cTnI N-TERMINAL DELETION: AN AGENT FOR RESCUING RESTRICTIVE CARDIOMYOPATHY, A DISEASE CAUSED BY MUTATIONS OF CARDIAC TROPONIN I

by

Cecile A. Getfield

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Xupei Huang, Department of Biomedical Science, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

SUPERVISORY COMMITTEE:

Xupei Huang, Ph.D.
Thesis Advisor

Howard Prentice, Ph.D.

Rui Tao, Ph.D.

Keith Brew, Ph.D.
Chair, Department of Biomedical Science

For: David J. Bjorkman, M.D., M.S.P.H.
Dean, The Charles E. Schmidt College of Medicine

Deborah L. Floyd, Ed.D.
Interim Dean, Graduate College

07/31/2014
Date

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ABSTRACT

Author: Cecile A. Getfield

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Restrictive cardiomyopathy (RCM) is represented in part by left ventricular stiffness and diastolic dysfunction. Missense mutations of the cardiac troponin I (cTnI) gene cause idiopathic RCM. These mutations are located in the C-terminus of cTnI and affect cardiac relaxation. Transgenic mouse models presenting the pathology observed in clinical patients with RCM have been generated previously and express the mutant cTnI in their hearts. RCM-linked mutations increase cardiac myofilament Ca^{2+} sensitivity and promote diastolic dysfunction in the heart. Previous studies using double transgenic mice (cTnI/R193H/ND) showed that ventricular relaxation is enhanced in the cTnI/R193H transgenic mice. In this study, another double transgenic mouse model, (cTnI/R193H/ND/KO), provides an avenue to investigate its rescuing effects on RCM-linked mutations in the cTnI /R193H/KO mouse. Use of molecular biological techniques, transgenic animal developments and murine echocardiography in this study has culminated into a greater understanding of RCM and diastolic dysfunction.

DEDICATION

This manuscript is dedicated to the members of my family, my mother Barbara Getfield, who always instilled in me discipline; with love, wisdom and kindness. As a teacher, she continuously emphasized the importance of an education. My late father Cecil Getfield, who taught me tenacity, and was a model of hopefulness in my life. My sister, Jacqueline Getfield, who has been resolute in her support of me throughout my life's endeavours.

cTnI N-TERMINAL DELETION; AN AGENT FOR RESCUING RESTRICTIVE CARDIOMYOPATHY, CAUSED BY MUTATIONS OF CARDIAC TROPONIN I

TABLES

FIGURES

CHAPTER 1: INTRODUCTION

1.1 The Heart: structure and function

The heart is muscular four-chambered organ, which is often considered as a pump. It is responsible for driving oxygenated and deoxygenated blood from the heart to the body and the lungs respectively. Consisting of the left and right sides (figure 1) each side of the heart is composed of the two chambers, an atrium and a ventricle. The walls of the ventricles are more muscular compared to that of the atria because they pump the blood to the body and the lungs. In particular, the left side of the heart receives oxygenated blood into the left atrium (LA) from the lungs and passes through the mitral valve into the left ventricle. The left ventricular (LV) chamber has two large papillary muscles, which are larger than those in the right ventricles. The papillary muscles are connected to both leaflets of the mitral valve. Filling of the left ventricle occurs when the mitral valve opens in early diastole. At this point normal diastolic filling occurs in the heart, one without alterations to preload. An increase in volume of the left ventricle causes a small rise in pressure, due to compliance of the heart muscle. Contraction of the left ventricle causes the left ventricular pressure to increase, when this pressure becomes more than the pressure in the left atrium the mitral valve closes. As this pressure rises, the LV volume does not change instantly because the aortic valve must open, the aortic valve separates the left ventricle from the aorta. This point is called iso-volumetric contraction (IVCT). When ventricular pressure equalizes with aortic diastolic pressure the ejection of

oxygenated blood occurs into the aorta. While ejection occurs, the LV volume decreases but the pressure continues to rise until relaxation takes place. LV relaxation marks the end ejection, and the ventricular pressure falls below the aortic pressure and the aortic valve closes. At this point the ventricle continues to relax as its pressure declines, but its volume remains constant because the mitral valve is not open. This phase is the iso-volumetric relaxation time. However, when the LV pressure is below the left atrial pressure, the mitral valve opens and the left side of the heart fills again.

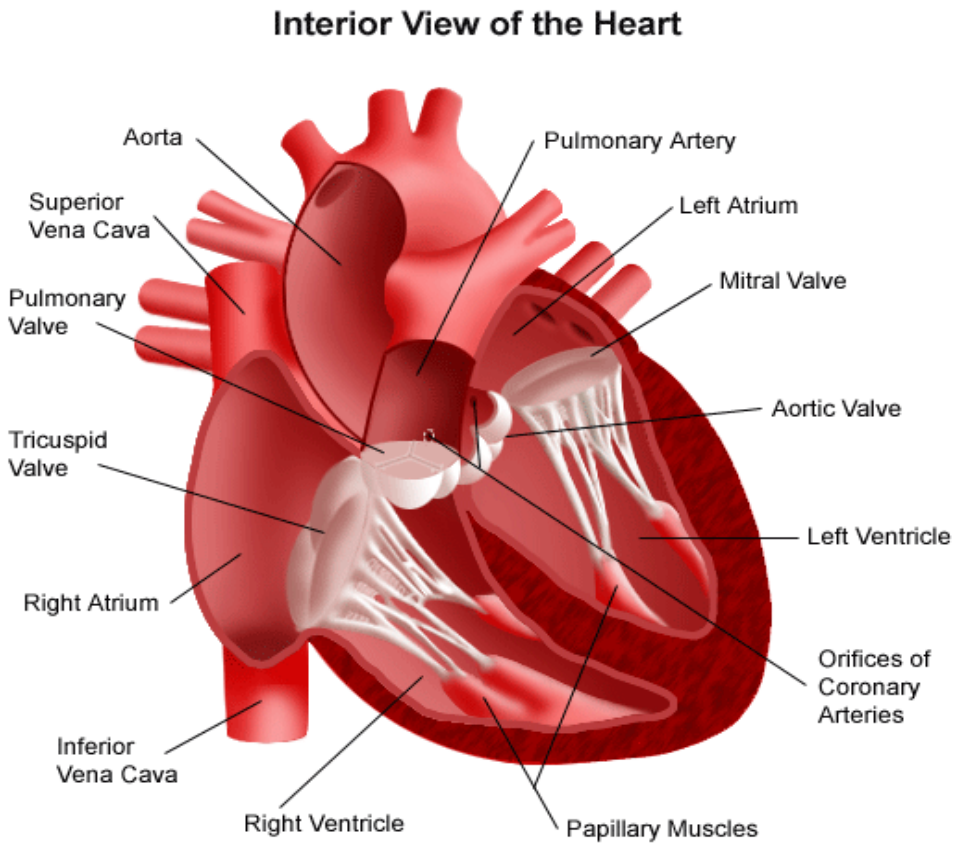

Figure 1: Interior view of the human heart.

1.2 Mechanism of cardiac function

Cardiac muscle is comprised of several hundred to thousands of myofibrils formed from repeating units of sarcomeres. Microscopically, sarcomeres appear as light and dark bands called myosin and actin filaments which are large polymerized protein molecules and slide pass each other and are responsible for muscle contraction and relaxation (figure 2). Myosin, the thick filament is composed of a long fibrous tail and a globular head. When the concentration of Ca2+ increases in the muscle cell, myosin which is bound to ATP uses it as a source of energy. It slides along actin, the thin filament during muscle contraction. Actin molecules are bound to the Z-lines which form the outer borders of each sarcomere. Cardiac muscles are striated like a typical skeletal muscle, contain actin and myosin filaments, and are almost identical to those found in skeletal muscle. However, in some ways cardiac muscles differ, one way is their mechanism of an action potential. In the cardiac myofibrils, an action potential is caused by the opening of two types of channels- fast sodium channels and slow calcium channels referred to as calcium-sodium channels. The calcium channels are slower to open and close, allowing for large quantities of calcium and sodium ions to enter into the interior of the cardiac muscle fiber. The consequence is a prolonged depolarization leading to the classic plateau observed in a cardiac action potential. It is the calcium ions which enter during the plateau phase that activate the muscle contractile process. The term ‘excitation-contraction coupling’ lends to the process in which an action potential causes the myofibrils to contract. The action potential spreads throughout the interior of the muscle fiber by way the transverse (T) tubules, prompting a release of calcium ions from the sarcoplasmic reticulum into the myofibrils. Troponin (Tn), a sarcomeric protein plays an

important role in the Ca^{2+} regulation in contraction and relaxation skeletal and cardiac muscles. It consists of three subunits, each having very distinct structure and function. cTnC binds calcium ions, undergoes a conformational change and triggers the contraction of the heart muscle because the action of cTnI is inhibited. The transduction between cTnC and cTnT during the inactivity of cTnI causes a conformational change in tropomyosin as well. The result is tropomyosin exposing the myosin binding site on the actin filament, allowing the myosin motor heads to crawl along the actin. The result is contraction of the muscle fibers. The intermolecular interaction in response to the increase concentration of Ca^{2+} plays an important role in normal cardiac pump function (Harada et al., 2004). TnT attaches troponin to tropomyosin (Tm) and actin. In its relaxed state Tm lies along the myosin binding sites on the actin filaments and prevents interaction of the myosin heads and actin. In turn, the sliding of the actin and myosin filaments along each other produces cardiac muscle contraction. Actin is closely associated with accessory proteins such as tropomyosin and troponin, a complex of three polypeptides, troponins T, I and C which are from three distinctive gene products.

1.3 Troponin Complex: troponin I and its role in muscle contraction

A heteromeric protein which plays a part in regulating cardiac muscle contraction, the troponin complex contains three different subunits: troponin I, C and T. Each has its unique responsibility in the function of the complex as a whole. Troponin is important in the cyclic transition from relaxation to contraction in the heart. Cardiac troponin C (cTnC) is the Ca^{2+} binding subunit, it plays the main role in Ca^{2+} dependent activation of

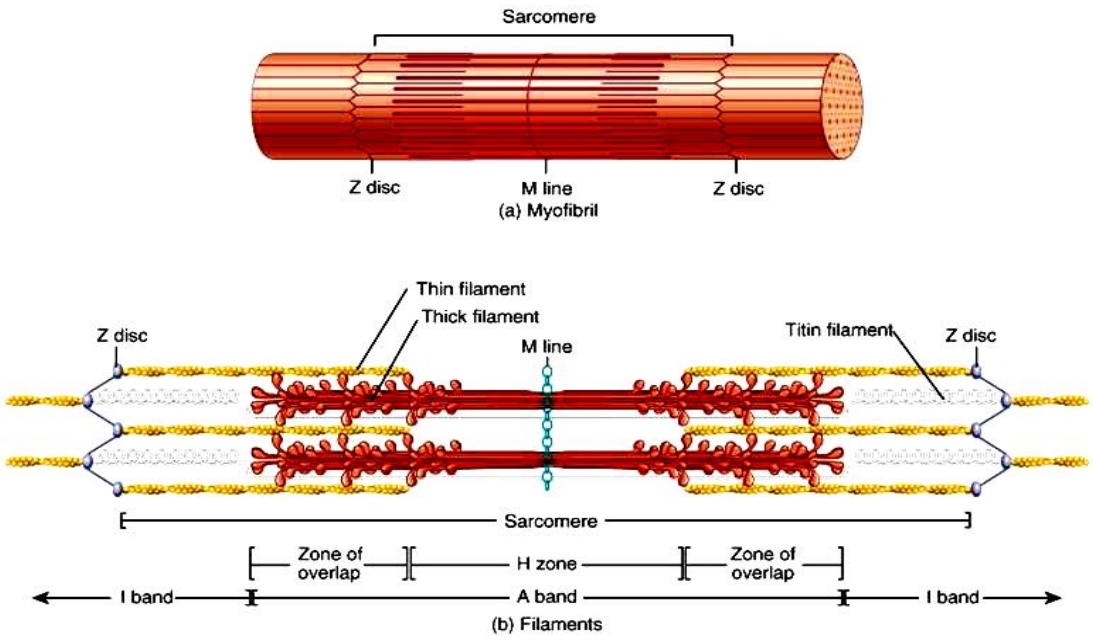

Figure 2: The sarcomere: the smallest functional unit of the cardiomyocyte.

cardiac muscle contraction. Muscle contraction is inhibited by the Tn-Tm interaction when Ca^{2+} concentration is sub-micromolar, at approximately 2x 10^{-7} M. When a micromolar concentration of Ca^{2+} exists, the N-terminal binding site of cTnC binds calcium and triggers cardiac troponin T (cTnT) to displace tropomyosin and expose the myosin binding site on actin. The myosin head then binds to actin in the cardiac myofilaments. cTnC does not directly interact with actin or tropomyosin , so the Ca^{2+} binding signal is transmitted indirectly to the thin filament by way of cardiac troponin I (cTnI) and cTnT. Cardiac troponin I, the inhibitory regulator of the troponin complex binds to actin and the actin-tropomyosin complex. It functions mainly to inhibit actin and myosin from interacting in the absence of Ca^{2+}. cTnT bound to tropomyosin and cTnI represents the cardiac muscle in a relaxed state where myosin is not bound to actin. cTnI, known for its

inhibitory character promotes cardiac relaxation by preventing ATPase activity of actomyosin by binding to actin and cTnT. When Ca^{2+} binds to cTnC, a conformational change takes place and causes cTnI to shift. In turn, through its interaction with cTnT, tropomyosin vacates the binding site for myosin on actin and causes force generating interactions or contractions. cTnI has only one isoform in the mammalian heart, with molecular weight 23.876 kilodaltons (kDa) and consists of 210 amino acid residues. This inhibitory subunit has several functional domains which include a cardiac specific N-terminal extension, an IT arm region, the inhibitory region, the switch region and C-terminal mobile domain. cTnI has two serine residues (Ser $_{23/24}$ in mouse cTnI) that are substrates for several different kinases during regulating muscle contraction under physiological and pathological conditions. Phosphorylated cTnI can be detected in a person's bloodstream and acts as reliable evidence for cardiac muscle injury.

1.4 Mutations of Cardiac Troponin I and cardiac disorders

As one of the sarcomeric proteins, cTnI plays a central role in the Ca^{2+} regulation of contraction in cardiac muscle. Over 200 mutations in cardiac sarcomeric proteins including myosin heavy and light chains, actin, troponin, tropomyosin, myosin binding protein-C and titin have been determined to cause various types of cardiomyopathies in human since 1990. Greater than 60 mutations in human cardiac troponin subunits are identified in dilated, hypertrophic and restrictive cardiomypathy (Harada et al., 2004). Studies on cardiac troponin mutations which include those of cTnT, cTnC and cTnI provide evidence that RCM-linked mutations increase cardiac myofilament Ca^{2+} sensitivity. cTnI is the main inhibitor of muscle contraction and its deficiency greatly

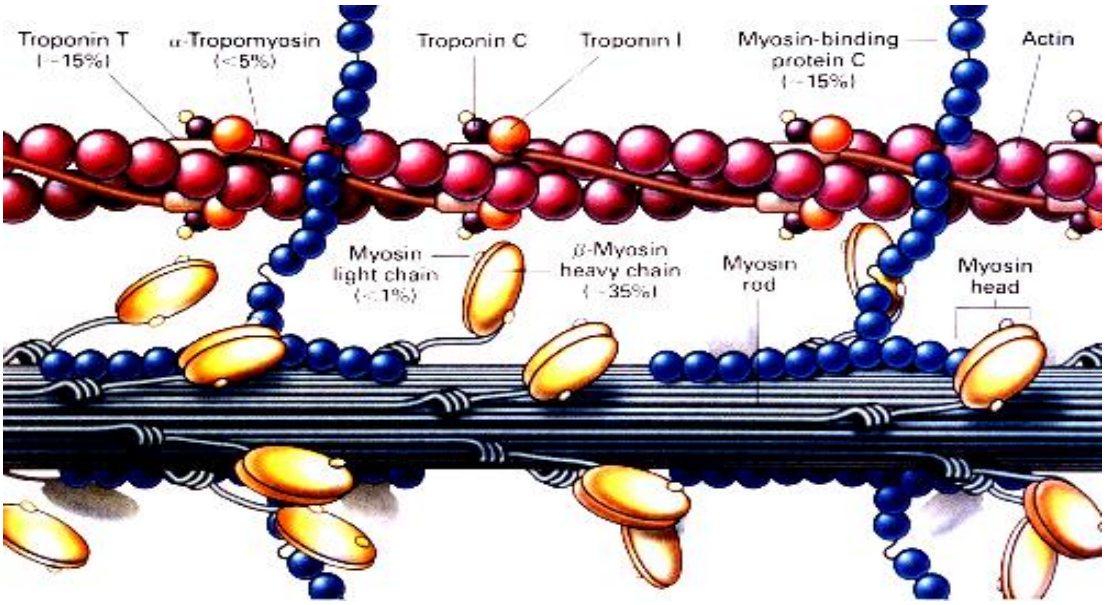

Figure 3: Troponin Complex and other contractile proteins.

impairs cardiac relaxation. According to Mogensen *et al.* six missense cTnI mutations are linked to RCM. They include L144Q, R145W, A171T, K178E, D190G and R192H. In humans the TNNI3 mutations are located within a highly conserved portion of the (cTnI) gene. All the cTnI RCM causing mutations are found to be located in the C- terminal region of cTnI , which contains the inhibitory region and two actin-tropomyosin sites (Jean Charles et al., 2011). The C-terminus of cTnI is the most conserved structure of cTnI and it interacts with tropomyosin based on the presence of Ca^{2+}. Mogensen *et al.*, also noted that R192H and K173E mutations occur *de novo* and are the worst missense mutations of cTnI, resulting in an earlier onset and a more severe disease manifestation. Missense mutations or point mutations are said to be the cause of inheritable restrictive cardiomyopathy. R192H, a point mutation in the C-terminus of human cTnI is caused by a single nucleotide change resulting in the codon which encodes histidine, not arginine. Most of these mutations are localized to specific regions of cardiac troponin and termed

as mutational “hotspots” (Paratvar et al,. 2010). These mutations affect muscle force generation and myofibrillar ATPase activity because of a Ca^{2+} sensitizing effect on muscle contraction. Ca^{2+} sensitizing effects of these six missense cTnI RCM mutations are much greater than the cTnI mutations causing HCM. RCM mutations result in an increased affinity for Ca^{2+} at pCa 4. These mutations in the C terminus of cTnI cause its conformational consequences to affect its interaction with other troponin subunits and tropomyosin, resulting in diastolic dysfunction (Akhter et al., 2013). Confirming the significance of these findings requires that investigations be performed *in vivo* and necessitates the development of animal models.

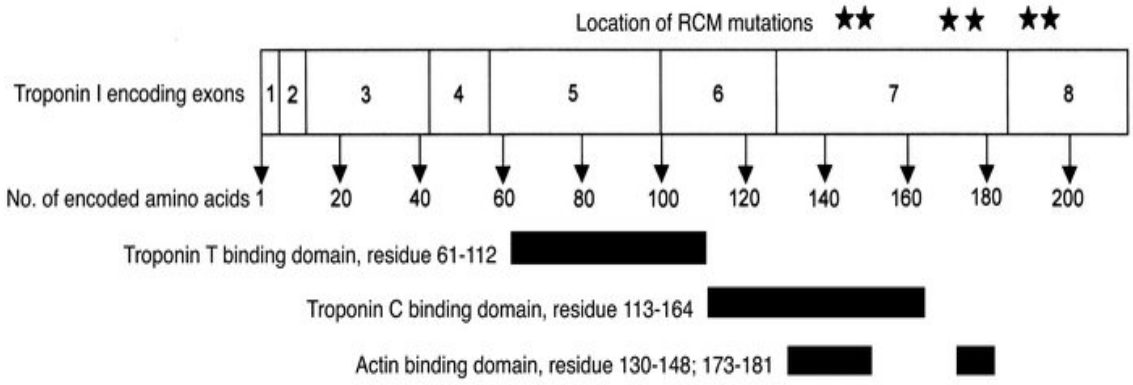

Figure 4: The locations of the six missense mutations in the C terminus of cardiac troponin I causing restrictive cardiomyopathy

1.5 Characterization of the NH_2 terminal extension of cTnI

Cardiac troponin I (cTnI) isoform contains a unique N-terminal extension which is found to modify activation of cardiac myofilaments. Troponin I is encoded by three homologous genes which produce isoforms which include fast skeletal, slow skeletal and cardiac TnI. Of the three, cardiac TnI is said to be the most evolve and possesses a unique

terminal peptide of 32 amino acids which functions to modulate cardiac muscle contraction. Compared to embryonic cardiac muscle which contains slow skeletal isoform of TnI (ssTnI), cTnI is specific to the adult heart and has an NH_2 terminal extension. Phosphorylation of the serine residues located in the NH_2 terminal (N-terminal) extension promotes cardiac relaxation by causing reduced affinity of cTnI for cTnC. Post translational modifications of sarcomeric proteins such as phosphorylation of ser 23and 24 occurs through the cAMP-dependent protein kinase A (PKA) and affect myofilament Ca^{2+} sensitivity. cTnI phosphorylation occurs in ser 23and 24 during β-adrenergic stimulaton and facilitates cardiac relaxation and decreases Ca^{2+} sensitivity in rodents (Kentish et al,. 2001)

How the NH_2 terminal extension affect the transduction of Ca^{2+} signaling between cTnI and the thin filament is not known. However, it is proposed that phosphorylation of cTnI induces a modified state of cTnI activity , and does not alter an intrinsic effect of the N-terminal extension during Ca^{2+} activation (Guo et al., 1994) This N-terminal extension contributes to the molecular conformation and function of cTnI (Shirin et al., 2011).

1.6 Role of NH2 terminal deletion in cardiac relaxation

By way of transduction, cardiac troponin I (cTnI) is essential in promoting the activation of myofilaments when calcium binds to cardiac troponin C (cTnC). Regions of the cTnI C-terminus, which are inhibitory peptides has proven to be essential in full inhibitory activity and Ca^{2+} sensitivity of cardiac myofilaments. While the heart undergoes cardiac remodeling, restrictive proteolysis of the N-terminal extension of cTnI alters myofilament

regulation (Biesiadecki et al., 2010). Cardiac N-terminal deletion is a structural change of 28-32 amino acids being removed with a functional consequence. Removal of this NH_2 terminal extension by way of restrictive proteolysis promotes functional adaptation to hemodynamic stress This N-terminal extension contributes to the molecular conformation and function of cTnI (Shirin et al., 2011). The N-terminus of cTnI binds to cTnC, however, little is known about its role in regulating and activation of cardiac myofilaments (Rarick et al., 1999). However, more recent studies have shown that physiologically occurring restrictive cleavage of the N- terminal of cTnI (cTnI ND) regulates myocardial function instead of destroying cTnI protein.

1.7 Deletion of the N-terminal extension- a therapeutic model for RCM

1.7.1 Structure and generation of N-terminal deletion mouse model

Cardiac N-terminal deletion is a structural change of 30 amino acids being removed with a functional consequence. A mouse model expressing cTnI ND in the absence of endogenous cTnI, contains 100% cTnI ND survived to adulthood and had normal activities (Feng et al., 2009). The transgenic cTnI- ND mouse model have altered cTnI phosphorylation and exhibits decreased β myosin heavy chain expression compared to the non-transgenic mice (Biesiadecki et al., 2010). Functional investigations of transgenic mice expressing cTnI with deletion of its N-terminal have shown improved diastolic function, with a better left ventricular compliance and a faster rate of relaxation compared to mice with normal hearts. All mammalian hearts investigated to date contain a small amount of the cardiac TnI N-terminal deleted (cTnI ND) molecule incorporated into the myofilaments. Proteolysis of the N-terminal of cTnI can be detected at low levels in the

normal heart and is said to occur as a functional adaptation to physiological, stress and simulated microgravity conditions (Yu et al., 2001). Therefore, the cTnI ND protein can be incorporated into the myofilament to produce a transgenic mouse model which rescues a cTnI null mutant. This cTnI ND mouse shows no indication of change in systolic function, but has a 100% rescued diastolic function.

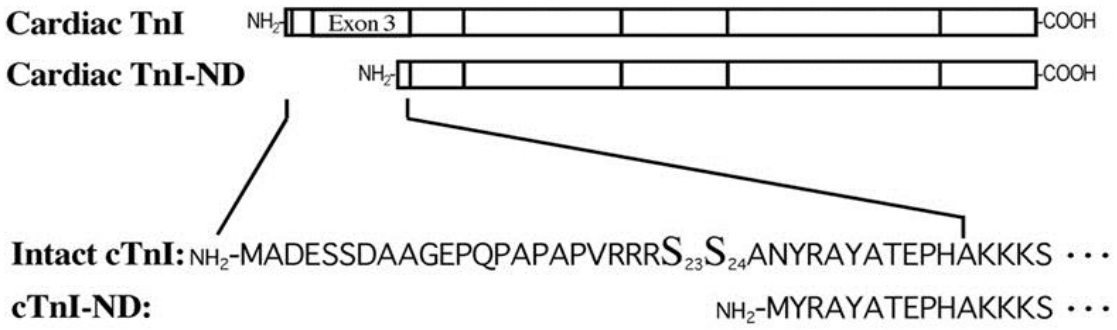

Figure 5: Structural maps of cardiac troponin I and cardiac troponin I ND.

The N-terminal deleted cTnI (cTnI ND) mutant does not contain the ser 23 and 24 amino acid residues, unlike the native cTnI isoform. However, the cTnI ND mutant has the same properties as the phosphorylated form of the native cTnI isoform, which shows reduced Ca^{2+} sensitivity of the cardiomyofilament and an increased rate of cardiac relaxation. This is similar to the physiological effects observed when WT cTnI mice undergo β adrenergic stimulation. Restrictive proteolysis of the N-terminal extension plays a compensatory role to improve diastolic dysfunction this feature is exploited in attempting to correct the effects of RCM.

1.8 RCM in transgenic mouse models

Investigations into restrictive cardiomyopathy (RCM) have led to transgenic animal models being generated to mimic the mutations of the disease observed in humans. Engineering these animal models is important in studying the patho-physiology of the RCM. The development of animal models having these single gene mutations will elucidate the knowledge needed to understand the mechanism of RCM, methods for its prevention and treatment as well as other diseases linked to diastolic dysfunction. The use of mouse models helps to confirm if the mutations observed are responsible for causing the restrictive patho-physiology seen in the diseased individual. In addition, it supports investigations of how gene mutations can affect the level of the mutant protein expression and the physiological function of the mouse. Due to the clinical heterogeneity observed in the causes of RCM in humans, transgenesis in mice helps to pinpoint the specific source(s) responsible for the disease, while adhering to the ethical standards of biomedical research. Having a short life-span compared to humans the progression of the disease can be fully monitored at various stages in the mouse models unlike in humans. The cTnI R193H transgenic mouse model provides an avenue for extensive evaluation as the disease progresses from impaired relaxation to manifesting diastolic dysfunction in one year old mice. It has been observed that the cardiac performance in particular, the cardiac ejection fraction (EF) worsened in mice at age 11 months compared to those 6-8 weeks old. In addition, histological samples were obtained, analyzed and studied using cellular assays to determine molecular, metabolic, morphological and functional changes in the mutant myofibrils and cardiomyocytes (Jean Charles et al., 2011). With the mutant protein being naturally incorporated into the mouse's genome and specifically in the thin

filament, the phenotypic effects are certain when determining characteristics of the disease. The argument for the importance of the RCM mouse model is further supported by the decreased variability among the murine subjects which contributes to consistency in data collection and the exclusion of external environmental factors which could influence data production. The diagnosis of RCM is based on the morphological appearances of the heart, but more so, on the functional properties of the working heart (Parvatiyar et al., 2010). The R192H mutation in the cTnI C-terminus, where arginine 192 is substituted for histidine in the human heart is modeled by heart specific expression of the mutated protein (cTnI R193H) in the transgenic mouse sequence (Du et al.,2006).

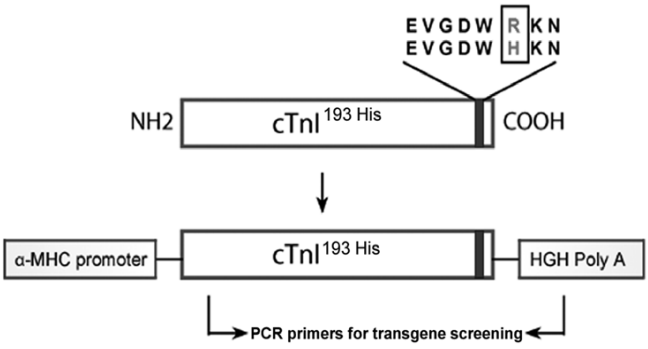

Figure 6: The transgene construct of the R193H mutation in the mouse genome, synonymous with the R192H mutation in the human genome

The main functional consequence observed by way of echocardiography in the cTnI R193H transgenic mice is impaired cardiac relaxation. Phenotypic indications of the disease include decreased left ventricular end diastolic dimension (LVEDD), increased dimensions in both atria at the end of diastole and a reduction in the ejection fraction compared to the wild-type mice. Cardiac function evaluated in cTnI R193H mice is best done with trans-thoracic echocardiography. Along with echocardiography, the RCM transgenic mouse model provides an excellent standard for evaluating the patho-

physiological evaluation of RCM and other cardiovascular diseases. The cTnI R193H mouse is critical in showing specific genetic consideration and factors which appear to be responsible for the progress of cTnI mutation-based cardiac disorders such as RCM.

1.9 Restrictive Cardiomyopathy and its clinical observations

As one of the cardiomyopathies studied, RCM has proven to be one of the most complicated and a rare disease of the heart. Genetic analyses of cardiomyopathies have shed some light on the familial inheritable and idiopathic causes of the disease. The results have provided us with answers indicating a large number of mutations located in genes encoding sarcomeric proteins. Mutations have been identified in cardiac genes encoding for desmin, α actin, troponin I and troponin T (Paratiyar et al., 2010). RCM familial inheritability has been linked to a novel missense mutation (D190H) in a conserved region of the cTnI gene (TNNI3) which has appeared in a separate group of family members presenting symptoms of the cardiac disease (Paratiyar et al., 2010). However, patients with idiopathic RCM have also been identified and studied. Genetic investigations have elucidated two different de novo mutations in children, (R192H and K173E) in the TNNI3 gene. In addition, cases of later age-related onset of RCM have been identified as (R145W, A171T and L144Q) TNNI3 mutations. These mutations have been discovered as localized in a specific region of cTnI in the C terminus, an area for actin/actomyosin binding sites. The common effect caused by the mutations of these sarcomeric proteins is alterations in cardiac performance. The result is abnormal diastolic filling, which can also be described as reduced ventricular compliance or increased stiffness of the cardiomyocytes. RCM is characterized as impaired ventricular filling with normal or decreased diastolic volume of either or both ventricles due to abnormal

rigidity. The disease causes an increase in end-diastolic pressure and normal or close to normal systolic function and sometimes wall thickness. In general, both atria become dilated due to a restriction in ventricular filling. The morphological appearance of the disease results from either fibrotic scarring of the endomyocardium or infiltration of the myocardium from foreign substances. Clinical evaluations of RCM patients show that there is difficulty breathing and pulmonary edema as a result of the lung involvement. Restrictive Cardiomyopathy causes severe cardiac dysfunction, has the worst prognosis and is associated with sudden death (Malcic et al., 2002). The incidence of mortality is high among RCM patients, more than half the patients diagnosed die within two years. Diagnoses are often made in children and represent about 2-5% of all pediatric cardiomyopathies (Jean-Charles et al., 2011). Although most of the RCM cases have been deemed idiopathic, several pediatric cases have been identified as familial, and so indicate autosomal dominant patterns. The causes of RCM are heterogeneous, so the potential for misdiagnosis occurs during the clinical evaluation of patients with RCM. The most common conditions which have similar features are amylodosis and constrictive pericarditis. Amylodosis is characterized by insoluble misfolded amyloid fibrils deposit within tissues including the heart. One form is hereditary amylodosis it is an autosomal dominant type of the disease which amyloid fibrils form from point mutations in the protein tranthyretin. This disease has systemic consequences and affects the vascular system, lungs, kidney, muscles as well as the heart. Cardiac involvement is pronounced by deposits of amyloid fibrils between myocardial fibers in the ventricles and atria. The infiltrating amyloid protein feature typically relates to the development of RCM as a secondary development. Even though amylodosis can progress into RCM, it

should emphasized RCM can occur without being secondary disease to amylodosis. Constrictive Pericarditis and RCM also share nearly identical symptoms, physical signs and hemodynamic profiles. The difference exists in the fact that constrictive pericarditis is correctable and RCM is rarely treatable. Differentiating the diseases relies on using diagnostic tools such as magnetic resonance imaging (MRI), computerized tomography (CT) and transvenous endomyocardial biopsies. CT and MRI can identify thickened pericardium seen in constrictive pericarditis, this is not appreciable in RCM. Treatments for RCM include salt restriction, calcium antagonists, β blockers, and diuretics, which improve systematic and pulmonary congestion. These therapeutic measures are modeled to target the symptoms of the disease, not RCM itself. β blockers reduce the heart rate and the mortality rate in post-infarct and heart failure (Jean-Charles et al.,2011), so this is a possible treatment for RCM with signs of myocardial ischemia. The challenge is that the treatment options that are available for diastolic dysfunction are not effective for RCM, and they are used only to relieve the symptoms that are secondary to the disease. Treating the patients with these medications have not been proven to prolong life, however the benefits are evident in alleviating of some symptoms. The stage of diastolic dysfunction or failure is a reliable indicator for the prognosis of abnormal relaxation as it relates to left ventricular compliance and the development of heart failure. A dependable assessment diastolic dysfunction is achieved with a non-invasive method such as Doppler echocardiography.

1.10 The Use of High Resolution Echocardiography in transgenic mice

Accurate evaluation of cardiac deficiency is done best using echocardiography. Murine models have become important in studying the molecular mechanisms of cardiac

dysfunction which are due to mutant gene products because of change in gene expression. Transgenic mouse models in this study are mutant mice which allow us to mimic the behavior and course of diastolic diseases. Restrictive cardiomyophathy (RCM) is one such disease in which the functional and structural alterations of the heart can be captured and evaluated with echocardiographic imaging. Impaired ventricular relaxation manifested by a decreased left ventricular end diastolic dimension (LVEDD) and increased diastolic dimensions in both atria are hallmarks of RCM. Trans-thoracic echocardiography provides an *in vivo* method for evaluation of these abnormal cardiac phenotypic characteristics. The trans-thoracic echocardiographic protocol used to evaluate RCM includes pulse wave Doppler (PWD) and tissue Doppler (TDI). Both provide dimensional measurements of the mouse heart and to quantify the degree of diastolic and systolic performance. M mode imaging is also used, it provides an opportunity to acquire images of the left ventricle in a one dimensional view to detect abnormal cardiac structure and movements of the left ventricle. Employing all three methods of the imaging protocol allows for longitudinal studies to be performed equally on the same animal, therefore ensuring consistency in data collection and evaluation. The use of transgenic mouse models for investigating the mechanism for cardiac growth and function demands that there is a development of non-invasive methods in assessing cardiac anatomy, physiology and size. More invasive methods of evaluating the left ventricular (LV) function include cardiac catheterization and necropsy, but these are more terminal methods and provide no information about chamber anatomy and dimensions (Gardin et. al., 1995). Non-invasive echocardiography is an alternative to the terminal methods mentioned and indirectly assesses intra-cardiac pressure/volume loop

measurements. Calculating the LV chamber dimensions with trans-thoracic M-mode echocardiography assesses the enlargement the chamber or increased wall thickness as reliable indications in various types of heart disease. Trans-thoracic echocardiography which includes M-mode imaging, PWD and TDI provides a comprehensive non-invasive evaluation of systolic and diastolic cardiac function.

The small size and rapid heart rates of up to 600 beats per minute (bpm) require high resolution imaging Acquiring highly detailed images using trans-thoracic echocardiography contributes to accurate functional assessment of diastolic dysfunction and requires the use of high frequency transducer technology. The Vevo 770 ™ High Resolution In Vivo Imaging System (Figure 8) was used in this study and provided the dynamic image acquisitions, optimal sensitivity, depth penetration, and resolution needed

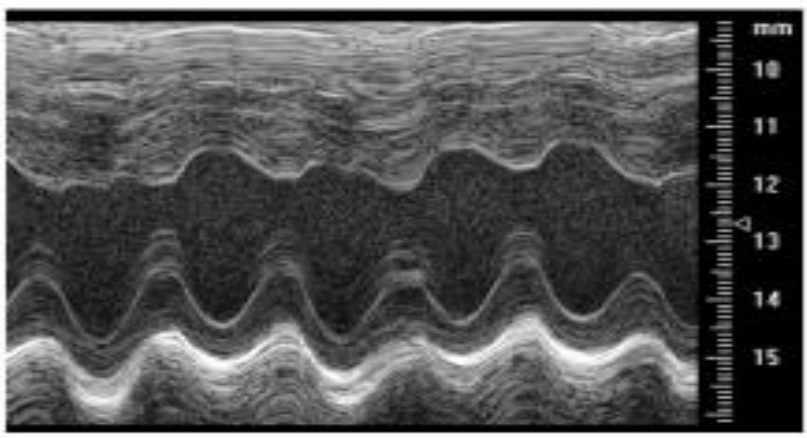

Figure 7: Echocardiographic image in M mode of the left ventricle in a wild type mouse.

in small animal imaging. In patients, echocardiography is a valuable tool in diagnosing because diastolic dysfunction is difficult to assess using clinical examinations which include chest radiography and EKG.

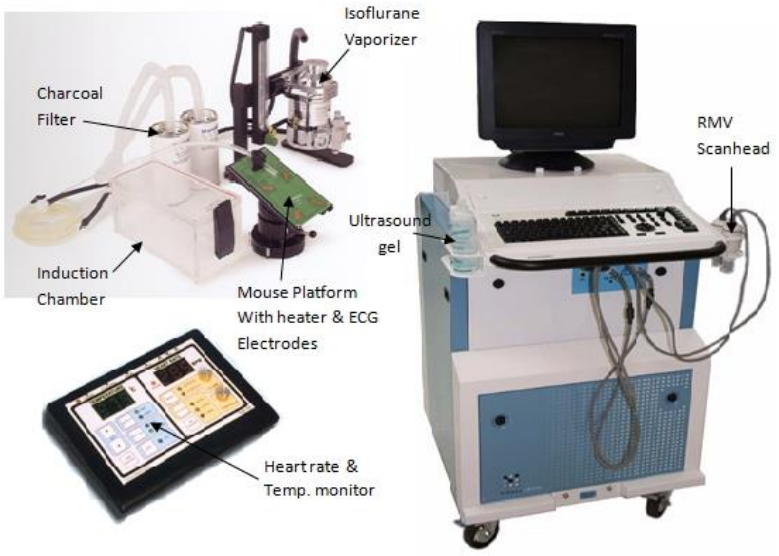

Figure 8: Visual Sonics Vevo 770 ® High Resolution Ultrasound scanner, with the accessory equipment used for trans-thoracic echocardiography

1.11 Summary of my Research Project

With use of transgenic mouse models, the goal of my study is to investigate whether or not the cardiac troponin I N- terminal extension deletion mutation corrects the debilitating effects of the R193H mutation in the cTnI R193H/KO mouse. The first restrictive cardiomyopathy (RCM) transgenic mouse model, cTnI R193H was created in our laboratory. From this transgenic mouse we found that diastolic dysfunction and heart failure was dose-dependently linked to the cTnI mutation. The cTnI R193H/KO transgenic mouse expresses the highest level of the mutant cTnI in the heart and causes in severe diastolic dysfunction, subsequent systolic dysfunction, heart failure and very early death. Previously in our laboratory it was found that by crossing the cTnI R193H mice with transgenic mice expressing an N-terminal truncated cTnI (cTnI-ND), the ventricular relaxation was enhanced. The presence of cTnI-ND significantly improved cardiac function in the double transgenic mouse as supported by echocardiogram data. In my study, by generating a new mouse model we can now investigate the effectiveness of the N terminal cTnI mutation on the high "dose" cTnI R193H mutation. The new double transgenic mouse model cTnI R193H/KO/ND reflects the genotype of these two transgenes with wild type cTnI null background. The hypothesis that introduction of N-terminal truncated cTnI into RCM mouse model will correct the impaired cardiac diastolic function and rescue the R193H high expressing transgenic mice was tested. By producing the cTnI R193H/KO and cTnI R193H/KO/ND mouse models, determining their genotypes using PCR-based assays, detecting and confirming the protein expression levels, and performing cardiac functional assessment with trans-thoracic

echocardiography, an investigation aimed at genetic and physiological assessment of RCM continues.

CHAPTER 2: RESEARCH DESIGN AND METHODS

2.1 Transgenic Mouse Generation

2.1.1 Welfare of Animals

This investigation conformed to the Guide for the Care and Use of Laboratory Animals (US National Institute of Health Publication No. 85-23, revised 1996) and in accordance with the protocols approved by the Institutional Animal Care and Use Committee at Florida Atlantic University.

2.1.2 Producing the cTnI R193H/KO and cTnI KO/ND mice

A series of crossbreeding steps among cTnI R193H, cTnI /HT and cTnI/ND mice were carried out in our laboratory. To produce the mice needed for this study, the cTnI KO/ND produced by restrictive proteolysis of the cTnI N-terminal and reconstituted with a null cTnI background was crossed with the original cTnI R193H mouse. The result of this crossbreeding provided us with the cTnI R193H/ND/HT mice from which a male and female were subsequently mated. Concurrently, a cTnI R193H transgenic mouse was mated with a cTnI HT mouse. The two sets of crossbreeding provided two intermediate mouse lines: cTnI R193H/HT (breeder1) and first generation of cTnI R193H/KO/ND (breeder2). Then, by mating breeder1 with breeder2, the cTnI R193H/KO and the cTnI R193H/KO/ND double transgenic mice needed in this study were produced. The mice

with the desired phenotype were tested and confirmed by genotyping. Following all experiments the cardiac samples were harvested and Western blotting was performed to evaluate the replacement rate of the mutant proteins into the genome and confirm the expression level of the mutant protein. Due to the loss in dose expression of R193H, the first generation cTnI R193H/ND/KO was not used in this study. The breeding strategy is presented in Figure 9.

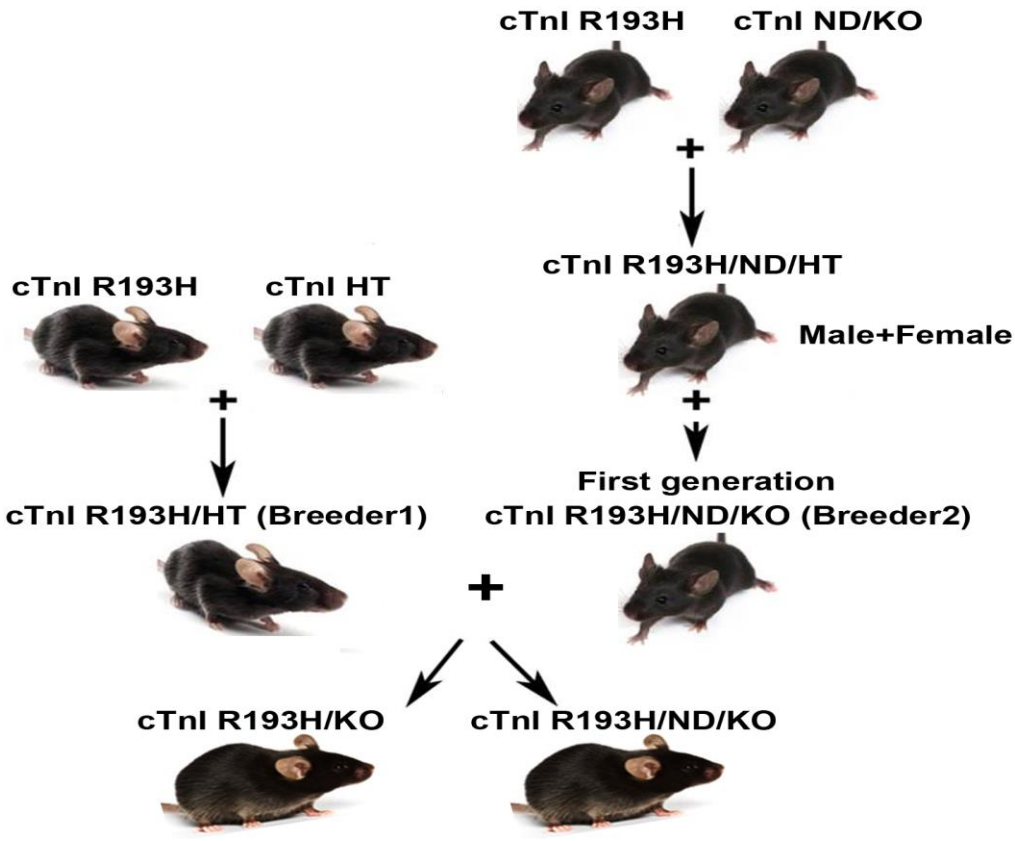

Figure 9: Breeding strategy used to produce the cTnI R193H/KO and cTnI R193H/KO/ND mice

2.2 Profile of the cTnI R193H/KO transgenic mouse

The genetic characteristics of the cTnI R193H/KO mouse results in a combined phenotypic profile of the cTnI KO and the cTnI R193H transgenic mice, which were both previously generated in our laboratory. The cTnI R193H/KO is termed 'high expression' and is reflective of the protein expression ratio of the R193H mutant and cTnI in the mice. In the cTnI KO mouse the cTnI isoform is deleted from the mouse's genome (Huang et al., 1999). Like the cTnI KO mouse, the transgenic cTnI R193H/KO mouse has a null cTnI background and therefore the regulatory cTnI subunit of the troponin complex is missing The R193H mutation in the cTnI R193H/KO is responsible for its RCM characteristics. These include increased end diastolic pressure, decreased left ventricular end diastolic dimension and biatrial enlargement at the end of diastole .Another similarity between the cTnI/KO and cTnI R193H/KO mice is they were born healthy with normal heart and body weight. The mice have phenotypic characteristics that are common to both transgenes. However it should be noted that the production the cTnI R193H/KO transgenic mouse is not merely due to cross breeding of cTnI KO and cTnI R193H mice. The cTnI R193H/KO mice used in this study were produced by cross breeding cTnI R193H/HT and cTnI R193H/KO/ND mice, as shown in figure 9.

2.3 Dose- dependent effect of the cTnI R193H mutant in the cTnI R193H/KO mice

The high expression, cTnI R193/KO mice were produced as described previously, by mating cTnI R193H/HT (breeder1) with cTnI R193H/KO/ND (breeder2). Previous experiments have determined that mutations of cardiac troponin I cause RCM which results in a large increase in Ca^{2+} sensitivity of tension, impaired ability to inhibit the

myosin ATPase activity and increased basal force (Parvatiyar et al., 2010). The physiological abnormalities contribute to diastolic dysfunction, heart failure and death. Diastolic dysfunction is the primary characteristic of RCM. The genotype of the cTnI/KO mice has a deleted cTnI gene, and therefore has a homozygous mutation. A feature of the cTnI/KO mice is that they are viable and healthy only because of the presence of the fetal isoform of TnI. The fetal isoform is identical to the ssTnI (the slow skeletal isoform) and compensate for the absence of cTnI (Huang et al., 1999). This compensatory process is temporary because ssTnI is depleted approximately two weeks after birth. Since there is insufficient TnI in heart to maintain normal cardiac function, the cTnI/KO mice dies 17-18 days after birth (Huang et al., 1999). When the R193H mutant is expressed in the cTnI/KO mice, the RCM mutant dose is expressed at a level higher than in the original cTnI R193H transgenic mouse. cTnI R193H/KO mice expresses the mutant cTnI R193H and no wild type cTnI background (Li et al., 2013).The phenotypic characteristics of RCM is amplified in the cTnI R193H/KO mutant mouse model and represents mice which have the highest level of the mutant protein. Prolonged relaxation time and delayed Ca2+ decay are observed in the mutant cardiomyocytes and correlates with the level of the mutant protein. Diastolic dysfunction caused by R193H mutation of cardiac troponin I is specific and shows dose dependency (Li et al., 2013). The high expression mouse having the highest R193H mutant dose, (figure 10) contains 80% R193H and 20% ssTnI, being that this mouse model has a null cTnI background.

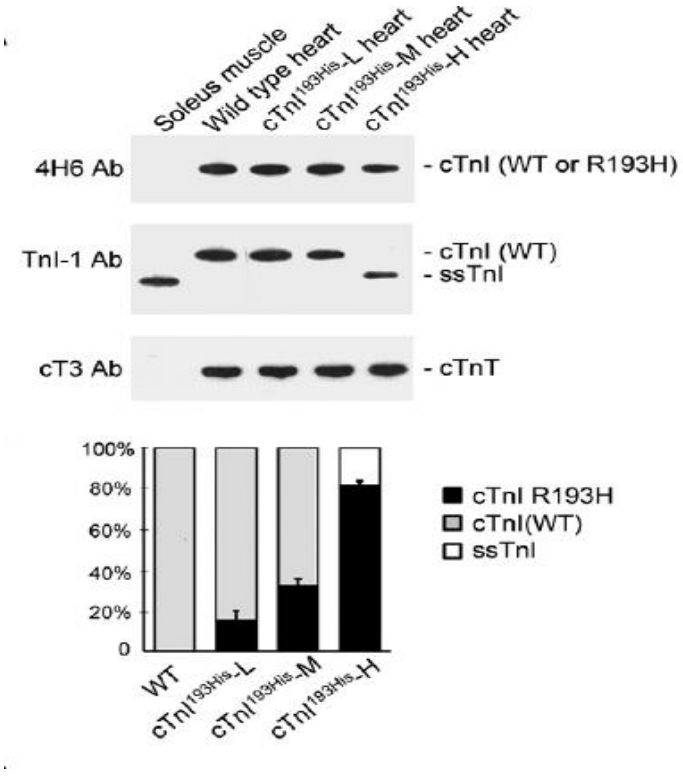

Figure 10: Western Blot showing varying levels of cTnI R193H mutant protein in the hearts of RCM transgenic lines.

2.4 Profile of the cTnI R193H/KO/ND double transgenic mouse model

The double transgenic mice (cTnI R193H/KO/ND) were produced as described previously in figure 9, by mating cTnI R193H/HT (breeder1) with cTnI R193H/KO/ND (breeder2). Breeder 1 and Breeder 2 were produced from an F1 generation of crossing cTnI R193H with cTnI HT mice and cTnI R193H with cTnI ND mice respectively. Mating breeder 1 with breeder 2 produced the double transgenic mice (DTG) expressing only R193H and N-terminal deletion mutants in the cardiac muscle during adulthood. PCR based assays were conducted to detect the presence of the transgenes. Western blotting was performed to confirm the presence and the level of expression for both transgenes and the absence of the cTnI wild type background. One aspect of the profile of

the DTG mice is they lack the cTnI gene and the 30 amino acid residue NH_2 extension that is specific to the adult heart. These mice which are genetically modified with the N-terminal extension deleted (cTnI ND) survive to adulthood and are maintained as distinct colonies in our laboratory. It is understood that cTnI ND mutant increases the relaxation rate of the ventricular muscle, also increases the ability of heart to decrease preload and cardiac output.

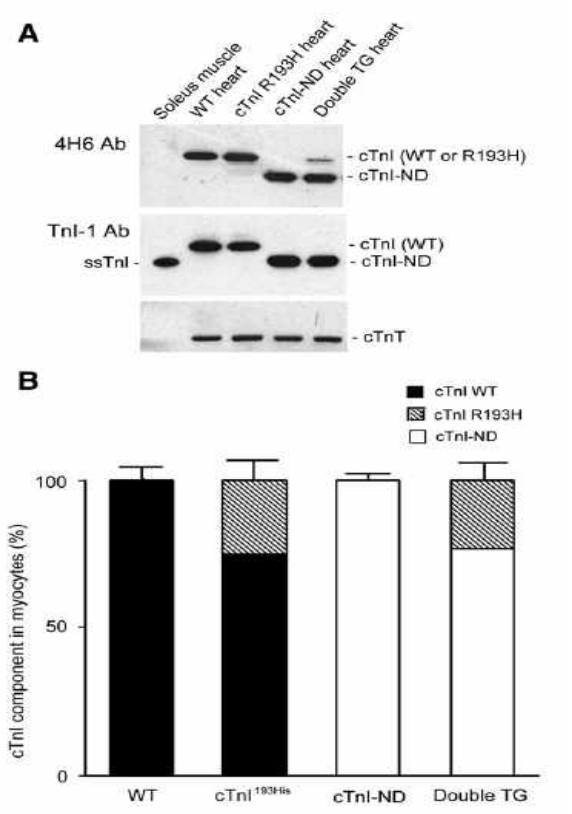

Figure 11: Western Blot showing protein expression level of cTnI wild type, cTnI ND and cTnI R193H among transgenic and double transgenic mice.

2.5 Genotyping

Offsprings obtained from the transgenic mouse lines were evaluated for genes expressed by PCR-based genotyping assays. PCR was not performed on the wild type mice. Genomic DNA was extracted from mouse tails by using Puregene Core Kit A (QIAGEN) following the manufacturer's instructions. Specific primers were designed to detect wild type cTnI gene, transgene cTnI R193H, and transgene cTnI-ND. Their primer sequences are demonstrated as follows: WT cTnI/KO: 5' primer, 5'-TAGGTGTGAGGACAGAAGGCCG (derived from cTnI gene upstream region), 3' primer 1, 5'-CCGTGAAGAGSGAAATCACTGATGGTGGTCC (derived from cTnI gene), 3' primer 2, 5'-GTGGAATGTGTGCGAGGCCA (derived from NEO); Transgene cTnI R193H: 5' primer, 5'-ACGCCGCTCCTCTGCCAACTACC (derived from cTnI transgene), 3' primer, 5'-CACAGGGATGCCACCCATCAAGC (derived from HGH polyA region); Transgene cTnI-ND 5' primer, 5'-GGCACCCTTACCCCACATAGACT 3' primer, 5'-GTGTGGGATCCATGGCTCAGCCCT. Amplified DNA fragments were separated on a 1-1.8% agarose gel and visualized under UV light after staining with ethidium bromide.

2.6 Western Blotting

Following the trans-thoracic echocardiography, the hearts of the WT, cTnIR193/KO and cTnIR193/KO/ND were excised to confirm and compare the cardiac troponin I and ssTnI proteins versus ND and R193H mutant concentrations in the heart using Western Blot. The myofibrils proteins were extracted from the mouse hearts with known genotypes. These samples were prepared from freshly isolated mouse hearts and were resolved in

NuPAGE 4-12% gradient Bis-Tris gels using an Xcell II sure lock Mini Cell gel system from Invitrogen. The proteins on the page gel were transferred to a nitrocellulose membrane using the Xcell II blot module purchased from Invitrogen. The transferred nitrocellulose membrane were be blocked with 5% dry fat milk in TBS-T solution and incubated with anti-cTnI antibodies. Anti-cTnI antibody which recognized cTnI and ssTnI was used at the dilution ratio of 1:15000. Purified cTnI and ssTnI were used as standard markers. The bound antibodies detected on the immunoblots were visualized by enhanced chemiluminescence (ECL detection kit from GE Healthcare) using Licor system.

2.7 Functional assessments of the transgenic and double transgenic mice

2.7.1 Trans-thoracic Echocardiography of WT, cTnI R193/KO and cTnI R193/KO/ND mice

All echocardiograhic images and measurements were performed using a Vevo 770® High Resolution In Vivo Imaging System (VisualSonics, Toronto, ON, Canada). A high resolution 30MHz transducer was used to measure and compare the cardiac function of the three mouse lines: wild type, cTnI R193H/KO and cTnI R193H/KO/ND. At 18-20 days the wild type and cTnI R193H/KO/ND had an average mass of 8g, while the cTnI R193H/KO had an average mass of 10g. Three mice from each group were evaluated, for consistency the same echocardiography protocol was used among them. All images were collected with all animals having an average heart rate of 332 beats per minute. At the start of the examination each experimental mouse was anesthetized with isoflurane at a concentration of 3% and then maintained at 1.0% isoflurane by a facemask during the

entire procedure. The bodies' temperatures were maintained at 37ºC, while remaining on a 40 ºC platform. Hair on the precordial region was cleanly removed with a Nair lotion hair remover (Church & Dwight Canada Corp, Mississauga, ON Canada) and the region was covered with pre-warmed ultrasound transmission Aquasonic gel (Parker Laboratory, Inc, Fairfield, NJ). The ECG was recorded using the Indus THM100. A comprehensive trans-thoracic echocardiography examination was performed for a duration of 20-30 minutes. The protocol included: (i) apical four chamber view and/or two chamber views in B-mode (2D imaging), (ii) para-sternal short axis view in M-mode, and finally (iii) apical four chamber view for acquisition of Pulse Wave and Tissue Doppler imaging.

2.7.2 Apical Four Chamber and Two Chamber View in B-mode

In B-mode imaging, the apical four chamber and two chamber views allow the anatomy to be visualized in 2D acquisition. When the structures are well defined and the axes are at the correct angle, the images showing both atria in the apical four chamber view were captured. In addition, an image of the left atrium and left ventricle in a two-chamber was collected and stored. The dimensions of both atria were compared among all three groups.

2.7.3 Para-sternal Short Axis View in M-mode

Para-sternal short-axis view is visualized by placing the transducer horizontally on the heart area and rotating it 90 degrees clockwise. This view is a cross section of the left ventricle and shows a completely round view of it. The M-mode measurement was obtained at the level of the papillary muscle. The M-mode echocardiography produces one dimensional information on a time-motion graph. The data was analyzed by measuring the left ventricular end diastolic dimension, left ventricular end systolic

dimension, inter-ventricular septum, the posterior wall, the fractional shortening and the ejection fraction with the software package featured in the Vevo 770® High-Resolution In Vivo echocardiography machine.

2.7.4 Apical Four Chamber View in Pulse Wave and Tissue Doppler Imaging

Acquisition of mitral flow velocity and Tissue Doppler Mode Imaging (TDI) requires that images are orientated in the apical four-chamber view. An apical four chamber view was obtained from the lower left side of the animals' thorax. In this view, the image is captured so that the viewer appears to be looking from an inferior to superior view, that is, at the heart from the apex to its base. The animal was also placed in a 60 °C left lateral decubitus position by elevating the right side of the mouse platform so that the heart is displaced maximally towards the animals left side of its thorax.

2.7.5 Pulse Wave Doppler Echocardiographic Assessment of diastolic properties

Pulse Wave Doppler (PWD) studies were obtained in the apical four chamber view. With the transducer angled and placed in a transverse position with its notch facing the left side of the mouse, images quantifying early diastolic peak filling velocity (E) wave and atrial peak velocity (A) wave were obtained. In this view a sample volume was assessed with the transducer placed slightly above the level of the mitral valve leaflets. Two diastolic waves, 'E' and 'A' are seen in normal mitral doppler in-flow patterns. Useful mitral in-flow parameters were assessed, they include: early filling peak velocity (E); atrial peak velocity (A); E/A ratio, isovolumetric relaxation time (IVRT) and isovolumetric contraction time (IVCT).

2.7.6 Tissue Doppler Imaging for Evaluation of Left Ventricular Relaxation

Tissue Doppler Imaging (TDI) permits the evaluation of myocardial motion using Doppler ultrasound imaging. The mitral annulus velocity is measured in the apical four chamber view. The velocity of diastolic movement of the mitral annulus away from the cardiac apex and towards the base has two peaks, E’ and A’. The peaks correspond roughly to the E and A waves of Doppler trans-mitral flow. The ratio of early filling trans-mitral peak velocity (E) to the DTI Mitral valve E Velocity (E') is a reliable guide to determine diastolic dysfunction.

CHAPTER 3: EXPERIMENTAL RESULTS

3.1 Breeding of the transgenic mice

Offsprings of the transgenic breeders needed for this study were produced successfully. After several rounds of cross-breeding between the cTnI R193H/HT and the cTnI R193H/ND/KO transgenic mouse models, multiple litters of pups were produced. Breeding among the wild type controls were also initiated. All the breeders were generative and produced the expected number of offsprings in an acceptable time frame. The expected number of mice for each group: cTnI R193H/KO, cTnI R193H/ND/KO and WT were produced. Within 10-12 days after birth tail biopsies were performed on each pup in the transgenic litters so that DNA could be extracted prior to determining their specific genotype. The presence of cTnI R193H/KO (high expression mutant) and cTnI R193H/ND/KO (double transgenic mutant mice) being among the litters of pups was confirmed using genotyping PCR-based assays.

3.2 Genotyping PCR-based assays

Using PCR-based assays, the genotype of each experimental group was confirmed. R193H, Knockout and ND primers were used to show bands with various sized DNA fragments and confirmed with a molecular marker. This study indicated that the R193H primer detected bands for the R193H mutant gene of size 600 base pairs (bp) for all the mice used in the study. The consequence of the R193H band indicates the successful

incorporation of the mutant into the mouse genome, and the expectancy of phenotypic characteristics related to the RCM mutant. Analogous to the R192H mutation in humans, the presence gives reason to expect the development of diastolic dysfunction due to the effect the mutation. At the molecular level, the main effect in the R193H mutant mouse is shortened cell length of the cardiomyocytes (Du et al., 2008) resulting in impaired myocardial relaxation. The transcriptional expression the R193H mutant is expected to be observed in the cTnI R193H/KO, cTnI R193H/ND/KO and not their WT counterparts. The knockout primer used to confirm the presence of the cTnI gene by way of the detection of wild type cTnI gene, represented by a band of 630 bp. The detection of the knockout cTnI gene is illustrated with this primer as a band of size 390 bp. The value of this primer is such that it detects heterozygosity of the cTnI gene on the chromosomes, indicated by both the wild type cTnI band of 630 bp and the KO band represented by 390 bp. In addition to having the R193H band, the transgenic cTnI R193H/KO mice was found not to have the cTnI gene on both alleles and is characterized as having a null cTn I background. Using the KO primer, it showed that these mice possessed only the KO band, and for this reason the cTnI R193H/KO mouse line has a null cTnI background and is characterized as high expression mice. Detecting the presence of the N-terminal extension deleted mutant gene necessitates using the ND primer in some PCR-based assays. The ND bands represented by 750 bp, indicates the presence of the N-terminal extension deletion mutant. In these experiments, the DTG cTnI R193H/KO/ND were positive for the N-terminal deletion and R193H mutant genes. These mice were also found to have a null cTnI background. With no evidence of having the wild type cTnI band of 630 bp, however, having the KO band represented by 390 bp, they were

characterized as not having the cTnI gene in their genome. At the end of the genotyping experiments, three mice were chosen from each group: cTnI R193H/KO, cTnI R193H/ND/KO and WT for the representative sample sizes.

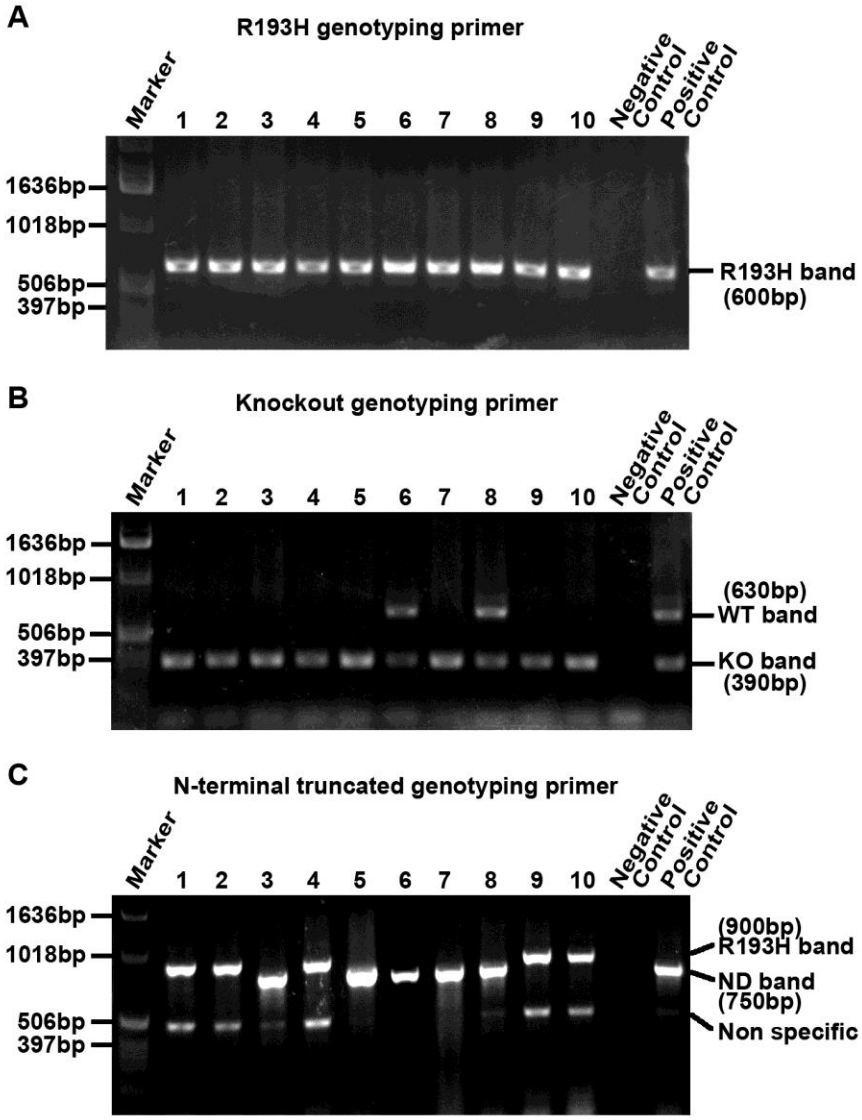

Figure 12: Genotyping results confirming transgene expressions. Specific primers used to detect wild type cTnI gene, R193H transgene and ND transgene

3.3 Western Blot

Evaluation of protein replacement of cTnI and ssTnI by the R193H and N-deletion mutants were performed with Western Blot assays after the echocardiograms. As observed in figure 13 extracts from the mice hearts and the soleus (leg) muscle, which was used as a control for ssTnI in this experiment, confirmed the dose and presence of cTnI and the ND mutant in all three groups: R193H/KO and R193/ND/KO and WT. The results of this Western Blot data showed that the monoclonal antibody (mAb) TnI1 detected the cTnI WT, cTnI ND and the ssTnI proteins but not R193H. The 4H6 mAb detected the R193H cTnI ND and WT cTnI but not ssTnI from the muscle. as observed in Figure 13.The cTnR193H mutant destroyed the TnI-1 epitope, but not the 4H6 epitope, allowing the distinction between cTnI WT and R193H proteins (Li et al., 2010). In figure 13 the hearts of the DTG mice suggests that there is a replacement of the cTnI protein by cTn ND and R1193H. The cTnI ND and ssTnI proteins have similar sized polypeptide chains, these proteins are often indistinguishable.

3.4 Trans-thoracic Echocardiography

Cardiac physiological and morphological comparisons of the three groups of 18 -20 days old wild type, high expression transgenic (cTnI R193H/KO) and double transgenic (cTnI R193H/KO/ND) mice showed variations specific to their genotype. B-mode, M-mode, Pulse Wave Doppler and Tissue Doppler images were collected from each mouse and evaluated. Three mice were examined from each group. The average body mass for the mice were 8.9g; WT, 8.97g; cTnR193H/KO and 8.97g; cTnR193H/KO/ND. Their heart

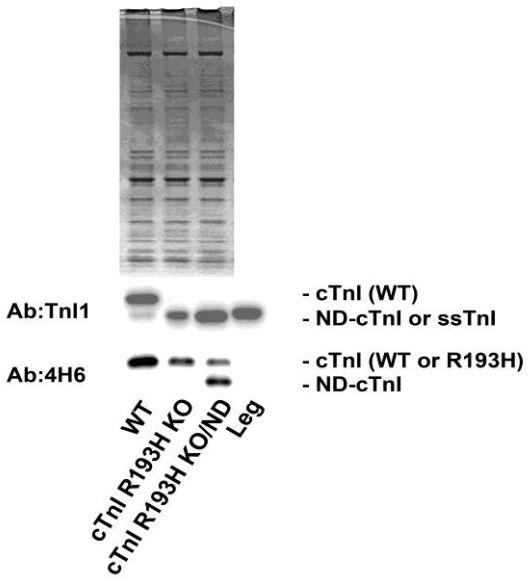

Figure 13: Western Blot of protein extracts from the hearts of WT, cTnI R193H/KO and cTnI R193/ND/KO mice. Protein expression levels are identified in all three groups using anti-cTnI (4H6) antibody and the TnI antibody

rates were maintained an average of 332 beats per minute.

3.4.1 B-Mode Imaging

The B-mode echocardiographic data indicate that the cTnI R193H/KO transgenic mice show significant morphological changes. The two chamber view shows an enlarged left atrium (figure 14) and the apical four chamber view show bi-atrial enlargement. Used as the control, the WT mice exhibits normal and similar sized atria compared to the DTG cTnI R193H/KO/ND group. The cardiac structure in double transgenic mouse did not show any difference compared to that of the wild type mouse group. This supports the likelihood of a functional correction by N-terminal deletion mutant in RCM phenotype.

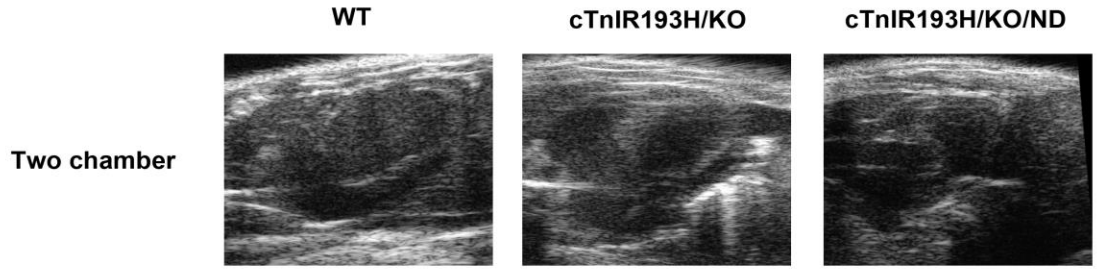

Figure 14: Two chamber echocardiographic images of the left ventricle and left atrium in B mode. An enlarged left atrium is seen in the cTnR193H/KO mice compared to normal left atrial dimensions in the WT and cTnR193H/KO/ND mice.

3.4.2 M-mode Imaging

High resolution echocardiographic imaging of the three groups: wild type, cTnI R193H/KO and cTnI R193H/KO/ND mice were performed in M-mode for a comprehensive evaluation of the left ventricular end diastolic dimension (LVEDD) and left ventricular end systolic dimension (LVESD), ejection fraction and fractional shortening. Table 1 summarizes the parameters used to evaluate diastolic dysfunction. The measurements showed evidence of left ventricular diastolic dysfunction among the high expression cTnI R193H/KO group. Among this group there was a marked decrease in the mechanical performance of the heart. The LVEDDs among the cTnI R193H/KO group showed significant reduction in dimensions compared to the WT and the cTnI R193H/KO/ND (figure15). No significant difference was seen in the LVEDD between the WT and cTnI R193H/KO/ND groups. The LVESD did not show much difference among all the animal models. The LVESD measured in the cTnI R193H/KO did not change, supporting the finding that RCM is not characterized by significant systolic abnormalities. Ejection fraction and fractional shortening among the WT and cTnI

R193H/KO/ND groups did not show significant differences. A slight reduction of 10% is seen among the cTnI R193H/KO group indicating the mice were experiencing heart failure during the examination. (Figure 15A)

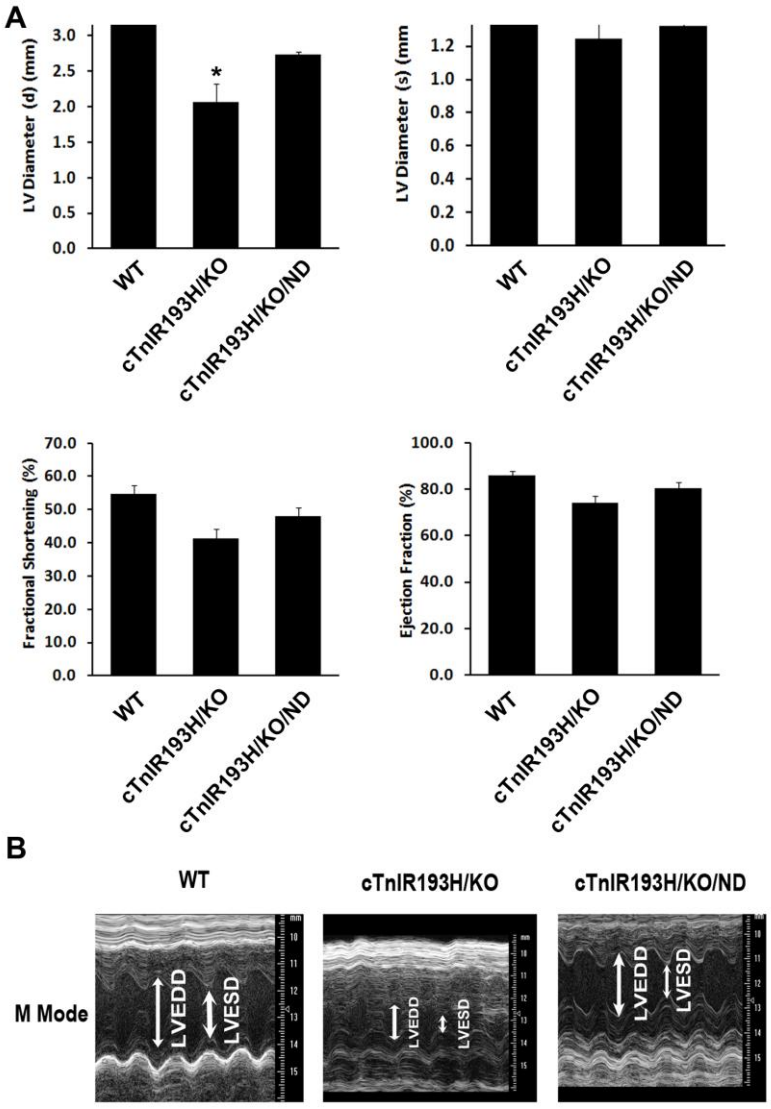

Figure 15: (A) Cardiac function measurements *in vivo* of LV Diameter (d) (left ventricular end diastolic dimension), LV Diameter (s) (left ventricular systolic dimension), FS (fractional shortening) and EF (ejection fraction), (B) M mode images of all three groups: WT, cTnR193/KO and cTnR193/KO/ND. Significant reduction in the LVEDD of the cTnR193/KO mice occurred while a normal LVEDD was seen in the WT and cTnR193/KO/ND mice.

3.4.3 Pulse Wave Imaging

Trans mitral flow evaluation of the left ventricle emphasizes the diastolic function above the ventricular performance as an indication of myocardial viability. Pulse Wave Doppler imaging evaluates blood flow direction and velocity through the mitral valve. Echocardiographic recordings of the mitral valve leaflets investigate mitral flow patterns and provide valuable information about LV relaxation, filling, compliance, end-diastolic pressure as well as left atrial function (Rusconi 2000). From the Pulse Wave Doppler protocol measurements of early peak filling velocity (E), and atrial filling velocity (A), iso-volumetric relaxation time (IVRT), isovolumetric contraction time (IVCT) are derived. The data obtained depicts a pattern with a significantly decreased E peak value compared to the A peak value among the mice in the cTnI R193H/KO group. The result is a graphical representation of reversed heights of the E and A waves (E<A) and a reversed E/A ratio among the high expression mice. Compared to the WT and cTnI R193H/KO/ND groups, this finding proves an abnormal early diastolic to late diastolic filling velocity ratio (E/A) among the cTnI R193H/KO mice. The severity of the diastolic dysfunction is illustrated by a E/A ratio that is less than 1, as seen in figure 16 .The E/A ratio easily distinguishes normal from abnormal diastolic function in mice (Du et al., 2008). Iso-volumetric relaxation time (IVRT), an early and dependable indicator of diastolic dysfunction was delayed in the high expression mouse cTnI R193H/KO compared that observed in the WT and cTnI R193H/KO/ND groups. A long IVRT is a common abnormality observed in RCM transgenic mice, this observation supports the compromised physiological factor typically seen in mice with diastolic dysfunction due to delayed relaxation. As the disease progresses LV compliance decreases and the left

ventricular pressure increases. The IVCT of the cTnR193/KO remained comparable to that of the WT and R193/KO/ND groups, indicating that systolic function was not significantly compromised.

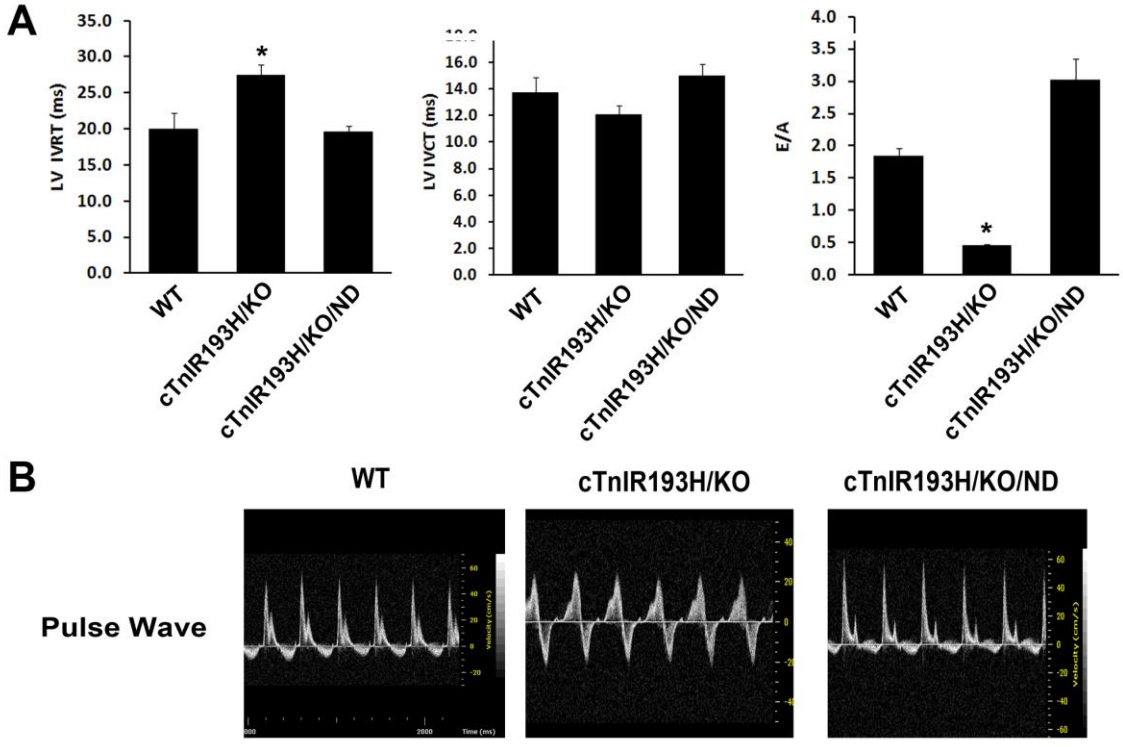

Figure 16: (A) Cardiac function measurements *in vivo* of LV IVRT (left ventricular isovolumetric relaxaxtion time), LV IVCT (left ventricular isovolumetric contraction time) E/A ratio (early filling peak velocity to atrial filling velocity); (B) Pulse Wave Doppler images of all three groups: WT, cTnR193H/KO and cTnI R193H/KO/ND. Normal mitral inflow pattern in WT and cTnI R193/KO/ND, E>A wave; Reversed E<A waves in cTnR193/KO mice.

3.4.4 Tissue Doppler Imaging

Doppler imaging is an important clinical tool which provides useful data for diastolic performance. Measured at the septal portion of the mitral annulus in a apical four

chamber view, Tissue Doppler imaging is a technique ideal for assessing early mitral annulus velocity. RCM patients have been observed to have a TDI imaging profile of reduced E' and A' waves. TDI is beneficial in distinguishing normal diastolic wave patterns from pseudo-normal and restrictive patterns. Data collected in figure 17 showed that E'/A' ratio is reduced significantly in the cTnI R193H/KO mice, having a ratio < 1.0. This reversed E'/A' ratio is indicative of impaired relaxation. Their WT and cTnI R193H/KO/ND counterparts have E'/A' ratios that are > 1.0, which appear roughly equal in measurements. A restrictive filling pattern is observed among the cTnI R193H/KO group compared to the WT and cTnI R193H/KO/ND as seen in figure 17. The reduced E/A ratio indicates that the early ventricular filling phase was affected more by impaired ventricular relaxation, than the atrial late filling phase which was affected less in the cTnI R193H mice (Du et al., 2008). The data in this study supports the finding that cTnI R193H mice with a null cTn I background possess these physiological abnormalities, while the cTnI R193/KO/ND do not, suggesting its correcting physiological effect.

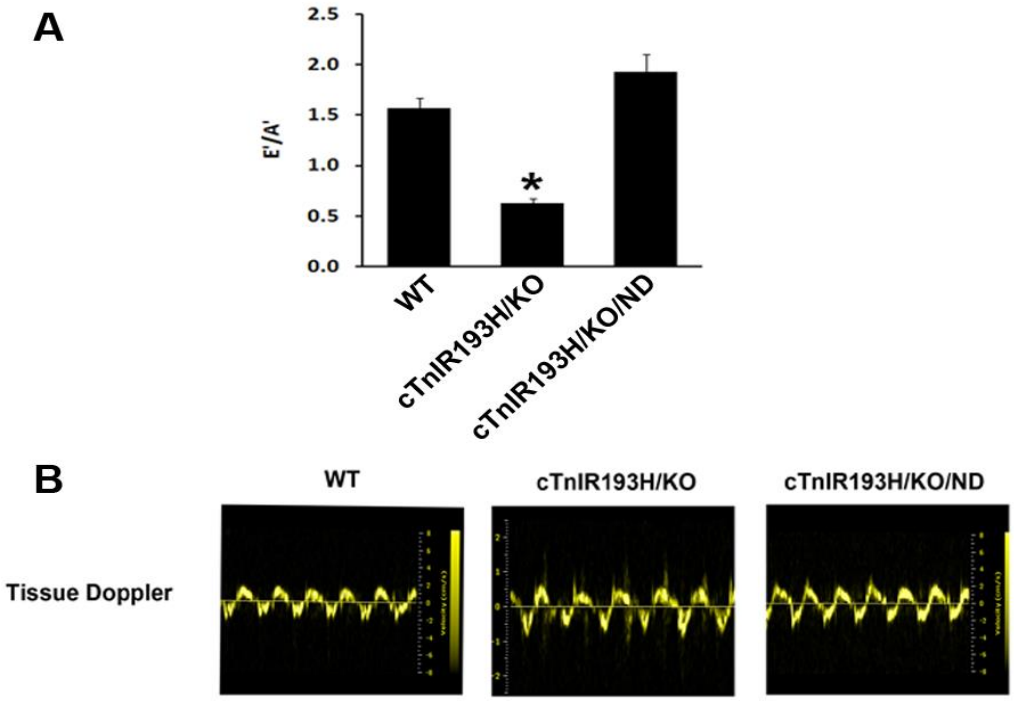

Figure 17: (A) Cardiac function measurements *in vivo* of E'/A' ratio (early filling peak velocity to atrial filling velocity). (B)Tissue Doppler images of all three groups: WT, cTnR193H/KO and cTnI R193H/KO/ND. Normal tissue Doppler pattern seen in the WT and cTnI R193H/KO/ND, a reversed E'/A' is observed the in cTnR193/KO mice.

TABLE 1. Echocardiographic measurements on WT, cTnI/R193H/KO and cTnI/R193H/KO/ND mice at 18-20 days old

Parameters	**WT**	**cTnI/R193H/KO**	**cTnI/R193H/KO/ND**
Body weight (g)	8.97 ± 0.42	8.97 ± 0.18	7.96 ± 0.17
Heart rate (bpm)	370 ± 16.7	306 ± 16.82	320.7 ± 4.24.
Echocardiography			
LV end diastole			
IVS (mm)	0.77± 0.03	0.9 ± 0.18	0.97 ± 0.02
PW (mm)	0.68 ± 0.04	0.60 ± 0.02	0.55 ± 0.03
LVEDD (mm)	3.19 ± 0.11	2.06 ± 0.27	2.73 ± 0.04
LV volume (μl)	41.97 ± 3.45	22.11 ± 4.56	28.94 ± 0.90
LV end systole			

IVS (mm)	1.44 ± 0.05	1.30 ± 0.20	1.21 ± 0.06
PW (mm)	1.25 ± 0.02	0.85 ± 0.02	0.93 ± 0.08
LVESD (mm)	1.35 ± 0.09	1.24 ± 0.20	1.32 ± 0.01
LV volume (μl)	5.95 ± 1.05	6.55 ± 1.63	5.75 ± 0.85
LV EF %	86.01 ± 1.81	73.96 ± 3.22	80.30± 2.68
LV FS %	54.73 ± 2.46	41.41 ± 2.74	48.00 ± 2.67
Mitral Doppler			
E (mm/s)	511.02 ± 3.34	194.55 ± 74.30	516 ± 20.09
A (mm/s)	295.74 ± 12.04	221.37± 42.70	184.31± 21.19
E/A	1.84 ± 0.12	0.71± 0.15	3.02 ± 0.33
IVRT (ms)	20.00 ± 2.20	27.50 ± 1.44	19.6 ± 0.87
IVCT (ms)	13.75 ± 1.10	12.08 ± 0.64	15.00 ± 0.83
Tissue Doppler			
E' (mm/s)	26.81 ± 1.42	15.04 ± 1.21	21.36 ± 1.45
A' (mm/s)	17.22 ± 0.25	22.70 ± 1.43	12.00 ± 1.76

Values are expressed as mean ± SE for each group. LV, left ventricle; EDD, end diastolic dimension; ESD, end systolic dimension; PW, posterior wall thickness of LV; IVS, Intra-ventricular septum; EF, ejection fraction of LV; FS, fractional shortening of LV; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; E, mitral Doppler E peak velocity; A, mitral Doppler A peak velocity; E', mitral annulus E peak velocity; A', mitral annulus A peak velocity. Statistical significance was determined by ANOVA followed by Student's t-test to determine statistical significance. Statistical significance was measured at $P<0.05$.

3.5 Whole Animal Observations

The gross morphological examinations of the heart of the all three mouse models were performed for comparison and study. Significant physiological changes are appreciated in the cTnI R193H/KO group compared to the WT and cTnI R193/KO/ND. The depictions of the morphological examinations support the physiological findings. Biatrial enlargement is observed in the cTnI R193H/KO group at age 18 days (figure 18). In the R193/KO/ND group the atrial size was in normal limits as compared to the mice in the WT group of the same age. There were no obvious morphological changes such as thickening or widening of the ventricles seen in any of the groups.

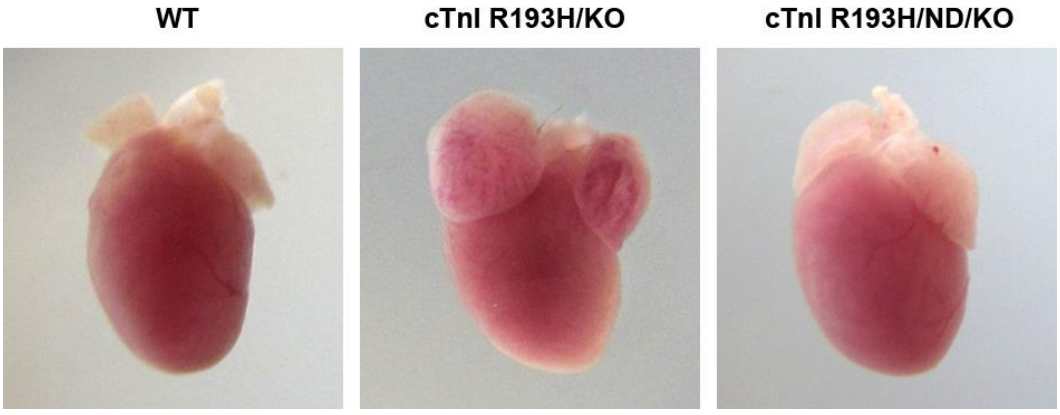

Figure 18: Heart morphology of the three groups: WT, cTnI R193H/KO and cTnI R193H/KO/ND. Enlargement of both atria seen in the hearts of the cTnI R193H/KO group but not in the WT and cTnI R193H/KO/ND groups.

CHAPTER 4: DISCUSSION

The RCM mouse model expresses the R193H mutant, which is similar to the mutation R192H mutation expressed in humans (Mogensen et al., 2003). These mouse models exhibit the phenotypic characteristics of the disease that are observed in the humans The cTnI R193H mutant and the cTnI Knockout mouse were previously generated and characterized in our laboratory. Since then successful generations of other transgenic models which include cTnI R193H/KO and cTnI R193H/KO/ND were produced in an effort to contribute to the study of restrictive cardiomyopathy. The R193H mutations involve the C terminus of cTnI and caused functional and structural consequences such as increased myofilament Ca^{2+} sensitivity, restrictive ventricles, increased ventricular pressure, enlargement of the atria and sudden cardiac death. These are the clinical characteristics of restrictive cardiomyopathy observed in patients carrying the R192H mutation. cTnI R193H/KO transgenic mice have a null cTnI background and possesses all the physiological characteristics of the RCM. With a genetic profile of 80% cTnI R193H mutant in a null cTnI background, (Li et al., 2013) the physiological and morphological onset of the disease is early. The cTnI R193H/KO mice died before their wean dates, between 19-20 days after birth. Unlike the cTnI R193H mouse model which experienced the earliest signs of RCM at 4 months and diastolic heart failure at 12 months (Du et al., 2008). The most obvious functional changes observed during trans-thoracic echocardiography occurred among the cTnI R193H/KO, an included impaired

left ventricular relaxation evidenced by prolonged IVRT, a significantly reduced E peak and reversed E/A and E'/A' ratios. Indicative of the disease's severity, some of the mice evaluated in this study at 18-20 days exhibited signs of heart failure, as seen in reduced ejection fraction and fractional shortening percentages among mice in the cTnI R193H/KO group. Like the cTnI R193H mouse model, among the cTnI R193H/KO group the earliest sign of RCM is prolonged IVRT, however these patho-physiological characteristics were manifested early in the cTnI R193H/KO mouse compared to the cTnI R193H mouse. At the same age the DTG cTnI R193H/KO/ND mice showed physiological functions similar to the WT controls. The data suggests that the differences in the genetic profile of the mice cTnI R193H/KO/ND and the cTnI R193H/KO translates into functional and morphological differences. Figure 13 illustrates a mutant R193H protein expression level of less than 30% and an N-terminal mutant protein expression level of over 50% in the DTG cTnI R193H/KO/ND mouse model. It is not possible to ascertain with certainty that the mouse's viability is solely due to the presence of the N-terminal mutant since a lesser percentage of the mutant R193H protein is confirmed this DTG mouse model compared to the cTnI R193H/KO mouse model. Previous functional studies have determined that cTnI ND mutant has increased relaxation velocity of the ventricular muscle, by desensitizing myofibril to Ca^{2+}. By extension, the result is an increased tolerance of cardiac function which occurs due to decreased preload and continued cardiac output via the Frank-Starling mechanism (Akhter et al., 2001). Physiological cleavage of cTnI at its N-terminus has been discovered to play a role in myocardial remodeling during stress conditions, so the presence of the cTnI N-terminal mutant has shown potential benefits that significantly improve the diastolic dysfunction

observed in cTnI R193H mice (Li et al., 2010). The results from this study suggest that cTnI ND mutant, like the cTnI R193H mutant is dose dependent. The DTG cTnI R193H/ND/KO mouse model used presently rescues diastolic dysfunction in mice with a higher protein expression level than the original cTnI R193H mouse which has the mutant protein expression level of less than 20%. Bands from the Western blot (figure 13) show the presence of ND protein, R193 H mutant protein and ssTnI protein in the DTG mouse. It is evident that the cTnI ND protein level in the cTnI R193/KO/ND mouse correlates with the rescuing effect on RCM in the cTnI R193/KO/ND mouse model even though its level of the R193H mutant protein is less than that of the cTnI R193H/KO mouse .Previous studies have shown an improved ventricular relaxation of the cTnI R193H mouse model. This data supports the finding that the presence of cTnI ND effectively rescued the deleterious phenotype of RCM by reducing the mortality rate of the cTnI R193H/KO mouse in a dose dependent manner.

It is noteworthy that the cTnI R193H/KO mice showed the presence of ssTnI as late as at 18 days old. These mice had a higher protein ssTnI expression than the cTnI/KO mouse at the same age. With an approximate 50:50 ratio of ssTnI to cTnI R193H the result is severe indications of the disease. It can be surmised that the presence of ssTnI compensated for the lack of cTnI and provided a counteracting effect against the cTnI R193H mutant introduced into the mouse genome for at least 4 days. In the cTnI/KO, ssTnI initially compensated for the lack of cTnI, but by day 14 after birth ssTnI depletion caused TnI deficiency leading to mechanical deterioration of the cardiac muscle performance (Huang et al., 1999). Conversely, in the cTnI R193H/KO mice the high expression level of ssTnI was high compared to the cTnI/KO mice even after day 14.

This could represent a compensatory or adaptive mechanism for these mice, however, it did not result in viability of the heart beyond 18 days. A precipitous cardiac function decline occurred because the R193H mutant protein level was higher than 40%, and the measure of protection from the ssTnI protein could not be sustained.

Evaluation of the mechanical performance of the left ventricle in all three groups using trans-thoracic echocardiography yielded results that showed deterioration in the cardiac function of the cTnI cTnI R193H/KO group as early as 18 days old. Similar to the clinical evaluations of RCM patients, the physiological investigations into the disease is largely based on echocardiography (Jean-Charles et al., 2011). The hypersensitivity to Ca^{2+} and prolonged relaxation of the cardiomyocytes in the R193H mouse is dependent on the concentration of the R193H mutant protein. Results from this study demonstrate non-typical measurements such as reduced LVEDD and prolonged IVRT among the cTnI R193H/KO mice which are strong indicators for diastolic dysfunction. The mitral Doppler results showed a reversed E/A ratio which illustrates abnormal mitral inflow patterns. The mitral inflow velocity profile is widely used to characterize ventricular diastolic dysfunction While Tissue Doppler Imaging illustrates restrictive filling as an indication of diastolic dysfunction evidenced by a reversed E'/A' ratio when the mitral annulus velocity was measured. Ventricular relaxation impairment in the cTnI R193H/KO mice is an evidence of the disease progression and dictates the mortality rate of these mice compared to the WT and cTnI R193H/KO/ND mice. The reduced ejection fraction and fractional shortening percentages points to the systolic function deterioration at the end stage of RCM as seen in the cTnI R193H/KO mice (figure 15). Comprehensive investigations and evaluations have revealed that the main impairment of RCM is

diastolic dysfunction with systolic function decline in the latter stages (Jean- Charles et al., 2011). Comparing trans-thoracic echocardiography data of the DTG cTnI R193H/KO/ND with that of the cTnI R193H/KO mice, left ventricular physiological improvements are observed in the DTG mice, this indicates improved diastolic dysfunction. The main functional and structural characteristics for diastolic abnormality, a delayed IVRT and enlarged atria were corrected in the cTnI R193H/KO/ND mice, as indicated in figures 16 and 18. The reason for this improvement is due to the presence of the N terminal deletion mutant in the DTG mouse model. Myofibril hypersensitivity to Ca^{2+} is the key cause of impaired ventricular relaxation in RCM. Correcting the diastolic impairment observed in RCM using restrictive proteolysis of the N-terminal extension is promising since cTnI ND lowers the hypersensitivity to Ca^{2+} in cTnI R193H cardiac muscle fibers. In response to patho-physiological stress, the improvement of cardiomyofilament sensitivity to Ca^{2+} by cTnI ND while conserving the cTnI core is significant. (Li et al., 2010)

This study provides a contribution in closing the gap between unknown information about RCM and its clinical manifestations. The development of several transgenic mouse models provides an opportunity to manipulate the mouse genome to create and study the progression of RCM. Although it is a rare disease, the impact RCM has on human patients is dire due to its poor prognosis. RCM, defined by hemodynamic characteristics leads to diastolic dysfunction and is associated with a high rate of mortality especially in the young (Li et al,. 2013) The R192H mutation is characterized as one having worst clinical phenotype so it requires continuing investigations and some promising solutions. Most experimental investigations and therapeutic treatments used in clinical settings

focus on Ca^{2+} sensitizing and enhanced contraction to help the performance of the already ailing heart (Li et al,. 2013). The goal is that the future therapeutic agents will include compounds that directly target diastolic dysfunction and not the conditions that are secondary to RCM. Compounds designed to model or enhance the process of endogenous N-terminal deletion of cTnI in the myocardium will prove beneficial. The double transgenic mouse model cTnI R193H/KO/ND has provided some answers about the N-terminal deletion mutant being an agent for correcting the effects of restrictive cardiomyopathy and other diastolic disorders.

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