Characterization of cardiac actin gene editing in zebrafish using CRISPR-Cas9 technology

by

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ABSTRACT

CHARACTERIZATION OF CARDIAC ACTIN GENE EDITING IN ZEBRAFISH USING CRISPR-CAS9 TECHNOLOGY

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University of Guelph, 2019

Advisor:
Dr. John Dawson

Cardiomyopathy is a common cause of heart failure. Two prevalent forms of cardiomyopathy are hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM), characterized by changes to the myocardium. The development of HCM and DCM has been associated with mutations found in genes encoding muscle proteins, including cardiac actin (ACTC1). My research focused on the development of zebrafish as an in vivo model to study the underlying molecular mechanisms leading to cardiomyopathy development. Three zfactc genes have been identified through literature and phylogenetic analysis: zfactc1a, cardiofunk (zfacta1b), and zfactc1c. To validate zfactc genes as cardiac-specific, I used CRISPR-Cas9 to characterize editing these genes in zebrafish embryos. Research about the role of cardiac actin in cardiomyopathy development using zebrafish is translatable to humans because zebrafish and human ACTC proteins share 99 % sequence identity. This work will determine the roles of zfactc genes in cardiac development and for future human ACTC rescue experiments.

Key words: CRISPR-Cas9, Cardiac actin, Zebrafish
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<th>Full Form</th>
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<tbody>
<tr>
<td>ACTC</td>
<td>Cardiac actin</td>
</tr>
<tr>
<td>ACTA1</td>
<td>Skeletal actin</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular valve</td>
</tr>
<tr>
<td>bp</td>
<td>Base-pairs</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR-associated protein 9</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilization</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-stranded break</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
</tr>
<tr>
<td>gRNA</td>
<td>Guide RNA</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>HDR</td>
<td>Homology-directed repair</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>Indel</td>
<td>Insertions and deletions</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>PAM</td>
<td>Protospacer adjacent motif</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sgRNA</td>
<td>Single guide RNA</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nucleases</td>
</tr>
<tr>
<td>YSL</td>
<td>Yolk syncytial layer</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc-finger nucleases</td>
</tr>
</tbody>
</table>
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Chapter 1 – Introduction

1.1 – Heart failure

Heart failure, characterized as a severe inability of the heart to function efficiently, is a growing epidemic in Canada (Dunlay and Roger, 2014). According to a recent report by the Heart and Stroke Foundation, 600,000 Canadians are living with this condition, and its rates are rising with an estimated 50,000 cases diagnosed per year (Mozaffarian et al., 2016). Almost half of all Canadians have been affected by heart failure, either by direct diagnosis or personal relationship. The lack of health promotion and continuous rise of heart failure has led approximately one-fifth of Canadians to misunderstand this condition as a normal process of aging (Mozaffarian et al., 2016). Heart failure is a burden to the Canadian economy costing more than $2.8 billion per year. Due to Canada’s aging population, our economic responsibility to treat heart failure patients significantly increases each year. Frequent and lengthy visits to the hospital result in reduced patient turnaround times, ultimately preventing optimal patient care.

Unfortunately, there is no cure for heart failure (Ponikowski et al., 2014). Depending on the severity of the symptoms and clinical indicators such as age, type of heart dysfunction and other comorbid conditions, approximately half of all patients do not survive more than five years of their diagnosis and the majority do not survive over a decade. Those living with damaged hearts suffer continuing challenges in their quality of life such as dyspnea, peripheral edema, and fatigue (Mozaffarian et al., 2016; Santos et al., 2009).
1.2 – Cardiomyopathy may lead to heart failure

The risk of developing heart failure is positively correlated to the time spent suffering a significant cardiovascular disease (Mozaffarian et al., 2016). Heart failure occurs from loss-of-function of cardiomyocytes, which may result from a pathological stressful stimulus, environmental stress or a gene response (Fuiju and Nagai, 2013). Heart failure is the result of all cardiovascular disease, such as myocardial infarction, hypertension, or congenital heart disease.

A common cause of heart failure is cardiomyopathy, characterized by changes to the heart muscle (myocardium) (van der Oost et al., 2014). Cardiomyopathy is a group of myocardial diseases that result in cardiac dysfunction. The myocardium is the histological layer in between the pericardium and endocardium. This layer is composed of cardiomyocytes, which carry a contractile function. In cardiomyopathy, the intrinsic ability of the myocardium to contract is reduced due to ventricular remodeling in response to the loss-of-function of cardiomyocytes, while others argue that increased contractility also leads to cardiomyopathy (Witjas-Paalberends et al., 2014). Consequentially, this defect leads to the inability of the heart to distribute adequate blood throughout the body leading to osmotic changes that cause lymphatic build-up, pedal edema and venous stasis (McNally et al., 2015).

1.3 Most prevalent types of cardiomyopathy - HCM and DCM

Two of the most prevalent types of cardiomyopathy are hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). HCM results from an enlargement of cardiomyocytes causing increased thickness of the myocardium (Ho, 2012).
This disease is the most common cause of sudden cardiac arrest. HCM can affect people of any age, including young athletes. DCM is characterized as emaciation of the heart muscle. DCM can affect people of any age and is the most common form of cardiomyopathy diagnosed in children (Ruppel et al., 2011; Fu and Eisen, 2018).

1.4 - Cardiomyopathy is linked to ACTC1 mutants

The development of HCM and DCM have been associated with mutations found in genes encoding muscle proteins (Sequeira et al., 2013). There are over 600 recognized mutations in genes encoding muscle proteins that are linked to the development of cardiomyopathy. These include the myosin heavy chain (MYH7) which accounts for a mutation frequency of 35% of all cases, the myosin binding protein C (MYBPC3) accounting for 30%, cardiac troponin T (TNNT2) accounting for 15%, tropomyosin α-1 chain (TPM1) accounting for 5%, and finally, cardiac actin (ACTC1) accounting for less than 2% of all cases. Cardiac actin is the muscle protein of focus.

Mutations in ACTC1 cause heart disease through distinctive molecular mechanisms (Dahari and Dawson, 2015). Cardiomyopathy development is associated with ACTC1 mutations, due to altered protein interactions at the actomyosin interface (Dahari and Dawson, 2015). To date, there are 16 known ACTC1 mutations in humans associated with cardiomyopathies, of which twelve are linked with HCM and the remaining four with DCM (Fatkin et al., 2010) (Figure 1a).
**Actin Isoforms**

<table>
<thead>
<tr>
<th><strong>α-striated muscle</strong></th>
<th><strong>Human</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha skeletal (ACTA)</td>
<td></td>
</tr>
<tr>
<td>Alpha cardiac (ACTC)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Smooth muscle</strong></th>
<th><strong>Human</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma smooth (ACTG2)</td>
<td></td>
</tr>
<tr>
<td>Alpha smooth (ACTA2)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Cytoplasmic</strong></th>
<th><strong>Human</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta cytoplasmic (ACTB)</td>
<td></td>
</tr>
<tr>
<td>Gamma cytoplasmic (ACTG1)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1: Actin.** A) 16 known ACTC mutations (yellow) linked with cardiomyopathies. Twelve associated with hypertrophic cardiomyopathy (labeled in black) and remaining four associated with dilated cardiomyopathy (labeled in red) on a ribbon model of G-actin monomer (blue). PDB: 1ATN. The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. B) Three major isoform types of actin (left column) are expressed by six actin genes in humans (right column).
1.5 - Why cardiac actin (ACTC1)?

A low frequency of mutations is preferred to study because it is more practical to assess the underlying molecular alterations leading to the development of cardiomyopathy. The frequency of mutations is correlated to the size of the gene and functional specificity of the protein (Dominguez and Holmes, 2011). Actin is small relative to other sarcomere proteins; specifically, 375 amino acids following posttranslational processing (Ennomani et al., 2016). Actin is multifaceted with an assembly of critical binding partners, participating in more protein-protein interactions than any known protein (Kristó et al., 2016). As a result, actin exhibits high selective pressure to conserve its sequence and so cannot endure many mutations. A single mutation in cardiac actin (ACTC1) may represent diverse pathways toward disease development (Ennomani et al., 2016; Khodyuchenko et al., 2015).

1.5.1 Three major isoform types of actin

Actin, weighing 42 kDa, is the most abundant protein in most eukaryotic cells (Kristó et al., 2016). The α-cardiac protein belongs to the actin family, which is comprised of three isoform classes: smooth muscle, α-striated muscle, and cytoplasmic actin.

In humans, there are six primary actin isoforms, α-skeletal (ACTA) and α-cardiac (ACTC1) belonging to the α-striated class, γ-smooth (ACTG2) and α-smooth (ACTA2) belonging to the smooth muscle class, and lastly, β-cytoplasmic (ACTB) and γ-cytoplasmic (ACTGI) belonging to the cytoplasmic actin class (Perrin and Ervasti, 2010) (Figure 1b).
A key property of actin is its ability to self-assemble into filaments called filamentous actin (F-actin) (Dawson et al., 2003; Morrison et al., 2010). This property is critical for life because these filaments are responsible for providing structural support for eukaryotic cells by forming a network called the cytoskeleton (Morrison et al., 2010; Roskoski, 2011). Actin interacts with myosin, a muscle motor protein, to form the contractile filaments of all muscle cells. Movement is generated by providing muscles with “tracks” for motor proteins to move along, such as the pumping of the heart (Greig and Jones, 2016).

1.6- Research Question and Gap

The anatomical pathology and histological understanding of cardiomyopathies is largely determined, but despite the critical importance of muscle and cytoskeleton for life, the underlying molecular basis for how changes in cardiac actin lead to the development of cardiomyopathy remains insufficient (Masters et al., 2016). One approach is to evaluate how the structure of actin proteins dictates function through a progression of biological complexity from the properties of ACTC proteins themselves to how their interactions change with other muscle proteins and finally the impact on the whole organism. My focus is to establish zebrafish as an *in vivo* model to connect the analysis of the biochemistry of ACTC changes at the molecular level to the development of cardiomyopathy in the whole organism. Generating a model for cardiovascular disease will allow scientists to target the associated dysfunctions with precision therapies leading to the betterment of patients and reducing the global burden of heart failure.
1.7 - Zebrafish as an *in vivo* model for human cardiomyopathy

The heart is the first organ to develop and function during zebrafish development, akin to humans (Bakkers, 2011). Due to their accelerated cardiovascular development, zebrafish exhibit a measurable heart beat at 24 hours post-fertilization (hpf) (Bakkers, 2011). During the first five days post-fertilization (dpf), the embryonic heart develops a functional cardiovascular system and adult configuration (Denvir et al., 2008).

1.7.1 Advantages

There are multiple advantages associated with working with zebrafish. This eukaryotic model is small in size, growing up to 4-6 cm in length; thus, the Hagen Aqua Lab facility at the University of Guelph can support thousands in a small space (Verkerk and Remme, 2012). They exhibit rapid development, reaching sexual maturity at 3 to 4 months; however, this period can be shortened to 2 months under ideal growth conditions (Kimmel and Meyer, 2016; Rocke et al., 2009). Adult zebrafish breed readily and yield a large number of embryos, specifically one female can produce approximately 100–200 embryos per week (Kimmel and Meyer, 2016; Nasiadka and Clark, 2012). They are transparent; the heart measuring approximately 1 mm in diameter can be visualized under standard light microscopy allowing for non-invasive analysis of development (Rocke et al., 2009). Importantly, zebrafish are ideal organisms to study gene function during cardiovascular development as they do not rely on a functional heart to pump oxygen throughout the blood for the first few days of development, by way of their tissues receiving oxygen through passive diffusion from the surrounding water. Due to this passive diffusion, zebrafish can live without a beating heart during the first few days of development (Rocke et al., 2009).
1.7.2 A valid, translatable model

71.4% of human protein-coding genes have minimally one homologous zebrafish gene, of which 82% are associated with genes related to human disease (Shih et al., 2015). Research about the role of cardiac actin in cardiomyopathy development using zebrafish is translatable to humans because zebrafish and human ACTC proteins share an incredible 99% sequence identity (Bakkers, 2011; Bertola et al., 2008).

As for differences in the genome, zebrafish have a partly duplicated genome, often possessing two copies of a gene that is present as a single copy in mammals (Hoshijima et al., 2016). For example, humans have a single ACTC gene in their genetic makeup, whereas zebrafish have more than one actin gene expressed in the heart. Due to this duplication, genetic redundancy is often observed in the zebrafish genome (Hoshijima et al., 2016).

Human and zebrafish hearts have fundamental structural similarities as they are both divided into atrial and ventricular chambers separated by cardiac valves and lined with an endocardium. Also, the heart is the first organ to develop in both organisms. However, the zebrafish heart does not undergo septation; the human heart has four chambers whereas the zebrafish heart only two. The zebrafish heart consists of two chambers attached to a sinus venosus and bulbus arteriosus which are separated by valves (Denvir et al., 2008). Zebrafish lack septation; however, they do not require a pulmonary system for oxygenation as they have gills (Santoriello and Zon, 2012). Despite this structural difference, the function of the heart remains the same.

Zebrafish and human cardiovascular structural and functional parameters scale with body size; specifically, zebrafish body size development positively correlates with heart growth (Dewey
et al., 2008). The average heart weight is \(1.37 \pm 0.59\) mg, up to 2 \% of the total adult zebrafish body mass (Vargas and Vásquez, 2016). The average heart weight of a human is approximately 310 grams, up to 0.3 \% of the total adult body mass for an average adult weighing approximately 80 kg (Zeek, 1942). Substantial evidence suggests cardiac dimensions and functional variables scale with body size within and across species due to an added cardiac demand (Dewey et al., 2008). Functional variables include metabolic rate (Dewey et al., 2008). Heart rate is correlated with the metabolic rate, specifically the rate of oxygen consumption and consequently the rate of energy output (Green, 2011). Therefore, it is critical to consider body size measurements in the assessment of cardiac structure and function during diagnosis and treatment of cardiomyopathy conditions in patients (Dewey et al., 2008).

1.7.3 Zebrafish embryo development

Spermatozoa enter the egg cytoplasm through a small opening called the micropyle. A single spermatid fertilizes the egg leading to the formation of the zygote. Embryo development occurs within 10 minutes of fertilization. Fertilization occurs through several periods (Table 1) (Kimmel et al., 1995).

1.7.4 Zebrafish heart development

Specification of myocardial and endocardial progenitor cells begins at 50 \% epiboly (5.5 hpf), marking the first cardiac development event in zebrafish. Before gastrulation, atrial and ventricular pools of myocardial progenitor cells are positioned bilaterally in the lateral marginal zone; the ventricle progenitor cells are located dorsally in the lateral marginal zone compared to the atrial progenitor cells. Distribution of atrial and ventricular pools of endocardial progenitor
cells throughout the marginal zone do not exhibit organization. During gastrulation, the cardiac progenitor cells migrate from the epiblast towards the anterior lateral plate mesoderm (ALPM). Assembly of the myocardial plate occurs at 8-10 somites (approximately 13 hpf). Cardiac differentiation is initiated in the imminent ventricle myocardial cells at 12-15 somites (approximately 16 hpf), followed by cardiac differentiation of imminent atrial myocardial cells. The endocardial and myocardial cells migrate from the ALPM towards the mid-line.
Table 1: Developmental stages of zebrafish embryos to larvae (Kimmel et al., 1995).

<table>
<thead>
<tr>
<th>Fertilization period</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygote Period (0-0.75 hpf)</td>
<td>The cytoplasm separates from the yolk toward the animal pole forming the blastodisc. The blastodisc divides from one cell to two cells</td>
</tr>
<tr>
<td>Cleavage Period (0.75-2.25 hpf)</td>
<td>The blastomeres divide quickly and synchronously in intervals of approximately 15 minutes. Generation of 64 cells from two cells occurs</td>
</tr>
<tr>
<td>Blastula Period (2.25-5.25 hpf)</td>
<td>The epiboly begins which is the dispersion of the Yoke Syncytial Layer (YSL) and the blastodisc over the yolk cell. Development from 128 cells to 50 % epiboly occurs</td>
</tr>
<tr>
<td>Gastrula Period (5.25-10.33 hpf)</td>
<td>Epiboly occurs and tail bud completely form. The primary germ layers are formed and positioned appropriately. The Shield Stage occurs which marks the dorsal side of the embryo. Development from 50 % epiboly to 1-4 somites occurs</td>
</tr>
<tr>
<td>Segmentation Period (10.33-24.0 hpf)</td>
<td>The dermis, skeletal and vertebrae muscle are developed. The embryo begins to move and elongation of the tail bud occurs, as well as the initial primary organ development. Development from 1-4 somites to 26 somites occurs.</td>
</tr>
<tr>
<td>Pharyngula Period (24.0-48.0 hpf)</td>
<td>The circulatory system develops marked by the first heart beat and blood circulation. The development of the notochord, Dorsal and Ventral Stripe, brain, and tactile sensitivity occur. Development from Prim 5 to Long-pec occurs.</td>
</tr>
<tr>
<td>Hatching Period (48.0 - 72.0 hpf)</td>
<td>The primary organ morphogenesis is completed and cartilage growth in the pectoral fin and development commences. The mouth becomes distinct and protrudes anteriorly in close proximity to the eye followed by asynchronous hatching. Development from Long-pec to Protruding-mouth occurs. Then development from Protruding-mouth to Day 30-44 occurs.</td>
</tr>
<tr>
<td>Larval Period (72.0 hpf- 30.0 dpf)</td>
<td>The swim bladder undergoes inflation, the internal organs become compound, and the larvae exhibit active avoidance and food-seeking manners</td>
</tr>
</tbody>
</table>
A cardiac disc structure is formed from fusion of the bilateral heart fields at the mid-line. The cardiac disc structure is composed of atrial myocardial cells at the periphery and ventricular myocardial cells at the circumference of the disc, with the endocardial cells located at the centre of the disc. Single, linear heart tube generation from the cardiac disc is initiated at 20 somites (approximately 19 hpf) endocardial cells. Then cardiac differentiation occurs; specifically, the arterial pole is located at the mid-line and the venous pole is positioned at the anterior left pole. Differentiation continues at the arterial pole. Tubular heart contraction is initiated at 26 somites (22 hpf). Cardiac looping formation is initiated at 33 hpf. Constriction at the AV canal is first observable at 36 hpf. At 48 hpf, endocardial fusion formation occurs. Cardiac looping continues into an S-shaped loop, followed by the formation of the epicardial layer from ellipsoid extracardiac pro-epicardial cells. The inner curvature of the atrium encompasses the pacemaker (de Pater et al., 2009; Glickman and Yelon, 2002; Poon and Brand, 2013).

1.8 - Isoform assignment in zebrafish

Zebrafish have a partially duplicated genome exhibiting multiple actc genes (referred to as zfactc genes here) and it is unclear which gene(s) are specific to the heart, whereas humans have only one cardiac actin gene. Direct assignment of cardiac actin isoforms in zebrafish has not been clear due to the high level of protein sequence conservation of actin sequences, producing mis-assignments during machine annotation of zebrafish actin gene names and expected function. To clarify the zebrafish cardiac actin isoforms, we conducted sequence analysis to determine which genes are the most likely candidates ACTC homologues. The human isoforms were compared with nine actin genes in zebrafish (Ojehomon et al., 2018) (Figure 2).
Figure 2: Phylogenetic tree of isoform assignment in zebrafish. The different types of actin have been presented in clades: red, α-striated actin genes; blue, smooth muscle genes; green, cytoplasmic actin genes. The human isoforms (red) are compared with nine actin genes in zebrafish (black). The genes that I will focus on are acta1b (cfk-13) and actc1c (Ojehomon et al., 2018).
Based on sequence similarity and the sparse literature focused on the functional analysis of these genes, the α-skeletal actin-1b (acta1b), otherwise known as cardiofunk (cfk) located on chromosome 13, and duplicate genes on chromosome 19 and 20, both called α-cardiac actin-1a (actc1a), are believed to be zebrafish α-cardiac actin genes. An additional actin sequence was found on chromosome 20, although at a different position. This previously undesignated zebrafish gene was assigned as α-cardiac actin-1c (actc1c) (Ojehomon et al., 2018).

Phylogenetic analysis of the nine zebrafish actin genes and human actin isoforms revealed that the actc1c gene product is most similar to the human α-cardiac actin sequence (Figure 2). This result is due to the non-N-terminal amino acid sequence alignment between the actc1c zebrafish α-actin and the α-cardiac actin, without inclusion of the common DDDE N-terminal motif in zebrafish actin sequences (Ojehomon et al., 2018).

Shih et al. (2015) characterized zebrafish homologues of human DCM associated genes, prioritizing those expressed with highest abundance in the heart. Of the zebrafish genes related to ACTC, actc1a was the most abundant homologue in both adult and embryonic hearts, contributing 74.58 % of the total actin gene transcripts, followed by acta1b at 20.22 %. In order of magnitude for expression in the heart, acta1b and actc1a are enriched compared to tail muscle, with a ratio of over 100-fold (248.67 % and 178.41 %, respectively) (Shih et al., 2015).

The actc1a and acta1b gene products are vital components for early zebrafish heart development. A common trend among mutants of these two isoforms is F-actin instability, providing insight into the role of actin in muscle development. Further investigation of disease
pathogenesis related to actin gene mutations is necessary for the development of novel treatments for cardiomyopathies (Sztal et al., 2015).

1.8.1 actc1a-19/20

Expression of the zebrafish actc1a gene is limited to the heart and somatic muscle (Glenn et al., 2012). However, actc1a does not demonstrate a major role in somatic trunk muscle development; thus, co-expression of other isoforms, including acta1a and acta1b, may occur in the somatic muscle (Glenn et al., 2012). Actc1a is the major actin isoform in both developing and adult hearts (Shih et al., 2015).

To understand the role of actc1a in heart structure and function, Glenn et al. (2012) targeted a point mutation in the zebrafish actc1a gene, the s434 mutation, which encodes a Y169S substitution mutation in the W-loop region of actin. The actc1a-s434 mutant alters hemodynamics within the heart, causes blood regurgitation at the atrioventricular canal (AVC), and a deficiency in the formation of endocardial cushion development from altered cardiac output, ultimately leading to pericardial edema and a thinner heart wall than that of wild-type (WT) embryos (Glenn et al., 2012).

Production of the F169S actin variant in yeast cultures indicates that actc1a-s434 mutants may also be associated with weak actin filaments, expressing the inability of the mutant actin monomer to reanneal after depolymerization. Phalloidin, an F-actin stabilizer, reverses these polymerization defects to a limited extent (Glenn et al., 2012).
1.8.2 acta1b/cfk

Endocardial cushion (EC) formation is a crucial step for ensuring valve morphogenesis; however; the interconnection between hemodynamics, cardiac function, and EC development remains unclear. In contrast to a previous hypothesis suggesting that shear stress on endocardial cells is a requirement for EC development, Bartman et al. (2004) show that EC development defects are primarily a result of myocardial dysfunction. Researchers utilized whole mount in situ hybridization (WISH) analysis to demonstrate expression of *acta1b* in myocardial cells.

An additional study broadened these findings by focusing on the cardiofunk mutation of *acta1b* which exhibits defects in EC development and regurgitant blood flow, demonstrating partial functional redundancy between *acte1a* and *acta1b* in the zebrafish heart (Glenn et al., 2012). This redundancy is supported by a WISH analysis of *cfk* expression during embryogenesis, indicating *cfk* expression in cardiac cells during the period in which *acte1a*-s434 mutants exhibit EC defects. Also, WISH analysis of embryos heterozygous for both *acte1a*-s434 and *cfk/acta1b* express comparable EC development defects. These results suggest that wildtype actin can compensate for the mutant actin genes, explaining why zebrafish heterozygous for both WT and mutant actin express partial functionality for a prolonged time (Glenn et al., 2012).

1.8.3 Zebrafish as a model for α-skeletal actin changes.

Sztal et al. (2015) focused on ACTA1 nemaline myopathy in humans to study the underlying mechanisms leading to skeletal muscle weakness through investigation of nemaline bodies in transgenic fish. Overexpression of ACTA1 through mRNA injection leads to cytoplasmic
actin aggregates causing functional impairment in skeletal muscle and actinin negative nemaline bodies near the myosepta.

Conversely, actin loss-of-function is associated with nemaline bodies within the myofibril and thickening of the Z-disk (Sztal et al., 2015). Although actin contributes to muscle function impairment through formation of cytoplasmic aggregates and nemaline bodies, actin itself is not the causal factor because ACTA1 knockout leads to the disease. Actc1a mutants of the actomyosin interface also lead to sarcomere impairments through reduced binding strength (Sztal et al., 2015). Overall, pathogenic consequences arise from actin imbalances in the skeletal muscle. (Sztal et al., 2015).

1.9 - CRISPR-Cas9 system to knockout zfactc genes

Knockouts of zebrafish cardiac actin genes will be generated using CRISPR-Cas9 technology. Development of the clustered, regularly interspaced, short palindromic repeat (CRISPR) technology has advanced targeted genome editing through engineered nucleases. This economical genetic tool involves many advantages including applicability to a wide array of cell types, accuracy, predictability, and reproducibility (Gagnon et al., 2014; Sander and Joung, 2014).

The CRISPR-Cas system utilized in this research is the original version that is well established and efficient. This version is composed of Cas9, a single polypeptide nuclease that cleaves the DNA, and a single guide RNA (gRNA) that guides the Cas9 nuclease to the target site in the genome. Double-strand cleavage occurs near a protospacer adjacent motif (PAM) sequence, often one to five nucleotides downstream of the target (Sander and Joung, 2014). The cell responds by imperfectly repairing the break, producing indels at the target site. If that target is a gene, the
indels result in the knockout of the gene. The CRISPR-Cas9 system is rapidly progressing in the study of cardiomyopathies as the number of loci targeted in zebrafish increase. This system can be utilized in zebrafish models for site-directed mutagenesis with detection of mutants by next-generation sequencing (Hwang et al., 2013).

All CRISPR-Cas systems are DNA-encoded, RNA-mediated, and mostly nucleic acid targeting. These systems vary in their mechanistic characteristics; for example, the sequence and structure of the guides can vary from single guides to dual guides or even single guides with multiple hairpins (Cong et al., 2013; Makarova et al., 2011; van der Oost et al., 2014). Recently, catalytically inactive Cas9 (dCas9) was used to bind target DNA sequences, creating a DNA:RNA R-loop that inhibits DNA polymerase and turns off transcription of the target (Agne et al., 2014; Ma et al., 2016). The transcriptome can also be modified by tethering dcas9 to an activator to turn on transcription locally (Balboa et al., 2015). Future directions include modifying Cas9 enzymes to recognize a broader spectrum of PAM sequences, thereby increasing potential target sites for genome modifications (Sander and Joung, 2014).

Earlier methods for genome targeting sites relied on protein-based systems, such as transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs), which are artificial fusion proteins (Sander and Joung, 2014). Although TALENs and ZFNs have aided in reverse genetics, these methods are not widely available, nor are they as predictable as the Cas9 system. The Cas9 system is superior as there is no need to modify the Cas9 enzyme, since directed genome mutagenesis can occur by merely replacing the sgRNA. This advantage has led to
numerous publications exhibiting Cas9 flexibility and precision for site-directed mutagenesis (Gagnon et al., 2014).

In addition to producing knockouts, newer CRISPR-Cas9 systems can be utilized in zebrafish models for site-directed mutagenesis with detection of mutants by next-generation sequencing. The CRISPR-Cas9 system can be used to assess allele diversity (Sander and Joung, 2014). Single-guide RNAs (sgRNAs) can vary in activity across orders of magnitude. Controlling the rate of mutagenesis activity enables scientists to produce specific outcomes, such as recognized null alleles inherited through the germline (Gagnon et al., 2014).

1.10 - Research objectives

1.10.1 Hypothesis and previous research

My hypothesis is that cfk-13 and actc1c are cardiac actin genes which undergo a developmental switch during early zebrafish development. Previous research from the Dawson Lab suggests that there is a genetic switch in zebrafish development between cfk-13 and actc1a at approximately 3 dpf with cfk-13 being more highly expressed during the first 3 dpf, after which actc1a becomes the dominant isoform expressed in the heart (Table 2).
Table 2: Expected results. Expected results for single gene knockout and triple knockout based on preliminary results.

<table>
<thead>
<tr>
<th></th>
<th>cfk-13</th>
<th>actc1a-19/20</th>
<th>actc1c</th>
<th>cfk-13 + actc1c + actc1a-19/20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before 3 dpf</strong></td>
<td>Decreased cardiac functionality</td>
<td>Close to normal cardiac function</td>
<td>Decreased cardiac functionality</td>
<td>Decreased cardiac functionality</td>
</tr>
<tr>
<td><strong>Post 3 dpf</strong></td>
<td>Close to normal cardiac functionality</td>
<td>Decreased cardiac functionality</td>
<td>Close to normal cardiac function</td>
<td>Decreased cardiac functionality</td>
</tr>
</tbody>
</table>

Based on these preliminary results, a cfk-13 knockout should result in reduced cardiac functionality during the first 3 dpf, while after 3 dpf cardiac functionality should improve. Conversely, an actc1a knockout should not significantly impact cardiac function in the first 3 dpf. However, after 3 dpf, deterioration of cardiac function is expected. Finally, a double knockout of both the cfk-13 and actc1a genes should result in reduced cardiac function for the entire experimental period, as both isoforms are no longer expressed in the heart. actc1c gene is previously undesigned and my work is the first functional analysis of this gene. In situ hybridization experiments performed by our lab shows that actc1c is expressed at 2 dpf (Ojehomon et al., 2018). Based on these results, an actc1c knockout should result in reduced cardiac functionality on 2 dpf, while after 2 dpf cardiac functionality should improve.

1.10.2 Research Proposal

The design of my research is divided into three stages. Each stage includes mock injected embryos from the same brood as the negative, internal control. The first and second stage involves creating knockouts of the individual cardiac actin gene (ACTC) isoforms, cfk-13 or actc1c. The
third stage involves a double knockout in which both *cfk-13* and *actc1c* are targeted together. I completed the first and second stage of my research plan.

The experiment begins with microinjections into zebrafish embryos. The sgRNA specific to the cardiac actin genes, *cfk-13* or *actc1c*, was premixed with the Cas9 protein and subsequently injected into the zebrafish embryo at the one-cell stage. Each day after injection, cardiac phenotype characterization was performed to assess rate of development, heart rate, viability, blood circulation, blood accumulation, and the presence of pericardial edema (Figure 4c). Characterization was carried out for seven days.

To study the role of cardiac actin in the development of human cardiomyopathies in zebrafish, our long-term strategy is to conduct a rescue experiment in which we knock out the zebrafish *actc* genes and replace them with human cardiac actin genes, followed by phenotype characterization (Figure 3). If human ACTC can fully or partially rescue zebrafish *actc* knockouts, then we can use this system to examine the impact of expressing mutant human ACTC on zebrafish development and physiology from the molecular to the organismal level.
Figure 3: Flowchart for Cas9-mediated mutagenesis.
1.10.3 Characterization of zfactc CRISPRs

The criteria for an established model include characterization of cardiac phenotypes at the embryo stage of development, including viability and the rate of overall development, heart rate, blood circulation, hemostasis, and cardiac morphology including the presence of pericardial edema (Figure 4c). Daily cardiac phenotype characterization was performed for seven days following injection, allowing me to track changes in zebrafish developmental milestones. These milestones were assessed through direct observation through a microscope with reference to the defined periods of embryogenesis (Kimmel et al., 1995).

1.11 - Summary and Significance

My objective is to establish zebrafish as a model system to study changes in ACTC. My approach is to knockout putative zebrafish actc genes using CRISPR-Cas9 technology. My work is part of a larger plan to establish an in vivo model for studying cardiac actin variants found in patients with cardiomyopathies. Using the CRISPR-Cas9 approach, I will clarify which zebrafish actin genes are functioning in the heart up to 7 dpf. My work will also help us characterize a potential developmental switch in actin genes during early zebrafish development. Finally, my work will be the first to examine the loss of function of the novel actc1c gene in zebrafish, determining the impact of its knockout.

Three zfactc genes have been identified through literature and phylogenetic analysis: zfactc1a, cardiofunk (zfactalb), and zfactc1c. I will focus on cardiofunk (zfactalb), and zfactc1c. Due to availability of embryos and time constraints, I was unable to study zfactc1a.
My work will be the crucial first step in determining if human *ACTC* can rescue zebrafish *actc* knockouts, thereby providing a model to study changes in the *ACTC* gene related to heart disease. A model provides a tool to dissect the molecular mechanisms of disease development as well as a tool for high-throughput screening for new therapeutics that aid in correcting the specific molecular dysfunction. Precision therapies will protect patients from harmful side effects and reduce the rising cost of heart disease in Canada.
Chapter 2- Materials and Methods

2.1 Reagents

Unless stated otherwise, reagents were from Fisher Scientific (Mississauga, ON). For RNA experiments, 4 % Sodium Dodecyl Sulfate (SDS) was used to wipe equipment and RNase-away (Molecular Bio-Products, California) was used to reduce RNAase contamination.

2.2 Target sequence identification and sgRNA synthesis

2.2.1 Identifying a zebrafish ortholog for a human cardiac actin gene

The following analysis was performed by Ojehomon et al. (2018). A Basic Local Alignment Search Tool (BLAST) search of the human \(\alpha\)-cardiac actin (ACTC) protein against the zebrafish actin protein sequences led to 18 actin protein sequences which were classified into eight distinct zebrafish actin genes. Human-directed sequence and functional analysis allowed for assignment of an unnamed cardiac actin gene associated with the zgc: 86709 entry as \(actc1c\).

2.2.2 Target sequence identification of putative \(zfactc\) genes

The following protocol is adapted from Gagnon et al. (2014). Candidate single-stranded guide RNA (sgRNA) targeting genomic sequences to the \(cfk-13\), and \(actc1c\) sequences to be deleted were identified using an online webtool called CHOPCHOP (https://chopchop.rc.fas.harvard.edu). CHOPCHOP uses an algorithm to detect off-target sites in the genome to increase specificity of the target site for the sgRNA. The CRISPR oligonucleotide and primers were selected based on an exon target on the respective zebrafish cardiac actin gene exhibiting low off-targets, >70 % G/C content, and low complementarity within the sgRNA and between the sgRNA
and RNA backbone. Other factors taken into consideration for the target site included a guanine base neighboring the PAM, and a location at the 5’ end of the open reading frame.

An earlier exon was selected as a target site to increase the probability of complete loss-of-function; specifically, the end of exon 1 of cfk-13 and exon 2 of actc1c. Several 20 nt target options were identified and scored by specificity values. A BLAST search of the chosen target site against the zebrafish genome was performed to verify specificity. The 20 nt target site chosen for cfk-13 was 5’-GTGACGGGGGGCAGCAACGATgg-3’ on chr13:24,504,299-24,504,477; and for actc1c was 5’-GCGAGGACGACCAACAATGGAGG-3’ on chr20:9,983,546 - 9,993,078. The CRISPR oligonucleotides and primers were purchased from Lab Services (Guelph, ON). The CRISPR oligonucleotides were used to produce sgRNA for cfk-13 and actc1c.

2.2.3 CRISPR single guide RNA synthesis

The following protocol is adapted from Gagnon et al., 2014. The appropriately designed CRISPR oligonucleotide was used to first produce genomic DNA (gDNA). The template for in vitro transcription of the sgRNA was produced by annealing the single stranded CRISPR oligo as designed above to a single stranded Constant oligo (5’ AAAAGCACCGACTCGGTGCCACTTT TTCAAGTTGATACCGACTAGCCTTATTTTTAACTTGCTATTTCTAGCTCTAAAAC -3’) (Integrated DNA Technologies, Coralville, IA). A PCR master mix cocktail was made including 1 µL CRISPR oligo (Lab Services, Guelph, ON) mixed with 1 µL Constant oligo and 8 µL MilliQ water for a total volume of 10 µL. Dilution of oligos was performed to achieve 100 µM final concentration. Thermocycler conditions for the PCR consisted of a denaturation at 95°C for 10 min, followed by annealing at 85°C for 1.5 s, then a hold at 25°C.

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T4 DNA polymerase was used to synthesize DNA in the overhangs generated from annealing the CRISPR oligo to the Constant oligo. A PCR master mix cocktail was made including 2.5 µL dNTPs diluted to a final concentration of 10 µM mixed with 3 µL water, 4 µL 5X T4 DNA Polymerase Buffer, and 0.5 µL T4 Polymerase for a total volume of 10 µL. This mixture was added to 10 µL Annealed CRISPR for a total of 20 µL followed by incubation at 12°C for 30 min.

After incubation, the template was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, and eluted in 50 µl water resulting in approximately 100-200 ng/µl DNA. Samples were run on a 1.2 % agarose gel for 30 minutes at 105 V, stained with EZ Vision Loading Dye (Amresco, Solon, OH) for diagnostic use.

The resulting linearized DNA template was used to transcribe sgRNA through \textit{in vitro} transcription using the MEGAscript® T7/SP6 kit (Ambion, Life Technologies, Carlsbad, CA). The transcription reaction was assembled including 5 µL 2X SP6 NTP CAP (Invitrogen, Carlsbad, CA), 2 µL Nuclease-free water, 1 µL linear template DNA, 1 µL SP6 enzyme mix (Invitrogen, Carlsbad, CA), and 1 µL 10X SP6 reaction buffer (Invitrogen, Carlsbad, CA). Following incubation at 37°C for 2 h, 1 µL TURBO DNAse (included in the Ambion transcription kit) was added to the mixture to degrade residual template DNA and further incubated at 37°C for 15 min.

The sgRNA transcription reaction was purified using ethanol/ammonium acetate. The solution was mixed with 10 µL 5 M ammonium acetate stop solution (included in the Ambion transcription kit), vortexed and centrifuged at 12,000 xg for 15 minutes at 4°C. The solution was mixed with 60 µL chilled 100 % ethanol and incubated at -80°C for a minimum of 30 min to precipitate nucleic acids, then centrifuged at 15,000 xg for 15 min at 4°C. The solution was
removed, and the pellet was washed with 1 mL chilled 70% ethanol to dissolve undesired salts, then centrifuged at 15,000 xg for 5 min at 4°C. The resulting supernatant was removed, and the remaining pellet was dried at room temperature for approximately 5 to 10 min before resuspending in 12 µL RNase-free water. 1 µL of the product was removed for analysis of potential contamination and to determine the RNA concentration using a Nanodrop spectrophotometer (Sigma-Aldrich, Oakville, ON), as reported by Clark et al. (Clark et al., 2011). An additional 1 µL of the product was removed for analysis on a 1% RNase-free agarose gel.

2.2.3.1 RNA Degradation

RNA degradation is a challenge when producing the gRNA. To prevent RNase contamination, it is important to improve RNase-free techniques, such as wearing gloves to prevent contamination from our own RNases. It is also important to change gloves frequently; especially if one has touched contaminated surfaces such as door handles. Lastly, it is important to ensure the environment is RNase-free by using an RNase cleaning product to clean all nearby surfaces including the bench, pipette, pipette tips, and electrophoresis equipment. In my experiment, I used 0.5% SDS as an alternative RNase cleaning product. An additional approach is to ensure that the reagents being used are RNase-free and functioning adequately (Houseley and Tollervey, 2009).

2.2.4 Generate Cas9 protein/sgRNA complex

The sgRNA specific to cfk-13 and actc1c was premixed with recombinant Cas9 protein (PNA BIO Inc, Thousand Oaks, CA). Using Cas9 protein instead of the conventional method of co-injecting Cas9 mRNA increases the efficiency of the knockout (Sander and Joung, 2014). The sgRNA and Cas9 protein was mixed at a 1:2 ratio with MilliQ water. The mixture was incubated
at room temperature for 10 min to allow formation of the Cas9 protein/sgRNA complex. The mixture was put in ice immediately after incubation. The mixture was aliquoted to prevent RNA degradation from the influence of freeze-thaw cycles and stored at -80°C. The Cas9 protein is tolerated up to 800 ng/µL; higher amounts of sgRNA and Cas9 protein can increase non-specific cleavage.

2.3 Zebrafish husbandry

Wild type (WT) zebrafish were obtained from AQUAlity Tropical Fish Wholesale, Mississauga, ON. The fish were maintained in a zebrafish system consisting of genotyping tanks (1.1 L each), nursery tanks (3.5 L each), and growth tanks (8.0 L each) installed in the Hagen Aqualab facility (University of Guelph, Guelph ON). Circadian rhythms were sustained though a consistent 14-hour light cycle. Fish were maintained in tanks with adequate space for free swimming in accordance to maximal housing densities in accordance to You et al. (2016). The zebrafish had a regular feeding routine consisting of feeding at 9 a.m. and a second feeding at 3 p.m. The fish were fed a diet consisting of Gemma 75 and 300 (Skretting, Stavanger, Norway) for larvae, and brine shrimp and TetraFin goldfish flakes to adulthood. The zebrafish were monitored during the regular feeding routine for abnormal swimming behaviour and physical appearance. Fish at sexual maturity were mated once per week.

Embryos and larvae were developed in egg water, a saline solution mixed with methylene blue (Sigma-Aldrich, St. Louis, MO). Larvae were maintained in sterile petri dishes with adequate egg water (petri plate should be over half full). All the larvae were maintained at 28°C under
standard conditions. The embryos and larvae were checked daily to replenish water and assessed for dead or dying embryos, which were discarded.

Larvae less than 7 dpf were euthanized in accordance with our Animal Use Protocol (University of Guelph #3407). The method of termination is a bleach solution (sodium hypochlorite bleach solution 6.15%; Lavo Pro 6, Montreal, QC) added to the zebrafish at a ratio of 1:5 bleach to water. During the early stages of development (<7 dpf), zebrafish larvae have not developed pain perception and thus do not feel distress (Matthews and Varga, 2012). Larvae more than 7 dpf and adult fish were euthanized by immersing in ice water and monitored until body movements desisted (approximately 20 min). The adult fish may alternatively be euthanized through treatment of ethyl 3-aminobenzoate methanesulfonate (Tricaine methanesulfonate).

Embryos minimally at the shield stage were transferred to a sterile Petri dish. Excess water was removed, and the embryos were rinsed with MilliQ water. 100 μg/mL Gentamycin (Sigma-Aldrich, St. Louis, MO) was mixed for 1 h at room temperature with agitation followed by a MilliQ water wash. 0.004 % bleach (Lavo Pro 6, Montreal, QC) was added for 5 min and mixed. The bleach was washed twice with egg water (without methylene blue) for 5 min intervals. The bleach and water rinses were repeated 2 times. Egg water with methylene blue was added to the sterilized embryos and they were incubated at 28°C. The following day, Pronase (EMD Milipore Corp, Massachusetts, US) was added to allow dechorination. The larvae were monitored daily until the yolk had been fully absorbed. Between 5 dpf and 6 dpf, the larvae were introduced into the system in the Hagen Aqualab facility where they were maintained under standard conditions.
2.3.1 Breeding and crossing

Zebrafish at sexual maturity were arranged for mating one day prior to injection. At approximately 3 p.m. one day prior to injection, males and females were placed into breeding tanks separated by a mesh net overnight. At approximately 9 a.m. the following morning, the mesh net was removed to allow breeding. On average, zebrafish take approximately 15 min to breed. The embryos fall through a mesh net at the bottom of the mating tank, which is used to prevent the zebrafish from consuming their embryos. Zebrafish produce approximately 100 healthy, fertilized embryos per week (Nasiadka and Clark, 2012). The embryos were removed from the breeding tank and transferred to a sterile petri plate containing methylene-blue egg water. The petri plate was transported from the Hagen Aqua Lab to the Van Raay laboratory in the Summerlee Science Complex. The petri plates were held steady while transporting to prevent embryo loss and limit embryo disruption by placing the plate on the base of the palm with the fingers gripping the lid.

2.3.2 Zebrafish Limitations

The main challenge associated with working with zebrafish is the amount and quality of eggs obtained. An ideal number of eggs is 100 from a single female in one week. A good quality egg is characterized by the rate of development at 24 hpf (Nasiadka and Clark, 2012). The rate of development is assessed through evaluation of characteristics expected at 24 hpf: recognizable median fin fold, and visible heart as a cone-shaped tube with a pericardial sac. Heart contraction is also expected at this stage but it may be irregular as this characteristic is developed just prior to 24 hpf.
To obtain healthy zebrafish eggs, proper maintenance of the zebrafish in the facility is required. This includes ensuring that their water is clean and feeding is regular with the proper food type and amount. The most important measure is to ensure a continuous turnover in the breeding fish so that they are not bred beyond 20 weeks post fertilization (Avdesh et al., 2012).

2.4 Microinjection of Cas9/sgRNA complexes

Collected embryos were observed though a dissecting microscope (Zeiss, Toronto, ON) to ensure quality; specifically, to ensure embryos are round, pale yellow with an even texture of the yolk, consistent in size, and without degradation. Unfertilized and low-quality embryos were removed. The fertilized embryos were positioned in a sterile petri plate containing a 3% agarose mold using forceps to prevent movement during microinjection. Egg water was added to the sterile petri plates. Approximately 1.5 µL Cas9/sgRNA complex was thawed and transferred by a microloader pipette (Eppendorf, Mississauga, ON) to a 1.0 mm outer diameter glass capillary needle (World Precision Instruments, Sarasota, FL) pulled using Flaming Brown Micropipette Puller (Sutter Instrument, Novato, CA). #5 Dumont Donostar forceps (Ladd Research, Williston, VT) were used to cut the needle to obtain a bolus size of 0.15 mm measured on a stage micrometer immersed in mineral oil.

The embryos were injected using a Picospritzer microinjection system composed of a micropipette attached to a micromanipulator. The Cas9/sgRNA complex was injected into the streaming yolk of an embryo during or prior to the 1 cell stage (approximately 30 min following fertilization). Mock-injected embryos from the same brood were included as a negative internal control, which were injected with MilliQ water. A few embryos were not injected as an additional
control for injection quality. For example, if the non-injected embryos survive while the CRISPR- and mock-injected embryos do not, the conclusion is that the injection method killed the embryos. If all three groups, non-injected, CRISPR- and mock-injected embryos, do not survive then that would indicate death due to poor egg quality.

Injected embryos were placed in an incubator set to 28°C (Thermo Scientific, Dubuque, Iowa) for approximately 1 h to allow recovery after perturbation. Following incubation, the embryos were gently removed from the agarose mold using a sterile probe and transferred to a sterile labelled petri dish containing fresh egg water, then placed back into an incubator set to 28°C for the remainder of experiment.

2.4.1 Analysis of microinjection

Microinjections are very efficient because hundreds of embryos can be injected per hour. It is important to prevent any delay in the injections during the one-cell stage so that the RNA can fully distribute throughout the embryo and be translated during development (Rosen et al., 2009).

The sgRNA specific to the cardiac actin genes, cfk-13 or actc1c, was premixed with the Cas9 protein and subsequently injected into the zebrafish embryo. This method is an improved adaptation of a previous approach involving the co-injection of mRNA for Cas9 with the sgRNA thus requiring the Cas9 to then be translated. The currently implemented protein for the Cas9 increases the efficiency of the knockout. Sander and Joung, 2014, produced high mutation rates with direct injections of Cas9 protein/sgRNA complexes, thus advancing our understanding of Cas9-mediated mutagenesis.
One challenge was acquiring operative skill with the microinjection equipment. A skilled operator can consistently inject the appropriate amount of material to all embryos by maintaining the injection pressure. Lethality is the most often result of injecting high concentrations of materials (Rosen et al., 2009).

2.5 Characterization of zfactc CRISPRs

2.5.1 Heart rate data collection and analysis

Heart rate data was collected and determined as outlined by Avey et al., 2017.

2.5.2 Phenotype characterization

Daily cardiac phenotype characterization was performed using zebrafish cardiac phenotype scoring criteria based on phenotypes in the injected embryos to track changes in zebrafish developmental milestones. The criteria included characterization of cardiac phenotypes at the embryo stage of development, including viability and the rate of overall development, tail bending, body curving, blood circulation, blood accumulation, yolk necrosis, hemostasis, heart rate and heart rate variability, and cardiac morphology including the presence of pericardial edema. Video recordings at 1080p resolution and 60 frames per second were taken of individual larvae at 1-day intervals over the first 7 dpf. The videos were taken for 20 s using an eyepiece adaptor (Carson Optical, Ronkonkoma, NY) for cellular devices such as the iPhone 5S camera (Apple, Toronto, ON) attached to a dissecting microscope (Zeiss, Toronto, ON). These videos were used to score phenotypes and to measure heart rate as outlined in Avey et al. 2017. Survival curves were
produced, using Kaplan-Meier analysis, to indicate the time until death for a period of 7 dpf for three trials for the control, \textit{cfk-13} and \textit{actc1c} CRISPR injections.

For an accurate reading of the heart rate and phenotype, the zebrafish must remain lateral for a minimum period of 30 s. 1.2 \% low melting point agarose (LMP; Sigma-Aldrich, St. Louis, MO) was used to hold the larvae during video recording. The LMP was heated to liquid, stirred and cooled to prevent zebrafish death from heat shock. 10 \mu L of the LMP was added as a base to a clean, dry well. The zebrafish larvae were individually added to the LMP base. Excess water was removed and an additional 10 \mu L LMP was added to the larvae. While the liquid LMP was being cooled into a gel, the zebrafish larvae were carefully positioned laterally using a blunt probe. The fish was acclimatized for 10 min minimum before video recording. The zebrafish were labeled by their associated well (e.g. well #A1 in a 24 well plate). Following video recording, the larvae were removed from the LMP by adding egg water to their well and gently slicing the LMP using a blunt probe to allow the larvae to swim out of the LMP. The larvae were placed back into the appropriate well in the 24 well plate. A minimum of 24 larvae were recorded for the injected group and 24 larvae for the control injection group. A common alternative to LMP is to use Tricaine methanesulfonate as an anesthetic, but this is not ideal as it decreases zebrafish heart rates and becomes toxic from repeated exposure (Denvir et al., 2008).
Chapter 3- Results

3.1 Statistical analysis of heart rates

The means of the heart rates of CRISPR-injected zebrafish embryos at different days post fertilization (dpf) were compared to those of mock-injected zebrafish embryos at the same dpf (Table 3). The null hypothesis is that there is no significant difference between the means. All statistical analyses were performed using SPSS® Statistics version 16.0 (IBM Corporation: Armonk, NY, USA).

3.2 Comparing control heart rates between cfk-13 and actc1c CRISPR experiments

The control groups’ average heart rate each day over the course of the experiment (7 dpf) was consistent within both cfk-13 CRISPR and actc1c CRISPR experiments, confirming reproducibility of results. In addition, the 6 control replicates (three from the actc1c CRISPR and three from the cfk-13 CRISPR experiments) were not statistically different at each time point. This reproducibility allowed me to combine the heart rate data for each individual experiment to determine an average heart rate per day.

3.2.1 Comparing cfk-13 and actc1c CRISPR to control heart rate

The average heart rate per day for 3 independent injections of the cfk-13 CRISPR was compared to the average heart rate per day for 3 independent control injections. There were statistical differences between the cfk-13 CRISPR and the mock-injected embryos on 2 dpf, 3 dpf, and 5 dpf when cfk-13 CRISPR-injected embryos exhibited lower heart rates (Figure 5c).
Table 3: The average heart rate for control and CRISPR-injected experiments. dpf, days post-fertilization; bpm, beats per minute. In some cases, movement of the embryo complicated heart rate measurements. The \( n \) value represents those embryos whose heart rates could be determined. *indicates statistical differences between the CRISPR-injected and the mock-injected control embryos.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>dpf</th>
<th>( cfk-13 ) bpm ± SEM ( (n) )</th>
<th>( actc1c ) bpm ± SEM ( (n) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>109 ± 2.23 (70)</td>
<td>110 ± 1.70 (62)</td>
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<tr>
<td></td>
<td>3</td>
<td>124 ± 2.28 (65)</td>
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<td>126 ± 2.32 (61)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>125 ± 2.14 (63)</td>
<td>113 ± 1.94 (64)</td>
</tr>
<tr>
<td>CRISPR</td>
<td>2</td>
<td>88 ± 2.18 (61) *</td>
<td>98 ± 2.66 (65) *</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>113 ± 2.79 (64) *</td>
<td>117 ± 1.68 (66)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>143 ± 2.52 (59)</td>
<td>124 ± 1.56 (63)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>149 ± 2.14 (55) *</td>
<td>136 ± 1.80 (61)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>122 ± 2.30 (47)</td>
<td>118 ± 2.13 (63) *</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>124 ± 2.84 (51)</td>
<td>110 ± 2.19 (61)</td>
</tr>
</tbody>
</table>
3.2.2 Comparing actc1c CRISPR to control heart rate

The average heart rate per day for 3 independent injections of the actc1c CRISPR was compared to the average heart rate per day for 3 independent mock injections. There were statistical differences between the actc1c CRISPR-injected and the mock-injected embryos on 2 dpf and 6 dpf when actc1c CRISPR-injected embryos exhibited a lower heart rate (Figure 6c).

3.3 Observed cardiac phenotypes

CRISPR-injected embryos exhibited cardiac phenotypes such as slowed rate of development, changes in heart rate, lethality, changes in blood circulation, hemostasis, blood accumulation, and the presence of pericardial edema (Figure 4c).

3.4 Phenotype comparison between CRISPR and control embryos

The CRISPR-injected embryos were grouped into 4 different trajectories based on their cardiac phenotype changes (or lack thereof) throughout the experiment: (1) Healthy, (2) Change in Vigor and Survive (CIV.S), (3) Change in Vigor and Gradual Decline (CIV.GD), (4) Gradual Decline (GD). Mock-injected embryos did not exhibit cardiac phenotypes in all experiments (Figure 4a and b).
Figure 4: Examples of phenotype trajectories and common cardiac phenotypes in CRISPR-injected embryos. A) Cfk-13 cardiac phenotype trajectories and progression throughout duration of experiment. B) Actc1c cardiac phenotype trajectories and progression throughout duration of experiment. C) Common cardiac phenotypes in CRISPR-injected embryos. Note: Varying levels of phenotype severity were observed but we only assessed for absence or presence for simplicity.
Healthy (H) (1): The embryo survives the course of experiment without presenting a cardiac phenotype. (2) Gradual Decline (GD): The embryo does not survive the course of the experiment and presents a cardiac phenotype that worsens as the experiment progresses. (3) Change in Vigor and Survive (CIV.S): The embryo survives the course of the experiment and presents a cardiac phenotype that is no longer present as the experiment progresses. Here we see a switch from the embryo looking unhealthy (cardiac phenotype) to looking healthy (no cardiac phenotype) within a day and the embryo is healthy for remainder of experiment. (4) Change in Vigor and Gradual decline (CIV.GD): The embryo does not survive the course of the experiment and presents a cardiac phenotype that is no longer present as the experiment progresses. (Figure 4a and b).

3.4.1 Phenotype comparison between cfk-13 CRISPR and control embryos

Among the cfk-13 CRISPR-injected phenotype trajectories, the Healthy and CIV.S trajectory survived through the course of the experiment (Figure 5b). The GD trajectory experienced lethality beginning at 3 dpf and survived up to 5 dpf. The CIV.GD trajectory experienced lethality beginning at 4 dpf and survived up to 7 dpf.

An Independent-Samples Kruskal-Wallis one-way analysis of variance was performed to test the differences in heart rate between the 4 different trajectories of cfk-13 CRISPR-injections and the Control group (Table 4; Figure 7a and b). On 2 dpf, the CIV.S and GD trajectories were statistically different than the Control group (Figure 7a). On 3 dpf, the GD trajectory was statistically different from the Healthy, CIV.S, and Control group. Also, the CIV.S trajectory and Control group were statistically different (Figure 7b).
Table 4: The average heart rate for cfk-13 trajectories on 2 dpf and 3 dpf.

<table>
<thead>
<tr>
<th>Phenotype trajectory</th>
<th>2 dpf bpm ± SEM (n)</th>
<th>3 dpf bpm ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIV.S</td>
<td>90 ± 2.41 (32)</td>
<td>118 ± 2.45 (30)</td>
</tr>
<tr>
<td>Healthy</td>
<td>97 ± 2.48 (15)</td>
<td>122 ± 3.22 (20)</td>
</tr>
<tr>
<td>CIV.GD</td>
<td>85 ± 8.72 (5)</td>
<td>122 ± 5.78 (4)</td>
</tr>
<tr>
<td>GD</td>
<td>66 ± 6.95 (9)</td>
<td>80 ± 9.54 (10)</td>
</tr>
<tr>
<td>Control</td>
<td>109 ± 2.23 (70)</td>
<td>124 ± 2.28 (65)</td>
</tr>
</tbody>
</table>

For 2 dpf and 3 dpf, the Healthy trajectory and Control group had the highest heart rate and GD had the lowest heart rate, compared to the other trajectories. This analysis could not be performed comparing all 4 trajectories after 3 dpf on GD and CIV.GD because the homogeneity of variance was violated due to a low number of samples from high lethality. CIV.S and Healthy trajectories were compared with each other for up to 7 dpf and were not statistically different. CIV.S and Healthy trajectories were then compared to the Control group for up to 7 dpf and were also not statistically different. However, we do notice trends in data (Figure 5a). For cfk-13 CRISPR-injections, GD had a lower heart rate than the other trajectories. The Healthy and CIV.S trajectory had the highest heart rates and were dispersed equally throughout the experiment. The CIV.GD trajectory was dispersed similar to GD on 2 dpf and 3 dpf and became similar to Healthy and CIV.S on 4 dpf, then the heart rate decreased on 5 dpf.
Figure 5: Heart Rate and Phenotype Characterization of *cfk-13* CRISPR-injected embryos. 
(A) Beats per minute (BPM) of CRISPR-injected zebrafish. Each point represents an individual embryo, (red is Healthy, blue is CIV.S, green is GD and black is CIV.GD). Lines of significance to show HR variability between the CRISPR group and Control group (black asterisks); bars represent standard deviation of the mean. 
(B) Proportion surviving of trajectory embryos living through days elapsed. Phenotype characterization of individual CRISPR-injected embryos show four trajectories coloured as A above. 
(C) *Cfk-13* CRISPR-injected embryos (blue) heart rate versus Control injected embryos (red). Error bars represent +/- 2 SE.
3.4.2 Phenotype comparison between *actc1c* CRISPR and control embryos

Among the *actc1c* CRISPR-injected phenotype trajectories, the Healthy and CIV.S trajectory survived through the course of the experiment (Figure 6b). The GD trajectory experienced lethality beginning at 3 dpf and survived up to 4 dpf. The CIV.GD trajectory experienced lethality beginning at 4 dpf and survived up to 7 dpf.

An Independent-Samples Kruskal-Wallis one-way analysis of variance test was performed for the heart rates of the *actc1c* CRISPR-injected phenotype trajectories and the control group (Table 5). At 2 dpf, the Healthy trajectory and Control groups were statistically different. Also, the CIV.S trajectory and Control were statistically different (Figure 7c).

**Table 5: The average heart rate for *actc1c* trajectories on 2 dpf**

<table>
<thead>
<tr>
<th>Phenotype trajectory</th>
<th>2 dpf bpm ± SEM (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIV.S</td>
<td>97 ± 3.75 (44)</td>
</tr>
<tr>
<td>Healthy</td>
<td>96 ± 2.89 (15)</td>
</tr>
<tr>
<td>CIV.GD</td>
<td>110 ± 2.10 (4)</td>
</tr>
<tr>
<td>GD</td>
<td>108 ± 4.48 (2)</td>
</tr>
<tr>
<td>Control</td>
<td>110 ± 1.67 (62)</td>
</tr>
</tbody>
</table>

On 3 dpf, there were no statistical differences within the 4 different trajectories, or between the trajectories and Control group. This analysis could not be performed comparing all 4 trajectories following 2 dpf with GD and CIV.GD trajectories because the homogeneity of variance
was violated due to a low number of samples from high lethality. CIV.S and Healthy trajectories were compared with each other for up to 7 dpf and were not statistically different. CIV.S and Healthy trajectories were compared to the Control group for up to 7 dpf and were not statistically different. However, we do notice trends in the data (Figure 6a), with GD dispersed evenly with the other trajectories but with a small sample size (n=2) so conclusions cannot be made without additional data. Similar to the cfk-13 CRISPR-injection results, the Healthy and CIV.S trajectories had the highest heart rates and were dispersed equally throughout the experiment.
Figure 6: Heart Rate and Phenotype Characterization of acte1c CRISPR-injected embryos. 
(A) Beats per minute (BPM) of CRISPR-injected zebrafish. Each point represents an individual embryo, (red is Healthy, blue is CIV.S, green is GD and black is CIV.GD). Lines of significance to show HR variability between the CRISPR group and control group (black asterisks); bars represent standard deviation of the mean. (B) Proportion surviving of trajectory embryos living through days elapsed. Phenotype characterization of individual CRISPR-injected embryos show four trajectories, coloured as in A above. (C) Acte1c CRISPR-injected embryos (blue) heart rate versus Control (red) injected fish. Error bars represent +/- 2 SE.
Figure 7: Independent-Samples Kruskal-Wallis one-way analysis of variance to test the differences in heart rate between the 4 different trajectories and the control group. There are two types of outliers, ‘out’ values (marked with a small circle) and ‘far out’ values (marked with a star). (A) cfk-13 on 2 dpf: Outliers are identified in the Change in Vigor and Gradual Decline (CIV.GD) trajectory and Control group. (B) cfk-13 on 3 dpf: Outliers are identified in the Healthy, Change in Vigor and Survive (CIV.S) trajectory, and the Control group. (C) actc1c on 2 dpf: Outliers are identified in the Control group. Letters are used to indicate differing means. Different letters represent statistically significant differences (P < 0.05).
Chapter 4- Discussion

4.1 Actin gene switch model

My results support an actin gene switch model where actc1c functions during 2 dpf, cfk-13 is expressed during 3-4 dpf, and preliminary results show that actc1a-19/20 expressed after 3-4 dpf.

Cfk-13 CRISPR injections resulted in reduced cardiac function during the first 3 dpf, but cardiac function did not improve after 3 dpf since the CRISPR embryos exhibited a significantly higher heart rate than the Control embryos on 5 dpf. Close to normal function after 3 dpf suggests a model where cfk-13 is not dominantly expressed after 3 dpf, when actc1a becomes the predominant actin isoform in the heart. However, the embryos exhibit a significant heart rate difference on 5 dpf. This difference may be the result of actc1a-19/20 gene expression after 3 dpf compensating for the cardiac phenotypes resulting from the downregulated expression of cfk-13 during the first 3 dpf. The knockout of cfk-13 may have induced lasting negative effects on heart development. One cfk-13 founder survived to adulthood but exhibited weak swimming behavior and blood accumulation. Often this individual laid on the bottom of the tank and would struggle to swim if disturbed by tapping on the aquarium glass. Future experiments should look at the long-term impact of the CRISPR to assess adult fish performance.

Actc1c CRISPR injections resulted in reduced cardiac function at 2 dpf, but cardiac function did not improve after 2 dpf since the CRISPR embryos exhibited a significantly lower heart rate than the Control embryos on 6 dpf. The decreased heart rate on 6 dpf may be a stress response in which the zebrafish is recovering from cardiac dysfunction on 2 dpf. Close to normal
function was observed after 2 dpf supporting our earlier finding that actc1c is only expressed during 2 dpf (Ojehomon et al., 2018). Perhaps the actc1a-19/20 gene is not expressed at a level needed to compensate for the cardiac phenotypes present on 2 dpf.

4.2. Phenotype trajectories

The heart rates of zebrafish in different trajectories could not be compared throughout 7 dpf because lethality was observed in some trajectories, leading to unequal group sizes. The zebrafish in the Change in Vigor and Gradual Decline (CIV.GD) and Gradual Decline (GD) trajectories both saw significant lethality following 3 dpf; therefore, the Independent-Samples Kruskal-Wallis one-way analysis of variance was only performed on 2 dpf and 3 dpf for cfk-13 and actc1c (Tables 4 and 5). One hypothesis is that the embryos in the Healthy trajectory do not possess observable cardiac defects because there was no CRISPR activity in these embryos.

For cfk-13 CRISPR injections on 2 dpf, there were no differences in heart rate between the Control and Healthy (H) and/or Change in Vigor and Gradual Decline (CIV.GD) trajectory. However, I was expecting to see a statistical difference between the Control and Change in Vigor and Gradual Decline (CIV.GD) trajectory because these embryos exhibited cardiac phenotypes leading to death. Perhaps CRISPR activity acting on cfk-13 is only maximal at 3 dpf or the sample size for the CIV.GD is too small, requiring a larger sample size to show significance.

For actc1c CRISPR injections on 2 dpf, the Change in Vigor and Survive (CIV.S) trajectory and Control are statistically different. These findings support a model where actc1c is required before 2 dpf for good cardiac function. On 3 dpf, there are no statistical differences within the 4 different trajectories, or between the trajectories and Control group. The Healthy trajectory
and Control groups are statistically different on 2 dpf, however there is a large variation between the sample size for the two trajectories.

The Healthy and Control group were not statistically different for 2 dpf and 3 dpf in the *cfk*-13 CRISPR injections, supporting the hypothesis that the Healthy group does not exhibit CRISPR activity. However, in the case of the *actc1c* CRISPR experiment, the Healthy trajectory and Control groups were statistically different on 2 dpf. This finding does not support the hypothesis that there is no CRISPR activity in the Healthy embryos because there should be no difference compared to the Control group. The mock control was injected with water alone, while the CRISPR-injected fish had sgRNA and Cas9 protein in water, the difference being the presence of the Cas9 protein and the sgRNA. Since the only variable between the *cfk*-13 and *actc1c* CRISPR experiments is the specific sgRNA, perhaps the statistical difference in the *actc1c* group is due to off-target effects from the *actc1c* sgRNA. Selecting a different target site for the *actc1c* CRISPR experiment will test for off-target effects.

### 4.3 Zebrafish and human fetal gene program

The expression of the *zfactc* genes through the stages of larval development may be comparable to the human fetal program when some sarcomeric genes undergo a switch from fetal to adult isoform expression following birth. Shih et al., identified genes exhibiting high expression in the larval stage that became downregulated during the adult stage, and vice-versa (Shih et al., 2015). Genetic reprogramming of human fetal and adult genes may be harnessed for heart disease therapy (Nandi and Mishra, 2015). The zebrafish heart may exhibit similar reprogramming in the
failing heart. The larvae exit the chorion on 3 dpf which is approximately when we see the switch in observed phenotypes.

Perhaps zebrafish in the CIV.S trajectory experience remodeling of the heart through expression of fetal genes to protect from heart failure (Nandi and Mishra, 2015). With this theory, the CIV.GD trajectory also undergoes fetal reprogramming but the switch does not reverse the cardiac dysfunction and the GD trajectory do not undergo fetal reprogramming. Regulation of the genes and miRNAs involved in fetal reprogramming would allow researchers to control the remodeling of the heart thus protecting the CIV.GD and GD trajectories from adverse remodeling.

4.4 Gene duplication in zebrafish affects phenotype severity

There are multiple *zfactc* genes in the cells of the zebrafish heart, including the *cfk-13* and *actc1c* genes, each with two alleles. When one *zfactc* gene or allele is knocked out by CRISPR activity, the others remain unaltered. Perhaps embryos in the CIV.S trajectory have CRISPR activity but actin redundancy allows them to survive. The GD and CIV.GD trajectory may have CRISPR activity on both alleles, or the knockout may be due to a combination other factors such as injection technique. Perhaps the Healthy trajectory does not exhibit CRISPR activity. Real-Time PCR (qPCR) will determine whether each trajectory possesses CRISPR activity.

4.5 Actin gene switch model for CRISPR efficiency

A significant limitation to the experimental design is that CRISPR knockouts are not 100% efficient (Lawson, 2016; Shalem et al., 2014); however, the resulting phenotypes can be used to predict which embryos experienced CRISPR activity. Based on the assumption that any phenotype
other than Healthy is the result of CRISPR activity, 69-77% of embryos in the first 2 or 3 dpf (before significant death occurred) had CRISPR activity (Table 6).

Considering that there are two alleles for each gene, a further model is that the severity of observed cardiac phenotypes reflects CRISPR activity on one or both alleles. Assuming that Change in Vigor and Gradual Decline (CIV.GD) and Gradual Decline (GD) are the result of CRISPR activity on both alleles, the frequency of having both alleles modified is 9-23% (Table 6). Assuming that modification of an allele has the same probability and is independent of the other allele, the probability of modifying one allele is the square root of the observed frequency of having both alleles modified, according to the multiplication of probabilities rule. By adding the frequencies of single and double allele modifications together, the total theoretical probability of observing single modification and double allele CRISPR modification can be calculated (Table 6).

Table 6. CRISPR efficiency calculations. CRISPR activity is based on the frequency of phenotype trajectories taken from Table 4 (cfk-13) and Table 5 (actc1c). Total activity is the percentage of all embryos expressing a phenotype other than Healthy, while double allele activity is the percentage of all embryos with a Change in Vigor and Gradual Decline (CIV.GD) or Gradual Decline (GD) phenotype.

<table>
<thead>
<tr>
<th></th>
<th>Total CRISPR Activity (Observed)</th>
<th>Double Allele CRISPR Activity</th>
<th>Single Allele CRISPR Activity (Theoretical)</th>
<th>Total CRISPR Activity (Theoretical)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfk-13</td>
<td>2 dpf</td>
<td>75%</td>
<td>23%</td>
<td>48%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>71%</td>
</tr>
<tr>
<td>cfk-13</td>
<td>3 dpf</td>
<td>69%</td>
<td>22%</td>
<td>47%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>69%</td>
</tr>
<tr>
<td>actc1c</td>
<td>2 dpf</td>
<td>77%</td>
<td>9%</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39%</td>
</tr>
</tbody>
</table>

The observed CRISPR efficiency rates for the cfk-13-targeting sgRNA are very similar to the theoretical rates based on the model that all phenotype trajectories other than Healthy experience CRISPR and that the Gradual Decline trajectories are due to CRISPR knockouts on
both alleles. The embryos may fall into 3 categories: no CRISPR activity, one allele knocked out, and then both alleles knocked out resulting in a complete knockout the gene.

On the other hand, the phenotypes observed for the actc1c knockouts might not be severe enough, even with both alleles modified, to result in Gradual Decline and hence even those embryos with both alleles modified are scored as Change in Vigor and Survive. This idea supports a model where the actc1c gene is a minor contributor to the development and function of the heart in zebrafish, as also seen in the low-level expression of the gene at 2 dpf (Ojehomon et al., 2018). Future qPCR measurements can confirm the levels of gene expression in the various phenotype trajectory groups to confirm the model proposed here.

Knocking out an actin gene may be lethal considering it is required for cardiac function. This lethality affects generating a knockout line because the CRISPR affected fish do not survive to adulthood. If my model proposed above is correct, we may not be able to generate homozygous cfk-13^-/- lines due to lethality; however, we could produce heterozygous cfk-13^wt/- lines and cross them to generate homozygous embryos for future study. Alternatively, the phenotypes of heterozygous cfk-13^wt/- zebrafish may be severe enough to perform future experiments with.

My model suggests that knocking out the actc1c gene entirely does not have a severe impact on viability, so a homozygous actc1c^-/- line could be produced. The zebrafish model may be utilized to determine improved cardiac function by screening for small molecules in collaboration with the Zebrafish Center for Advanced Drug Discovery in the Li Ka Shing Knowledge Institute of St. Michael’s Hospital in Toronto. Future experiments will also look at the long-term impact of the CRISPR on adult fish to assess their performance.
With newer CRISPR systems, we might mutate the $zfactc$ genes to create a point mutation and assess their effects. In my work, we are using the earliest CRISPR-Cas9 system that can be detected with fragment analysis to determine mutation success; site-directed missense mutagenesis requires direct sequencing. Also, we already possess human $ACTC$ constructs, making it convenient to produce transgenic zebrafish lines expressing them or inject $zfactc$ knockout zebrafish lines with human $ACTC$ mRNA.

4.6 Confirmation of Hypothesis

My hypothesis was that $cfk-13$ and $actc1c$ are cardiac actin genes which undergo a developmental switch during early zebrafish development. Knocking out genes encoding cardiac actin isoforms in zebrafish would result in observable cardiac phenotypes, including reduced cardiac function and increased mortality. Reduced cardiac function in the knockout fish during the time of gene expression confirm that $cfk-13$ and $actc1c$ genes are cardiac actin genes in zebrafish. Analysis of observed phenotypes and heart rate variability support an actin gene switch model in which $actc1c$ functions during 2 dpf, $cfk-13$ functions during 3-4 dpf. Future work will examine the contribution of the third $zfactc$ gene ($actc1a-19/20$) to cardiac structure and function during zebrafish development.

4.7 Analysis of heart rate protocol

The employed heart rate analysis procedure is a simple, rapid and noninvasive method to measure the heart rate in zebrafish (Avey et al, 2017). Traditional methods involve stopwatch counting that is time-consuming, labor-intensive and can introduce human error in the counting
The Fiji software used is sensitive, so any macroscopic movement, from either the zebrafish or bumping the cell phone, can reduce accuracy of the results. Measures must be implemented to ensure the video frame of focus is steady throughout the recording, including a microscope eyepiece cell phone adaptor, video cropping and optimal capture settings. Utilization of a microscope eyepiece cell phone adaptor is a crucial measure to reduce video disturbance. However, the adaptor moves when handled, such as when starting and stopping a recording. An alternative is to record lengthy videos, minimally 20 seconds, so that both ends of the recording can be cropped with a video-editing tool in MPEG StreamClip software and/or utilizing Siri to start and stop the recording with your voice alone. Another measure for good video data is to modify cellular device settings to block auto-focusing thereby preventing the camera from zooming in and out during the recording and affecting the depth gradient of the Z-axis profile.

Recent methods extend our ability to assess zebrafish embryonic heart size and cardiac function, from cardiac output estimates from 2-dimensional imaging of ventricular shape which does not represent diastolic function, to three-dimensional imaging of the zebrafish embryonic heart (Denvir et al., 2008). For example, a hypercholesterolemic female zebrafish has been whole-body imaged through synchrotron X-ray micro-computed tomography (SR-µCT) enabling precise 3D visualization of whole-volume zebrafish tissues. This technique allows for analysis of microstructures of different organs and can be used to identify blood vessels (Seo et al., 2015). However, the introduction of inexpensive, time-efficient, and widely available techniques to assess cardiac function remains a challenge (Denvir et al., 2008).
Continued advances in technological methods for assessing morphological defects will lead to improved downstream modifier screens and detections of small molecules, in turn establishing zebrafish as a model for high throughput screening of compounds that can reduce the impact of or prevent heart disease (Jeanray et al., 2015; Hoage et al., 2012). Therefore, continued improvements to methodologies will entail decreases in time and workload, as well as increases in impartiality and reproducibility for classifying zebrafish embryos, (Jeanray et al., 2015).
Chapter 5- Future Work

5.1 Increasing potential target sites for genome modifications

Future directions may include modifying Cas9 enzymes to recognize a broader spectrum of PAM sequences, thereby increasing potential target sites for genome modifications (Sander and Joung, 2014). CRISPR technology may be used to correct mutations in living cells, thereby preventing cardiac defects. CRISPR-Cas9 editing has been utilized to correct a cardiac myosin binding protein-C mutation in human preimplantation embryos (Ma et al., 2017).

5.2 Validating gene knockout through Real-Time PCR

Real-Time PCR (qPCR) may be used to determine the absolute or relative quantity of PCR products. To examine gene expression, RNA is extracted and reverse transcribed into cDNA which becomes the template for qPCR analysis. This high-throughput process is ideal for examining gene expression due to high precision and speed (it requires a few hours). SYBR Green Dye, an intercalating fluorescent dye, binds to the minor groove of double stranded DNA. The accumulation of amplification product at each PCR cycle is monitored by the fluorescence emitted during the reaction. A minimum of three biological replicates are required because the expected differential expression is high. Performing qPCR on CRISPR-injected embryos will tell us whether the cardiac phenotypes observed are the result of decreased gene expression and aid in confirming the absence of off-target effects. This process may be performed at Genomics Facility at the University of Guelph using a Bio-Rad real-time PCR detection system (Heid et al., 1996; Lederman, 2008).
5.3 CRISPR-derived off-target effects

The interpretation of results with CRISPRs is compromised by off-target effects. Future studies may include designing and injecting a new gRNA targeted to a different region of the zfactc gene of interest to control for off-target cleavage events. Similar heart rate and phenotype results between the different gRNAs would confirm that the observed effect is target-specific. Off-target mutations may be mitigated by regulating the cell cycle stage at the target site and by using a purified recombinant Cas9 protein (Ma et al., 2017). Alternatively, deep sequencing or a T7 Endonuclease assay may be employed to detect off-target effects (Hegedus et al., 2009; Hua et al., 2017).

5.4 Long-term work

While generating and characterizing the zebrafish zfactc knockout strains, others in the Dawson lab are producing transgenic zebrafish lines expressing human ACTC and its variants (Clark et al., 2011; Kawakami, 2005; Suster et al., 2009). Following the successful knockout of zebrafish actc1a, cfk-13, and actc1c genes, the long-term goal is to cross these CRISPR lines with the human ACTC transgenic lines to determine if human ACTC can rescue the zebrafish actc knockout. A rescue would establish a zebrafish model to study the impact of ACTC variants related to cardiomyopathy development. Evaluating when and where gene expression occurs during zebrafish development provides insight for when to express human ACTC together with the gene knockout line. The impact of ACTC rescue of actc1a, cfk-13, and actc1c knockout would be evaluated using the cardiac phenotype measures described in my work.
It is important to establish two independent lines for each gene knockout, as this will allow us to determine potential therapeutics. Stable zebrafish lines expressing CRISPR zfactc gene knockouts will be characterized through histology, ultrastructure work, molecular markers of cardiac dysfunction, metabolism measurements, and phenotype scoring.

The details of the proposed zfactc gene switch in zebrafish cardiovascular development may be clarified in future experiments examining the underlying signaling mechanisms of that switch. This genetic switch may also prove to be advantageous for our long-term experiment: if one of the isoforms is dominant during the first 3 dpf, we can perform our experiments during that time period.
Chapter 6- Conclusions

The research outlined here is focused on the cardiac actin protein (ACTC1) to understand how changes in interactions of muscle proteins lead to the development of cardiomyopathies. My work is part of a larger plan to establish an in vivo model for studying cardiac actin variants found in patients with cardiomyopathies. By producing zfactc knockout lines, we will clarify which zebrafish actin genes are functioning in the heart. My work also contributes to characterizing a potential developmental switch in actin genes during early zebrafish development.

Future work will include establishing zebrafish (Danio rerio) as a model to study the role of cardiac actin mutations in the development of cardiomyopathies (Bakers, 2011) where a human ACTC variant protein compensates for an endogenous zfactc knockout. Such a model provides a tool to dissect the molecular mechanisms of disease development and for high-throughput screening for new therapeutics that aid in correcting the specific molecular dysfunction (Dahari and Dawson, 2015). Precision therapies will protect patients from harmful side effects seen in currently prescribed broad medications and reduce the rising cost of heart disease in Canada.

Overall, my work is a crucial first step in developing the tools to determine if human ACTC can rescue zebrafish actc knockouts, thereby providing a model to study changes in the ACTC gene related to heart disease. Finally, my work is the first to characterize the novel actc1c gene in zebrafish, determining the impact of its knockout.
Bibliography


