Effects of the Runx Gene Family on Tooth and Bone Development in Zebrafish

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Abstract:
The transcription factor Runx2 plays an important role in the skeletal development of vertebrates. Zebrafish (*Danio rerio*) possess two orthologs of the Runx2 gene, *runx2a* and *runx2b*. These two genes present slightly different expression patterns in developing zebrafish (Flores et al., 2003). The role of the Runx2 transcription factor has been studied in odontogenesis in mice (Camilleri et al., 2006), and here I aim to explore its role in zebrafish odontogenesis using green fluorescent protein (GFP) transgenic zebrafish. The CRISPR/Cas9 system mediated the insertion of GFP so that it was controlled by the promoter of the target gene, in my case either *runx2a or runx2b,* creating GFP knock-in transgenic fish. I found that the two genes showed slightly differing expression patterns in tooth development, although both could be seen in the dental mesenchyme in early development and then in the dental epithelium in later development. The results show both zebrafish orthologs of the Runx2 transcription factor likely play a role in odontogenesis in this model vertebrate species.

Objective:
The objective of this project was to create and study stable 'reporting' mutant lines of the *runx2a* and *runx2b* genes. These 'reporting' mutant lines descend from a single GFP knock-in transgenic fish. Starting with fish that had been injected with the CRISPR/Cas 9 system at the one cell stage the goal was to produce heterozygous mutant fish for both the *runx2a* and *runx2b* genes. These fish could then be used to create homozygous mutants for each gene in addition to creating double mutants of both genes. It was hoped that studying these fish and their GFP expression patterns could help explain the role of each gene in tooth development.

Methods:
The CRISPR-injected fish were crossed with wildtype fish in crossing tanks. Embryos were observed under a fluorescence microscope to detect GFP expression. To visualize tooth development some embryos were fixed with formaldehyde. A GFP-HRP antibody was applied to the fixed embryos as well as TSA substrate to aid in visualizing the GFP. Embryos were also stained with DAPI and alizarin red to view surrounding cells and the calcified teeth, respectively. These stained embryos were photographed with a fluorescence microscope. The z-stacks were processed with FIJI. To analyze the genetics of the embryos, genomic-DNA samples were extracted with the New England BioLabs Monarch DNA extraction kit. Primers for the PCR were designed on Geneious. PCR reactions were done and gels run to see if the GFP plasmid was inserted in the correct section of genomic DNA.

Results Obtained:
runx2b
Some offspring of a cross between a fish that had been injected with the GFP plasmid and CRISPR/Cas 9 system at the one cell stage and a wild-type were GFP positive. These fish were heterozygous mutants of the *runx2b* gene. The GFP expression appeared in the cleithrum and in the pharyngeal region in these crosses, as would be expected for a Runx2 gene (Figure 1). Select GFP positive offspring were raised to be able to breed in the coming months. Other GFP positive offspring were fixed and stained in a

sequence at various ages to compare stages of development. The embryos were stained in sequence twice throughout the work to catch various stages of formation of the larval dentition. In the first sequence the embryos were 58, 82, 104, and 122 hpf. In the second sequence the embryos were 61, 73, 104, and 120 hpf. In these sequences the GFP appeared in the dental mesenchyme of teeth early in their development, which was seen from 61 to 104 hpf, and possibly could be observed before and after this window. To know exactly when the *runx2b* gene turns on and off in developing tooth germs, this would have to be studied further. There was no expression observed in the dental mesenchyme by 120 hpf, but some expression was visible in the dental epithelium. Expression was also visible in the dental epithelium at 104 hpf in addition to the mesenchymal expression (Figure 2). The zebrafish tooth develops through an interaction between the dental mesenchyme and epithelium. The mesenchymal and epithelial cells work together in the formation of the tooth but play slightly different roles in the process (Verstraeten et al., 2010). Knowing whether *runx2b* is expressed in the mesenchyme, epithelium, or both is important in understanding its role in tooth development.

The PCR reactions visualized with gels showed that the plasmid had been inserted into the genomic DNA in the reverse orientation. Knowing the orientation of the GFP plasmid allows for the proper modeling of the genetic makeup of the transgenic fish, which in turn allows for the correct design of primers to locate the ends of the transgene. Using a variety of primers that flanked the insertion site, the 5' end was 'located' but the 3' end of the insertion could not be located. This suggests the GFP plasmid may not have been inserted exactly as designed, which could alter results.

runx2a

Offspring of a male *runx2a* F0 fish that had been injected with the GFP plasmid and CRISPR/Cas 9 system also showed GFP expression. The GFP was also expressed in the cleithrum and pharyngeal region of these crosses (Figure 3). Again, some were selected to raise to be bred and others were fixed. There was not time to fix these in a sequence as the GFP-positive fish were discovered at the end of the research period. Instead, embryos were fixed at 135 hpf. These embryos showed GFP expression in the dental mesenchyme early in development, and later in development, the expression was seen in the dental epithelium (Figure 4).

The PCR reactions of the *runx2a* rk01 DNA that were visualized with gels showed that the plasmid had again inserted in the reverse orientation. Both ends of the plasmid were 'located".

Significance and Interpretation of Results:

runx2b

The significance of the results visualized under the microscope depend on the accuracy of the plasmid insertion. If the plasmid is not inserted at the correct locus, the patterning of the GFP is irrelevant to this investigation. For the *runx2b* mutants, the gels showed that one side of the plasmid was inserted at the correct locus, but it is unknown how accurately the entire plasmid was inserted as the other side could not be located. If there are significant deletions on that end, the patterning seen may not best represent the expression of *runx2b*. If it is assumed that the plasmid was accurately inserted to fully replace the *runx2b* gene in the genomic DNA, the results of the apotome imaging show that the *runx2b* gene is expressed early in tooth development in the tooth germ and is less expressed as the tooth develops. *Runx2b* is primarily expressed in the dental mesenchyme, but is also expressed slightly in the dental epithelium (Figure 2). While these results show *runx2b* is present in initial tooth development and that it plays a role in both the mesenchyme and epithelium, more research would need to be done to show its

exact implications in tooth development. Creating a double mutant for *runx2b* would be an important next step in this work.

runx2a

The same goes for the *runx2a* mutant, as the results of the apotome imagine are only significant if the plasmid was inserted to accurately replace the gene. The gels showed both ends of the plasmid insertion could be located, so it is with confidence that the GFP patterning correctly imitates where *runx2a* would be expressed.

The GFP patterning visualized with the apotome microscope showed that *runx2a* is also expressed in the tooth germ early in development and is then primarily expressed in the dental mesenchyme as the tooth develops (Figure 4). Again, more work building off of these findings would need to be done to understand the exact role that *runx2a* plays in development, and again creating a double mutant would be a good next step. Further research could also fix *runx2a* mutants in a series as I did with *runx2b* mutants to understand how *runx2a* is expressed as development progresses.

This study was successful in exploring when and how both *runx2a* and *runx2b* are expressed in the development of the teeth, but more work would have to be done to understand the implications of the genes in tooth development and the roles they play.

Figures/Charts:

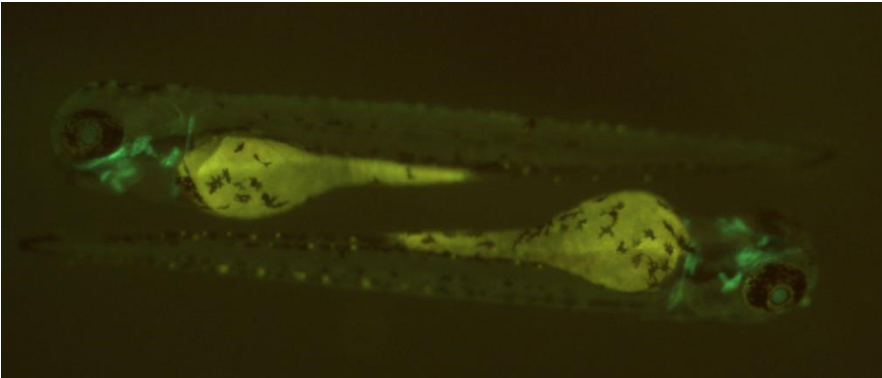

Figure 1. Expression pattern of offspring of a GFP knock-in transgenic runx2b mutant crossed with a wildtype fish fixed at 73 hpf.

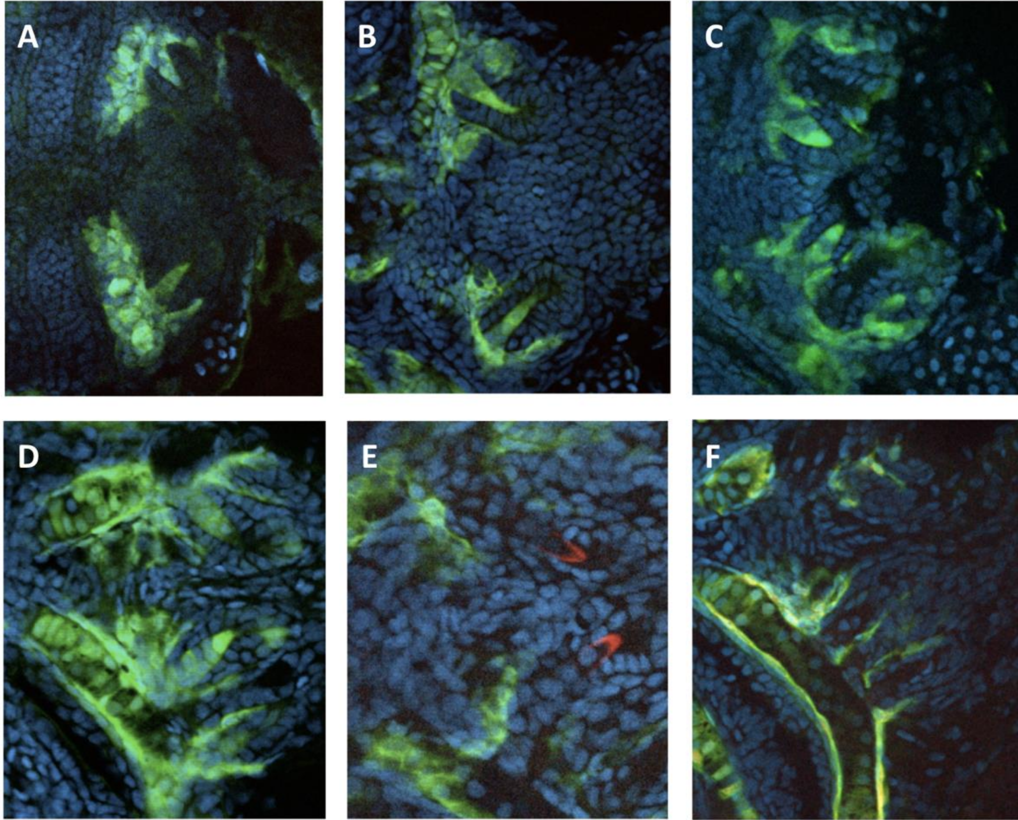

Figure 2. Sequence of cross sections of GFP reporting runx2b mutant stained with a GFP-HRP antibody and TSA substrate (green), a dapi stain (blue) and an alizarin stain (red). Images are oriented ventral side up with the anterior to the left. Fish were fixed at (A) 61 hpf, (B) 73 hpf (C) 82 hpf, (E) 104 hpf, and (F) 120 hpf.

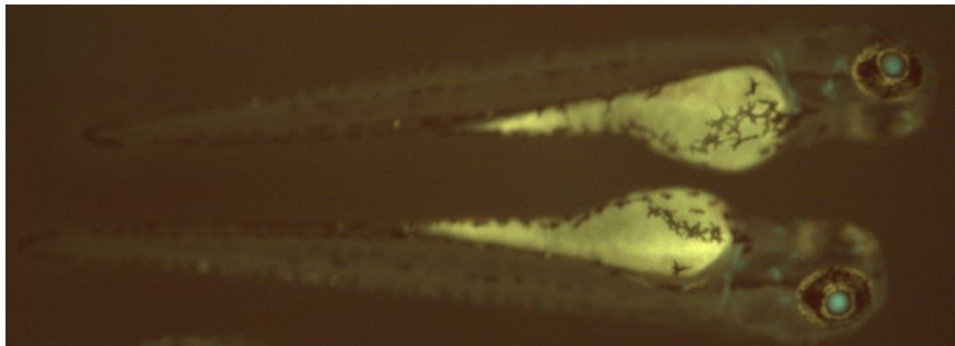

Figure 3: Expression pattern of offspring of a GFP knock-in transgenic runx2a mutant crossed with a wildtype fish fixed at 99 hpf.

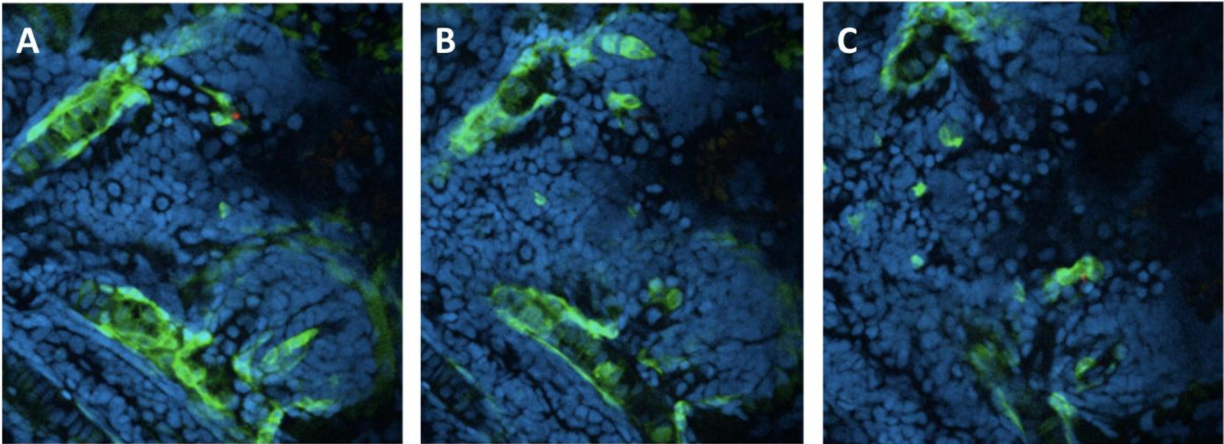

Figure 4: Cross sections of GFP reporting runx2a mutants fixed at 135 hpf. Fish are oriented ventral side up with the anterior on the left. Cross sections move dorsally from left to right, with A being most ventral and C most dorsal.

Acknowledgments and References:

Works Cited

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