Editing *twist2* via CRISPR/Cas9 to Determine its Role in Zebrafish Development

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Introduction

Embryonic Mesoderm is a precursor to various types of tissues, such as the somatic mesoderm, also known as somites. These somites are a segmented tissue that gives rise to the sclerotome, along with myotome in all vertebrates. This sclerotome further develops into the axial skeleton (Stickney, Barresi, and Devoto 2000) . Somites are easily observable under low power microscopy during fish development. In fish, great strides have been made in studying the developmental fate of somites (Stoiber, Haslett, and Sänger 1999; Stickney, Barresi, and Devoto 2000) . Zebrafish have been studied in many labs due to their similarities with higher vertebrates, while still maintaining a multitude of advantages over Mus musculus (Laboratory mice). These advantages include their inexpensive cost of care, external fertilization, high fecundity, embryonic transparency, and quick organ development (Nusslein-Volhard, Dahm 2002; Hoon 2009) .

Both vertebrate and invertebrate genomes contain a family of genes known as the Twist genes.These genes are essential for embryonic development and survival and have been shown to have expression in sclerotome development and formation (Germanguz et al. 2007) . Twist genes play an important role in embryonic research due to their ability as transcription factors to activate and repress other gene expressions. Much is still unknown about the role that Twist genes, specifically Twist2, plays in sclerotome development (Hoon 2009; Germanguz et al. 2007) . By utilizing CRISPR/Cas9 to edit an animal's genome, it has become possible to further study the role that these genes play during development (Ansai and Kinoshita 2014) . Genetic compensation has been shown to occur if a portion of a gene remains after editing, which complicates studies aiming to remove a gene's function (El-Brolosy and Stainier 2017) . By attempting to fully knock out Twist2, genetic compensation will hopefully be avoided, and further research on Twist2's impact on sclerotome development can occur.

Materials and Methods

1. Researched background of *twist2.*
2. Researched targeted sites for *twist2.*
3. Utilized CHOPCHOP to design and order gRNAs from Integrated DNA Technologies (IDT) that could cut out the entire gene (Labun et al. 2019).
4. gRNA Synthesis
   a. Generate gRNA scaffold
      i. Use a PCR reaction to generate scaffold.
      ii. Purify product with Promega's Wizard® PRC Clean Up Kit.
      iii. Run Gel to verify generation of correct product.
   b. Amplify gRNA scaffold
      i. Assemble second PCR reaction to amplify scaffold.
      ii. Purify product with Promega's Wizard® PRC Clean Up Kit.

iii. Use Beckmann Spectrophotometer to measure concentration.

c. *In vitro* transcription
   i. Setup transcription reaction.
   ii. Incubate reaction at 37C for 2 hours.
   iii. Remove DNA template with DNAse.
   iv. Stop Reaction with Ammonium Acetate.
   v. Purify Reaction with Isopropanol Precipitation.
   vi. Use gel electrophoresis to verify product.
   vii. Use Beckmann Spectrophotometer to measure product concentration.

Results

Twist2 Targeted gRNA Region

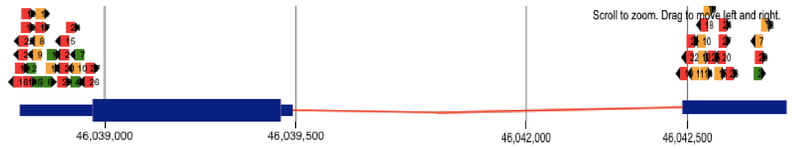

Figure 1. Using CHOPCHOP gRNA was designed on both ends of the gene in order to cut out the entire gene sequence. Figure 1 shows *twist 2* and the associated gRNA binding locations that CHOPCHOP suggested. Three guides were selected, 1 on the 5' side and 2 on the 3' side to target the entire region of *twist2.*

gRNA Synthesis PCR

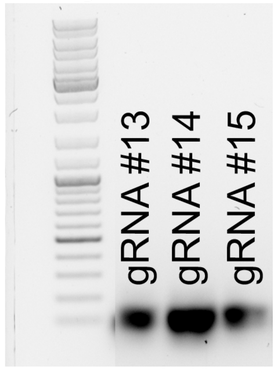

Figure 2. Results from the first PCR reaction. Results show that generation of the gRNA scaffold was successful for all three sequences. DNA ladder is the Thermo Gene Ruler (going from 100bp to 10,000bp).

gRNA Amplification PCR

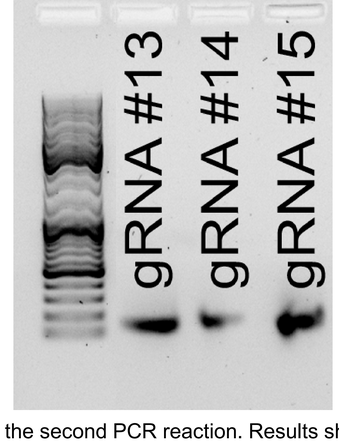

Figure 3. Results form the second PCR reaction. Results show that the transcription reaction was successful for all three sequences.

Twist2 Expression

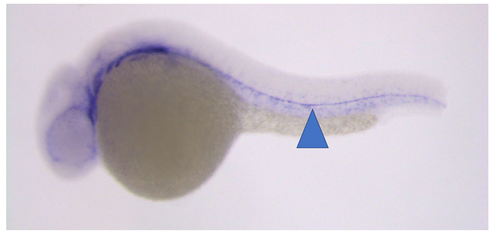

Figure 3. Expression of *twist2* in zebrafish, demonstrating its expression in the sclerotome, the ventral medial portion of the trunk at 22 hours post fertilization (hpf) (arrow).

Conclusions/Future Directions

Based on the PCRs from the synthesis and amplification reactions, our reactions were successful and the scaffold was successful for all sequences.

Moving forward, injection of the gRNA into zebrafish embryos is the next step. Doing so, twist2 could be knocked out and expression of the gene halted. Afterwards PCR could be done to confirm expression or non-expression of the gene in order to determine if the research was successful.

Acknowledgements

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