The Winthrop McNair Research Bulletin

Volume 3 *The Winthrop McNair Research Bulletin Volume 3*

Article 7

2017

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Targeted Mutagenesis of FOX0 Transcripts Using the Model Organism *Ciona intestinalis* Utilizing the CRISPR/Cas9 System

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ABSTRACT

The chordate, *Ciona intestinalis*, has become an excellent model organism for the study of cardiac development. *C. intestinalis* is a member of the phylum Tunicata, which is the sister phylum to vertebrates. Since both phyla share a common ancestor, an orthologous gene has been identified in *C. intestinalis* and vertebrates, the FOX0 transcript. Multiple reasons makes *Ciona intestinalis* an interesting model organism. For instance, ease of access to the animal, close relation to vertebrates, and mapping of the genetic code of *Ciona intestinalis* makes this an excellent model organism. The FOX0 family of transcripts plays an important role in cardiac, muscular, and neural development in humans. Humans have four copies of the transcript while *Ciona intestinalis* only has one. However, the function of FOX01 in *Ciona intestinalis* is unknown. This study aims to investigate the function of FOX0 transcripts in *C. intestinalis* by incorporating transgenic DNA into embryos using fertilization, dechorionation, electroporation and the CRISPR/Cas9 system.

INTRODUCTION

The development, modification, and utilization of the CRISPR/Cas9 system has revolutionized biomedical research. Clustered Interspaced Short Palindromic Repeats (CRISPR) refer to short segments of DNA that contain repeated base pair sequences, which are then followed by short segments of spacer DNA. Through the addition of short guide RNA (sgRNA) segments, Cas9 is guided to the target DNA (Stolfi et al., 2014). Through the utilization of this 'genomic editing tool', researchers can now 'cut' genes at a desired loci and 'paste' the desired gene in its place. This can allow for the development of new drugs, new vaccines, and more efficient methods of treatment for genetic diseases. This system also allows for the manipulation of genes to determine their functions. Here, we seek to use the CRISPR/Cas9 system to alter the function of a specific gene in *Ciona intestinalis* in order to determine the role of this gene during cardiac development. *C. intestinalis* is a sessile, marine organism. They filter water, food, and other nutrients through the buccal siphon and expel excess water and metabolic wastes through their atrial siphon. This filter feeder process has earned them the nickname 'sea squirts' because through the process of expelling water, it 'squirts' water out of their bodies. http://www.marlin.ac.uk/assets/images/marlin/species/web/o_cioint4.jpg

Figure 1: A picture of a *Ciona intestinalis*. A.) The buccal siphon, which is also called the incurrent siphon, draws water in the body. B.) The atrial siphon, which is also called the excurrent siphon, expels water from the body.

C. intestinalis have been used in developmental, molecular, and regenerative biological research for more than one hundred and twenty five years (Jeffery, 2014). There are numerous reasons why *C. intestinalis* make excellent model organisms for biological research. The first of which is its unique position on the phylogenetic tree (Cirino et al., 2002). Tunicates are the sister phyla of

Vertebrata. This allows for certain genes to be highly conserved throughout the evolution that both humans and *Ciona intestinalis* have in common. Such as, the FOX0 transcription factor that this study wants to investigate. Humans have four copies of the transcript and *Ciona intestinalis* has only one. This study aims to investigate the role of the FOX0 transcript in cardiac development and may gain insight into human cardiac development. The following figure illustrates the close proximity of vertebrates and tunicates.

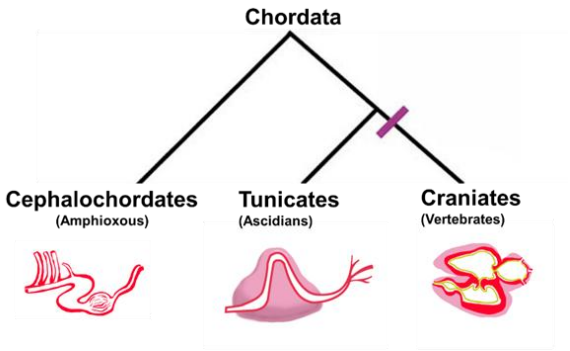

Figure 2: Phylogenetic position of Tunicates and Vertebrates.

The ease of access to *Ciona intestinalis* is another reason that makes these organisms so attractive as a model organism. *Ciona intestinalis* is relatively easy to obtain on both coasts of the United States. Also, they are readily available in most all marine environments and some estuaries. This ease of access makes them attractive to researchers because of their abundance for research projects. Furthermore, *Ciona intestinalis* may also be cultivated and maintained as colonies in a laboratory setting. *Ciona intestinalis* are transparent and only have three tissue layers. This makes dissection easy for researchers as well. Moreover, the entire genome of *C. intestinalis* has been sequenced and annotated.

Another compelling reason researchers are attracted to *C. intestinalis*, is due to the rapid development of fertilized embryos to mature adult organisms. The first cell division becomes visible approximately forty-five minutes post-fertilization. The prospect of controlling all *in vitro* conditions is appealing and almost essential for researchers. This control allows researchers the ability to reduce extraneous variables and ensure optimal growing conditions for the sensitive organism. All of these reasons combine to make *Ciona intestinalis* an attractive model organism for researchers.

The FOX0 transcription factor plays an important role in cardiac development. However, there has been very little investigation into the function of this gene. Through genetic breakthroughs, the entire genome of *Ciona* intestinalis has been mapped and orthologous gene identified in both vertebrates and chordates (See Appendix A). The research that has been completed on this gene shows that it plays an important role in cardiac, muscular, and neural development, but the exact function is not known. As stated earlier, *Ciona intestinalis* has only one copy of the transcript while humans have four copies of the gene. It is logical that humans have four copies of the gene because of FOX0's role in not only cardiac but muscular and neural development as well. *Ciona intestinalis* does not have a muscular system like humans nor do they have a brain or central nervous system. This investigation aims to define the function of the FOX0 transcript in cardiac development in *Ciona intestinalis*.

The processes of fertilization, dechorionation, electroporation and the CRISPR/Cas9 system will allow manipulation of the DNA of *Ciona intestinalis*. Fertilization will allow this study to have ample specimens to manipulate because some may not develop to the juvenile stage. Dechorionation will enable this study to manipulate the DNA of *Ciona intestinalis*. Electroporation will allow this study to put the desired DNA strand into the DNA of *Ciona intestinalis*. The CRISPR/Cas9 system will allow this study to isolate the FOX0 transcript and examine its function through the process of 'knocking out' the gene. After these steps are completed, then the development of *Ciona intestinalis* can then be monitored to see the role FOX0 plays in cardiac development of *Ciona intestinalis*.

MATERIALS & METHODS

CRISPR/Cas9 System

In order to investigate the function of FOX0 in *C. intestinalis* using CRISPR/Cas9, a

specific *C. intestinalis* FOX0 sequence must be identified. The protocol for designing and cloning a single guide RNA (sgRNA) expression vector for use in the CRISPR/Cas9 system was adapted from Stolfi et al, 2014. In order to design the sgRNA guide construct, a series of nineteen nucleotides, 5' to Protospacer Adjacent Motif (PAM) is identified. Due to the ease of use of the CRISPR systems, the vast amount of research using it, and the complete annotation of the genetic information of Ciona intestinalis, E CRISP is a tool that researchers can use to identify potential target sequences for their gene of choice. In order to make sure that the targeted gene has been specifically identified and to reduce the chance of non-specific 'off targets', sequences will be sorted using the Basic Local Alignment Search Tool or BLAST using (https://blast.ncbi.nlm.nih.gov). Once the target sequence has been located and isolated, then a proto-spacer nucleotide 'G' and an overhang sequence 'agat' will be inserted to the 5' terminus end using PCR; this will then become the top oligo. Another overhang sequence of 'aaac' will be inserted on the 3' terminus end of the reverse complement strand of the target sequence; this will become the bottom oligo that is necessary to anneal to the top oligo for cloning.

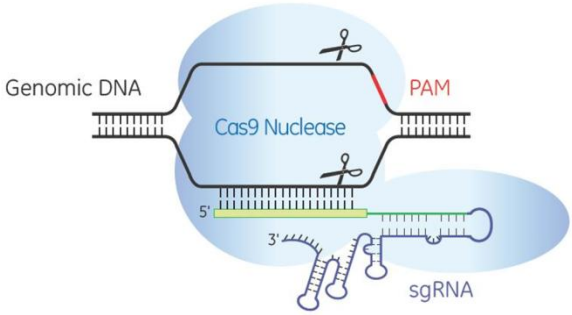

Figure 3: An illustration of the CRISPR/Cas9 process isolating and annealing the desired target sequence in place.

The target sequence will be incorporated into a vector that also includes a promoter sequence 5' to the inserted target sequence as well as a sgRNA(F+E) scaffold at the 3' end. The vector will be used for amplification of the target sequence to produce abundant plasmid DNA that can then be electroporated into *C. intestinalis* embryos that will express the exogenous DNA along with the CRISPR/Cas9 components. In order to get embryos that can be electroporated, two processes must occur: in vitro fertilization and dechorionation of fertilized eggs.

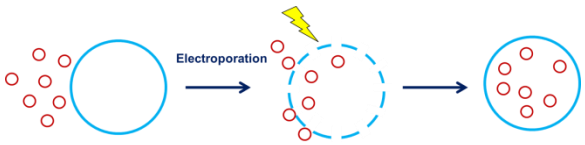

Figure 4: An illustration of the process of electroporation of a desired plasmid into vector DNA.

Ciona intestinalis Maintenance and Husbandry

C. intestinalis are sensitive organisms and pH, water temperature, salinity and overall water quality must be strictly maintained. Optimal pH is approximately seven. Water temperature must be maintained at 56°F and the optimal salinity is 3.5%. Strict attention to the overall quality of the water must be maintained after the biological load (i.e. a batch of *Ciona* intestinalis) was added to the aquarium. This was achieved using a water quality testing kit that tested for ammonia, nitrite, and nitrate levels. These levels must be maintained because these products of the natural biologic processes of *Ciona intestinalis* are harmful and toxic to the animals. Ammonia, nitrite, and nitrate levels were monitored using an API Salt Water Quality. Salinity was measured using a refractometer. Artificial seawater was made using Instant Ocean. The organisms were obtained from M-Rep in San Marcos, California.

Figure 3: A.) Protein filter that detoxifies the metabolic wastes from the biological load. B.) Chiller that moderates the temp of the water C.) *C. intestinalis*

organisms D.) Digital thermometer that constantly monitors water temp.

Ciona were received from M-REP every 2 weeks. Once received, they were allowed to acclimate to the aquarium for at least 24 hours prior to initiating any procedures. In addition, gamete production in *C. intestinalis* is affected by periods of darkness, a process called light induced spawning (Wantanbi et al., 1973). To alleviate this problem, a light is added to the tank to provide constant illumination in order to stimulate gamete production prior to in vitro fertilization.

In vitro Fertilization

Cross fertilization was achieved following the Cold Spring Harbor protocols (CSHP, 2009). The eggs and sperm of the organism were collected and placed in separate dishes. The eggs were collected in an egg basket that was then placed in the sperm dish that contained filtered artificial sea water, Tris 9.5 buffer, and sperm. The eggs and sperm were allowed to mix for one minute and thirty seconds and then sent through a series of washes to remove any excess sperm. Some of the fertilized eggs were then pipetted into a plate and their development was monitored. The rest of the fertilized eggs were then pipetted into a formaldehyde coated plate. The plate was prepared according to Cold Spring Harbor Protocols for coating dechorionation plates. This coating keeps bacterial growth to a minimum and also coats the plates for dechorionated eggs. Dechorionated eggs are extremely susceptible to rupturing if they come into contact with anything plastic. They will also attach to and grow if not properly placed. Then the eggs could proceed to the next step, dechorionation.

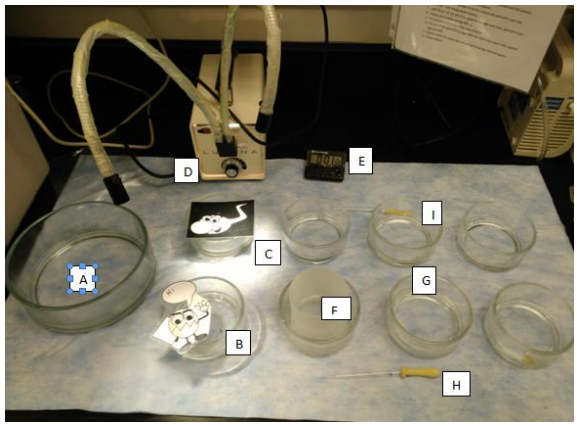

Figure 4: A.) Dissection bowl B.) Egg collection dish C.) Sperm collection dish D.) Moveable light E.) Timer F.) Egg collection filter G.) Six subsequent H.) Egg pipette I.) Sperm pipette

Dechorionation

Dechorionation was achieved following the Cold Spring Harbor Protocol (CSHP, 2009). The fertilized eggs were collected in a conical tube that had been altered to include a filter in the exposed end of the tube. This would allow for the fertilized eggs to come into contact with the dechoirionation solution and the ensuing washes that occurred. The eggs were then passed through the series of washes in formaldehyde coated plates. As stated earlier, the intention of the formaldehyde coating was to keep bacterial growth to a minimum. The plates were also coated with filtered artificial seawater and the dechorionation solution in an effort to keep the fertilized eggs submerged in water. The eggs were then sent through five subsequent washes to remove excess solution. Some of the eggs were then plated on a formaldehyde coated plate lined with FASW. It was then placed in an incubator held at 18°C and monitored for development. The other eggs were then sent through the next process, electroporation.

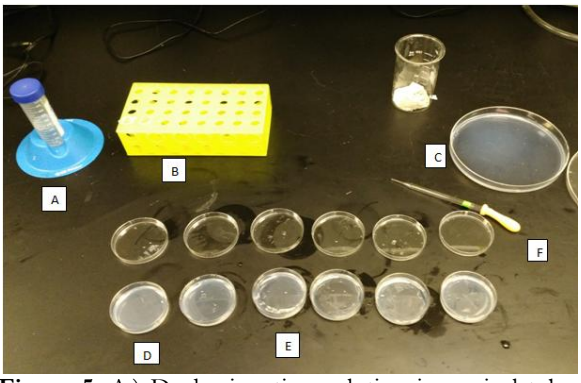

Figure 5: A.) Dechorionation solution in conical tube holder B.) Cuvette holder C.) 1X formaldehyde coated plate for collection D.) Egg collection dish E.) Subsequent washes F.) Glass pipette

Electroporation

Electroporation was achieved using the Cold Spring Harbor Protocol (CSHP, 2009). DNA constructs are then placed in a cuvette along with embryos, mannitol and topped off with FASW if necessary to ensure that all samples contained the same volume. The machine was turned on, the voltage was set at 0.05 kV, 1.00 μF X 1000, and samples were then placed in the Gene Pulser electroporator. The construct was then electroporated into fertilized embyros.

TRIzol® Reagent

Isolation of RNA was achieved using the TRIzol® Reagent protocol from Thermo-Scientific (THSP). RNA was extracted from specimens at different stages of development; larval, juvenile, and the first ascidian stage to detect when FOX0 is expressed in *C. intestinalis*. After adding phenol and choloroform to RNAse free tubes, the samples were then incubated on ice for ten minutes and then centrifuged. After, the aqueous supernatant that contains RNA was removed and placed in another RNAse free tube and isopropyl alcohol was added to the tube. The samples were then incubated for ten minutes and centrifuged for ten minutes. The pellet was then air-dried under the dryer hood for ten minutes and then the pellet was dissolved in RNAse free water and then incubated again for ten minutes. The results were then obtained using PCR.

RESULTS

CRISPR/Cas9

At the time this paper was written, the constructs were unavailable. However, the expected results from this investigation are that there will be altered cardiac development through the manipulation of the FOX0 transcript.

In vitro Fertilization

The results of *in vitro* fertilization were as expected. The embryos reached the 2-cell stage 45 minutes post-fertilization at 18°C (Figure 6) and then progressed to the larval stage by 12-18 hours post-fertilization (Figure 7). Larvae were then moved to uncoated plates to develop into juveniles. Juveniles were evident 3 days post-fertilization (Figure 8).

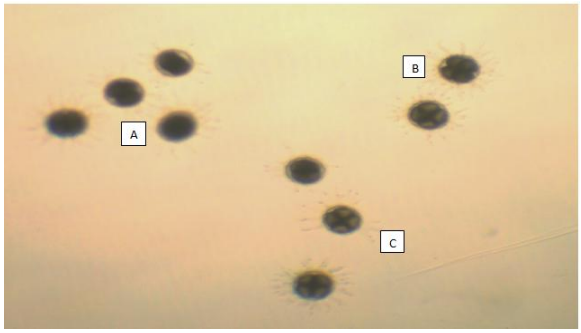

Figure 6: A.) 2 cell stage embryonic division B.) Chorion of fertilized egg C.) 4 cell stage embryonic division

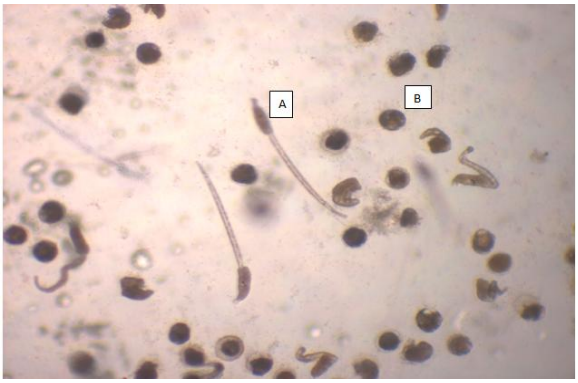

Figure 7: Larval stage of development of *C. intestinalis* A.) Lavae of *Ciona intestinalis*. B.) Undeveloped *Ciona intestinalis*.

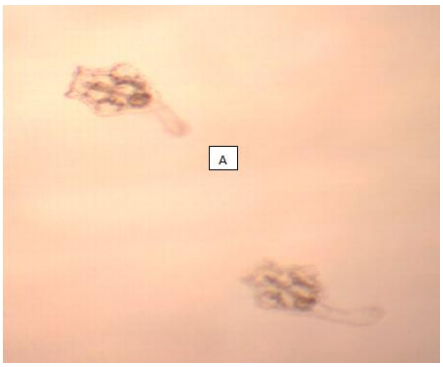

Figure 8: A.) 3 day old juvenile *C. intestinalis*

Dechorionation

During the process of fertilization, a chorion is formed to protect the fertilized egg. However, the chorion protects the fertilized egg so much so that it inhibits any manipulation to the fertilized egg. To circumvent this process, the chorion is removed during dechorionation. The fertilized eggs were moved to a container with a 10 micron filter to keep the fertilized eggs in the dechorionation solution. The solution was comprised of sodium thioglcolate and sodium hydroxide was placed in a series of petri dishes to perform a serial wash of the fertilized eggs to remove the chorion.

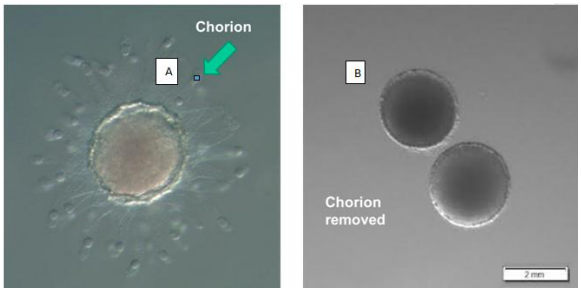

Figure 9: A.) A fertilized egg with chorion in tact. B.) A fertilized egg after dechorionation.

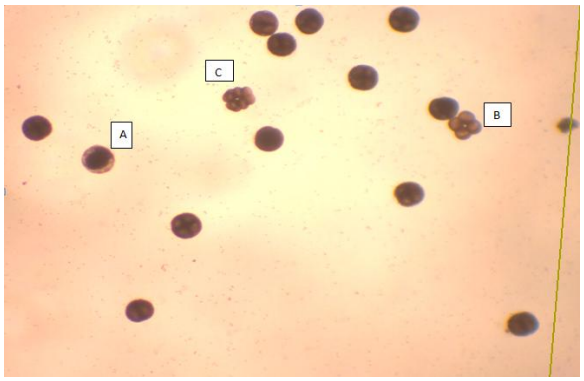

Figure 10: A.) Fragmented chorion of dechorionated eggs. B.) 4 cell stage dechorionated embryonic development. C.) Multi-cell stage dechorionated embryonic development

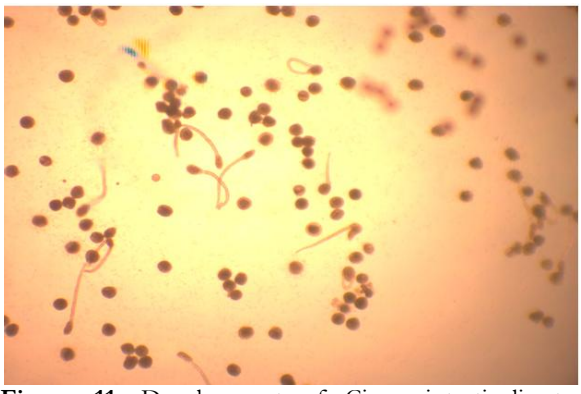

Figure 11: Development of Ciona intestinalis to juvenile.

FUTURE DIRECTION OF INVESTIGATION

Although this research was not completed in one investigational time period, there are still plans to continue this study. The aims of this study will be to identify the role of the FOX0 transcription factor in cardiac development of *Ciona intestinalis*. This study can give researchers keen insight into the not only the role that FOX0 plays in cardiac development in *Ciona intestinalis,* but also to perhaps gain insight into our own cardiac development. There are many directions that this investigation can take. All of them will be beneficial to our current understanding of cardiac development.

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APPENDIX A

APPENDIX

Appendix 1: pMiCiTnIG (PMIC) Construct Sequence

Gray: SV40ter

Green: GFP

Blue: NLS

Yellow: promoter

Pink: Minos ITRs

GGTACCAAGTGCTTGAAATGCTAAATGTTTTCAATTTTTCGCCATTAAGACAA
GCCTACACAAATGCTTCTATAAATTATGCCAAGCACGTTAGCAGCTTCTACGA
GCCCCAACCACTATTAATTCGAACAGCATGTTTTTTTGCAGTGCGCAATGTTT
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Appendix 2: pMiCiTniGCiprmG (PMIC cip) Construct Sequence

Gray: SV40ter

Green: GFP

Blue: NLS

Yellow: promoter

Pink: Minos ITRs

GGTACCAAGTGCTTGAAATGCTAAATGTTTTCAATTTTTCGCCATTAAGACAA
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