The Physiological Consequences of Hypertrophic Cardiomyopathy (HCM) and Restrictive Cardiomyopathy (RCM) Related Mutations in Human Cardiac Troponin I

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THE PHYSIOLOGICAL CONSEQUENCES OF HYPERTROPHIC CARDIOMYOPATHY (HCM) AND RESTRICTIVE CARDIOMYOPATHY (RCM) RELATED MUTATIONS IN HUMAN CARDIAC TROPONIN I

By

Yuhui Wen

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

THE PHYSIOLOGICAL CONSEQUENCES OF HYPERTROPHIC
CARDIOMYOPATHY (HCM) AND RESTRICTIVE CARDIOMYOPATHY (RCM)
RELATED MUTATIONS IN HUMAN CARDIAC TROPONIN I

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An arginine (R) to a glycine (G) mutation at position 145 in the highly reserved inhibitory domain of cardiac troponin I (cTnI) is associated with hypertrophic cardiomyopathy (HCM), an autosomal dominant disease characterized by left ventricular hypertrophy. An arginine (R) to tryptophan (W) mutation at the same position in cTnI is associated with restrictive cardiomyopathy (RCM), a disease characterized by diastolic dysfunction with normal left ventricular size and normal systolic function. In this study we addressed the functional consequences of the human cardiac troponin I (hcTnI) HCM R145G mutation and hcTnI RCM R145W mutation in transgenic mice.

Simultaneous measurements of the ATPase activity and force in skinned papillary fibers from hcTnI R145G transgenic mice (Tg-R145G) versus hcTnI wild type transgenic mice (Tg-WT) showed a significant decrease in the maximal Ca$^{2+}$ activated force without changes in the maximal ATPase activity and an increase in the Ca$^{2+}$ sensitivity by both ATPase activity and force development. No difference in the cross-bridge turnover rate was observed at the same level of cross-bridge attachment (activation state) showing that changes in Ca$^{2+}$ sensitivity were not due to changes in cross-bridge kinetics. Energy cost calculations demonstrated higher energy consumption in Tg-R145G fibers compared to Tg-WT fibers. The addition of 3mM BDM at pCa 9.0 showed that there was
approximately 2–4 percent of force generating cross-bridges attached in Tg-R145G fibers compared to less than 1.0 percent in Tg-WT fibers, suggesting the mutation impairs the ability of the cardiac troponin complex to fully inhibit cross-bridge attachment under relaxing conditions. Prolonged force and intracellular [Ca\(^{2+}\)] transients in electrically stimulated intact papillary muscles were observed in Tg-R145G compared to Tg-WT. These results suggest that the phenotype of HCM is most likely caused by the compensatory mechanisms in the cardiovascular system which are activated by: 1) higher energy cost in the heart resulting from a significant decrease in average force per cross-bridge; 2) incomplete relaxation (diastolic dysfunction) caused by prolonged [Ca\(^{2+}\)] and force transients; and 3) an inability of the cardiac TnI to completely inhibit activation at low levels of diastolic Ca\(^{2+}\) in Tg-R145G.

Simultaneous measurements of the ATPase activity and force in transgenic skinned papillary fibers from hcTnI R145W transgenic mice (Tg-R145W) versus Tg-WT showed that there was a ~13 to ~16 percent increase in the maximal Ca\(^{2+}\) activated force and ATPase activity, respectively. The rate of dissociation of force generating cross-bridges (g) and energy cost (ATPase/force) was the same in all groups of fibers. These results suggest that the increase in force and ATPase activity is associated with an increase in the number of force generating cross-bridges attached at all activation levels. Additionally, there was a large increase in the Ca\(^{2+}\) sensitivity of force development and ATPase activity. In intact fibers, the mutation caused prolonged force and intracellular [Ca\(^{2+}\)] transients, as expected due to the increased Ca\(^{2+}\) sensitivity (slower dissociation rate of Ca\(^{2+}\) from cTnC). The above cited results suggest that: 1) there would be an increase in resistance to ventricular filling during diastole resulting from the prolonged
force and \( \text{Ca}^{2+} \) transients, especially at high heart rates; 2) there would be a decrease in ventricular filling (diastolic dysfunction); and 3) an increase in contractility during systole that would off-set the negative effect of a decrease in diastolic filling on ventricle stroke volume thus allowing the heart to maintain normal stroke volume despite the compromise in RCM (Tg-R145W) heart.
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Chapter 1

Introduction

1.1 Overview of Muscle Contraction

There are three major muscle types in the human body: skeletal muscle, smooth muscle and cardiac muscle. Both skeletal muscle and cardiac muscle are called striated muscle due to the presence of a cross-striped appearance when viewed under the microscope. The basic functional and anatomical unit of the striated muscle is the sarcomere. The center portion of the sarcomere is called the A-band. The A-band is composed of thick filaments which primarily are composed of the protein myosin and thin filaments which are composed of actin, tropomyosin, and the troponin complex (troponin I, troponin C and troponin T). There is also a region of the sarcomere consisting of thin filaments and titin called the I-band. The Z-line (Z-disc) marks the middle of the I-band and delineates the boundaries of the sarcomere.

The primary function of the muscle is to contract. During contraction, the muscle forms cross-bridges that generates force and shortening allowing muscle to participate in body functions like pumping blood, breathing, locomotion, and so on. Ringer discovered that the presence of external calcium triggers the heart muscle to contract (Ringer et al. 1927).
1882; Ammash et al. 2000). We now know that Ca$^{2+}$ is the most important regulator of striated muscle contraction, especially for cardiac muscle.

The action potential generated along the surface of the myocyte initiates the contractile process in the heart. The action potential spreads from the sarcolemma down to the transverse tubules (t-tubules). The action potential descends down the t-tube into triadic junctions composed of a single t-tubule and two terminal cisternae that form the ends of sarcoplasmic reticulum (SR). The depolarization causes the opening of voltage-gated L-type calcium channels and the entering of Ca$^{2+}$ from the t-tubules. This small influx of calcium causes *calcium-induced calcium release* from the sarcoplasmic reticulum, which contains the calcium release channels-RyR channel. The increase in myoplasmic free Ca$^{2+}$ concentration causes muscle contraction (Fabiato and Fabiato 1975).

Ca$^{2+}$ binds to the Ca$^{2+}$ specific regulatory site in troponin C (TnC) initiate muscle contraction. TnC which binds to Ca$^{2+}$ is one of the subunits of the troponin (Tn) complex. There are two other subunits: troponin T (TnT), which binds to tropomyosin (Tm) and troponin I (TnI), which binds to actin and inhibits actomyosin ATPase. The Tn and Tm forms the Ca$^{2+}$ regulatory system of the muscle contractile apparatus (Gergely 1974; Zot and Potter 1987). The interaction of cross-bridges with the thin, actin containing filaments couple ATP-consumption to the generation of force. This interaction is regulated by Tm and Tn in a Ca$^{2+}$ dependent manner. Ca$^{2+}$ binds to the specific binding site of TnC when myoplasmic free Ca$^{2+}$ concentration rises following the release of Ca$^{2+}$ from the sarcoplasmic reticulum. This binding of Ca$^{2+}$ allows the C-terminus of TnI to interact with the N-terminus of TnC causing the dissociation of the TnI from the actin-
tropomyosin complex, allowing myosin to bind to actin. TnI is the main regulator of the actomyosin ATPase activity (Tobacman et al. 1996).

1.2 Thick and Thin Filaments Protein

1.2.1 Myosin

The thick filament is the bipolar polymer of the motor protein myosin, which interacts with the thin filament protein actin to form cross-bridges, and to produce force and sarcomere shortening. It also includes other large proteins, such as titin, C protein and M protein. Myosin II is composed of two heavy chains with molecular masses of ~200kDa each and four light chains, two ~18kDa and two ~27kDa. Four light chains are categorized into two different types: one called regulatory light chain (also called LC2 or RLC), the other called essential light chain (also called LC1 or ELC). The heavy chains form a parallel two chain coiled-coil structure over most of their length except for the large, globular NH2-terminal regions, termed myosin head or S1 (subfragment 1). The globular head contains the ATPase site which provides energy for muscle contraction and also has the actin binding site. The S1 head structure projecting from the backbone of the thick filament interacts with actin to form the cross-bridge, produces force and filament sliding. One pair of light chains (one ELC and RLC) binds to each S1 (Margossian and Slayter 1987). It is not very clear what the functions of RLC and ELC are for. However, in smooth muscle RLC regulates actomyosin interactions by RLC phosphorylation.
Other non-motor proteins have been shown to play an important role in the thick filaments. The M protein connects the thick filament together at the center of the A band (H-zone) in the sarcomere. Titin is a large elastic molecule which play an important role in the passive tension and active elasticity as well as sarcomere stability (Wang et al. 1996). C protein bundles the myosin together in the filament and constrains the motion of the myosin heads to keep them close to the surface of the thick filament (Hofmann et al. 1990).

### 1.2.2 Actin

Actin is the most abundant thin filament protein. Actin monomer (G-actin) is a single polypeptide with a molecular weight of 42,000 and globular structure. G-actin binds to ATP and Ca\(^{2+}\), reversibly. G-actin can spontaneously polymerize to form F-actin, the backbone of the thin filament in presence of physiological ionic strength and with ATP bound. F-actin has a two-stranded long pitch helical structure. The atomic model of cardiac actin structure shows that actin is composed of four subdomains and surrounded by a metal-nucleotide binding pocket (Kabsch et al. 1990). Between these subdomains, subdomain 1 contains both the NH\(_2\) and COOH terminus and plays a important role in interacting with myosin (Egelman and Orlova 1995).

The primary form of actin is \(\alpha\)-actin in the adult skeletal and cardiac myofilament. Muscle actin is a highly conserved amino acid sequence. Also there is a high degree of homology between skeletal \(\alpha\)-actin and cardiac \(\alpha\)-actin. Both skeletal and cardiac \(\alpha\)-actin
genes are coexpressed in muscle development but exhibit distinctive tissue-specific patterns of expression. Skeletal α-actin is the only actin isoform in the adult skeletal muscle. Cardiac α-actin is the only isoforms in the adult mouse cardiac muscle. However, in the adult human heart, both skeletal α-actin and cardiac α-actin coexist (Minty et al. 1982; Mayer et al. 1984).

1.2.3 Tropomyosin

Tropomyosin (Tm) is a ~42nm long highly extended molecule formed as a dimer by two α-helical chains arranged as a coiled coil. The stability of the coiled coil is produced by hydrophobic interactions between nonpolar side chains contributed by amino acids in each chain. Each chain is 284 residues long and extends across seven actin monomers on each strand of the F-actin filament. The neighboring tropomyosins overlap in a head-to-tail pattern with periodicity of 38.5nm along the thin filament. The overlapping region of Tm provides much of the stability of the binding to F-actin. This is illustrated by the greatly reduced affinity of Tm for actin when the overlap region has been deleted (Mak and Smillie 1981). Tn further stabilizes Tm binding to F-actin. Tn binding extends from overlap region to near the Tm Cys-190, one third of the way from the COOH-terminal end of Tm toward the NH₂-terminal end. The position of Tm on actin depends on Ca²⁺ concentration in sarcoplasma. During low Ca²⁺ concentration, Tm blocks the binding of the actin molecule to myosin and thus inhibits actomyosin ATPase activity. During muscle contraction, Ca²⁺ ions bind to the regulatory domain of TnC,
resulting in structural changes in Tn complex and the movement of Tm deeper into the
groove of the thin filament, thus allowing actin binding site to be revealed and thus
allowing weak and some strong cross-bridge binding to actin (Gordon et al. 2000).

There are two different Tm isoforms, α-Tm and β-Tm. Each chain of coiled coil is the products of these two genes with variable expression in the different muscle types.
The two isoforms differ by 39 amino acids. In smaller mammals, cardiac Tm more closely resembles fast skeletal muscle Tm, with an mixture of α- and β- isoforms. In larger mammals, α- Tm is the predominant isoform in cardiac muscle (Tobacman et al. 1996).

1.2.4 Troponins

The troponin complex (Tn) is composed of three interacting subunits: troponin C (TnC) binds with Ca\(^{2+}\), troponin T (TnT) anchors the Tn complex to the Tm and troponin I (Tnl) binds to actin or TnC in a Ca\(^{2+}\) dependent manner and inhibits the actomyosin ATPase activity in the low concentrations of Ca\(^{2+}\).

(Takeda et al. 2003) showed the crystal structure of the core domain of cardiac troponin (cTn) is divided into two major subdomains. One is the regulatory head, which contains the N-terminal of cardiac troponin C (cTnC) (residues 3-84) and the regulatory site of cardiac troponin I (cTnl) (downstream of the inhibitory region, residues 150-159). The other subdomain is called Tnl-TnT arm (IT arm) and is composed of the C-terminal of cTnC (residues 93-161), C-terminal of cardiac troponin T (cTnT) (residues 203-271)
and two long cTnI α-helices (residues 42-136). IT arm is large and rigid, being conserved between species and having no direct interaction with actin–tropomyosin. It bridges the two Ca\(^{2+}\) independent domains in TnT, TnT1 and C-TnT, which attaches to actin–tropomyosin. The D/E linker (the linker connecting the two lobes of the cTnC) of cTnC and the inhibitory region of cTnI form a flexible linker that is likely involved in the regulatory mechanism by allowing the two domains to rotate and alter their relative orientation (Figure 1.1). This crystal structure suggests that during muscle contraction, the cTnI regulatory domain (C-terminal region of cTnI, residues 137-210) undergoes major changes in both conformation and position depending on the Ca\(^{2+}\) concentration. At high Ca\(^{2+}\) concentration, the regulatory domain is detached from actin–tropomyosin and the H3 helix (residues 150-159) is associated with the N-terminus of cTnC. At low Ca\(^{2+}\) concentration, the regulatory domain of cTnI forms an extra attachment to actin–tropomyosin, except for TnT1 and C-TnT, so that the troponin strand is tied down onto the actin filament (Figure 1.2). This formation of the third attachment itself, as well as the rotation of the IT arm, might change the properties of the tropomyosin strand on the actin filament.
Figure 1.1: *Ribbon model of the crystal structure of the core domain of the Tn complex determined by Takeda et al. (2003).* cTnC shows in orange; cTnT shows in blue; cTnI shows in green (Kobayashi and Solaro 2005).
Figure 1.2: *A schematic representation of the interactions between troponin and other thin filament components in the absence and presence of Ca$^{2+}$ ions.* The actin and the tropomyosin strands are in green and brown, respectively. TnT is showed in yellow; TnC is showed in red; TnI is showed in light blue and dark blue depends on different domains. TnT1 and C-TnT are yellow ellipsoids; the inhibitory region (IR) and the C terminus of TnI (C-TnI) are blue ellipsoids. The black arrows indicate the interactions between troponin and tropomyosin–actin (Takeda et al. 2003).
1.2.4.1-Troponin C

Troponin C (TnC) is the Ca\(^{2+}\) sensor in skeletal and cardiac muscle contraction. It is a highly acidic protein and a member of EF-hand Ca\(^{2+}\) binding family. There are two isoforms of TnC: skeletal TnC (sTnC) that exists in fast skeletal muscle and cardiac TnC (cTnC) that exists in both cardiac muscle and slow skeletal muscle. Both sTnC and cTnC are highly homologous. Molecular weight of both TnC isoforms is 18kDa. The crystal structure of TnC showed a dumbbell-shaped molecule with two globular domains, an NH\(_2\) and COOH terminal, which connected by a long central helix, called D/E helix (Houdusse et al. 1997; Spyracopoulos et al. 1997). Each domain contains two possible Ca\(^{2+}\) binding sites called site I to site IV from N-terminus to C-terminus. The EF hand Ca\(^{2+}\) binding sites consists of an \(\alpha\)-helix-coil-\(\alpha\)-helix structure motif. The NH\(_2\)-terminal Ca\(^{2+}\) binding sites (site I and site II in sTnC, site II in cTnC) bind Ca\(^{2+}\) with lower affinity (\(~10^{-5}\) M\(^{-1}\)) and high selectivity of Ca\(^{2+}\) over Mg\(^{2+}\) (Potter and Gergely 1974; Potter and Gergely 1975). Thus, these Ca\(^{2+}\) specific binding sites are able to exchange Ca\(^{2+}\) fast enough to activate muscle contraction in the presence of Ca\(^{2+}\). The N-terminal region in cTnC contains only a single Ca\(^{2+}\) binding site II, because Ca\(^{2+}\) binding to site I is inactivated due to a replacement of non-conservative amino acid (van Eerd and Takahshi 1976; Johnson et al. 1981). The COOH-terminal sites (III-IV) have high Ca\(^{2+}\) affinity (10\(^{-7}\) M\(^{-1}\)) and low Mg\(^{2+}\) affinity so that Mg\(^{2+}\) is normally occupied these sites under relaxed conditions. III-IV Ca\(^{2+}\) binding sits play a structure role in anchoring TnC tightly to TnI, since Mg\(^{2+}\)/ Ca\(^{2+}\) binding enhances the interaction of TnC with TnI and binding of TnC to the thin filament (Zot and Potter 1987).
TnC binds with TnI irrespective of Ca\(^{2+}\) concentration, but interaction strength is increased in the presence of Ca\(^{2+}\) (Potter and Gergely 1974). TnC complex with a short peptide of TnI (residues 1-47) x-ray structure suggested that the C-terminus of TnC interacts with the N-terminus of TnI (Vassylyev et al. 1998). In addition, the inhibitory region downstream peptide (residues 150-159) of TnI binds with the hydrophobic cleft in the N-terminus of TnC in the presence of Ca\(^{2+}\) (Takeda et al. 2003). The C-terminus of TnC also interacts with the C-terminus of TnT and binding of Ca\(^{2+}\) to TnC stabilizes the interaction between TnC and TnT.

1.2.4.2 Troponin T

Troponin T (TnT) is the largest and longest molecule in the Tn complex. The primary function of TnT is to glue all components in the thin filament together. It interacts with Tm, TnI and TnC and anchors troponin complex to the actin. It acts not only to assist in binding TnC-TnI to Tm-actin and Tm to actin, but in cooperative activation of the thin filament. TnT is a highly asymmetric molecule and the sequence contains high percentage of charged residues, which are unevenly distributed, with a very rich region of acidic residues in the N-terminus and a basic residues region in C-terminus.

TnT can be cleaved into two fragments, TnT1 and TnT2, by treatment with chymotrypsin. Fragment TnT1 weighted 19kDa contains 158 residues in N-terminal. This fragment is the major Ca\(^{2+}\) insensitive anchoring site of troponin onto tropomyosin. The TnT1 fragment binds to the site located near to the C-terminus of the Tm dimer and
overlaps the region of contact of two Tm dimers interacting head to tail (Mak and Smillie 1981). The interaction of TnT1 with neighboring overlapping Tms provides the cooperative activation. Fragment TnT2 weighted 13kDa contains 101 amino acid in C-terminal. TnT2 fragment interacts strongly with Tm, TnC and TnI. The interaction with Tm in TnT2 fragment is Ca\(^{2+}\) dependent and is stronger at low rather than high Ca\(^{2+}\) concentration (Pearlstone and Smillie 1981; Pearlstone and Smillie 1982; Pearlstone and Smillie 1983).

Other than binding to Tm, the TnT2 fragment also binds to TnC and TnI. Ca\(^{2+}\) strengthens the TnC-TnT2 interaction while weakening the TnT2 binding to Tm and actin. Because the NH\(_2\)-terminal region of TnT2 may inhibit the actomyosin ATPase (Malnic et al. 1998). The Ca\(^{2+}\) induced interaction of TnC-TnT2 may aid in activating the thin filament. Even though the Tm-Tn to actin affinity decrease is small, this provides a second site for Ca\(^{2+}\) regulate the Tm position on the actin (in addition to the Ca\(^{2+}\) medicated decrease in TnI-actin binding). The binding of TnT2 to TnI is not affected strongly by Ca\(^{2+}\), although there may be changes in the regions of TnI interacting with TnT (Tripet et al. 1997).

TnT has many isoforms with a hyper variable region and a number of alternatively spliced variants. Three different isoforms, encoded by distinct genes are present in fast skeletal, slow skeletal and cardiac muscle respectively (Cooper and Ordahl 1985; Smillie et al. 1988). Multiple isoforms of TnT are present in the cardiac muscle. The adult isoform of human cardiac TnT (hcTnT) consists of 288 amino acids. Exon 4 and exon 5 can be alternatively sliced into 4 different isoforms TnT1 \(\rightarrow\) 4 with decreasing molecular weight. TnT1 is the primary fetal isoform while TnT3 is

1.2.4.3 Troponin I

Troponin I (TnI) is the subunit that binds to actin, Tm, TnT and TnC. Many of these interactions are regulated by Ca\(^{2+}\). TnI is also the major subunit that inhibits the actomyosin ATPase. During relaxation (low Ca\(^{2+}\) concentration), TnI binds to the actin-Tm complex and inhibits strong cross-bridge interaction between myosin and actin. When Ca\(^{2+}\) is high it binds to TnC and causes a conformational change in the whole Tn complex, C-terminal of TnI binds tightly to N-terminal of TnC but weakly binds to actin-Tm (N-terminal of TnI binds to C-terminal of TnC in a Ca\(^{2+}\) independent manner in relaxing muscle). This allows Tm to move away from the actin groove and exposes the myosin binding site on actin thereby allowing myosin to bind to actin to form cross-bridges (Lehrer and Geeves 1998). A positively charged peptide from TnI (96-116 residues in skeletal TnI and 128-148 residues in cardiac TnI) is responsible for binding to the N-terminal region of actin and the inhibition of actomyosin ATPase in a one-to-one manner. The binding of TnI to actin is not responsible for preventing myosin binding to actin directly, because TnI is only present in a 1:7 ratio to actin, but acts through tropomyosin.
There are three isoforms of TnI, slow skeletal TnI (ssTnI), fast skeletal TnI (fsTnI) and cardiac TnI (cTnI), which is relatively abundant in slow skeletal, fast skeletal and cardiac muscle respectively. cTnI is larger than the other two isoforms due to an additional 32 amino acids at the N-terminal end (Leszyk et al. 1987; Murphy et al. 1991; Perry et al. 1999). The importance of this N-terminal extension in cTnI is that it contains two serine amino acids which can be phosphorylated by protein kinase A (PKA) (Solaro et al. 1976). The phosphorylation of N-terminal cTnI is important to decrease Ca\(^{2+}\) sensitivity and may play a major role in accelerating cardiac muscle relaxation (Zhang et al. 1995).

Myofilaments from fetal and adult heart show a difference in Ca\(^{2+}\) sensitivity and respond to different acidic pH’s. Solaro et al. (Solaro et al. 1986) reported that these changes in Ca\(^{2+}\) sensitivity is not due to the shift in isoforms of TnC or myosin heavy or light chain. Also, numbers of researchers showed that ssTnI is the main isoform in the neonatal heart and cTnI does not become a dominant form until after birth (Saggin et al. 1989; Westfall et al. 1997; Huang et al. 1999). This developmental transition has significant functional roles. Westfall et al. (Westfall et al. 1997) found that ssTnI has lower threshold for Ca\(^{2+}\) activated contraction than cTnI and a better resistance to the PH change. Huang et al. (Huang et al. 1999) found that mice lacking cTnI were born healthy because of the compensation of ssTnI form in fetal, but died of acute heart failure after 18 days of birth because ssTnI expression began to decline at 15 days after birth.

Human cardiac TnI (hcTnI) (210 amino acids; ~24kDa) can be classified into five main functional regions: (a) an N-terminal cardiac-specific extension that contains PKA-phosphorylation sites (Ser-23 and Ser-24); (b) an N-terminal region that binds to the C-
lobe of TnC; (c) a region (residues 90-135 in hcTnI) that binds to the C-terminal of TnT; (d) the inhibitory region or first actin-binding region (residues 137-148 in hcTnI); (e) second actin-binding region (residues 192-210 in hcTnI). As mentioned earlier, the main function of TnI is to inhibit actomyosin ATPase activity through inhibitory peptide of TnI (137-148 residues in hcTnI) (Figure 1.3).

Figure 1.3: *A schematic representation of the human cardiac troponin I with the number of corresponding amino acids encoded by the gene and interaction sites with other sarcomeric contractile proteins.* The stars indicate mutations identified in patients with restrictive cardiomyopathy in numerical order. The red circle indicates the position of the 145 residue in the hcTnI. The black bars indicate functional domains of hcTnI (Mogensen et al. 2003).

The importance of cTnI in Ca$^{2+}$ regulation of muscle contraction is clearly highlighted by the mutations in cTnI that are shown to cause hypertrophic cardiomyopathy (HCM) and restrictive cardiomyopathy (RCM) (Lang et al. 2002; Mogensen et al. 2003; Gomes and Potter 2004; Gomes et al. 2005). To date, around 20 mutations and 6 mutations in cTnI have been reported as a cause of HCM and RCM
respectively. Among them, the different missense at residue 145 causes different cardiomyopathies. R145G/Q (positively charged arginine replaced by an uncharged glycine/glutamine) mutation is correlated with HCM and R145W (positively charged arginine replaced by tryptophan) is correlated with RCM. The R145G mutation was found in 5 members in a 18-member family. Four carriers had typical ventricular hypertrophy. The R145W mutation was found in two patients in two families. Both of them were diagnosed with RCM in their late fifties and had symptom of heart failure. The functional consequences of the R145G mutation have been studied extensively in many labs (Elliott et al. 2000; Takahashi-Yanaga et al. 2000; Takahashi-Yanaga et al. 2001; Lang et al. 2002; Westfall et al. 2002; Kruger et al. 2005; Sanbe et al. 2005). Previously, our lab and others have shown that the hcTnI R145G mutation increases the unregulated force in the absence of $\text{Ca}^{2+}$ (also called as basal force), decreases the maximal force recovery and significantly increases the $\text{Ca}^{2+}$ sensitivity of force development when compared with the wild type (WT) hcTnI. These results suggest that the diastolic dysfunction and somewhat decreased contractility would be prominent clinical features and that hypertrophy may arise as a compensatory mechanism. On the other hand, not much is known about the consequences of the hcTnI R145W mutation causing RCM. The aim of this study is to investigate the functional consequences of hcTnI R145W and R145G mutations by using transgenic mice.
1.3 Inherited Cardiomyopathies

Cardiomyopathies are diseases of the heart muscle that are associated with cardiac dysfunction. Cardiomyopathies are classified according to morphological and functional criteria into three categories: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) and restrictive cardiomyopathy (RCM). Figure 1.4 illustrates three types of cardiac remodeling. The cardiomyopathies can be a primary myocardial disorder due to heritable gene mutations or can develop as a secondary consequence of a variety of conditions, including myocardial ischemia, infection, metabolic and nutritional deficiency, increased myocardial pressure or volume load and toxic agents. Over the last decade, the importance of gene defects in the etiology of primary cardiomyopathies has been recognized. Monogenic inherited cardiomyopathies, caused by mutations in a single gene, have been largely focused because these disorders enable patho-physiological processes applicable to a wide range of more commonly occurring heart diseases to be evaluated. In 1989, Christine and Jon seidman showed the first association between β myosin heavy chain and familial hypertrophic cardiomyopathy (FHC) (Geisterfer-Lowrance et al. 1990). Since then, missense genes have been associated with DCM, RCM and HCM. The mutations were found in all kinds of proteins, such as sarcomere proteins, nuclear membrane proteins, mitochondrial proteins and so on (Schonberger and Seidman 2001). Around 90% of the heritable gene mutations which cause cardiomyopathies are inherited in an autosomal dominant fashion. Two potential mechanisms can exert their dominant effect. When both normal protein and the mutant proteins are both expressed and incorporated into the sarcomeric structure, the mutant
protein could act as a poison peptide and disrupt the function of normal protein. Most of the mutations cause loss of function of a gene through this mechanism (Wolska et al. 2001). Alternatively, a dominant mutation may functionally inactivate a gene and reduce the protein concentration by 50%. Even though molecular genetic studies have enabled us to identify molecular triggers of different cardiomyopathies, the functional consequences of gene mutations and precise details of the signaling pathways that lead to dilation, hypertrophy and contractile function remain to be elucidated.

Figure 1.4 Types of cardiomyopathy. Normal heart is shown in the upper of the figure. Compared to normal heart, DCM has an enlarged ventricle; HCM has a thickened ventricular wall and RCM has a stiff but not thickened ventricular wall.
1.3.1 Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a primary myocardial disorder with an autosomal dominant pattern of inheritance. HCM is characterized by hypertrophy of both ventricles, especially left ventricle, with histological features of myocyte hypertrophy, interstitial fibrosis and myofibrillar disarray. HCM is one of the most common inherited cardiac disorders, with the prevalence in young adults of 1 in 500 (Maron et al. 1995). It is often caused by mutations in contractile proteins. In approximately 50% of patients, over 200 mutations have been identified in 10 genes as a cause of familial hypertrophic cardiomyopathy (FHC). Most of these genes encode saromeric proteins, beta myosin heavy chain, cardiac myosin binding protein C, essential/regulatory myosin light chain, alpha-Tm and cardiac troponin complex (cTn): cTnT, cTnC and cTn I. Diagnosis of the disease is based on unexplained cardiac hypertrophy (ventricular wall thickness >13mm). The extent of left ventricular hypertrophy varies between different genes. Unusual forms of hypertrophy have been reported, localized to the left ventricular apex in some cardiac troponin I mutations (such as Arg162Trp and Gly203Ser) (Kimura et al. 1997). The clinical manifestations of HCM vary from a benign asymptomatic course to that of severe heart failure and sudden cardiac death. Sudden cardiac death is often the first manifestation of the death in young competitive athletes.

To determine the impact of genetic mutations on the phenotypic expression of the disease, genotype-phenotype correlation studies have been performed in many proteins, beta myosin heavy chain, cTnT, cTnC and cTnI. In general, cardiac disease in individuals with mutations in beta myosin heavy chain and myosin binding protein C manifest at
younger age and are with a high incidence of sudden cardiac death (Fatkin and Graham 2002). However, individuals with cTnT mutations exhibit less hypertrophy and a high incidence of sudden cardiac death (Knollmann and Potter 2001). *In vitro* and *in vivo* functional studies on these mutations have shown that abnormal cardiac myocyte function is a precipitating factor towards disease progression. Abnormal cardiac myocyte function may occur due to mechanical defects (alterations in cross bridge kinetics, actomyosin interactions and maximum power output of the muscle etc), biochemical defects (altered Ca\(^{2+}\) sensitivity and ATPase activity) or structural defects. The functional consequences of cTnI mutations causing HCM have been extensively characterized (Elliott et al. 2000; Takahashi-Yanaga et al. 2000; Takahashi-Yanaga et al. 2001; Lang et al. 2002; Sanbe et al. 2005). Among these cTnI mutations, Arg145Gly (R145G) mutation, which induced typical HCM symptoms in patients (Kimura et al. 1997) is studied widely. In the hcTnI R145G exchanged porcine cardiac myofibrils reconstituted system, Takahashi-Yanaga et al. (Takahashi-Yanaga et al. 2000; Takahashi-Yanaga et al. 2001) showed no change in the maximal force, while Lang et al. (Lang et al. 2002) reported a decrease in the maximum force. At the same time, both of them showed an increase in the Ca\(^{2+}\) sensitivity of force development. In the reconstituted actin-Tm-activated myosin ATPase assay, Takahashi-Yanaga et al. (Takahashi-Yanaga et al. 2000; Takahashi-Yanaga et al. 2001), Lang et al. (Lang et al. 2002) and Elliott et al. (Elliott et al. 2000) reported that this specific mutation reduced the ability of cTn to inhibit the actomyosin ATPase activity in the absence of Ca\(^{2+}\) and reduced the maximal ATPase activity in the presence of Ca\(^{2+}\). Kruger et al. (Kruger et al. 2005) found a slight decrease in Ca\(^{2+}\) sensitivity of force development in myofibrils from mouse cardiac TnI R146G (mcTnI
R146G) transgenic mice but no change in hcTnI R145G exchanged murine cardiac myofibrils. They also observed a significant upward shift of the passive force-sarcomere length curve at pCa 7.5 which can be rescued by addition 2, 3-butanedione monoxime (BDM) in both reconstituted myofibrils and transgenic mouse myofibrils. James et al. (James et al. 2000) constructed transgenic mice carrying mcTnI R146G, which showed increased Ca$^{2+}$ sensitivity, but never showed hypertrophy or pathology except in parous females. In contrast, Sanbe et al. (Sanbe et al. 2005) found increased Ca$^{2+}$ sensitivity and significant levels of interstitial fibrosis and myocyte disarray even at low protein expression in a transgenic rabbit model which carrying rabbit cardiac TnI R146G (rcTnI R146G). Indeed, the high replacement of this TnI mutation in rabbit models caused premature death.

1.3.2 Restrictive Cardiomyopathy

Restrictive cardiomyopathy (RCM) is a myocardial disorder characterized by impaired diastolic filling (rapid early filling and slow late filling of the left ventricle), and reduced diastolic volume of either or both ventricles with normal or near normal systolic function and wall thickness. Among all cardiomyopathies, RCM responds worst to treatment and is associated with the greatest morbidity and mortality (Artz and Wynne 2000). RCM can be classified as primary or secondary to some systemic disorder, such as a metabolic disorder (Fabry’s disease) or infiltrative disorder (amyloidosis) (Ammash et al. 2000; Mogensen et al. 2003). Primary RCM includes endomyocardial fibrosis, Loeffler’s endocarditis and idiopathic RCM. The latter is non-infiltrative and the only
detectable histological abnormality is interstitial fibrosis of the myocardium. Very recently, a linkage study on 6 RCM patient’s family and some of their surviving relatives has demonstrated that idiopathic RCM was part of the clinical expression of cTnI mutation (Mogensen et al. 2003). All these mutations on cTnI (L144Q, R145W, A171T, K178E, D190G and R192H) appeared in conserved and functionally important domains of the gene, especially R145W that is in the inhibitory region of cTnI. Mogensen et al. (Mogensen et al. 2003) had reported that two patients with R145W mutation had typical features of RCM in their late fifties and had the symptom of heart failure with both left and right atrial enlargement. A number of researchers have studied the effects of the six cTnI RCM-related mutations on actomyosin ATPase and contractility in reconstitute system and transgenic system. Gomes et al. (Gomes 2005) showed that five RCM-related cTnI mutations (L144Q, R145W, A171T, K178E, and R192H) increased the Ca\(^{2+}\) sensitivity of force development in reconstituted porcine skinned fibers and decreased the ability of cTnI to inhibit actomyosin ATPase activity in the presence of 1 mM EGTA in an actin-Tm activated myosin-ATPase assay. Among these mutations, the hcTnI R145W mutation has the poorest ability to inhibit actomyosin ATPase activity and force development in the absence of Ca\(^{2+}\) and the lowest maximal force in the presence of Ca\(^{2+}\). Yumoto et al. (Yumoto et al. 2005) also showed that all RCM-related cTnI mutations increase the Ca\(^{2+}\) sensitivity of force development in reconstituted rabbit skinned fibers. Davis et al. (Davis et al. 2007) showed increased Ca\(^{2+}\) sensitivity of force development and reduced basal sarcomere length in R193H-transduced rat cardiac myocytes. Du et al. (Du et al. 2006) showed reduced left ventricular end diastolic volumes in the hcTnI R192H transgenic mice. Interestingly, different point mutations at the 145 residue in cTnI
linked to different cardiomyopathies. The hcTnI R145G mutation and R145W mutation correlate with HCM and RCM, respectively. To further study mutations at residue 145, measurements of physiological function were made using hcTnI R145G transgenic mice (Tg-R145G) and hcTnI R145W transgenic mice (Tg-R145W) which were compared to hcTnI wild type transgenic mice (Tg-WT).

The working hypothesis is that the R145W RCM and R145G HCM cTnI mutations both alter myosin cross-bridge kinetics and Ca\(^{2+}\) sensitivity of contraction in different manners as to cause different diastolic filling patterns and systolic function. The objective is to comprehensively study the impaired TnI inhibitory functions, Ca\(^{2+}\) sensitivity of contraction, maximum force and ATPase activity, fractional cross-bridge attachment, kinetics of cross-bridge turnover rate, energy cost and force and calcium transients in the Tg-R145G and Tg-R145W compared with Tg-WT. Comparative analysis of the effects of the RCM and HCM mutations is expected to allow better understanding of the pathogenesis of the distinct diseases. Specific aim 1 is to characterize the pathology of transgenic mice carrying either the HCM mutation hcTnI R145G or the RCM mutation hcTnI R145W. Specific aim 2 is to elucidate the possible mechanisms underlying the distinct phenotypes observed in HCM and RCM through the study of transgenic mice.
Chapter 2

Materials and Methods

2.1 Generation of Transgenic Mice

2.1.1 Cloning of Human Cardiac Troponin I – Wild type and Mutant Isoforms

The cDNA of human cardiac troponin I (hcTnI) was previously cloned in our lab by the reverse transcriptase-polymerase chain reaction using a template of total RNA from human myocardium and oligonucleotide primers specific for the 5’ and 3’ regions of the respective coding sequences (Zhang et al. 1995). Two mutations, R145G and R145W, were introduced into hcTnI by sequential overlapping PCR (Ausubel et al. 1995).

Amplified cDNA fragments of hcTnI R145G, R145W and WT were subcloned into Nco/BamH1 site of the pET3d expression vector. The WT, R145G and R145W cDNAs were released from pET-3d and subcloned into the Sall site of the plasmid, α-myosin heavy chain (α-MHC) clone (a gift from Dr. J. Robbins, Cincinnati Children's Hospital Medical Center) by using the blind end ligation method. The subcloned DNA was transformed into E.coli DH5α cells. The transformed cells were added onto LB-
Ampicillin plates and incubated at 37°C overnight for the development of transformed colonies. Two positive clones were picked by RCR with hcTnI R204C 3’ and hcTnI A2V 5’ primers and the right orientation of plasmid DNA was determined by XhoI and HindIII restrictive enzymes, which digested the correct oriented plasmid DNA to a ~500bp fragment and the incorrect oriented plasmid DNA to a ~100bp fragment. The gene construction was isolated from those correct oriented plasmid DNA and purified by Qiagen plasmid DNA purification kit. All mutations were verified by sequencing and the correct inserts were used for transgenic mice preparation.

2.1.2 Construction of hcTnI WT/R145G/R145W Transgene

α-MHC clones (a gift from Dr. J. Robbins, Cincinnati Children's Hospital Medical Center) with the correct insertion of plasmid DNA were digested by NotI restrictive enzyme. The resulting gene construction contained about 5.5 kb of the mouse α-MHC promoter, including the first two exons and part of the third, followed by hcTnI WT/R145G/R145W-cDNA and a downstream 630-base paired 3’-untranslated region (3’-UTR) of the human growth hormone (hGH) enhancer (Figure 2.1). This total ~7.0 kb fragment was used for the microinjection to generate hcTnI WT/R145G/R145W transgenic mice (Tg-WT/ Tg-R145G/Tg-R145W).
Figure. 2.1. *hcTnI* transgene construction. A schematic diagram of the *hcTnI* transgene to generate *hcTnI* wild type, R145G and R145W mutant expressing mice. Sequences corresponding to the α-myosin heavy chain (α-MHC) promoter/ hGH enhancer sites are shown as purple and white boxes, respectively. Sequences corresponding to the *hcTnI* cDNA are shown as blue box. The point mutation R145G and R145W introduced into the *hcTnI* cDNA, relevant restriction sites and transcription start site are indicated.

2.1.3 Purification of *hcTnI* WT/R145G/R145W Transgene for Microinjection

The total ~7.0 kb fragment released from α-MHC clone by NotI was purified by agarose gel electrophoresis, followed by electroelution and resuspension in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA at a final concentration of 20μg/ml for transgenic microinjection.

Run the digested DNA on an agarose gel and stain with ethidium bromide. Visualize with long wave uv light to avoid degradation and excise the band containing the gene construction. Use a Qiagen gel extraction kit to free the DNA from the agarose gel. Add 1/10 volume of 3M acetate and ethanol precipitate in 2-2.5 volumes of absolute ethanol. Centrifuge the DNA at 4˚C and wash well with 70% ethanol. Resuspend the DNA in the low salt buffer (0.2M NaCl, 20mM Tris HCl and 1.0mM EDTA, PH 7.4) and pass the DNA through elutip-D mini-column sold by Schleicher and Schuell. Wash the minicolumn by the low salt buffer. Elute the DNA from the minicolumn by passing the
high salt buffer (1.0M NaCl, 20mM Tris HCl and 1.0mM EDTA, PH 7.4) through the minicolumn. Ethanol precipitate and wash well with 70% ethanol. Be careful to evaporate the ethanol completely and resuspend the DNA in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA at a final concentration of 20μg/ml for transgenic microinjection.

2.1.4 Strategy to Generate Transgenic Mice

Since the first gene transfers into mouse was successfully executed in 1980, transgenic mice allow researchers to observe experimentally what happens to an entire organism during the progression of a disease. Transgenic mice have become models for studying human diseases and their treatments. Transgenic mouse is an organism that has DNA introduced into one or more of its cells artificially. There are two major ways to generate transgenic mice. One method of producing transgenic animals (knock-in mice), is accomplished by introducing the DNA into embryonic stem (ES) cells and selecting for cells in which the DNA has undergone homologous recombination with matching genomic sequences. Another method, which I used, is injected purified transgene (hcTnI/R145G/R145W) into the fertilized mouse eggs. Embryos were implanted in the uterus of a surrogate mother. The cDNA can integrate anywhere in the genome in this method, and multiple copies often integrate in a head-to-tail fashion. There is no need for homology between the injected DNA and the host genome. The selected gene will be expressed by some of the offspring (Figure 2.2). Offspring, also called founder mice, were identified by southern blot and PCR using tail clip DNA, probed with the hcTnI cDNA fragment. For the southern blot, EcotI restrictive enzyme was used to digest the tail clip DNA. There are several EcotI restrictive sites on the murine cardiac troponin I (mcTnI) introns,
which digest 2.4Kb and 1.4Kb restriction fragment. In addition, there are two restrictive sites on the injected transgene, one is on the a-MHC promoter and the other one is on hGH enhancer, which can digest a 3.0Kb restrictive fragment. So basically only the mice have the injected transgene have the 3.0 Kb fragment. However, all mice should have the 2.4 and 1.4Kb fragments. The PCR method also used to confirm the result. Paired primers, one from a-MHC promoter and the another one from R192 residue of the hcTnI cDNA were used to amplify a 600bps fragment. Each PCR reaction also included a set of primers specific for the mouse-actin gene to confirm successful genomic amplification (eliminating false negative animals and producing a 200-bp PCR product).

**Transgenic Mice**

Figure. 2.2. *Strategy to generate transgenic mice.* Injected embryos are implanted in the uterus of a surrogate mother. The selected gene will be expressed by some of the offspring.
2.2 Analysis of mRNA Expression in Transgenic Mouse Hearts

The expression of messenger RNA (mRNA) in the transgenic mouse hearts was determined by real time PCR. The left ventricles of mouse hearts were dissected and immerse in RNA later (Qiagen®) overnight in -4°C. Total RNA was extracted by using RNeasy® Fibrous Tissue kit (Qiagen®). After extraction, the quantity and quality of total RNA was identified by spectrophotometer and 12% agarose gel electrophoresis, respectively. In order to measure mRNA, reverse transcriptase PCR was performed to convert mRNA to cDNA, which was then amplified by PCR. 100ng RNA in 20μl total volume (according to RNA concentration calculated from the spectrophotometer) was used to perform reverse transcriptase PCR to get 100ng/1μl cDNA. 100ng of RT product cDNA was used to prepare 20μl target sample. 1ng of cDNA was used to prepare 20μl endogenous control 18s sample. In target sample and endogenous 18s master mix, TagMan Gene Expression Assay for human TNNI3 (cardiac) (Applied Biophysics®) and TagMan endogenous control human 18s (Applied Biophysics®) was used respectively. Run triplicates for each sample in real time PCR. The relative quantity (RQ), which indicates fold change, is equal to $2^{\Delta\Delta Ct}$. $\Delta\Delta Ct$ (test sample)=Average $\Delta Ct$ (test sample)-Average $\Delta Ct$ (calibrator sample). The calibrator sample in my study is NTg. $\Delta Ct = Ct(target)-Ct(endogenous\ control\ 18s)$. Threshold cycle (Ct) represents the PCR cycle at which the SDS software first detects a noticeable increase in reporter fluorescence above a baseline signal.
2.3 Analysis of hcTnI Protein Expression in Transgenic Mouse Hearts

The expression of hcTnI protein in the transgenic mouse hearts was determined by Western blot analysis. The experimental mouse hearts and control human heart tissue were minced in a solution of 1% (v/v) β-mercaptoethanol, 1% (w/v) SDS, 1 mM PMSF, 1 mM EDTA, and Protease Inhibitor Cocktail (Sigma). These samples were homogenized in 20 mM Tris-HCl, pH 7.4, 1% SDS, 1% β-mercaptoethanol, 10% glycerol on ice, and the total protein concentration of each cleared homogenate was determined by Bio-Rad Coomassie Plus assay. SDS-PAGE gels (15%) were run with a total of 2 μg of protein for each lane. Proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, GA, USA). The membrane was blocked in Rockland blocking buffer for an hour at room temperature. A monoclonal antibody 6F9 (Fitzgerald Industries international Inc.) was used at 1:4,000 dilution for an hour to detect both human cardiac TnI (hcTnI) and mouse cardiac TnI (mcTnI). The 6F9 Mab recognizes the epitope 189-195 (amino acids: DWRKNID). This epitope is the same in both hcTnI and mcTnI, as shown in Figure 2.3 (the comparison of hcTnI and mcTnI), so 6F9 Mab recognize both proteins (Katrukha and Voipio-Pulkki 1998). The hcTnI has 211 amino acids and mcTnI has 210 amino acids. There are 16 amino acids difference between them. The gel mobility of hcTnI is faster than that of mouse because of its lower molecular mass (24 kDa versus 25 kDa, respectively). Immunoreactivity was detected using goat anti-mouse IgG antibody labeled with CY5.5 fluorescent dye at 1:3000 dilution for an hour in room temperature and the reaction signal blot was scanned with Odyssey Infrared Imager (LICOR) and analysis was carried out on Dell Pentium Computer using Scion Image software. The percentage of transgenic protein expression was calculated as:
{Transgenic human TnI / (Endogenous mouse TnI+ Transgenic TnI)} X100

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Figure 2.3 *The amino acids difference between hcTnI and mcTnI*. The red amino acids indicate the differences in the human cardiac TnI (hcTnI) compared to mice cardiac TnI (mcTnI).

### 2.4 Histopathological Characterization

Different age groups (4 months, 6 months, 12 months, over 15 months) of mice were used. After euthanasia, the hearts were excised, weighted and immersed in 10% (v/v) buffered formalin. Slides of whole hearts were prepared by American Histolabs, Inc., Gaithersburg, Maryland. The paraffin embedded, longitudinal sections of whole hearts stained with hematoxylin & eosin (red muscle fibers and blue nucleus) and masson trichrome (blue collagen and red muscle fibers) were examined for overall morphology, hypertrophy, myofilament disarray and fibrosis by using zeiss microscope and 40x/0.65 Plan Apos objective. To assess potential mutation-mediated hypertrophy, the heart weight to body weight ratios were determined for all sacrificed mice.
2.5 Functional Studies in Skinned Papillary Muscle Fibers

2.5.1 Apparatus

Mouse skinned and intact papillary muscle preparations were used in the Guth muscle research system (Figure 2.4) that allows to make simultaneous mechanical and optical measurements. In skinned papillary fibers, force and actomyosin ATPase (measured by NADH fluorescence changes) were measured simultaneously while subjecting the skinned fibers to a continuously increasing gradient of Ca\(^{2+}\). Similarly, in intact papillary muscle, force and intracellular Ca\(^{2+}\) transients were both measured simultaneously.

A simplified diagram of the Guth Muscle Research System used is shown in Figure 2.4. It consists of a mechanical system depicted by the two boxes labeled force transducer and linear motor. The intact or skinned muscle depicted in red was attached at one end to the force transducer and at the other end to the linear motor. The muscle preparation was enclosed in either a 3 mm diameter quartz cuvette (for intact fibers) or 1 mm diameter cuvette (for skinned fibers). The cuvette was then perfused by a computer controlled perfusion system with test solutions. The force transducer measures the force. The optics system consists of a light source depicted as a light bulb, a filter wheel spinning at 250 Hz containing the excitation filters used in the experiment, an objective to collect the fluorescence (NADH for skinned fiber, Ca Green-2 for calibration, or Fura-2 for intact fiber), a beam splitter for directing the emission light to two photomultiplier tubes (PMTs), through two emission filters (Em Filter). The optical part of the system also has a signal sorter which sorts out the fluorescence signals associated with the excitation
filter in the light path. For example in the case of Fura-2 (fluorescence indicator for Ca\(^{2+}\)) it is excited at two different excitation wavelengths (excitation filters in the wheel are 340 and 380 nanometers). The fluorescence light associated with 340 and 380 nanometers passes through one of the emission filters (500 nanometer long pass cutoff) to one PMT and is captured by the signal sorter. Associated voltage is read out to the A-D board of the computer every 1/250 of a second (4 milliseconds). The computer automatically takes the ratio (340/380 fluorescence ratio) and displays it on the computer monitor screen in time of calcium. For the NADH fluorescence measurements a UG11 (ultraviolet) excitation filter was used and the emission filter was a 500 nanometer cutoff filter. For the calcium Green-2 fluorescence measurements, a 480 nanometer excitation filter was used and the emission filter was a 515 nanometer OG cutoff filter. The filter wheel limits the time resolution of the optical measurements to 4 milliseconds, which is the time it takes the filter wheel to make one revolution.
2.5.2 Simultaneous Force and ATPase Measurement

Mouse papillary muscles, ~1mm long and 60-90 μm in diameter, were dissected from excised mouse heart in relaxing solution and then skinned in relaxing solution containing 1% (v/v) Triton X-100 for 30 minutes at room temperature. Standard solution contains 85 mM K$^+$, 2 mM MgATP$^{2-}$, 1 mM Mg$^{2+}$, 7 mM EGTA, 10$^{-9}$ M to 10$^{-3.4}$ M Ca$^{2+}$, and propionate as the major anion. Ionic strength is adjusted to 0.15 and pH is maintained at 7.00 ± 0.02 with imidazole propionate. Relaxing solutions are solutions with no added Ca$^{2+}$. Maximal contracting solutions are solutions that give maximal tension beyond which increasing Ca$^{2+}$ further does not increase tension. For ATPase measurements...
solutions contain in addition to the constituents described in the above section 5 mM phosphoenol pyruvate (PEP), 0.4 mM NADH, 100 units/ml pyruvate kinase (PK), 140 units/ml L-lactic dehydrogenase (LDH). The concentrations of the various ionic species in the maximal contracting and relaxing solutions are determined by a computer program using binding constants from the literature (Donaldson et al. 1975). The Ca\(^{2+}\) concentration in the cuvette perfusing the skinned preparation was varied by use of a gradient maker (Scientific Instruments GmbH, Heidelberg) to mix two solutions (relaxing and contracting solutions) of known Ca\(^{2+}\) and ionic composition together. A complete description of the method is given in Allen et al. (Allen et al. 2000). The Ca\(^{2+}\) concentration in the cuvette perfusing the skinned preparation was varied continuously from pCa 9 to pCa 3.4. The fluorescent Ca\(^{2+}\) indicator, Calcium Green-2 (Molecular Probes), was used to calibrate and calculate the [Ca\(^{2+}\)] during measurements. Calcium Green-2 changes its fluorescence over the range of [Ca\(^{2+}\)] required for activation of contraction and ATPase activity. The \(K_d\) of Calcium Green-2 used to calculate pCa was \(10^{-5.53}\) M. The concentration of Calcium Green-2 in the gradient solution was 1.0 μM. The Calcium Green-2 fluorescence was excited at 480nm and the fluorescence measured with a cut-off filter at 515 nm.

The skinned fibers were placed in a quartz cuvette and mounted in the Guth Muscle Research System (Guth and Wojciechowski 1986), which allowed for simultaneous measurements of force and ATPase activity (Wang et al. 1999; Ward et al. 2004; Hernandez et al. 2005). The sarcomere length of the fibers was set by removing the slack from the fiber and stretching the fiber 10%. The solution in the cuvette was changed every 20s using a peristaltic pump triggered by a computer. The hydrolysis of ATP was
measured by the NADH fluorescence method, in which ATP is regenerated from ADP and PEP by the enzyme PK (Reaction 2.1) (Takashi and Putnam 1979; Guth and Wojciechowski 1986). This reaction is coupled to the oxidation of NADH (fluorescent) to NAD (nonfluorescent) and the reduction of pyruvate to lactate by L-lactic dehydrogenase (LDH) (Reaction 2.2) (Takashi and Putnam 1979; Guth and Wojciechowski 1986; Wang et al. 1999). In this reaction 1.0 mol of PEP and NADH are used to produce 1.0 mol of ATP and NAD. The solution surrounding the fiber in the quartz cuvette was illuminated at 340 nm and the decrease in NADH concentration detected by a decrease in the fluorescence signal at wavelength 450 nm. The slope of the linear decrease in NADH concentration was used to calculate ATPase activity. The solution in the cuvette was changed every 20s to replenish the solution in the cuvette. In this manner, a continuous increasing $\text{Ca}^{2+}$ gradient is achieved and fresh un-oxidized NADH solution is introduced into cuvette every 20s, and the fluorescence change taking place between each solution change was converted to the rate of ATP hydrolysis by comparison to NADH standards. An sample of such data is shown in Figure 2.5.

\[
\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate} \quad 2.1
\]

\[
\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD} \quad 2.2
\]

Pk-pyruvate kinase; LDH-lactate dehydrogenase; PEP-phosphoenol pyruvate
Figure. 2.5. *Typical raw data of the NADH fluorescence change and force during Ca\(^{2+}\) activation in mouse skinned papillary fiber.* The upper trace is the NADH fluorescence. The lower trace is the force. The skinned fiber is subjected to an increasing Ca\(^{2+}\) gradient. The ATPase rate and force increase with increasing Ca\(^{2+}\) concentration. The saw tooth appearance of NADH fluorescence results from the solution change that refreshes the NADH in the cuvette every 20 seconds. The falling slope of the NADH fluorescence is used to calculate the ATPase rate.

2.5.3 Fractional Cross-bridge Attachment at Maximal Ca\(^{2+}\) Activation

At the end of each experiment, 10 mM MgADP was substituted for 2 mM ATP in the maximal Ca\(^{2+}\) activating solution in the cuvette perfusing the fiber. This substitution forces all cross-bridges into the force generating state producing the maximal force that the fiber can develop. The maximal Ca\(^{2+}\) activated force is then divided by the maximum force the muscle can develop in the presence of 10 mM MgADP. This ratio is the fractional cross-bridge attachment at maximal Ca\(^{2+}\) activation.
2.5.4 Cross-bridge Turnover Rate (g)

The force that a muscle develops can be characterized by the apparent rate of the formation of force generating states (f) and the dissociation of force generating states (g) according to the two state model of Huxley (1957), as shown in the scheme below (Kerrick and Xu 2004).

\[ \text{Non-Force} \xrightarrow{f} \text{Force} \xleftarrow{g} \text{Non-Force} \]

Based on this kinetic scheme, g can be calculated by the following equation:

\[ g = \frac{\text{ATPase}}{\text{total cross-bridges attached}} \]

Total cross-bridges attached = [myosin subfragment1 head] x fractional cross-bridge attachment any Ca\(^{2+}\) concentration. The fractional cross-bridge attachment at any Ca\(^{2+}\) concentration equals to the fractional cross-bridge attachment at maximal Ca\(^{2+}\) activation times the normalized force. The total intracellular myosin subfragment1 head concentration in muscle is approximately 154 µM (Ferenczi et al. 1984). In this study, g was calculated based on the above equation.

2.5.5 Energy Cost (used energy/ unit force)

It is generally accepted that the steady state force a muscle can develop is proportional to the number of cross-bridges in the force generating state and the ATPase activity is proportional to the rate of cross-bridge cycling. Thus, force and ATPase
activity can be expressed as equation 2.3 and 2.4. Where \( S_{1/2}A[M] \) equals to the total number of myosin cross-bridges in a half sacromere length of the muscle and \( F_{avg} \) is the average force/cross-bridge. Therefore, energy cost (used energy/unit fiber), which equals to \( ATPase/force \) can be expressed as equation 2.5.

\[
\text{Force}=AS_{1/2}[M]f_{avg} \quad 2.3
\]

\[
\text{ATPase}=AS_{1/2}[M]g(L/S_{1/2}) \quad 2.4
\]

\[
\text{ATPase/force (Energy cost)}=(L/S_{1/2}) (g/f_{avg}) \quad 2.5
\]

\( A \): cross-section area of fiber; \( S_{1/2} \): half sacromere length
\( L \): fiber length; \( f_{avg} \): average force/cross-bridge
\( [M] \): myosin S1 head concentration

2.5.6 Force-Length Measurements Under Relaxing Conditions

In this experiment, 2,3-butanedione monoxime (BDM) was used to test if there are higher numbers of cross-bridges attached in the mutant skinned fibers compared to controlled skinned fibers under relaxing conditions. BDM is an effective, fast acting and fully reversible inhibitor of cardiac contractility in man (Mulieri et al. 1989; Schwinger et al. 1994). Brixius and Schwinger (Brixius and Schwinger 2000) found that BDM exerts negative inotropic activity by reducing the number of force generating cross-bridges, possibly by increasing the cross-bridge detachment rate as well as by reducing force
generation per cross-bridge in human myocardium. From previous study, it was learned that BDM affects striated muscle contraction of at least three levels: in the sarcoplasmic reticulum, in the thin filament regulatory mechanisms and during actomyosin interaction. In cardiac muscles, it was reported that the addition of BDM reduced peak calcium release from the SR (Blanchard et al. 1990; Gwathmey et al. 1991). Since the skinned fiber was used in this experiment, the effect of BDM we are interested in is during the thin filament regulatory mechanisms and actomyosin interaction. Many of these studies suggest that the suppression of muscle contraction is mainly due to a direct action of BDM on the myosin head. Kawai and Zhao (Zhao and Kawai 1994) found that BDM affects two major steps in the cross-bridge cycle: the nucleotide binding and the cross-bridge attachment (force-generating) steps.

The force-length experiment was performed by using mouse papillary muscle, which was skinned and mounted as described above (Guth and Wojciechowski 1986). After mounting the fiber, it was perfused in the pCa 9.0 solution. Once mounted in the tweezers the slack from the fiber was removed. The force measurement at this point was set as zero. The fiber was then stretched to 10% of its original length. When the steady state force was established, data points were recorded as shown in Figure 6a. Each data point was taken at 10s intervals. After several data points were collected, the pCa 9.0 perfusing solution in the cuvette was changed to pCa 9.0 with the 3mM BDM solution and force data was again recorded. Without changing the solution, the fiber was stretched to 20% of its original length. After the force reached steady state, we again recorded the force before changing the pCa 9.0 3mM BDM to the pCa 9.0 minus BDM solution. The procedure was repeated at the 30% and 40% stretch of its original length. After 40%
stretch, we released fiber to its original length and changed the solution to maximum activating pCa 3.4 solution to contract the fiber. The force at each stretch was normalized to the maximum force the fiber can develop at pCa3.4.

2.6 Functional Studies in Intact Papillary Muscle Fibers

Following CO₂ euthanasia, hearts were removed quickly and soaked in ice-cold saline (0.9% (w.v) NaCl). Intact papillary muscle was dissected quickly from right ventricles in oxygenated Krebs–Henseleit solution (119 mM NaCl, 4.6 mM KCl, 11 mM glucose, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2mM MgSO₄ 1.8mMCaCl₂) containing 30mM BDM and mounted in the Guth Muscle Research System (Wang et al. 1999) (Figure 2.4). The effect of BDM on the calcium transient was small and not enough to account for the tension change in the intact fiber (Horiuti et al. 1988). Fibers were loaded in oxygenated Krebs-Henseleit solution without 2, 3-butanedione monoxime for an hour at room temperature to allow the fiber to adapt to the extracellular environment. The fibers were then loaded with 5 μM Fura-2 AM (AM, acetoxymethyl ester form) for 1 h at room temperature in oxygenated Krebs–Henseleit solution containing 0.5% (v/v) of the non-cytotoxic detergent Cremophor, which increases the solubility of Fura-2 AM. Fura 2 was used only to measure the time course of the intracellular Ca²⁺ transients, but not the actual calculations of intracellular Ca²⁺. Because Baylor and Hollingsworth (Baylor and Hollingworth 2000) showed that Fura-2 is sufficiently hydrophobic that it binds to proteins which in turn alter the Kd of Ca²⁺ for Fura-2. Since there is no way to know the ratio of calcium bound to unbound Fura-2 is in the cytoplasm, so the Kd of Fura-2 for
Ca\textsuperscript{2+} cannot be known. In addition the two other important parameters of Fura-2 are greatly altered, Fmin and Fmax. Therefore Fura-2 was not used to measure intracellular calcium. However the off-rate of Ca\textsuperscript{2+} from Fura-2 is fast enough to measure the time course of Ca\textsuperscript{2+} transients in cardiac muscle (Baylor and Hollingworth 2000). Therefore Fura-2 was used to measure the time course of the intracellular Ca\textsuperscript{2+} transients.

The intact papillary muscle was stimulated at 1.0 Hz through the two tweezers attached to the ends of the preparation. Once the preparation was mounted in the apparatus, muscle length was adjusted until maximum active twitch force was obtained. Force and fluorescence corresponding to either 340 nm or 380 nm excitation were recorded by the computer. The fluorescence 340 nm/380 nm ratio was calculated and plotted along with force. Force and calcium transients were normalized and then averaged and plotted as a function of time.

2.7 Statistical Analysis

Data are expressed as the average of n experiments ±SE (standard error). Statistically significant differences were determined using an unpaired Student's \textit{t}-test (Sigma Plot 8.0), with significance defined as * \( P<0.05 \) and ** \( P<0.01 \).
Chapter 3

Characterization of Transgenic Mice and Histopathology Results

3.1 Characterization of Transgenic Mice

3.1.1 The hcTnI cDNA Expression in Tg-WT/R145G/R145W

**Southern Blot**-EcoT1 restrictive enzyme was used to digest the tail clip DNA in southern blot. There are several EcoT1 restrictive sites on the murine cardiac troponin I (mcTnI) introns, which digest the 2.4Kb and 1.4Kb restriction fragments. Also there are two restrictive sites on the injected transgene, one is on the α-myosin heavy chain promoter (α-MHC) and the other one is on human growth hormone enhancer, which digest a 3.0Kb fragment. Basically, only mice have the injected transgene show the 3.0 Kb band, but all mice should show 2.4 and 1.4Kb bands, as shown in Figure 3.1.

Two lines of human cardiac troponin I (hcTnI) wild type transgenic mice (Tg-WT), two lines of hcTnI R145G transgenic mice (Tg-R145G) and four lines of hcTnI R145W transgenic mice (Tg-R145W) were produced (Figure 3.1). However, line 1 of
Tg-R145G and line 3 and 4 of Tg-R145W could not be studied in detail because of the early death and paucity of transgenic offspring. The remaining lines were examined.

Figure. 3.1. *A representative of Southern blot*. Two positive lines of Tg-WT and four positive lines of R145W are shown. Tg-RW represents the Tg-R145W. The number after the Tg-WT and Tg-RW indicates the line number.

**PCR Method**-Paired primers, one from α-MHC promoter and another one from R192 residue of the hcTnI cDNA were used to amplify a 600bps fragment. Each PCR reaction also included a set of primers specific for the mouse-actin gene to confirm successful genomic amplification (eliminating false negative animals and producing a 200-bp PCR product) (Figure 3.2).

Figure. 3.2. *A representative of PCR results*. The upper 600bps fragment was amplified by two primers from α-MHC promoter and R192 residue of the hcTnI. The lower 200bps fragment was amplified by actin primers as loading control.
3.1.2 The Gene Expression in Tg-WT/R145G/R145W

Real Time PCR: The messenger RNA (mRNA) expression of the transgene in Tg-WT/Tg-R145G/Tg-R145W is detected by real time PCR. The transgenic threshold cycle (Ct) values of endogenous control 18s was normalized to non-transgenic mice (NTg) Ct value to show the changes in endogenous gene expression (Figure 3.3). As shown in Figure 3.3, there are no changes in endogenous gene expression in all groups of mice. The relative quantity (RQ), which indicates the fold change in cDNA level of transgenic heart compared to that of NTg, was used to show the transgene expression changes in all groups of transgenic mice (Figure 3.4). As shown in Figure 3.4, Tg-WT line 1, Tg-R145W line 1 and line 2 all have same level of cardiac troponin I (cTnI) gene expression compared to NTg, indicating that there are no significant increases in transgene expression. However, gene expression in Tg-WT line 2 and Tg-R145G line 2 was increased 2.7 and 1.9 fold respectively compared to NTg.
Figure. 3.3. **Summary of endogenous control 18s gene expression in Tg-WT, Tg-R145G and Tg-R145W lines normalized to NTg.** The number indicates the line number. The results are the mean $\pm$ SD of 3 measurements.

Figure. 3.4. **Summary of target gene (cTnI) expression in Tg-WT, Tg-R145G and Tg-R145W lines normalized to NTg.** The number indicates the line number. The results are the mean $\pm$ SD of 3 measurements.
3.1.3 The hcTnI Protein Expression in Tg-WT/R145G/R145W

**Western Blot**-Mutant protein expression is driven by the α-MHC promoter in the mouse heart and were measured by Western blot analysis. The hcTnI WT, R145G and R145W protein expression in thin filament was normalized to total cTnI content. The human cTnI replaced the murine cTnI in the heart, so the murine cTnI was reduced. Two lines of Tg-WT express 9.84% ± 2.37 (Line1) and 66.4% ± 2.26 (Line2) of hcTnI in mouse heart, respectively. Two lines of Tg-R145G express 2.34% ± 0.47 (Line1) and 35.8% ± 2.0 (Line2) of mutant protein, respectively. The remaining two lines of Tg-R145W express 11.20% ± 1.44 and 9.57% ± 1.5 of mutant protein respectively (Figure 3.5 and Figure 3.6). The relationship between gene expression and protein incorporation is linear as shown in Figure 3.7, indicating that the mutant protein incorporation to the thin filament is proportional to the gene expression. Line 2 of Tg-WT, line 2 of Tg-R145G and line 1 of Tg-R145W consistently produce expected transgene product levels and are chosen for further histopathology and functional studies.

![Western Blot Image]

Figure. 3.5. *A representative Western blot of heart extracts from Tg-WT, Tg-R145G and Tg-R145W lines versus NTg and Human Heart extraction.* The protein expression levels in transgenic mice were assessed on the basis of densitometry. Western blots labeled with monoclonal troponin I 6F9 antibody and followed by a goat anti-mouse secondary antibody.
Figure 3.6. *Summary of mutant protein express in Tg-WT, R145G and R145W lines.* The results are the mean ± SE of 5 measurements.

Figure 3.7. *Relationship between the mRNA expression level and the mutant protein (human cardiac TnI) incorporation level.* The incorporated mutant protein is proportional to the gene expression.
3.2 Histopathology Studies

3.2.1 Histopathology Results for Tg-R145G

In the histopathology studies, different age-groups (2 months, 6 months, 12 months, 15 months and 18 months) were used for all groups of mice. The heart weight to body weight ratio (R) with increasing the age of mice is evaluated. No statistically significant difference between NTg, Tg-WT and Tg-R145G was observed in R value (Table 3.1). However, it is known that HCM patients carrying mutations in cTn or Tm do not necessarily present with cardiac hypertrophy (Earing 2003; Gomes and Potter 2004; Gomes and Potter 2004), meaning that the increase of the heart weight/body weight ratio is not a good parameter to be considered as a HCM phenotype in mice. Also, no gender and age differences were found in the heart weight to body weight ratio between NTg, Tg-WT and Tg-R145G (Figure 3.8). The heart tissue morphology of NTg, Tg-WT and Tg-R145G are presented in Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12. Figure 3.9 shows hematoxylin & eosin (H & E) stain of longitudinal section of whole mouse hearts of 14-15 month old NTg (Figure 3.9a), Tg-WT (Figure 3.9b) vs Tg-R145G (Figure 3.9c). There are no dramatic morphology changes in Tg-R145G heart compared to those of NTg and Tg-WT hearts. Figure 3.10a and 3.10b show the left ventricular wall and septal sections of these hearts stained with H & E. No cellular myocardial disarray was found in either left ventricle or septum of Tg-R145G heart compared to control hearts. Figure 3.11a and 3.11b show the left ventricular wall and septal sections of these hearts
stained with masson trichrome. In masson stain, no fibrosis was observed in all groups of hearts. As shown in Figure 3.12, the papillary muscles of these mice show no myofibrillar disarray compared to NTg and Tg-WT littermates. These papillary muscles are used in the skinned fiber and intact fiber studies. Since no on-set information about the cTnI R145G related HCM, the oldest 18 month old mice were used in this histological studies and no myocardial disarray and fibrosis was found. Basically, there were no apparent histological differences in the Tg-R145G hearts.

Table 3.1: Heart weight to body weight ratios (R) in Tg-R145G versus Tg-WT and NTg

<table>
<thead>
<tr>
<th>Age (m)</th>
<th>Gender</th>
<th>NTg H(g)</th>
<th>Tg-WT H(g)</th>
<th>Tg-R145G H(g)</th>
<th>NTg B(g)</th>
<th>Tg-WT B(g)</th>
<th>Tg-R145G B(g)</th>
<th>R(10^-3)</th>
<th>R(10^-3)</th>
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<td>0.103</td>
<td>0.129</td>
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Rav = 4.82±0.21, Rav = 5.37±0.18, Rav = 5.12±0.23

N=12, N=13, N=16

A, age of the animal (months); G, gender; H, heart weight (g); B, body weight (g); R= H/B. NTg controls are from the littermates of the Tg-R145G line 2. The average ratios (R) are means of n experiments ±SE. None of the differences between NTg, Tg-WT and Tg-R145G are statistically significant (P>0.05).
Figure 3.8. *Heart weight to body weight ratio (R) between Gender (a) and Age (b) in NTg, Tg-WT and Tg-R145G.* There are no statistically significant differences between gender and age in all groups of heart (P>0.05). The results are the mean ± SE of n measurements (n=5~10).
Figure 3.9 a) H & E stain of longitudinal section of NTg (15 months) heart
Figure 3.9 b) H & E stain of longitudinal section of Tg-WT (14 months) heart

Figure 3.9 c) H & E stain of longitudinal section of Tg-R145G (15 months) heart
Figure 3.9 *Hematoxylin & eosin (H&E) stain of longitudinal section of whole mouse hearts.* a) 15 month old female NTg heart. b) 14 month old female Tg-WT heart. c) 15 month old female Tg-R145G heart. Whole Tg-R145G heart showed no dramatic morphology change compared to those of Tg-WT or NTg littermates.
Figure 3.10 a) LV section (H &E Stain)

NTg (15 months)

Tg-WT (14 months)

Tg-R145G (15 months)
Figure 3.10 b) Septum (H &E Stain)

NTg (15 months)

Tg-WT (14 months)

Tg-R145G (15 months)

Figure 3.10 *Left ventricular wall (a) and septal sections (b) of hearts showed in Figure 3.9 stained with hematoxylin & eosin.*
Figure 3.11 a) LV section (masson trichrome stain)

NTg (15 months)

Tg-WT (14 months)

Tg-R145G (15 months)
Figure 3.11. Left ventricular wall (a) and septal sections (b) of hearts showed in Figure 3.9 stained with masson trichrome.
Papillary muscle fiber (H&E stain)

NTg (15 months)

Tg-WT (14 months)

Tg-R145G (15 months)

Figure 3.12. *Hematoxylin & eosin stain of papillary muscle of Tg-R145G versus NTg and Tg-WT.*
3.2.2 Histopathology Results for Tg-R145W

Same as the histopathology studies for Tg-R145G, I separated mice with 2 month, 6 month, 12 month, and 15 month old groups. Since patients developed cTnI R145W associated RCM at their late fifties, which corresponds to mice around 15 months old, the oldest Tg-R145W examined was 15 month old. The heart weight to body weight ratio (R) with increasing the age of Tg-R145W is evaluated. No statically significant difference was found between NTg, Tg-WT and Tg-R145W (Table 3.2). Also, no gender and age differences were observed in the R value between NTg, Tg-WT and Tg-R145W (Figure 3.13). The heart tissue morphology of NTg, Tg-WT and Tg-R145W is presented in Figure 3.14, Figure 3.15, Figure 3.16 and Figure 3.17. Figure 3.14 shows the hematoxylin & eosin stain of longitudinal section of whole mouse hearts of 15 month old Tg-R145W. There was no observable morphology difference between Tg-R145W heart(Figure 3.14) compared to NTg (Figure 3.9a) and Tg-WT hearts (Figure 3.9b). Figure 3.15a and 3.15b show the left ventricular wall and septal sections of these hearts stained with hematoxylin & eosin. In H&E stain, no myocyte disarray (H&E stain) was found in both left ventricle and septum of Tg-R145W compared to control hearts. Figure 3.16a and 3.16b show the left ventricular wall and septal sections of these hearts stained with masson trichrome. In masson stain, a small extent of fibrosis was observed in the left ventricular of Tg-R145W heart compared to NTg and Tg-WT hearts. As shown in Figure 3.17, the papillary muscles of these mice show no myofibrillar disarray compared to NTg and Tg-WT littermates. These papillary muscles are used in the skinned fiber and intact fiber studies, as presented in later chapters.
Table 3.2: Heart weight to body weight ratios (R) in Tg-R145W versus Tg-WT and NTg

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<th>A(m)</th>
<th>G</th>
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\[ R_{av} = 4.82 \pm 0.21 \quad R_{av} = 5.37 \pm 0.18 \quad R_{av} = 4.92 \pm 0.18 \]

A, age of the animal (months); G, gender; H, heart weight (g); B, body weight (g); R = H/B. NTg controls are from the littermates of the Tg-R145G line 2. The average ratios (R) are means of n experiments \( \pm SE \). None of the differences between NTg, Tg-WT and Tg-R145W are statistically significant (P>0.05).
Figure 3.13. *Heart weight to body weight ratio (R) between Gender (a) and Age (b) in NTg, Tg-WT and Tg-R145W.* There are no statistically significant differences between gender and age in all groups of heart (P>0.05). The results are the mean ± SE of n measurements (n=5~10).
Figure 3.14 *Hematoxylin & eosin stain of longitudinal section of Tg-R145W heart.* Whole Tg-R145W heart showed no morphology difference compared to Tg-WT or NTg littermates, as shown in Figure 3.9a and 3.9b.
Figure 3.15 a) LV section (H &E Stain)

NTg (15 months)

Tg-WT (14 months)

Tg-R145W (13 months)
Figure 3.15 b) Septum (H &E Stain)

NTg (15 months)

Tg-WT (14 months)

Tg-R145W (13 months)

Figure 3.15 *Left ventricular wall (a) and septal sections (b) of hearts showed in Figure 3.9 and Figure 3.14 stained with hematoxylin & eosin.*
Figure 3.16 a) LV section (masson trichrome stain)

NTg (15 months)

Tg-WT (14 months)

Tg-R145W (13 months)
Figure 3.16. Left ventricular wall (a) and septal sections (b) of hearts showed in Figure 3.9 and Figure 3.14 stained with masson trichrome. A small extent of fibrosis in Tg-R145W heart compared to Tg-WT and NTg heart.
Figure 3.17. *Hematoxylin & eosin stain of papillary muscle of Tg-R145W versus NTg and Tg-WT.*
Chapter 4

Skinned Papillary Fiber Studies

4.1 Skinned Fiber Studies for Tg-R145G

4.1.1 The Ca$^{2+}$ Sensitivity Study in Skinned Fibers

Ca$^{2+}$ sensitivity is usually expressed as the Ca$^{2+}$ concentration at half maximum force or ATPase activity. A number of different factors can affect the Ca$^{2+}$ sensitivity of force or ATPase rate, such as Pi, pH, MgADP, sarcomere length, and lattice spacing, etc. These factors affect the Ca$^{2+}$ sensitivity mostly through influencing either Ca$^{2+}$ binding to cTnC or cross-bridge binding, which in turn affect TnC-calcium affinity (Gordon et al. 2000).

To investigate whether the hcTnI R145G mutation influences Ca$^{2+}$ sensitivity, simultaneous force and ATPase measurement was conducted to explore Ca$^{2+}$ sensitivity. Simultaneous ATPase-pCa and force-pCa measurements were performed in freshly skinned papillary muscle fibers from hcTnI R145G transgenic mice (Tg-R145G) compared to non-transgenic mice (NTg) and hcTnI wild type transgenic mice (Tg-WT) under isometric conditions. 3-7 month old mice were used. Measurements were also performed with 12.0±1.0 month old mice. No functional differences were observed in
these different age-groups. The ATPase activity and force were plotted as a function of pCa. Each curve is the averaged data, as shown in Figure 4.1.

An increased Ca\(^{2+}\) sensitivity in Tg-R145G fibers compared to Tg-WT fibers was seen in the steady-state force development measurements with \(pCa_{50} = 5.26 \pm 0.02\) (\(n = 10\)) and \(pCa_{50} = 5.16 \pm 0.02\) (\(n = 13\)) for Tg-R145G and Tg-WT, respectively. The \(\Delta pCa_{50} = 0.11\) was statistically significant (\(P < 0.05\)). The Hill coefficient of force in Tg-R145G fibers (\(n_H = 1.86 \pm 0.14\)) was significantly lower than in Tg-WT fibers (\(n_H = 2.52 \pm 0.12\)) (Figure 4.1a). Figure 4.1b showed that the Ca\(^{2+}\) sensitivity measured by ATPase activity was also increased in Tg-R145G fibers compared to Tg-WT fibers. The \(pCa_{50}\) was \(5.58 \pm 0.02\) (\(n = 10\)) and \(5.38 \pm 0.02\) (\(n = 13\)) for Tg-R145G and Tg-WT fibers, respectively. The \(\Delta pCa_{50} = 0.20\) between Tg-R145G and Tg-WT fibers was statistically significant (\(P < 0.05\)). As expected, there was no significant difference in \(pCa_{50}\) and Hill coefficient between the Tg-WT and NTg fibers as shown in Figure 4.1.

4.1.2 Maximum Ca\(^{2+}\) Activated Force and ATPase

Maximal force values (\(10^5\) N/m\(^2\)) and maximal ATPase rates (s\(^{-1}\) per myosin S1 head) were also presented for NTg, Tg-WT and Tg-R145G skinned fibers in simultaneous force and ATPase measurement (Figure 4.2). As shown in Figure 4.2a, there is \(~14\%\) decrease in maximal Ca\(^{2+}\) activated force between Tg-R145G fibers (0.60 \(\pm 0.03\)) and Tg-WT fibers (0.69 \(\pm 0.02\)). No statistically significant difference was observed in maximum Ca\(^{2+}\) activated ATPase between Tg-WT fibers (5.33 \(\pm 0.24\)) and Tg-R145G fibers (5.29 \(\pm 0.21\)).
Figure 4.1. The effects of hcTnI R145G mutation on Ca\(^{2+}\) sensitivity of force and ATPase activity were measured simultaneously in isometric fibers. (a) The force-pCa relationship in Tg-R145G (n= 10) versus Tg-WT (n = 13) and NTg (n = 11) fibers; (b) The ATPase-pCa relationship between Tg-R145G (n = 10) versus Tg-WT (n = 13) and NTg (n = 11) fibers. A significant leftward shift in Ca\(^{2+}\) sensitivity of both force and ATPase in Tg-R145G compared to Tg-WT. No significant difference between NTg fibers and Tg-WT fibers in both force-pCa curve and ATPase-pCa curve. The pCa values and Hill coefficients for force curve and pCa values for ATPase curve are shown. Data are expressed as mean of n experiments ± SE.
Figure 4.2. The effect of hCTnI R145G mutation on (a) maximal force (10⁵ N/m²) and (b) maximal ATPase activity (s⁻¹ per myosin head) in transgenic papillary muscle fiber. The maximal force is ~14% lower in Tg-R145G fibers compared to Tg-WT fibers. There is no significant difference in maximal ATPase in Tg-R145G fibers compared to Tg-WT fibers (P > 0.05). No statistically significant difference in both maximal Force and ATPase between NTg and Tg-WT fibers (P > 0.05). * represent statistically significant difference (P < 0.05). Data are expressed as mean of n experiments ± SE.
4.1.3 Fractional Cross-bridge Attachment at Maximal Ca$^{2+}$ Activation

At the end of each simultaneous force/ATPase experiment, the substitution of 10 mM MgADP for 2 mM ATP was made to the maximal Ca$^{2+}$ activating solution in the cuvette perfusing the fiber. After this substitution, all cross-bridges were forced into the force generating state producing the maximal force that the fiber can develop. The maximal Ca$^{2+}$ activated force is then divided by the maximum force the muscle can develop in the presence of 10 mM MgADP. This ratio is the fractional cross-bridge attachment at maximal Ca$^{2+}$ activation (Figure 4.3). This ratio indicates how many percentage of cross-bridge actually attached at the maximum Ca$^{2+}$ activation in skinned fiber.

![Graph showing fractional cross-bridge attachment at maximal Ca$^{2+}$ activation in NTg, Tg-WT and Tg-R145G fibers.](image)

Figure 4.3. *Fractional cross-bridge attachment at maximal Ca$^{2+}$ activation in NTg, Tg-WT and Tg-R145G fibers.* There are no significant differences in all groups of fibers (P > 0.05). Data are expressed as mean of n experiments ± SE.
4.1.4 The Rate of Cross-bridge Turnover (g)

Figure 4.4 shows the rate of cross-bridge turnover (g) as a function of activation state (fraction of maximum activated force). There are no statistically significant differences in g value in all groups of fibers. These results suggest that the hcTnI R145G mutation does not affect the cross-bridge turnover rate at any Ca$^{2+}$ activating levels.

Figure 4.4. *The rate of cross-bridge turnover (g) as a function of activation state (fraction of maximum activated force) in Tg-R145G skinned papillary muscle fibers compared to NTg and Tg-WT fibers.* As shown, the rate of dissociation of cross-bridge (g) is the same in Tg-R145G (n = 10), Tg-WT (n = 13) and NTg (n = 11) fibers. Data are expressed as mean of n experiments ± SE.
4.1.5 Energy Cost (used energy/unit force)

The energy cost in Tg-R145G and Tg-WT, NTg fibers were investigated by using ATPase activity divided by force in all groups of fibers. As shown in Figure 4.5, energy cost (ATPase/force) as a function of activated state in Tg-R145G skinned fibers is statistically significantly higher than that in Tg-WT and NTg skinned papillary fibers. Since energy cost is proportional to g/f_{avg} (where f_{avg} is the average force per cross-bridge), the only explanation for the increase in energy cost is that there is a decrease in f_{avg}, because g does not change as shown in Figure 4.4.

**Figure. 4.5.** The energy cost (ATPase/force) as a function of activated state (fraction of maximum activated force) in Tg-R145G skinned papillary muscle fibers compared to NTg and Tg-WT fibers. As shown, the Tg-R145G (n=10) fiber has statistically significant higher energy cost (ATPase/force) than that in Tg-WT (n=13) and NTg (n=11) fibers. Statistically significance defined as * P<0.05. Data is expressed as mean of n experiments \( \pm \) SE.
4.1.6 Force-Length Relationship

To investigate if the hcTnI R145G mutation impairs the ability of cTnI to inhibit the actomyosin ATPase activity under relaxing conditions, a force-length experiment was performed. Representative experiments for both Tg-WT and Tg-R145G fibers are shown in Figure 4.6a. The force due to 10~40% stretch under relaxing conditions (pCa 9.0) in Tg-R145G skinned papillary fibers was shown to be slightly increased compared to the Tg-WT (Figure 4.6b). In the presence of BDM, the Tg-R145G force-stretch curve shifts downward to superimpose with the Tg-WT force-stretch curve. This suggests that there is active cross-bridge attachment which is responsible for the separation of the two force-stretch curves. As shown in Figure 4.6c, Tg-R145G has two to four times more cross-bridges attached than that in the Tg-WT fibers under relaxing conditions. In agreement with Kruger et al. (Kruger et al. 2005) in murine cardiac TnI (mcTnI) transgenic mice and myofibrils, our hcTnI transgenic mice results suggest that under relaxing condition the hcTnI R145G mutation impairs the inhibition of the actomyosin cross-bridge cycling.
Figure 4.6. *Relative force in Tg-R145G skinned fiber due to different stretch levels under relaxing conditions with/without 3mM BDM.*

(a) Representative experiments for both Tg-WT and Tg-R145G fibers. Force at each stretch was normalized to the maximum Ca^{2+} activated force at pCa 3.4. The dash lines are the breaking point to change the pCa 9.0 solution with or without 3mM BDM at each stretch. (b) Tg-WT (n = 5) versus Tg-R145G (n = 3) force due to fiber stretch in the presence and absence of 3mM BDM. (c) Force differences between the presence and absence of BDM in fibers at each stretch. Differences in Tg-WT fibers and Tg-R145G fibers were shown in black and gray bars, respectively. Statistically significance defined as * P<0.05 and ** P<0.01. Data is expressed as mean of n experiments ± SE.
4.2 Skinned Fiber Studies for Tg-R145W

4.2.1 The Ca\(^{2+}\) Sensitivity Study in Skinned Fibers

Like the studies in Tg-R145G skinned fibers, the Ca\(^{2+}\) sensitivity of force development and ATPase activity, maximum Ca\(^{2+}\) activated force and maximum Ca\(^{2+}\) activated ATPase, fractional cross-bridge attachment at maximal Ca\(^{2+}\) activation, cross-bridge turnover rate (g), energy cost and force-length relationship in hcTnI R145W transgenic mice (Tg-R145W) skinned fiber were also explored. Simultaneous ATPase-pCa and force-pCa measurements were performed in freshly prepared skinned papillary muscle fibers under isometric conditions (Figure 4.7-Figure 4.12). The 3-7 month old mice were used. Measurements were also performed with 12.0±1.0 month old mice. No difference in functional studies was observed between these different age-group. The force and ATPase activity were plotted as a function of pCa. Each curve is the averaged data.

A significant increase in Ca\(^{2+}\) sensitivity of force development in Tg-R145W fibers compared to Tg-WT fibers was seen with pCa\(_{50}\) = 5.336 ± 0.02 (n = 15) and pCa\(_{50}\) = 5.157 ± 0.017 (n = 13), respectively. The ΔpCa\(_{50}\) = 0.179 was statistically significant (P < 0.05). This increase is even 0.079 unit higher than the Ca\(^{2+}\) sensitivity of force development in Tg-R145G fiber, suggesting that Tg-R145W fibers are more sensitive to Ca\(^{2+}\) than Tg-R145G fiber. Unlike Tg-R145G fiber, which has a significant lower Hill coefficient than Tg-WT fiber, there was no significant change in the Hill coefficient of force in Tg-R145W fibers (n\(_H\) = 2.47 ± 0.14) compared to Tg-WT fibers (n\(_H\) = 2.52 ±
0.16) (Figure 4.7a). This indicates that hcTnI R145W mutation does not interfere the neighboring protein interaction. Figure 4.7b showed that the Ca\(^{2+}\) sensitivity measured by ATPase activity was also increased in Tg-R145W fibers compared to Tg-WT fibers. The pCa\(_{50}\) was 5.64 ± 0.02 (n = 14) and 5.38 ± 0.02 (n = 13) for Tg-R145W and Tg-WT fibers, respectively. The ΔpCa\(_{50}\) = 0.26 between Tg-R145W and Tg-WT fibers was statistically significant (P < 0.05).

4.2.2 Maximum Ca\(^{2+}\) Activated Force and ATPase

Maximal force values (10\(^5\) N/m\(^2\)) and maximal ATPase rates (s\(^{-1}\) per myosin S1 head) were also presented for NTg, Tg-WT and Tg-R145W skinned fibers in simultaneous force and ATPase measurement (Figure 4.8). The increased maximum Ca\(^{2+}\) activated force suggests that there is either a change in the average force/cross-bridge or a change in the number of cross-bridge attached at the maximum Ca\(^{2+}\) activation. Figure 4.8a shows there is ~13% increase in maximal Ca\(^{2+}\) activated force between Tg-R145W fibers (0.78 ± 0.03) and Tg-WT fibers (0.69 ± 0.02). At the same time, there is ~16% increase in maximum Ca\(^{2+}\) activated ATPase in Tg-R145W fibers (6.17 ± 0.19) compare to Tg-WT fibers (5.33± 0.24) (Figure 4.8b). The increased maximum Ca\(^{2+}\) activated ATPase indicates that there is a change either in the rate of cross-bridge turnover or in the number of cross-bridge attached at the maximum Ca\(^{2+}\) activation. As shown, there is no change in the maximum force values (10\(^5\) N/m\(^2\)) and maximal ATPase rates (s\(^{-1}\) per myosin S1 head) between the NTg fiber and Tg-WT fiber.
Figure 4.7. The effects of hcTnI R145W mutation on Ca\textsuperscript{2+} sensitivity of force and ATPase activity were measured simultaneously in isometric fibers. (a) The force-pCa relationship in Tg-R145W (n = 13) versus Tg-WT (n = 13) and NTg (n = 11) fibers; (b) The ATPase-pCa relationship between Tg-R145W (n = 13) versus Tg-WT (n = 13) and NTg (n = 11) fibers. A significant leftward shift in Ca\textsuperscript{2+} sensitivity of both force and ATPase in Tg-R145W compared to Tg-WT. The pCa values and Hill coefficients for force curve and pCa values for ATPase curve are shown. Data is expressed as mean of n experiments \pm SE.
Figure 4.8. The effect of hTnI R145W mutation on (a) maximal force ($10^5$ N/m$^2$) and (b) maximal ATPase activity (s$^{-1}$ per myosin head) in transgenic papillary muscle fiber. The maximal force is ~13% higher in Tg-R145W fibers than in Tg-WT fibers. The maximal ATPase in Tg-R145W fibers is ~16% higher compared to Tg-WT fibers. * represent statistically significant difference (P < 0.05). Data are expressed as mean of $n$ experiments ± SE.
4.2.3 Fractional Cross-bridge attachment at Maximal Ca\textsuperscript{2+} Activation

As discussed earlier, the fractional cross-bridge attachment at maximal Ca\textsuperscript{2+} activation equals the maximal Ca\textsuperscript{2+} activated force divided by the maximum force the muscle can develop in the presence of 10 mM MgADP. In another words, the fractional cross-bridge attachment at maximal Ca\textsuperscript{2+} activation indicates what percentage of cross-bridges actually attached at maximum Ca\textsuperscript{2+} activation in Tg-R145W fiber compared to NTg and Tg-WT fibers. As we can see, there is no significant difference in the fractional cross-bridge attachment at the maximal Ca\textsuperscript{2+} activation in all groups of fibers (Figure 4.9).

![Figure 4.9](image_url)

**Figure. 4.9.** Fractional cross-bridge attachment at maximal Ca\textsuperscript{2+} activation in NTg, Tg-WT and Tg-R145W fibers. There are no significant differences in all groups of fibers (P > 0.05). Data are expressed as mean of n experiments $\pm$ SE.
4.2.4 The Rate of Cross-bridge Turnover (g)

Figure 4.10 shows the rate of cross-bridge turnover (g) as a function of activation state (fraction of maximum activated force) in NTg, Tg-WT and Tg-R145W fiber. There are no statistically significant differences in g values in all groups of fibers (P>0.05). These results suggest that the hcTnI R145W mutation does not affect the turnover rate of the cross-bridge at any Ca^{2+} activating levels.

Figure 4.10. The rate of cross-bridge turnover (g) as a function of activation state (fraction of maximum activated force) in Tg-R145W skinned papillary muscle fibers compared to NTg and Tg-WT fibers. As shown, the dissociation of cross-bridge rate (g) was the same in Tg-R145W (n = 13), Tg-WT (n = 13) and NTg (n = 11) fibers. Data is expressed as mean of n experiments ± SE.
4.2.5 Energy Cost (used energy/unit force)

As mentioned before, energy cost equals to ATPase divided by force and is also proportional to g divided by average force per cross-bridge. Energy cost were investigated by using ATPase activity divided by force in all groups of fibers. As shown in Figure 4.11, energy cost (ATPase/force) as a function of activated state in Tg-R145W skinned fibers has no significant difference compared to Tg-WT and NTg skinned papillary fibers.

![Energy Cost (energy used/unit force)](image)

Figure 4.11. *The energy cost (ATPase/force) as a function of activated state (fraction of maximum activated force) in Tg-R145W skinned papillary muscle fibers compared to NTg and Tg-WT fibers.* As shown, the Tg-R145W (n=13) fiber is the same compared to Tg-WT (n=13) and NTg (n=11) fibers (P>0.05). Data is expressed as mean of n experiments ± SE.
4.2.6 Force-Length Relationship

The ability of WT and mutant TnIs to inhibit the actomyosin ATPase activity under relaxing conditions (pCa=9.0) in the Tg-R145W fiber was investigated by stretching the skinned fiber to various lengths in the presence and absence of BDM. Representative experiments of both Tg-WT fiber and Tg-R145W fiber were shown in Figure 4.11a. The force due to 10~40% stretch under relaxing conditions (pCa 9.0) showed no significant difference in Tg-R145W compared to the Tg-WT (Figure 4.11b). In the presence of BDM the Tg-R145W force-stretch curve showed no difference compared with the Tg-WT force-stretch curve. This indicates that there is no active cross-bridge attachment in the Tg-R145W fiber at the $10^{-9}$ Ca$^{2+}$ concentration as in the Tg-WT fiber, as shown in Figure 4.11c. These result suggest that under relaxing conditions the hcTnI R145W mutation does not impair the inhibition of the actomyosin cross-bridge cycling.
Figure 4.12. **Relative force in Tg-R145W skinned fiber due to different stretch levels under relaxing conditions with/without 3mM BDM.** (a) Representative experiments for both Tg-WT and Tg-R145W fibers. Force at each stretch was normalized to the maximum Ca$^{2+}$ activated force at pCa 3.4. The dash lines are the breaking point to change the pCa 9.0 solution with or without 3mM BDM at each stretch. (b) Tg-WT (n = 5) versus Tg-R145W (n = 6) force due to fiber stretch in the presence and absence of 3mM BDM. (c) Force differences between the presence and absence of BDM in fibers at each stretch. Differences in Tg-WT fibers and Tg-R145W fibers were shown in black and gray bars, respectively. No statistically significance was found in all groups of fibers (P>0.05). Data is expressed as mean of n experiments ± SE.
Chapter 5

Intact Papillary Fiber Studies

5.1 Overview

The total cTnC concentration is approximately 100 μM in myocyte, thus the total Ca^{2+} that can bound to cTnC is about 100 μM in cardiac muscles (only one Ca^{2+} specific binding site in cTnC) (Yates and Greaser 1983). In contrast, the free intracellular Ca^{2+} varies from 0.1-0.2 μM at rest to 1-10 μM at maximal activation. The intracellular Ca^{2+} transient in intact muscles can be measured by different Ca^{2+} indicators depending upon the purpose of the experiment or the interest of the investigator (Takahashi et al. 1999). In this thesis study, the fluorescent Ca^{2+} indicator, Fura-2, was used to simultaneously measure the intracellular Ca^{2+} transient and force in intact papillary muscle as was previously described for cardiac muscle (Wang et al. 1999; Wang and Kerrick 2002). The reason for using Fura-2 is that it is a ratiometric Ca^{2+} fluorescent indicator, so it can minimize the movement artifacts. Additionally, it has the right affinity for Ca^{2+} to measure the changes in the intracellular Ca^{2+} transient during a twitch, which results from the electrical stimulation. The actual calculations of intracellular Ca^{2+} were not done in this thesis for the following reasons. Baylor and Hollingsworth (Baylor and Hollingworth
2000) showed in a paper dedicated to measurement and interpretation of cytoplasmic Ca\(^{2+}\) that Fura-2 could not be calibrated to measure accurately intracellular Ca\(^{2+}\). The primary reason is that Fura-2 is sufficiently hydrophobic that it binds to proteins which in turn alter the Kd of Ca\(^{2+}\) for Fura-2. Since there is no way of knowing what the ratio of calcium bound to unbound Fura-2 in the cytoplasm, so the Kd of Fura-2 for Ca\(^{2+}\) cannot be known. In addition, the other two important parameters of Fura-2 are greatly altered, Fmin and Fmax. Therefore Fura-2 was not used to measure intracellular Ca\(^{2+}\). However the off-rate of Ca\(^{2+}\) from Fura-2 is fast enough to measure the time course of Ca\(^{2+}\) transients in cardiac muscle (Baylor and Hollingworth 2000). Therefore Fura-2 was used only to measure the time course of the intracellular Ca\(^{2+}\) transients.

5.2 Intact Papillary Fiber Studies in Tg-R145G

In this experiment, simultaneous force and [Ca\(^{2+}\)] transient measurements were performed in electrically stimulated intact papillary muscles from 3-7 month old mice (Figure 5.1, Figure 5.2, Figure 5.3 and Table 5.1). Intact papillary muscles from hcTnI R145G transgenic mice (Tg-R145G), hcTnI wild type transgenic mice (Tg-WT) and non-transgenic mice (NTg) were examined. The muscle was stimulated at 1.0 Hz and force as well as fluorescence signals were recorded simultaneously. Each force and [Ca\(^{2+}\)] transient was normalized and averaged. Figure 5.1 demonstrates the averaged force and [Ca\(^{2+}\)] transients for all groups of intact fibers as a function of time. Table 5.1 summarizes the t\(_{50}\) and t\(_{10}\) values in milliseconds from the peak to 50% and 10% of force and [Ca\(^{2+}\)] in relaxation, respectively. Force transient (Figure 5.1a) and [Ca\(^{2+}\)] transient
(Figure 5.1b) are significantly prolonged in Tg-R145G compared to Tg-WT or NTg. The $t_{50}$ and $t_{10}$ values of force transient are $\sim$1.7-fold and $\sim$2.0-fold longer in Tg-R145G muscle compared to those of Tg-WT, respectively (Table 5.1 and Figure 5.2). At the same time, the $t_{50}$ and $t_{10}$ values of $[Ca^{2+}]$ transients in Tg-R145G muscle are both $\sim$28% longer than in Tg-WT muscle (Table 5.1 and Figure 5.3). As expected, Tg-WT and NTg fibers had no differences in force and $[Ca^{2+}]$ transients (Figure 5.1).

Table 5.1: $t_{50}$ and $t_{10}$ values of both force and $[Ca^{2+}]$ transients in intact papillary muscles from NTg, Tg-WT and Tg-R145G

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<td>361.3±13.3$^a$</td>
<td>215.2±5.0$^a$</td>
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$t_{50}$(Duration) is the time from peak to 50% of force or Ca$^{2+}$ in relaxation. $t_{10}$ is the time from peak to 10% of force or Ca$^{2+}$ in relaxation. Data are the mean of the n experiments ±SE.

$^a$ significantly different compared with Tg-WT (P<0.05)
Figure 5.1. Force and Ca$^{2+}$ transients in NTg, Tg-WT and Tg-R145G intact transgenic papillary muscle fibers. (a) Force and (b) [Ca$^{2+}$] transients in Tg-R145G versus Tg-WT and NTg intact papillary fibers. Intact papillary muscle fibers were stimulated at 1.0 HZ. Fluorescence produced by Fura-2 is recorded at the same time. As shown, Force and [Ca$^{2+}$] transients are both prolonged in Tg-R145G fibers compared to Tg-WT fibers. Data are expressed as mean of n (5 ~ 9) experiments ± SE.
Figure 5.2. $t_{50}$ and $t_{10}$ values of force transient in NTg, Tg-WT and Tg-R145G intact fibers. a) $t_{50}$ value of force transient is ~1.7-fold longer in Tg-R145G papillary muscles compared to that of Tg-WT. b) $t_{10}$ value of force transient is ~2.0-fold longer in Tg-R145G papillary muscles compared to that of Tg-WT. Data are the mean of the n experiments ±SE. *significantly different compared with Tg-WT (P<0.05)
Figure 5.3. $t_{50}$ and $t_{10}$ values of Ca$^{2+}$ transient in NTg, Tg-WT and Tg-R145G intact fibers. a) $t_{50}$ value of force transient is ~28% longer in Tg-R145G papillary muscles compared to that of Tg-WT. b) $t_{10}$ value of force transient is ~28% longer in Tg-R145G papillary muscles compared to that of Tg-WT. Data are the mean of the n experiments ±SE. * significantly different compared with Tg-WT (P<0.05)
5.3 Intact Papillary Fiber Studies in Tg-R145W

Same as the Tg-R145G intact fiber experiment, intact papillary muscles from 3-7 month old hcTnI R145W transgenic mice (Tg-R145W), Tg-WT and NTg were examined for both force and [Ca$^{2+}$] transients. (Figure 5.4, Figure 5.5, Figure 5.6 and Table 5.2). Figure 5.4 demonstrates normalized force and [Ca$^{2+}$] transients for all groups of intact fibers as a function of time in milliseconds. Each curve is the normalized and then averaged data. In figure 5.4a, the normalized Tg-R145W force transient (green solid line) shifts to right compared to the Tg-WT.

It is not possible to measure force per cross-sectional area in the intact mouse papillary muscles because of their irregular shapes and cross-section along the length of the fiber. Therefore we looked for an indirect method for determining the relative transient force differences during a twitch for the Tg-WT and mutant papillary muscles. Unique to this study, from the skinned fiber experiments, we know that the rate of cross-bridge dissociation ($g$) was the same for Tg-WT and Tg-R145W fibers for a given level of activation (Figure 4.10). Additionally we determined that the fraction of force generating cross-bridges attached is the same for both types of fibers (Figure 4.9). Since the fraction of force generating cross-bridges attached is equal to $f/(f+g)$ (Huxley, 1957) then $f$ should be the same for Tg-WT and Tg-R145W papillary muscles at all levels of activation, because $g$ is the same at all levels of activation (Figure 4.10). Thus for an identical Ca$^{2+}$ transient the transient force response would be the same, since rates of rise and fall in force would be determined by cross-bridge rate parameters $f$ and $g$. Additionally we know that the intracellular Ca$^{2+}$ transients is the same for both the Tg-
R145W and Tg-WT papillary muscles up to the peak of the Ca\(^{2+}\) transient. It is only during the falling phase of the Ca\(^{2+}\) transient that the Ca\(^{2+}\) transients diverge, and the Tg-R145W transient decays more slowly than the Tg-WT. Thus by normalizing the Tg-R145W and Tg-WT force transients and adjusting the amplitude of the Tg-R145W transient, the normalized rate of rise of normalized force transients can be made to coincide during the initial time period where the Ca\(^{2+}\) transients are identical, the relative magnitude of the force transients for the Tg-R145W and Tg-WT papillary muscles can be observed (Figure 5.4a, dash green trace). This analysis shows that the Tg-R145W force transient is approximately 40% higher than the Tg-WT. The increase in time to peak force for the Tg-R145W papillary muscle results from the fact that the Ca\(^{2+}\) transient is longer, causing the papillary muscle to contract longer before the force starts to decay. This type of analysis only works when the initial phase of the Ca\(^{2+}\) transient is identical in the two muscle types. This analysis does not take into account the analysis of the skinned fiber data presented previously that there is a 13 percent increase in the number of cross-bridges that interact with the actin in the Tg-R145W fibers. This would add an additional 12 - 13 percent increase in relative force to the previous cited 40 percent increase for a total of 53 percent increase in relative force for the Tg-R145W fibers compared to Tg-WT.

Since g did not change between Tg-R145W and Tg-WT fibers, the increase in Ca\(^{2+}\) sensitivity in the Tg-R145W skinned fibers was due to an increase in the affinity of Ca\(^{2+}\) for troponin C. The question is how much of an effect would this increase in Ca\(^{2+}\) affinity have on the amount of Ca\(^{2+}\) bound to TnC. The answer is very little, approximately a 5.0 percent increase in Ca\(^{2+}\) bound to TnC. The reason is as follows:
Assume that there is 100 uM of TnC Ca\(^{2+}\) binding sites, and only 50 uM of total Ca\(^{2+}\) is released from the sarcoplasmic reticulum (SR) with each twitch. In order to calculate the change in CaTnC the following equation needs to be solved:

\[
[Ca_{\text{total}}] (Ca^{2+} \text{ released from SR}) = [CaTnC] + [Ca^{2+}]
\]

Hill equation: \(\frac{[Ca^{2+}]^n}{([Ca^{2+}]^n + Kd^n)} = \text{fraction of TnC}_{\text{total}} \text{ bound to Ca}^{2+}\)

\((n \text{ is Hill coefficient and Kd is the dissociation constant or the } [Ca^{2+}] \text{ associated with the } pCa_{50} \text{ force})\)

Therefore \([CaTnC] = TnC_{\text{total}} (\frac{[Ca^{2+}]^n}{([Ca^{2+}]^n + Kd^n)})\)

Solving this equation \([Ca_{\text{total}}] (Ca^{2+} \text{ released from SR}) = [CaTnC] + [Ca^{2+}]\) for the CaTnC associated with the Tg-WT and Tg-R145W fibers shows that CaTnC would only increase by approximately 5.0 percent, thus the force transient would be expected to increase at most by only 5.0 percent as a result of the increased affinity of TnC for Ca\(^{2+}\). Finally this 8 percent increase in force would have to be added to the 53 percent increase in transient force for a total of approximately a 58 percent increase in Tg-R145W force relative to Tg-WT transient force.

Table 5.2 summarizes the \(t_{50}\) and \(t_{10}\) values in milliseconds from the peak to 50% and 10% of force and \([Ca^{2+}]\) in relaxation, respectively. Force (Figure 5.4a) and \([Ca^{2+}]\) (Figure 5.4b) transients are significantly prolonged in Tg-R145W compared to Tg-WT and NTg. Also, the time to peak force is increased in Tg-R145W force transient compared to Tg-WT force transient. The \(t_{50}\) (the time from peak to 50% of force in relaxation) and \(t_{10}\) (the time from peak to 10% of force in relaxation) value of force transients are both \(~1.5\)-fold longer in R145W papillary muscles compared to those of Tg-WT (Table 5.2 and Figure 5.5). At the same time, the \(t_{50}\) (the time from peak to 50%
of Ca$^{2+}$ in relaxation) and $t_{10}$ (the time from peak to 10% of Ca$^{2+}$ in relaxation) value of [Ca$^{2+}$] transients in Tg-R145W papillary muscles are both ~13% longer than in Tg-WT papillary muscles (Table 5.2 and Figure 5.6).

Table 5.2: $t_{50}$ and $t_{10}$ values of both force and [Ca$^{2+}$] transients in intact papillary muscles from NTg, Tg-WT and Tg-R145W

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$t_{50}$ (Duration) is the time from peak to 50% of force or Ca$^{2+}$ in relaxation. $t_{10}$ is the time from peak to 10% of force or Ca$^{2+}$ in relaxation. Data are the mean of the n experiments $\pm$ SE.

$^a$ significantly different compared with Tg-WT (P<0.05)
Figure. 5.4. **Force and Ca\[^{2+}\] transients in NTg, Tg-WT and Tg-R145W intact transgenic papillary muscle fibers.** (a) Force and (b) [Ca\[^{2+}\]] transients in Tg-R145W versus Tg-WT and NTg intact papillary fibers. In figure (a), green solid line indicates normalized Tg-R145W force transient and green dash line indicates Tg-R145W force transient normalized to Tg-WT (matching the rate of rise of Tg-R145W (green solid line) and Tg-WT (blue line)). As shown, Force and [Ca\[^{2+}\]] transients are both prolonged in Tg-R145W fibers compared to Tg-WT fibers. Also, the Tg-R145W force transient normalized to Tg-WT is about 40% higher compared to Tg-WT. Data are expressed as mean of n (5 ~ 9) experiments ± SE.
Figure 5.5. $t_{50}$ and $t_{10}$ values of force transient in NTg, Tg-WT and Tg-R145W intact papillary muscle fibers. a) $t_{50}$ value and b) $t_{10}$ value of force transient are both ~1.5-fold longer in Tg-R145W papillary muscles compared to that of Tg-WT. Data are the mean of the n experiments ± SE. * significantly different compared with Tg-WT (P<0.05)
Figure 5.6. $t_{50}$ and $t_{10}$ values of Ca$^{2+}$ transient in NTg, Tg-WT and Tg-R145W intact papillary muscle fibers. a) $t_{50}$ value and b) $t_{10}$ value of force transient are both ~13% longer in Tg-R145W papillary muscles compared to that of Tg-WT. Data are the mean of the n experiments ±SE. * significantly different compared with Tg-WT (P<0.05)
Chapter 6

Discussion

6.1 Summary for *in vitro* studies

Different mutations in human cardiac TnI (hcTnI) at the residue 145 cause different cardiomyopathies. The R145G (positively charged arginine replaced by an uncharged glycine) mutation is associated with hypertrophic cardiomyopathy (HCM) and the R145W (positively charged arginine replaced by aromatic uncharged tryptophan) mutation is associated with restrictive cardiomyopathy (RCM). Unlike HCM, which is described by the heart morphology change (hypertrophy), RCM is characterized by impaired ventricular filling, reduced diastolic volume and increased end diastolic pressure in the presence of normal systolic and normal or near normal myocardial thickness and systolic function (1980). However, some patients with RCM-associated cTnI mutations (such as D190G) demonstrate features of HCM, RCM or both, depending on the genetic background of the subject and possibly on the specific mutation.

Since Kimura et al. (Kimura et al. 1997) reported that the R145G mutation linked with HCM and Mogensen et al. (Mogensen et al. 2003) reported that the R145W mutation associated with RCM, many researchers had studied these two mutations in
reconstituted system, cardiomyocytes or even transgenic animals. Using the reconstituted system, Lang et al. (Lang et al. 2002) showed that the hcTnI R145G mutation impaired the ability of TnI to inhibit the ATPase activity in the reconstituted actin-tropomyosin-myosin ATPase system in the presence of 1.0 mM EGTA in the absence of Ca\(^{2+}\) and decreased the ATPase activation in the same reconstituted system in the presence of Ca\(^{2+}\). Using skinned cardiac porcine muscle fibers reconstituted with hcTnI R145G, Lang et al. concluded that there was an increase in the unregulated force in 10\(^{-8}\) M Ca\(^{2+}\) (also designated basal force), a decrease in the maximal force recovery and a significant increase in the Ca\(^{2+}\) sensitivity of force development when compared with the wild type hcTnI. Also, using the reconstituted system, Gomes et al. (Gomes et al. 2005) showed that the hcTnI R145W mutation impaired the ability of cTnI to inhibit the ATPase activity in the reconstituted actin-tropomyosin-myosin ATPase system in the absence of Ca\(^{2+}\) (1 mM EGTA) and decreased the ATPase activation in the same reconstituted system in the presence of Ca\(^{2+}\). Using skinned cardiac porcine muscle fibers reconstituted with hcTnI R145W, Gomes et al. concluded that there was an increase in the unregulated force in 10\(^{-8}\) M Ca\(^{2+}\), a decrease in the maximal force recovery and a significant increase in the Ca\(^{2+}\) sensitivity of force development when compared with the wild type hcTnI. From these experiments, we can conclude that the hcTnI R145W mutation affects most items in same direction, but it has more severe effects than the hcTnI R145G mutation (Figure 6.1). To investigate what are the underlying mechanisms in different phenotypes of HCM and RCM, in vivo system- transgenic mice models were addressed in this study.

To elucidate the mechanism by which the hcTnI R145G mutation results in ventricular and/or septal hypertrophy and the hcTnI R145W mutation results in restrictive
filling pattern, increased end-diastolic pressure with normal wall thickness and systolic function, transgenic mice expressing hcTnI R145G, R145W and hcTnI wild type (as control) in the murine heart (Tg-R145G/Tg-R145W/Tg-WT) were generated. What makes this study unique is that it uses transgenic mice expressing both the hcTnI mutation and the proper control hcTnI wild type. Using Tg-R145G, Tg-R145W versus Tg-WT made it possible to extend our knowledge to what effects the hcTnI R145G and R145W mutation have on physiological function. Another unique aspect of this study is that it used simultaneous measurements of force and actomyosin ATPase in the skinned fibers. Since force is proportional to the number of force generating cross-bridges attached and ATPase is proportional to cross-bridge turnover rate, these measurements make it possible to calculate the rate of cross-bridge turnover, energy cost and force per cross-section. In addition, simultaneous measurements of intracellular Ca^{2+} and force transients in intact papillary muscles allowed for further observations of changes in physiological function caused by the hcTnI R145G and R145W mutation that causes HCM and RCM in humans, respectively.
Fig. 6.1. *Comparison of in vitro results for hcTnI R145G and R145W mutations with wild-type.* The compared items are inhibition of ATPase activity, maximum ATPase activity, basal force, maximum force, calcium sensitivity and cooperativity of force. *hcTnI* R145W and R145G mutation affect most items in same direction, but R145W had more severe effects than R145G. Results from Lang et al. (Lang et al. 2002) and Gomes et al. (Gomes et al. 2005).
6.2 Pathological Changes in Tg-R145G, R145W Hearts

To test the pathological changes in the Tg-R145G and Tg-R145W hearts, longitudinal sections of the whole hearts were stained with Hematoxylin & eosin and masson trichrome. James et al. (James et al. 2000) showed no discernible pathology at any age of α-myosin heavy chain promoter driven mcTnI_{R146G} mice which had 40% transgenic protein expression, which is in agreement with our results that no myofibrillar disarray (H&E stain) and fibrosis (masson stain) were observed in Tg-R145G heart (Figure 3.7 and Figure 3.8). However, only 25% replacement of transgenic protein in β myosin heavy chain promoter driven rabbit model showed significant levels of interstitial fibrosis and myocyte disarray (Sanbe 2005). The ratio between α-myosin heavy chain and β-myosin heavy chain in the mouse is 16:1 (Ng 1991) compared to 1:4 in rabbit (Sinha et al. 1982). Krenz and Robbins showed that increasing the ratio of β- to α-myosin heavy chain are disadvantageous to mouse and human hearts under severe cardiovascular stress (Krenz and Robbins 2004). Also, Metzger (Metzger et al. 2007) demonstrated that the β-myosin heavy chain is a negative inotrope. They found that the gene-transfer based replacement of α- with β- myosin heavy chain attenuated contractility in a dose dependent manner (Herron et al. 2007).

No difference was seen in the heart weight/body weight ratios in the Tg-R145G compared to NTg and Tg-WT, which would indicate a lack of potential for hypertrophy (Table 3.1). However, it is known that HCM patients carrying mutations in cTn or Tm do not necessarily present with heart hypertrophy (Earing et al. 2003; Gomes and Potter 2004), meaning that the increase of the heart weight/body weight ratios is not a good
parameter to be considered as a HCM phenotype. Also our morphology of hematoxylin &
eosin stained longitudinal sections of whole Tg-R145G heart showed no dramatic
difference compared to those of Tg-WT or NTg (Figure 3.6), even compared the 18
month old littermates. These results suggest that there are no apparent phenotypes in Tg-
R145G heart.

Although hypertrophy was not found in our transgenic mice model, we know that
the functional properties between mouse heart and human heart are significantly different.
For example, mouse hearts are much smaller and have a much faster heart beat than that
of humans. Secondly, there are a number of troponin mutations that have distinct
phenotypes in humans, that do not show up in transgenic mice. Thirdly, according to the
literature, other laboratories have shown that the higher the expression of mutant proteins,
the more likely the murine heart will develop morphological changes. James et al. (James
et al. 2000) reported that the mcTnI-R146G transgenic mice that displayed mutant protein
expression levels of over 50% have clear phenotypes. Finally, in the case of diastolic
dysfunction, the phenotype may only occur when the diastolic dysfunction is severe
enough that the heart can no longer adjust stroke volume to accommodate for the
diastolic dysfunction and then compensatory hypertrophy may develop in the mouse
model.

For the hcTnI R145W mutation, our pathology result showed that the hematoxylin
& eosin stained longitudinal sections of whole Tg-R145W heart has the same left
ventricular wall thickness compared to those of Tg-WT or NTg littermates (Figure 3.6
and Figure 3.11). Also, no difference was seen in the heart weight/body weight ratios in
the Tg-R145W compared to NTg and Tg-WT, which would indicate a potential for
hypertrophy (Table 3.2). There are no myofibrillar disarray showed in the H&E stain from the left ventricle and septum of the Tg-R145W heart (Figure 3.12). A small extent of fibrosis is observed from both left ventricle and septum of the Tg-R145W heart in the masson trichrome stain (Figure 3.13). The fibrosis will cause less compliant heart and increase the resistant to filling during diastole.

6.3 Physiological Changes in Tg-R145G Fibers

To elucidate the potential mechanisms by which the hcTnI R145G mutation results in ventricular and/or septal hypertrophy and alters cardiac muscle contraction in humans, transgenic mice expressing hcTnI R145G in the murine heart were generated.

The physiological changes caused by the hcTnI R145G mutation in transgenic mouse fibers were as follows: 1) There was a large increase in Ca\(^{2+}\) sensitivity of force development and ATPase activity (especially in the low activating range of Ca\(^{2+}\)) as well as a significant decrease in the Hill coefficient of force (Figure 4.1); 2) There was a significant decrease in the maximal Ca\(^{2+}\) activated force, but no change in the maximal Ca\(^{2+}\) activated ATPase (Figure 4.2); 3) There was no significant difference in the fractional cross-bridge attachment at maximal Ca\(^{2+}\) in all groups of fibers (Figure 4.3); 4) The rate of cross-bridge turnover as a function of activation state (fraction of maximum activated force) was unchanged in Tg-R145G fibers compared to Tg-WT fibers (Figure 4.4); 5) Energy cost (ATPase/ force) as a function of active state was higher in Tg-R145G fibers than in Tg-WT fibers (Figure 4.5); 6) In the presence of 10\(^{-9}\) M Ca\(^{2+}\), 2–4 percent of the force generating cross-bridges remained attached in the Tg-R145G fibers.
compared to less than 1.0 percent in Tg-WT fibers suggesting that the hcTnI R145G mutation does not allow the complete inhibition of force generating cross-bridges under relaxing conditions (Figure 4.6); 7) Both force and intracellular Ca\(^{2+}\) transients were prolonged with the force transient prolongation being more pronounced than in the Ca\(^{2+}\) transient (Figure 5.1).

The significant decrease in the maximum Ca\(^{2+}\) activated force in transgenic mice expressing hcTnI R145G (in this study), transgenic mice expressing mcTnI R146G (James et al. 2000) and reconstituted porcine skinned fiber study (Lang et al. 2002) could be explained by a change in the cross-bridge kinetics or a decrease in the average force per cross-bridge, since the maximum force a fiber can generate is equal to the number of cross-bridges attached at maximum Ca\(^{2+}\) activation times the average force/cross-bridge (Kerrick and Xu 2004). Using simultaneous measurements of force and ATPase activity in the transgenic skinned fibers in this study, it was found that a significant 14% decrease in maximum Ca\(^{2+}\) activated force in Tg-R145G fibers was due to the decrease in the average force per cross-bridge rather than a change in cross-bridge kinetics, because the cross-bridge turnover rate (g) did not change. This means the Tg-R145G muscle would have to use more ATP to exert the same force as the Tg-WT muscle. This is also consistent with our result that Tg-R145G fibers have higher energy costs compared to Tg-WT fibers, as shown in figure 4.5. This fact alone may cause the R145G heart to hypertrophy in humans because it would be equivalent to causing the WT muscle to work at a higher rate, as for example during exercise. It is well known that exercise can cause cardiac muscle to hypertrophy in humans (Abergel and Oblak 2006).
As can be seen in Figure 4.1, there was a large increase in Ca\(^{2+}\) sensitivity in the low range of Ca\(^{2+}\) activation. Also very noticeable in Figure 4.1a, there was a large decrease in the slope of the force-pCa curve showing a decrease in the cooperative protein-protein interactions within the thin filament during activation that is known to be responsible for the steepness of muscle force-pCa relationship. The decrease in the \(n_{	ext{Hill}}\) indicated that the hcTnI R145G mutation might interfere with the Ca\(^{2+}\) effects of the nearest neighbor regulatory or cross-bridge binding to the nearest neighbor regulatory units. Previous cTn mutations related to HCM have been reported by our labs to cause changes in cooperativity of the thin filament, but the mechanism by which they cause these changes is still poorly understood (Szczesna et al. 2000; Harada and Potter 2004). These results lead us to the following question: what is responsible for the increase in Ca\(^{2+}\) sensitivity?

There are two major possibilities: 1) a decrease in the cross-bridge turnover rate (Robinson et al. 2002) and 2) a decrease in the off-rate of Ca\(^{2+}\) from the cTnC (Wang et al. 1999; Wang and Kerrick 2002). No change in cross-bridge turnover rate was observed (as shown in Figure 4.4), thus it is reasonable that the off rate of Ca\(^{2+}\) from cTnC was changed by the hcTnI R145G mutation. It is well known that TnI binding to TnC decreases the off rate of Ca\(^{2+}\) from TnC (Johnson et al. 1980; Solaro and Rarick 1998). Our results are also in accordance with previous fluorescence data showing that this specific mutation leads to an increase in the Ca\(^{2+}\) affinity for cTnC in reconstituted thin filaments (Kobayashi and Solaro 2006). This increase in Ca\(^{2+}\) sensitivity in Tg-R145G, especially in the low range of activating Ca\(^{2+}\), is probably responsible for the prolongation of the Ca\(^{2+}\) and force transients observed in the intact papillary muscle (Figure 5.1). This is because cardiac TnC is one of many Ca\(^{2+}\) buffers in the cardiac
muscle myoplasm and thereby influences Ca$^{2+}$ uptake by the sarcoplasmic reticulum during the relaxation phase of muscle contraction. So, a decrease in the off rate of Ca$^{2+}$ from troponin C would lead to prolonging the Ca$^{2+}$ and force transients as predicted by a computer model in Lang et al. (Lang et al. 2002).

The relative force due to 10~40% stretch of the fiber length under relaxing conditions (pCa 9.0) in Tg-R145G skinned papillary fibers was shown to be slightly increased compared to the Tg-WT (Figure 4.6b). In the presence of BDM the Tg-R145G force-stretch curve shifts downward to superimpose with the Tg-WT force-stretch curve. This shows that there is active cross-bridge attachment which is responsible for the separation of the two force-stretch curves, and is expected based on the incomplete inhibition of force and ATPase activity seen previously (Lang et al. 2002). As shown in Figure 8c, Tg-R145G had two to four times more cross-bridges attached than that in the Tg-WT fibers under resting conditions. In agreement with Kruger et al. (Kruger et al. 2005) and Lang et al (Lang et al. 2002), our hcTnI transgenic mice results suggest that under relaxing conditions the hcTnI R145G mutation impairs the inhibition of the actomyosin cross-bridge cycling.

As can be seen, the hcTnI R145G mutation causes three problems, all of which would be expected to adversely affect contractility. The first problem is a decrease in the average force per cross-bridge, resulting in a decrease in the force per cross-sectional area of ventricular muscle, which would cause a decreased stroke volume in R145G hearts. The second problem is an increase in the Ca$^{2+}$ sensitivity of force probably due to a decrease in the Ca$^{2+}$ off-rate from cTnC. This results in prolonged force and Ca$^{2+}$ transients, which cause an increased resistance to filling the ventricle during diastole,
especially at high heart rates. The third problem is an incomplete inhibition of cross-
bridge attachment at $10^{-9}$ M Ca\textsuperscript{2+}. This would also result in an increase in resistance to
filling the ventricle during diastole. These effects on diastolic filling (preload) would
result in a decreased stroke volume (SV) as well.

These three physiological effects of the hcTnI R145G mutation would cause the
cardiovascular system to react by increasing the heart rate to maintain a constant cardiac
output (CO=HR x SV). From the muscle’s point of view, energy cost (ATP hydrolysis)
would increase because of this high heart rate as it does during exercise. Overtime a
compensatory increase in muscle mass (hypertrophy) would substitute for the increase in
heart rate in order to maintain cardiac output. Thus all of the above physiological changes
caused by the hcTnI R145G mutation would be predicted to be pro-hypertrophic in
humans.

In summary, the hcTnI R145G mutation decreases the average force per cross-
bridge, increases Ca\textsuperscript{2+} sensitivity in the low activating range of Ca\textsuperscript{2+}, impairs the cTnI
ability to inhibit cross-bridge attachment under relaxing conditions and prolongs force
and intracellular Ca\textsuperscript{2+} transients. These physiological changes give insights into how the
hcTnI R145G mutation might trigger hypertrophy and also for the poor prognosis of
patients with this HCM mutation.
6.4 Physiological Changes in Tg-R145W Fibers

Among all the cardiomyopathies, RCM is characterized by a restrictive filling pattern (prominent rapid filling wave (E), shortened deceleration time and reduced isovolumic relaxation time due to increased left atria pressure), reduced end-diastolic volume and increased end-diastolic pressure with normal or near normal systolic function and wall thickness (1980). Recently, six mutations were reported to be associated with restrictive cardiomyopathy (RCM) (Mogensen et al. 2003).

Different point mutation at residue 145 in cTnI can be associated with different cardiomyopathies. The hcTnI R145G and hcTnI R145W mutation correlate with HCM and RCM respectively. Based on previous studies, R-G and R-W mutations at position 145 in cTnI often produce similar biochemical and physiological properties (Lang et al. 2002; Gomes et al. 2005). For example they both result in an increase in Ca\(^{2+}\) sensitivity of force and a decrease in maximum force in porcine skinned fibers reconstituted with either the R-G or R-W cTnI mutation. *In vitro* actomyosin systems reconstituted with either the R-G or R-W mutation there was a decrease in maximum ATPase activity, an increase in Ca\(^{2+}\) sensitivity of ATPase activity, and a decreased ability of the cTnI to inhibit ATPase activity in 1 mM EGTA (Figure 6.1). These similar biochemical and physiological findings give no insights as to why these two mutations result in different cardiomyopathies. Because of the well known limitations of *in vitro* systems and the use of skinned fiber reconstitution experiments it was decided that the use of transgenic mouse models expressing either WT or mutant R145W, R145G would be a good approach to elucidate why these two mutations result in different cardiomyopathies.
Using the same experiments under the same conditions as mentioned in Tg-R145G, the physiological consequences of Tg-R145W include: 1) a large increase in Ca\(^{2+}\) sensitivity of force and ATPase activity development, as well as no significant change in the Hill coefficient in the force-pCa relationship (Figure 4.7); 2) a significant \(~13\%-16\%\) increase in both maximal Ca\(^{2+}\) activated force and maximal Ca\(^{2+}\) activated ATPase (Figure 4.8); 3) no change in the fractional cross-bridge attachment at maximal Ca\(^{2+}\) activation (Figure 4.9); 4) no change in the rate of cross-bridge turnover as a function of activation state (fraction of maximum activated force) (Figure 4.10); 5) no significant difference in the energy cost (ATPase/force) (Figure 4.11); 6) prolonged force and intracellular Ca\(^{2+}\) transients (Figure 5.4); and 7) force transient normalized to rate of activation of Tg-WT showed there was approximately a 40\% higher peak force in Tg-R145W papillary muscle than that in Tg-WT (Figure 5.4a).

As can be seen in Figure 4.7, there was a large increase in Ca\(^{2+}\) sensitivity with no change in the slope of the force-pCa curve. No change in the \(n_{\text{Hill}}\) indicated that hcTnI R145W mutation does not interfere with the interaction of the nearest neighbor regulatory units on the actin filament (Robinson et al. 2002). The large increase in Ca\(^{2+}\) sensitivity of force shown in the skinned papillary fibers from Tg-R145W is similar to what was previously showed in skinned porcine fibers reconstituted with mutant R145W cTnI when compared to WT cTnI. These results lead us to the following question: what is responsible for the increase in Ca\(^{2+}\) sensitivity? There are two major possibilities: 1) a decrease in the cross-bridge turnover rate (Robinson 2002) and 2) a decrease in the off-rate of Ca\(^{2+}\) from the cardiac troponin C (Wang et al. 1999; Wang and Kerrick 2002). Since in these studies simultaneous ATPase and force measurements were made in the
same fibers it was possible to determine the reason for the increase in Ca$^{2+}$ sensitivity of force and ATPase. It is well known that cross-bridge turnover rate can affect the Ca$^{2+}$ sensitivity of skinned fibers (Johnson et al. 1980; Guth and Potter 1987; Solaro and Rarick 1998). Figure 4.10 shows that the rate of cross-bridge turnover $g$ is not affected by the mutation so that fact eliminates changes in $g$ as a reason for the increase in Ca$^{2+}$ sensitivity of force and ATPase. The most likely explanation for this increase is that hcTnI R145W mutation results in an altered conformational change in the cTnC-TnI complex resulting in a decrease in the off-rate Ca$^{2+}$ from TnC. This conclusion is also in accordance with previous fluorescence data showing that this specific mutation leads to an increase in the Ca$^{2+}$ affinity for cTnC in reconstituted thin filament (Kobayashi and Solaro 2006).

The question is how a decrease in the off-rate of Ca$^{2+}$ would translate into a physiological defect that would result in RCM? If the off-rate of Ca$^{2+}$ was decreased sufficiently in vivo it would be expected resulting in prolonged Ca$^{2+}$ and consequently force transients. This is because cardiac TnC is one of many Ca$^{2+}$ buffers in the cardiac muscle myoplasm and thereby influences Ca$^{2+}$ uptake by the sarcoplasmic reticulum during the relaxation phase of muscle contraction. So, a decrease in the off rate of Ca$^{2+}$ from cTnC would lead to prolonging the Ca$^{2+}$ and force transients (Wang 1999). Figure 5.4 shows that in intact papillary muscles both the Ca$^{2+}$ and force transients are lengthened in Tg-R145W muscle compared to Tg-WT. Thus diastolic Ca$^{2+}$ would be increased, especially at high heart rates, resulting in more active force during diastole. This would be expected to cause an increase resistance to filling (RCM). This increased filling resistance would cause diastolic dysfunction by decreasing filling of the ventricle.
This is well known to result in a decrease in stroke volume. Compensatory hypertrophy (HCM) would be expected in order to maintain stroke volume. The problem is that in RCM there is no hypertrophy associated with it by definition.

Therefore the hcTnI R145W mutation must somehow improve the ability of the heart to eject blood in order to maintain stroke volume despite the decrease in preload associated with the increased resistance to filling. Figure 4.8 shows that there is a 13–16 percent increase in force per cross-section as well as an increase in ATPase activity. This data indicates that there is an increase in the ability of the ventricle to contract that could off-set the diastolic dysfunction and maintain stroke volume. Lengthened time course of Ca\(^{2+}\) transient is also helpful in maintaining stroke volume (Figure 5.4b). The longer the Ca\(^{2+}\) transient the longer the time course of the contraction. As reported in the literature (Brenner 1988), the fraction of force generating cross-bridges attached is equal to \(f/(f+g)\). Since the fraction for force generating cross-bridges attached was constant (Figure 4.9) for both Tg-WT and Tg-R145W fibers and cross-bridge kinetics was not changed (Figure 4.10), \(f\) would also have to be constant for both fibers. Therefore the rate of rise of force would also have to be the same for both Tg-R145W and Tg-WT fibers. Also, calcium transient of Tg-R145W and Tg-WT with same shape and magnitude (Figure 5.4b) would cause the same rate of rise in force. By matching the rise time of the force transients (matching green solid line to the blue solid line), it can be seen in Figure 5.4a that the peak force of Tg-R145W (green dash line) translates into a 40 percent increase compared to Tg-WT as a result of an increase in the duration of the Ca\(^{2+}\) transient.

In conclusion, the increase in contractility for the hcTnI R145W mutation during a twitch comes from three sources: 1) The largest increase comes from the increase in the
duration of the $\Ca^{2+}$ transient. Analysis shows that would cause approximately a 40% increase in twitch force compared to WT (Figure 5.4a, green dash trace); 2) Skinned fiber data analysis shows that there would be an additional increase in force of 13 percent due to extra cross-bridge attachment; 3) Finally calculations show there is an 5 percent increase in force due to the increase in $\Ca^{2+}$ sensitivity of cTnC for $\Ca^{2+}$. These three increases in force add up to a predicted 58 percent increase in the R145W cardiac muscle twitch force compared to WT as shown in Figure 6.2. This large increase in contractility for the R145W cardiac muscle is the most important difference between the R145G and R145W muscles containing these mutations.

Figure 6.2. *The predicated force twitch in Tg-R145W compared to Tg-WT.* The higher peak force in Tg-R145W dues to longer calcium transients, more cross-bridge attached and increased calcium sensitivity.
What is the reason for this increase in force per cross-section? There are at least three obvious reasons. First there could be an increase in the force per cross-bridge. This however cannot be the case since figures 4.10 and 4.11 respectively show that there is no change in cross-bridge turnover rate and energy cost respectively. Energy cost equals to ATPase rate/force and is proportional to \( g/f_{avg} \) (cross-bridge turnover rate \( g \)/ average force per cross-bridge \( f_{avg} \)). Since both \( g \) and energy cost are not changed, therefore \( f_{avg} \) cannot have changed. A second reason for an increase in force would be a decrease in the rate of dissociation of force generating cross-bridges which is not the case as shown in Figure 4.10. The third reason is that there was an increase in the number of cross-bridges that can interact with actin. This is the most likely reason since the first two cited reasons can be ruled out. The argument for an increase in the number of cross-bridges that can form is that both maximum force and ATPase statically increase proportionately (Figure 4.7). The reason for this increase in the number of cross-bridges can only be speculative. Since TnI is part of the thin filament regulatory complex, it may be that in the presence of \( \text{Ca}^{2+} \) the hcTnI R145W mutation changes the equilibrium between TnI binding to actin and TnC such that more myosin binding sites on actin are exposed to interact with myosin. All the above results mean that the Tg-R145W muscle would have higher contractility compare to Tg-WT muscle in systole. The increased contractility may compensate for the diastolic dysfunction which is due to prolonged force and \( \text{Ca}^{2+} \) transients, suggesting that the Tg-R145W heart does not need to hypertrophy in order to maintain the same stroke volume compared to the Tg-WT heart. This assumption also is confirmed by our result that there is no change in energy cost (ATPase/force) in Tg-R145W fiber compare to Tg-WT fiber, as shown in Figure 4.11. As a consequence, the
Tg-R145W heart does not hypertrophy even with severe diastolic dysfunction. This is different from our findings in Tg-R145G heart, where hypertrophy compensates for the decrease in average force/cross-bridge if severe enough to affect stroke volume.

Why RCM has such a high mortality rate can only be speculative. Even though under normal conditions RCM heart is able to maintain normal stroke volume, they always start out with a decrease in preload (decreased end diastolic volume) compared to normal heart. This will decrease cardiac reserve and the maximum volume that can be ejected will also be decrease. Because an increase in cardiac output is required during exercise, cardiac reserve will also be further decreased and limited by this fact. Once this limit is approached increases in heart rate normally used to increase cardiac output will only cause summation of end diastolic Ca$^{2+}$ resulting in a further decrease in cardiac reserve and high end diastolic Ca$^{2+}$ and diastolic force. This defect in Ca$^{2+}$ homeostasis could result in disruption of cell to cell conduction through the gap junctions caused by high diastolic intracellular Ca$^{2+}$ resulting in defective pathways of depolarization causing fibrillation and sudden death in humans where the length of the pathways of depolarization are much greater than they are in the mouse (De Mello et al. 1983; Spray et al. 1985). The defect in Ca$^{2+}$ homeostasis could also result in apoptosis in myocytes, for calcium is the second messenger for many signaling pathways. Apoptosis causes scar tissue-fibrosis in heart muscle, which makes the heart less compliant and hard to fill during diastole. Additionally, lack of cardiac reserve in conditions of hormonal stress caused by fear or excitement could result in a loss of blood pressure as a result of a decrease in peripheral resistance and a complete loss of cardiac reserve finally resulting in heart failure and sudden death.
In Summary, restrictive cardiomyopathy caused by the hcTnI R145W mutation results in diastolic dysfunction without in general causing hypertrophy in humans. The question is why? The results reported above using transgenic mice expressing hcTnI R145W suggest the following. First the diastolic dysfunction is caused by a decrease in the off-rate of Ca$^{2+}$ from cTnC that translates into increased intracellular Ca$^{2+}$ during diastole and results in an increase in resistance to ventricular filling. Second there is an increase in the number of cross-bridges attached at all levels of Ca$^{2+}$ activation. The combination of increased Ca$^{2+}$ sensitivity and the increase in the number of cross-bridges that can attach during diastole results in diastolic dysfunction which by itself if severe enough could cause hypertrophy in order for the heart to maintain stroke volume. However, the combination of a prolonged Ca$^{2+}$ transient and an increase in the number of force generating cross-bridges that can attach during systole results in a large increase in contractility of the heart muscle. This increased contractility could allow the R145W heart to maintain normal systolic function despite severe diastolic dysfunction without hypertrophy. However the combination of severe diastolic dysfunction and increased contractility should result in decreased cardiac reserve capacity and if severe enough result in heart failure.

Previous studies (Gomes et al. 2005) showed that there was a decrease in maximum Ca$^{2+}$ activated force, a decrease in the Hill coefficient and an inability of TnI to completely inhibit force at 10$^{-8}$ M Ca$^{2+}$ in skinned porcine fibers reconstituted with the hcTnI R145W and R145G mutation. These finding are in contrast to our findings which showed an increase in force per cross-section in Tg-R145W. No evidence was found suggesting that skinned transgenic mouse fibers containing the hcTnI R145W mutation
were not completely relaxed in $10^9$ M Ca$^{2+}$. Skinned fibers observed floating in relaxing solution under the microscope showed no tendency to contract. Additionally, fibers set to 10% of its non slack length (starting length of fiber experiments in the Guth Muscle Research System) showed no change in force when exposed to plus and minus 3 mM BDM (Figure 4.12). The reason for the above discrepancies (summarized in Figure 6.3) is most likely because of differences in the reconstituted \textit{in vitro} experiments and transgenic mice fiber experiment. Presumably the use of transgenic mouse skinned fibers is more physiological since the mutant proteins were incorporated into the muscle fibers \textit{in vivo}.

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**Figure 6.3.** Comparison of \textit{in vitro} results for hcTnI R145G and R145W mutations with wild-type in reconstituted actomyosin ATPase systems and porcine skinned fibers and \textit{in vivo} results for Tg-R145G and Tg-R145W fibers with Tg-WT fibers. The red arrows and green arrows are the differences between results from reconstituted system and transgenic system.
Chapter 7

Summary of Conclusions

This study has provided the first animal model for the hcTnI R145W mutation which can cause RCM, the first investigation of histopathology in the Tg-R145W, the first comparison of the hcTnI R145G and the hcTnI R145W mutation under the same condition with hcTnI wild type. The histological results and physiological results for Tg-R145G and Tg-R145W compared with Tg-WT are shown in Table 7.1 and Table 7.2 respectively. The conclusions of this dissertation are as following:

1. No discernible phenotypes in Tg-R145G heart.
2. A little extent of fibrosis was found in Tg-R145W heart.
3. A large increase in Ca\(^{2+}\) sensitivity of force development and ATPase activity in both Tg-R145G and Tg-R145W skinned papillary fibers. The increase in Tg-R145W skinned fiber is even larger than that in Tg-R145G skinned fiber.
4. A large decrease in hill coefficient in force-pCa relationship in Tg-R145G skinned fiber, but no change in hill coefficient in Tg-R145W skinned fiber.
5. A significant 14% decrease in maximal calcium activated force in Tg-R145G skinned fiber, but a significant 13% increase in maximal calcium activated force in Tg-R145W skinned fiber.
6. No significant change in maximal calcium activated ATPase activity in Tg-R145G skinned fiber, but a significant 16% increase in maximal calcium activated ATPase activity in Tg-R145W skinned fiber.

7. No significant change in the fractional cross-bridge attachment at maximal calcium activation in both Tg-R145G and Tg-R145W skinned fibers.

8. No significant change in the cross-bridge turnover rate in both Tg-R145G and Tg-R145W skinned fibers.

9. A significant increase in energy cost in Tg-R145G skinned fiber, but no change in Tg-R145W skinned fiber.

10. A higher number of cross-bridge attached under relaxing conditions in Tg-R145G skinned fiber, but no change in Tg-R145W skinned fiber.

11. Prolonged calcium transients and force transients in both Tg-R145G and Tg-R145W intact papillary fibers.

12. Higher peak force and longer time to peak in Tg-R145W force transient compared to Tg-WT and Tg-R145G due to longer Ca$^{2+}$ transient.

Based on these findings, we conclude that the hcTnI R145G mutation decreases average force per cross-bridge, which the hcTnI R145W mutation does not. The hcTnI R145W mutation increases the number of cross-bridge attached, which the hcTnI R145G mutation does not. Also, both mutations may impair the diastolic function. These physiological findings can relate with different cardiomyopathy phenotypes in humans. In R145G heart, hypertrophy may cause by two factors. One is diastolic dysfunction due to the increased calcium sensitivity and incomplete inhibition of cross-bridge attachment during diastole. Another one is the decreased systolic function due to the decease in the
average force/cross-bridge. Both of these factors cause a decrease in the stroke volume which if severe enough would cause the heart to hypertrophy (increase force/cross section muscle) in order to maintain cardiac output. In R145W heart, the resistance to filling (like R145G) would be due to an increase in calcium sensitivity and prolonged calcium and force transients. However, there is an increase in systolic contractile function due to an increase in number of cross-bridge attached during systole and prolonged calcium transient that would off-set the decrease in stroke volume of a normal heart caused by diastolic dysfunction. Thus, the stroke volume has a tendency to be maintained in spite of diastolic dysfunction, resulting in little change in cardiac output. Therefore, the R145W heart does not need to develop hypertrophy in order to maintain cardiac output.

Table 7.1 Summary of Tg-R145G and Tg-R145W histological studies

<table>
<thead>
<tr>
<th></th>
<th>Tg-R145G</th>
<th>Tg-R145W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>HCM</td>
<td>RCM</td>
</tr>
<tr>
<td>Heart weight/Body weight (R)</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Left ventricle wall thickness</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Myocyte disarray</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

All items are compared with Tg-WT. There are no significant differences in all items between NTg and Tg-WT.
Table 7.2 Summary of Tg-R145G and Tg-R145W physiological studies compared to Tg-WT

<table>
<thead>
<tr>
<th></th>
<th>Tg-R145G</th>
<th>Tg-R145W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) sensitivity of force and ATPase</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>n(_{\text{Hill}})</td>
<td>Decreased</td>
<td>No change</td>
</tr>
<tr>
<td>Maximum Ca(^{2+}) activated force</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Maximum Ca(^{2+}) activated ATPase</td>
<td>No change</td>
<td>Increased</td>
</tr>
<tr>
<td>Fractional cross-bridge attachment at maximal Ca(^{2+}) activation</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Rate of cross-bridge turnover (g)</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Energy cost (energy used/unit force)</td>
<td>Increased</td>
<td>No change</td>
</tr>
<tr>
<td>Basal force (10(^{-9}) Ca(^{2+}))</td>
<td>Increased</td>
<td>No change</td>
</tr>
<tr>
<td>Force Transient</td>
<td>Prolonged</td>
<td>Prolonged</td>
</tr>
<tr>
<td>Ca(^{2+}) Transient</td>
<td>Prolonged</td>
<td>Prolonged</td>
</tr>
<tr>
<td>Average force/cross-bridge</td>
<td>Decreased</td>
<td>No change</td>
</tr>
<tr>
<td>Total number of cross-bridge attached</td>
<td>No change</td>
<td>Increased</td>
</tr>
<tr>
<td>Calcium off-rate from cTnC</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Diastolic function</td>
<td>Impaired</td>
<td>Impaired</td>
</tr>
<tr>
<td>Systolic function</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
</tbody>
</table>

All items are compared with Tg-WT. There are no significant differences in all items between NTg and Tg-WT. Black items in the left column are the physiological functions I actually measured. Blue items are the physiological results concluded from the measured physiological functions. Yellow shadow indicates Tg-R145G and Tg-R145W have different effects.
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