Curriculum Vitae
Mitsu Rakesh Shah
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Education

University of Maryland, Baltimore (UMB) August 2013 – Present
Master of Science in Medical and Research Technology Expected – May 2015
Concentration: Biomedical Research
Relevant Coursework:
Biochemistry, Biostatistics, Lab Management and Regulations, Quality Control and Assurance, Scientific and Technical Writing, Research Design, Responsible Conduct of Ethical Research, Lab Sciences.

University of Maryland, Baltimore County (UMBC) August 2009 – May 2013
Bachelors of Science in Biological Sciences
Relevant Coursework:
Biology I and II with lab, Chemistry I and II with lab, Organic Chemistry I with lab, Genetics with lab, Cellular and Molecular Biology, Microbiology with lab, Microbial Genetics, Developmental Biology with lab, Physics I and II, Statistics, Computer Programming I.

Research Experience

The Johns Hopkins Medical Institutes (JHMI) December 2013 – Present
600 North Wolfe Street,
Baltimore, MD 21287
Researcher – Heart and Vascular Institute; Department of Cardiology
Principal Investigator: Roselle Abraham, M.D.

- Investigate mechanisms underlying energy and calcium signaling dysfunction by examining differences in protein expression in transgenic mice of Hypertrophic Cardiomyopathy using Western Blot

University of Maryland, Baltimore County May 2012 – May 2013
1000 Hilltop Circle,
Baltimore, MD 21250
Researcher – Department of Bioinformatics and Computational Biology
Principal Investigator: Maricel Kann, Ph.D

- Uncover protein-protein interactions by using concepts of interlogs and a text mining method by extracting mutations using Extraction of Mutations (EMU) and MetaMap/MeSH index searches to map diseases
- Build a database of human mutations related to disease by using PubMed

- Utilize PubMed and Online Mendelian Inheritance in Man to identify the binding sites of two mouse genes to understand their interactions and roles in protein expression

The George Washington University May 2011 – January 2012
2121 I Street Northwest,
Washington, DC 20052

Researcher – The Cancer Institute
Principal Investigator: Donald E. Henson, M.D.

- Identify mortality and incidence rates of the papillary adenocarcinoma of the extrahepatic bile duct and intrahepatic bile duct from Surveillance Epidemiology and End Results program (SEER) by using statistical methodology.

Teaching and Tutor Experience

University of Maryland, Baltimore August 2014 – December 2014
Teaching Practicum – Clinical Chemistry Laboratory

- Teach lab lecture and guide students during execution of experiments in weekly lab sessions in topics including, electrophoresis, immunoassays, endocrinology, and various technical skills

University of Maryland, Baltimore County August 2012 – May 2013
Teaching Assistantship – Molecular Genetics Laboratory

- Teach lab lecture and guide students during execution of experiments in weekly lab sessions in topics including, transcription and translation, mechanisms in gene control, signal transduction pathways relating to model organisms such as, yeast, C. elegans, drosophila, and mice.

University of Maryland, Baltimore County January 2012 – May 2013
Tutor – Biological Sciences Learning Resource Center

- Clarify difficult concepts, summarize lectures, assist in homework and incorrect quiz answers, and prepare students for exams.
- Produced practice problems for additional practice.
- Tutoring subjects included, Biological Sciences, Genetics, and Ecology and Evolution.

University of Maryland, Baltimore County January 2011 – May 2013
Teaching Assistantship – Introductory Psychology

- Facilitate small group discussion during lecture.
- Clarify difficult concepts, summarize lectures, assist in homework and incorrect quiz answers, and prepare students for exams.
- Produced practice problems for additional practice.

Publications

Published as contributor in Abstract and Acknowledgements in the Fasical on "Cancers of Extrahepatic Biliary System" of 2012 by the George Washington Cancer Institute.

Skills

Computer

- Microsoft Word, Excel, PowerPoint, ImageJ analysis software

Laboratory

- Basic: Pipette, microscopy, electrophoresis, dilutions, centrifuge, microbiology and sterile techniques.
- Advanced: Western Blotting, protein quantification assay, protein isolation, and mitochondria protein isolation.

Conference and Presentations

Graduate Research Conference — March 2014
Presenter — 620 West Lexington Street, Baltimore, MD 21201

- Hosted by the Graduate Student Association of UMB.
- Poster presentation of my previous research of Bioinformatics at UMBC.

Undergraduate Research and Creative Achievement Day — UMBC
Presenter — April 2013

- Hosted by the Undergraduate Student Association of the UMBC.
- Poster presentation of my research in the field of Bioinformatics at UMBC.

Phage Hunter's Prey Poster — UMBC
Guide — 2008 - 2014

- University of Maryland, Baltimore County Phage Hunter's Prey.
- Supported by the UMBC Department of Biological Sciences, the UMBC Honors College, and the Howard Hughes Medical Institute.

Volunteer and Extracurricular Activities

JACQUES Initiative Health Fair — UMB
Volunteer — June 2014

- Hosted by the Institute of Human Virology at the University of Maryland School of Medicine with the Gallery Church of Baltimore and HopeSprings.
- Provided information on safe health care, screen for blood pressure, educated on healthy lifestyle education, medication, and oral health education for National HIV Testing Day.

Women Involved in Learning and Leadership UMBC
Member August 2011- May 2013

- Practice leadership skills that promote academic excellence and community engagement.
- Meet with renewed speakers and activists committed to diverse gender and social justice issues and to work with feminist and other social change organizations.

Abstract

Title of Thesis: Protein Expression Differences in Hypertrophic Cardiomyopathy Transgenic Mice

Mitsu Shah: Master of Science, 2015

Thesis Advisor: Dr. Roselle Abraham, M.D.
Co-Director, Hypertrophic Cardiomyopathy Center of Excellence
Assistant Professor of Medicine
Department of Cardiology
The Johns Hopkins Medical Institutions
Baltimore, MD 21218

Hypertrophic Cardiomyopathy (HCM) is the most common genetic cardiac disease, occurring in 1 out of every 500 individuals. Lacking concrete treatment and cure, the underlying mechanisms and normal pathogenesis of the disease is extremely understudied. HCM is characterized by various clinical phenotypes such as left ventricular hypertrophy, myocyte disarray, and fibrosis. These phenotypes arise from protein mutations of the cardiac sarcomere; however the direct pathway of how these mutations lead to variable phenotypes needs to be elucidated. Abnormalities in energy and calcium handling have been studied in experimental models and have shown to be a consequence of causal mutations, leading to HCM. This study focuses on transgenic mice containing the R403Q mutation of the Myosin Heavy Chain and R92W mutation of Troponin T. AMPK, CamKII, and MCU relative protein expression levels were studied, as these proteins play a role in energy and calcium ion regulation, facilitating proper signaling under normal conditions.

Methods:

Each transgenic mouse heart of age 5 weeks and 24 weeks was harvested and homogenized in 2% Triton-Ripa buffer. Protein from whole heart homogenate was isolated by centrifugation and subjected to Western Immunoblotting.

Results:

In vitro studies have shown protein expression differences in all three transgenic mouse models at both age 5 weeks and 24 weeks. Total target protein, AMPK, CamKII, and MCU were equally expressed in all three models; however there was a 25% increase in phosphorylated AMPK protein expression in R403Q-MHC transgenic mice than R92W-TnT transgenic mice of age 5 weeks and 24 weeks. Phosphorylated CamKII had 38% increase in protein expression in R92W-TnT transgenic mice than R403Q-MHC transgenic mice of age 5 weeks. Phosphorylated MCU had 13% increase in protein expression in R92W-TnT transgenic mice of age 5 weeks and 26% increase in R92W-TnT transgenic mice of age 24 weeks than R403Q-MHC transgenic mice of age 5 weeks and 24 weeks. Although pCamKII lacked expression in transgenic mice of age 24 weeks, this result can lead us to further investigations to gain additional insight.

Conclusion:

The R403Q mutation of MHC results in abnormal energy caused by conditions of low ATP, while the R92W mutation of the TnT results in high calcium sensitivity by an increase of calcium ions. Results confirm that there is disruption in energy and calcium handling due to the respective mutations compared to that of control. At 5 weeks, in the early development of disease, pAMPK protein was expressed 15% lower than at 24

weeks, showing more energy stress at a later stage of disease. However, the opposite holds true for pCamKII, as it was expressed 100% higher at age of 5 weeks, as it lacked expression at age of 24 weeks. This shows that there may be abnormal calcium handling at an early developing stage of disease, but not when the disease is well developed.

Protein Expression Differences in Hypertrophic Cardiomyopathy Transgenic Mice

By:
Mitsu Rakesh Shah

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2015

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for your lifetime full of support and love.

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List of Abbreviations

HCM – Hypertrophic Cardiomyopathy

MHC – Myosin Heavy Chain

TnT – Troponin T

PBS – Phosphate Buffered Saline

TBST – Tris Buffered Saline and Tween 20

BSA – Bovine Serum Albumin

LDS – Lithium Dodecyl Sulfate

DTT – Dithiothreitol

BCA – Bicinchoninic Acid Assay

PVDF - Polyvinylidene Fluoride

GAPDH – Glyceraldehyde 3-phosphate Dehydrogenase

HRP – Horseradish Peroxidase

AMPK – AMP-activated Protein Kinase

pAMPK – Phosphorylated AMP-activated Protein Kinase

CamKII- δ – Calcium/Calmodulin Dependent Protein Kinase II delta

pCamKII-δ – Phosphorylated Calcium/Calmodulin Dependent Protein Kinase II delta

MCU – Mitochondrial Calcium Uniporter

pMCU – Phosphorylated Mitochondrial Calcium Uniporter

CaM – Calmodulin

Ca^{2+} - Calcium Ions

MPTP – Mitochondria Permeability Transition Pore

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I. Introduction and Background

1.1 An Overview of Hypertrophic Cardiomyopathy Disease:

Hypertrophic Cardiomyopathy is the most common genetic cardiac disease, with an incidence rate of 1 out of every 500 individuals, and is also the leading cause of sudden cardiac death in the young [1]. Family studies have demonstrated that HCM is a heritable disease that is transmitted as an autosomal dominant trait [2]. Usually, HCM patients do not portray obvious symptoms, however most affected individuals experience angina and heart palpitations depending on the stage of disease and other contributing factors. HCM is hypothesized to arise from mutated proteins on the sarcomere, which include mutations on the cardiac myosin heavy chain, troponin T, troponin I, tropomyosin, myosin-binding protein C, regulatory myosin light chain, and actin proteins [3]. Lacking concrete treatment and cure, the pathway and mechanism of how these causal genes cause variable penetrance of HCM and disruption in signaling is understudied. Sarcomeric protein mutations cause variable clinical phenotypes and histopathology characteristics such as, left ventricular hypertrophy, myofibrillar disarray, and fibrosis, depending on the mutation acquired and stage of disease [1].

The cardiac sarcomere, responsible for cardiac muscle contraction and relaxation, is composed of a thick filament and thin filament. Proteins on these filaments allow sarcomere to facilitate its function primarily by exerting energy in the form of ATP and regulating calcium ions. This promotes proteins on the filaments to slide and lead to sarcomere contractility. The main source of energy that is responsible for the contractile function of the sarcomere is the hydrolysis of the ATP molecule. However, when sarcomere proteins have mutations such as the R403Q mutation of MHC and the R92W mutation of TnT, there becomes a lack of energy and disruption in calcium homeostasis, which leads to abnormal contractility of the sarcomere.

There are several key signaling proteins that facilitate proper sarcomere contractility, however during the course of this research, AMPK, CamKII, and MCU proteins were studied as these are directly involved with ATP and calcium. In order to study the impact of HCM causal protein mutations of the sarcomere, HCM transgenic mice containing the R403Q-MHC mutation and R92W-TnT mutation were studied. Both transgenic mouse models of HCM are identical to the human HCM disease. Gaining insight on all aspects of HCM can provide better understanding of underlying mechanisms and pathways, eventually leading to a novel therapy.

1.2 Cardiac Sarcomere:

The cardiac sarcomere is the basic contractile unit of the heart and is comprised of a thick filament and a thin filament, which allow the sarcomere to contract and relax when proper sliding occurs. The sarcomere units are aligned in parallel and are composed of proteins that facilitate sarcomere function [1]. The main protein of the thick filament is the MHC, while the main protein of the thin filament is actin, but is also embedded with tropomyosin, and troponins I, C and T proteins (Figure 1). Following activation by calcium ions, a series of events involving the troponin-tropomyosin complex, results in sliding of the thick and thin filaments, facilitating cardiac muscle contraction.

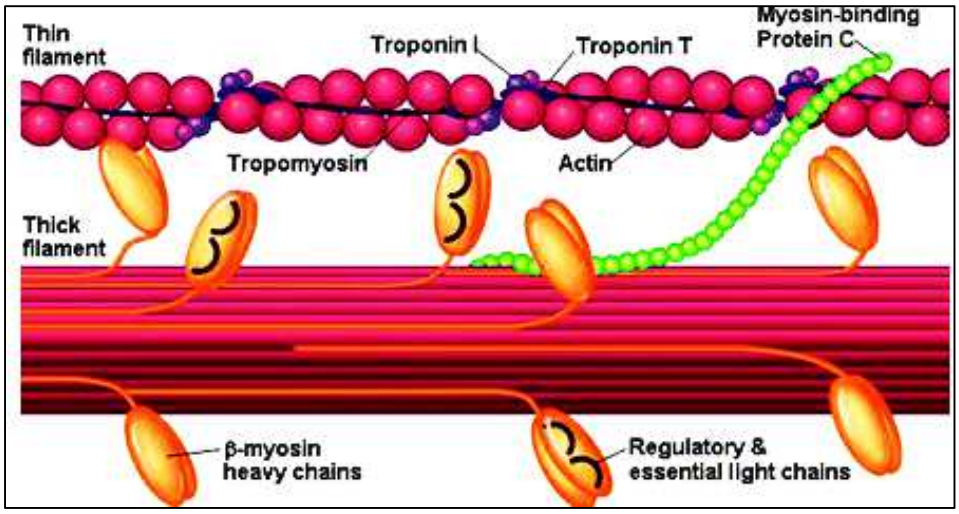


Figure 1. Cardiac Sarcomere. The cardiac sarcomere contains several proteins that facilitate sarcomere contractile function. Some of which contain causal HCM mutations. Desai MY., et al. Circulation. 2011.

The regulatory function of the cardiac sarcomere resides in the thin filament. Muscle contraction depends on the access of the myosin head to the actin filament, which is regulated by the tropomyosin–actin complexes upon the binding of calcium ions, as well as exerting energy in the form of ATP [4-5]. Abnormal interactions between the filaments result in contractility dysfunction. In normal conditions, the myosin protein head of the MHC hydrolyzes ATP into ADP and Phosphate ion. This leads to the myosin head of the thick filament binding to actin protein on the thin filament. The myosin head can only bind to actin when tropomyosin uncovers actin's binding sites in the presence of high calcium ions and high ATP levels [6]. With proper conformational changes in filaments by energy and calcium ions, the sarcomere contracts and then relaxes when another myosin head binds to another ATP molecule. The troponin tertiary complex of the thin filament modulates the calcium ion dependence of muscle contraction by coupling the level of cytoplasmic calcium to the production of mechanical force by ATP.

This ensues sliding of the filaments and proper sarcomere contractility (Figure 2, Figure 3).

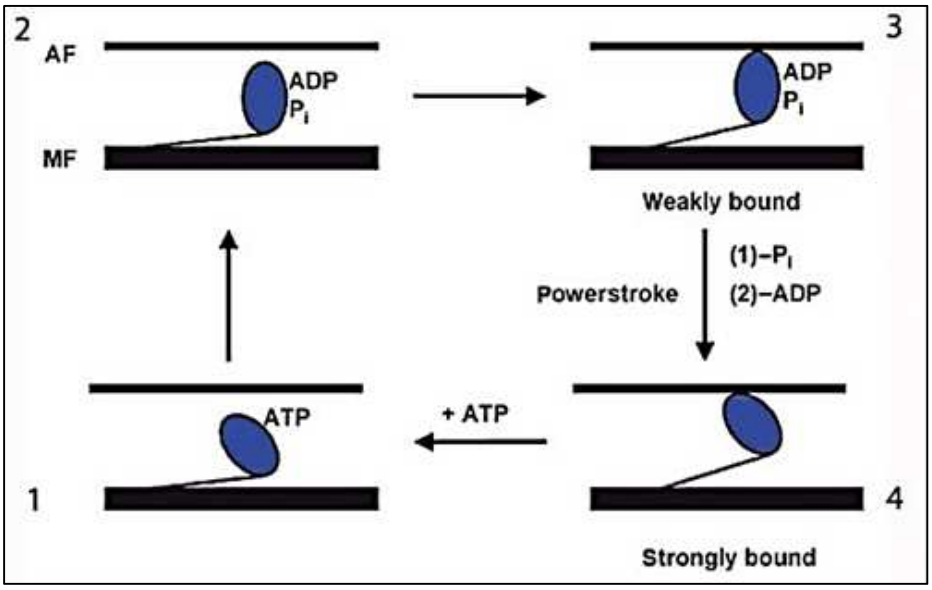


Figure 2. Sarcomere contraction cycle. ATP hydrolysis releases the energy required for myosin to do its job. AF: actin filament. MF: myosin filament. Goody RS. Nature. 2003.

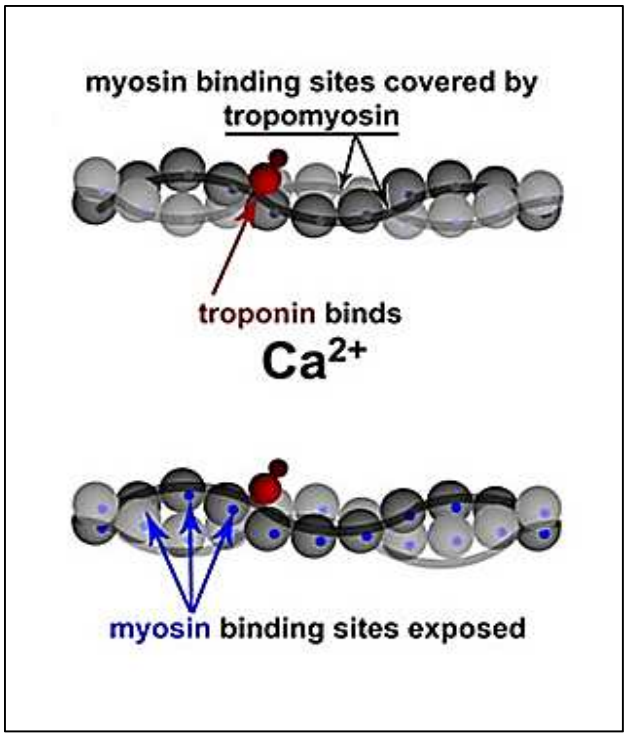


Figure 3. Troponin and Tropomyosin regulate contraction via calcium binding. Upon binding calcium, troponin moves tropomyosin away from the myosin-binding sites on actin, effectively unblocking it. Lehman W., et al. Nature. 1994.

Genetic mutations affect sarcomeric protein structure and expression level that affects molecular and cellular functions, such as the interaction between actin and myosin proteins [6]. This has led to the hypothesis that HCM is a disease of the sarcomere. However, the exact relationship between mutant contractile proteins and the phenotype remains unclear. Characterizing HCM pathogenesis at a cellular and molecular level may provide the information needed to address this question [7].

The initial genetic discoveries placed emphasis primarily on HCM caused by mutations in the protein constituents of the thick and thin filaments of sarcomeres [6]. The R403Q mutation of the MHC and the R92W mutation of TnT have raised many questions about the cell biology of cardiac muscle, such as the mechanism by which a cardiac

specific phenotype results from mutations in sarcomere genes and which intracellular signaling pathways are triggered by expression of gene defects [1]. Studies have elucidated a diverse array of functional defects including altered calcium ion sensitivity caused by the R92W-TnT mutation and energy disruption caused by the R403Q-MHC mutation [8-21].

In the heart, ATP supply is tightly regulated to meet energetic demands of the myofilaments [22]. The mechanisms by which cardiac energetics is still a matter of considerable debate, but there is emerging consensus on the importance of two regulators, calcium and ATP [23-24]. It was noted that the hyper contractile phenotype in HCM would lead to inefficient contraction at rest, requiring the consumption of more ATP than usual and compromising the cardio myocyte to maintain energy levels [25]. The suggestion that energy depletion underlies HCM is supported by the HCM-like phenotype found with the R403Q-MHC mutation [26]. Primary biochemical studies on R403Q-MHC mutants showed that actin filament translocation and force generation were reduced, requiring more ATP [27-28]. The increased demand compromises the capacity of cardio myocytes to maintain energy levels responsible for contraction and calcium ion homeostatic functions. The ensuing myocyte dysfunction results in hypertrophy [29-30] (Figure 4).

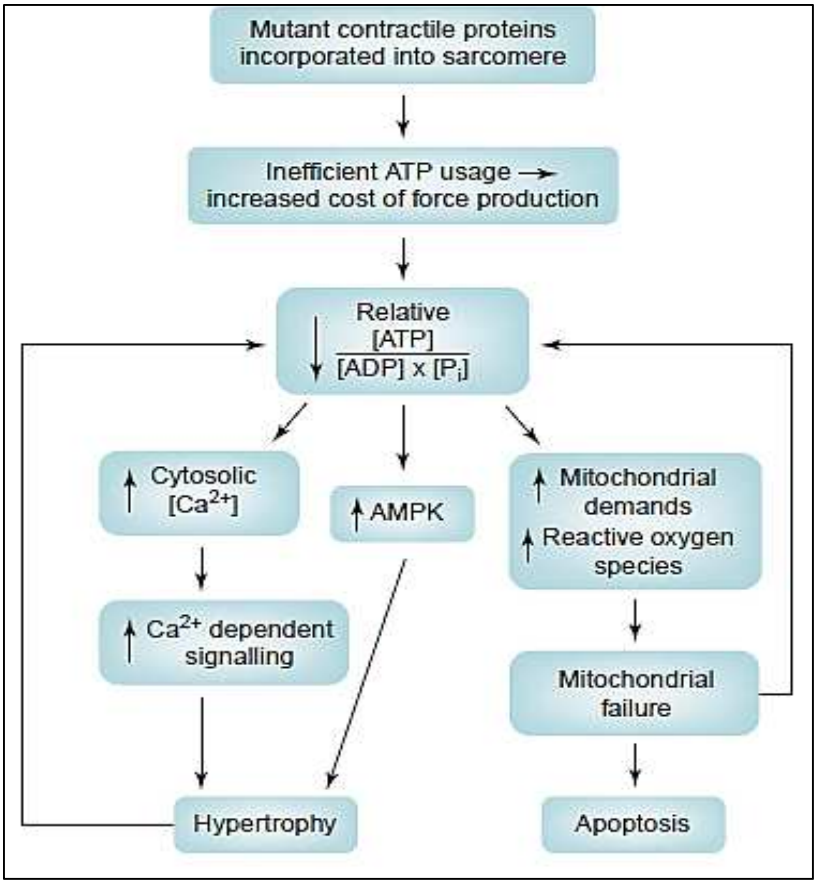


Figure 4. Energy depletion model of HCM caused by sarcomeric protein mutations. The proposed abnormality is energy depletion in compartments of the cardio myocyte leading to failure to maintain energy levels sufficient for contraction and critical homeostasis functions, such as Calcium ion reuptake. Energy depletion, myocyte dysfunction, elevation of calcium ion, and AMPK activation results in hypertrophy. Progressive mitochondrial dysfunction exacerbates the energetic compromise. Ashrafian H., et al. Trends in Genetics. 2003.

1.3 Causal Mutations:

Although it's hypothesized that HCM is a disease of the sarcomere due to causal mutations arising from sarcomere proteins, there are several knowledge gaps regarding these causal mutations. Not all proteins residing in these genes are known and not all mutations of the known proteins are known. However, it is known that approximately 35% of HCM result from cardiac MHC gene missense mutations. More than 80 unique mutations have been reported that alter one amino acid residue in the globular head of the MHC. These mutations are expressed with a high degree of penetrance and about 90% of affected individuals exhibit significant myocardial hypertrophy on two-dimensional echocardiography studies by 20 years of age [31]. Despite significant hypertrophy, survival in HCM caused by a cardiac MHC mutation varies and is, in part, mutation-specific. For example, individuals with the R403Q-MHC missense mutation, a mutation resulting from Glutamine amino acid replacing Arginine amino acid at codon 403, have shortened life expectancies, with the average death being at age 45 years. A variety of cardiac TnT gene defects result from missense mutations, small deletions and mutations in splice signals, causing approximately 15% of all HCM [32]. The R92W-TnT missense mutation, results from Tryptophan amino acid replacing Arginine amino acid at codon 92. The cardiac phenotype portrayed by these gene defects is characterized by substantially less hypertrophy, but more prominent fibrosis, myocyte disarray, large papillary muscles, and a higher rate of sudden cardiac death than that observed with R403Q-MHC mutation [33]. Other understudied mutations of the myosin binding protein C, tropomyosin and troponin I proteins causes approximately 20-30% and less than 5% of HCM, respectively. The direct link between mutations in the structural components of the

cardiac sarcomere and the resultant complex clinical phenotype remains unknown [34]. In vitro biochemical approaches have demonstrated that mutations in the MHC have defects in actin-activated ATPase activity. This reduction in motor activity triggers a compensatory hypertrophic response, explaining the high frequency of hypertrophy in patients with MHC mutations [35].

Transgenic mice have been created for mutations in the cardiac MHC and TnT, and these models have both similarities and differences with each other and with the human disease. Both models exhibit many of the histological features of the disease; one exhibits significant ventricular hypertrophy, whereas the other exhibits no ventricular hypertrophy [36- 37]. Structurally, TnT interacts directly with Tropomyosin within the thin filament and promotes the ordered assembly of the Troponin-Tropomyosin complex onto the actin filament [38]. In low calcium ion states, when the sarcomere relaxes, TnT binds to Troponin I-Troponin C complex. This effect is overcome at higher calcium ion concentration, when the sarcomere contracts, where the binding of calcium ion to Troponin C favors the reformation of the Troponin I-Troponin C complex. Therefore, the ability of the thin filament to respond to shifts in calcium ion within the myocyte is modulated by multiple protein-protein interactions that occur along the TnT molecule [39].

1.4 R403Q-MHC Transgenic Mouse Model:

In order to study the impact of contractile protein mutations of HCM, transgenic mice of HCM with R403Q-MHC mutation and R92W-TnT mutation were studied during the course of this research. Leinwand *et al*. created transgenic mouse lines which phenotypically are virtually identical to human R403Q-MHC of HCM, allowing the impact of contractile protein mutations on the heart to be analyzed, as well as the factors

contributing to the disease's natural history [7]. The transgenic mouse model of HCM mimics several aspects of HCM in humans, specifically the appearance of myocellular disarray, fibrosis, and cardiac dysfunction [40]. Based on these findings, the transgenic mice are suitable models for HCM, as their analysis provides insight into the mechanisms involved in developing the disease phenotype [41-42]. R403Q-MHC mice develop predicted histopathological changes of HCM, such as myocyte hypertrophy, disarray and fibrosis by age 15 weeks. At age 30 weeks, left ventricular hypertrophy is detectable by echography [43]. This mimics the human disease very closely (Figure 5, 6, 7).

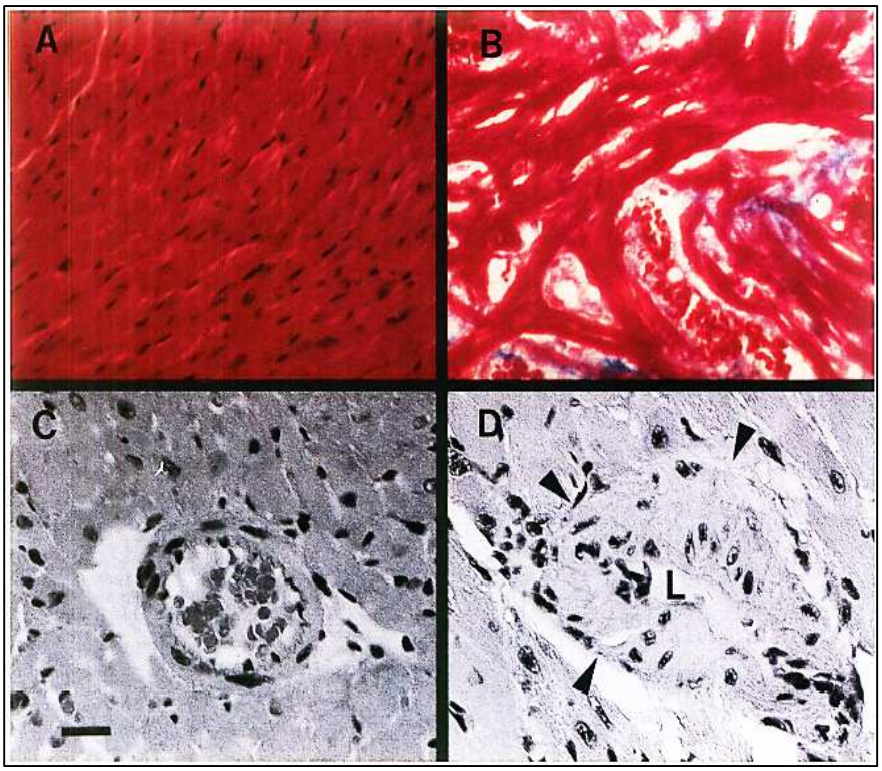


Figure 5. Transgenic mouse hearts exhibit histopathologic features of HCM. Paraffin sections of hearts from control (A and C) and transgenic (B and D) mice were stained with Masson's trichome (A and B) or hemotoxylin/eosin (B and D). Evidence of HCM was seen in the left ventricle and included focal myocyte hypertrophy and myocellular disarray with increased matrix accumulation. Histopathologically, this is seen as thickening of the smooth muscle layer of the small coronary vessels. Magnification at 100x (A) and 200x (B). Leinwand LA., et al. Molecular Medicine. 1996.

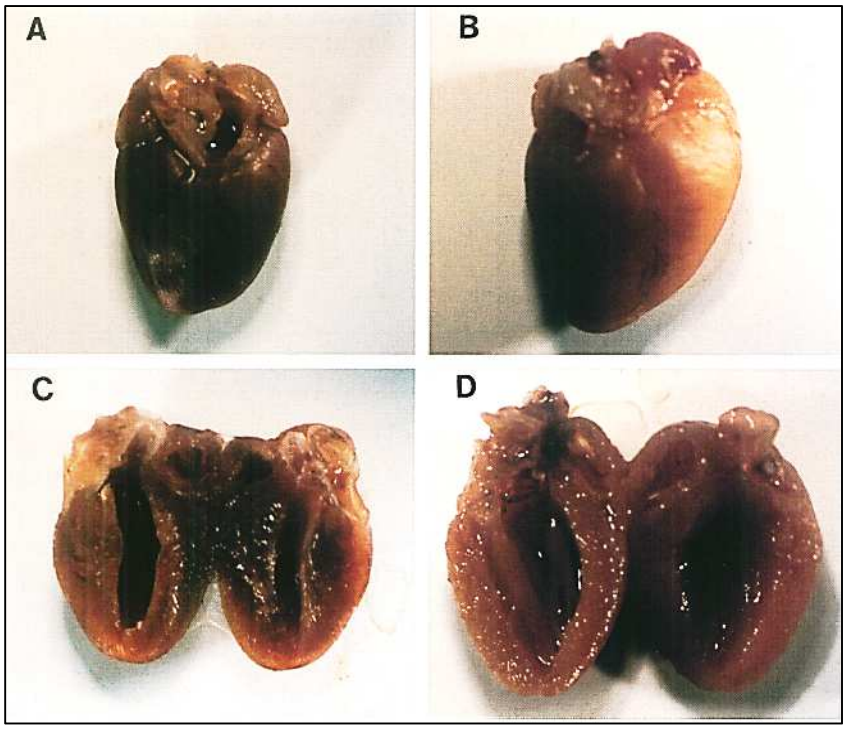


Figure 6. Chamber dilation in 8-month-old transgenic mice. Formaldehyde fixed hearts, both control (A and C) and transgenic (B and D), were bisected axially by an incision through the anterior face of the left ventricle. The two halves of the heart were splayed open to reveal the left ventricle cavity (C and D). Leinwand LA., et al. Molecular Medicine. 1996.

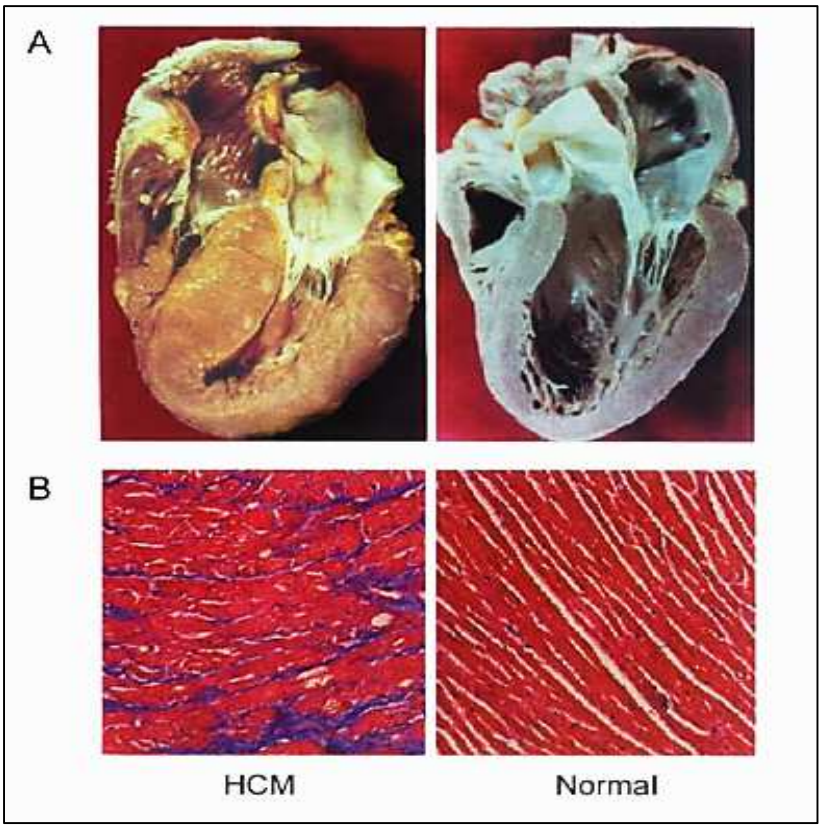
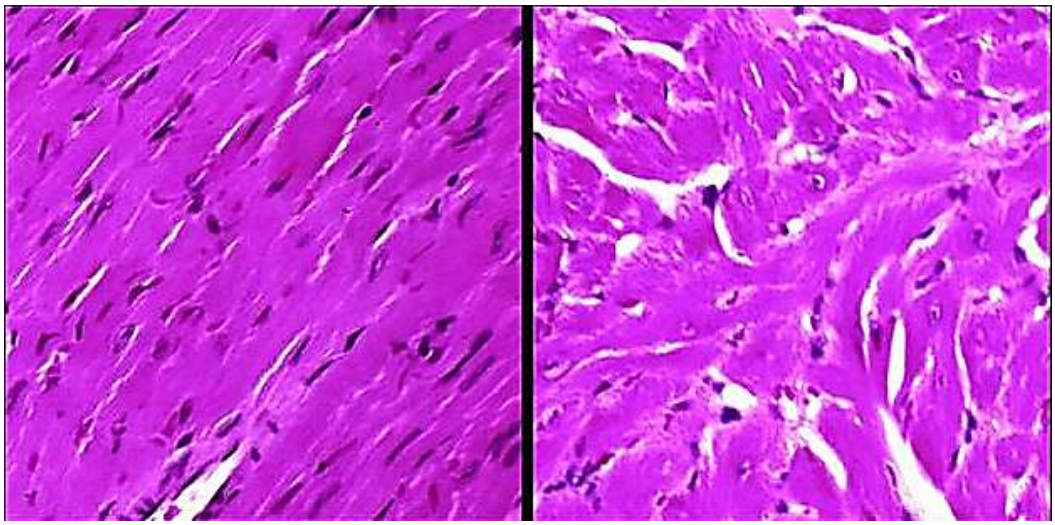


Figure 7. Pathology of HCM. (A): Post-mortem examination of the heart from an individual with HCM demonstrating massive asymmetric left ventricular hypertrophy, with associated reduction in left ventricular cavity size, compared to a normal heart. (B): Histopathology of heart sections stained with Massons trichrome showing significant myofibre disarray and fibrosis in HCM. Chung MW., et al. Cell. 2003.

1.5 R92W-TnT Transgenic Mouse Model:

HCM caused by R92W-TnT mutation is characterized by a high frequency of early sudden cardiac death, often in the absence of evident ventricular hypertrophy [44]. Tardiff *et al*. created transgenic mouse lines that phenotypically are virtually identical to human R92W-TnT of HCM. R92W-TnT adult mice revealed a broad range of histopathology consistent with that found in human mutant TnT hearts, including myocyte disarray and degeneration, mild inflammatory cell infiltration, occasional hypertrophied cells, and minimal fibrosis [45] (Figure 8). Hearts from R92W-TnT transgenic mice exhibited a more widespread histopathology that was detectable at the age of 2 months. R92W mice alone exhibit a significant decrease in myocyte size that results in a decrease in ventricular mass [46]. It is important to note that the early activation of the hypertrophic gene program in the R92W mice not only did not lead to overt ventricular hypertrophy, but these hearts and myocytes were smaller than the wild type mouse hearts. These results support the hypothesis that closely related changes in TnT structure can result in distinct cardiovascular phenotypes in vivo and provide a basis for the phenotypic variation seen in human HCM [47-48].



A. **B.**

Figure 8. 26 week R92W-TnT transgenic mouse heart histology. A: Control mouse heart with no myocyte disarray. B: Transgenic mouse heart shows disarray. Magnification: 40x. Tardiff JC., et al. Proceedings of the National Academy of Sciences. 2005.

Studies using myosin isolated from R92W-TnT mouse model of HCM revealed an increase in the energy cost of tension development in whole hearts [49-50]. The hearts of these animals are significantly smaller than those of controls. The reduction in ventricular mass could be explained by the observation that there are fewer myocytes and are smaller than controls. In addition, the hearts develop severe diastolic dysfunction and milder, but significant, systolic dysfunction [35]. Consequently, these mutational alterations in TnT structure and function have severe implications for the cardiac sarcomere, contractility, and calcium ion handling. At the age of 2 months, only R92W mice showed a significant difference in resting intracellular calcium ion levels compared with wild type mice. By age of 6 months, R92W myocytes exhibited significantly decreased resting intracellular

calcium ion levels [51-52]. Changes in resting intracellular calcium ion may be due to age-dependent changes in calcium ion buffering capacity of R92W mutant cardiac myocytes have hypothesized that the increased myofilament calcium ion sensitivity inherent to R92W mutation has additional effects on cardiac contractility, such as possibly interfering with intracellular calcium ion cycling and reuptake [53]. In this study, transgenic mice of age 5 weeks and 24 weeks will be studied, as age 5 weeks will represent an early pre hypertrophic stage of disease and age 24 weeks will represent a hypertrophic developed stage of disease. This will provide us with more insight on how different proteins are expressed at different stages of disease.

1.6 Significance of Mouse Models:

Answering such questions is limited in human studies due to a variety of factors, such as broad genetic backgrounds, environmental factors, small numbers of individuals with the same mutation, and the relative difficulty in obtaining human cardiac samples as well as inadequate methods of maintaining human heart tissue in cell culture systems. For these reasons, a variety of biochemical, cell and animal models have been engineered to more fully dissect the consequences of human sarcomere mutations on muscle structure and function [54]. The development of transgenic mice of HCM has been useful since genetic and environmental backgrounds can be controlled and obtaining tissue samples is unlimited. Transgenic mice that over-express mutant forms of MHC, TnT, myosin-binding protein C, or troponin I, as well as a model that physiologically expresses a particular myosin mutation, R403Q have been studied [55-58]. All these models exhibit histopathology comparable to that observed in human HCM including myocyte disarray, hypertrophy and myocardial fibrosis. The first and most extensively studied mouse model

of R403Q-MHC of HCM illustrates how the human disease is replicated in mice [59-60]. This provides greater insight in addressing the issues of molecular pathogenesis, signaling mechanisms, and modifying factors.

Since transgenic mice are excellent tools for analyzing phenotypic changes over time, it was studied that older male and female mice exhibit distinct phenotypes. While the cardiac hypertrophy was increased in older female animals, male mice exhibited significant dilation of the left ventricle. Although sex-specific differences in the HCM phenotype have not been reported in humans, clinical heterogeneity is a hallmark of this disease. In addition, HCM patients often exhibit progressive wall thinning or relative ventricular dilation as they age [61- 64]. Although cardiac disease in a small animal model is inherently different from that seen in humans, the mouse allows analyses of disease at molecular and cellular levels impossible in humans. One recently described mouse model for HCM genetically most closely resembles the human disease; phenotypically, it displays substantial differences, namely left atrial enlargement in the absence of ventricular hypertrophy [65]. The difference in phenotype may be due to the nature of the mutation, the genetic background, or both. Insight into the basic mechanisms underlying cardiac hypertrophy may be gained from comparing the different models [66]. Most importantly, these transgenic mice of human HCM provide evidence that a mutation in a sarcomeric gene is indeed the primary cause of HCM.

1.7 AMPK as an Energy Sensor:

The discovery of AMPK dates back to the 1970s and was demonstrated that it is expressed in the heart [67]. The key chemicals within the cell that facilitate proper sarcomere contractility are ATP and ADP, which are interconverted by the reaction ATP,

ADP, and phosphate ion. ATP generation needs to remain in balance with ATP consumption, and regulatory proteins that sense ATP and ADP levels would be a logical way to achieve this [68-69]. Decrease in cellular energy is associated with increases not only in ADP, but also AMP. Proteins sensing cellular energy status should monitor the ADP/ATP ratio, AMP/ATP ratio, or both [70]. The main energy sensor in most eukaryotic cells is AMPK. Consistent with a role in maintaining energy homeostasis, when AMPK is activated by energetic stress, it switches on catabolic pathways that generate ATP while switching off biosynthetic pathways that consume ATP [71]. Under decreased intracellular ATP levels, AMP or ADP can directly bind to the δ regulatory subunits of AMPK, leading to a conformational change that promotes AMPK phosphorylation at Threonine amino acid at codon 172 and also protects AMPK from dephosphorylation to ensure it remains activated [72-74]. There is emerging evidence that AMPK might modulate the development of cardiac hypertrophy [75-78].

In addition to its actions on cardiac energy metabolism, AMPK also has important actions on the cardiac hypertrophic process [79-80]. When activated, AMPK inhibits cardiac protein synthesis and the cardiac hypertrophic process. Recent results with muscle-specific AMPK double knockout mice lack any detectable AMPK activity and have supported the idea that AMPK activation is significant during muscle contraction. In a study by Tian's group, AMPK activity was increased in rat hearts and demonstrated energetic changes. However, isolated cardio myocytes developed hypertrophy but did not demonstrate increased AMPK activity. These results raise the possibility that the AMPK response during hypertrophy might vary depending on the model or rate of development of the pressure overload stress. AMPK activation and inactivation have been shown to

contribute to cardiac hypertrophy [81-92].

AMPK phosphorylation is a key mechanism controlling AMPK activation in the heart. Phosphorylation of Threonine amino acid at codon 172 activates AMPK during times of metabolic stress [80, 93]. While the relationship between cardiomyopathy caused by defects in AMPK and HCM remains to be delineated, sarcomeric HCM and AMPK mutations convene on a common final path, implicating AMPK and altered energy homeostasis is involved in the pathogenesis of hypertrophy [26]. Although the precise involvement of AMPK in cardiac hypertrophy is currently unknown, many studies support the concept that during times of energy depletion, AMPK is activated and protein synthesis may be inhibited in order to conserve cellular energy status [80].

1.8 CAMKII and MCU Regulate Calcium Ions:

Hypertrophy in HCM is an energetic compromise, and is marked by disordered cellular calcium ion homeostasis. With calcium ion constituting a sensor for cellular energy balance, CaMKII contributes to alteration in intracellular calcium ion homeostasis and responds to these very changes by increasing its activity. If energy generation cannot match demand, calcium ion re-uptake and removal at the end of each contractile cycle is compromised. Failure to meet the extreme energy requirements leads to abnormal sarcomere contractile [80, 94].

The multifunctional CaMKII is an intracellular protein that is present at the cell membrane, in the cytoplasm, and in the nucleus of many cells including cardiac myocytes. CaMKII plays important functional roles in all of these locations and has the capability of linking cellular responses to changes in cytoplasmic calcium ion levels throughout the cardiac myocyte [94-95]. There are 3 types of CaMK: I, II, and IV. Type II

is the most abundant in heart, while type IV is absent or present in such low abundance as to be unlikely to play any significant role. The role of CaMKI is unclear in heart; however, unlike CaMKII, CaMKI does not appear to be increased in the setting of structural heart disease [96]. There are 4 isoforms of CaMKII, alpha, beta, delta, and gamma. Expression of the alpha and beta isoforms is largely restricted to neuronal tissue, while the delta and gamma isoforms are both present in diverse tissues including heart [97]. The δ isoform is the prominent cardiac isoform, and the relative expression of different δ variants changes throughout cardiac development and disease. All CaMK types and isoforms are activated by calcium bound CaM (Ca^{2+} /CaM) [98-103]. Activation by Ca^{2+} /CaM means that CaMKII is responsive to changes in intracellular calcium ions. Once CaMKII is activated by Ca^{2+}/CaM, CaMKII is a substrate for itself and autophosphorylation can ensue or inhibit CaMKII activity [103-107].

CaMKII activity and expression are reported to increase in genetic and surgical models of structural heart disease, while a lesser number of reports show a reduction in CaMKII activity [108-110]. Mice with cardiac-specific CaMKII expression of the predominant δ isoform have cardiac hypertrophy. These studies provide essential information that builds a case for CaMKII serving as a disease determinant in structural heart disease. Another key piece of information will need to come from studies demonstrating that CaMKII inhibition can reverse disease phenotypes, such as arrhythmias, hypertrophy, and dysfunction in models of structural heart disease [111-112]. Preliminary findings using mice with cardiac expression of a CaMKII inhibitory peptide suggest that CaMKII inhibition can reduce arrhythmias and mortality and improve myocardial function even in mice with severe cardiomyopathy due to over expression of

the Ca^{2+} /CaM activation. Additional preliminary reports show that CaMKII inhibition can reduce left ventricular dilation and improve myocardial function in mice [113]. These findings that CaMKII activity and expression are increased in structural heart disease, CaMKII over expression recapitulates key structural heart disease phenotypes, and CaMKII inhibition improves many of these same phenotypes are strong evidence that CaMKII is an important potential therapeutic target [111-112]. However, CaMKII over expression can cause cardiomyopathy and is an area that still needs better understanding. It was shown that CaMKII levels increase in patients with end stage cardiomyopathy from a variety of causes CaMKII is one of many signals that is being reported to change in heart disease but such a change may not indicate a causal role for CaMKII in determining disease phenotype [114-115].

CaMKII is activated in numerous cardiac diseases, and contributes to the development of heart failure, and arrhythmias [116-117]. Sustained activation of CamKII in the presence of ATP results in auto phosphorylation across subunits at Threonine codon 287 [118-119]. This phosphorylation event prevents the reassociation of the catalytic domain resulting in the persistence of enzyme activity even in the absence of Ca^{2+}/CaM. Over expression of CaMKII in the heart leads to deranged calcium ion homeostasis and heart failure. Excessive CaMKII activity results in opening of MPTP and over production of ROS [120-122]. Additionally, CaMKII phosphorylates the MCU, resulting in increased cytoplasmic calcium ion currents into mitochondria [123-126].

It has been shown that the targeted inhibition of CaMKII activity results in impairments in cardiac contractility and Ca^{2+} handling. However, the long-term, chronic effects of CaMKII activation have been shown to become pathogenic and may be

involved in the disease mechanism of R92W mouse models, which remains significantly impaired, compared with that of wild type models [127-129]. A more complete understanding of CaMKII regulation in the heart will help to understand the normal physiology of the heart, how it changes during diseases, and may identify new potential treatments. We are just starting to understand the important subcellular mechanisms in which CaMKII is involved [130].

1.9 Calcium Sensitivity:

Attention was focused on the higher calcium ion sensitivity early on in the study of HCM and this has been supported by subsequent experimentation. Increased calcium ion sensitivity has been reported in R92W-TnT mutation of HCM [131-133]. It is remarkable that two MHC mutations also increase calcium ion sensitivity indicating that small changes in myosin–actin interactions exert allosteric effects upon the thin filament switch to change calcium ion sensitivity [134-136]. During the course of this research, R403Q-MHC transgenic mice, R92W-TnT transgenic mice, and wild type mice will be studied to gain a better understanding of HCM. AMPK, CamKII, and MCU proteins will be tested from whole heart. Transgenic mice of age 5 weeks will be studied to represent an adolescent pre hypertrophic stage and transgenic mice of age 24 weeks will be studied to represent a mature hypertrophic stage.

II. Hypothesis

R403Q mutation of Myosin Heavy Chain and R92W mutation of Troponin T in Hypertrophic Cardiomyopathy transgenic mice result in AMPK, CamKII, and MCU protein expression differences in proteins from whole heart at age 5 weeks and age 24 weeks.

III. Objectives

In this study, transgenic mice with R403Q-MHC and R92W-TnT mutations of HCM will be studied. Transgenic mice of age 5 weeks and 24 weeks will be studied. 5 week old transgenic mice will represent a pre hypertrophic early stage of disease, while 24 week old transgenic mice will represent a hypertrophic developed stage of disease. Studying protein expression in different age groups will allow us gain insight on significant differences between different stages of disease and how energy and calcium homeostasis are affected at these stages.

1. To examine differences of AMPK, CamKII, and MCU relative protein levels from whole heart homogenate of three different transgenic mouse models at age 5 weeks with HCM.
2. To examine differences of AMPK, CamKII, and MCU relative protein levels from whole heart homogenate of three different transgenic mouse models at age 24 weeks with HCM.

IV. Materials and Methods

Ethical Aspects of the Proposed Research: The protocol was approved by the Committee on the Ethics of Animal Experiments of the Johns Hopkins Medical Institutions. All the animal care and use conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

4.1 Materials:

Routine reagents were purchased from Sigma Aldrich (Louis, MO), Life Technologies (Grand Island, NY), and Cell Signaling Technology (Beverly, Massachusetts) unless specified. Antibodies against AMPK, pAMPK, pCamKII, and pMCU were obtained from Cell Signaling Technology (Beverly, Massachusetts). Antibodies against CamKII and MCU were obtained from Abcam (Cambridge, United Kingdom).

Male C57/BL6 littermate transgenic mice containing mutations R403Q-MHC, R92W-TnT, and control of 5 weeks and 24 weeks of age were obtained. R403Q-MHC transgenic mouse line was constructed by Leinwand et al (Boulder, Colorado) [7]. R92W-TnT transgenic mouse line was constructed by Tardiff et al (Tuscon, Arizona) [45].

Biological Replicates:

16 transgenic mice from each age group were used to study protein expression from whole heart (n=4).

8 control mice, 4 R403Q-MHC mice, and 4 R92W-TnT mice age of 5 weeks were obtained.

8 control mice, 4 R403Q-MHC mice, and 4 R92W-TnT mice age of 24 weeks were obtained.

4.2 Experimental Approach:

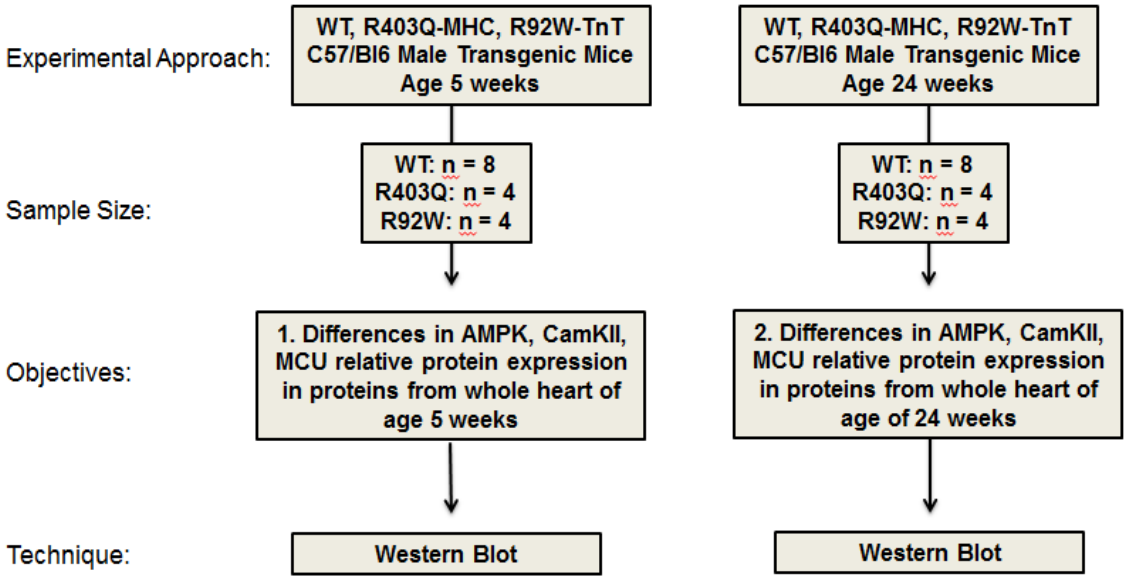


Figure 9. Experimental Approach. C57/Bl6 male transgenic mice containing mutations of R403Q-MHC, R92W-TnT, and their respective littermate controls were obtained. Leinwand et al. constructed transgenic mouse model containing R92W-TnT mutation [7] and Tardiff et al. construct transgenic mouse model containing R403Q-MHC mutation [45]. Sample size of 5 week aged transgenic mice was n = 4 for each mutation and control. Differences in AMPK, CamKII, MCU relative protein expression in proteins from whole heart were tested by Western immunoblotting. Sample size of 24 week aged transgenic mice was n = 4 for each mutation and control. Differences in AMPK, CamKII, MCU relative protein expression in proteins from whole heart were tested by Western immunoblotting.

4.3 Heart Harvest:

All mice were anesthetized and euthanized by cervical dislocation. Mice were dissected and each heart was isolated and washed in PBS buffer. Each heart was placed in a 1 ml sample tube and flash frozen until protein isolation.

4.4 Protein Isolation from Heart:

Each whole heart was homogenized and lysed with 2% Triton-Ripa buffer, Protease Inhibitor cocktail, and PhosSTOP EASYpack tablets (Roche; Basel, Switzerland), in its own 1.5 ml sample tube. Heart homogenate was left at room temperature for 15 minutes and then on ice at 4 degrees Celsius for 1 hour. Homogenates were centrifuged at 10,000 rpm for 15 minutes. Pellet was discarded and supernatant was aliquot into new sample tubes and frozen. BCA assay was used to determine protein concentration (Thermo Scientific Pierce, Rockford, IL). SoftMax Pro version 5.4.5 software on SpectraMax M2 (Molecular Devices; Sunnyvale, California) was used to measure protein concentration from BCA assay. Equal amounts of protein were subjected to Western Blot.

4.5 Sample Preparation:

Homogenates were prepared with dH2O, LDS 4X sample buffer, and 1 M DTT in centrifuge eppendorf sample tubes. Samples were vortexed and then centrifuged at 10,000 rpm for 2 minutes. Samples were denatured at 70 degrees Celsius for 10 minutes on heat block and then cooled at room temperature for 5 minutes. Equal volume of total loading sample was loaded for Western Blot.

4.6 Western Blot Immunoblot:

Denatured proteins were loaded in 4-12% Bis-Tris buffer gels, with MES SDS Running Buffer, and run at 165 V for 1 hour in 4 degrees Celsius. Proteins on gel were transferred to PVDF membranes with iBlot Transfer apparatus (Invitrogen R; Waltham, Massachusetts) at Program 0 for 8 minutes. Each membrane was incubated with 10 ml of 5% nonfat dried milk blotting grade blocker in TBST or 5% BSA in TBST for 2 hours at room temperature on a shaker. Membranes were incubated with appropriate primary antibody at 1:1000 dilution overnight at 4 degrees Celsius on a shaker. AMPK, CamKII, and MCU were diluted with 2% milk-TBST and pAMPK, pCamKII, and pMCU were diluted with 2% BSA-TBST. The following day, membranes were incubated with anti-GAPDH loading control for protein at 1:12,000 dilution for 1 hour at room temperature on a shaker. Anti-GAPDH was diluted with 2% milk-TBST for AMPK, CamKII, and MCU. Anti-GAPDH was diluted with 2% BSA-TBST for pAMPK, pCamKII, and pMCU. Membranes were then followed by incubation of HRP-conjugate secondary antibody at 1:24,000 dilution for 1 hour at room temperature on a shaker HRP was diluted with 2% milk-TBST for AMPK, CamKII, and MCU. HRP was diluted with 2% BSA-TBST for pAMPK, pCamKII, and pMCU. Electro chemiluminescence using Femto followed to visualize bands.

V. Analysis

The experiment was performed several times to optimize the technique. Following optimization of technique, experiment for AMPK and pAMPK protein targets was repeated 5 times, experiment for CamKII and pCamKII protein targets was repeated 7 times, and experiment for MCU and pMCU protein targets was repeated 7 times. Each result represents the best representation of all repeated experiments and is expressed as mean values ± standard error of mean. Student's t test is quantitated by densitometry and normalized with that of GAPDH loading control. Asterisk (*) indicates $P < 0.05$ relative to control. Sample size (n) for each mutation is $n = 4$. The ratio of band intensities of pAMPK and total AMPK is also determined. The representative western blots below show the significant collective data obtained.

VI. Results

pAMPK Protein Expression is in R403Q-MHC 5 week Transgenic Mice

In order to confirm that the R403Q mutation of MHC results in energy stress, we tested anti-AMPK and anti-pAMPK antibodies in 5 week transgenic mice. There is no significant difference in total AMPK protein expression in transgenic mice at 5 weeks. However, there is 25% increase in pAMPK protein expression in R403Q-MHC transgenic mice than R92W-TnT transgenic mice, as there may be some degree of energy stress due to low ATP (Figure 10, Figure 11).

Key:

C: Control

M: R403Q-MHC

T: R92W-TnT

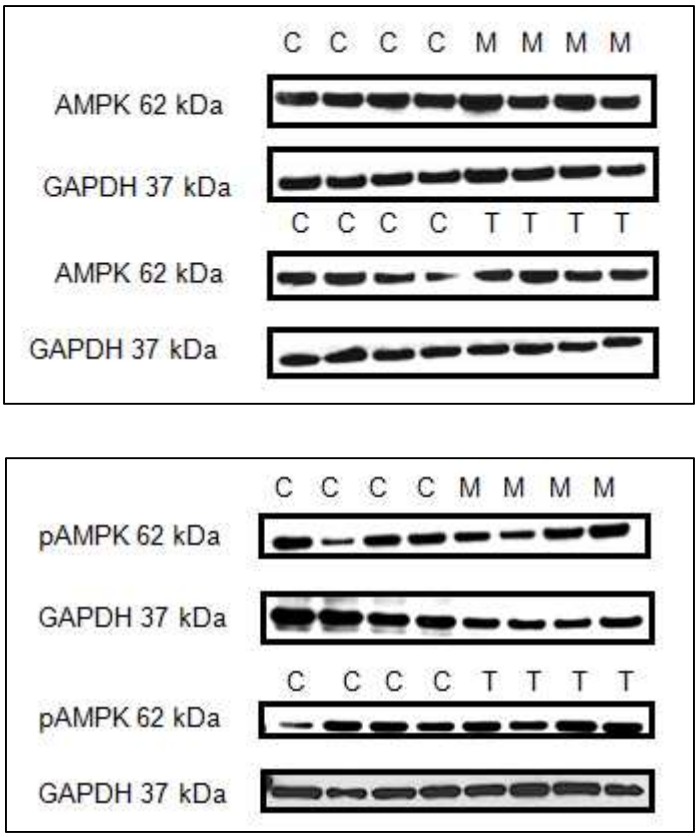
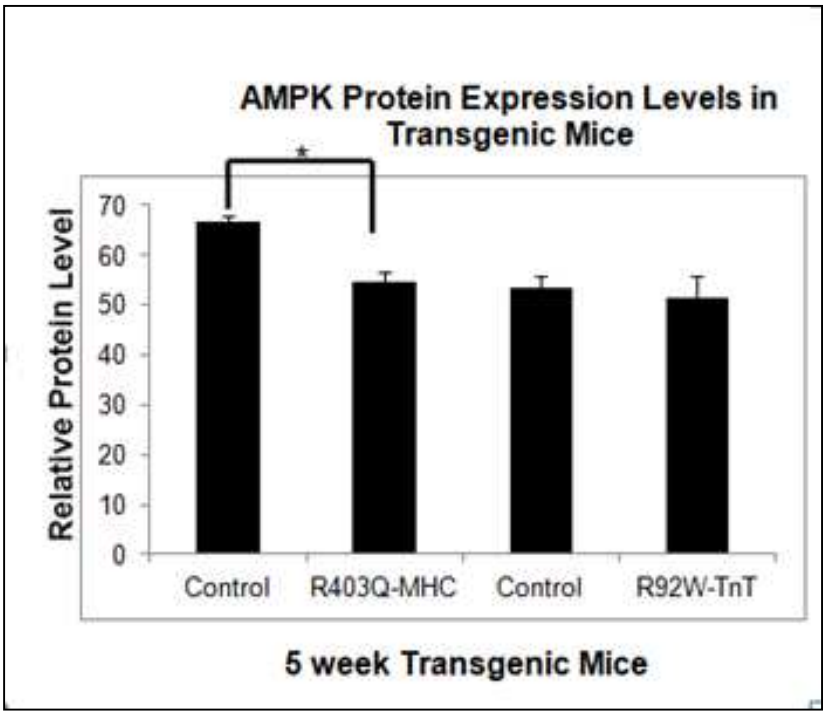


Figure 10. Western Blots for AMPK and pAMPK in 5 week Transgenic Mice.



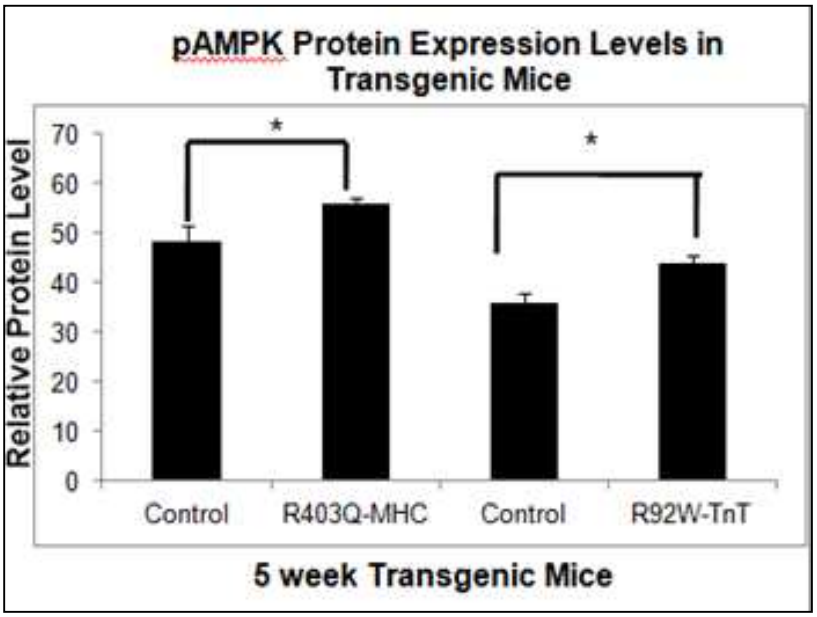


Figure 11. Western Blots for analysis of AMPK and pAMPK protein expressions in 5 week transgenic mice. 7 experiments yielded similar results and the figure above represent the best representation out of all collected experiments. Data are expressed as mean values ± standard error of mean.

*P < 0.05 relative to control. n = 4.

pAMPK Protein Expression Highest is R403Q-MHC 24 week Transgenic Mice

In order to confirm that the R403Q mutation of MHC results in energy stress, we tested anti-AMPK and anti-pAMPK antibodies in 24 week transgenic mice. There is no significant difference in total AMPK protein expression in the transgenic mice at 24 weeks. However, there is an 18% increase in pAMPK protein expression in R403Q-MHC transgenic mice than R92W-TnT transgenic mice. There is also an 18% increase in pAMPK protein expression from 5 weeks to 24 weeks in R403Q-MHC transgenic mice. There may be a further decrease of ATP at 24 weeks than 5 weeks resulting in higher energy stress. (Figure 12, Figure 13).

Key:

C: Control

M: R403Q-MHC

T: R92W-TnT

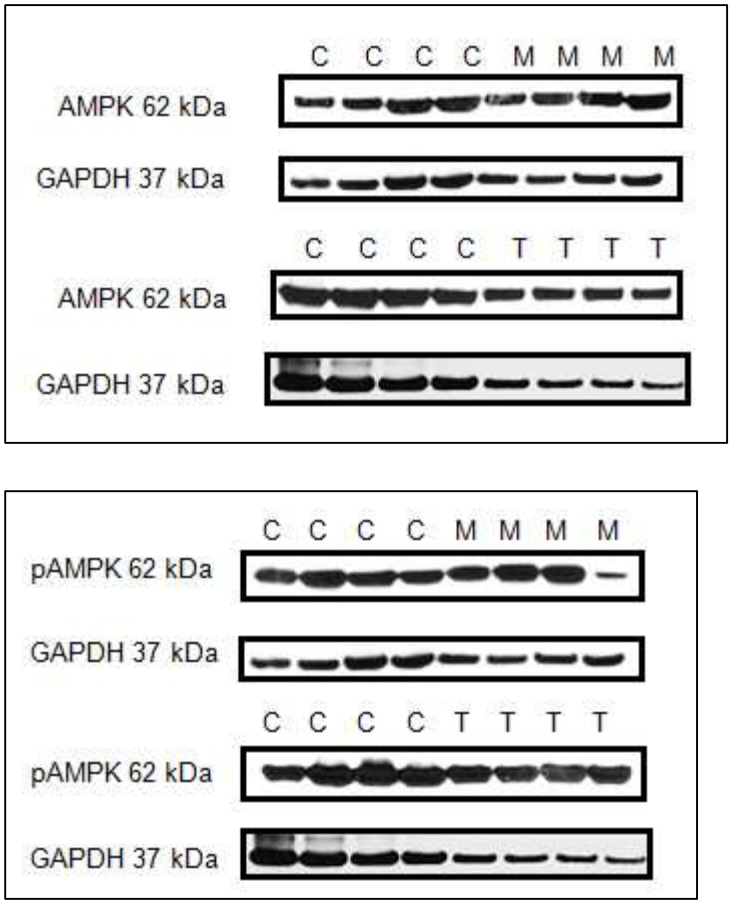
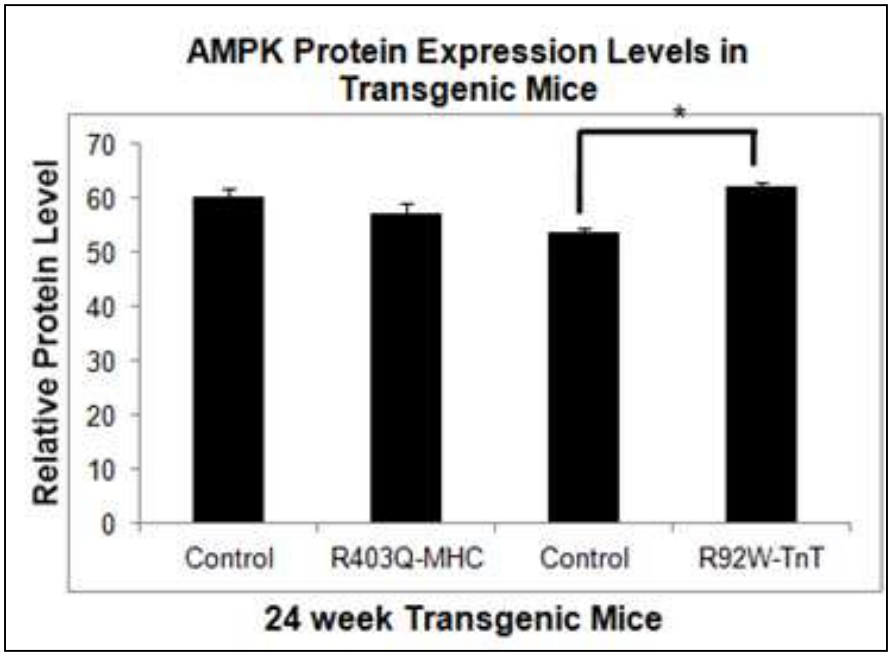


Figure 12. Western Blots for AMPK and pAMPK in 24 week Transgenic Mice.



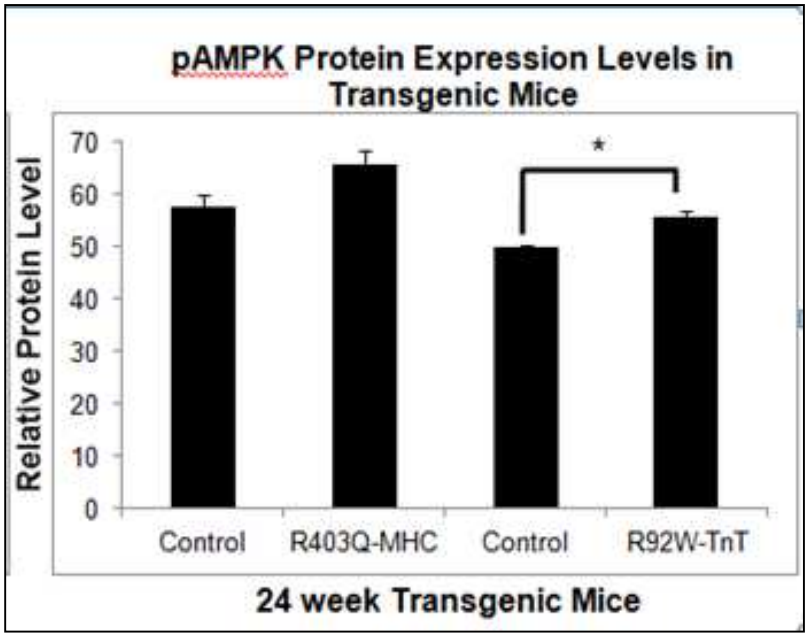
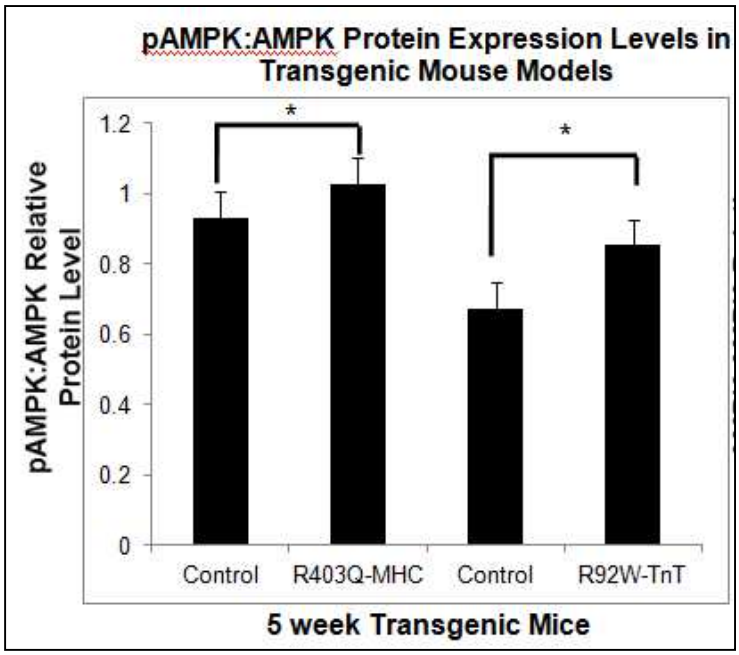


Figure 13. Western Blots for analysis of AMPK and pAMPK protein expressions in 24 week transgenic mice. 7 experiments yielded similar results and the figure above represent the best representation out of all collected experiments. Data are expressed as mean values ± standard error of mean.

*$P < 0.05$ relative to control. $n = 4$.

pAMPK and AMPK Protein Expression Ratio in 5 week and 24 week Transgenic Mice

In order to confirm that the R403Q mutation of MHC expresses high pAMPK due to low ATP causing energy stress, we measured the relative protein level ratio of pAMPK to AMPK in all three transgenic mice at 5 weeks and 24 weeks of age. As presumed, there is a 14% increase in pAMPK:AMPK ratio protein expression in R403Q-MHC transgenic mice of age 5 weeks to 24 weeks. There may be more energy stress at an early developing stage of disease than a later developed stage (Figure 14).



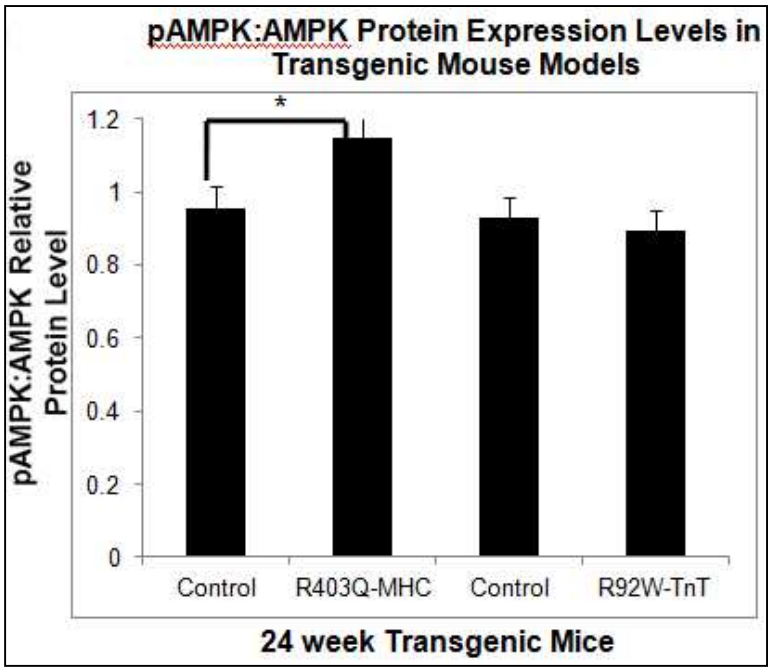


Figure 14. Analysis of pAMPK to AMPK ratio protein expressions in 5 week and 24 week transgenic mice. 7 experiments yielded similar results and the figure above represent the best representation out of all collected experiments. Data are expressed as mean values ± standard error of mean. *P < 0.05 relative to control. n = 4.

pCamKII Protein Expression Highest is in R92W-TnT 5 week Transgenic Mice

In order to confirm that the R92W mutation of TnT results in high calcium sensitivity, we tested anti-CamKII and anti-pCamKII antibodies in 5 week transgenic mice. There is no significant difference in total CamKII protein expression in the transgenic mice at 5 weeks. However, there is a 38% increase in pCamKII protein expression in R92W-TnT transgenic mice than R403Q-MHC transgenic mice, as there may be some degree of abnormal calcium handling, leading to HCM (Figure 15, Figure 16).

Key:

C: Control

M: R403Q-MHC

T: R92W-TnT

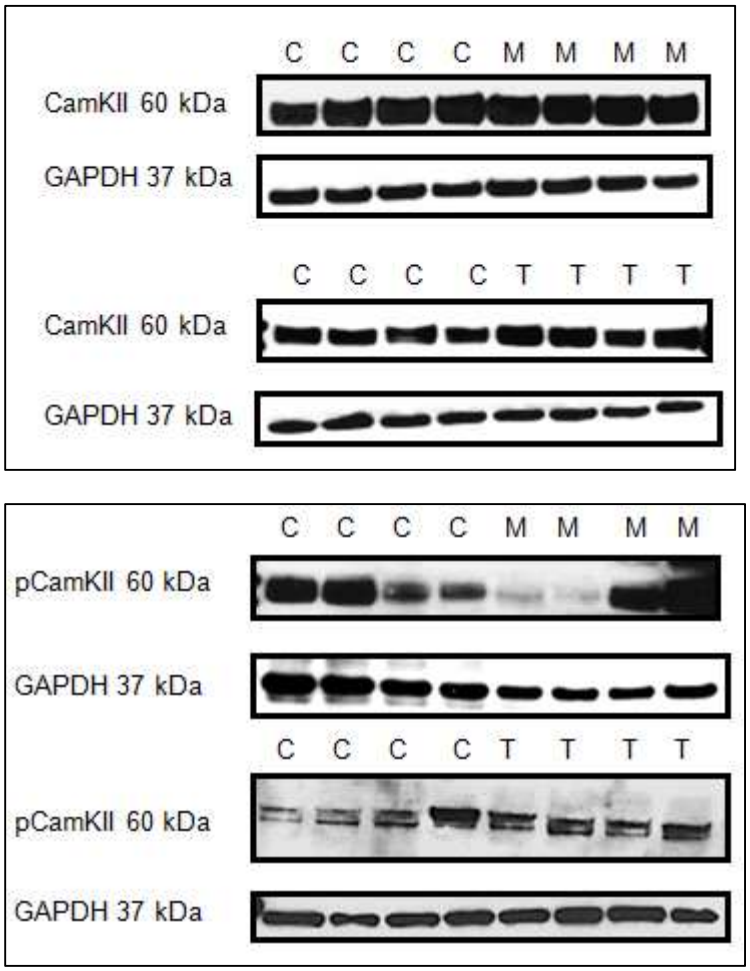
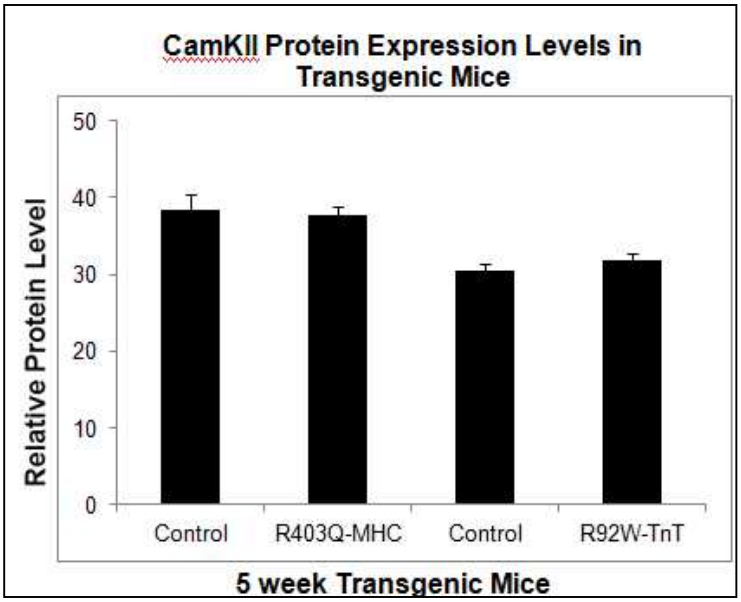


Figure 15. Western Blots for CamKII and pCamKII in 5 week Transgenic Mice.



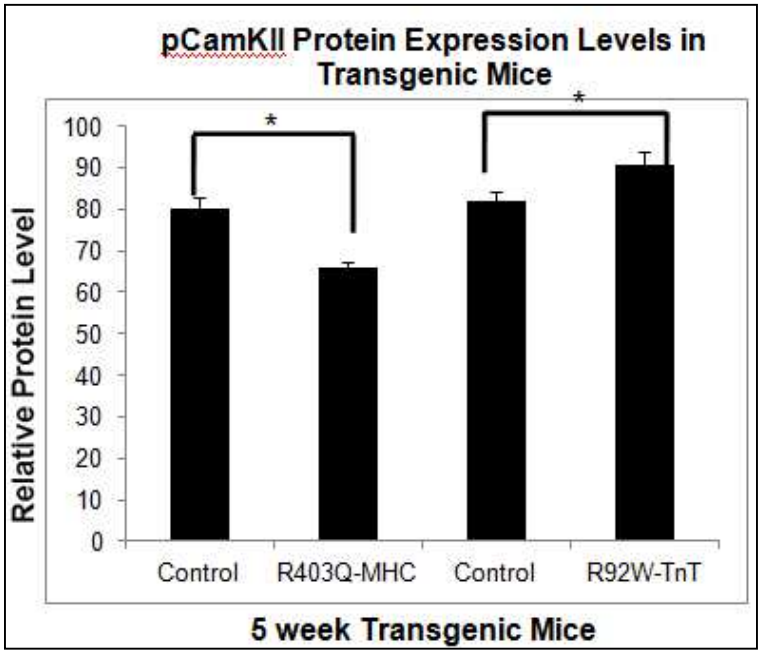


Figure 16. Western Blots for analysis of CamKII and pCamKII protein expressions in 5 week transgenic mice. 5 experiments yielded similar results and the figure above represent the best representation out of all collected experiments. Data are expressed as mean values ± standard error of mean.

*P < 0.05 relative to control. n = 4.

pCamKII Protein Expression is Not Expressed in 24 week Transgenic Mice

In order to confirm that the R92W mutation of TnT results in high calcium sensitivity, we tested anti-CamKII and anti-pCamKII antibodies in 24 week transgenic mice. There is no significant difference in total CamKII protein expression in the transgenic mice at 24 weeks. It is also surprising to see that there is pCamKII is not expressed in any of the transgenic mice. This could be the result of CamKII unphosphorylating and becoming auto inhibited following a decrease in calcium ions (Figure 17, Figure 18).

Key:

C: Control

M: R403Q-MHC

T: R92W-TnT

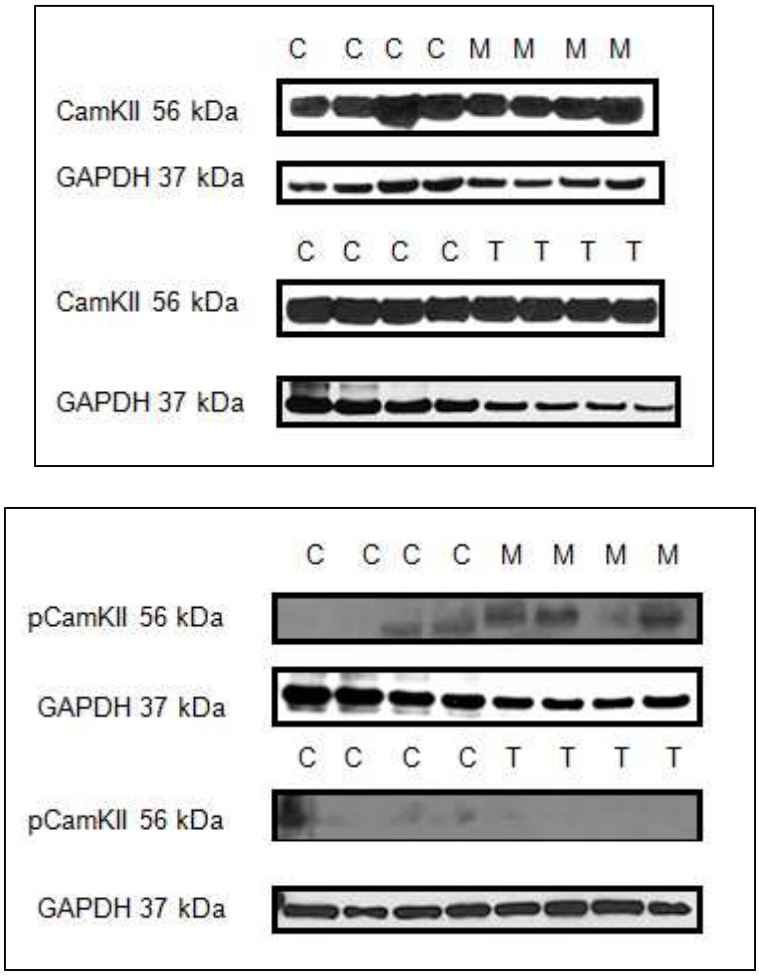


Figure 17. Western Blots for CamKII and pCamKII in 24 week Transgenic Mice.

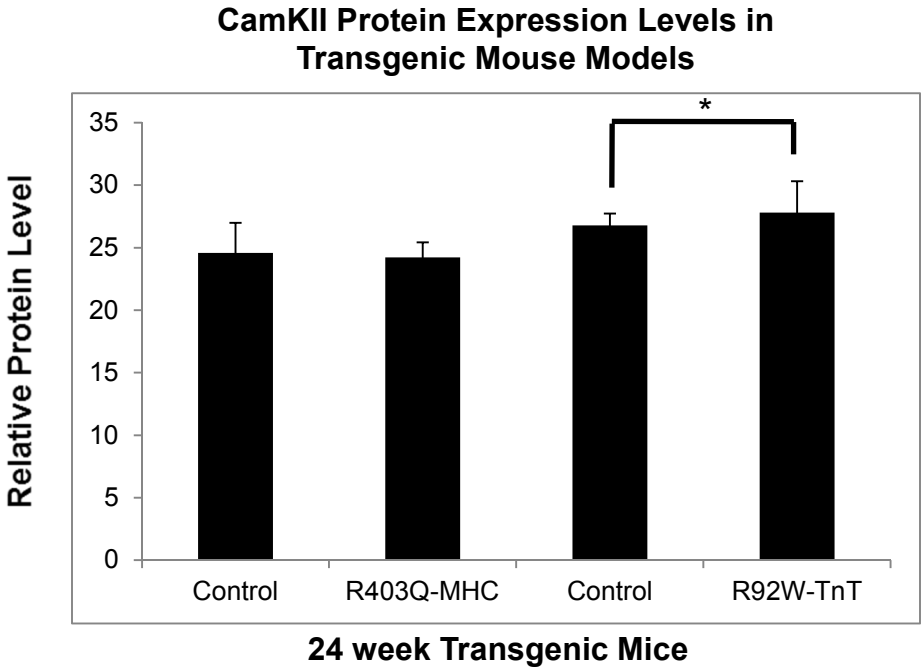


Figure 18. Western Blots for analysis of CamKII and pCamKII protein expressions in 24 week transgenic mice. 5 experiments yielded similar results and the figure above represent the best representation out of all collected experiments. Data are expressed as mean values ± standard error of mean.

*P < 0.05 relative to control. n = 4.

pMCU Protein Expression Highest is in R92W-TnT 5 week Transgenic Mice

In order to confirm that the R92W mutation of TnT results in abnormal calcium handling, we tested anti-MCU and anti-pMCU antibodies in 5 week transgenic mice. There is no significant difference in total MCU protein expression in the transgenic mice at 5 weeks. However, there is a 13% increase in pMCU protein expression in R92W-TnT transgenic mice than R403Q-MHC transgenic mice, as there may be an overload of calcium ions passing through the MCU as well as the presence of pCamKII (Figure 19, Figure 20).

Key:

C: Control

M: R403Q-MHC

T: R92W-TnT

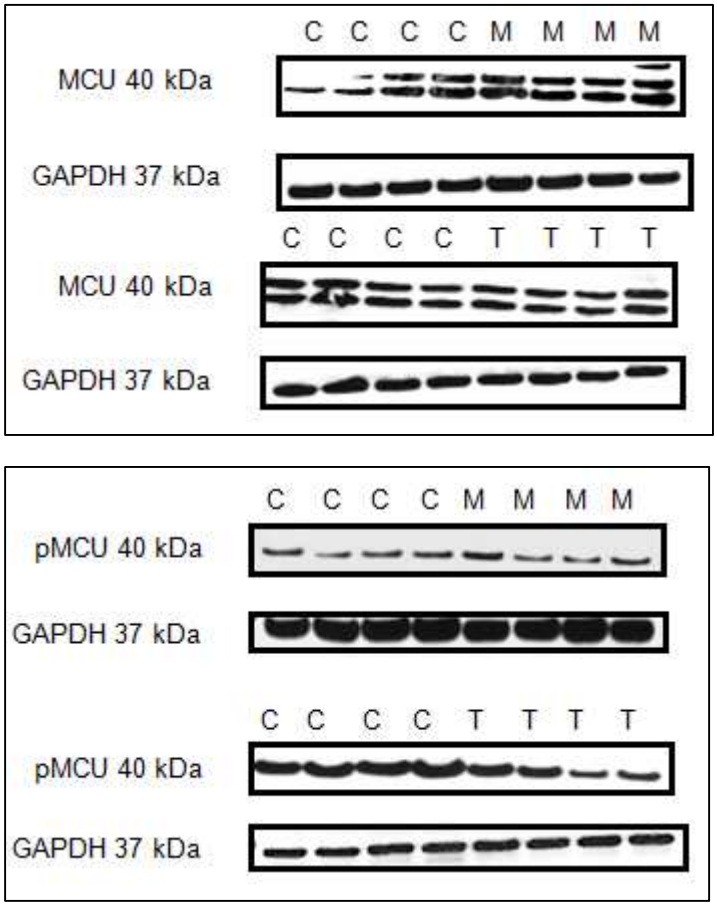
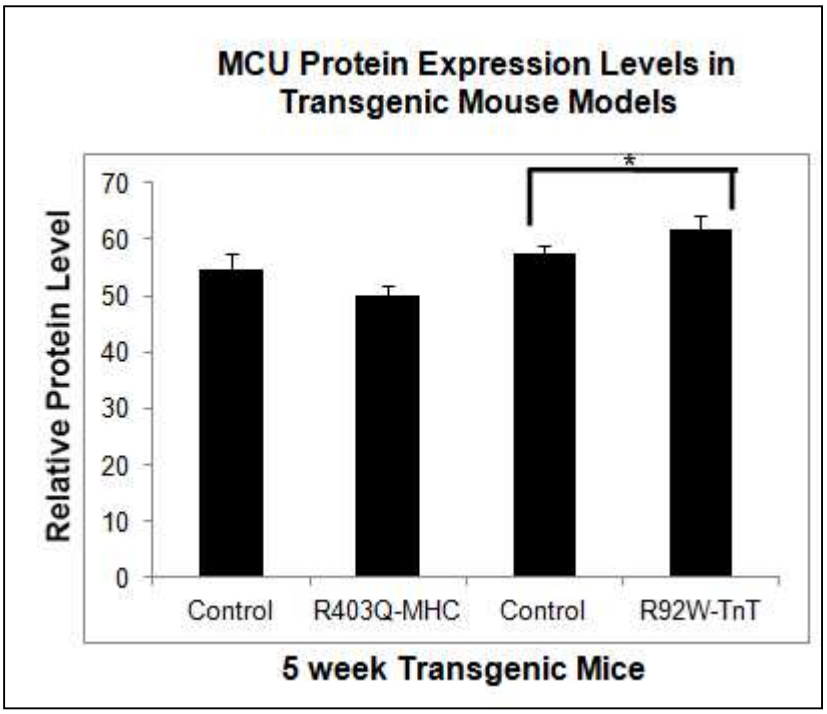


Figure 19. Western Blots for MCU and pMCU in 5 week Transgenic Mice.



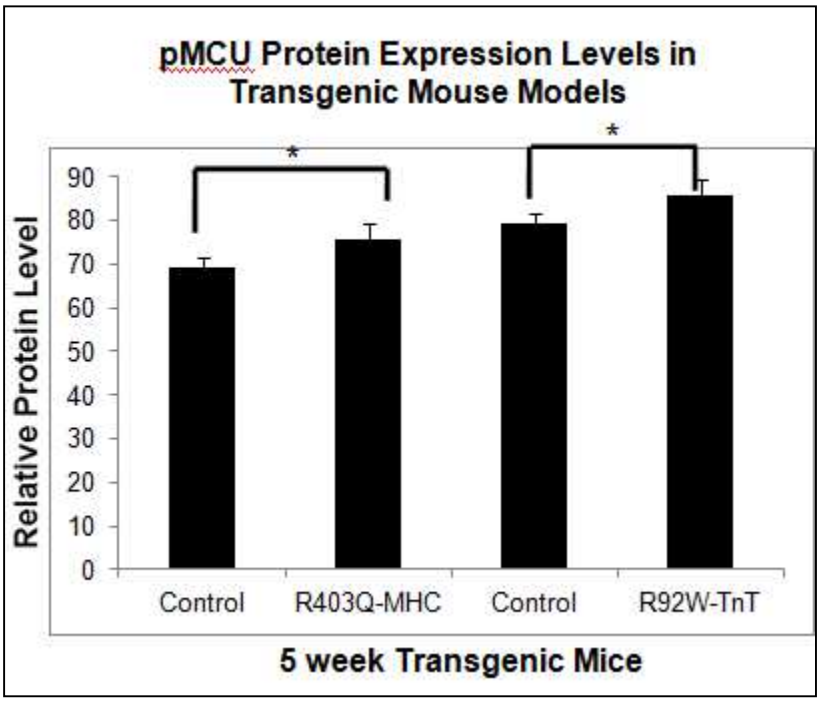


Figure 20. Western Blots for analysis of MCU and pMCU protein expressions in 5 week transgenic mice. 5 experiments yielded similar results and the figure above represent the best representation out of all collected experiments. Data are expressed as mean values ± standard error of mean. *$P < 0.05$ relative to control. $n = 4$.

pMCU Protein Expression Highest is in R92W-TnT 24 week Transgenic Mice

In order to confirm that the R92W mutation of TnT results in abnormal calcium handling, we tested anti-MCU and anti-pMCU antibodies in 24 week transgenic mice. There is no significant difference in total MCU protein expression in the transgenic mice at 24 weeks. However, there is a 28% increase in pMCU protein expression in R92W-TnT transgenic mice than R403Q-MHC transgemic mice. There is also a 14% decrease in pMCU protein expression in R92W-TnT transgenic mice from age 5 weeks to age 24 weeks, as there may be a greater increase in calcium ions passing through MCU as well as an over-regulated CamKII, which becomes activated at an earlier stage of disease (Figure 21, Figure 22).

Key:

C: Control

M: R403Q-MHC

T: R92W-TnT

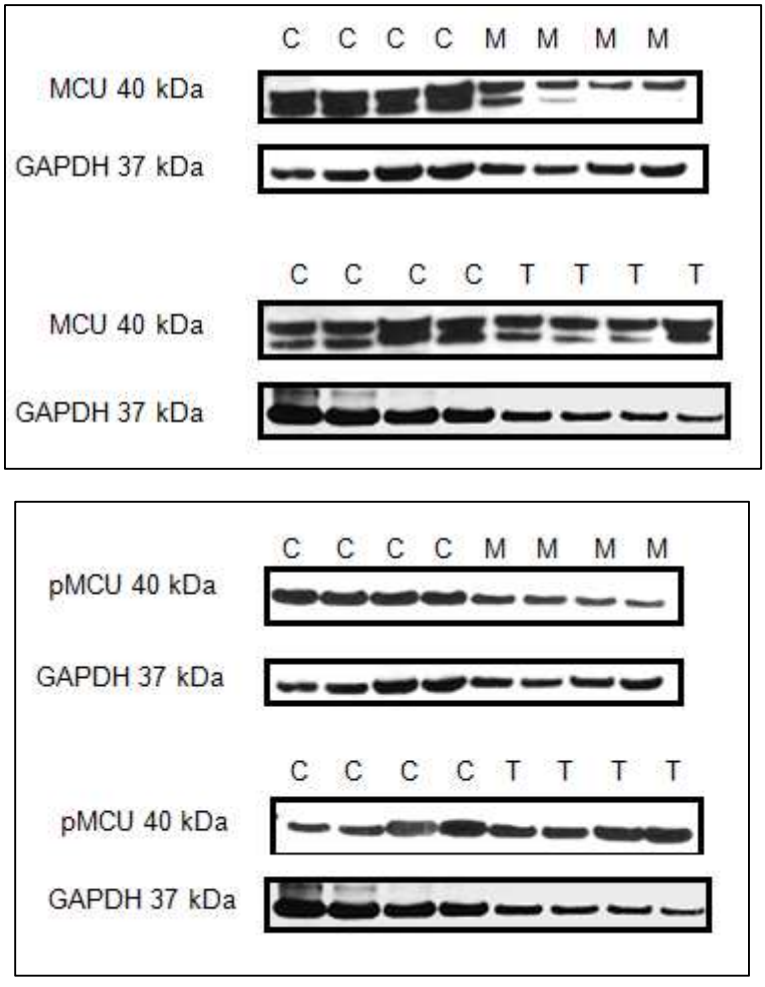
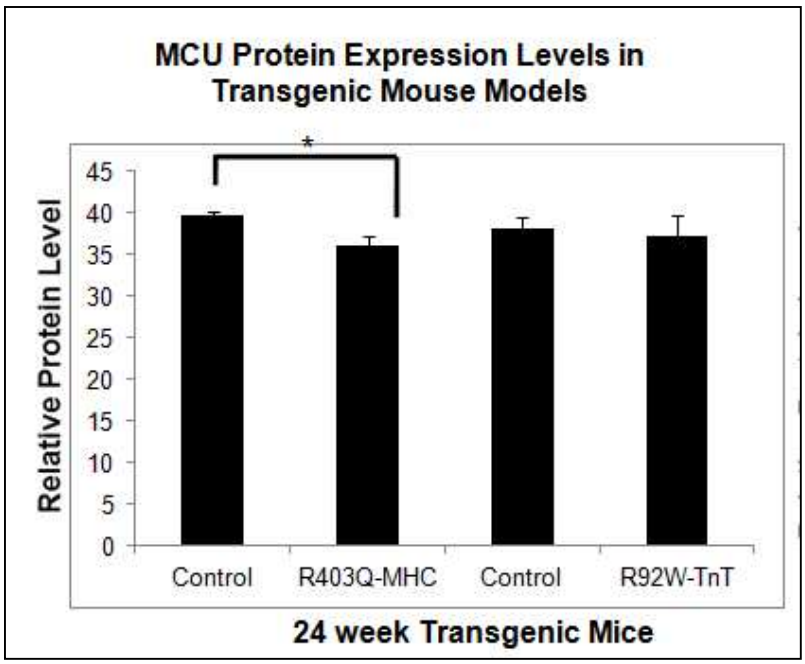


Figure 21. Western Blots for MCU and pMCU in 24 week Transgenic Mice.



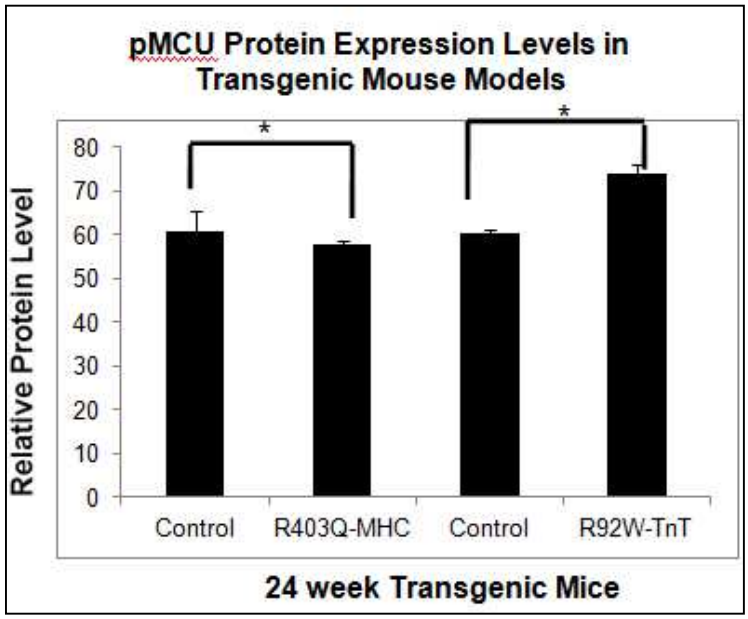


Figure 22. Western Blots for analysis of MCU and pMCU protein expressions in 24 week transgenic mice. 5 experiments yielded similar results and the figure above represent the best representation out of all collected experiments. Data are expressed as mean values ± standard error of mean.

*P < 0.05 relative to control. n = 4.

VII. Discussion

These findings have provided us confirmation that there are abnormalities in energy in the R403Q-MHC mutant and calcium handling in the R92W-TnT mutant. Based upon the results, protein expression differences exist in transgenic mice of R403Q-MHC, R92W-TnT, and control at age 5 weeks and age 24 weeks. Total target protein, AMPK, CamKII, and MCU were expressed equally in all three models. It was shown that phosphorylated AMPK protein had a 25% increase in protein expression in R403Q-MHC mice. We know that AMPK acts as an energy sensor and is activated in conditions of low ATP, when there is deficiency in energy. This confirms that the R403Q-MHC mutation causes energy stress and demands more ATP than there is supply. At both 5 weeks and 24 weeks, there is a 25% increase in protein expression in R403Q-MHC transgenic mice, however there is an 18% increase in protein expression from 5 weeks to 24 weeks in R403Q-MHC transgenic mice. This presumes that there is more energy stress due to conditions of low ATP at a hypertrophic stage of disease than there is at 5 weeks, pre hypertrophic stage of disease.

Phosphorylated CamKII protein had a 38% increase in protein expression in R92W-TnT transgenic mice than R403Q-MHC transgenic mice at 5 weeks. We know that CamKII becomes activated when there is high calcium ion present, so this confirms that the R92W-TnT mutant results in high calcium sensitivity. It is surprising to see that pCamKII is not expressed in any of the transgenic mice at 24 weeks. This may be due to the fact that there is a decrease in calcium ions, which in turn can inactivate CamKII and become auto inhibited again. As HCM disease progresses into the hypertrophic stage, it could be possible that there becomes low calcium sensitivity.

Phosphorylated MCU protein had a 13% increase in protein expression in R92W-TnT transgenic mice than R403Q-MHC transgenic mice at 5 weeks, and a 28% increase in protein expression at 24 weeks. We know that MCU modulates the influx of calcium ions from the cytoplasm and into the mitochondria. There was a 14% decrease in protein expression of pMCU in R92W-TnT transgenic mice from age 24 weeks to age 5 weeks. This indicates that MCU is activated due to unregulated influx of calcium ions. This indicates that there could be a higher influx of calcium ions at a pre-hypertrophic stage than a hypertrophic stage. It is surprising to see that pMCU is expressed in 24 week R92W-TnT transgenic mice even though pCamKII is not expressed in 24 week R92W-TnT transgenic mice. This can lead us to investigate further and gain additional insight.

Studying underlying mechanisms and pathogenesis of human disease is very complex, since all areas have to be acknowledged to fully understand the disease. Studying AMPK, CamKII, and MCU protein expression from whole heart of transgenic mice models of 5 weeks and 24 weeks of age containing R403Q-MHC mutation and R92W-TnT mutation only covered a small portion. There are several additional aspects that need to be studied in order to provide proper understanding of energy and calcium disruption. Future directions include studying protein expression from isolated mitochondria, studying other target proteins that play a role in energy and calcium handling, as well as studying other post translational modifications in addition to phosphorylation.

The vital role of mitochondria as providers of energy, in the form of ATP, for the high demands of cardiac contractility is well recognized [137]. Impairment of mitochondrial function and morphological disorganization has been reported in mouse

models and in HCM patients. However, a systematic study of mitochondrial function is lacking as it is unclear whether mitochondrial abnormalities are a primary event or secondary event in HCM [138-140].

Since mitochondria occupy more than 30% of the cardio myocyte volume, there should be strong energy signaling pathways existing to ensure energy utilization [141]. The nature and function of these signals are still unclear, however ATP and calcium changes have all been considered to play a role. Their relative contribution to energy metabolism homeostasis, if any will depend on the metabolic conditions the heart has to respond to [142-144]. Isolated mitochondria allow assessment of the specific activity of mitochondria. The low yield of cardiac mitochondria isolation, the selection bias during isolation and the changes in mitochondrial regulation limit the extrapolation of isolated mitochondria studies to the living heart. It has been shown that errors in these events either by protein mutation or deficiency can result in a protein not reaching its final destination, ultimately leading to a disease state in humans often associated with characteristic features of HCM [145, 146].

Mitochondria are the major site of energy production in the cell, thus it is not surprising that energy-dependent tissues such as the heart are particularly sensitive to mitochondrial dysfunction. Accumulating evidence suggests that mitochondria dysfunction, reflected in the structure, function, and number of mitochondria within cardiac myocytes, leads to decrease in energy production, loss of myocyte contractility, and increased cell death during the development of heart failure [147]. However, despite extensive animal studies, the fundamental mechanisms behind mitochondria dysfunction contributing to the development and progression of left ventricular failure in humans

have not been fully elucidated [148]. Studying protein expression in proteins isolated from mitochondria will allow us to gain better understanding of its signaling mechanisms during different stages of the disease as some proteins are mostly within the mitochondria and not cytoplasm [149].

It is important to study various proteins that play a role in energy and calcium homeostasis. Beyond focusing on AMPK, CamKII, and MCU protein expression, protein expression of Acetyl-CoA carboxylase (ACC) in protein from heart and isolated mitochondria is just as important. It was founded that there is a reciprocal relationship between AMPK activity and ACC, providing a mechanism for the acceleration of fatty acid [78]. Phosphorylation of ACC by AMPK has been well documented and although controversial, it has been suggested that it is also a phosphorylation target of AMPK. The oxidation of fatty acids in muscle is an important source of energy and growing evidence supports a key role for AMPK in the regulation of ACC [150]. Inhibition of ACC reduces the level of malonyl CoA. Oxidized-CamKII also plays a role in calcium handling [151]. When CamKII becomes constitutively activated, it becomes oxidized and causes an unregulated influx of calcium ions to pass through the MCU. This causes an overload of calcium ions and decrease in energy production, eventually leading to hypertrophy. By studying oxidized-CamKII, we would also be able to study an additional post translational modification, oxidation. Oxidants can generate stable post translational modifications on proteins, as it can remove reactive oxygen species in mitochondria [152].

VIII. Conclusion

Findings have shown that there are protein expression differences in transgenic mice of R403Q-MHC and R92W-TnT mutations of HCM, both at 5 weeks of age and 24 weeks of age. We can confirm that the R403Q-MHC mutation results in abnormal energy caused by conditions of low ATP, while the R92W-TnT mutation results in high calcium sensitivity by an increase of calcium ions. Results confirm that there is disruption in energy and calcium handling due to the respective mutations compared to that of control. Although there is energy stress in transgenic mice of age 5 weeks, a pre hypertrophic stage, there is slightly more energy stress in transgenic mice of age 24 weeks, a hypertrophic stage as pAMPK had an 18% increase in expression in the R403Q-MHC transgenic mice of age 24 weeks than 5 weeks. This holds true for abnormal calcium handling as pCamKII had a 38% increase in expression in R92W-TnT transgenic mice than R403Q-MHC transgenic mice of age 5 weeks. This result was surprising because the protein that modulates calcium ions, MCU was activated in R92W-TnT transgenic mice of age 5 weeks and 24 weeks, even when pCamKII was not expressed in transgenic mice of 24 weeks.

IX. References

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