Spring 5-1-2018

Characterizing Pitx2 Tooth Morphology and Candidate Genes of Axenfeld- Rieger Syndrome in Zebrafish

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Characterizing Pitx2 Tooth Morphology and Candidate Genes of Axenfeld- Rieger Syndrome in Zebrafish

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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Spring 2017

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Abstract

Axenfeld-Rieger Syndrome (ARS) has been shown to be correlated with the Pitx2 mutation in both humans and zebrafish (Ji et al, 2016). ARS is classified as a rare autosomal dominant disorder that includes defects in the eyes, teeth and craniofacial design (Dressler et al 2010). The earliest signs of a Pitx2 mutation have yet to be defined in tooth morphogenesis. In this review, the earliest signs of the mutation are defined at 4dpf but, it is understood that further research needs to be done in order to classify that this mutation does not start to appear earlier.

The second goal was to analyze expression of candidate genes that were found, specifically sidekey269.114, MyHz2 and CyP4v7, in pitx2 mutants in order to see if certain gene expressions are changed. Using In Situ Hybridization (ISH) experiments, it was found that the sidekey269.114 gene may show early signs of expression or increased expression in pitx2 mutants. No mutants were found for MyHz2 and CyP4v7. No obvious differences in expression were seen between heterozygous (HET) and wild-type (WT) pitx2 embryos.

Executive Summary

One of the mechanisms of tooth evolution, relevant to the human phylum, has been proven to be decent of pharyngeal teeth most commonly found in zebrafish (McCollum & Sharpe, 2001). Using zebrafish as a model for tooth development, I studied the Pitx2 mutation and characterized its earliest sign of tooth and craniofacial defects. Pitx2 is correlated with Axenfeld-Rieger Syndrome (ARS) in humans and the Pitx2 mutation in zebrafish is 100% identical to the human genome. This justifies using pitx2 embryos from zebrafish as a way to characterize the mutation and its correlation with other genes.

To characterize the tooth morphology, pitx2 embryos were fixed at 4 days post fertilization (dpf) and at 6dpf. These days were chosen based on past research that had indicated the earliest signs of the mutation showed at 5dpf (Ji et al, 2016). However, since tooth development in zebrafish begins at 2dpf, I decided to look further into when the earliest indications are. Bone and cartilage double staining solutions were used to mark dentition development and craniofacial structures. At 2dpf, no signs of bone were seen so we next looked at 4dpf to narrow down the development time for Pitx2. At 4dpf, no mutants were found however, there were signs of hypertelorism in heterozygous (HET) pitx2 embryos. It is important to classify any defects in HET embryos because Pitx2 is an autosomal dominant disorder, meaning HET embryos are likely to show some phenotypes. Hypertelorism is classified as a craniofacial malformation that appears as an extensive broad, flat nasal bridge (Tümer & Bach-Holm, 2009). 6dpf embryos were also examined and 3 mutants were found. At this point in development 2 sets of teeth are developed and are seen as so in wild-type (WT) pitx2 embryos.

In each mutant found, the second set of teeth were all missing. While this seems significant, it cannot be stated as such because of the small number of embryos that were obtained.

Candidate genes associated with Pitx2 were found by a previous grad student in the lab, Yonchang Ji, and probes were developed to show expression of these genes during tooth development at 2dpf. Sidekey269.114, MyHz2 and CyP4v7 were the genes used to define expression in pitx2 embryos. However, only when using the sidekey269.114 probe were mutants found. For both MyHz2 and CyP4v7 no mutants were found so, no expression could be defined. In their HET embryos, no obvious defects were seen. For the sidekey269.114 gene, there seemed to be increased or early expression in pitx2 mutants at 2dpf. However, only one mutant was found so this conclusion cannot be made. If time had permitted, I would have liked to repeat all of these experiments to find more samples in order to supplement with quantitative data.

Introduction

The first vertebrate species did not possess the evolutionary benefits of jaws and teeth like most vertebrates have today. The first species to even own a jaw were gnathostomes, a more successful species of fish descendent from agnathans, the first vertebrate species which lacked a jaw (McCollum & Sharpe, 2001). More so, there are two standing mechanisms for how the evolution of dentition occurred. The first, relevant to human phylum, states that teeth most likely formed independently of the jaw and more importantly from pharyngeal denticles, commonly found in many fish species like zebrafish (McCollum & Sharpe, 2001). Because of the close proximity and connectedness of the body, even the evolution of the head has depended on correct tooth development. As the teeth are an essential part of all craniofacial development in vertebrates, without the appropriate tooth shape and connection to the proper bones, abnormalities are likely to occur and will affect the organism's overall health.

In humans, craniofacial features include the skull, eyes, nose, jaw, teeth and complete appearance of the face. Any malformations of these elements will result in not only facial defects, but health issues as well. Mammalian teeth are special because natural selection favored rooted teeth that are connected by periodontal ligaments and alveolar sockets to the jaw (Jheon et al 2012). The typical tooth of a mammal is surrounded by the periodontium which provides support to the tooth and both are consistent of all mineralized tissue; cementum, bone, enamel and dentin (Jheon et al 2012). Periodontal ligaments connect the teeth to the alveolar bone while the inside of the tooth contains pulp, a rich supply of blood vessels, nerves and putative odontoblast stem cells (Jheon et al 2012). Overlaying the alveolar bone are the gums or the gingiva tissue (Jheon et al 2012). Human teeth are classified as heterodonts, meaning they have four different types of teeth (Jheon et al 2012). In order, from the midline of the mouth to the

distal end of the mouth, the teeth types are incisors, canines, premolars and molars (Jheon et al 2012).

The tissue, to create such dynamic features, is sourced from two primary regions. The pharyngeal endoderm provides the majority of the epithelium and the cranial neural crest cells form the mesenchyme, which creates the prominent facial features (Jheon et al 2012). The epithelium and mesenchyme are the primary regulators of tooth morphogenesis (Jheon et al 2012). In humans, it is the cooperation between these dental epithelia and neural crest mesenchymal cells that are vital to correct formation of the teeth (Huang & Chai, 2012). For example, the Hertwig's epithelial rooth sheath (HERS), comprised of a double epithelial layer, initiates and ensures proper tooth root development (Huang & Chai, 2012). More so, on either side of the HERS are the neural crest derived mesenchyme cells responsible for the formation of the dental papilla and eventually dental follicles (Huang & Chai, 2012). These cells are critical for appropriate tooth and dental tissue development.

Molecular Regulation of Tooth Development

Tooth development begins with the activation of epithelial FGF8 and BMP4, which cause the expression of many vital transcription factors including my primary focus, the pituitary homeobox 2 gene (Pitx2) (Tümer & Bach-Holm, 2009). Both BMPs and FGFs are peptide growth factors and more specifically, BMPs are bone morphogenetic proteins while FGFs are fibroblast growth factors (St.Amand et al, 2000). FGF8 is one of the first genes expressed by the oral epithelium accompanied by BMP4, a mediator between the epithelial and mesenchymal tissue signaling (Cho et al, 2015). FGF8 and BMP4, are the chief regulators in tooth

development and more importantly, regulate each other. When FGF8 is activated, BMP4 signaling is decreased and vice versa (St.Amand et al, 2000).

By the time tooth bud development begins, it is the mesenchyme cells that take over direction in tooth development (St.Amand et al, 2000). In accordance with my work, St.Amand et al found that FGF8 is a positive regulator of the Pitx2 transcription factor and BMP4 is actually a negative regulator of the transcription factor (St.Amand et al, 2000). BMP4 has been known to be restricted to epithelium once the beginning of tooth development is initiated and since Pitx2 is also restricted to the oral epithelium, it was found that expression of BMP4 represses Pitx2 expression (St.Amand et al 2000). Hayashi et al also found that BMP4 is a repressor of Pitx2 expression and goes on to state that if BMPs are inhibited, the overexpression of Pitx2 causes repressed Osterix expression (Hayashi et al, 2007). The Osterix gene is a transcription factor that codes for osteoblast differentiation, meaning without osteoblasts bone cells cannot reach maturation (Hayashi et al, 2007). Because overexpression of Pitx2 can have this effect, the dental and craniofacial defects associated with Axenfeld-Rieger Syndrome will occur.

Pitx2 in craniofacial development

Pitx2 is a homeodomain transcription factor, meaning it contains a 60-amino acid DNA-binding domain that can control the function of many other genes (Tümer & Bach-Holm, 2009). Mutation of the highly conserved Pitx2 causes many craniofacial defects that characterize Axenfeld-Reiger Syndrome (ARS) (Tümer & Bach-Holm, 2009).

ARS is classified as a rare autosomal dominant disorder that includes defects in the anterior segment of the eye as well as dental features (Dressler et al 2010). The eye defects

observed are iridocorneal adhesions, iris hypoplasia, corectopia, embryotoxon posterius and polycoria (Dressler et al 2010). These defects usually cause 50% of patients with ARS to also have Glaucoma that can end up leading to blindness (Dressler et al 2010). The dental abnormalities are also extensive including hypodontia/oligodontia of the primary and permanent teeth, short roots, microdontia, abnormally shaped teeth and taurodontism (Dressler et al 2010). The outcome of Pitx2 mutations also shows a very extensive broad, flat nasal bridge termed hypertelorism (Tümer & Bach-Holm, 2009). A small percent of ARS patients also have reported abnormalities in the pituitary gland, deafness in the middle ear, malformation of the heart and even mental retardation (Dressler et al 2010). However, if organ malformation in the heart occurs the mutation can become embryonic lethal and it does not survive through development. The effects of Pitx2 mutations have proven to be extremely detrimental and this is an important area of study for researchers.

Zebrafish as a model to study Pitx2 function

Zebrafish have recently become a useful model organism for studying the effects of Pitx2 mutations. Because zebrafish embryos are small in size, are optically transparent, and develop externally, they are ideal for examining the function of Pitx2. The Pitx2 homeodomain in zebrafish is also 100% identical to that of the human PITX2, making it a successful ARS animal model for in vivo studies. The Amack Lab was the first to develop a successful ARS animal model using zebrafish genetics (Ji et al 2016). Ji et al classified three different types of mutations with the HD allele of Pitx2, $pitx2HD^{sny6}$, $pitx2HD^{sny7}$, and $pitx2HD^{sny15}$(Fig 1) (Ji et al, 2016). When compared to the wild-type sequence, both the $pitx2HD^{sny6}$ and $pitx2HD^{sny7}$ alleles have deleted nucleotides while the $pitx2HD^{sny15}$ allele has an insertion of

nucleotides (Fig. 1A) (Ji et al 2016). However, every pitx2HD mutation causes a frameshift and subsequently a premature stop codon within the homeodomain at the second helix (Fig. 1B) (Ji et al 2016). Thus, it is predicted that these mutations lead to a loss of Pitx2 function.

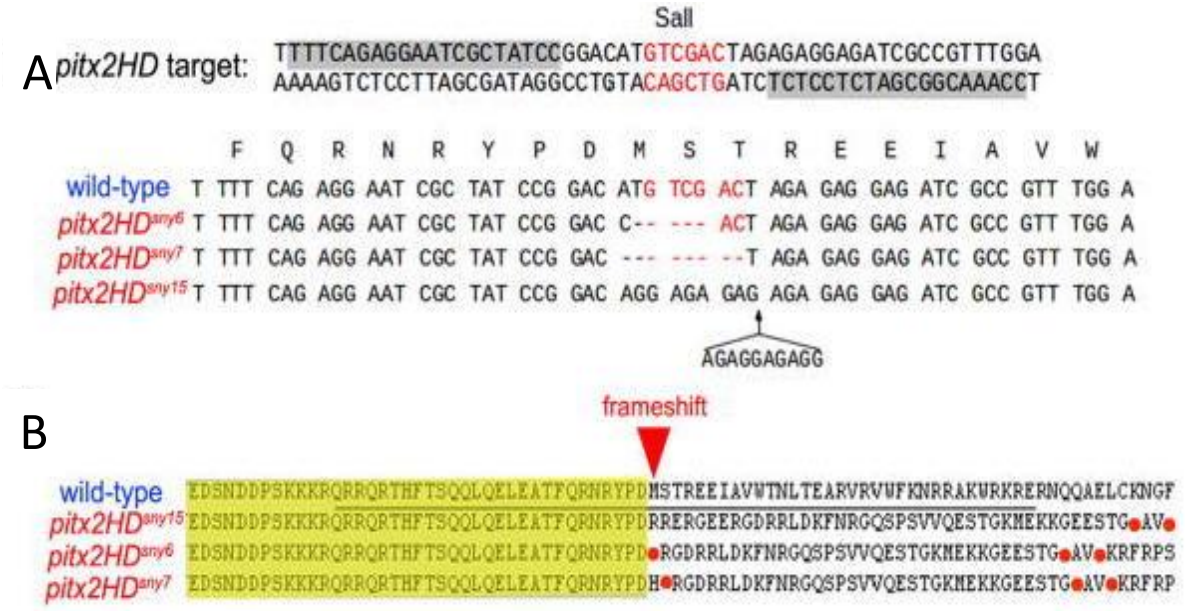

Fig. 1. Pitx2HD mutations in sequence. The deletions in the sequence for sny6 and sny7 shown using red dashes (A). The insertion sequence is show for the sny15 (A). Each mutation causes a premature stop codon and a frameshift (B).

Zebrafish have pharyngeal teeth that appear first at 2 days post fertilization (dpf). While they are not identical to the morphology of human teeth, the same conserved genes regulate their development. At the 2dpf point in the development of the zebrafish, it is in the peak development of the pharyngeal arches (Kimmel et al, 1995). Seven pharyngeal arches will form with a distinct boundary prevalent between the second and third arches (Kimmel et al, 1995). The significance of this boundary becomes important to the formation of the mandibular and hyoid arches which form anterior to the boundary (Kimmel et al, 1995). The correct development of these anterior arches is essential to the correct formation of the operculum and jaw (Kimmel et al, 1995). The branchial arches are located posterior to the boundary and are responsible for proper gill formation (Kimmel et al, 1995). Creating a model of the tooth morphology progression in mutant

Pitx2 zebrafish compared to that of normal zebrafish will be the first step in characterizing the mutation related to ARS.

Goals of this project:

1. The first goal is to characterize tooth development at different stages in zebrafish pitx2 mutants. This is done by using a cartilage and bone staining at different stages of development to determine when tooth defects first appear in mutants.
2. The second goal is to characterize the expression of candidate Pitx2 target genes. This is accomplished using RNA In Situ Hybridization (ISH). In this process, probes, which are used to mark the expression of certain genes, are used in order to visualize gene expression at certain points in development and determine whether there is a difference in appearance between wildtype, heterozygous and homozygous mutant pitx2 embryos.

Results: Part 1

Identification of pitx2 embryos by genotyping

For all of the experiments in this project, pitx2 heterozygous parents were crossed to produce embryos for analysis. It is expected that 25% of the embryos will be homozygous wild-type (WT), 50% will be heterozygous (HET) and 25% will be homozygous mutant (MUT). However, throughout embryogenesis, WT, HET and MUT embryos appear similar and cannot be distinguished by phenotype. Thus, it was necessary to use genotyping to identify WT, HET and MUT embryos. To genotype a DNA extract, PCR, restriction digest, and gel electrophoresis must be performed. It is important to address how essential is it to have proper PCR product for the restriction digest to work. There are many factors of the PCR reaction that can affect and alter the product to create null results. For PCR to amplify a DNA extract, denaturation, annealing and extension of the sequence is repeated for 40 cycles to supply enough DNA for the restriction digest. After a lot of problem solving, I found that the annealing temperature in the PCR program was too high and effecting my product. New primers had been ordered and although they were the same JYC 128, 129 primers needed, the new primers had slightly lower boiling point temperatures. To compensate for the issue, I established a new PCR program with a different annealing temperature that would allow both primers to function correctly (TALENHDA).

The schematic (Fig. 2) displays how the restriction enzyme cuts the amplified DNA from the PCR and ultimately how the gel is read to distinguish between genotypes. The SalI enzyme cuts the WT allele (+/+) at one location along the sequence creating two bands that appear when they are separated out using gel electrophoresis. These bands are lengths of 225 base pairs (bp) and 125bp (Fig. 2). The MUT allele (-/-) does not get cut because the mutation changes the

sequence of the SalI site such that the enzyme no longer recognizes it. In HET embryos (+/-), there are three bands because they contain both a WT (cut) and MUT (uncut) allele (Fig. 2).

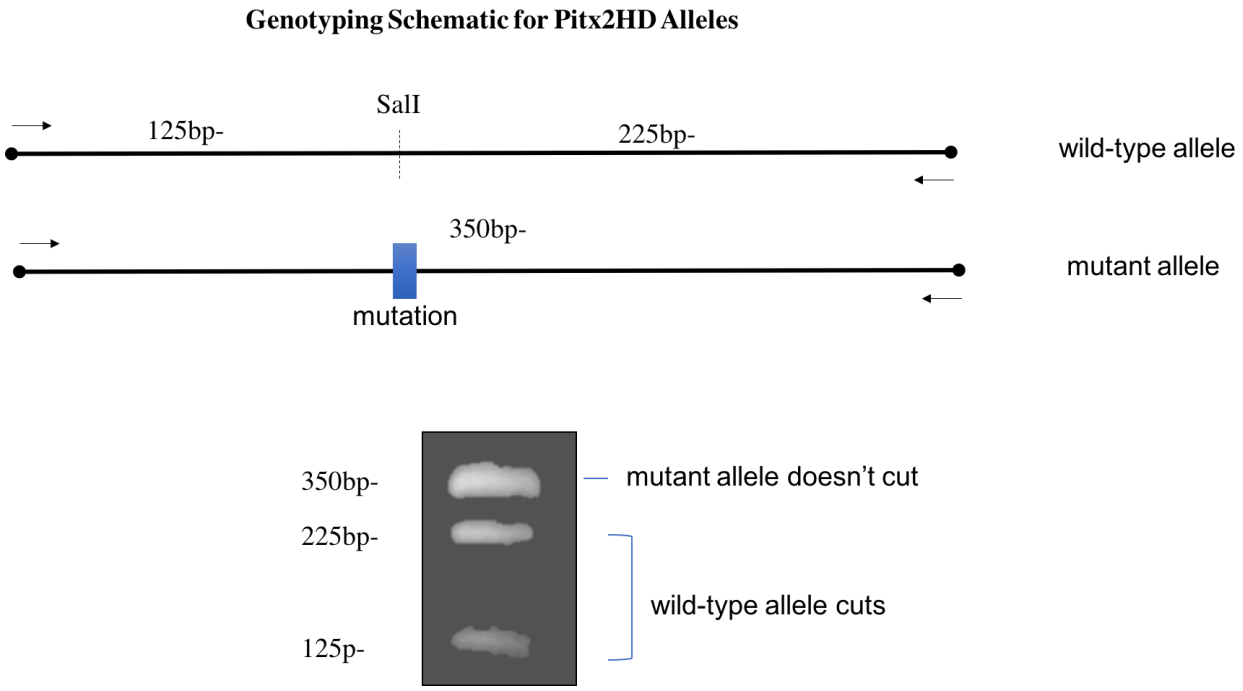

Fig. 2. Schematic showing genotyping process of Pitx2HD alleles. Segments represent DNA sequence. Arrows show direction of reverse and forward primers. Gel electrophoresis band examples shown using a heterozygous genotype.

Tooth development 4dpf and 6dpf in pitx2 zebrafish embryos

To characterize tooth development of pitx2 mutants, alcian blue and alizarin red double staining was used to visualize the bone and cartilage of the embryos (Fig.3). I fixed embryos for staining at the 4dpf and 6dpf stage. Previous work has shown tooth morphology in mutants at 5dpf and 9dpf (Ji et al, 2016). However, the earliest effects of the mutation had not been found. Since tooth development begins at 2dpf in zebrafish, staining was also done at 2dpf, but showed no staining in any of the embryos. This indicates that the mutation's effects do not appear at the 2dpf stage in development. At 4dpf, although no MUT embryos were found, because pitx2 is autosomal dominant we still need to consider the phenotypic differences in the HET embryos

(Fig 3C, D). In the WT embryos, we can see that the earliest mineralized teeth have already formed indicated with red arrows (Fig. 3B). These teeth are coming off of the 5th ceratobranchial cartilage, a part of the 5th branchial arch. These teeth are also evident in the HET embryo as well (Fig. 3D). Although there are no tooth differences, it is important to note there does seem to be the beginning of craniofacial defects based on the broadened 2nd pharyngeal arch (Fig. 3C).

Once at 6dpf, also attached to the 5th ceratobranchial cartilage, there is a second pair of mineralized teeth (Fig. 3F). While this second pair of teeth is present in the 6dpf HET, they appear to be not as mineralized (Fig. 3G, H). In MUT embryos, there is a complete absence of these teeth (Fig. 3I, J). Also, evident in the MUT embryos, there are signs of craniofacial abnormalities seen where the cartilage staining overlaps the eyes marked by a red arrow (Fig. 3I).

The table shown is used to quantify how many embryos of each genotype and at each stage in development have missing teeth (Table 1). To create the table, each embryo was blindly observed at 4dpf and if it did not have one or both of the first pair of teeth on the 5th ceratobranchial cartilage it was counted as a disruption in tooth morphogenesis (Fig. 3B, D). The observations were then compared with their genotype and the ratios are given accordingly (Table 1). The same process was used to find the ratios for 6dpf. However, for tooth morphogenesis disruption to be counted the embryo has to have the second pair of teeth or tooth, which stems off the same ceratobranchial cartilage as the first pair, to be missing (Fig. 3F, J). While there are not enough embryos for adequate statistical power, it is important to point out all 3 mutants found at 6dpf were missing the complete set of second teeth on the 5th ceratobranchial cartilage (Table 1). Together, these results suggest tooth development may be delayed at 4dpf in some

HET embryos, but then catches up by 6dpf. Additional experiments are needed to analyze tooth developmental defects in MUT embryos at 4dpf.

Bone and Cartilage Staining Using pitx2HD^{sny6} Embryos

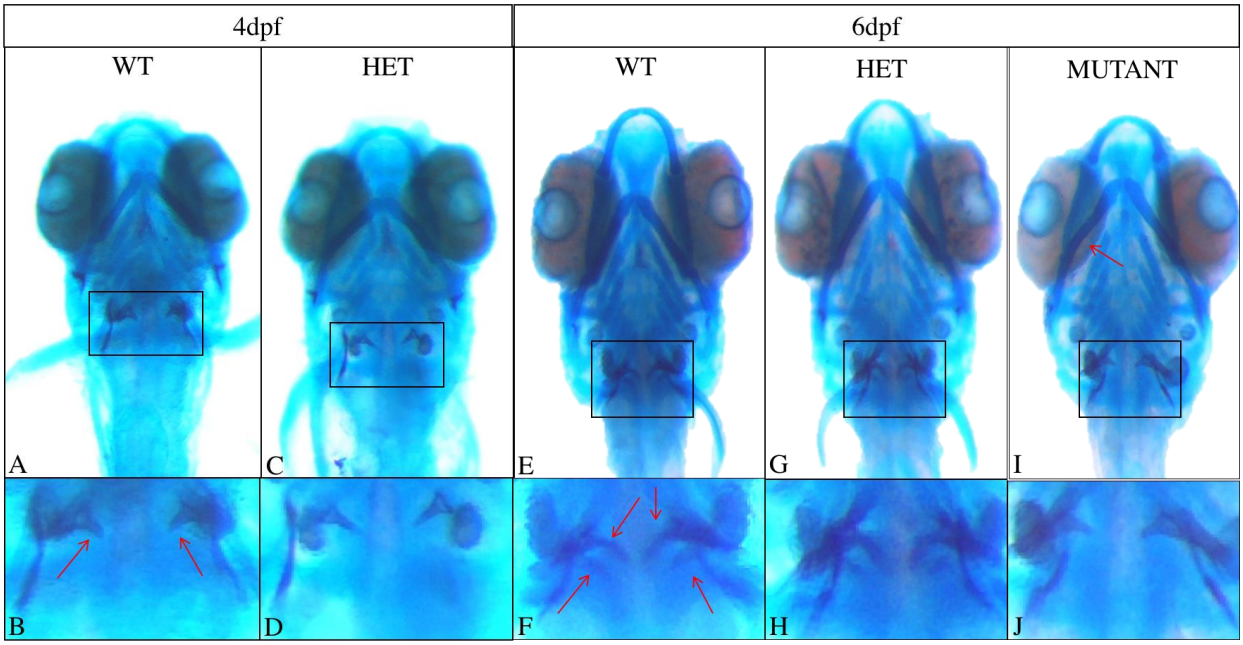

Fig.3. Tooth morphogeneis in 4dpf and 6dpf pitx2 zebrafish embryos (A-J). 4dpf embryos showing first teeth budding from 5th ceratobranchial cartilage (A-D). Red arrows show teeth in 4dpf (B). 6dpf embryos show first and second pair of mineralized teeth indicated with red arrows in panel F (E-H). Mutant shows missing second pair of teeth and slight hyperterletorism (I-J). Ventral view (A, C, E, G, I). Zoom in of box indicated in above panel (B, D, F, H, J).

Tooth Morphogenesis Disruption in pitx2HD^{sny6}

Stage	Allele	Genotype	# of embryos with missing teeth/total # of embryos counted
4dpf		+/+	0/1
		+/-	2/7
		-/-	0/0
6dpf		+/+	0/2
		+/-	0/6
		-/-	3/3

Table 1. Ratios of disruption in tooth morphogenesis pitx2 zebrafish embryos. All alleles are Pitx2HD^{sny6}. Number of embryos with missing teeth counted based on teeth present in WT of the dpf indicated. Embryos genotyped for confirmation of phenotypes.

Results: Part 2

Transcriptome analysis of pitx2 mutant zebrafish

To identify candidate Pitx2 target genes, we used RNA-sequencing (RNA-seq) to analyze mRNA transcripts (e.g. the transcriptome) in WT and pitx2 mutant zebrafish. A previous graduate student in the lab, Yongchang Ji, isolated total RNA from the head of individual embryos from heterozygous pitx2$^{+/-}$ parents. This included craniofacial structures, eyes and teeth. The tail of each embryo was used for genotyping. RNA from pitx2$^{sny7/sny7}$ mutants, pitx2$^{sny15/sny15}$ mutants and wild-type siblings were used to generate cDNA libraries that were sequenced on an Illumina MiSeq platform. RNA-Seq data analysis was performed using CLC Genomics Server 6.0. Sequence data for each sample was aligned to a Danio rerio reference genome (Ensembl Danio rerio Zv9.74). Comparing results between pitx2$^{sny15/sny15}$ mutants and wild-type siblings identified 23 transcripts that were showed statistically significant expression levels between mutants and wild-type. Further analysis revealed that 6 of these transcripts were also differentially expressed between pitx2$^{sny7/sny7}$ mutants and their wild-type siblings (Table 2). cDNA fragments of selected genes were PCR amplified and cloned into pCRII TOPO vectors to generate constructs that were used to produce antisense mRNA probes for RNA in situ hybridization analysis. For this project, we selected known genes myhz2, cvp4v7 and unannotated gene si:dkey-269i1.4 (sidekey269.114) for further analysis.

Table 2. Differentially expressed transcripts in pitx2 mutants vs. wild-type siblings

	pitx2HD^{sny15}		**pitx2HD^{sny7}**	
Gene	**Mutants**	**WT siblings**	**Mutants**	**WT siblings**
ctslb	62.21	148.42	61.28	157.85
myhz2	9.04	17.00	6.52	18.67
si:dkey-269i1.4*	2.74	13.78	2.63	16.62
cyp4v7	4.14	16.07	0.22	15.89
zgc:153409*	0.00	1.76	0.15	0.96
si:ch211-281l24.3*	0.35	1.68	3.90	6.10

zgc:158463*	191.91	1,534.39	332.03	244.33
crybb1	231.97	499.21	454.59	307.65
si:rp71-36a1.5*	0.00	0.79	1.90	0.00
si:dkeyp-87e3.1*	80.25	22.04	88.05	128.04
zgc:113364*	3.13	0.00	0.88	3.37
5_8S_rRNA	56.51	1,194.10	119.88	30.10
NFASC	1.01	2.32	1.61	1.64
slc26a2	2.89	6.48	6.01	2.70
CABZ01077217.1*	0.00	0.47	0.75	0.00
si:dkey-153m14.1*	370.98	1,882.64	583.91	378.24
Metazoa SRP	15.33	206.64	0.30	0.00
si:ch211-57n23.4*	1.19	3.39	1.16	1.29
ptgs2b	1.90	4.38	2.26	2.38
gdpd2	5.31	10.30	9.17	6.87
cps1	1.88	3.49	3.22	2.66
otud4	234.86	2,910.14	203.89	174.74
rn7sk	6.01	56.43	12.84	8.89

Table 2. Expression values in FPKM (fragments per kilobase of transcript per million reads)
Shaded boxes indicate significant expression differences between mutant and wild-type (WT)
*Denotes an unannotated gene.

Candidate gene: Sidekey269.114

Since sidekey269.114 is an unannotated gene, it is still unknown what it encodes for. Sidekey269.114 is located on chromosome 12 but, its sequence is associated with a cluster of genes on this chromosome and it is cited as possibly being a tandem duplicate of cstl. Cstl is characterized as expressed in hatching gland cells that migrate into the mesoderm. The gene begins to be expressed at 2dpf so, ISH experiments were done using embryos fixed at 2dpf. Sidekey269.114 probes detected expression in the hatching gland that wraps around the yolk just below the head of the embryo (purple staining in Fig. 4). No qualitative difference in expression level was observed between WT, HET and MUT embryos. However, in the WT, no cells appeared to be migrating into the head region. In the HET embryo, there is 1 cell that migrating into the head region. In the MUT embryo, 4 cells can be counted that are migrating into the head

region (arrows in Fig. 4). More analysis would need to be done in order to classify this is finding is significant.

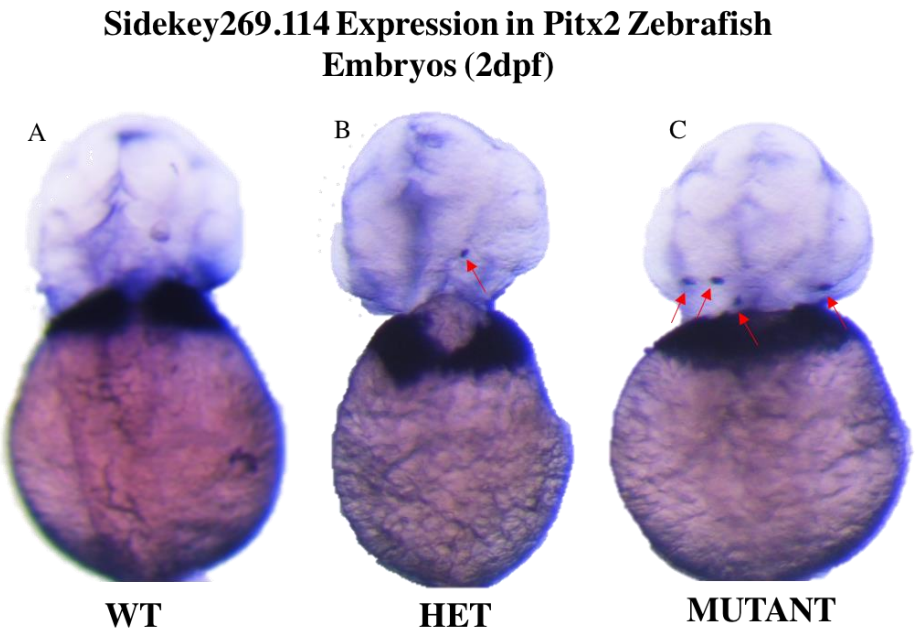

Fig. 4. Sidekey269.114 probe used to stain 2dpf pitx2HD^{sny7}embryos (A-C). Ventral view (A-C). Arrow point to migrating hatching gland cells (B-C).

Candidate gene: MyHz2

MyHz2 is a classified as a fast muscle cell that will appear in the cephalic muscles of the head. This candidate gene is located on chromosome 5 in the genome and first starts to show expression 2dpf in zebrafish development. No mutants were found when genotyping ISH embryos and no apparent differences in expression were seen between WT and HET pitx2 embryos (Fig. 5). Additional experiments are needed to test MUT embryos.

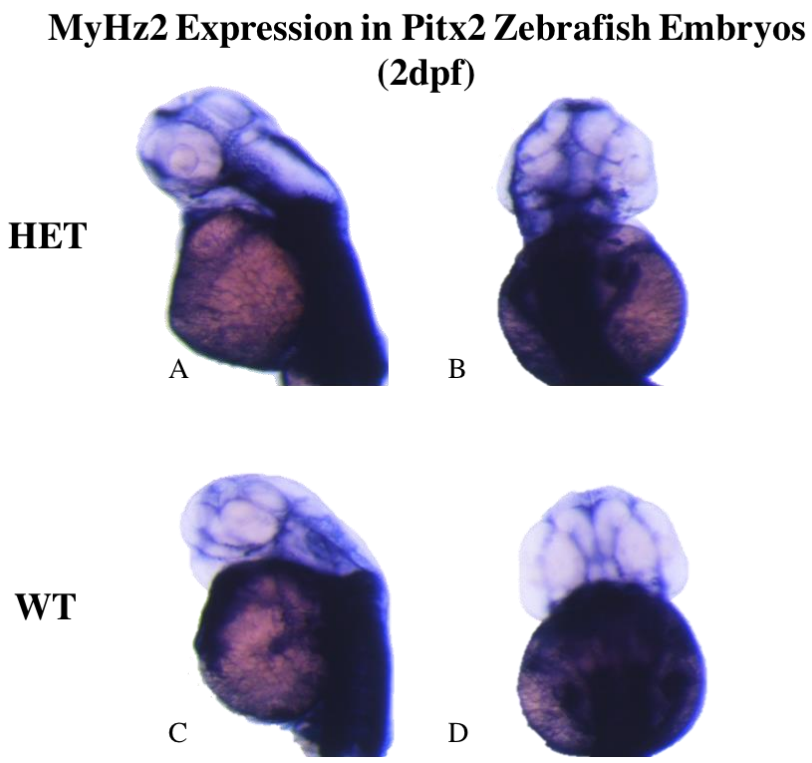

Fig. 5 MyHz2 probe used to stain 2dpf pitx2HD^{sny7}embryos (A-D). Profile view (A & C). Ventral view (B & D).

Candidate gene: CyP4v7

CyP4v7 is a classified as a gene important for oxidation-reduction processes, meaning metabolic functions. It is located on chromosome 14 and also begins to show 2dpf in zebrafish development. No mutants were found when these ISH embryos were genotyped post imaging. However, there is does not appear to be any significant differences between WT and HET embryos (Fig. 6). Additional experiments are needed to test MUT embryos.

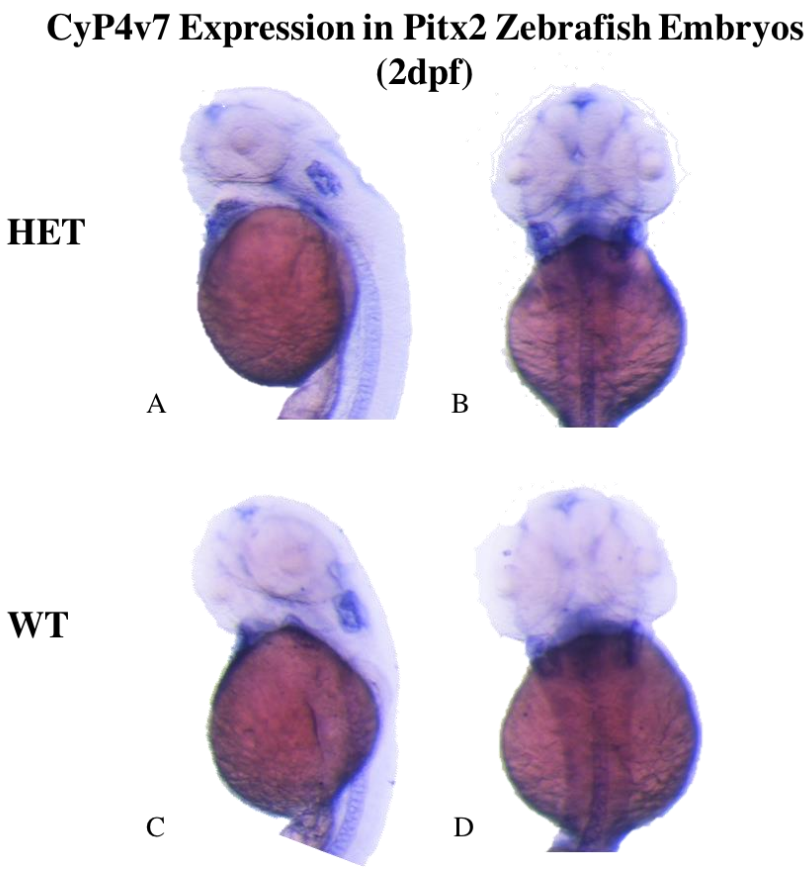

Fig. 6. CyP4v7 probe used to stain 2dpf pitx2HD^{sny7}embryos (A-D). Profile view (A & C). Ventral view (B & D).

Materials and Methods

Zebrafish Tail Clipping

Pitx2 zebrafish tail clips were used to genotype the adults used. Each fish was anesthetized one at time in 6mL tricaine stock (.4% solution) diluted in 150mL of water from the system. Once anesthetized, the were placed on parafilm and 1 square mm of one tail fin was cut using a scalpel. All instruments were sterilized before they were used. The tail clip is then deposited into 50μL of 50mM sodium hydroxide in a polymerase chain reaction (PCR) tubes. Tail clipped zebrafish were placed in isolation for a one week recovery before returning to their original tank. DNA from tail clips were then extracted using the HotSHOT DNA Extraction Protocol.

HotSHOT DNA Extraction

PCR tubes of tail clips or embryos were rinsed with two washes of a 1x concentration of phosphate-buffered saline (PBS). Each sample had 100μL of 50mM sodium hydroxide added followed by 30 seconds of a brief vortex and centrifuge. The samples were then incubated at 95°C for two intervals of 20 minutes. During the interval, samples were vortexed and centrifuged to ensure complete DNA extraction. After the last 20 minutes of incubation, 10μL of Tris- HCl of pH 8 was added to each sample once samples were cooled. Vortex and centrifuge were then used briefly to collect all undigested DNA at the bottom of the tubes. These samples were stored at -20°C.

Polymerase Chain Reaction (PCR)

To prep each sample for PCR 12.5μL of EconoTaq PLUS Green 2x Master Mix was added to 4μL of extracted DNA followed by 1μL of the forward and reverse primers and 6.5μL of

nuclease free water. The primers used for Pitx2 HD zebrafish were JYC 128 (5′-TGAAGCTTGTTCCTCTGC-3′) and JYC 129 (5′-AAAATTTAGGGTTATATCACATA-3′). For Pitx2c zebrafish JYC 152 and JYC 153 primers were used. A PCR program was developed to coincide with temperatures necessary for the amplification of the DNA. Program 1 (TALENHD2) applies 3 minutes of incubation at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. The last 5 minutes of the program is at 72°C. Program 2 (TALENHDA) is the same as program 1 except the annealing temperature is 50°C. TALENHDA was best to amplify Pitx2 HD samples and TALENHD2 was best to amplify Pitx2c samples.

Restriction Digest

Following the PCR reaction, 2.5μL of the selected buffer was added to 4μL of raw PCR product followed by 18.5μL of nuclease free water and .25μL of the restriction enzyme. For Pitx2 HD samples the buffer is CutSmart from BioLabs and the corresponding restriction enzyme is SalI-HF from BioLabs. For the Pitx2c samples the buffer is also CutSmart and the corresponding restriction enzyme is BamHI-HF. After brief vortex and centrifuge, the samples are incubated overnight at 37.5°C.

Gel Electrophoresis

To make the 2% gels, 4g of Agarose were added for every 200mL of 1x concentration of TBE (Tris-borate-EDTA). Once dissolved, 6μL of ethidium bromide was added. The solution was poured into the molds with well combs and allowed to cool until solid. To prep the gel for electrophoresis, 20μL of the restriction digest samples were loaded into individual wells. A

1Kb+ purple loading dye was used for the DNA Latter. The gel was allowed to run for 45 minutes and samples were genotyped based on basepair length. Wild type showed two bands (125bp and 225bp). Heterozygotes showed three bands (125bp, 225bp, 350bp). Mutants showed one band (350bp).

Alcian Blue/Alizarin Red Double Tooth Staining

The double staining protocol was adapted and conducted from previous papers (Walker and Kimmel, 2007) (Ji et al, 2016). Pitx2HD^{sny6} embryos were fixed at 4dpf and 6dpf using a 4% paraformaldehyde/1x PBS for 2 hours rocking using a notator. After fixing, 50% ethanol dehydration was used. Post dehydration, the double staining solution (.005% alizarin red/.02% alcian blue and 60mM $MgCl_2$ in 70% ethanol) was added to the embryos and they were allowed to rock on the notator overnight. Next, embryos were bleached to remove background staining and cleared using .25% KOH in glycerol. Imaged in 100% glycerol.

In Situ Hybridization (ISH)

ISH protocols used and followed as described (Ji et al, 2016). Ji et al made gene-specific probes using cDNA amplified from zebrafish cDNA library and cloned with TOPO TA cloning kit. Embryos were then genotyped to confirm phenotypes (2016).

Discussion

In collaboration with previous research done on pitx2 tooth development, characterizing the morphology at 4dpf and 6pf pitx2 embryos adds to the database of knowledge about specific points in tooth development the mutation phenotypes start to appear. However, these results are not enough to end here. If time had permitted, I would have bred more embryos and repeated staining at 2dpf, 4dpf and 6dpf. This is essential for sufficient statistical power because I did not find have enough embryos of each genotype to support my figures with quantitative data. Also, I was not able to find mutants at 4dpf to see if the mutation does begin to show this early in development. Data that I found for 6dpf, seems promising that mutations are prominent at this point however, yet again I would need to be able to image and genotype more embryos to confirm this.

The candidate gene sidekey269.114 also shows results that are very interesting. The migrating cells were only imaged in HET and MUT embryos and could be showing either elevated levels of expression or early expression in pitx2 zebrafish. I would also have liked to repeat this experiment to have a larger sample population. There were more of these embryos that I could have imaged and genotyped. Unfortunately, there was an error made in the lab and samples of each candidate gene ISH embryos were lost.

Since the sidekey269.114 candidate gene appears to be worth further research, I would like to perform more experiments focusing on its correlation with pitx2. Although ISH experiments are vital to observing the expression of genes, it is a qualitative experiment and should be supplemented with quantitative studies to confirm any significant findings.

Experiments like quantitative PCR (qPCR) and protein western blots could be used to count the differences of protein expression between WT and MUT embryos.

Because mutants were also not found in MyHz2 ISH experiments, I would also like repeat these experiments to find a sample size large enough to show if data are significant or not. Similarly, CyP4v7 ISH experiments also did not yield mutants and repeating these experiments in the same way would help to bring conclusions to the question of whether or not this candidate gene is correlated with the pitx2. The MyHz2 is of particular interest because it was thought that since pitx2 mutants have defects in the eyes that the cephalic muscles surrounding the eyes were being affected and possibly correlated with the pitx2 mutation.

While my results were inconclusive, it is important to address what this experience has taught me. From my time in the Amack Lab, I have learned how to problem solve like a true biologist and how to create a project worth researching. Research does not always turn out the way in which we would like however, the skillset I can take away from this experience will definitely serve me well in the future.

Acknowledgements

I would like to thank Dr. Jeffrey Amack and the entirety of the Amack Lab for not only supporting me through this process but, helping me grow into a biologist that can contribute to the science community. I would also like to thank my honors reader, Dr. Paul Gold for being a vital asset to my career at SU. Lastly, thank you to the Renée Crown Honors Program for always providing opportunities that help its students reach for their ambitions.

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