Epha4b Expression in the Craniofacial Development of African Cichlid Fishes

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Epha4b gene expression may contribute to variances in facial formation including functional differences such as nose shape and clinical conditions such as cleft palate. During craniofacial development, neural crest cells migrate to the pharyngeal arches then differentiate to form bone, muscle, and cartilage cells. The Eph/ephrin signaling pathway guides the streams of migrating neural crest cells into the pharyngeal arches; *epha4b*, a gene encoding an Eph receptor, contributes to this signaling pathway. To determine how *epha4b* expression differs between species at different developmental stages, in situ hybridization, a process that stains areas of gene expression, was performed. African cichlid fish are an ideal model because species have evolved various morphologies based on their feeding. For instance, species such as *Labeotropheus fuelleborni* evolved a short mandible for biting and species such as *Maylandia zebra* evolved a long mandible for suction feeding. *M. zebra* embryos demonstrated low *epha4b* expression in pharyngeal arch one while *L. fuelleborni* demonstrated more *epha4b* expression, suggesting a negative correlation between level of *epha4b* expression and mandible length. Understanding the factors contributing to craniofacial development and variation will help discover treatments for facial birth defects and conditions.

Introduction

Craniofacial anomalies are birth defects such as cleft palate that cause structures of the head and face to form incorrectly[1,2]. Two out of every three babies born with congenital birth defects demonstrate phenotypes caused by craniofacial malformations[3]. Craniofacial birth defects range from mild to severe; one example is cleft palate where the roof of the mouth separates, which can cause issues with breathing, speech, and language development[1,2]. Scientists do not know the identity or role of all the environmental exposures, genes, or signaling pathways that contribute to facial development and anomalies such as cleft palate[1,2,4]. In addition to studying craniofacial malformations, it is also important to study the evolution and development of normal variations of facial structure. Humans, and many other vertebrates, have slight differences in facial features such as nose size or shape, that do not result in clinical conditions. Discovering which specific environmental exposures, genes, signaling pathways, and other factors contribute to craniofacial formation and variation will possibly help to better prevent and treat facial malformations. While researchers have not discovered all the factors involved in the development of craniofacial conditions, they have established that it occurs as a result of the formation, migration, and differentiation of special cells called neural crest cells in an area of the embryo known as pharyngeal arches in vertebrates[5,6,7,8,9].

To begin the process of craniofacial development, neural crest cells separate into streams to migrate from the rhombomeres to the pharyngeal arches[8,10]. Many signaling molecules are responsible for controlling the migration of these cells[10]. The Eph/ephrin signaling pathway, for example, negatively controls neural crest cell migration by restricting the migrating neural crest cells to stay within the proper stream[10,11]. Eph/ephrin interactions induce a signal transduction pathway that causes changes in the actin cytoskeleton along which neural crest cells move[12]. When Eph receptors on a cell's membrane are activated by ephrin ligands, the cell is repelled, restricting neural crest cell migration to the proper path or stream in the cytoskeleton[10,12,13]. Once the neural crest cells have migrated according to the chemical and environmental cues, the cells differentiate to form the bones, skin, muscle, and neurons of the face[5,14]. While scientists understand the general process of craniofacial development, they lack a complete understanding of normal facial variation and complex, non-Mendelian conditions such as cleft palate. These conditions are difficult to understand due to the multitude of genes, regulatory regions, signaling pathways, and other factors involved[7]. Discovering which specific genes, pathways, and environmental interactions affect craniofacial development can help to better understand and possibly treat these anomalies.

African cichlid fishes are ideal model for studying the craniofacial biology and evolution of both normal variations and abnormalities. There are between 1,450 and 1,750 cichlid species in the Great Lakes of Africa that have undergone rapid evolution[15]. During their evolutionary radiation period, some cichlid species developed different craniofacial morphologies for feeding[15,16]. For example, Lake Malawi cichlids evolved a spectrum of different jaw morphologies based on how they feed – longer mandibles for suction feeding (e.g. *Maylandia zebra*) and shorter mandibles for biting (e.g. *Labeotropheus fuelleborni*)[15,16]. The functional differences in cichlid craniofacial morphology are comparable to human craniofacial variations because cichlids have some common genes, signaling pathways, and other factors that may contribute to facial formation in humans[17,18]. Eph/ephrin genes and signaling pathways are conserved between fish and humans and may explain variations in craniofacial development. The roles of Eph/ephrin signaling in producing these variations in craniofacial morphologies have not been extensively studied in cichlids.

Genes are expressed differently in each cell, directing the formation of unique characteristics that define the function of a cell. This study examined where the gene *epha4b* is expressed in pharyngeal arch one, the area responsible for mandibular development, and the levels of expression in different species of cichlid fish. Higher expression of *epha4* results in the production of a greater number of receptors. If there are more receptors, more neural crest cells will be repelled, causing fewer to migrate into pharyngeal arch one, the area responsible for mandibular development. The growth of craniofacial structures from each arch is limited by the amount of neural crest cells available to develop the structure. We hypothesized that *Maylandia zebra* will have less *epha4b* expression in pharyngeal arch one contributing to the development of a longer mandible and *Labeotropheus fuelleborni* will have more expression producing a shorter mandible. In situ hybridization, a process of staining areas of gene expressions, showed the areas and levels of expression of *epha4b* in *M. zebra* and *L. fuelleborni* at two different developmental stages. *Epha4b* expression was studied during neural crest cell migration in embryos at 2 days post fertilization (dpf) and after the completion of migration in embryos at 3 dpf. The results support the hypothesis; *M. zebra* demonstrate less *epha4b* expression and *L. fuelleborni* demonstrate more *epha4b* expression. The causes of differences in expression remain unknown, but future DNA sequence comparisons may reveal which areas of the genome are responsible. The particular regions that will be studied are enhancers and silencers because they are known to promote or repress gene expression when transcription factors bind to these regions of the DNA[5]. Determining which genes, regulatory sequences, signaling pathways, and other factors affect craniofacial development and the roles they play, will further our understanding of craniofacial variation and possibly lead to treatments for craniofacial malformations.

Methods

In Situ Hybridization Probe Design and Synthesis

In situ hybridization stains areas of expression by binding an RNA probe to the mRNA in the cells. Some of the uracil bases in the probe have a molecule called digoxigenin bound to them so an antibody can recognize digoxigenin and bind to it. An enzyme called alkaline phosphatase, which is attached to the antibody, reacts with two chemicals called NBT and BCIP and turns them purple. Areas of purple show gene expression because alkaline phosphatase had to be present to create the purple stain which means it bound to the antibody which is bound to the digoxigenin molecules on the probe which is bound to the mRNA. Areas with more mRNA appear darker purple.

RNA probes were designed and synthesized by Kara E. Powder as described below. Three different probes were used for this project: (1) a 647 base pair (bp) probe that was the reverse complement of *epha4b* mRNA ("antisense," experimental), (2) a 647 bp probe to *epha4b* that was the same region as the antisense probe, but matched the mRNA sequence ("sense," a negative control), and (3) a 347 bp probe that was the reverse complement of the *sox9b* mRNA sequence ("antisense," a positive control). The *epha4b* sense probe functions as a negative control because the sequence of the probe matches the mRNA sequence so it doesn't bind since like base pairs don't bind. The *sox9a* probe was previously used and known to work in lab to demonstrate gene expression through the process of in situ hybridization.

The sequence for the F2R2 *sox9a* probe is:

5'-
ctgtaaagaacgggcagagcgagggcgaggacggcagcgagcagacgcacatttccccaaacgccatcttcaaagctctccagcaggcagactcccctgcatccagcatgggagaggtgcattctcctggagagc
actcaggctcccagggtccccctaccctcccactaccccaaagactgatgtcagctcaggcaaggtcgatctaaaacgcgaagtaggcctccgctccctgcctgatggtcccggtgggcgacagctcaacattgactt
ccgtgacgtggacatcggcgagctgagcagcgatgtcatctcccatattgagaccttcgatgtcaacgagtttgaccagtacctccctcccaatggccacccggggtccaccaacgcagcgccagtaagttacactggc
agctacagcatcagcagcggcggcgctccagtcagcccccagtcaggaggcacctggatggctaaaagcccgaaccagcagggacaacagcagcagcagcacaccctgaccaccctggggagcagtggggcctt
cagatggcgctcagacccagcacaggacccagatcaagacagagcagctgagcccgagccactacagcgagcagcagggctccccacagcacgtcagcccctacagcccttcaacctgcagcactacagcccc
tcctcttcctacccggccatctccagggcacagcagcagcagtacgactaccctgaccaccaggggggaggcactacaactgcctcctcctactacagccacgcgggggcggggcagagctcggggctgtactcaa
ctttcagctacatgagcagcccaaaccagaggcccatgtaca-3'

The sequence for the *cEpha4b* F1R1 probe is:

5'-
atgaactgcgtcaccttggagtgggcaccaccagagacaccggaggcagaggagacatcatctacaacctcaattgcacaaagtgctctggtgatggcaagaagtgcacaaaatgcggtagcaacgtccattttgttcc
caggaactttggtctgtcatcaacaaccgtgttggttaccgacttggagccagactccaactacagcttcacaattgagagtgagaatggagtgtcggccttaagtccaacacccaagggaaaagtcgtgataaatgtcac
aacatcgcagacagtcagcgtagtgttgaaggaaaggcggagcaaggacatgttgactttggcctggcagggcccagagagacccaacggagtgatcgtggaatatgaggttatctactatgagaagaaccaacgag
aacagaactatacagttctgaagactcgctccaacatgatgacagtggaagggctcaaacctgggaccacttatgtgttcagggtccgggctcggactgatgggggtatgggagctatggtggacagattgagctaga
aacaagtcacgaa 3'

The sense *Epha4b* negative control probe sequence is the reverse complement of the *cEpha4b* F1R1 sequence.

To produce the probes, cDNA was used as a template for PCR amplification. First the DNA was denatured by a heat shock at 95°C for 30 seconds, followed by 35 cycles of the following series: 30 seconds at 95°C (denaturation), 30 seconds at 56 °C (annealing), 30 seconds at 72 °C (extension), and a final extension at 72 °C for 5 min. The PCR product was purified with the New England Biolabs DNA Cleanup kit, eluting in 15uL of sterile water. Then another round of PCR was performed using 1uL of that purified PCR product as a template to add a promoter for T7 RNA polymerase to the PCR product to make templates for transcription.

For the transcription process, the following were mixed in a 1.5mL tube: 25uL of VWR Nuclease free sterile water, 4uL of Purified T7 PCR product, 4uL of 10X transcription buffer, 4uL of DIG rNTP mix, 1uL RNase Inhibitor enzyme, and 2uL T7 RNA polymerase. Then the tube was incubated at 37°C for 4 hours. To remove the DNA template, 5uL of DNaseI was added to the tube and the contents were mixed by pipetting then incubated at 37°C for 30 minutes.

The following components were added then mixed by pipetting for probe precipitation: 4uL of 0.2M EDTA, 5uL of 4M LiCl, and 150uL of ice cold 95% ethanol. Then the tube was incubated at 37°C overnight. The tube was centrifuged at 13000rpm for 30 minutes at 4°C. The supernatant was pipetted off, then the tube was inverted to dry at room temperature until the RNA pellet turned mostly clear, which took approximately seven minutes. The pellet was then re-suspended in 20uL of VWR nuclease-free sterile water. Then the tube was incubated at 37°C for five minutes.

Because the probe was longer than 350bp it was fractionated to split the RNA into smaller fragments for easier mobility and migration through cell membranes. To fractionate the probe, the following components were added and mixed in a 1.5mL tube: 20uL of the RNA probe, 12uL of VWR nuclease free sterile water, 4uL of 0.4M sodium bicarbonate ($NaHCO_3$), and 4uL of 0.6M sodium carbonate (Na_2CO_3). Then, to break the bonds between nucleotide bases of the RNA, the tube was incubated in a 60°C water bath for a time based on the following equation: Time(min) = (Starting kb – Desired kb) / (.11 x Starting kb x Desired kb) making sure that sizes are in kb and using a desired kb value of 0.35.

The fractionated probes were then precipitated by adding and mixing the following components: 40uL of VWR nuclease free sterile water, 8uL 3M sodium acetate, 1.04uL glacial acetic acid, and 150uL ice cold 95% ethanol. Then, the probes were incubated at -80°C overnight. The probes were centrifuged at 13000rpm for 30 minutes at 4°C. After 30 minutes, the tube was inverted then centrifuged for another 20 minutes. The supernatant was pipetted off, then the tube was inverted to dry until the RNA pellet turned mostly clear, which took approximately 7 minutes. The pellet was then re-suspended in 20uL of VWR nuclease free sterile water. The tube was incubated at 37°C for 5 minutes.

3uL of the probe was run on a 1% agarose gel, to check if the RNA was successfully fractionated. Because gel electrophoresis separates DNA fragments by size, RNA probes that were correctly fractionated should produce a smear instead of a clear band because fractionation produces a range of DNA fragments that are approximately, but not exactly, the same size. 1mL of hybridization solution (HS), see below, was added to the remaining 17uL of probe.

In Situ Hybridization

To determine areas of mRNA expression, in situ hybridization was performed. All washes, unless otherwise specified, were performed at room temperature. Embryos were fixed in 4% paraformaldehyde (PFA) (50mL sterile 10X PBS, 450 mL sterile water, and 20g Paraformaldehyde) for 2-7 days at room temperature. The embryos were washed three times in 1x PBST (137mM sodium chloride, 27 mM potassium chloride, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 0.1% Tween) for 10 minutes each time. The embryos were dehydrated by washing once, for 10 minutes in each solution: 25%, 50%, 75% methanol/PBST, then twice for 10 minutes in 100% methanol. They were stored in 100% methanol at -20°C.

The embryos were rehydrated by washing once, for 5 minutes in each solution: 75%, 50%, 25% methanol/PBST, then twice for 5 minutes in PBST. After these washes, the embryos were transferred to a clean well of a 12 well plate. The embryos that were 3dpf were digested with proteinase K at 3:2000 dilution for one hour but the 2dpf embryos were not digested with proteinase K and instead remained in PBST. The embryos were carefully rinsed, then re-fixed with 4% PFA for 30 minutes. The embryos were washed three times for 5 minutes each with PBST then transferred into a sterile 2mL tube, with a maximum of 15-20 embryos per tube. The embryos were prehybridized in 2mL of prehybridization solution, PHS, (50% formamide, 5X SSC, 9.2mM citric acid, 0.01% Tween-20, and sterile water) and allowed to settle to the bottom of the tube. After the embryos had settled, the PHS was replaced with 2mL of fresh PHS and incubated for 2-3 hours in the 70°C water bath. The PHS was replaced with probe after the incubation period. The embryos were incubated in probe overnight at 70°C in the water bath.

In the morning, the embryos were washed twice for 5 minutes each time in PHS at 70°C in the water bath. Then they were washed for 5 minutes in 25% PHS/ 75% 2x SSC (300 mM sodium chloride, 30 mM trisodium citrate) at 70°C in the water bath. Then they were washed for 10 minutes in 2x SSC at 70°C in the water bath. Then they were washed three times for 30 minutes each time in 0.2x SSC (30 mM sodium chloride, 3 mM trisodium citrate) at 70°C in the water bath. The embryos were washed twice for 5 minutes each time in MABT (distilled water, 100mM maleic acid, 150mM sodium chloride, 0.1% Tween) at room temperature. Fresh blocking solution was made for the blocking and for the antibody solution. For every 1mL of blocking solution: 13.34uL of heat inactivated horse blood sera, 6.66uL heat inactivated sheep blood sera, 20uL of 10% Boehringer Mannheim Blocking reagent were added, and then the tube was filled up the rest of the way to 1mL with MABT. Half of the blocking solution was pipetted onto the embryos then the embryos were incubated for 2-3 hours at 37°C. The anti-DIG-AP antibody was added at 1:3000 dilution to the remaining half of the blocking solution, and then shaken for 2-3 hours. The blocking solution was replaced with antibody solution and incubated overnight on the orbital shaker at 4°C.

In the morning, the embryos were washed twice, for 5 minutes each time, in MABT. Embryos were washed six times, for one hour each time, in TST (2.5mL 1M NaCl, 500uL 1M Tris-HCl (pH 9.0), 500uL 10% Tween-20, and sterile water to the 50mL mark). The TST was replaced with fresh TST and the embryos were incubated overnight on the orbital shaker at 4°C.

The embryos were washed twice in NTMT (for every 10mL of NTMT needed: 1mL 1M NaCl, 1mL 1M Tris-HCl (pH 9.0), 0.5mL 1M $MgCl_2$, 1mL 10% Tween-20, and sterile water to the 10mL mark). Then, NTMT was replaced with color solution (for every 1mL of color solution needed: 3.5uL 50mg/mL BCIP, 4.5uL 100mg/mL NBT, and NTMT to the 1mL mark). The embryos were enclosed in foil so the reaction could occur in the dark.

In order to test that all of the elements for the in situ hybridization were added and reacted under the right temperature and light conditions, 0.5mL of color solution was added to a test beaker with all the other solutions from the earlier washes. If everything was added and reacted properly, the solution in the test beaker turned purple. If something was not added or did not react properly, the solution in the test beaker would remain colorless. If the color reaction did not occur in the test beaker, the experiment was determined unsuccessful and would have to be redone.

If the color reaction occurred in the test beaker, the embryos were checked for color under the microscope every 15 minutes. When the color reaction was complete, the reaction was stopped by washing the embryos twice in PBST for 5 minutes each time. The embryos were dehydrated for 10 minutes in each solution: 25%, 50%, 75% methanol/PBST, then twice in 100% methanol. The embryos were stored at least overnight at 4°C in 100% methanol.

In the morning, the embryos were rehydrated for 10 minutes in each solution: 75%, 50%, 25% methanol/PBST then washed twice for 5 minutes each time in PBST. The embryos were washed for at least one hour each in 25%, 50%, 80% glycerol in PBST. The yolk was dissected off. Then embryos were put into fresh 80% glycerol to minimize the yolk debris for imaging. Then the embryos were stored at 4°C in 80% glycerol in PBST.

DNA Sequencing

Samples were prepared by combining 12.5uL of Green Go Taq Master Mix by Edvotek, 0.5uL of forward primer, 0.5uL of reverse primer, 0.5uL of an *M. zebra* DNA template, and 11uL of nuclease free water. The samples for the FR intron 14 primer and F2R2 intron 14 primer were amplified with PCR using the following program: 95°C for 4 minutes, thirty cycles of 95°C for 30 seconds, 58°C for 30 seconds, 68°C for 4 minutes, then after the thirty cycles 68°C for 5 minutes. The samples for the F3R3 intron 14 primer were amplified with PCR using the following program: 95°C for 4 minutes, thirty cycles of 95°C for 30 seconds, 59°C for 30 seconds, 68°C for 4 minutes, then after the thirty cycles 68°C for 5 minutes. Then, 10uL of these PCR products were checked using gel electrophoresis. The gel was made using 70mL of 1X TAE buffer, 1.05g of Agarose, and 7uL of DNA gel stain. The gel was run at 100V for 35 minutes and 1uL of loading dye was added to each of the 10uL PCR products.

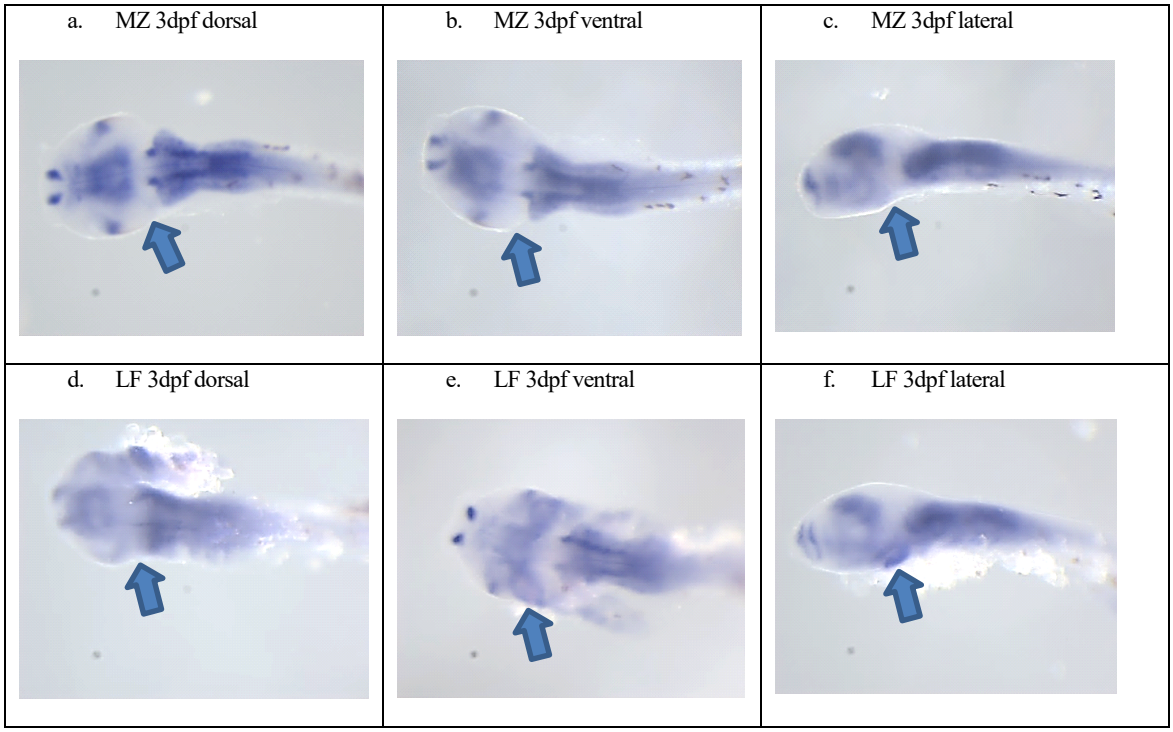
The remaining 15uL of the PCR samples that produced clear bands on the gel electrophoresis were submitted for sequencing with Eton Bioscience. The PCR reactions were transferred to a 1.5mL tube, labeled with the sample name, and sealed with parafilm. 2uL of the working stock of primer for each sequencing reaction were also sent for sequencing. All of the tubes were mailed to the company and an online order was placed.

Results

Genes are expressed differently in each cell, producing unique characteristics that direct the development of cells. A series of in situ hybridization tests, where darker staining indicates more expression, were performed to illustrate the amount and locations of *epha4b* expression. One or two embryos from each species at different developmental stages were analyzed to compare the expression of *epha4b* in pharyngeal arch one, where mandibular development occurs.

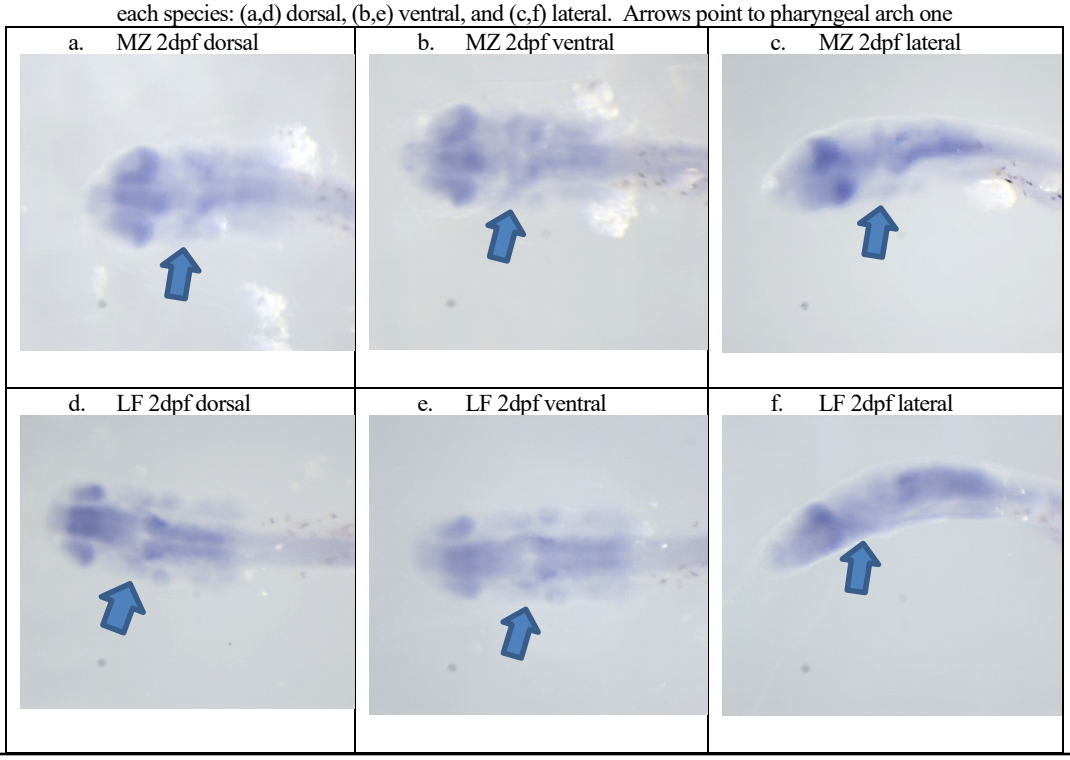
To examine species-specific differences in *epha4b* expression at 3 days post fertilization (dpf), when neural crest cell migration has finished, *M. zebra* and *L. fuelleborni* embryos were stained using in situ hybridization. *M. zebra* embryos at 3 dpf demonstrated lower expression of *epha4b* in pharyngeal arch one compared to *L. fuelleborni* embryos (as indicated by the arrows in Figure 1).

Figure 1. (a-c) *M. zebra epha4b* antisense 3dpf (d-f) *L. fuelleborni epha4b* antisense 3dpf. Three different views of each species: (a,d) dorsal, (b,e) ventral, and (c,f) lateral. Arrows point to pharyngeal arch one.



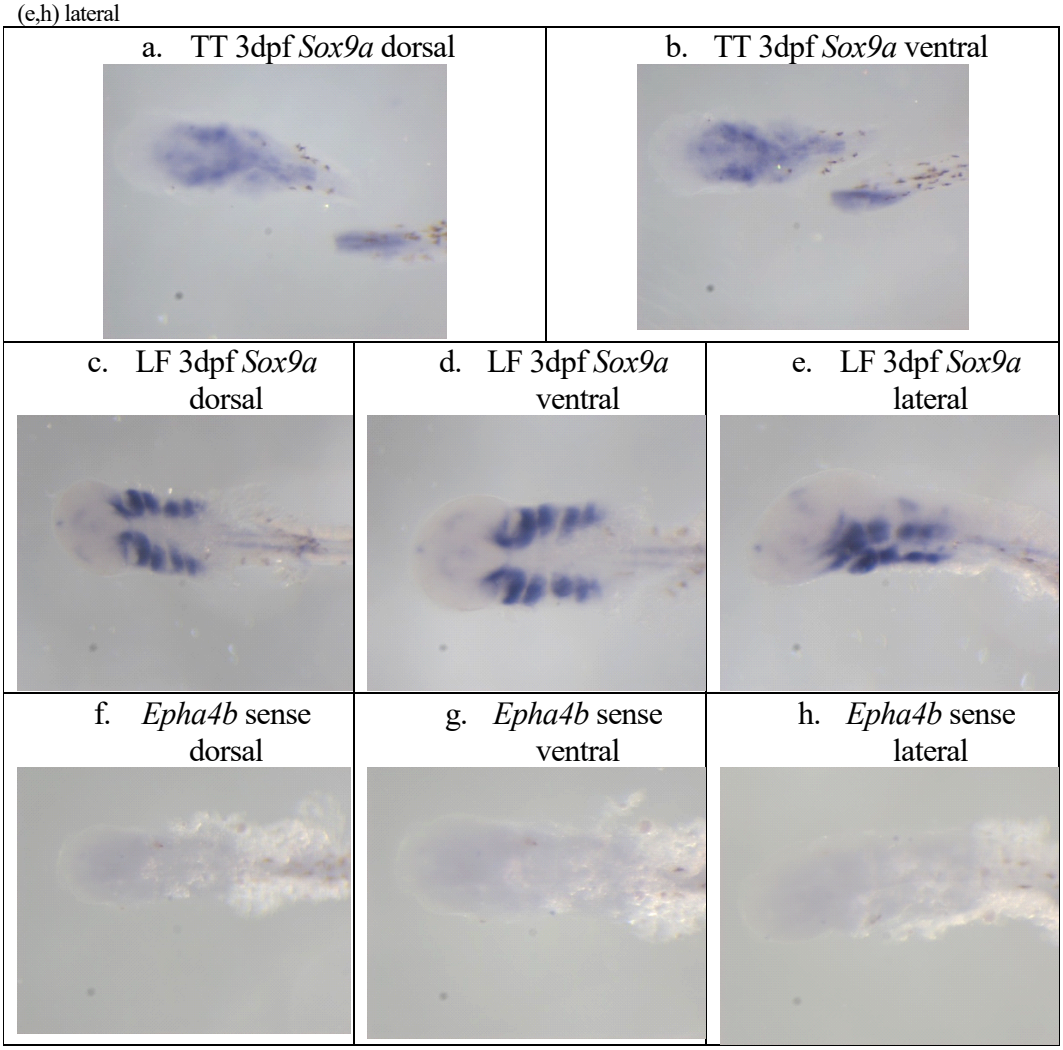
To compare *epha4b* expression at 2dpf, when neural crest cell migration is still in progress, *M. zebra* and *L. fuelleborni* embryos were also stained using in situ hybridization. *M. zebra* and *L. fuelleborni* embryos at 2dpf showed similar patterns and levels of expression of *epha4b* in pharyngeal arch one (as indicated by the arrows in Figure 2).

Figure 2. (a-c) *M. zebra epha4b* antisense 2dpf (d-f) *L. fuelleborni epha4b* antisense 2dpf. Three different views of each species: (a,d) dorsal, (b,e) ventral, and (c,f) lateral. Arrows point to pharyngeal arch one



During each of the two in situ hybridization experiments, both positive and negative controls were also tested. *Sox9a* was used as a positive control because it is expressed in all neural crest cells. The positive *sox9a* controls demonstrated specific staining showing that all the components of the color reaction worked correctly (Figure 3). The *epha4b* sense probe was the negative control. It was included to show that the antisense probe used in Figure 1 and Figure 2 demonstrated specific staining. It demonstrated no specific staining (Figure 3).

Figure 3. (a-b) positive control *T. tropheops sox9a* 3dpf (c-e) positive control *L. fuelleborni sox9a* 3dpf (f-h) negative control *L. fuelleborni epha4b* sense 3dpf. Three different views of each species: (a,c,f) dorsal, (b,d,g) ventral, and (e,h) lateral



The results from the sequencing of *M. zebra epha4b* intron 14 using the forward primers F, F2, and F3 and the reverse primers R, R2, and R3 are listed below.

The sequence of *epha4b* intron 14 from the F primer for a *M. Zebra* cichlid is:

5’ -
gatgggcgagtgtctactccgtctgactcgctgactctactaggcggcggtttaaacagtcgccgacttacaagtagaaggcggagtattgctttgagtaaaataagttaggtgggtataataacacccgctggcacgtgt
agttgtctgtgattccactccacaaagcttcatgcttaaaatgaatactttcaaagagtctgaactcctgtaaaaaaacatttaattttcaagtctaatttcttatttttgcaactagtcgctaattcaaaaaatattagacaagatga
aatttttttttttgcggggtctaaaaacacctttgataaccgccccatttattgcttgtgcccgtgcattcgataatgaattacttgtataagaagtggagacaggagatatgttaactgttggcggcattggtgtcttgatcttgttgtt
acatgaagtacaataggagcctttgttgactaattatctaaatggctatgatgtgccaatagtaaaagcactataagggtctttaggaaaagtgtggctgattctaccatagttgtgttagtagtgacaaatctggaacaaaact
ggcacaatcttctgactatttgggttgggggatgtgtgg – 3’

The sequence of *epha4b* intron 14 from the R primer for a *M. Zebra* cichlid is:

5’ -
nnnntnntcaatgttttctattccgttcaccctcaccaatttcgccccgttttacctccccacttatatcctcggatccctttattaaatagtgggattccccctccaggtgtgctgtgcagtccaatccacaaagctttctggatatt
cagaatactttcctcaaggggacaggaaaaaaaaaaaacacctcttgttcaagggcacaaaatcacacatttaaaactaatcactaatttcttcaaaattttgacaagaggtaaatttttttcttgcagggtttaacatctctcttg
tagaccgccctttttttttctttgcgcgtgagttcgagaatgtattactttcatcacaaagggacacaggagaattggtaactgttcactgcattggttgttcctgagcttgtattacataaaaatgatacatgagacttttagtact
tattatcaaaaaagctggaatggacattggg – 3’

The sequence of *epha4b* intron 14 from the F2 primer for a *M. Zebra* cichlid is:

5’ -
nnagnnaaatnnnantnctcatacgttgcatgttttcgcgttttccccaccatttttctctaacccctttagacggaatgacctgttcaccctaaggtaccaggaaatgtgctactgacgtaggcatatacatacctggctctttg
taccagtgagcgttggcaggaacaaggacacaccgccaccagagctccgaggaggccgttaagacggaacaacgtgtcgctgttggttttctcatcgaattctccgtctataaactcttcaaaaagagagagaaggtct
gtaggggccccccccccccccccgggggggggggggggtgaaaccccccccccccccccc – 3’

The sequence of *epha4b* intron 14 from the R2 primer for a *M. Zebra* cichlid is:

5’ -
nnnannaagcnngttctatgtgtctatgcaattgcccatttcggtccgtttttcacgtcttaaacgaattcatcacccctacagcgaccgtgtcgctacggctcgccttatggaggcggtgtgtgcttgttcctgccaacgct
cactggtacaaagagccaggtatgtatatatgggggacgtcagaagcacagtgtccttgtagctttactttaagttggatcattcacgtctcaaatggtttaaactatacagagatggcttttgttgctctctccccccagcatg
ggggctcaggcaggtgtgaatccctcccaccccccccaaca – 3’

The sequence of *epha4b* intron 14 from the F3 primer for a *M. Zebra* cichlid is:

5’ -
nnnantgccaggactccgtttcgtgtcctagttgccttttttttcctcccttatgccttctctataatattccagctgctgccccagatttttggtcactgtgttttcagataaggataacttgtataatttctcatcatattttagaaaatg
gctgggctctgcctcattgttttacaaatacttagaaggagtgagggagaagaatgttgtcagcaggcctcaccgcccaccgaagaaaatccaattctctttcgcttttataacggagacgaaggttactggcccagaaga
caccaccaccagagaccggcctactctggttgtgtgagatgttaacgaagtagcgagaaacagaaatcatttaaggcgatgtatcactgattttacaaactgtcttagtgggggcttcgggggggttcgttccttggttctccc
gatatggttgaaattgtcaatcaaaaaccaacacacgggggggggggcgtctctgactg – 3’

The sequence of *epha4b* intron 14 from the R3 primer for a *M. Zebra* cichlid is:

5’ -
taggctcactcgctcagatttgtcctggttccccttttttccccttaatctgtccttggtttcgttcgccagattttgtcctgcttgggcggtacttgtgtgtcttaactttcaaatatgcctggtctctgcctcatctcctgcctcatcta
ggggggggggtggggagggggttgccagcaggcctcgccctccgcctgagaaattccgagtgtctttgcttgaagcacgcagactaaggctgatggccccgaagacaccagcacccgagaccggccgactgtggct
tgggtgtgtgtgtgtttgatgtggtgggattcagaatccctctcatgcgatctctcgctatttttacctacggtcgttctgggggcgttgggggggtgt – 3’

Discussion

The areas demonstrating expression of *epha4b* and the levels of expression vary among two different species of cichlids at three days post fertilization (dpf), when neural crest cell migration has terminated. *Maylandia zebra* embryos at 3dpf have less *epha4b* expression in pharyngeal arch one compared to *Labeotropheus fuelleborni*. However, the same trend was not observed in *M. zebra* and *L. fuelleborni* embryos at the 2 dpf stage where neural crest cell migration is still in progress. The results of this experiment support the hypothesis and the previously described trend that Eph/ephrin signaling negatively controls neural crest cell migration (Smith et al., 1997). Eph/ephrin interactions determine the path of neural crest cells by changing the cytoskeleton along which neural crest cells migrate (Poliakov et al., 2004). When Eph receptors are activated by ephrin ligands, the cytoskeleton extends, pushing neural crest cells in the opposite direction of the cell bound to the activated eph receptor (Poliakov et al., 2004). Therefore, if *epha4b* is highly expressed, fewer precursor neural crest cells migrate to an area to differentiate and form structures, so less development occurs in that area. Similarly, low *epha4b* expression causes more neural crest cell migration to an area leading to more development, producing phenotypes such as a longer mandible in *M. zebra*. Variations of the levels and areas of *epha4b* expression along with other genes could contribute to the differences in adult phenotypes such as a longer mandible in *M. zebra* or a shorter mandible in *L. fuelleborni*.

Repeating the in situ hybridizations with larger sample sizes would decrease the effect of random error and confirm the results. Additional in situ hybridization experiments can be performed to determine the levels of *epha4b* expression in additional species of cichlids and other organisms. Determining the different levels of *epha4b* expression in various cichlid species will further the understanding of the evolution of cichlids as well as the evolution of different craniofacial morphologies.

Determining how *epha4b* expression is controlled in cichlids and how it relates to different craniofacial morphologies will help future studies determine how *epha4b* expression affects craniofacial development in humans. Future studies will be conducted to study the causes of the varied levels of expression across species by using gene sequencing to compare the sequences of certain regulatory regions such as enhancers and silencers in different species of cichlids. A deeper understanding of the complex Eph/ephrin signaling pathway could lead to the development of treatments for craniofacial malformations using techniques such as gene therapy to alter the expression of genes such as *epha4b*.

Acknowledgements

Clemson University, the Calhoun Honors College, and the South Carolina Governor’s School for Science and Mathematics are acknowledged for providing this opportunity through the Summer Program for Research Interns. Dr. Kara E. Powder: Assistant Professor Department of Biological Sciences, Clemson University and Principal Investigator of the Powder Lab is acknowledged for assisting in the design, development, data collection, and data analysis of the project. Stephen Groves: lab technician, Clemson University is acknowledged for providing information about the project and assisting in data collection. Katlyn Taylor: lab technician, Clemson University is acknowledged for assisting in data collection. Dr. Jennifer Taylor: Scientific Investigations instructor and Science Chair of the South Carolina Governor’s School for Science and Mathematics is acknowledged for assisting in the design, development, and data analysis of the project.

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