Investigating the effect of cyp26 onset timing in *Danio rerio* tooth development

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Abstract

Zebrafish (*Danio rerio*) serve as model species that researchers observe to learn more about developmental pathways that are similar between *Danio* and humans. One such pathway is the development of teeth. The Jackman lab aims to determine what specific molecules, such as retinoic acid (RA), do during tooth development to understand exactly how teeth are formed in *Danio*, as well as why they form the way that they do. The knowledge of how the RA signaling pathway works in *Danio* can hopefully be eventually translated to humans due to their developmental similarities to *Danio*. In the long term, this research could help prevent human tooth morphogenesis issues such as Cleidocranial Dysplasia. This summer, I analyzed the effect of cyp26, an enzyme that degrades RA, on *Danio* tooth development. Two lines of *Danio* were selected for their differences in cyp26 onset timing and compared using antibody labeling, dissections, and fluorescence microscopy. The results of this experiment indicate a need to look earlier in *Danio* development to see exactly when cyp26 onset begins.

Project Objectives

In *Danio rerio*, RA is necessary in the development of teeth (Gibert, Y. et al.). According to preliminary data from the Jackman lab, *Danio* introduced to excess RA developed longer, thinner teeth than wild type *Danio*. Cyp26, an enzyme that degrades RA in normal *Danio* tooth development, is potentially a determining factor of *Danio* tooth shape by inhibiting RA to create the shorter, wider teeth seen in wild type *Danio.* The current hypothesis is that cyp26 helps regulate tooth shape by inhibiting RA, and thus that a later introduction of cyp26 would lead to longer, thinner teeth developing. To compare with wild type *Danio*, the lab looks at the mountain minnow *Tanichthys. Tanichthys* are a fish with a similar tooth development pattern to *Danio,* except that they begin expressing cyp26 later in development than *Danio. Tanichthys* also display significantly longer and thinner teeth than *Danio* (Gibert, Y. et al.), like the *Danio* exposed to excess RA. Later cyp26 expression in *Tanichthys* means that RA is uninhibited for a longer time in *Tanichthys* than in *Danio*, potentially indicating a link between the regulation of cyp26 expression and tooth shape. The genetic difference that controls this difference in cyp26 timing between species is unknown. By labeling the cyp26 enhancer, we are testing if the timing is controlled by the cyp26 enhancer identified by the lab in intron2.

Prior to my time in the lab, Professor Jackman created a stable line of transgenic *Danio* with a *Tanichthys* cyp26 enhancer attached to a GFP coding sequence. Additionally, a line of *Danio* was created with a GFP coding sequence attached to a *Danio* cyp26 enhancer. Both lines were created using CRISPR Cas-9. My objective this summer was to observe GFP expression in embryos from both lines to determine if there was a difference in expression between the *Danio/Tanichthys* (pBJ131) and the wildtype *Danio* (pBJ126). From there, I aimed to determine if the cyp26 onset timing (as shown by GFP) was different between the two types of fish.

Methodology Used

Embryo raising and care

In crosses were obtained from the pBJ131 fish and the pBJ126 fish and stored in an incubator at 28ºC. Embryos were cleaned and observed twice per day on days zero, one, and two post-fertilization. At 72 hours post fertilization (hpf), pBJ131 and pBJ126 embryos were selected for GFP expression viewed

under a Leica fluorescence microscope (Figure 1) and fixed using 4% formaldehyde 1x PBS solution with a small amount of 20% tween-20 added. An additional set of each embryo type was selected and fixed at 77hpf to compare GFP expression at different stages of development.

Embryo staining
The embryos were permeabilized using Ethanol and the Proteinase K enzyme and bleached using hydrogen peroxide. The stains added for observation under fluorescence were the GFP-HRP antibody, DAPI, and Alizarin red.

Dissecting, mounting, and imaging
Insect pins were used to clean excess tissue from the tooth area of the embryos prior to decapitating the embryos. The embryo heads were mounted onto microscope slides and then observed under fluorescence with an Apotome microscope. Stack videos were created with the different stains fluorescing under the light.

Image analysis
Composite images of the tooth region (Figure 2) were created from the Apotome images using FIJI. Images were discussed collaboratively with the lab to determine tooth development progress from the developed teeth and neighboring tooth germs. The images were labeled with their relative developmental stage.

Results Obtained
All embryos tested displayed some GFP expression in the cells of developing tooth germs at 72hpf and 77hpf. At 72hpf, both the *Tanichthys* embryos and the *Danio* embryos displayed similar GFP expression to each other (Figure 2). The 77hpf embryos displayed a similar phenomenon of about equal GFP expression in the tooth region no matter the line of fish (Figure 2). The brightness of the GFP expression varied slightly, with the *Tanichthys* generally having a brighter green hue than the *Danio*, regardless of the time of fixing. However, the location of GFP expression and area of GFP expression were about the same between both lines of fish.

Significance and Interpretation of Results
The presence of some GFP expression in all embryos at 72hpf and 77hpf indicates that cyp26 is active beginning at least at 72hpf in *Tanichthys* and *Danio*. However, because GFP was observed in similar locations to a similar degree between the *Tanichthys* and *Danio*, no conclusion can be drawn about the effect of cyp26 on tooth shape due to cyp26 having similar (if not identical) activity across both lines during the time periods studied. The next steps for this project will be looking at earlier developmental stages (younger than 72hpf) of both lines and comparing the GFP expression between them. According to in situ hybridizations completed for the grant proposal of this project, the main difference in RA expression between *Tanichthys* and *Danio* occurs between 50hpf and 56hpf. Experimentation and observation around these time periods could potentially illuminate when cyp26 initially activates in both lines, and if there is a difference in this timing. If a difference is found, then tooth shape could be further analyzed to determine if there is a link between cyp26 and tooth shape.

Another possible conclusion is that RA is not biologically responsible for determining tooth shape. While RA injections did show the creation of a longer, thinner tooth in preliminary trials, it is possible that this is not what RA naturally does within the organism. RA could affect tooth shape in the same way that another biological molecule does that has yet to be analyzed. If this is the case, then changes to cyp26

regulation and timing would have no correlation to tooth shape. However, more data is needed to determine if this is a viable theory.

Figures/Charts/Pictures

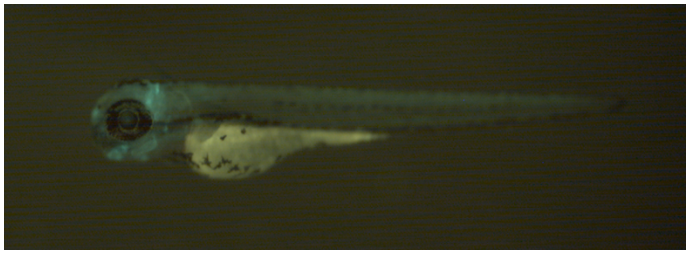

Figure 1. Preferred GFP expression for the *Tanichthys* embryos. Embryos with this GFP expression pattern were selected from the dishes, fixed, dissected, and imaged according to protocol.

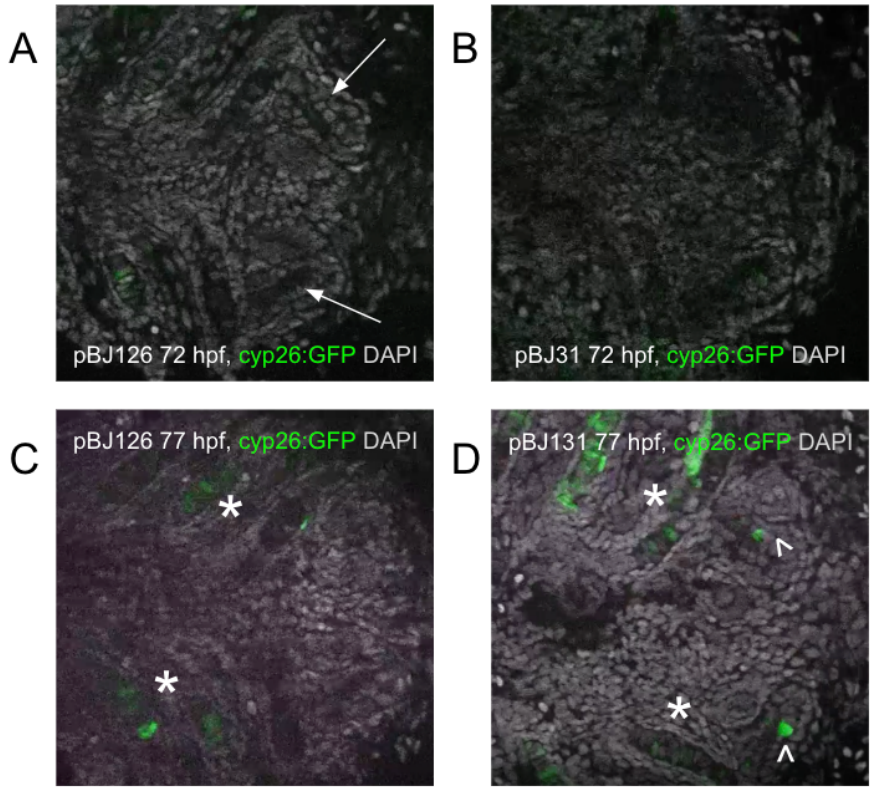

Figure 2. Sample Apotome images of the tooth region of stained, dissected embryos, imaged using fluorescence. Arrows in A indicate tooth location. Bright green indicates the activation of the cyp26 enhancer/GFP transgene present in all embryos studied. Grey indicates DAPI nuclear stain. A, B) *Danio* (A) and *Tanichthys* (B) embryos fixed at 72 hpf. A small amount of GFP expression is observed in both embryo images. C, D) *Danio* (C) and *Tanichthys* (D) embryos fixed at 77 hpf. Here, more GFP expression is seen in bones surrounding teeth (denoted by asterisks), as well as some expression in the tips of the *Tanichthys* teeth (denoted by carets). The GFP expression is brighter in the *Tanichthys* embryo, but still present in the *Danio* embryo.

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