus, and Labeotropheus Fuelleborni. Embryos from mouth brooding females were recorded in days post-fertilization (dpf) starting with 0 dpf for the first day of mouth brooding. Embryos were removed at the desired dpf and fixed or raised in separate culture tanks.

Embryos or juvenile fry were anaesthetized using 0.17mg/ml MS 222 tricaine (Sigma) preceding fixation (Westerfield 1995). Fry were fixed for at least 24 hours in 4.0% Paraformaldehyde in PBS and stored at 4ºC before any analysis. All analyses were performed after hatching stage from the corion.

2.0 Whole Mount Preparations

2.1 Dissections

Specimens were skinned and cleaned using a common razor and forceps. The lower pharyngeal jaw of adult lake samples were removed from the gill slit. Attached muscle and tissue was removed and jaws were air dried prior to any measurements. The ventral pharyngeal arches were detached through an incision from the Temporal Mandibular Joint to the point where the operculum meets, exposing the mandible and lower pharyngeal jaw. Finer dissections on embryos and juvenile were made using Dumont 55 stainless steel forceps.

2.1 Alizarin Red and Alcian Blue Staining

Adult specimens were removed from ethanol and placed into a solution of alacian blue for a period of 6 hours to stain cartilage. After the stain the specimens were washed with distilled water for at least two days; the water was changed twice daily until the odour of ethanol was undetectable. Specimens were then placed in trypsin to digest tissue for 4 hours. Specimens were then placed in alizarin red bone stain, for around 2 hours or until the tips of the fins were visibly stained. The stained fish were then placed in a 4% solution of KOH in dH_2O for 4-5 days to clear; the solution was changed daily. The cleared and stained fish were then placed in glycerol. Once the glycerol absorbed the remaining excess stain, it was replaced with fresh glycerol and a few crystals of thymol were added to prevent microbial growth. Specimens were stored at room temperature in thymol treated glycerol.

The protocol was modified for fry to reduce stress on the more delicate tissue. Juvenile or embryos were removed from 4%PFA/PBA solution after at least a day of fixation and placed into dH_2O for one hour. Either the alizarin red bone stain or the alcian blue cartilage stain was performed to reduce damage to the tissue and maintain high resolution.

The bone stain was generally performed after 6-7dpf. For earlier specimen, the alcian blue cartilage preparation was used because ossification had not yet taken place. For the bone stain, fry were placed in trypsin solution for one hour and then moved to alizarin red. Staining took between 20 and 40 minutes, with larger specimen requiring a longer stain time. Once the tips of the fins were stained red, the fry were moved to the KOH solution for a period of 24 hours. The cleared and stained fry were then stored in glycerol with 2-3 crystals of thymol at room temperature.

Tissue that had not ossified in cichlid embryos was viewed using alcian blue stain. Specimens were taken from dH_2O and placed in a 50% ethanol in dH_2O solution for one hour. Fry were then moved into a 75% ethanol solution for one hour and then 100% ethanol for another hour. Fry were then transferred to alcian blue solution for 2 hours. After the stain, the fry were worked back into dH_2O using 50% dH_2O in ethanol for 1 hour, 75% dH_2O for 1 hour, and finally pure dH_2O for a period of 24 hours, replacing with fresh dH_2O three times. The trypsin digest was skipped for embryos under 8dpf. Older fry were digested in trypsin for 1 hour. Specimens were then placed into 2%KOH for 12-24 hours, depending on the robustness of the specimen. The cleared and stained fry were then stored in glycerol with 2-3 crystals of thymol at room temperature.

2.3 Microscopy

Whole mount preparations were analyzed using a Leica Microsystems stereo microscope (MZ16). Specimens were photographed with a Leica digital camera attachment and images were viewed using Adobe Photoshop (v. 9.0). Fry from *In Situ* hybridization were placed in either NTMT or PBS over a 4% agarose medium in a 15ml Petri dish. Specimens in glycerol were either glued to the container or left floating for whole mount microscopy. High-magnification microscopy was performed on either histological sections or dissected tissue using a Leica DC2500 compound DCI microscope. The specimens were mounted on glass slides and 1 drop of 90% glycerol/PBS was added. A glass cover slip was gently placed over the specimen with the drop and excess glycerol solution was wiped away. The cover slip was fixed with N.Y.C.® clear nail polish. Specimens were analyzed and photographed between 10X to 60X magnification with a Leica digital camera in .jpeg format and viewed using Adobe® Photoshop (v. 9.0).

3.0 Molecular Methods

3.1 RNA Extraction

RNA was extracted from broods 5 dpf of development to encapsulate expression of odontogenic activity. An entire brood of cichlid embryos was anaesthetized using 0.17mg/ml MS 222 tricaine (Sigma) and snap frozen while fresh in 2ml screw cap tubes submerged in liquid nitrogen for 1 minute. Snap freezing was not a necessary step but aided in the breakdown of tissues during homogenization. A single snap frozen brood was homogenized in 1 ml of TRIzol Reagent (GibcoBRL) using a glass-Tephlon® homogenizer. The insoluble and un-homogenized

tissue was separated by centrigugation at 13,000 X g for 10 minutes at 4°C. The RNA containing supernatant was poured into a 2ml vial and incubated for 5 minutes at room temperature. 0.2 mL of chloroform was added; the mixture was shaken by hand, and incubated at room temperature for 2-3 more minutes. The mixture was then centrifuged at 13,000 x g for 15 minutes at room temperature to separate the RNA into the upper colorless aqueous layer. The aqueous phase was extracted using a micropipette and added to 0.5ml of isopropanol. The mixture was incubated at room temperature for 10 minutes before centrifuging at 13,000 x g for 10 additional minutes at room temperature to form a gel-like pellet. The pellet was washed with 1ml 75% ethanol, vortexed briefly, and centrifuged for 5 minutes. The ethanol was removed using a pulled glass pipette and left to air dry for 5 minutes. Finally, the isolated RNA was re-suspended in 100μL RNase-free H_2O and stored at -70°C.

3.2 cDNA Synthesis

First strand cDNA containing only exons was synthesized from previously isolated cichlid RNA. 2μl of total RNA (~2μg) was added to 1μl of Oligo-DT (Promega) and 11μl of Nuclease-free H_2O. The mixture was incubated at 72°C for 5 minutes and then placed on ice for 5 minutes. 5μl of AMV reverse transcriptase 5X reaction buffer (Promega), 2.5μl dNTP mix (Promega), 40 units of RNasin® Ribonuclease Inhibitor (40mM), 2.5μl sodium pyrophosphate (40mM; prewarmed to 42°C), and 30 units of AMV (avian malony virus) RT (Promega) in Nuclease-free H_2O were then added to make up a total volume 25μl. The mixture was gently stirred and incubated at 42°C for 1 hour. The final cDNA was then stored at -20°C.

3.3 Degenerate Primer design

Primers for suspected genes of interest in odontogenesis were designed for *in situ* hybridization. Degenerate primers were assembled using sequence data from vertebrate taxa using the publically available database Genbank (www.nbci.nlm.nih.gov). The sequence for a given gene was matched to partial sequence data available for one of six cichlid species at http://cichlids.biology.gatech.edu/blastpage.php (Loh in review). The identified partial sequence was then verified using the Genbank website BLAST 'Basic Local Alignment Search Tool' (Altschul et al. 1990). Sequences were aligned using the OPERON website (https://www.operon.com). Sense and antisense primers were chosen between 18 and 24 nucleotide bases with a melting point between 55ºC-60ºC to amplify a region of cichlid sequence containing the genes of interest of around 700 base pairs. A primer pair was chosen to amplify the 3' and 5' end of the gene. Primers were then constructed through OPERON for PCR.

3.4 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify a gene of interest using a pair of degenerate primers. The 0.5 µL of each primer from a pair was combined with 12.5 µL GoTAQ, 10.5 µL nuclease free H_2O, and 1 µL of isolated cDNA. Heat controlled cycles of PCR annealing and dissociation were run for 3 hours to amplify the target cDNA region. Amplified fragments were separated by base pair length with a 1% agarose/TBE gel containing 0.5µg/mL ethidium bromide (Sigma). The sizes of the isolated fragments were estimated using a 1kilo base ladder run along the PCR products and compared to predicted target sequence length. Gels were viewed and photographed under Ultra Violet (UV) light. Products were cleaned by adding 12.5 µL ExoSap and running a second round of PCR.

3.5 Amplification of PCR Products

PCR products were amplified by inserting them into a vector, transforming the complete plasmid into a bacterial host, and amplifying the bacterium along with the product containing plasmid. DNA inserts were first ligated into a pGEM®-T Easy Vector System I (Promega). 3μl of ExoSap purified insert DNA were added to 1μl of vector, 5μl of 2X Rapid Ligation Buffer, and lastly 1μl of T4 DNA Ligase enzyme. The solution was gently mixed and incubated at room temperature for 1 hour.

3μl of complete plasmid ligation product were added into 30μL competent Escherichia coli cells (XL-1 Blue; Stratagene) that were kept on ice. The plasmids in cells were mixed very gently and incubated on ice for 30 minutes. The cells were then heat shocked at 42ºC for 60 seconds and immediately placed back on ice for 2 minutes. Transformation was completed by adding 300μl of LB (Luria-Bertani) media to the heat shocked cells and incubating at 37°C for 1 hour. The transformed cells at this point could be stored at 4°C for up to one week.

40 μL xGAL and 12 μL IPTG were spread onto dry LB agar plates containing ampicillin (50μg/ml) using an L-shaped glass rod sterilized with heat and ethanol. The plates were allowed to soak up the xGAL mixture at 37°C for 30 minutes in order to later identify transformed colonies. 100μL of the transformed cells were then spread onto the xGAL soaked plate using the sterilized L-shaped glass rod and incubated overnight at 37°C. Colonies that had not transformed the plasmid could be identified by a blue color from the xGAL solution. 8 to 10 colonies were selected that were not blue xGAL positive and did not have satellite colonies. Selected colonies were spotted onto a labeled LB agar replica plate and soaked in μL ExoSAP. Plasmids

transformed in colonies were amplified with PCR and analyzed for purity on a 1% agarose/TBE electrophoresis gel containing 0.5μg/ml ethidium bromide (Sigma).

3.6 DNA Sequencing

Plasmid DNA was sequenced in the Environmental Science and Technology Building at the Georgia Institute of Technology. Sequences were displayed with % confidence using the Sequencher® USB drive tool. High purity sequences were verified using the National Center for Biotechnology Information (NCBI) BLAST tool (www.ncbi.nih.nlm.gov) to confirm the identity of the sequenced gene.

3.7 Linearization of Plasmid DNA

Plasmids containing the target gene DNA, verified through sequencing, were lineralized to create RNA probes. 10μg plasmid DNA, μL DEPC-H_2O, μL 10X reaction buffer, and lastly 20 units Enzyme specific to each plasmid, for a final volume of 50μL, were added to a 2 mL reaction tube. The plasmid was incubated with the specific enzyme and buffer for 1 hour at 37°C to complete digestion. Linearized plasmid DNA was purified with a 50:50 phenol:chloroform containing 3M sodium acetate (NaOAc). 200 μL 100% ethanol was added to precipitate the DNA. The mixture was centrifuged at 13,000 x g to form a DNA gel-like pellet. Ethanol was removed from the DNA pellet using a pulled sterile glass pipette and re-suspended in 10μl DEPC-H_2O to give an approximate concentration of 1μg/μl. Both the lineralized and complete

plasmid were run alongside each other with a 1 kilo base ladder on a 1% agarose/TBE gel containing 0.1µg/ml ethidium bromide to confirm linerization.

3.8 Synthesis of DIG-labelled RNA probes

RNA probes were synthesized from the lineralized DNA plasmids for use in *in situ* hybridization. 1µg linearized plasmid DNA, 5X transcription buffer, 10mM DTT, 10X DIG-RNA labelling mix (Roche), 20 units RNase Inhibitor (Roche), ~20 units polymerase were combined for a total volume of 20µL. The reaction mixture was incubated at 37°C for 2 hours, 20 units of DNase I (Roche) was added, and the mixture was incubated at 37°C for an additional 15 minutes. 100µl TE (10mM Tris (pH 8); 1mM EDTA in dH_2O) , 10µl 4M LiCl, and 300µl absolute ethanol were added and the mixture was centrifuged at 13, 000 x g for 10 minutes at room temperature. The solution was removed from the resulting pellet and 100µl 70% ethanol in dH2O was added as a wash. The pellet was re-centrifuged for 2 more minutes and the ethanol was removed from the pellet using a sterile finely pulled glass pipette. The pellet was finally breifally air dried and resuspended in 100µl TE resulting in an approximate concentration of 0.1µg/µl. The product was run on a 1% agarose/TBE gel containing 0.5µg/ml ethidium bromide against a 1 kilo base ladder to confirm the presences of the RNA probe.

3.9 In situ hybridization using RNA probes

In situ hybridization was taken from a protocol published by Fraser (Fraser 2004) that was adapted from Xu et al (Xu et al. 1994). The reactions for the protocol were performed in

individual wells of a 24-well titer plate. After embryos were fixed in 4% PFA they were worked into methanol. Specimen were washed 3X 10 minutes in PBST (1% tween in phosphate buffer solution) and then one 10 minute wash at 25%, 50%, 75% methanol in PBST. Embryos were rinsed 2X with 100% methanol and stored in methanol for at least 24 hours at -20˚C, where they could be left for storage. Embryos were then rehydrated by rinsing with a graded series of methanol in PBST at 1X 5 minutes of 75%, 50%, and 25% methanol in PBST. Embryos were rinsed 2X in 100% PBST and digested with proteinase K. The concentration of proteinase K was dependent on the age of the embryo at a 1:1000 dilution of 10μg/mL proteinase K. For example, a 7dpf embryo was washed at a 7:1000 dilution. The specimen were rinsed once in PBST and refixed in 4% PFA for 30 minutes. The specimens were then washed 3X 5 minutes in PBST. Prehybridization solution was made up of 25mL formamide, 12.5mL 20X SSC, 460μL citric acid, 500μL tween-20, and 24mL DEPC-H$_2$O. Fish were covered and soaked in preybridization solution until they settled to the bottom of the well. The solution was replaced with fresh prehybridization solution and the well was placed in a 70˚C water bath for 2-3 hours. At this point, all reactions were carried out at 70˚C until the addition of MABT to reduce background staining. Hybridization solution was prepared using 25mL formamide, 12.5mL 20X SSC, 460μL citric acid, 500μL tween-20, and 24mL DEPC-H$_2$O, 125μL tRNA, and 100μL heparin. The prehybridization solution was replaced with hybridization solution the desired probe was added a dilution of 10μL for each mL of hybridization solution. The reaction was allowed to take place overnight.

The following day the probes were taken off and stored and embryos were washed with prehybridization solution 2X for 5 minutes. Embryos were then washed 25% prehybridiation solution in 2X SSC for 5 minutes, 100% 2X SSC for 10 minutes, and 0.2X SSC for 3X 10

minutes. After the third wash the embryos were removed from the 70˚C water bath and rinsed

2X for 5 minutes at room temperature with MABT. Blocking solution was prepared with 5mL

goat serum, 5mL BBR, and 40mL MABT. Specimens were incubated with fresh blocking

solution for 2-3 hours at room temperature on a rocker. The blocking solution was replaced with

a fresh aliquot and AP Fragments Anti-dig antibody was added at a 1:6000 solution. The reaction

was incubated at 4˚C overnight on a rocker.

The following day the embryos were washed 2X 5 minutes with TST, followed by 6X 60

minutes with TST. TST was prepared with 500μL NaCl, 250μL 2M 7.5 pH tris HCl, 50μL

tween, and 49mL DEPC-H_2O. The specimens were washed 2X 5 minutes in NTMT. NTMT was

prepared with 1mL NaCl, 2.5mL 2M 7.5 pH tris HCl, 500μL tween, 1.25mL 2M $MgCl_2$, and

45mL DEPC-H_2O. NBT/BCIP was added at a dilution of 20μL to every mL of NTMT. The color

reaction was allowed to take place for as many days as necessary to develop without

overstaining. Once the full expression signal was detected, the embryos were rinsed 2X in PBST,

refixed with 4% PFA, rinsed PBS, and transferred to glycerol with a few crystals of thymol for

storage at 4˚C.

3.10 Albumin Sections

Embryos that were hybridized with RNA probes were embedded in gelatin-albumin (2.2g

gelatin in 450 ml PBS; 135g chick egg albumin; 90g sucrose; stored at -20°C) solution and

sectioned for histological analyses. Embryos were oriented according to the desired plane of

section in approximately 1 ml gelatin-albumin solution in wax coated molds. 2.5%

gluteraldehyde fixative was added and quickly mixed throughout the gelatin-albumin before

setting without disturbing the embryo orientation. The filled molds were covered in cellophane bags with a moist towel and left overnight at 4°C to set. The embryo embedded albumin blocks were cut from the mold and placed in 4% PFA/PBS for 1 hour as a post fix. The blocks were then soaked in PBS for at least 30 minutes before sectioning. The finished block was oriented and mounted onto the removable stage of a Leica Microsystems VT1000 'vibratome' and adhered using cyanide based glue. 15-25µm sections were cut through the embryo and placed onto glass slides according to the procedures outlined in the microscopy section.

4.0 Chemical Treatment

4.1 Cyclopamine Mutagenesis

Cyclopamine (LC labrotories) mutagenesis cultures were performed in order to alter shh gene mediated development. Cyclopamine is a dangerous teratogen that inhibits hedgehog transduction by binding to the patched membrane protein. The treatment protocol was adapted from previous literature (Yammamoto 2004), but a different solvent was chosen because of previously unpublished late day developmental defects resembling fetal alcohol syndrome in ethanol treated control groups. A brood was selected based on developmental stage (usually between 3-6dpf) and its species specific dentition to study a certain aspect of odontogenesis. A brood was divided into three groups. One control group was incubated in the fish room under normal culture conditions with no treatment. A second control group was incubated in 1% DMSO (40 µL DMSO in 4.0mL of egg water) in a 5mL Petri dish at 28ºC for 24 hours shaking at 130 rpm on a Barnstead Lab-Line Max 4000 culture incubator. Several holes were punctured in the lid of the Petri dish to allow for proper air circulation and the dish was wrapped with

parafilm to prevent spilling. A third group was treated with 50µM cyclopamine incubated in a 5mL Petri dish at 28ºC for 24 hours shaking at 130 rpm. A 5mM stock solution of cyclopamine in DMSO was produced by dissolving 10mg of cyclopamine in 4.84mL of DMSO for 30 minutes at 55ºC. Stock solution was stored at -20ºC and redissolved at 55ºC for 30 minutes before use. 40 µL of the 5mM cyclopamine/DMSO stock solution was added to 4.0mL egg water in a 5ml Petri dish to make the 50µM cyclopamine solution. After the 24 hour treatment, the DMSO control and the cyclopamine treatment group were rinsed several times with fresh egg water and moved to the fish room in the Ford Environmental Science and Technology building at the Georgia Institute of Technology. The three groups were cultured under normal conditions at 28ºC with fresh egg water daily. A fraction from each group was anesthetized using 0.17mg/ml MS 222 tricaine (Sigma) and fixed in 4% PFA/PBS at 6-7dpf in order to investigate early stages of odontogenesis. The remaining fry from each group were cultured and fixed at 12-14dpf to examine later stages of odontogenesis. Fixed fish from each group were either analyzed with *in situ* hybridization or cleared and stained using alacian blue or aliziran red.

4.2 SU5402 Mutagenesis

SU5402 mutagenesis cultures were performed in order to alter fibroblast growth factor (fgf) gene mediated development. SU5402 is a dangerous teratogen that blocks fgf signaling by binding to fgf receptors (fgfr). The treatment protocol was adapted from previous literature (Jackman et al. 2004). A brood was selected based on developmental stage (usually between 3-6dpf) and its species specific dentition to study a certain aspect of odontogenesis. A brood was divided into three groups. One control group was incubated in the fish room under normal

culture conditions with no treatment. A second control group was incubated in 0.5% DMSO (20 µL DMSO in 4.0mL of egg water) in a 5mL Petri dish at 28ºC for 24 hours shaking at 130 rpm on a Barnstead Lab-Line Max 4000 culture incubator. Several holes were punctured in the lid of the Petri dish to allow for proper air circulation and the dish was wrapped with parafilm to prevent spilling. A third group was treated with 25µM SU5402 incubated in a 5mL Petri dish at 28ºC for 24 hours shaking at 130 rpm. A 5mM stock solution of SU5402 in DMSO was produced by dissolving 1.0mg of SU5402 in 0.6784mL of DMSO for 10 minutes at 37ºC. Stock solution was stored at 4ºC and redissolved at 37ºC for 10 minutes before use. 20 µL of the 4.975mM SU5402/DMSO stock solution was added to 4.0mL egg water in a 5ml Petri dish to make the 25µM SU5402 solution. After the 24 hour treatment, the DMSO control and the SU5402 treatment group were rinsed several times with fresh egg water and moved to the fish room in the Ford Environmental Science and Technology building at the Georgia Institute of Technology. The three groups were cultured under normal conditions at 28ºC with fresh egg water daily. A fraction from each group was anesthetized using 0.17mg/ml MS 222 tricaine (Sigma) and fixed in 4% PFA/PBS at 6-7dpf in order to investigate early stages of odontogenesis. The remaining fry from each group were cultured and fixed at 12-14dpf to examine later stages of odontogenesis. Fixed fish from each group were either analyzed with *in situ* hybridization or cleared and stained using alacian blue or aliziran red.

Results

Cyclopamine disrupts odontogenesis

Cyclopamine was applied at 5dpf at a concentration of 50µM to inhibit SHH signaling to the first developing tooth. An equivalent amount at 1% of ethanol was applied to a control group according to previously published methods (Yammamoto 2004). While in situ hybridization experiments showed promising differential expression of *shh* and *dlx2* in the teeth and brain, late day bone stain preparations revealed gross phenotypic differences between the ethanol control and the wild type, suggesting the need for a new solvent. 1% DMSO was chosen as a control for 5mM stock of cyclopamine in DMSO experiments. Embryos were treated for 24 hours with the cyclopamine or control DMSO solvent for 24 hours, at which point the water was replaced with fresh egg water and the treatments were allowed to recover for another 24 hour period. Some of each brood was fixed at 7dpf for *in situ* hybridization. *In situ* hybridization revealed that cyclopamine treated embryos exhibited two *shh* expressing placodes in the dentary while the control DMSO embryos expressed the normal 3 demarcated placodes seen in wild type experiments (figure 1). Furthermore, the cyclopamine treated embryos lacked a clear zone of inhibition (ZOI) between the developing teeth and the following second odontogenic band. Finally, the two halves of the secondary odontogenic band marked by *shh* expression in the mandible were fused. Embryos from the same treatments and controls were allowed to further develop to 12dpf, the stage where the second row of teeth has erupted in C. *afra*, and stained with alizarin red. While the control 1% DMSO treated individuals exhibited a normal dentition composed of multiple teeth in two rows, the treated embryos expressed complete anadontia. The remnants of three detached shards of teeth were present in the loose epithelium.

SU5402 reduces tooth number and increases tooth size

SU5402 was applied at 5dpf to inhibit fgf signaling of the first developing tooth. While blocking Fgf receptors with SU5402 did not completely remove the dentition of treated embryos, it did reduce the number of teeth that developed (figure 2). Interestingly, while the number of teeth was reduced, the size of the tooth buds and subsequently the size of the developed teeth were larger than that of untreated wild type and control DMSO treated embryos. *In situ* hybridization of *shh* gene expression was performed not because of anticipated effects downstream of SU5402 treatment, but as a marker for the tooth placodes. *Shh in situ* hybridization revealed that the buds were larger and expression was overlapping the odontogenic band of the developing juxtaposed anterior row. dlx2 is a downstream target of fgf's in zebrafish tooth development (Jackman 2004). Expression of *dlx2* was almost absent in the dental competent region of the dentary in SU5402 treated embryos. However, *dlx2* expression continued to demarcate the lateral ridges of the mandible as well as other *dlx2* expressing organs such as the brain. Control embryos with DMSO and wild type maintained normal *dlx2* expression patterns surrounding the developing teeth as well as at the lateral ridges of the mandible.

Effects of teratogens on replacement

While cyclopamine aborted all developing teeth and did not allow for recovery, fgf inhibition through SU5402 allowed the initiated dentition to develop into fewer, but larger teeth. Whole mount images of juvenile cleared and stained treated specimen against the control

revealed that replacement teeth were either severely reduce or absent (figure 3). Coronal sections revealed that while the treated embryos still had replacement teeth, they were very small and underdeveloped. The DMSO control replacement teeth were approximately the same size or slightly larger than their corresponding initial erupted teeth. The replacement teeth of SU5402 treated specimen appeared as shards that were much smaller than the corresponding erupted teeth. These teeth were likely not functional and would either erupt and abort or fail to erupt and replace all together.

Differential of SU5402 effects between species

Preliminary results between predator morphs of Malawi cichlids and non-piscavore Mbuna and non-Mbuna revealed differential effects of treatment SU5402 on the dentition. As reported above, SU5402 treated specimen of C. *afra* and M. *Zebra Albino* exhibited fewer, more robust teeth. Interestingly, this dental phenotype mimics that of what is seen in wild type piscavore Malawi cichlids. M. *labrosus*, one such piscavore Malawi cichlid, was treated under the same concentrations and conditions as the other specimen. However, the dentition did not seem to be greatly affected by the Fgf inhibition through SU5402 treatment. While the treated M. *labrosus* exhibited few, large teeth as in the other treated species; this phenotype was not a departure from the wild type.

Discussion

Choosing a solvent system for the teratogen

The imperative choice of an ideal control is often easy to overlook. Many treatments with cyclopamine were performed using ethanol as a solvent delivery system. Ethanol was chosen because of previously published literature and because it was suggested by the manufacturer as the solvent of choice. While cyclopamine is very soluble in ethanol and less volatile than other solvents such as methanol, it is also the key ingredient behind fetal alcohol syndrome. As reported by others, differential expression patterns were seen between ethanol control groups and individuals treated with cyclopamine from an ethanol stock solution. However, because low concentrations were used, the embryos were viable for examination of effects later in development. Both the control embryos treated with 1% ethanol and the cyclopamine treated specimen had large differences craniofacial and tooth development compared to that of wild type, including a missing operculum, reduced forebrain, and large protruding teeth. Because these phenotypic effects are only seen later in development, they have not yet been reported. However, a proper positive control group should not exhibit any differences from the wild type so only isolated effects of the signal altering chemical treatment are seen. DMSO is suggested not only as an alternative, but as an improved solvent for cyclopamine delivery. While cyclopamine is not very soluble in concentrations above 4 mg/mL of DMSO, it goes into solution faster and at a lower temperature than it would in ethanol at concentrations suitable for *in vivo* studies. Furthermore, DMSO has no observable effect on the developing dentition.

SHH is required for cichlid tooth development and the importance of first tooth

Previous studies of the mouse indicate (Cobourne 2001, Sakar 2000) that inhibition of SHH signalling during the initiation of the first tooth placode destroys the competence of the dentition, arresting the developing teeth at the tooth bud stage. Inhibition of hh signalling by treatments of the Malawi cichlid C. *afra* with cyclopamine during the development of the first tooth confirms this. While the developing first tooth was given a stage to recover, during which time *shh* expression was detected in the first three placodes, those teeth eventually aborted. Furthermore, then dentition never recovered after the stage-specific treatment. By destroying the patterning of the first tooth, the iterative copy-paste mechanisms (Fraser et al in press) of determining the development for subsequent teeth and rows is obliterated. Even though teeth in later rows and lateral teeth within the first row were not initiated during the time of cyclopamine treatment, no teeth formed. The lack of recovery of the subsequent dentition suggests that the first tooth is essential for laying down the pattern of subsequent teeth.

Preliminary results with lower concentrations at later days in different species suggest that if the first tooth does indeed initiate, a reduced dentition will develop. Treatment with cyclopamine was performed on 6dpf, after the initiation of the first few teeth (data not shown). While the number of teeth was reduced, teeth were still present proportional to the age of the specimen. This suggests that treatment with cyclopamine does not permanently deactivate the hedgehog signalling pathway and can become unbound to smoothened receptors, allowing the dentition to recover. However, when examining the pre-maxilla, which is one day delayed in the developing cichlid dentition, we discovered that the dentition had been deleted. This observation further supports the notion that initiation of the first tooth is essential for patterning the dentition while also providing for a good internal comparison group for future studies.

Effects of SU5402 on the dentition and its' replacement

SU5402 treatments blocked the fgf receptor for all members of the fgf pathway applied in the epithelial thickening stage of the developing C. *afra* tooth. *In situ* analysis of *shh* expression was performed because *shh* demarcates the developing tooth placodes. Placodes of those embryos treated with SU5402 had larger, more robust patterns of *shh* expression compared to that of the controls. When these treated individuals were allowed to grow to a stage equivalent to that of a wild type with multiple rows and multiple teeth with an initiated replacement system, several effects were noted. The most obvious difference was the presence of fewer, larger teeth. While, the number of teeth was reduced, they were quantifiably more robust and functional. This difference between normal odontogenesis in C. *afra* is likely due to inhibition of fgf members activated early in the thickening and bud stage of the developing cichlid tooth. While *fgf8* and *fgf9* have been indicated as key initiators of the murine molars and dental lamina, fgf8 is not expressed in the early development of the pharyngeal zebrafish (Jackman 2004) or either of the trout dentition, both oral and pharyngeal (Fraser 2006). *fgf8* is similarly not expressed in the oral or pharyngeal dentitions in cichlids (Fraser et al, in press). It is possible that other fgf molecules, such as fgf9 is acting to initiate or shape the dentition, but *fgf9* in osteichthyans does not share the homology seen between *fgf8* and *fgf9* in the mouse. In contrast, *fgf9* is more homologous to *fgf20* but may still act in the initiation of the cichlid and possibly the zebrafish dentition. Primers have been designed for *in situ* analyses and probes will be developed shortly. Alternatively, another member of the Fgf pathway may be acting early on in the cichlid dentition.

Dlx2 expression was analyzed to confirm the inhibition of fgf's. dlx2 is proposed to lie downstream of fgf8/9. Therefore, inhibition of signaling from Fgf family members would result in down-regulation of *dlx2* expression in the developing presumptive tooth field. This is in fact the case seen in SU5402 treated fish. While expression patterns of *dlx2* are normal in those embryos treated with control DMSO solvent, the expected expression between the developing placodes is absent in treated embryos. On the other hand, lateral mandibular expression of dlx2 is maintained in treated embryos. This pattern of expression suggests that Fgf/Dlx2 interactions are specific to the presumptive tooth field in the developing cichlid mandible and a yet unidentified fgf is acting upstream of Dlx2 in odontogenesis.

Histological sections were performed to determine effects of SU5402 on the underlying replacement teeth. While the replacement teeth were not initiated at the time of treatment, those embryos that were exposed early during the initiation of the first tooth exhibited a reduced first round of replacement teeth. There are several possible reasons for their reduction. One hypothesis is that the erupted tooth signals the underlying developing replacement tooth. If such signaling exists, supported by preliminary data concerning *pitx2* and *dec1* expression (Fraser and Streelman unpublished), alteration of the development of the first teeth could result in deformations of the replacement mechanism. An alternative would be that the competence of the mesenchyme or epithelium that contributes to the replacement dentition is affected by early fgf inhibition.

Late day treatments on C. *eucinostomas* demonstrated that application of SU5402 for longer times could potentially wipe out the entire replacement mechanism. fgf10 and fgf3 are indicated in the maintenance of the stem cell niche of in the rodent incisor. By inhibiting both or either of genes at the fgf receptor, the stem cell mechanisms needed for the lifetime replacement

of cichlid teeth could be interrupted. BrdU labeling could be used to measure levels of apoptosis that might be associated with the interruption of these stem cells. Additional late day treatments as well as similar experiments that allow for further development of the treated embryos are needed to further elucidate the role of fgf's in the cichlid dentition.

Conclusion

Cichlids provide a diverse pattern of dentitions that act as "natural mutants" for developmental studies. Coupling the variance seen inter-specifically in Malawi cichlids with the imposed manipulation of expression through chemical modulators allows for a powerful model for odontogenic studies. This coupling provides an almost limitless array of factors to examine that play a part in the complex development of the dentition. By inhibiting shh signaling during the development of the first tooth, we have shown that this tooth is essential for the development of the ensuing dentition and without it or an implemented rescue procedure, anadontia is inevitable. Furthermore, we have demonstrated a role for fgf's in the cichlid dentition. This role affects downstream dlx2 signaling and the pattern of the degenerate dentition. While some fgf's are necessary at tooth initiation, others are important for the regulation of the stem cell populations responsible for maintaining proper replacement cycles.
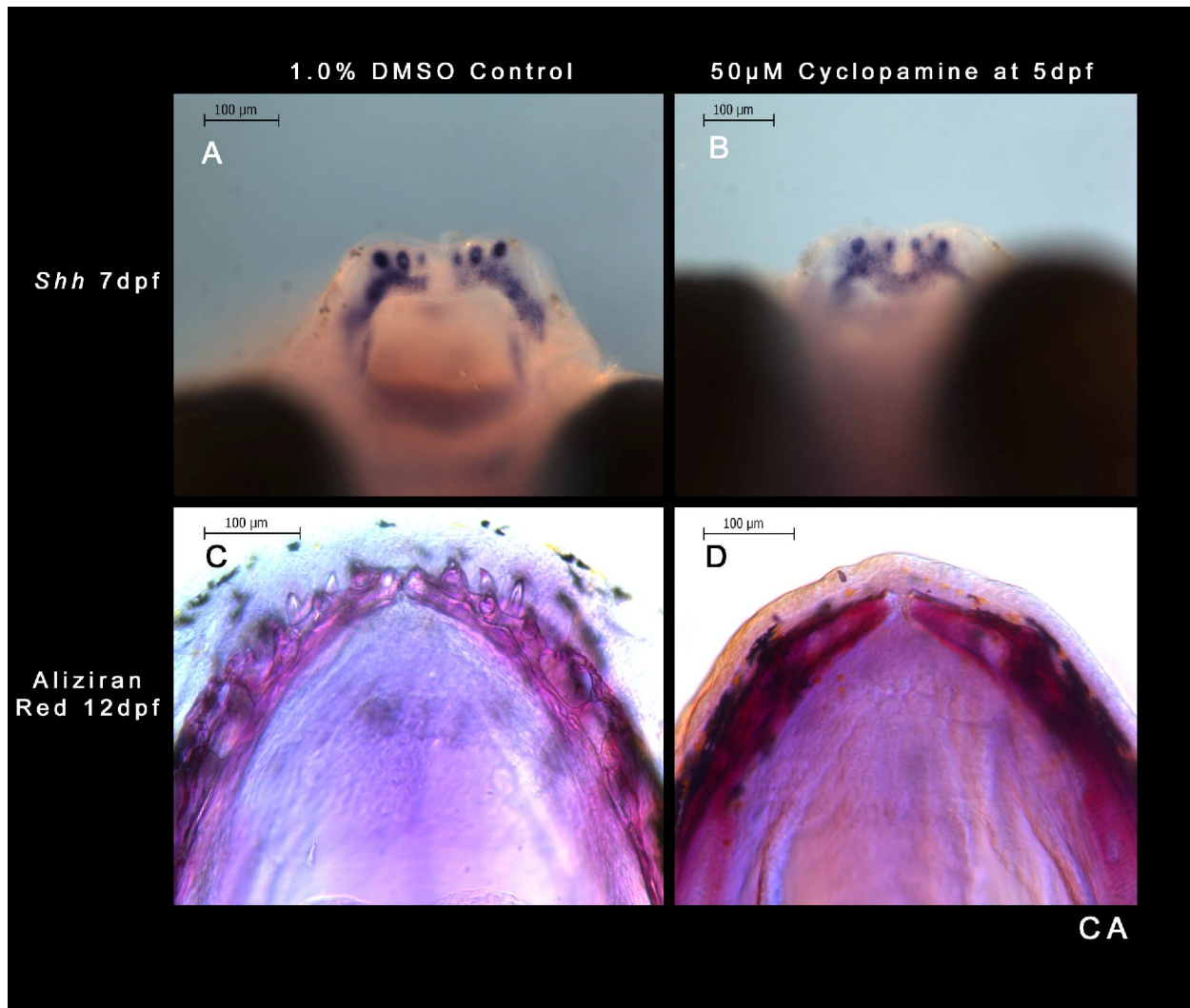
Figure 1 - Hedgehog pathway is essential for patterning the entire dentition in Malawi cichlids. Control (DMSO) treated (A) and cyclopamine treated (B) C. afra embryos (7 dpf) showing shh expression in the lower jaw dentition. Cyclopamine (and DMSO) was administered to C. afra embryos at 5 dpf for 24 hours, embryos continued to develop for a further 24 hours under standard conditions and fixed at 7dpf. Weaker levels of shh expression in cyclopamine-treated fish along with a disturbed initial pattern of tooth placodes and second odontogenic band (B) compared to the DMSO control (A). Note three tooth buds on each half of the dentary in the control (A) compared to two tooth buds on each half of the dentary in embryos treated with cyclopamine (B). C. afra that were treated at 5 dpf (as above) continued to develop for a further 6 days (12 dpf) through into stages where teeth of the first row are expected to erupt and teeth should be developing in the second tooth row. Compared to the DMSO control (C) the cyclopamine-treated C.afra (D) failed to develop a dentition, a single tooth shard is

seen unattached within the epithelium above the ossified lower jaw (alizarin red preparation).
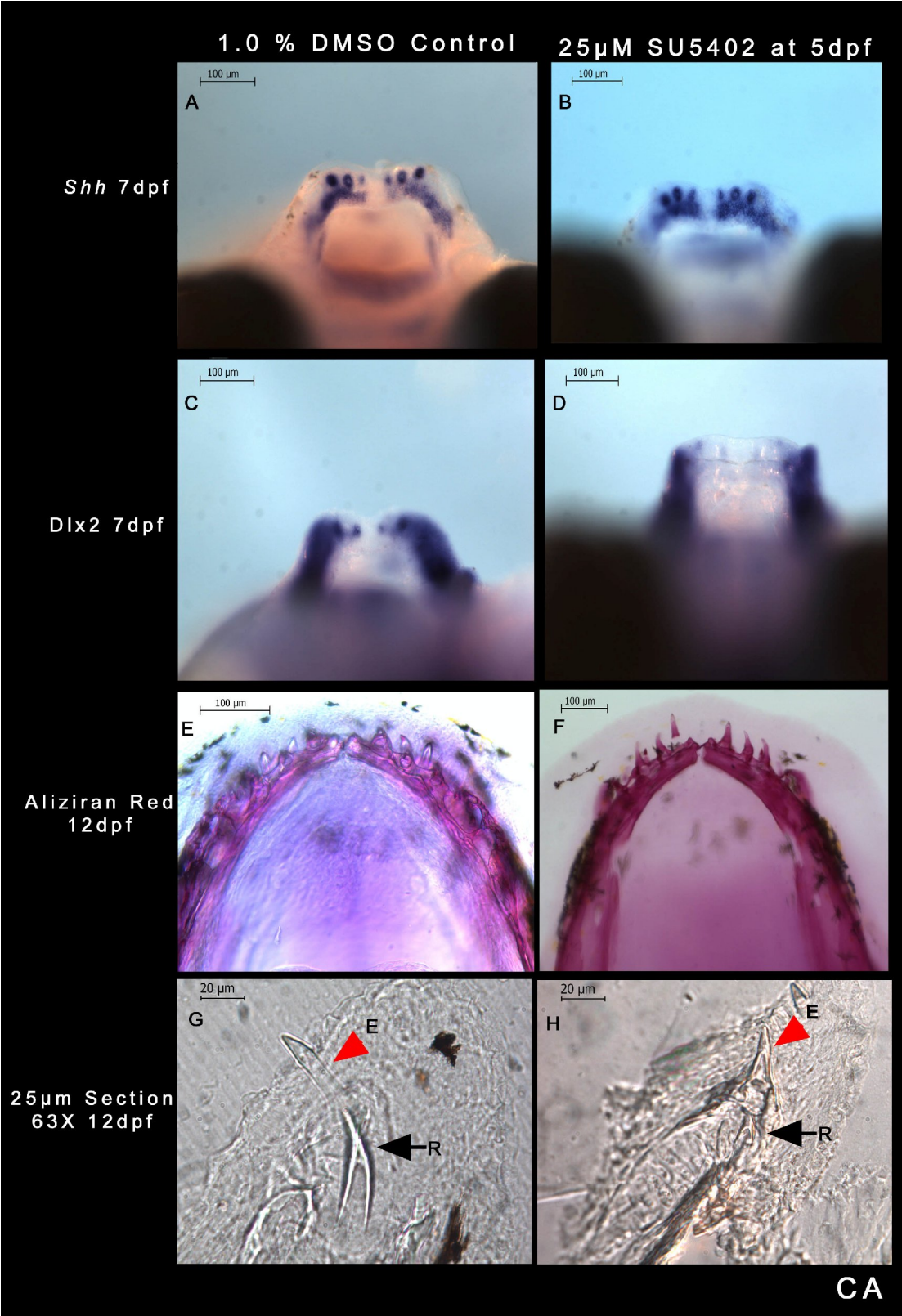
Figure 2 – FGF pathway maintains tooth size and replacement. Control (DMSO) treated (A) and SU5402 treated (B) C. afra embryos (7 dpf) showing shh expression in the lower jaw dentition. SU5402 (and DMSO) was administered to C. afra embryos at 5 dpf for 24 hours, embryos continued to develop for a further 24 hours under standard conditions and fixed at 7dpf. Expanded levels of shh expression in SU5402-treated fish in tooth placodes overlaps into the second odontogenic band (B) compared to the DMSO control (A). Note three demarcated tooth buds on each half of the dentary in the control (A) compared to three enlarged overlapping tooth buds on each half of the dentary in embryos treated with SU5402 (B). Dlx2 expression (C, D) surrounds the developing placodes and is expressed in the lateral dentary in the DMSO control (C) while medial placode surrounding expression is lost in SU5402 treated specimen. C. afra that were treated at 5 dpf (as above) continued to develop for a further 6 days (12 dpf) through into stages where teeth of the first row are expected to erupt and teeth should be developing in the second tooth row. Compared to the DMSO control (E), the SU5402-treated C.afra (F) had a dentition limited to one row with fewer, larger teeth in the lower jaw (alizarin red preparation). 25 micron coronal histological sections (G, H) of the alizarin red preparation specimen (E, F) viewed at 63X magnification revealed that SU5402-treated C. afra (H) had severely reduced underlying replacement teeth (black arrow) far from the above erupted tooth (red arrow). The DMSO control (G) exhibited a normal replacement tooth (black arrow) that was larger than the erupted functional tooth (red arrow).
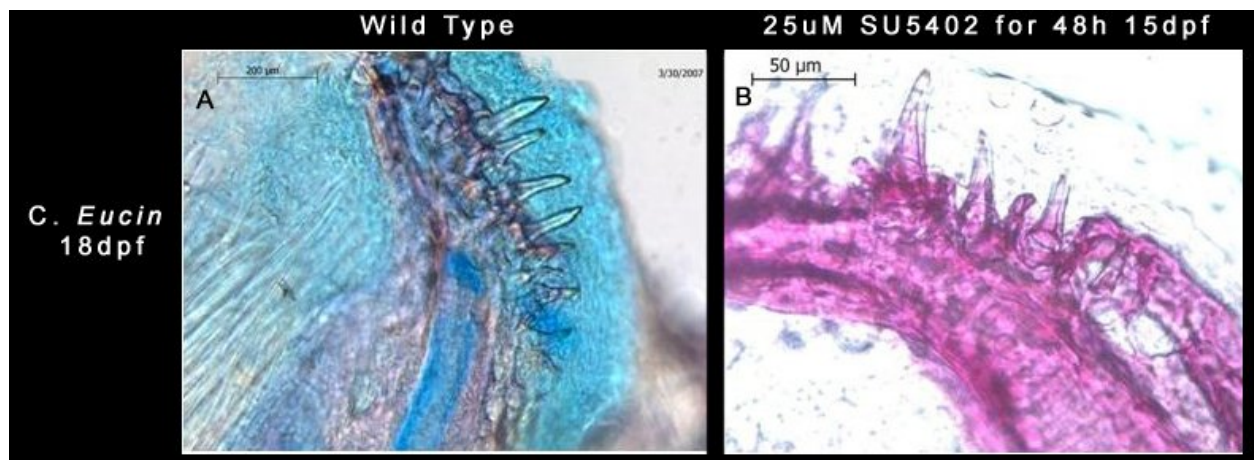

Figure 3 – Late FGF signaling sustains the replacement program. Wild type C. eucinostomas at 18dpf (A) posses a row of replacement teeth in the underlying mesenchyme of the functioning erupted teeth. C. eucinostomas treated with SU5402 for 48 hours (B) that were allowed to develop for an additional 24 hours exhibited a complete lack of underlying replacement teeth. The long treatment times could have interrupted the presumptive replacement dentition stem cell niche.
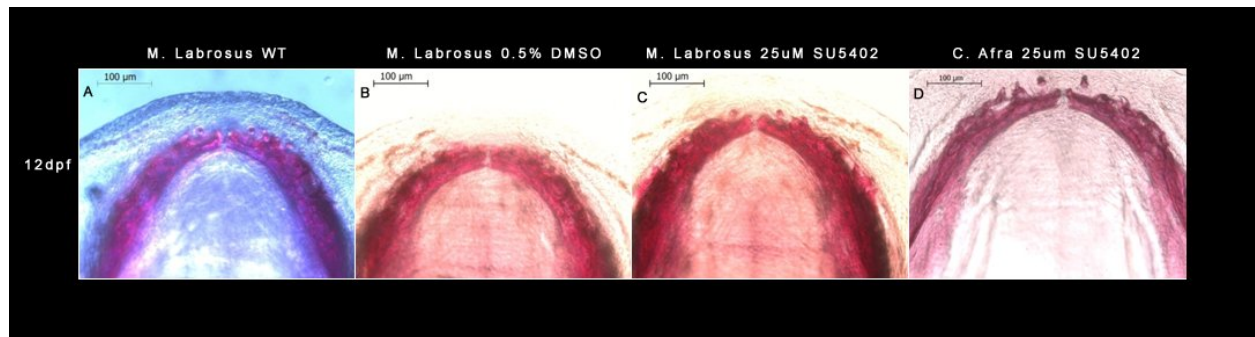
Figure 4– FGF inhibition results in a piscavore -like dentition. Alizarin red preparations of wild type, DMSO control, and SU5402-treated M. labrosus (A, B, C respectively) and SU5402-treated C. afra (D) all at 12dpf showing similar dentitions. SU5402-treated at 5dpf M. labrosus (C) did not exhibit a difference between the wild type (A) and DMSO control (B) M. labrosus in the erupted dentition. SU5402-treated at 5dpf C. afra exhibited a very similar dentition with fewer, larger teeth. Reduction in Fgf signaling results in a piscavore dentition, suggesting a molecular model for species divergence.

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