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The Role of Fibroblast Growth Factor 20a in the Craniofacial Development of Zebrafish

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The Role of *Fibroblast Growth Factor 20a* in the Craniofacial Development of Zebrafish

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in Biology with Honors

May/2008

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Abstract

Fibroblast growth factors are a family of intercellular signaling molecules that participate in the normal growth of the craniofacial skeleton. Advances in molecular genetics have revealed that fibroblast growth factors are involved in the regulation of bone growth by maintaining a balance between the differentiation of osteoblasts and the proliferation of mesenchyme (Morriss-Kay and Wilkie, 2005). The focus of my research is *fibroblast growth factor 20a* because it seems to play a significant role in the normal development of the skull. At birth, the skull is made up of bony elements, that meet at fibrous membranes called sutures. Throughout development, those bony elements fuse together at different rates to form solid bone. Previous research has shown that zebrafish deficient in *fgf20a*, which are referred to as *dob* mutants, have abnormal suture patterning and exhibit several craniofacial defects that are similar in presentation to the defects seen in individuals with craniosynostosis (Albertson, unpublished data). Craniosynostosis is a birth defect that is caused by the premature closure of sutures and is characterized by an irregular craniofacial shape. Using whole mount *in situ* hybridization (WISH) methods and advanced microscopic imaging, I gained insights into the pathological mechanisms that lead to aberrant suture formation in humans. Our research results suggest that *fgf20a* directly affects suture and skull development. We have demonstrated this in two ways. Firstly, we showed that in the absence of *fgf20a*, zebrafish have abnormal suture patterning. Secondly, we showed that the *fgf20a* gene is normally expressed in the cranial sutures of wild-type zebrafish through WISH methods. Our results are important because this is the first instance where an fgf ligand is implicated in craniosynostosis. Furthermore, we have established a molecular foothold (via *fgf20a*) into skull and suture development, and we have established a model system for studying the developmental origins and mechanisms of craniosynostosis.

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I would like to thank the Albertson Lab, Dr. Craig Albertson, Dr. Jim Cooper, and Sarah Collins, because without their help this project would have never been possible. They have contributed greatly to my academic growth and scientific understanding.

Introduction

Craniosynostosis, a birth defect occurring in 1 of 2,500 live births, is characterized by an irregular craniofacial shape that often presents challenging clinical problems (Morriss-Kay and Wilkie, 2005). At birth, the skull is made of bony elements that gradually fuse together into solid bone throughout development. These bony elements meet at sutures, which are fibrous membranes, and fuse together at different rates. This mechanism allows for the normal expansion of the brain and soft tissues during infancy and childhood since a newborn has only forty percent of her adult brain volume (Kabbani and Raghuveer, 2004). Craniosynostosis is caused by the premature fusion of sutures and this can have detrimental effects on the child's intelligence since brain growth is restricted. The disease also results in an abnormal head shape and an increase in intracranial pressure that often has devastating physiological consequences (Morriss-Kay and Wilkie, 2005).

The recent discovery of mutations in FGFR1, FGFR2, and FGFR3 in syndromic and non-syndromic cases of craniosynostosis has led to the hypothesis that the disease involves the failure of the fibroblast growth factor signaling system (Morriss-Kay and Wilkie, 2005). Fibroblast growth factors are a family of intercellular signaling molecules that are involved in cell proliferation, differentiation, and migration during early stages of development. They also participate in a wide array of tissue interactions, including the growth and development of the craniofacial skeleton.

The focus of our research is *fibroblast growth factor 20a* because it seems to play a significant role in suture patterning (Albertson, unpublished data). The objectives of our research are to identify the anatomical consequences of a null *fgf20a* mutation, determine at what stage of development the defect arises, and to gain a better understanding of the pathological mechanisms that lead to the defect.

Anatomy of the Craniofacial Skeleton

The mammalian skull vault is made up of the neurocranium, which surrounds and protects the brain, and the viscerocranium, which forms the face. The skull consists of two frontal bones, two parietal bones, and one occipital bone. These bones are joined by five major cranial sutures: metopic, sagittal, coronal, lambdoid, and squamosal. Also, newborns have an anterior fontanelle and a posterior fontanelle which enable the plates of the skull to flex (Morriss-Kay and Wilkie, 2005). Throughout development, the sutures and fontanelles close at different times to form one continuous plate of bone, but they are still considered pairs of bone because of their paired developmental origin (Table 1).

Table 1- Closure of Sutures and Fontanelles*

Type of Suture/fontanelle	Closure Time
Metopic Suture	Nine months to two years (may persist into adulthood)
Coronal, sagittal, lambdoid sutures	40 years
Anterior fontanelle	Nine to eighteen months
Posterior fontanelle	Three to six months

*Adapted from Kabbani and Raghuvver, 2004

Zebrafish (*Danio rerio*) are an important emerging model system in the study of human diseases and are an appropriate model to study craniofacial defects. The anatomy of the zebrafish cranial vault and suture patterning is similar to that of mammalian organisms (Figure 1). Also,

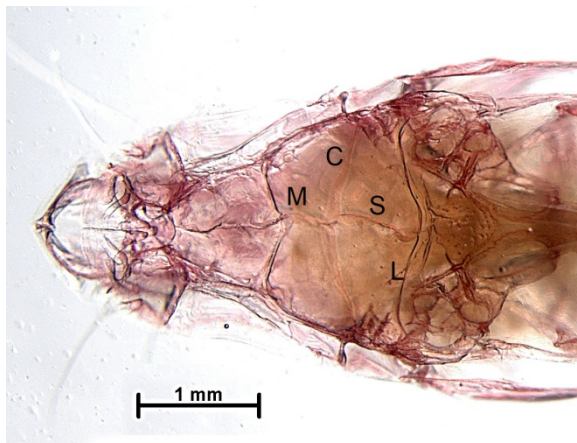


Figure 1- The dorsal view of the wild-type zebrafish with normal suture patterning. The metapoic (M), sagittal (S), lambdoid (L), and coronal (C) sutures are clearly seen.

zebrafish produce a large number of embryos that are transparent and develop externally. We can therefore analyze their phenotypic traits without using invasive techniques and their genetics are easily manipulated.

Previous Research

Previous research in mice has shown that a broad repertoire of *Fgfs*, including *fgf20*, is expressed in the cranial sutures (Hajihosseini et al. 2001) (see Table 2). Furthermore, recent studies in zebrafish have shown that a null mutation in *fgf20a* has several phenotypic consequences including an irregular shape of the skull (Albertson, unpublished data). Zebrafish that are deficient in *fgf20a* are known as *devoid of blastema*, *dob*, mutants because of their inability to regenerate their tail due to the loss of blastema cells. A blastema is a mass of cells that are

undifferentiated and, thus, are capable of regenerating tissues and organs (including the tail fin in fish).

Table 2- Previous FGF/FGFR Research

FGF/FGFR	Species	Tissue(s)	Process	Mutation
FGFR1	Mice	Sutures	Accelerated osteoblast differentiation	Mild form of Pfeiffer syndrome (Morriss-Kay and Wilkie, 2005)
FGFR2	Mice	Sutures	Premature loss of proliferation in osteoprogenitor cells	Crouzon, Pfeiffer, Apert syndromes (Wilkie, 1997)
FGFR3	Mice	Sutures	Negative regulator of endochronal ossification	Growth disorders of long bones (dwarfism), Muenke syndrome, and rare variant of Crouzon syndrome (Wilkie, 1997)
FGF1	Mice	Coronal suture	Cause suture obliteration and fusion (Morriss-Kay and Wilkie, 2005)	
FGF2	Mice		Overexpression leads to increased apoptosis	Chondrodysplasia (shortened long bones) (Rice et al, 2000)
FGF4	Mice	Embryonic osteogenic fronts	Accelerates suture closure, stimulates cell proliferation (Rice et al, 2000)	
FGF10	Mice	Calvarial Sutures		
FGF20	Mice	Coronal sutures and limb buds	Suggests role in proliferation, differentiation, and apoptosis (Hajihosseini and Heath, 2002)	

*Fgfs 3,4,5,6, and 8 are not expressed in cranial sutures

** In general, undifferentiated (immature) osteoblast cells respond to FGF treatment with increased proliferation, whereas in differentiating cells FGF causes apoptosis.

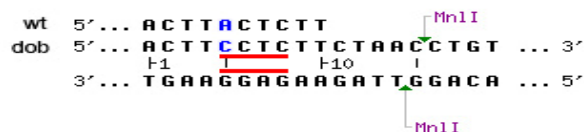
Previous research has also revealed that craniosynostosis is linked

to fibroblast growth factor receptors, but little is known about the signal molecules, ligands, that bind to receptors to trigger cells to proliferate, differentiate, and/or migrate (Hajihosseini et al. 2001). Thus, in an effort to identify one of the cognate ligands, we examined the expression pattern of *fibroblast growth factor 20a* in the developing zebrafish craniofacial skeleton by using polymerase chain reaction (PCR) and whole mount in situ hybridization (WISH) methods.

Methods

Polymerase Chain Reaction

We genotyped the mutant *dob* zebrafish by using Polymerase Chain Reaction (PCR) and the restriction enzyme, *mn1*. PCR primers were designed to amplify the *fgf20a* locus, and *mn1* was chosen because it recognizes the mutated form of the gene (see Figure 2). In this way



homozygous recessive,

heterozygous and wild-type animals could be identified.

Figure 2- The restriction enzyme, *mn1*, recognizes the sequence CTC and then cuts seven base pairs downstream.

We extracted the DNA by cutting the tip of a zebrafish tail

and placing it in a microcentrifuge tube with 50 μ l of Extraction solution

and 12.5 μ l of Tissue Preparation Solution. We then incubated the tube for 10 minutes at room temperature and then for 3 minutes at 95 °C. Once the zebrafish tail had been sufficiently digested, we added 100 μ l of Neutralization Solution B and mixed thoroughly. We then added 4 μ l of water, 10 μ l of REExtract-N-Amp PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO), 1 μ l of Forward Primer (Invitrogen, Carlsbad, CA), 1 μ l of Reverse primer (Invitrogen, Carlsbad, CA), 4 μ l of Tissue Extract and mixed gently. The forward and reverse primers used for the amplification of the *fgf20a* locus were CTGTCAGCCGAGTGTGTGTT and ACGTCCCATCTTTGTTGAGG, respectively. We then performed thermal cycling (see Table 3).

Table 3- Thermal Cycling

Step	Temperature	Time (minutes)	Cycles
Initial Denaturation	94 °C	3	1
Denaturation	94 °C	1	35
Annealing	65 °C	1	35
Extension	72 °C	1	35
Final Extension	72 °C	10	1
Hold	4 °C	Indefinitely	

*Adapted from Sigma-Aldrich

After the DNA was successfully amplified, we then combined 20 μ l of the PCR product, 3 μ l of 10x BSA, 3 μ l of #2 Buffer, 3 μ l of distilled water, and 1 μ l of the restriction enzyme, *mnI1* (NE Biolabs, Ipswich, MA). We let the mixture sit overnight at room temperature and then ran the digested product on a 5% agarose gel (Figure 3).

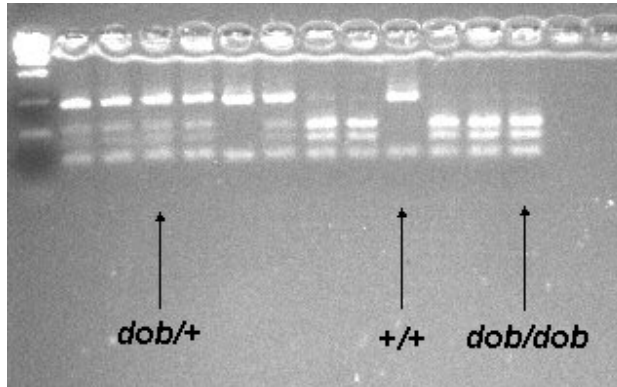


Figure 3- The large DNA fragment in wild-types is cut in animals that carry the *dob* allele.

In *dob* mutants the *mnI1* restriction enzyme cut the target sequence resulting in three DNA fragments in *dob/dob* individuals

compared to two fragments in wild-type (*+/+*) individuals.

The heterozygous carrier of the *dob* mutation, *dob/+*, has one allele with the *fgf20a* mutation and one normal allele, so four fragments were formed upon the *mnI1* digest.

Whole Mount In Situ Hybridization

Using WISH methods and an antisense *fgf20a* riboprobe, we were able to localize *fgf20a* mRNA to particular regions of the developing skull. We fixed juvenile (64 day) wild-type zebrafish in 4% paraformaldehyde (PFA) overnight at 4°C. Then, we dehydrated the embryos by washing them with different concentrations of methanol and phosphate buffered saline + tween (PBSt). The next day, we rehydrated the embryos by using

the following protocol: 2 x 10 mins in 50% methanol/ 50% PBSt, 1 x 10 mins in 25% methanol/ 75% PBSt, 2 x 10 mins PBSt. Then we removed the eyes and guts of the juvenile fish and digested the remaining tissue by using a 1:5000 dilution of a 50 mg/mL stock of proteinase K⁺ in PBSt. After 30 minutes, we rinsed the sample in PBSt and refixed it in 4% PFA for half an hour.

Then, we placed the samples in 1.5 mLs of Prehybridization solution (PHS) and put it in a water bath at 70°C for 3 hours. Then, we replaced the PHS with 70°C Hybridization solution (HS) plus the riboprobe and let it incubate overnight in the water bath. The next day, we performed a series of washes at 70°C: 1 x 10mins in 75% PHS/ 25% 2x SSC, 1 x 10mins in 50% PHS/ 50% 2x SSC, 1 x 10mins in 25% PHS/ 75% 2x SSC, 1 x 10mins in 2x SSC. Then, we dropped the temperature of the waterbath to 68°C and washed the sample with 0.2x SSC followed by two washes with Maleic Acid Buffer (MAB) at room temperature.

To block the zebrafish, we moved the specimen to a plate with 2 mLs of blocking solution at room temperature for three hours.

Simultaneously, we pre-blocked the anti-DIG antibody in the blocking solution. After three hours, we combined the specimen with the anti-DIG solution and incubated it at 4°C overnight.

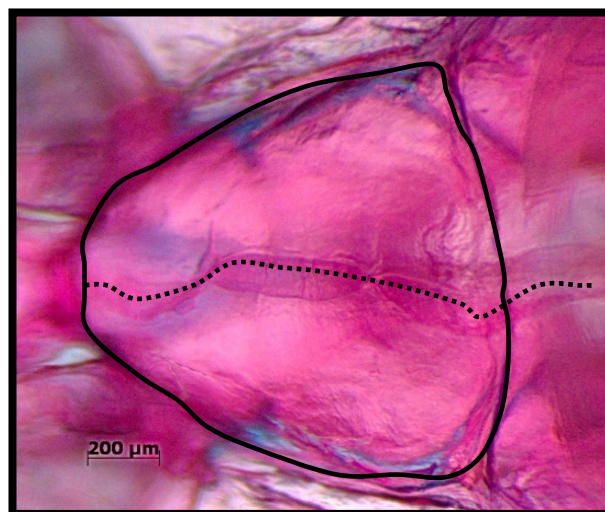
The next day, we performed another series of washes: 1 x 5mins in MAB, 2 x 10mins in MAB, 1 x 30mins in MAB, 1 x 1hr in MAB, 3 x 5mins in AP Buffer. Then, we stained the embryos by removing the excess AP

Buffer and adding approximately 500 μ l of staining solution. We kept the sample in a dark room and after 20mins we washed it with 2x 5mins in PBSt to stop the reaction. Then, in order to facilitate the clearing of non-specific staining, we performed the following washes: 1 x 10mins in 25% methanol/75% PBSt, 2 x 10mins in 50% methanol/ 50% PBSt, and then stored them in 100% methanol overnight at 4 °C. The next day, we rehydrated the specimens and performed the following glycerol series in order to facilitate clearing: 4hrs 25% glycerol/ 75% PBSt, 6 hrs 50% glycerol/ 50% PBSt, and then we stored them in 75% glycerol/ 25% PBSt.

Results

Identifying the Defect

By staining wild-type and *dob/dob* mutant zebrafish with alizarin red, it is evident that adult *dob* mutants have defective suture patterning (Figures 4-6).



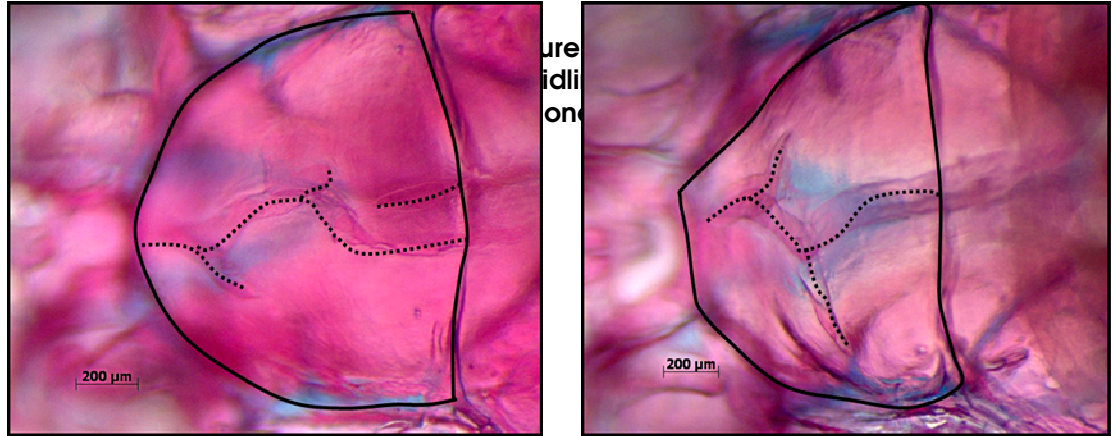
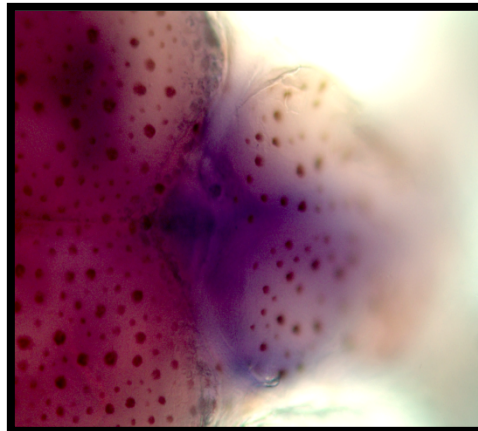


Figure 5- The patterning of the sagittal suture in *dob* mutants is irregular (dashed lines) as is the shape of the

Figure 6- The sagittal suture of this *dob* mutant deviates in several places from the midline (dashed lines) and the frontal

Expression of *fgf20a* in Cranial Sutures

We used an antisense *fgf20a* riboprobe with a purple label to localize *fgf20a* mRNA *in situ*. Our results from the WISH analyses suggest that *fgf20a* skull and



plays a primary role in suture development.

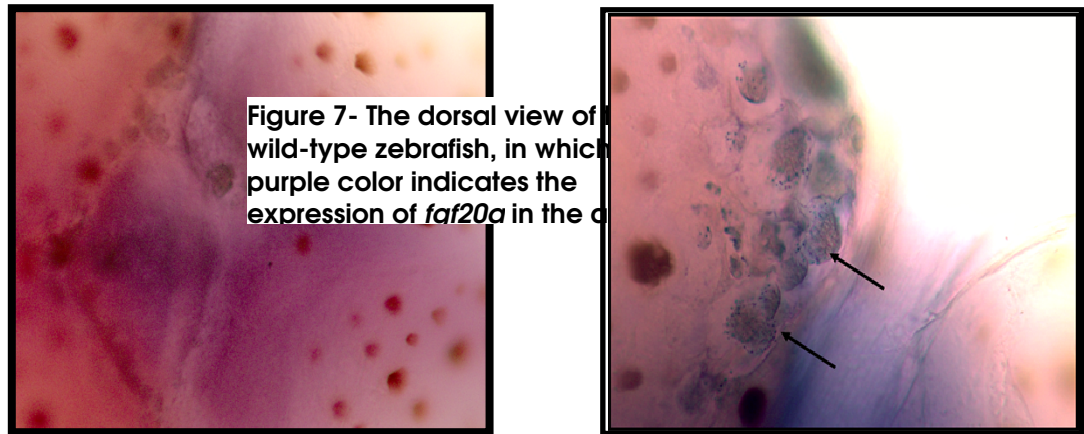


Figure 7- The dorsal view of wild-type zebrafish, in which purple color indicates the expression of *fgf20a* in the d

Figure 8- This is a closer view of the fontanelle where *fgf20a* is obviously expressed.
Figure 9- This is a closer look at the suture boundary with putative osteoblast cells expressing *fgf20a*

Conclusion

Our research results suggest that *fgf20a* directly affects suture and skull development. We have demonstrated this in two ways. Firstly, in the absence of *fgf20a* zebrafish have abnormal suture patterning (Figures 5 and 6). Secondly, the *fgf20a* gene is normally expressed in the cranial sutures (Figures 7-9). The next step is to determine when this aberrant suture pattern arises in the *dob* mutant. The future direction of our research is to compare the craniofacial development in wild-type and *dob* mutant zebrafish at different stages of development. Below is an image of a wild-type zebrafish stained with calcein (Fig. 10). Calcein, also known as fluorexon, is used to visualize the developing bone in live embryos. Calcein binds to free Calcium in the bone matrix, which results in green fluorescence under UV light.

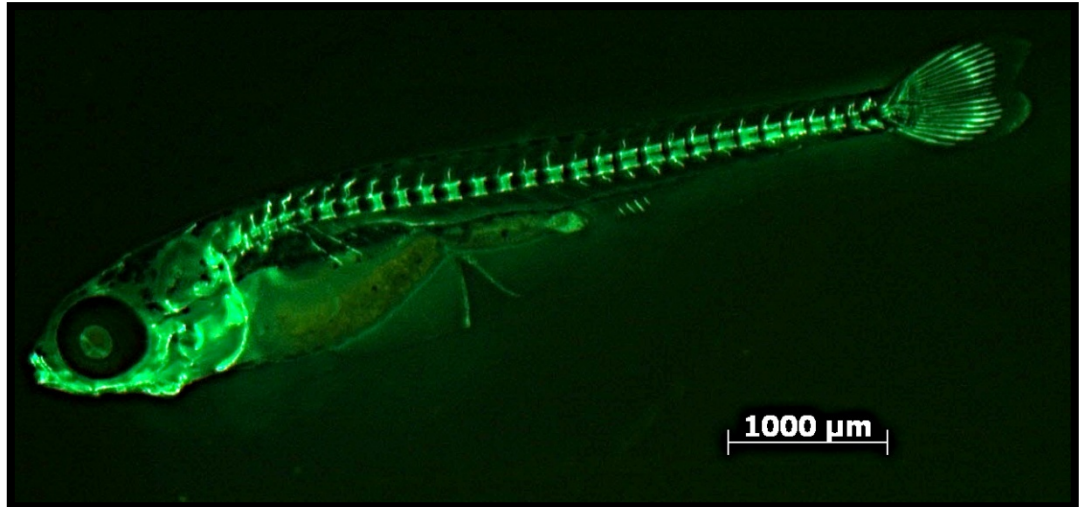


Figure 10- Image of live larval wild-type zebrafish stained with Calcein and viewed under UV light.

Our results are important because we have identified a developmentally tractable model (zebrafish) to study the developmental origins of human craniosynostosis and this model system has proven to be very advantageous for understanding human health and disease. Also, we have established a molecular foothold (via *fgf20a*) into skull and suture development.

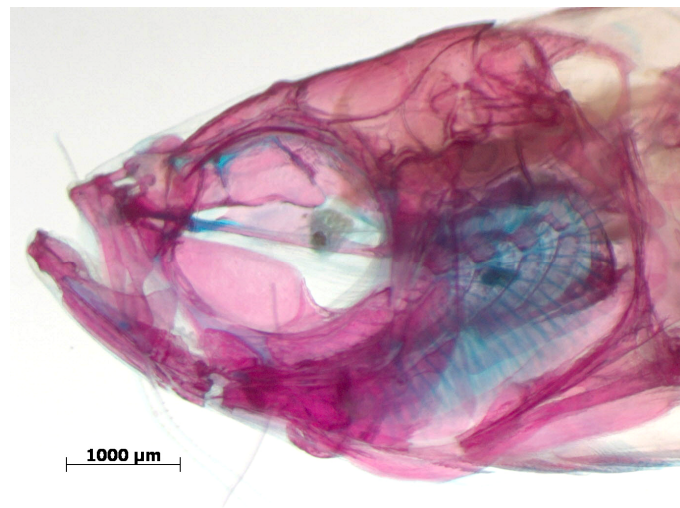


Figure 11- A lateral view of a zebrafish stained with Alizarin red. We have established that zebrafish are an excellent model system for studying developmental origins and mechanisms of craniosynostosis.

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Written Capstone Summary

The Role of Fibroblast Growth Factor 20a in the Craniofacial Development of Zebrafish

The development of a multicellular adult organism is dependent on the individual's genetic makeup. Genes are considered the blueprint for the human body because they tell the body how to build a specific structure. Thus, to understand development, we often study the effect of particular genes. The focus of our research project is *fibroblast growth factor 20a*, also known as *fgf20a*, because it seems to play a significant role in the normal development of the skull. Fibroblast growth factors are a family of small proteins that bind to receptors on other cells to signal them to multiply, differentiate, and/or migrate. Throughout early development, cellular specialization and tissue growth are due in large part to the *fgf* intercellular signaling process. Previous research has revealed that many human growth defects are linked to defective *fgf* receptors, including craniosynostosis (Morriss-Kay and Wilkie, 2005).

Craniosynostosis, is a serious birth defect that occurs in approximately 1 of 2,500 live births and is characterized by abnormal craniofacial shape (Morriss-Kay and Wilkie, 2005). At birth, the skull is made of bony elements that gradually fuse together into solid bone throughout development. Furthermore, the bony elements meet at

sutures, which are fibrous membranes, and fuse together at different rates. This mechanism allows for the normal expansion of the brain and soft tissues during infancy and childhood since a newborn has only forty percent of her adult brain volume (Kabbani and Raghuveer, 2004).

Craniosynostosis is caused by the premature fusion of sutures and this can have detrimental effects on the child's intelligence since brain growth is restricted. The disease also results in an abnormal head shape and an increase in intracranial pressure that often has devastating physiological consequences (Morris-Kay and Wilkie, 2005). The recent discovery of mutations in FGFR1, FGFR2, and FGFR3 in several cases of craniosynostosis has led to the hypothesis that the disease involves the failure of the fibroblast growth factor signaling system (Morris-Kay and Wilkie, 2005). Furthermore, previous research has shown that zebrafish deficient in *fgf20a* exhibit several craniofacial defects that are similar in presentation to the defects seen in individuals with craniosynostosis (Albertson, unpublished data).

The objectives of our research are to identify the anatomical consequences when *fgf20a* is absent, determine at what stage of development the defect arises, and to gain a better understanding of the mechanisms that lead to the defect. Essentially, we want to answer the questions, What?, When?, and How?.

We chose zebrafish (*Danio rerio*) as our model system to study craniofacial defects because the anatomy of the zebrafish cranial vault

and suture patterning is similar to that of mammalian organisms. In fact, the five major cranial sutures present in humans, metopic, sagittal, coronal, lambdoid, and squamosal, are also found in zebrafish. Also, zebrafish produce a large number of embryos that are transparent and develop externally. We can therefore analyze their phenotypic traits without using invasive techniques, and their genetics are easily manipulated.

Previous research in mice has shown that a broad repertoire of *Fgfs* is expressed in the cranial sutures including *fibroblast growth factor 20a* (Hajihosseini et al. 2001). Furthermore, recent studies in zebrafish have shown that when *fgf20a* is absent there are several phenotypic consequences including an irregular shape of the skull (Albertson, unpublished data). Zebrafish that are deficient in *fgf20a* are known as *devoid of blastema, dob*, mutants because of their inability to regenerate their tail due to the loss of blastema cells. A blastema is a mass of cells that are undifferentiated and, thus, are capable of regenerating tissues and organs (including the tail fin in fish).

Previous research has also revealed that craniosynostosis is linked to fibroblast growth factor receptors, but little is known about the signal molecules, ligands, that bind to the receptor to trigger the cells to proliferate, differentiate, and/or migrate (Hajihosseini et al. 2001). Thus, in an effort to identify one of the cognate ligands, we examined the expression pattern of *fgf20a* in the developing zebrafish craniofacial

skeleton using whole mount in situ hybridization (WMISH) methods.

In our study, we amplified the *fgf20a* DNA sequence using Polymerase Chain Reaction (PCR), and then used a restriction enzyme that recognized the mutated form of the gene to cut this sequence in mutant animals. In this way we could identify animals that lacked a functional copy of the gene. Then, once we identified the *dob* mutants, we used advanced microscopic imaging to characterize the phenotypic consequences of the mutation, which included an expanded frontal region of the skull and aberrant suture patterning.

We used whole mount in situ hybridization to localize *fgf20a* mRNA to particular regions of the developing skull in wild-type zebrafish. A synthesized mRNA complementary strand with a purple label was used to bind to the target sequence *in situ*. Thus, we were able to determine where the gene is normally expressed in the developing craniofacial skeleton.

Our research results suggest that *fibroblast growth factor 20a* directly affects suture and skull development. We have demonstrated this in two ways. Firstly, in the absence of a fully functional *fgf20a* gene zebrafish have abnormal suture patterning. Secondly, we have shown that the *fgf20a* gene is normally expressed in the cranial sutures through the WMISH methods. The next step will be to determine when this aberrant suture pattern arises in the *dob* mutant. The future direction of this

research will include comparing the craniofacial development in wild-type and *dob* mutant zebrafish at different stages of development.

Our results are important because this is the first instance where an fgf ligand is implicated in craniosynostosis. Furthermore, we have established a molecular foothold (via *fgf20a*) into skull and suture development, and we have established a model system for studying developmental origins and mechanisms of craniosynostosis.