**Introduction**

About 610,000 people die of cardiovascular disease in the United States every year (CDC). It is the number one leading cause of death in the United States and is responsible for 17% of the United States national health costs (Heidenreich, et al). It is imperative to find better and more effective solutions to combat Cardiovascular disease in the United States to save lives and money. One possible solution to combat Cardiovascular disease is heart regeneration. Unlike humans, many lower vertebrates when injured, are able to regenerate limbs, appendages or internal organs. The Zebrafish is a perfect model organism to use for this study because they are one of the few organisms that possess the unique ability of cardiac regeneration and accel in it as well (Lepilina et al). Zebrafish hearts are able to fully regenerate following cardiac injury of which corresponds to a loss of approximately 20% of the total ventricular mass. In the low-pressure fish heart, this large wound is effectively sealed by an initial fibrin clot, which is gradually replaced by de novo regenerated heart tissue (Laflamme and Murry). Unfortunately, injury to the human heart does not regenerate but rather results in scarring (Laflamme and Murry). Conveniently, zebrafish are also transparent in the embryo stage which makes it very easy to study heart development or heart regeneration. The T-box family of transcription factors is a group of genes that play important roles in development of both vertebrate and invertebrate embryos (Smith). These T-box genes take part in controlling gastrulation, development of arms or legs, and the development of the heart. Mutations in these transcription factors are known to cause heart defects and can inhibit development (Horb et al). Due to the vast number of t-box genes, this study will focus only on the t-box gene, tbx5. Tbx5 has already been extensively studied and known to be expressed in the developing of the heart. However, this study, through the use of the Dominant Negative Tbx5, will determine if the transcription factor, Tbx5, is necessary for myocardial regeneration in response to surgical injury.

A Dominant negative mutation is a type of mutation that alters the protein so that it interferes with the function of the wild-type protein produced by the other allele (Veitia). For example, **figure 1** shows a normal Genotype vs a Dominant Negative Genotype. Within these Punnett squares, the **A** represents receptors, ligands, transcription factors, RNA or a protein that functions as a dimer. The **A’** represent the poisoned or mutated dimers. F**igure 1** illustrates that the Dominant Negative Genotype will produce only 25% of the desired dimer. This can produce damaged binding or signaling sites that greatly decrease the function of the receptors, ligands, transcription factors, or RNA. However, in the case of tbx5, the Dominant negative is interfering by competing with the wild-type protein for binding to DNA regulatory elements of target genes.

**Figure1.** Veitia RA., 2007

Tbx5 is directly involved in the initiation of vertebrate limb and heart development (Takeuchi, et al). The expression of the Tbx5DN before development of the heart began would kill the zebrafish (Gupta, et al). Before the Tbx5DN could be expressed, the zebrafish needed to survive till the adult stage which is about 60 days post fertilization (dfp). In order to do so, a process called Crelox Recombination that allows temporal control of when the Tbx5DN is expressed can be performed.

**Experiment**

The wild type zebrafish (cre) and the zebrafish embryos were purchased from the zebrafish international research center. The Dominant negative Tbx5 was created similarly if not identically to the Dominant negative gata4 that was created in Jiang et al (1999)’s experiment. The tbx5 Dominant Negative was constructed using standard cloning procedures by ligating a repressor domain of the *Drosophilia Engrailed* gene into the pSLAX plasmid. This was done by creating a transgenic line known as Tg(bactin2:loxP-mTagBFP-STOP-loxP-mCherry-2a-EnR-tbx5). Due to the low frequency of transgenesis when DNA alone is injected into the one-cell stage embryo, the transgene construct was designed with recognition sites for I-SceI and co-injected with this enzyme. I-SceI is a meganuclease that cuts both strands of the DNA at its recognition site, maintaining it in linear form and thus increasing the transgenesis frequency. After the dominant negative was constructed, injected into the embryos, and allowed to grow to adult stage, The cre zebrafish and transgenic line zebrafish were then bred together. The offspring were then separated into three categories: wildtype zebrafish (cre), lox-tbx5DN zebrafish, or a combination of both genetic traits. The wildtype zebrafish and lox-tbx5DN zebrafish served as the control. An attempt to induce the combined traits zebrafish to express the tbx5DN (Dominant negative tbx5) was performed through crelox recombination.

 Crelox Recombination is a process that functions at the transcriptional level. This process attaches the protein, Cre, to the loxP sites which then excises or removes the STOP sequence, allowing the Tbx5DN to be expressed. The cmlc2 or Cardiac Myosin Light Chain 2 is what gives spatial control and restricts Cre to the heart. However, this process can only be achieved through the distribution of the hormone, 4-hydroxytamoxifen. The ER in CreER is the hormone binding domain of the estrogen receptor, which causes the fusion protein (CreER) to be regulated in the same way as the native estrogen receptor. The CreER will be confined to the cytoplasm unless the 4-hydroxytamoxifen hormone

(4HT) is present. Because of this, temporal control of when Tbx5 is expressed was achieved. The fish were then able to grow 60 days, passing the juvenile stage. The 4HT hormone was then able to be injected into the water. This then induced the expression of Tbx5DN and allowed the experimentation on adult zebrafish heart regeneration without them dying before the experiment could be carried out.

**Figure3.**

After the dominant negative has been expressed, ventricular resection surgeries were conducted and the zebrafish were staged as described in Lepilina’s study. About 20% of the ventricular muscle was taken from the apex with iridectomy scissors (Lepilina, et al). The zebrafish were then euthanized by immersion in MS-222 (tricaine) at 2 g/L water. To prevent any decomposition of the organs, the heart was fixated by perfusion. This was done by perfusing the heart with phosphate buffered formalin for 24 hours. Before the tissue could be viewed under a slide, Cryosectioning was performed. The temperature of the tissue was first equilibrated to the temperature of the cryostat which was about -20°C. The tissue was then placed onto the cryostat specimen disk. Sections of about 5 µm thick were cut, placed on a Fisher Superfrost slide, and dried overnight at room temperature (RT). The slides were then fixed by immersion in cold acetone (-20°C) for 2 minutes, air dried at RT and

proceeded to be stained. Acid Fuchsin-Orange G staining and immunofluorescence was performed on 10-12 µm cryosections. This then enabled the ability to distinguish the difference between muscle and callogen. This is illustrated in **figure 5.** All animal procedures were performed in accordance with Virginia Commonwealth University guidelines.

**Discussion**

After the experiment, if all goes well, one of two things can happen. If the resected hearts scar instead of regenerate, this indicates that tbx5 is not is not necessary for myocardial regeneration. If the resected hearts regenerate sucessfully when expressing the tbx5DN, this indicates that tbx5 is not necessary for myocardial regeneration.

From Gupta experiment, we can assume that when the Dominant negative is expressed within the juvenile zebrafish, their hearts do not develop properly and causes the zebrafish survival rate to drop. **Figure 5** illustrates sections of the 30 days post amputation (dpa) ventricular wall. The top half of **figure 5** represents the control (non-induced zebrafish), whereas the bottom half represents an induced zebrafish that expresses tbx5DN. The Gupta paper researchers found that the control zebrafish had their ventricular walls intact after 30 dpa. Contrast to the control group, the induced fish developed scarring on the ventricular wall. If this result is present after the experiment has been conducted then it is safe to state that Tbx5, is necessary for heart regeneration in response to surgical injury. If this is not seen after the experimental procedure, then we know that tbx5 is only necessary for development.

**Figure5.** Gupta et al, 2013

A potential problem with this experiment could be the possible difference between expressing a dominant-negative tbx5 versus a conditional knockout of tbx5. This is probably the way this experiment would have been conducted in mice -- instead of using cre-lox to induce expression of a transgene one could use it to induce excision of the endogenous gene, if that gene is flanked by loxP sites. It's straightforward to modify the endogenous gene in this way in mice, but the methods for doing this in zebrafish and xenopus haven't been worked out yet. This is why people use the dominant negative approach in those particular organisms. Another potential problem is if the conditional knockout approach was conducted in zebrafish and a different result from the dominant negative approach emerged.  Which would be the "real" result?

**References**

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