INVESTIGATIONS INTO THE EFFECTS OF ALTERED TITIN ISOFORM
EXPRESSION IN STRIATED MUSCLE

By

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“The heart of the prudent acquires knowledge, and the ear of the wise seeks knowledge.” (Proverbs 18:15)
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INVESTIGATIONS INTO THE EFFECTS OF ALTERED TITIN ISOFORM EXPRESSION IN STRIATED MUSCLE

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Under the Supervision of Professor Marion L. Greaser

At the University of Wisconsin-Madison

As a critical regulator of striated muscle physiology, the giant protein titin performs a variety of functions within the sarcomere. These roles are modulated through the expression of several titin isoforms, which differ throughout the body. Cardiac muscle expresses smaller, stiffer N2B; larger, compliant N2BA; or a combination, depending on the species, location, and developmental stage. A recently characterized rat mutation (Hm) was found to have an inoperative Rbm20 gene, the splicing factor which regulates titin isoform expression. Without the splicing action of Rbm20, Hm rats possess more middle Ig and PEVK regions, resulting in a new titin isoform. Hm rats express a giant N2BA titin (N2BA-G) molecule, but adult wild type rats present predominantly N2B titin. In an investigation into the sarcomeric profile of Hm rats, it was found that titin is the major protein affected, while other proteins were unchanged in this rat mutation model. However, N2BA-G titin phosphorylation levels were similar to that of wild type titin. Thyroid hormone levels were manipulated through drug intervention, and myosin heavy chain proportions were altered. Wild type rat titin shifted toward a more compliant isoform pattern in both the hyperthyroid and hypothyroid states. In contrast, Hm rats continued to express only N2BA-G. Finally, familial hypercholesterolemic (FH) swine, which demonstrate ischemia and atherosclerosis, were
analyzed for titin isoform expression. Ischemia is known to increase compliant titin presence, and FH pigs provide a useful model for studying the human disease state.

Following limit-feeding of FH pigs, a novel titin isoform was discovered (N2BA-NE), which was not present in conventional swine. Rbm20 levels were similar across all FH treatment groups, making it possible that Rbm20 functions differently in swine titin alternative splicing. FH mini-swine were generated, and they were also found to express NE titin. Investigations into the titin splicing hotspots (middle Ig domains) showed similar expression to that of conventional pigs, but the presence of additional PEVK exons may be responsible for NE titin expression. Titin isoform expression is influenced by a number of internal and external factors, which can lead to multiple changes to the protein and the sarcomere at-large.

Approved: ________________________

Date: ________________________
INTRODUCTION

Titin is the largest known protein at approximately 3 MDa in size, depending on the isoform expressed. In order for proper function to occur within the cardiac and skeletal muscle system, titin isoform expression must be precisely modulated. When titin expression is disrupted and inappropriate isoforms are present, various cardiomyopathies will result. To aid in the understanding of this process, we have utilized a mutant rat model which does not present a normal titin isoform pattern; rather, only a single giant titin isoform is manifested. This rat mutation leads to ventricular dilation and other negative effects. In the present study, we investigated the effects of this mutation on cardiac physiology, as well as a hypercholesterolemic pig model which influences titin isoform expression.

Objectives

The first objective was to study the effects of the rat model on titin expression and phosphorylation, and to determine whether this mutation has any impact on other myofibrillar proteins. The second objective of this thesis was to examine the relationship between thyroid hormone levels and titin expression in the wild type rat and the mutant rat, to see if thyroid hormone could influence the titin profile. The last objective involved the characterization of a hypercholesterolemic swine model, which undergoes ischemia, a dysfunction which is known to alter titin expression.

Thesis Organization
This thesis is comprised of five chapters. The first chapter is the literature review, which encompasses prior knowledge of titin, the rat mutation, and various titin disease states. Chapter two is an investigation into the effects of this mutation on titin phosphorylation and other myofibrillar proteins. Chapter three pertains to the influence which thyroid hormone has on titin and the cardiovascular system in normal and mutant rats. Chapter four describes the familial hypercholesterolemic swine model and its effects upon titin. Lastly, chapter five provides a general summary of the results presented herein.
CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

The giant myofibrillar protein titin, also referred to as connectin, is the largest known protein and performs a wide variety of roles in the striated muscle sarcomere. These functions are fulfilled in a very specific manner, dictated by the expression pattern of titin. Depending on the developmental stage and animal species, titin can exist as an array of alternative splice variants to properly confer its effects upon the sarcomere. A larger, more compliant titin isoform will result in a phenotype with lower resting tension, while the smaller and stiffer isoforms predominate in adult animals and exhibit high passive tension. A recently discovered mutation has been described [Greaser et al., 2008] which ablates titin alternative splicing, leading to a 3.83 MDa giant cardiac titin isoform. This rat model has a partial deletion of the Rbm20 gene, a splicing factor that controls titin isoform expression [Guo et al., 2012]. The dysregulation of titin splicing will lead to various disease states, including dilated cardiomyopathy. In addition to Rbm20, titin’s expression profile can be affected by other physiological alterations. Thyroid hormone has been shown to affect titin expression, and changes to its levels can drastically modify the cardiovascular system.

1.2 Striated muscle sarcomere

The striated muscle sarcomere is an excellent prototype showing the precision and order of a biological system. Since the 1950’s, it has been demonstrated that the cross-
striations of actin and myosin endow contractile ability upon the sarcomere [Huxley and Hanson, 1954]. However, the sarcomeric periodicity is maintained by its multiple filament systems: the thick, thin, and titin filaments. Most important is the transient binding of the myosin thick filament to the actin thin filament, commonly referred to as the sliding filament model [Huxley and Niedergerke, 1954; Huxley and Hanson, 1954]. These systems demonstrate a large number of protein-protein interactions to preserve the integrity of the myofilament. With a multitude of interactions, force generation is tightly conserved and regulated [Clark et al., 2002].

Myosin, the major protein of the thick filament, will bind to titin and form a stabilized structure. In order to specify contractile speed and endurance, several myosin isoforms are expressed in striated muscle. A shift in the isoform composition will strongly affect the muscle fiber’s shortening velocity [Shroff and Motz, 1989]. The isoform classification is dictated by the type of heavy chain the myosin possesses, and cardiac and skeletal muscles possess unique isoform combinations.

In cardiac tissue, myosin heavy chain (MHC) content is binary: α-MHC and β-MHC are the only existing isoforms. In the prenatal ventricle, α-MHC dominates, and subsequent development shows a gradual suppression of this isoform [Lompre et al., 1984]. The ATPase activity of α-MHC is higher than its companion, and α-MHC predominates in young mammals. As aging occurs, β-MHC gradually replaces the other isoform, until it is the primary isoform expressed [Pope et al., 1980]. Larger mammals (i.e., pigs) will express larger proportions of β-MHC, while rodent ventricular tissue is comprised heavily of α-MHC [Clark et al., 1982]. Adult human ventricles also express
predominantly β-MHC (approximately 95%), and in diseases such as dilated cardiomyopathy, β-MHC proportions will increase even further [Miyata et al., 2000; Reiser et al., 2001]. Cardiac MHC expression is very sensitive to external stimuli, which will induce changes in the MHC ratio. Chronic exercise [Rupp, 1989] and cardiac hypertrophy [Kinugawa et al., 2001] will both increase α-MHC levels in the heart. Additionally, detrimental effects such as cardiomyopathy, heart failure, and thyroid hormone levels can alter normal MHC expression [Everett et al., 1984; Hoh et al., 1978; Miyata et al., 2000; Reiser et al., 2001].

Adult skeletal muscle can express four distinct MHC isoforms, which are listed by slowest- to fastest-contracting: type I, IIA, IIX, and IIB. Typically, skeletal muscle contains at least two of these isoforms depending on the contractile needs of the muscle [Lu et al., 1999]. While the protein sequences of these isoforms are very similar, they differ in their actin-binding and ATPase domains, which account for the differences in contractile speed and metabolic nature [Weiss et al., 1999]. The MHC isoform profile in skeletal muscle acts as a continuum, and these isoform are able to transition like cardiac MHC. Changes in the mechanical, neural, and hormonal state of the animal will induce isoform shifts toward either a slower or faster phenotype. For example, chronic exercise will incite a shift toward the slower, metabolically oxidative fiber types, while disuse will induce atrophy and a shift to the faster, more glycolytic phenotype [Staron and Pette, 1993]. Just as titin is affected by changes to the thyroid hormone level, MHC isoform profile will also be altered by drug treatment in both types of striated muscle [Fitzsimons et al., 1990].
Prior to its placement in the muscle ultrastructure, titin must be appropriately spliced to perform its precise function in the sarcomere. Regardless of its location in the body, titin can present itself as a wide array of splice variants [Guo et al., 2010]. The titin isoform nomenclature is: skeletal muscle only expresses the N2A isoform, while cardiac muscle can express an N2B or an N2BA isoform, should the latter contain an N2A unique sequence in addition to an N2B sequence. Depending on the animal, developmental range, and disease state, the expression pattern of these isoforms is controlled precisely to prevent a pathological state [Neagoe et al., 2003].

1.3 Titin structure and function

Due to its immense size, titin was not discovered until the mid-1970’s [Maruyama, 1976; Wang et al., 1979]. Titin is made up of 363 exons and 38,138 amino acid residues in humans, equating to a potential total size of approximately 4.2 MDa [Bang et al., 2001]. Alternative splicing plays a major role in titin expression, resulting in a titin molecule that is between 3.0 and 3.7 MDa in cardiac and skeletal muscle [Freiburg et al., 2000]. Until recently, it was difficult to ascertain the specific isoform pattern of titin, but SDS-agarose gel electrophoresis has allowed for accurate measurement of titin’s expression [Warren et al., 2003b]. As a protein which spans an entire half-sarcomere (Fig. 1.1), titin’s structure is facilitated so that it may confer a variety of functions upon the muscle ultrastructure.

The tightly-regulated environment of the sarcomere has been stated above, but specific regions of titin bind to specific proteins to support the myofibril. Along most
points of its length, titin has at least one binding partner [Linke, 2008]. At the Z-disk, titin’s N-terminus will interact with a number of proteins, including telethonin, obscurin, and alpha-actinin to stabilize the structure [Gregorio et al., 1998; Young et al., 2001; Young et al., 1998]. There are a series of sequence motifs and Ig domains that allow formation of these complexes, and signaling pathways may act through titin in this area.

The A-band region is highly conserved across muscle types and species, in which Ig and fibronectin domains are arranged in super-repeating sequences [Labeit and Kolmerer, 1995]. Because of this repetitive structure, these regions provide a strong interaction between titin and the thick filament, conferring stabilization in the middle of the sarcomere. In a similar way, titin’s C-terminus helps to sustain the M-line structure by binding to M-protein and myomesin, as well as myosin [Obermann et al., 1997].

Titin termini are pivotal to the myofibrillar sense and stretch response. Z-disk (N-terminal) titin provides structural and mechanical functions by binding to multiple proteins at the ends of the sarcomere, and the telethonin-titin complex anchors the Z-disk. Also, through titin’s interaction with α-actinin, a large cross-linked network is formed near the Z-disk to provide more stability to the muscle ultrastructure. Cytoskeletal support is aided by obscurin’s grasping of titin to confer additional support [Linke, 2008].

At the other end of the titin molecule, its function at the C-terminus has been proven vital by experimentation. Close to the M-line, titin possesses a kinase domain, and when this was deleted, sarcomeres could not form [Musa et al., 2006]. Just as its N-terminus, titin’s COOH end acts as a stretch-sensor. The titin kinase’s target substrate is telethonin, but its specific role is unspecified [Linke, 2008]. Because sarcomeres cannot be formed
without a functional titin kinase, its activation is vital to the developing myocyte in creating a healthy M-line. The mature sarcomere’s stability hinges on the titin-myosin-myomesin complex, which also forms near the M-line. Myomesin acts as a cross-linker between C-terminal titin and myosin, creating an elastic binding to allow for M-line flexibility [Lange et al., 2006]. Antibody injection experiments performed by Wang et al. [Wang et al., 1998] showed that when titin’s C-terminus is blocked with antibody, M-protein and myomesin cannot be incorporated, and the M-line structure could not be assembled. In addition to simple protein-protein interactions, the titin kinase domain activity is critical during myofibrillogenesis, which, when removed, prevents sarcomeres from being produced. This condition also renders the association of titin to myosin and myomesin impossible, strongly showing that M-line titin is important for muscle ultrastructure as well as in signaling pathways [Miller et al., 2003]. When the M-line sequence is deleted, embryonic lethality results. Experiments using a skeletal muscle-specific deletion of titin kinase showed an extreme phenotype: animals lived only until around 5 weeks of age and were subject to progressive muscle weakness [Gotthardt et al., 2003]. Titin’s extreme size and length permit it to participate in many roles throughout the sarcomere, but it is the I-band region which has been shown to be a hotbed of activity.

I-band titin has been the subject of many areas of study, due to the fact that it possesses a wide variety of splice variants to modulate its extensibility and functionality in muscle tissue [Anderson and Granzier, 2012]. Titin is responsible for maintaining passive tension in striated muscle, which explains its flexibility and extensible abilities. These domains are found within titin I-band region, and they include tandem
immunoglobulin-like (Ig) domains, a PEVK region which is rich in proline, glutamate, valine, and lysine (P, E, V, and K, respectively), and N2-unique sequences. These I-band domains function as stretch-sensors and can extend and collapse as needed. Circular dichroism spectra of titin PEVK have shown that this region is highly disordered [Duan et al., 2006] and is thus capable of extending and contracting. When the sarcomere is relaxed and unstretched, titin’s extensible domains are collapsed. Upon sarcomere stretch, Ig domains will be straightened, and, as sarcomere length extends even further, PEVK segments will become extended as well [Linke et al., 1996]. At the upper limits of physiological sarcomere length, the N2-unique sequences will lengthen [Watanabe et al., 2002], making N2B a third adjustable spring in this titin region in cardiac muscle [Helmes et al., 1999].

Across mammalian species, titin molecules in striated muscle are classified based on their N2-unique domain expression. Myocardial tissue can express the N2B (approximately 3.0 MDa) or the N2BA (between 3.2 and 3.7 MDa) isoform in differing proportions, depending on species and developmental stage. The N2BA isoform will decrease in proportion during development, and the N2B isoform will predominate in the mature animal [Cazorla et al., 2000; Freiburg et al., 2000; Labeit and Kolmerer, 1995]. N2B size remains consistent throughout life, while different subtypes of N2BA will be present based on their expression pattern of the middle Ig and PEVK domains. In rat fetal tissue the N2BA bands will appear at 3.71 MDa, but at adulthood the N2BA isoforms will be 3.22 and 3.39 MDa in cardiac titin [Warren et al., 2004]. Prior to birth, mammalian fetal heart compliance is blunted by constraint outside the heart, so in order
to maintain a lower passive tension, high proportions of N2BA are present. After birth, N2B isoform is upregulated, limiting extensibility and preventing overextension. Increased collagen expression provides an additional stabilizing role [Grant et al., 2001]. In cases where this transition does not occur, disease states such as dilated cardiomyopathy may result [Wu et al., 2002]. These changes will be discussed further. The normal titin switching from N2BA-predomination to one of high levels of N2B is crucial to optimize cardiac function.

Further details regarding the N2B unique domain and its roles have been revealed. In addition to the extensible nature of the Ig and PEVK regions of the I-band, N2B phosphorylation state will contribute to passive tension maintenance as well. Both protein kinase A (PKA) [Yamasaki et al., 2002] and PKG [Kruger et al., 2009] have been shown to phosphorylate the Ser469 residue of the N2B domain, which leads to decreased passive tension in the heart. This decrease was more pronounced at shortened sarcomere length [Fukuda et al., 2005], where the Ig and PEVK do not have a large contribution to passive force. Conversely, N2B hypophosphorylation in a heart failure model demonstrated increased tension [Borbely et al., 2009]. The species and time differences of N2B isoform expression have been discussed, but when N2B is expressed in higher proportions, the extent of passive force decrease is enhanced. This phosphorylation event can also occur in hearts containing an N2BA titin [Fukuda et al., 2005]. In a similar way, PKC has the ability to phosphorylate titin, although differences are evident. Two PKC phosphorylation sites exist within the PEVK domain of N2B titin, and hyperphosphorylation of both serine residues showed an increase in passive stiffness.
[Hidalgo et al., 2009]. The I-band’s three major domains act in concert to provide passive force and tension, however. N2B unique domain knockout mice demonstrated increased passive tension and reduced slack sarcomere length, with the Ig and PEVK regions showing increased extension at slack length [Radke et al., 2007]. Subsequent studies on the N2B knockouts showed increased length-dependent activation and active tension; Frank-Starling mechanism effects were more pronounced, as well [Lee et al., 2010]. The titin I-band region is an area of the molecule worth a great deal of attention because of its roles in passive force determination and alternative splicing.

In conjunction with its role in passive force determination, titin is critical to diastolic function in myocardial tissue as well. It begins in early diastole, as soon as the transition from systole occurs [Helmes et al., 1996], to facilitate left ventricle chamber filling. Titin is a main contributor to the diastolic properties of the ventricle and ventricular filling [Bell et al., 2000]. Because of this, titin plays an important role in the Frank-Starling mechanism by determining the sarcomere stretch needed for ventricular filling [LeWinter et al., 2007]. The Frank-Starling law is controlled partially by the titin molecule, which is defined by sarcomere length dependence on myocardial activation. That is, because titin controls ventricular filling, this in turn increases ventricular volume, leading to increased force necessary to contract and expel blood from the left ventricle [Katz, 2002]. When pCa-force curves are created at differing passive forces, sarcomere length has a larger impact at higher levels of passive force [Cazorla et al., 2001]. Titin isoform expression profile is important in this relationship. Different levels of passive force are found in differential isoform expressions [Fukuda et al., 2003], showing that
titin has a role in length-dependent activation of the Frank-Starling Law [Fukuda et al., 2001]. Therefore, the role of titin in determining passive force requires that titin is responsible for filling and expulsion of blood from the left ventricle. Titin is also an important protein which modulates active force generation in myocardial tissue [Cazorla et al., 2001]. The N2BA and N2B expression in myocardium is important to preserve proper ventricular function, and disruptions to this expression pattern can often be severe.

In contrast to cardiac tissue, skeletal muscle only expresses the N2A titin isoform and does not possess an N2B unique domain, but its size varies across muscles and species [Prado et al., 2005]. Depending on the muscle, rat skeletal titin can be smaller, such as 3.29 MDa in the tibialis anterior, or larger, up to 3.66 MDa in the external oblique. Experiments detailing the rate and quantity of skeletal titin splicing have been investigated, and in mice and rabbits, the soleus was found to transition very rapidly postnatal, while the tibialis anterior took a longer amount of time to shift to a smaller isoform [Ottenheijm et al., 2009]. Skeletal muscle titin is spliced in a similar fashion as cardiac titin, in that prenatal and perinatal rats will express a 3.70 MDa isoform across all muscles, but aging will decrease titin sizes to varying degrees [Li et al., 2012]. Further, the splicing changes may not manifest themselves until after 20 days postnatal, which is slower than the transition observed in cardiac titin.

While similar in most other respects, skeletal and cardiac titin differs in the I-band region: N2B titin will express less Ig domains and a shortened PEVK, while the N2A will present more Ig domains and a longer PEVK region [Freiburg et al., 2000]. The PEVK region contains two types of sequences: so-called 28 residue PPAK repeats and poly E
repeats (rich in glutamic acid residues) [Greaser, 2001]. The 28 residue repeats may appear as seven module superrepeating structures in certain regions [Greaser, 2000; Gutierrez-Cruz et al., 2001]. However, skeletal muscle titin also undergoes alternative splicing in its extensible I-band region leading to different titin sizes [Freiburg et al., 2000], and changes occur mainly in the I-band’s middle Ig and PEVK domains [Labeit and Kolmerer, 1995]. The Ig domains will extend first, followed by the PEVK regions in a hierarchical manner to provide local flexibility and fine motor control of skeletal muscle. PEVK domain provides two types of extensibility: at shorter sarcomere lengths it acts as a loose, compliant spring, but develops stiffness at higher extensions [Linke et al., 1998]. The N-terminal PEVK region will extend first, followed by the C-terminal end, and lastly, the middle PEVK [Nagy et al., 2005]. Since isoform differences are mainly seen in this extensible region, passive stiffness will vary depending on the size. Neonatal skeletal muscle will have lower passive tension which will increase with age as skeletal muscle becomes stiffer [Ottenheijm et al., 2009]. As in cardiac tissue, skeletal muscle titin has been shown to be at least partially responsible for passive tension development. Early titin research by Horowits et al. showed that when skeletal muscle titin function was disrupted with irradiation, passive tension generation was greatly reduced. The authors also found that titin keeps the skeletal muscle thick filament aligned in the sarcomere’s center [Horowits et al., 1986]. Later research has indicated that while skeletal muscle titin is important in passive force development, the relationship between its size and the sarcomere’s passive tension was not highly correlated, and a combination of extramyofibrillar and titin-based stiffness will dictate skeletal muscle
resting tension [Prado et al., 2005]. Skeletal muscle titin performs the same roles as cardiac titin, but the isoform expression is slightly different in the I-band.

1.4 Rbm20 and rat mutation

Over the last decade, a novel rat mutation model has been studied and the source of the mutation elucidated. Initially, this model was discovered when some rats did not undergo the normal developmental titin isoform shift, and two larger isoforms were observed in what was later revealed to be the heterozygous genotype [Greaser et al., 2005]. The heterozygotes demonstrated an intermediate effect of this mutation between that of the wild type and homozygous condition. Heterozygotes presented N2BA titins of larger size (3.5 MDa) relative to normal rats, and further breeding led to the generation of a homozygous phenotype.

Homozygotes showed the novel N2BA-G isoform (3.83 MDa), which, rather than being alternatively spliced to smaller isoforms as in wild type rats, persisted through adulthood [Greaser et al., 2008]. As a result of this condition, homozygous cardiovascular function is severely altered. Because titin is a major contributor to passive tension, the longer I-band regions of the homozygote [Li et al., 2012] will correlate with lower passive tension [Greaser et al., 2008]. Muscles with larger titin sizes generally have lower passive tension, while those containing smaller, stiffer titins will have higher passive stiffness [Anderson and Granzier, 2012; Granzier and Labeit, 2002]. Homozygous rats’ cardiomyocytes could be stretched to more than 100% of slack length without breakage, whereas wild type cells would rupture at lengths past 2.5 µm. In
addition to collagen, other sources of passive tension have been hypothesized, such as the intermediate filament network or the existing sarcolemma structure [Greaser et al., 2008]. N2BA-G expression also affects other contractile properties in the homozygous mutants. Even though passive force was higher in wild type rats, Ca$^{2+}$-activated force was lower in mutant rats. Due to N2BA-G’s increased extensibility, the rate of myocardial force redevelopment was significantly lower in the mutant phenotype as well [Patel et al., 2012].

This spontaneous mutation in Sprague-Dawley rats was found to be a partial deletion of the RNA-binding motif protein 20 (Rbm20) gene. Sequence analysis comparing normal and homozygous animals revealed a 95 kb deletion which removed exons 2-14 of the Rbm20 gene, rendering it non-functional [Guo et al., 2012]. This led to the ablation of titin alternative splicing, creating the N2BA-G isoform, which is significantly larger than wild type N2B (3.83 vs. 2.97 MDa, respectively). N2BA-G titin contains Ig and PEVK domains which are normally spliced out of the wild type adult. The necessity of Rbm20 in the titin alternative splicing events was shown through adenoviral rescue experiments, as delivery of an Rbm20-containing adenovirus proved sufficient to re-express the normal N2B isoform in mutant cardiomyocytes [Guo et al., 2012].

Subsequent analysis demonstrated that while the Rbm20 deletion did affect approximately 30 other genes in striated muscle, its presence was confined to the striated muscle system. Western blot analysis confirmed that Rbm20 exists exclusively in striated muscle, where it can affect titin alternative splicing. Analyses showed that
Rbm20 performs a similar role in both skeletal and cardiac muscle. Rbm20-deficient rats possess a 3.75 MDa N2A-G isoform at adulthood in all skeletal muscles [Li et al., 2012]. This size is slightly smaller than N2BA-G titin, due to the lack of an N2B region in skeletal muscle titin.

The Rbm20-deletion rat model demonstrated multiple changes to the musculoskeletal system, resulting in severe physiological effects. Echocardiographic analysis showed that heterozygous and homozygous mutants had left ventricular dilation and increased diastolic dimensions. Systolic dimensions and contractility were not altered by this mutation. Sudden death was also observed in homozygous animals. Other proteins’ alternative splicing profiles were altered by this mutation, including calmodulin-dependent kinase II δ, LIM domain-binding protein 3, and Cacna1c [Guo et al., 2012].

Rbm20 is a serine/arginine-domain-containing (SR) protein. Such proteins often dictate inclusion or exclusion of exons of immature transcripts and regulate the alternative splicing pattern [Long and Caceres, 2009]. SR proteins play critical roles in pre-mRNA splicing and regulation of mRNA translation and expression. A large number of SR proteins have been characterized, but to be classified as such, the protein must contain several specific domains in its modular structure. These include an RNA recognition motif (RRM), an arginine-serine-rich domain (RS domain), and must participate in constitutive or alternative splicing [Lin and Fu, 2007]. Classical SR proteins will bind to exonic splicing enhancers and ensure the correct linear exon presence in mature mRNA. However, there are two mechanisms by which SR proteins
can act to achieve this: a recruitment model or an inhibitor model. The recruitment model states that SR domains will stabilize the spliceosome machinery and perform exon definition; the inhibitor model suggests that SR proteins will antagonize hnRNP proteins and recognize exonic splicing silencers to get correct expression [Long and Caceres, 2009]. Some SR proteins, such as Rbm20, are present exclusively in specific tissue type (i.e., striated muscle) [Guo et al., 2012], while others such as Rbm4 can be expressed ubiquitously in mammalian tissue [Markus and Morris, 2009]. SR proteins have been found across many species and in many tissues [Zahler et al., 1992] to perform roles in alternative splicing. When SR protein function is disrupted, cancer and other serious physiological effects are observed [Long and Caceres, 2009].

Because of its structure, Rbm20 is considered to be a classical SR protein. Its cDNA sequence contains 14 exons, which when translated produces an approximately 130 kDa protein [Brauch et al., 2009]. In addition to RRM and RS domains, Rbm20 is comprised of a C-terminal proline-rich region and two zinc-finger domains. The serine residues in the RS domain in many SR proteins are reversibly phosphorylated to activate the splicing ability [Lin and Fu, 2007]. It has been shown that a single point mutation to one of the serine residues in the RS domain of RBM20 is sufficient to cause disease [Guo et al., 2012]. As an SR protein, it resides in the nucleus, localizing to nuclear speckles [Li et al., 2013]. The phosphorylation state of an SR protein regulates its cellular localization as well as its activity. RS domain serine residues can be phosphorylated by one of several kinase classes, including: SR protein kinases, the Clk/Sty kinase family, and topoisomerase I [Long and Caceres, 2009]. The phosphorylation of an SR protein is
required for spliceosome assembly to begin [Xiao and Manley, 1997], but their
dephosphorylation will precipitate the splicing event [Cao et al., 1997].

Rbm20 has been recently identified as the protein responsible for some cases of
dilated cardiomyopathy (DCM) [Li et al., 2010]. Human patients without a functional
Rbm20 gene and the homozygote mutant rats present a similar pathology, including
increased fibrosis, electrical abnormalities, arrhythmias, and sudden death [Brauch et al.,
2009; Guo et al., 2012; Li et al., 2010]. Genome-wide linkage analysis was utilized in
human patients suffering from idiopathic DCM to discover Rbm20, and it was discovered
to be an autosomal dominant mutation [Brauch et al., 2009]. The mutations were
primarily found within the RS domain, which demonstrates the importance of this region
to regulate gene splicing. A mutation of one of the residues in the exon encoding the RS
domain was sufficient to bring about a cardiac pathology [Brauch et al., 2009]. Indeed,
the majority of mutations to the Rbm20 gene have been contained within the RS domain.
These point mutations will often replace an active amino acid residue, typically an
arginine or a serine. The removal of these crucial residues is sufficient to manifest DCM
[Brauch et al., 2009; Guo et al., 2012; Li et al., 2010]. Li et al. did find a point mutation
in the RRM region of Rbm20 which also contributed to DCM [Li et al., 2010]. It was
hypothesized by the two initial studies [Brauch et al., 2009; Li et al., 2010] that the
downstream effects of these mutations will target a specific set of genes, and indeed, this
was the case. Rbm20 mutations led exclusively to cardiac dysfunction, mostly those
characteristic of DCM.
1.5 Titin and disease

The critical functions of titin have been summarized above; due to its extreme length and ubiquitous presence in the sarcomere, it preserves myofibrillar integrity, signals both inside and outside the myocyte, and maintains resting stiffness [Granzier and Labeit, 2002]. Its role in diastolic function must also be carefully monitored, since titin controls ventricular filling and stiffness [Bell et al., 2000]. Therefore, mutations to the titin molecule typically have drastic effects on muscle ultrastructure. Multiple disease states have been cited as a result of titin dysfunction, which can vary from simple sequence deletions to inappropriate alternative splicing and isoform expression.

Dilated cardiomyopathy (DCM) is a myocardial disease which is denoted by left ventricular dilation and impaired contractility. It is a severely debilitating disease with a 50% mortality rate [Dec and Fuster, 1994]. Most DCM cases are idiopathic, although over 30 genes have been implicated as the root cause. A mutation to one, or several, of these genes will typically be responsible for DCM onset, and the majority of these genes encode either sarcomeric proteins, contractile proteins, or cytoskeletal structural proteins [Hershberger et al., 2009]. These genes include titin, Rbm20, troponin T, troponin C, tropomyosin, telethonin, and myosin heavy chain [Rampersaud et al., 2010]. Myosin heavy chain gene expression may be altered in DCM cases; disease onset increased the proportion of β-MHC, while improvement of cardiac function was associated with increased α-MHC levels [Abraham et al., 2002]. Indeed, DCM patients’ left ventricles did not present any detectable levels of α-MHC [Reiser et al., 2001]. If the giant
myofibrillar protein titin experiences a direct mutation or an alteration to its isoform expression, DCM can result.

Mutations to the titin gene (TTN) will cause DCM. An autosomal dominant mutation has found TTN mutations to be highly heritable, which also appears to be the case regarding DCM. Multiple familial cases of DCM have been reported [Gerull et al., 2006; Gerull et al., 2002; Siu et al., 1999]. Initial genomic analysis tracked the DCM patient mutation to chromosome 2q31, where the TTN gene resides [Siu et al., 1999]. A human pedigree showed a frameshift mutation which truncates titin within its A-band region, while another family had a truncated titin molecule shown to be only 1.1 MDa, significantly shorter than its normal 3.0 MDa size [Gerull et al., 2002]. Eventually, those patients presenting a TTN mutation were afflicted with congestive heart failure. In a recent, large-scale analysis, Herman et al. found that approximately 25% of cases of idiopathic DCM were due to TTN mutations [Herman et al., 2012]. These errors were nonsense mutations, frameshift mutations, or alternative splicing errors. The nonsense and frameshift variants in DCM were significantly more frequent than those resulting in hypertrophic cardiomyopathy. There was found to be 17 TTN splice variants in 19 patients which would alter RNA splicing, 11 of which altered fully conserved splice-site nucleotides [Herman et al., 2012]. The titin truncations resulting in DCM were distributed predominantly within titin’s A-band region and were absent from the Z-disk and M-line [Herman et al., 2012]. When titin is truncated, it still becomes incorporated into the developing myofibril, but it would then be unable to anchor the M-line proteins as it would in the healthy state [Obermann et al., 1997]. Additionally, the removal of M-
line titin would preclude it from sensing and modulating sarcomeric force [Kontrogianni-Konstantopoulos et al., 2009]. Studies have been performed that indicate the importance of titin for myofibril assembly [van der Ven et al., 2000] and a structurally sound M-line [Miller et al., 2003]. A mouse knock-in model containing an insertion to TTN mimics human DCM, and this insertion mutation led to embryonic lethality in homozygote animals. This genotype truncated titin within the A-band, showing the necessity of a titin molecule that reaches from the Z-disk to the M-line [Gramlich et al., 2009]. Each region of titin is unique and interacts with its binding partners using specific and intimate methods.

However, it is not only titin truncations that will cause DCM. Mutations which generate elongated titins or those that express higher levels of compliant N2BA titin in cardiac muscle can also lead to ventricular dilation. Rbm20 mutations in human patients have been shown to induce DCM [Brauch et al., 2009; Li et al., 2010]. Indeed, it was through familial genetic analysis in which Rbm20 mutations were discovered to be the common thread in some DCM patients. Further, the deletion of Rbm20, which results in DCM onset, will block titin alternative splicing and lead to N2BA-G isoform expression [Guo et al., 2012]. A pacing tachycardia model of DCM in dogs has shown that an increase in the ratio of N2B/N2BA expressed [Bell et al., 2000]. Unsurprisingly, when N2B isoform is upregulated, there is a concomitant increase in titin-based and collagen-based stiffness in this DCM model. The shifting of the isoform expression ratio permits titin to modulate passive properties in cardiac tissue [Wu et al., 2002]. Ultimately, the increased stiffness of these pacing-induced DCM hearts will reduce dilatation in the
short-term, but may increase diastolic pressure and prove detrimental to healthy function in the long-term.

In human patients with non-tachycardia-induced DCM, the titin isoform expression was flipped in the more compliant direction. An increased N2BA:N2B ratio is found in DCM patients [Makarenko et al., 2004; Nagueh et al., 2004]. These studies both showed significant increases in N2BA expression at the expense of N2B, but Makarenko et al. used human DCM transplant hearts and observed an increase of N2BA proportion of 0.12 [Makarenko et al., 2004]. Nagueh et al. used patient hearts that had already undergone heart failure, and they found a much larger N2BA increase. The ratio of N2BA to N2B increased from 0.56 in control hearts to 0.97 in DCM heart failure cardiac tissue [Nagueh et al., 2004]. Both studies demonstrated that DCM patients experienced decreased passive tension as a result of titin isoform shift, and that the reduced tension was due exclusively to titin, and not collagen or extracellular changes [Makarenko et al., 2004; Nagueh et al., 2004]. However, it was shown by Makarenko et al. that as passive stiffness goes down in DCM hearts, the proportion that titin contributes to passive stiffness also decreases [Makarenko et al., 2004]. Therefore, with higher levels of compliant titins in the heart, collagen becomes responsible for a greater percentage of passive tension. Experiments conducted on a DCM model of turkey myocardium demonstrated that those birds with DCM did not undergo an increased level of N2BA titin when treated with furazolidone to induce DCM. However, as a result of the DCM phenotype, the birds did exhibit increased passive stiffness, which was due to increased collagen-based stiffness [Wu et al., 2004]. The avian study suggests that
because DCM was brought about by drug administration, left ventricular dilation will occur, but collagen can be upregulated to increase passive stiffness. The cooperation between titin and collagen is important in maintaining normal heart physiology.

Cardiac titin isoform shifts are frequently observed when physiological dysfunction occurs. Myocardial titin will express varying proportions of a stiffer N2B isoform and a more compliant N2BA isoform in an age- and species-specific manner. In a spontaneously hypertensive rat model, those rats had a higher passive stiffness compared to their control counterparts. The hypertensive rats also had a higher heart weight to body weight ratio, indicating that they had undergone cardiac hypertrophy to some degree. While there was no direct difference in N2BA to N2B ratios between groups, the titin isoform ratio was correlated with heart weight:body weight ratios in the hypertensive, but not the control, rats [Warren et al., 2003].

Ischemic cardiomyopathy can occur together with left ventricular diastolic changes, and it can also demonstrate altered titin isoform ratios. In human patients suffering from coronary artery disease (CAD) following ischemia, significant titin isoform changes were noted. CAD hearts had significantly higher proportions of N2BA titin, which subsequently led to decreased passive tension [Neagoe et al., 2002]. Despite the fact that collagen and desmin have been shown to increase left ventricular wall stiffness in CAD [Hein et al., 2000; Weber, 1997], titin shows increased compliant titin expression. This indicates that titin is not responsible for the stiffening inherent to long-term ischemia.
1.6 Thyroid hormone

The thyroid gland is responsible for the production of thyroxine (T4) and triidothyronine (T3), both classified as thyroid hormone. These tyrosine-based hormones, which affect nearly every cell in the body, play a critical role in metabolism, and have a large effect on the cardiovascular system. Thyroid-stimulating hormone (TSH) is released from the pituitary gland as part of a positive feedback loop. High levels of TSH are found in low concentrations of T4, in an attempt to increase the concentration of thyroid hormone. When thyroid hormone levels need to be increased, TSH signals the thyroid gland to synthesize T4 [Klein and Danzi, 2007]. As the thyroid gland produces and releases high levels of T4 into the bloodstream, it is converted to T3 by several deiodinases [Berry and Larsen, 1992]. T3 is the active form of thyroid hormone and plays a variety of roles throughout the body. Once T3 has been manufactured, it will enter its target cell, translocate to the nucleus, and interact with its nuclear receptors (TR). Following this interaction, transcription of the target DNA sequence will be stimulated by TR binding to thyroid response elements (TRE) [Kahaly and Dillmann, 2005]. TREs exist in genes that are positively regulated by thyroid hormone, but TRs and TREs will interact regardless of thyroid hormone presence. When TRs are complexed with thyroid hormone, TRs will enhance transcription levels; without T3 present, transcription is blocked [Wu and Koenig, 2000]. This genomic mechanism is responsible for multiple genes’ expression levels in the heart.

A large number of structural and regulatory genes are controlled by thyroid hormone levels, making T3 critical to heart function. One of the most notable cardiac
genes affected by thyroid hormone levels are the myosin heavy chain genes. For many years, it has been known that thyroid hormone treatment influences cardiac MHC composition [Klein and Hong, 1986]. Cardiac muscle is comprised of two MHC isoforms: α-MHC and β-MHC. Long-standing knowledge indicates that α-MHC gene expression is upregulated in the presence of thyroid hormone, which is coupled to a concomitant decrease in β-MHC. It has been shown that T3 acts directly upon the α-MHC promoter and indirectly on the β-MHC promoter through non-coding RNA [Haddad et al., 2010]. When T3 is absent or at low levels in the body (hypothyroidism), β-MHC experiences increased transcription levels [Klein and Danzi, 2007]. A study investigating the regulation of α-MHC gene expression through T3 treatment found that administration of T3 increased α-MHC mRNA levels to euthyroid status in rats that had been thyroidectomized [Danzi et al., 2005].

Skeletal muscle MHC has long been known to be affected by thyroid hormone, too. The effect of young rat skeletal MHC upon thyroid hormone manipulation was investigated and it was reported that fast-twitch muscle will express adult-type fast MHC following thyroid hormone treatment. Slow-twitch muscle treated with T4 will not change the transition from neonatal-specific MHC to adult-specific MHC [Gambke et al., 1983]. Subsequent reports have shown the effect of hyper- and hypothyroidism on the MHC distribution in skeletal muscle, and two common themes have emerged. First, an increase or decrease in thyroid hormone levels will induce change mainly on slow-twitch, oxidative muscles (e.g., soleus muscle), while the fiber type distribution of fast-twitch, glycolytic muscles (e.g., tibialis anterior) is relatively unchanged. Second, elevated
thyroid hormone levels shifted the MHC profile toward a faster-contracting phenotype, while hypothyroidism induces a shift in the direction of increased slow-type MHC [Caiozzo et al., 1991; Fitzsimons et al., 1990]. Therefore, the MHC isoform changes that arise in T4- and propylthiouracil (PTU)-treated animals occur in a similar manner to other physiological changes, that is, a shift in MHC isoform content from fast-to-slow or slow-to-fast. Hyperthyroid and hypothyroid animals will experience increased or decreased, respectively, exercise abilities depending on thyroid hormone levels [Baldwin et al., 1980; Baldwin et al., 1981]. Old rats’ skeletal MHC was responsive to thyroid hormone to the same degree as young animals following treatment [Larsson et al., 1994]. Additionally, the age-related decline in contractile speed in old rats can be attenuated by T3 treatment [Li et al., 1996].

There are additional, critical genes that are controlled by thyroid hormone through traditional genomic pathways. Other genes of important note that are upregulated by T3 include the $\beta_1$-adrenergic receptors, $\text{Na}^+/\text{K}^+$ ATPase, and voltage-gated potassium channels. $\beta$-adrenergic receptors were downregulated in the hypothyroid state, which led to decreased receptor ability to recognize its agonist [Stiles et al., 1981]. Conversely, adenylyl cyclase types V and VI, the T3 nuclear receptor, and $\text{Na}^+/\text{Ca}^{2+}$ exchangers are downregulated in the presence of T3 [Klein and Ojamaa, 2001]. Calcium handling and signaling is also impacted by thyroid hormone levels. Calsequestrin, which maintains a $\text{Ca}^{2+}$ pool, is upregulated in the presence of high thyroid hormone levels and downregulated in low thyroid hormone levels [Novak and Soukup, 2011]. Calcium transporter proteins are most definitely responsive to thyroid hormone, as their expression
levels have been shown to increase following T3 treatment [Shenoy et al., 2001].
Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) expression is also beholden to thyroid hormone levels, and it is upregulated when T3 levels are high [Hartong et al., 1994]. SERCA acts to regulate intracellular calcium signaling to allow for the calcium influx responsible for contraction. In contrast, phospholamban blunts the effects of SERCA; phospholamban levels are elevated when thyroid hormone is low [Kiss et al., 1994]. SERCA and phospholamban are responsible for contractile function and cardiac diastolic relaxation, and they are one of the mechanisms through which T3 acts to regulate the contractile nature of the heart.

In recent years, non-genomic, non-nuclear, TRE-independent T3 actions have been elucidated which exert effects on the cardiac system. It appears that when T3 binds to its receptor (TR), physiological changes may be demonstrated, without the involvement of TRE. Such changes occur more rapidly than those involving genomic pathways, and can affect multiple regions of the cell. T3 can quickly regulate membrane potential, contractile activity, and membrane depolarization by regulating ion channels [Sen et al., 2002; Sun et al., 2000]. In addition to these roles, T3 acts non-genomically on cell surface proteins [Zhou et al., 1992], plasma membrane transport proteins [Huang et al., 1999], and signal transduction pathways [Davis et al., 2000]. A review by Davis and Davis compiled the multiple signaling pathways affected by thyroid hormone: the mitogen-activated protein cascade, protein kinase A and inositol phosphate pathways, and the calmodulin-Ca\(^{2+}\) pathway [Davis and Davis, 2002]. These pathways use thyroid hormone which may bind directly to the first effector of the pathway, or that effector may
complex with a thyroid hormone-bound TR to start signaling. Recent evidence has shown that the phosphatidylinositol 3-kinase (PI3K)/protein kinase Akt pathway (PI3K/Akt) can be affected in a non-genomic manner by thyroid hormone. Immediately following T3 administration in mice, Akt activity levels were significantly increased [Hiroi et al., 2006]. Subsequent analyses have shown that, following Akt phosphorylation and activation, downstream PI3K/Akt pathway proteins are also stimulated by thyroid hormone treatment [Kuzman et al., 2005b].

T3 impacts the PI3K/Akt signaling in a non-genomic, canonical manner. Thyroid hormone will bind to a TR or to a receptor protein kinase, which will activate PI3K. Activated PI3K will convert PIP$_2$ to PIP$_3$, which can in turn bind phosphatidylinositol-dependent kinase (PDK1). Excited PDK1 can phosphorylate Akt, which in turn phosphorylates downstream targets. Akt is phosphorylated at two residues (Ser473 and Thr308) upon activation with thyroid hormone. When endogenous thyroid hormone is blocked with PTU, Akt shows very low phosphorylation levels [Kuzman et al., 2005b].

When Akt is phosphorylated, though, it will phosphorylate its downstream target, mammalian target of rapamycin (mTOR). mTOR will regulate downstream protein synthesis [Ojamaa, 2010]. In the cardiomyocyte, this protein synthesis is accomplished through phosphorylation of the p70 variant of S6 kinase (p70$^{S6K}$). Upon phosphorylation of p70$^{S6K}$, the 40S ribosomal subunit protein S6 will consequently be phosphorylated, and the ribosomal machinery will associate with its mRNA target, stimulating translation [Kenessey and Ojamaa, 2006]. Thus, T3 has successfully induced protein synthesis in its
target cells. It has been shown that T3 activation of the PI3K/Akt pathway will stimulate cardiac hypertrophy [Crie et al., 1983; Kenessey and Ojamaa, 2006].

The importance of thyroid hormone and Akt signaling is obvious. In serum-starved heart cells, the effects of T3 were investigated. Neonatal cardiomyocytes were subjected to serum starvation-induced cell death, and then treated with T3. T3 was successful in preventing cell death following treatment, via activation of Akt signaling [Kuzman et al., 2005a]. Blockage of the PI3K/Akt pathway using wortmannin, a T3 inhibitor, also prevented protein synthesis in cardiac cells [Kenessey and Ojamaa, 2006]. Insulin can also regulate the PI3K/Akt pathway in cultured rat cardiomyocytes. In the presence of insulin, the PI3K/Akt signaling pathway was activated in the same manner as T3, resulting in cardiac hypertrophy [Krueger et al., 2010]. More pathologically, thyroid cancer can be initiated by constitutive activation of PI3K/Akt signaling [Saji and Ringel, 2010]. Akt, also known as protein kinase B, can phosphorylate SR proteins which lead to their activation. Its effects are similar to other SR protein kinases’ activity, which leads to exons being removed from the mature mRNA [Blaustein et al., 2005b]. Lastly, the PI3K/Akt signaling pathway via thyroid hormone stimulation has been shown to affect the titin alternative splicing event [Krueger et al., 2008].

The effects of thyroid hormone on cardiovascular function are multitudinous. High thyroid hormone levels reduce systemic vascular resistance, increase contractility, and increase cardiac output. Elevated thyroid hormone levels also demonstrate increased blood volume, increased left ventricular systemic and diastolic function, and increased rates of isovolumic relaxation [Klein and Ojamaa, 2001]. Thyroid hormone increases the
basal metabolic rate throughout the body; summarily, high thyroid levels create a physiological response that is similar to exercise [Klein and Danzi, 2007]. Low levels of thyroid hormone in the bloodstream will decrease contractility and cardiac output, as well as induce brachycardia [Brent, 1994]. In more general terms, hyperthyroidism increases cardiac output anywhere from 50% to 300%, while hypothyroidism drops cardiac output by 30% to 50% [Klein and Danzi, 2007]. Thyroid disorders can arise from hyperthyroid or hypothyroid status, and each carries with it a unique set of physiological manifestations and concerns.

Hyperthyroidism, found in approximately 1.3% of the United States population, is classified by elevated free thyroid hormone (T4 and T3) levels and decreased TSH [Hollowell et al., 2002]. The increased cardiac output comes as a result of the increased cardiac preload and decreased vascular resistance [Biondi and Cooper, 2008]. The decrease in vascular resistance causes the vasculature to be unable to account for the increased stroke volume, thus leading to hypertension. Elderly patients are at a higher risk of developing systolic hypertension [Saito and Saruta, 1994]. Tachycardia and atrial fibrillation are the two most common effects of hyperthyroidism. Tachycardia is usually a concern, as patients will present a resting heart rate above 90 beats per minute. A chronic state of tachycardia will lead to hypertrophic cardiomyopathy, and high-output heart failure can often be an end result of prolonged hyperthyroidism [Klein and Ojamaa, 1998]. Atrial fibrillation increases in prevalence with patient age, and even subclinical hyperthyroidism can demonstrate an increased risk for atrial fibrillation. Fortunately, there are a number of characteristics that are predictive for atrial fibrillation. In addition
to patient age, these predictors include history of cardiac failure, elevated blood pressure, diabetes, and left ventricular hypertrophy [Osman et al., 2002]. Fortunately, atrial fibrillation can be treated with β-adrenergic blocking agents [Biondi et al., 2002]. As a result of hyperthyroid-induced tachycardia, exercise intolerance is another common side effect [Biondi and Cooper, 2008]. Hyperthyroidism also brings about left ventricular hypertrophy (LVH), and in the short term it will improve diastolic function [Dorr et al., 2005a]. However, chronic thyrotoxicosis and LVH will eventually lead to diastolic dysfunction [Mintz et al., 1991]. Other cardiac symptoms of hyperthyroidism are palpitations, anginal chest pain, peripheral edema, and congestive heart failure [Klein and Danzi, 2007]. General physiological symptoms include fatigue, weight loss, diarrhea, and heat intolerance [Rhee and Pearce, 2011]. In addition to beta-blockers, multiple drug options are available for hyperthyroid patients. Antithyroid medicines such as PTU, radioactive iodine ablation, and thyroid gland surgery are all viable options for those suffering from hyperthyroidism [Biondi and Cooper, 2008]. An overactive thyroid gland producing excessive amount of thyroid hormone can affect the cardiovascular system in profound ways.

Hypothyroidism afflicts a larger proportion (4.6%) of the United States population than hyperthyroidism and is characterized by an increase in serum TSH concentration and a corresponding decrease in thyroid hormone levels [Hollowell et al., 2002]. Many of the cardiovascular changes induced by hyperthyroid status will be altered in hypothyroidism, but in the opposite physiological direction. Cardiac output is decreased by more than 30%, coupled with decreased stroke volume and heart rate
Low thyroid hormone levels heavily impact the left ventricle, leading to diastolic dysfunction. Slowed myocardial relaxation time and impaired ventricular filling are commonly seen in hypothyroid patients, and systemic vascular resistance is elevated as a result of the increased diastolic pressure [Biondi and Klein, 2004]. Physical exertion causes systolic and diastolic dysfunction, but resting LV diastolic characteristics are altered, as well [Rhee and Pearce, 2011]. Diastolic heart failure in elderly patients is more common, as deterioration of cardiac structure and function renders them more vulnerable to this event [Biondi and Klein, 2004]. Chronic hypothyroidism also causes increased arterial resistance, thus leading to diastolic hypertension in 20% of hypothyroid patients [Klein and Ojamaa, 2001]. This hypertension can be relieved with thyroid hormone administration. Brachycardia will begin in the early stages of hypothyroidism and leads to the decreased cardiac output described above. The hypothyroid condition is not subject to the atrial arrhythmias that hyperthyroidism presents [Rhee and Pearce, 2011]. Additional cardiovascular concerns resulting from overt hypothyroidism include accelerated athlerosclerosis, coronary artery disease, and hypercholesterolemia. Reduced cardiac preload and decreased ejection fraction and stroke volume also occur [Fazio et al., 2004]. Hypothyroidism has multiple physiological effects in the body: fatigue, weight gain, constipation, cold intolerance, goiter, and depression will most likely result in the event of long-term low thyroid hormone levels [Rhee and Pearce, 2011]. There are multiple causes of hypothyroidism, most commonly iodine deficiency. Other reasons include irradiation of the neck, tumors in the pituitary gland, and drugs such as lithium carbonate [Klein and Danzi, 2003].
Thyroxine is the gold standard for treatment of hypothyroidism and is usually very effective at reversing its effects. In otherwise healthy patients, T4 is provided at the necessary dosage, and problems rarely arise. Older patients require a gradual increase in T4 administration for the body to acclimate to the changes, or to repair the heart, if underlying ischemia is present [Crowley et al., 1977].

Several reports have been published which indicate that titin isoform expression is another factor that can be regulated through thyroid hormone presence. Krueger and colleagues have reported that treatment of embryonic rat cardiomyocytes with T3 has altered titin isoform expression [Krueger et al., 2008]. These authors demonstrated that T3-treated cells increased the proportion of N2B isoform at the expense of N2BA titin, and immunoblotting results showed that this isoform shift is due to PI3K/Akt pathway signaling. Conversely, if thyroid hormone production is blocked through PTU administration, adult rats will demonstrate an increased ratio of N2BA:N2B titin, and a concordant decrease in passive tension is shown [Wu et al., 2007]. These two reports illustrate the dependence of proper titin isoform expression on thyroid hormone levels, providing yet another critical role that thyroid hormone plays in the cardiovascular system.

Thyroid hormone physiology is a complicated biological phenomenon that has an impact throughout the body. Hormone levels must be precisely maintained to convey proper functioning, and the cardiovascular system is heavily reliant on this regulation. Widespread effects on the cardiac system are observed in the presence or absence of thyroid hormone [Klein and Ojamaa, 2001], which affect the contractile profile and
hemodynamics of the heart. Indeed, in some cases thyroid dysfunction may only be manifest through the cardiovascular system, making a serum TSH analysis all the more critical in screening for heart disease.

1.7 Dilated cardiomyopathy and hypothyroidism

DCM [Dec and Fuster, 1994] and hypothyroidism [Biondi and Klein, 2004] are both characterized by left ventricular dilation and diastolic dysfunction. Therefore, in accordance with the wide array of roles that thyroid hormone can play on the heart, it should not be surprising that patients suffering from DCM may also be afflicted with hypothyroidism. The link between cardiac failure and low thyroid hormone levels has been shown [Hamilton et al., 1998]: a recent study in dogs has begun the investigation of the physiological consequences of thyroid hormone treatment on DCM [Tidholm et al., 2003]. There are many results which suggest that hypothyroidism may lead to the reduced blood flow in DCM, affecting the cardiac system’s functionality [Khalife et al., 2005].

Long-term idiopathic DCM patients will frequently show subclinical thyroid disorders. Bauerlein and colleagues showed that DCM can be due to thyrotoxicosis [Bauerlein et al., 1992]. A subsequent study of chronic DCM patients found that 97% of patients possessed low thyroid hormone levels, and that length of DCM was highly correlated with thyroid levels [Fruhwald et al., 1997].

In a pair of studies, Moruzzi and others discovered that treatment of idiopathic DCM with T4 was successful in improving cardiac and exercise performance [Moruzzi et
Following medium-term T4 treatment, the DCM patients did not exhibit hyperthyroidism, and diastolic ventricular function was restored to normal. Cardiac performance was enhanced, and cardiac output was restored to healthy levels [Moruzzi et al., 1996]. T4 administration has also been successful in treating a cardiomyopathic hamster model. These hamsters demonstrate a DCM-like phenotype, mimicking human DCM effects. Originally, these animals were presented with subclinical hypothyroidism, but thyroid hormone treatment was able to improve cardiovascular and hemodynamic function [Khalife et al., 2005].

There have been multiple case reports describing hypothyroidism inducing the onset of DCM. Ladenson and colleagues describe a 22 year-old hypothyroid man that was also afflicted with DCM, and prescribed a nine-month T4 regiment. Over the course of this treatment the man experienced cardiovascular improvements; the authors showed that DCM changes are reversible, when low thyroid levels are a cause [Ladenson et al., 1992]. A long-standing hypothyroid patient demonstrated brachycardia and high TSH levels, and upon study, was found to have left ventricular dilation. This patient was discovered to have DCM-type heart failure, as a result of the low circulating thyroid hormone levels. Treatment with T4 was successful in reversing these effects [Stanescu et al., 2007]. Two DCM patients analyzed by Khochtali and others were both found to have low T4 and high TSH levels. Cardiovascular function was also depressed, but T4 was again successful in relieving hypothyroidism and DCM effects [Khochtali et al., 2011]. Although heart failure as a direct result of hypothyroidism is somewhat uncommon, patients’ T4 and TSH levels should be checked in the event of dilation. Multiple studies
have concluded that thyroid hormone supplementation is capable of alleviating DCM’s effects.

For several decades, the knowledge that DCM and low thyroid levels are linked has increased. It has been shown that patients suffering from long-term hypothyroidism demonstrate an increased risk of developing heart problems, including DCM [Stanescu et al., 2007]. The depressed cardiac functionality and left ventricular dilation are typical consequences of these two abnormalities; it was unknown until recently, however, that the link between DCM and hypothyroidism was so intimate. Fortunately, the detrimental effects of idiopathic DCM as brought about by hypothyroidism can be alleviated by simple T4 administration.

1.8 Hypercholesterolemia and cardiovascular disease

As the leading cause of death in the United States [Rosamond et al., 2007], cardiovascular disease (CVD) encompasses many different classifications and occurs through malfunctions to the heart and blood vessels. The vast majority of CVD are found to be cases of coronary artery disease, atherosclerosis, and ischemia, brought about through genetic predisposition and obesity [Freedman et al., 2007]. Hardening or blockage of the arteries can be caused by fatty deposits or inadequate clearing of lipid from the bloodstream [Ross, 1999], resulting in ischemia and infarct. High fat levels can lead to hyperlipidemia, and elevated low-density lipoprotein cholesterol (LDL) has been shown to be the chief component associated with an increased risk of CVD [Rhoads et
al., 1976]. This, in combination with decreased myocardial contractile ability, will lead to heart failure [Holubarsch et al., 1996].

Titin is one of the major proteins responsible for this contractile action, and alterations to its levels and isoform expression has been associated with a variety of diseases, including those listed above. Cardiac ischemia is the final result of unresolved atherosclerosis, in which the myocardium receives inadequate blood. Following ischemic onset, increased diastolic pressure occurs, stiffening the heart. Titin is one of the major proteins responsible for diastolic pressure maintenance [LeWinter and Granzier, 2010a], such that a shift to a smaller, stiffer titin isoform profile will lead to increased pressure during diastole. It has been shown that an increase in left ventricular wall stiffness brought about by chronic ischemia will induce a shift in titin isoforms [Neagoe et al., 2002]. Left ventricles from patients afflicted with extensive CAD demonstrated an increased level of extensible N2BA titin relative to non-failing donor hearts. Neagoe and colleagues also performed titin isoform analysis on a rat model by inducing myocardial infarction through left anterior descending coronary artery ligation. Whereas normal rat left ventricles are comprised almost exclusively of N2B titin, these myocardial-infarcted failing rat hearts showed increased expression of an N2BA molecule, confirming a titin isoform shift in ischemia-induced CVD [Neagoe et al., 2002].

Genetic mutations which cause elevated lipid and cholesterol levels can also result in severe cases of CVD. Familial hypercholesterolemia (FH) is a genetic defect characterized by elevated cholesterol levels and hyperlipidemia at birth, in which the individuals have a malfunctioning LDL receptor. These mutations render the patients
unable to process and clear LDL from circulation [Jansen et al., 2005], which leads to atherosclerotic plaques and partial blockage. The depressed functionality of the blood vessels will in turn increase the likelihood of CVD.

FH swine have been developed as a model for human hypercholesterolemia, since it closely resembles the human disease state [Hasler-Rapacz et al., 1995; Prescott et al., 1995; Prescott et al., 1991]. FH pigs will demonstrate high levels of circulating cholesterol and develop atherosclerosis [Rapacz et al., 1986]. Because of the physiological proximity to humans and swine, Rapacz FH pigs provide a great model system for elucidating the roles that diet, lifestyle, and cholesterol levels have on cardiovascular health.

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**Figure 1.1. Structure of titin in sarcomere.** N-terminus is at the Z-disk, and C-terminus is at the M-line. Titin spans an entire half-sarcomere, and its isoforms are shown at bottom. Titin I-band domains are comprised of Ig domains, a PEVK region, and unique sequences (N2B, N2A). FCT: fetal cardiac titin. Image from Anderson et al. 2012.
CHAPTER TWO: EFFECTS OF RBM20 MUTATION ON TITIN
PHOSPHORYLATION AND MYOFIBRILLAR PROTEIN ISOFORM
EXPRESSION IN STRIATED MUSCLE

Abstract

The giant protein titin is found primarily in striated muscle, where it is responsible for a number of roles. In order to modulate passive tension and provide structural support within the sarcomere, titin is subjected to multiple alternative splicing events and is present as several different isoforms. The extent of titin phosphorylation also controls resting tension in the myocardium. Recently, it was reported that Rbm20 is the splicing factor responsible for titin alternative splicing, and its deletion leads to the expression of a giant titin isoform. Homozygous mutant (Hm) rats lack a functional Rbm20, and the sarcomeric proteins’ isoform expression was compared to wild type (Wt) rats. Titin phosphorylation was also examined. We report no difference between titin phosphorylation in Wt and Hm rats, as the optical density vs. protein load was similar (0.13 vs. 0.17, respectively). Further, the cardiac myosin heavy chain (MHC) isoform expression was similar between Wt and Hm. Wt rats expressed 66.9% α-MHC and 33.1% β-MHC, and Hm expressed 60.4% α-MHC and 39.6% β-MHC. The MHC content of the tibialis anterior (TA) skeletal muscle was also studied, and the proportions of type IIX and type IIB MHC were similar across genotypes as well. TA sarcomeric proteins’ expression level was measured, and no differences in expression of actin, myosin light chain (MLC)-1, MLC-2, MLC-3, tropomyosin, troponin C, troponin I, or troponin T were
found to exist. Thus, it appears that the Hm rat mutation primarily shows differences to the titin protein, while the remaining sarcomeric proteins are unchanged. At present, the lack of a working Rbm20 gene only seems to affect titin alternative splicing.

2.1 Introduction

The striated muscle ultrastructure is dependent upon multiple protein-protein interactions, both permanent and transient [Clark et al., 2002]. To provide this stability, several filament systems are set down which allow for precise control during the contractile process. For nearly 60 years, it has been known that the myosin thick filaments slide over the actin thin filaments in the “sliding filament” model [Huxley and Hanson, 1954]. Myosin is responsible for the contractile event, and its speed of contraction is dictated by the isoform present, regulated by developmental timeframe and disease state [Lompre et al., 1984]. The myosin protein is composed of two heavy chains (MHC) and four light chains (MLC), and MHC domains perform the workload of the molecule, which can be attenuated by the isoform present. MHC isoform expression is controlled by many factors, chief among them: species, age, location, and health. Cardiac tissue may contain either α-MHC, β-MHC, or a combination [Hoh et al., 1978], while skeletal muscle is more complex and is able to express a cohort of four MHCs: type I, type IIA, type IIX, and type IIB depending on the muscle physiology [Schiaffino and Reggiani, 2011]. Proper myosin expression is necessary, because a shift will alter the kinetics of the contractile apparatus and, in the heart, can result in cardiac failure [Gupta, 2007]. The interplay between the myosin thick filament and the actin thin filament is imperative for the contractile event to occur. In the healthy heart, the actin filament is
formed by the polymerization of many globular actin monomers, and it is capped at both ends to prevent inappropriate breakdown of the thin filament [Littlefield et al., 2001].

To provide further support to the thick filament and sarcomeric elasticity, the giant protein titin, approximately 1 µm in length, will stretch from the Z-disk to nearly the M-line in the sarcomere [Nave et al., 1989]. As yet another filament-type protein found within striated muscle, titin (or connectin) plays a number of critical roles on the muscle structure [Maruyama, 1976; Tskhovrebova and Trinick, 2003; Wang et al., 1979]. Because of its 3 MDa size, titin is comprised of many different domains, all of which are critical for maintenance of the muscle. Its N-terminus is present at the Z-disk, where it binds to multiple partners to strengthen that region of the sarcomere [Gregorio et al., 1998; Young et al., 1998]. The A-band region of titin is responsible for the close interaction between titin and the myosin thick filament; there are many super-repeating immunoglobulin (Ig) and fibronectin domains that will bind to the myosin filament [Labeit and Kolmerer, 1995]. The titin-myosin complex is therefore strong enough to stabilize the thick filament in the middle of the sarcomere and it has been shown that disruption of the titin molecule in single fibers is sufficient to prevent thick filaments from organizing in the center of the sarcomere [Horowits et al., 1986]. At the M-line, titin’s C-terminus is responsible for both stretch-sensing and signaling of tension-related processes [Linke, 2008]. The presence of titin’s C-terminal end is responsible for incorporation of the M-line proteins such as myomesin and M-protein [Wang et al., 1998]. This portion of titin also contains a kinase domain, which is required for myofibrillogenesis to occur. Besides phosphorylating telethonin [Linke, 2008], titin
kinase permits the association of titin, myosin, and myomesin in the central sarcomere [Miller et al., 2003].

The I-band region of the titin molecule has been subjected to considerable research, and the mechanism by which this extensible portion of titin functions to confer elasticity to the sarcomere has been elucidated. I-band titin contains only Ig domains and unique sequences, which include the PEVK and N2-unique regions. However, this particular region can exhibit diverse alternatively spliced variants, to the point where significant protein size differences are demonstrated. As stated above, it is the I-band region which allows for extensibility and flexibility during stretching and relaxation of the sarcomere, and the titin I-band will extend in a step-wise fashion. The muscle’s passive force, dependent upon titin size, is specified by this region. When the sarcomere is first stretched, the Ig domains will become extended. Then, as sarcomere length increases further, the PEVK domain will elongate. Finally, the titin N2-unique sequences will extend, which will control the overall passive stiffness of the sarcomere [Linke et al., 1999].

The nomenclature of titin isoforms is specified by the unique sequence domains that are contained in the I-band, and their presence or absence is controlled by tissue type and alternative splicing events. The two types of unique sequences are N2B and N2A, which are found in cardiac and skeletal muscle, respectively [Labeit and Kolmerer, 1995]. In some cases, however, myocardium can also exhibit an N2BA isoform, a larger titin subtype which contains both N2B and N2A sequences. Summarily, skeletal muscle titin always includes N2A segments, and cardiac titin always includes N2B segments.
[Cazorla et al., 2000; Freiburg et al., 2000]. The relationship between titin size and passive stiffness is observed here: myocardium can co-express N2BA and N2B titin to adjust passive tension [Neagoe et al., 2003], while different skeletal muscles can express differently-sized N2A titins depending on the number of inserted Ig domains [Prado et al., 2005]. Since I-band titin is responsible for passive stiffness determination, different splice variants of the titin molecule will demonstrate differing levels of passive tension. As the ratio of N2BA:N2B decreases in cardiac tissue, passive tension will increase [Wu et al., 2002]. Recently, it has been ascertained that Rbm20, a splicing factor protein, is responsible for the titin alternative splicing events [Guo et al., 2012].

In a rat model which lacks a functional Rbm20 gene, a significantly larger titin isoform was found in both cardiac and skeletal muscle. In homozygous mutant rat hearts, a 3.83 MDa giant N2BA isoform (N2BA-G) was expressed, in contrast with primarily a 2.97 MDa N2B isoform found in normal wild type animals [Greaser et al., 2008]. Similar results were observed in skeletal muscle; wild type rats express skeletal titins between 3.29 to 3.66 MDa, but the mutant phenotype expresses a 3.75 MDa N2A titin [Li et al., 2012]. Further, this animal model was found to have lower passive tension at the similar sarcomere lengths relative to wild type animals [Greaser et al., 2008]. The cardiomyocytes of homozygous rats could also be stretched far beyond the normal physiological work range, and their significantly larger titins prevented rupture. Splicing of at least 30 proteins appears to be affected by Rbm20 [Guo et al., 2012]. Of the sarcomeric proteins, it appears that Rbm20 acts primarily on the titin mRNA to control its expression pattern.
In addition to the influence that titin size performs on passive stiffness, phosphorylation state of this protein will also modulate the muscle’s physiological characteristics. The cardiac N2B domain was experimentally determined to be a substrate for protein kinase A (PKA), and this phosphorylation leads to the lowering of passive tension [Yamasaki et al., 2002]. Following PKA treatment, skinned rat cardiomyocytes showed 40% reduced passive tension compared to control values, and an expressed N2B segment could also be phosphorylated in vitro. Atomic force microscopy of the N2B segment showed decreased passive force following PKA treatment [Leake et al., 2006]. Reports have also been made regarding human heart failure in cardiomyocytes; a low phosphorylation state of the N2B sequence showed increased passive force [Borbely et al., 2009]. Subsequent studies have investigated other kinases and the importance of N2B’s presence for this effect to be observed. Fukuda and others have shown that in muscles with a larger N2B:N2BA ratio and PKA treatment, larger reductions in passive force were demonstrated, and that the extent of the decrease is controlled by the relative isoform amounts [Fukuda et al., 2005]. PKA and protein kinase G (PKG) were both found to phosphorylate Ser469 residue of the human N2B unique titin region, and PKG-induced phosphorylation had a similar effect on lowering passive tension as PKA [Kruger et al., 2009]. Conversely, in vitro experiments investigating protein kinase C’s (PKC) role in titin phosphorylation yielded increased passive tension [Hidalgo et al., 2009]. The authors observed that PKC was able to phosphorylate 2 sites within PEVK region, but the phosphorylation events led to a passive tension that was elevated 15-20%. This area of research has thus far determined the modulation of titin
force generation, but the timing and extent of titin posttranslational modifications have yet to be fully elucidated.

Here the effects of the rat mutation lacking a functional Rbm20 gene [Greaser et al., 2008] on titin phosphorylation and sarcomeric protein isoform expression were investigated. From this data, it appears that this particular rat model primarily impacts titin among the sarcomeric proteins.

2.2 Materials and Methods

2.2.1 Animals and tissues

Rats used in this study were from a mixed-breed colony, consisting of 50% Brown Norway, 25% Fisher 344, and 25% Sprague-Dawley. Adult wild type (Wt) and homozygous mutant (Hm) rats lacking a functional Rbm20 gene were used in this study and have been previously described [Greaser et al., 2008]. Rats (n=6 for both Wt and Hm) were euthanized using CO₂, and left ventricles and tibialis anterior (TA) were excised and immediately placed in liquid nitrogen. Tissues were stored at -80°C until use. All procedures involving animal care and handling were in accordance with protocols approved by the University of Wisconsin-Madison Animal Care and Use Committee. A small piece of frozen tissue (~100 mg) was homogenized until dissolved using a Dounce homogenizer in SDS sample buffer (8 M urea, 2 M thiourea, 3% SDS, 75 mM dithiothreitol) as described [Warren et al., 2003b].

2.2.2 Titin isoform gel electrophoresis and phosphoprotein staining

To analyze titin isoform expression, left ventricle tissue was electrophoresed on a vertical SDS-agarose gel electrophoresis system [Warren et al., 2003b]. Following
electrophoresis, gels were subjected to phosphoprotein and total protein staining using Pro-Q Diamond and SYPRO Ruby, respectively (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol as modified [Chen et al., 2010; Stelzer et al., 2006]. SDS-agarose gels were stained first with Pro-Q Diamond and then with SYPRO Ruby so as not to confound the phosphoprotein staining. Gels were analyzed after each staining procedure. To standardize protein loading differences between Wt and Hm samples, a range of concentrations per sample was loaded onto the gel and then the slope of integrated optical density (IOD) vs. concentration (µg) for both phosphoproteins and total proteins was compared. Phosphoprotein and myofibrillar protein band intensities were detected using UVP BioImaging System (UVP, LLC, Upland, CA), and bands were quantified using LaserPix software (Bio-Rad Laboratories, Hercules, CA).

2.2.3 Myosin heavy chain gel electrophoresis

For determination of cardiac myosin heavy chain (MHC) isoform expression, left ventricles were electrophoresed on 16 × 18 cm large-format glass plates containing 8% SDS-PAGE gels (30% acrylamide, 50:1 acrylamide:bisacrylamide ratio) modified from the previous methods [Talmadge and Roy, 1993; Warren and Greaser, 2003a]. Bisacrylamide, not N,N'-diallyl-tartardiamide, was used as a cross-linker. Following electrophoresis, gels were stained with Coomassie brilliant blue (CBB) and de-stained (10% methanol, 7.5% acetic acid) to detect MHC isoforms. The upper band was determined to be α-MHC and lower band was β-MHC. The MHC bands were quantified and analyzed using GelBandFitter [Mitov et al., 2009]. Skeletal MHC isoform expression was performed on a small-format electrophoresis system using the method
previously described [Talmadge and Roy, 1993], except for several changes. Gels were run at 4°C with a constant 130 V, and β-mercaptoethanol was added to the upper reservoir buffer at the beginning and at 12h into electrophoresis. These changes allowed for increased clarity of MHC isoform bands. Following electrophoresis, gels were stained with CBB and de-stained as above. Band intensities were quantified and analyzed using NIH Image software (Version 1.63, National Institutes of Health, Bethesda, MD).

2.2.4 Myofibrillar protein gel electrophoresis

TA muscle was run on small-format 12% SDS polyacrylamide gels to assess small myofibrillar protein expression as previously described [Giulian et al., 1983]. Gels were electrophoresed at 40 mA constant current, stained with CBB, and de-stained as above.

2.2.5 Statistical analysis

Band intensities were analyzed using computer software indicated above, and care was taken to eliminate background interference. In the case of titin phosphorylation and total protein, slopes were obtained and compared. Cardiac MHC was measured with GelBandFitter to ascertain the ratio of alpha to beta MHC isoforms. For analysis of samples containing multiple bands, band intensities were totaled and a percentage of the total expression was determined. Then, Wt was normalized to 1 for each protein, and Hm bands were compared relative to Wt. All data presented appears as means ± SE. Statistical analysis was performed using student’s t-tests in Microsoft Excel. P values of < 0.05 were deemed to be significantly different.
2.3 Results

2.3.1 Titin phosphorylation state

Previous studies have shown the ability of cardiac titin to be phosphorylated by a variety of protein kinases [Hidalgo et al., 2009; Kruger et al., 2009; Yamasaki et al., 2002], but the mutant rat’s phosphorylation state has not been explored. Hm rats express a N2BA-G titin (3.83 MDa) while Wt rats express predominantly the N2B titin (2.97 MDa). Figure 2.1A shows a representative gel image of a Pro-Q Diamond stained SDS-agarose gel of both Wt and Hm left ventricle titin. Note that both the N2B (Wt) and N2BA-G (Hm) bands showed phosphorylation. However, the T2 band, a titin proteolytic fragment that extends from the PEVK region of the I-band to the carboxyl terminus in the M line, showed no staining. This suggests that the major phosphorylation sites in titin reside between the Z-line and middle I-band region.

To measure the level of phosphorylation, IOD was plotted versus the concentration of the samples (60, 120, 180, 240 µg) on both the Pro-Q Diamond and SYPRO Ruby-stained gels. After determining the slope of IOD vs. protein concentration, the ratio of phosphoprotein:protein band weight was obtained. Because of the large difference in titin size between genotypes, a factor of 1.29 was used with Hm samples to correct the slopes of phosphoprotein and total protein and thus bring comparisons to an essentially equivalent mole basis. Hm N2BA-G titin had higher slope values for both the phosphorylated and total protein as compared to Wt N2B titin. Hm phosphoprotein slope was 0.17 relative to Wt’s phosphoprotein slope of 0.13 (Fig. 2.1B), and Hm total protein was 5.73 versus 4.63 for Wt total protein (Fig. 2.1C). However, Hm slopes were not
significantly different, and the ratio of phosphoprotein:protein was similar between Wt and Hm (0.038 vs. 0.035, respectively, Fig. 2.1D). Thus, after accounting for the titin sizes, no differences in phosphorylation levels were observed between Wt and Hm.

2.3.2 Cardiac myosin heavy chain analysis

MHC isoform expression in myocardium will vary based on location [Carnes et al., 2004], developmental timeframe [Fitzsimons et al., 1999], and species [Reiser and Kline, 1998]. Generally, larger adult mammals, such as pig and human, express predominantly α-MHC in atrial tissue and β-MHC in both ventricles. However, smaller mammals, such as rodents, express a mixture of both isoforms that persists through adulthood. Mature rats demonstrate the presence of α- and β-MHC in their ventricular regions, while atria predominantly express the α-MHC isoform. Large-format SDS-PAGE according to [Talmadge and Roy, 1993] allowed for separation of both isoforms, and a representative gel image is depicted in figure 2.2A. The left ventricle MHC contained both α-MHC and β-MHC in Wt and Hm rats. MHC isoform expression was quantitatively similar in Wt (66.9% ± 3 α-MHC and 33.1% ± 3 β-MHC) and Hm (60.4% ± 3 α-MHC and 39.6% ± 3 β-MHC) ventricles; no statistical difference was observed in isoform proportions (Fig. 2.2B).

2.3.3 Skeletal muscle protein analysis

TA muscle was selected for analysis to observe if the Rbm20 deletion had any effect on the MHC and other myofibrillar proteins’ expression pattern. It has already been established that the Hm phenotype will lead to titin splicing alterations in cardiac and skeletal muscle [Greaser et al., 2008; Li et al., 2012]. SDS-agarose gel
electrophoresis shows two N2A isoforms bands in Wt TA, a lower band at 3.29 MDa, and an upper band at 3.44 MDa. When Rbm20 function was negated, all Hm skeletal muscle titin was 3.75 MDa in size. It has yet to be reported if this mutation will change the skeletal muscle phenotype in other aspects beyond titin isoform expression.

MHC expression in TA was analyzed using small-format SDS-PAGE as previously reported [Talmadge and Roy, 1993], and this method was sufficient in differentiating between MHC isoforms. TA muscle expresses the two fastest-contracting MHC fiber types, type IIX and type IIB. NIH Image was used to analyze the proportions of type IIX and IIB MHC, and a representative gel image is shown (Fig. 2.3A). No difference was reported between Wt and Hm MHC percentages, as genotype did not impact the fast-twitch TA muscle (Fig. 2.3B). Wt (17.3 ± 4% type IIX and 82.7 ± 4% type IIB) demonstrated similar MHC levels relative to Hm (20.4 ± 2% type IIX and 79.6 ± 2% type IIB). This rat model did not convey any alterations to skeletal muscle myosin heavy chain proportions.

Next, the smaller proteins of the myofibril were electrophoresed and analyzed on a typical SDS-PAGE system [Giulian et al., 1983]. This system was used for detection and quantitation of the following proteins: actin, MLC-1, MLC-2, MLC-3, Tm, TnC, TnI, and TnT. Quantities of these myofibrillar proteins was determined by densitometric analysis. Wt band intensities were normalized to 1, and Hm was compared to Wt bands. SDS-PAGE (Fig. 2.4A) shows the full complement of myofibrillar proteins. No differences were discerned in any of the proteins listed above (Fig. 2.4B). There was no change in expression of actin, myosin light chains (MLC-1, MLC-2, and MLC-3), and
thin filament regulatory proteins (Tm, TnC, TnI, and TnT). All proteins studies showed similar expression levels across genotypes, which is supported by prior knowledge that thin filament [Yates and Greaser, 1983] and contractile protein stoichiometry [de Tombe and Solaro, 2000] is precisely controlled. A change to the titin isoform expression profile does not alter other sarcomeric protein expression.

2.4 Discussion

For this study, experiments were conducted to observe the effects of a rat splicing factor mutation [Greaser et al., 2008; Guo et al., 2012] on sarcomeric proteins. Recent evidence has indicated that this rat model also exhibited blockage of skeletal muscle titin splicing [Li et al., 2012]. However, since this rat model resembles the pathology of human patients suffering from dilated cardiomyopathy, a clearer picture must be assembled to ascertain how this model can be utilized in human health applications. Therefore, building on previous knowledge about the Rbm20-deleted Hm rat should prove to be an excellent route to study cardiac dysfunction.

2.4.1 Effects of Rbm20 mutation on titin phosphorylation

Multiple studies have been published regarding the effects that phosphorylation to titin’s I-band region has on cardiac passive stiffness, but Hm rats permit a new avenue in elucidating the extent to which passive stiffness can be modulated. After accounting for the difference in titin size between Wt and Hm ventricles, the slope of phosphorylated titin was similar, as was that of total protein. The phosphoprotein:protein ratio was used to determine if there were differing phosphorylation states; no differences were found, indicating that despite its significantly greater titin isoform size, Hm rats are
phosphorylated to approximately the same extent as Wt. The initial model description by Greaser and colleagues reported that Hm have decreased passive stiffness [Greaser et al., 2008], mainly caused by additional I-band titin domain inclusion. Coupled with this previous study, the current work strongly supports the hypothesis that the decreased cardiac passive stiffness is a consequence of increased titin size, and not of a change due to the titin phosphorylation state. Rbm20’s splicing hot spots are contained within the I-band region [Li et al., 2012], which is responsible for conferring extensibility to the molecule. The extra length of the Hm titin thus appears to be the major reason for decreased passive tension in Hm rats. Several studies have reported that titin phosphorylation sites are also contained within the I-band, and these authors describe that passive tension drops when phosphorylation is high [Hidalgo et al., 2009; Kruger et al., 2009; Yamasaki et al., 2002]. From a functionality standpoint, it can be assumed that increased Hm titin extensibility and increased N2B phosphorylation in Wt animals can perform similar roles in decreasing passive tension in the heart.

2.4.2 Effects on cardiac MHC expression

Since the Hm rat does show signs reminiscent of DCM, it is reasonable to infer that changes to other myofibrillar proteins may occur as a result of this mutation. Following investigation of cardiac MHC isoform profiles, no changes between Wt and Hm were observed. While MHC expression patterns can be attenuated by, among other things, age and species, the two rat genotypes studied showed both isoforms, but a higher proportion of α-MHC (Fig. 2.2). It is important to note that the animals utilized in this study were approximately 6 months of age, a maturity level at which rats still express
more α-MHC. Analyses investigating the relationship between rat age and MHC expression pattern has been explored [Fitzsimons et al., 1999], and our results support those findings of Fitzsimons and colleagues. These authors also showed a general decrease in α-MHC proportion as aging occurs. Based on the present findings, Wt and Hm do not show a difference in their cardiac MHC isoforms, and we hypothesize that, despite Rbm20’s deletion, there should be no difference in MHC throughout the life cycle. Previous studies have demonstrated that rat ventricles will present both isoforms [Reiser and Kline, 1998], which is confirmed by this study. Cardiomyopathies and heart failure can certainly induce alterations to the MHC profile [Gupta, 2007], but an Rbm20 deletion does not appear to play a role in cardiac MHC isoform determination.

### 2.4.3 Effects on skeletal muscle myofibrillar proteins

The lack of titin alternative splicing in Hm rats extends to the skeletal muscle system as well [Li et al., 2012], and Hm skeletal muscle titin is 3.75 MDa in all analyzed muscles. The tibialis anterior (TA) was chosen as a representative muscle, and its myofibrillar protein profile was examined. TA MHC isoform pattern is shown in figure 2.3, and its corresponding graph indicates that Wt and Hm present similar MHC percentages. In a similar manner to cardiac MHC profiles, skeletal muscle MHC can be adjusted and modulated based on a variety of stimuli. Muscle fiber type composition is dictated by numerous effects, including mechanical, neuronal, and hormonal factors [Pette and Staron, 2000], and the MHC complement is extremely plastic in nature [Schiaffino and Reggiani, 2011]. While there are a multitude of factors that modulate MHC, the Hm genotype does not interact with these myosin-specific mechanisms. From
the evidence provided here, Rbm20 does not seem to affect skeletal muscle MHC proportions. The expression levels of the critical myofibrillar proteins were also investigated here. Quantitation of the thin filament regulatory proteins showed similar protein levels regardless of genotype, and the representative gel (Fig. 2.4A) illustrates that Hm is devoid of changes in these lower molecular-weight sarcomeric proteins relative to Wt rats. There were no changes in any of the 8 analyzed proteins (Fig. 2.4B), and it again appears that Rbm20 deletion effects are primarily confined to titin. This was expected, as the precision inherent to the striated muscle sarcomere is well-established [Clark et al., 2002], and a correct sarcomeric protein stoichiometry is necessary for a healthy phenotype [de Tombe and Solaro, 2000].

As the Hm rat has only been reported recently and is in the midst of being characterized, these findings can provide a baseline for future comparisons. When this phenotype was first described [Greaser et al., 2008], the results showed an N2BA-G cardiac titin isoform and decreased passive tension, but no change to TnT expression. More recently, this mutation has also been discovered to affect skeletal muscle titin [Li et al., 2012], but myosin and most of the other sarcomeric proteins have yet to be studied. The information provided here demonstrates that, at present, the non-functional Rbm20 rat causes changes to titin size and its downstream effects. However, the phosphorylation state of its I-band region, shown in the past to modulate passive stiffness, is not altered by this mutation. Because of the novelty of this rat model, further research is needed for a complete characterization, but the phosphorylation state and other myofibrillar proteins, aside from titin, do not seem to be affected by this mutation.
References


A. Phosphoproteins stained with Pro-Q Diamond

B. Slope of phosphorylated titin (IOD/µg)

C. Slope of titin protein (IOD/µg)

D. Ratio (Phosphorylated titin:titin protein)
Figure 2.1. Phosphorylation and total protein stain of Wt and Hm left ventricles. A. Representative large-format SDS-agarose gel stained with Pro-Q Diamond to detect phosphoproteins and SYPRO Ruby to detect total proteins. Amount of protein load (µg) is shown at the bottom of the gel. B. Phosphoprotein slope shows analysis of plots of integrated optical density (IOD) vs. protein concentration. C. Slope of total proteins (C) represented by IOD vs. protein concentration. No differences were observed between Wt and Hm for phosphorylation or total protein slope for titin. D. The ratio of phosphoprotein:total protein was similar between Wt and Hm. Bars represent the means ±SE.
A

B

![Graph showing percent MHC for Alpha and Beta with error bars]

- **A**
  - Wt Wt Wt Wt Hm Hm Hm Hm
  - α-MHC β-MHC

- **B**
  - Percent MHC
  - Alpha Beta
  - Wt Hm
Figure 2.2. SDS-PAGE analysis of cardiac myosin heavy chain isoforms. A. Typical expression profile of Wt and Hm ventricles following Coomassie blue staining. Gel depicts α-MHC as the upper band and β-MHC as the lower band. B. Graphical analysis of MHC percentages in Wt and Hm shows no significant difference in cardiac MHC isoform expression. Bars represent means ± SE.
Figure 2.3. SDS-PAGE analysis of tibialis anterior myosin heavy chain isoforms. A. Representative gel image depicting typical expression profile of TA in Wt and Hm adults were examined as described by Talmadge and Roy (1993) with Coomassie blue staining. The fast-twitch TA expresses primarily type IIX (upper band) and type IIB (lower band) MHC. B. Analytical results showed no difference between Wt and Hm expression profile. Bars represent means ± SE.
Figure 2.4. SDS-PAGE analysis of tibialis anterior myofibrillar protein isoform composition. A. Representative image depicting myofibrillar proteins on a standard 12% SDS-PAGE gel of TA samples. B. No significant differences in protein expression were observed between Wt and Hm across any myofilament contractile proteins studied. MHC: myosin heavy chain; α-A: α-actinin; TnT: troponin T; Tm: tropomyosin; MLC-1: myosin light chain-1; TnI: troponin I; TnC: troponin C; MLC-2: myosin light chain-2; MLC-3: myosin light chain-3. Bars represent means ± SE.
CHAPTER THREE: THYROID HORMONE EFFECTS ON THE TITIN
EXPRESSION OF NORMAL AND MUTANT RATS

Abstract

The effect of thyroid hormone status on heart and skeletal muscle properties was determined in wild type rats (Wt) and those lacking the Rbm20 splicing factor (homozygote mutants, Hm). Rats were made hyperthyroid by daily T3 injects (21 days) or hypothyroid using propylthiouracil (PTU) for four months. Echocardiographic results showed that hyperthyroid rats (WtT, HmT) had left ventricular hypertrophy, increased heart rate, and decreased ejection time, while hypothyroid rats (WtP, HmP) exhibited depressed cardiac output and functionality. The cardiac myosin heavy chain (MHC) content was also influenced by thyroid hormone levels; WtT and HmT showed increased α-MHC, while WtP and HmP shifted to more β-MHC content. Cardiac titin isoform expression was influenced by thyroid hormone levels as well, since WtT and WtP showed a significantly higher proportion of N2BA titin relative to WtC (of total titin, 21.3% and 36.1% vs. 12.1%, respectively). However, the HmT and HmP titin profile did not change and only showed expression of a single giant N2BA, suggesting that Rbm20 is still necessary for the isoform shift brought about by thyroid hormone changes.

3.1 Introduction

Titin (also known as connectin), a ~3 MDa sarcomere protein discovered in 1970’s, plays a prominent role in maintaining sarcomere structure [Maruyama, 1976; Wang et al., 1979] and defining passive force [Horowits et al., 1986; Maruyama et al.,]
in striated muscle. Because of its large size, titin spans an entire half-sarcomere, from the Z-disk to nearly the M-line [Tskhovrebova and Trinick, 2003]. To provide structural support and flexibility to the muscle, titin contains several domains in its I-band region which perform these functions. I-band titin possesses three segments that are extended during stretch [Linke et al., 1999]. These are: Ig-domain regions, a PEVK region rich in proline (P), glutamic acid (E), valine (V), and lysine (K), and an N2B unique sequence in myocardium [Freiburg et al., 2000]. Differential splicing occurs in the Ig and PEVK regions and leads to multiple isoforms which are expressed in a tissue- and time-specific manner [Freiburg et al., 2000; Labeit and Kolmerer, 1995]. In the heart, the expression of the two major classes of isoforms, a smaller and stiffer N2B isoform and a larger and more compliant N2BA isoform, varies in species-specific and tissue-specific manner: the expression is predominantly N2B in the ventricles of small rodents, with a small percentage of N2BA [Cazorla et al., 2000; Neagoe et al., 2003]. The molecular weight of N2B titin is similar in all species (approximately 3 MDa), but different N2BA-containing isoforms can range from 3.2 MDa up to 3.4 MDa [Freiburg et al., 2000]. Furthermore, the N2BA isoform is predominantly expressed during embryonic and early postnatal stages [Lahmers et al., 2004; Opitz et al., 2004] and as development progresses, the expression shifts from N2BA to N2B [Warren et al., 2004]. This shift in titin isoform expression, resulting from alternative splicing events, is essential to provide adequate stiffness and the structural organization required for proper function of the developing heart. Thus, any disruption in alternative splicing of the titin gene would prevent the shift in titin isoform expression and lead to abnormal contractility.
of the heart, such as dilated cardiomyopathy [Makarenko et al., 2004]. For example, in the rat, a deletion of the RNA-binding motif protein 20 (Rbm20) gene, responsible for alternative splicing of titin, allows the expression of a giant N2BA isoform to persist into adulthood [Greaser et al., 2005; Greaser et al., 2008] and the resultant effect is ventricular dilation [Guo et al., 2012].

Altering the thyroid hormone status is known to have profound effects on the expression pattern of titin isoform in the heart: an increase in thyroid hormone (hyperthyroidism) elevates N2B expression in rat cultured embryonic cardiomyocytes [Krueger et al., 2008] whereas a decrease in thyroid hormone (hypothyroidism) elevates N2BA expression in adult rat ventricles [Wu et al., 2007]. Thyroid hormone also exerts control over the myosin heavy chain (MHC) content of cardiac tissue; altering thyroid hormone levels can shift the isoform profile from β-MHC to α-MHC in hyperthyroidism or α-MHC to β-MHC in the case of hypothyroidism [Danzi et al., 2005]. Importantly, hypothyroidism can bring about cardiac dysfunction, including dilated cardiomyopathy [Klein and Danzi, 2007]. Treatment of this affliction with thyroid hormone has been shown to alleviate the symptoms [Khochtali et al., 2011; Ladenson et al., 1992]. It has been hypothesized that the differential pattern of titin isoform expression in the heart results from thyroid hormone-induced changes in the PI3K/Akt signaling pathway [Krueger et al., 2008; Ojamaa, 2010].

The primary goal of the present study was to examine the effects of thyroid hormone-induced changes in the expression of titin isoform in skeletal and heart muscle of wild-type rats (Wt) and homozygous mutant (Hm) rats lacking a functional Rbm20
gene [Greaser et al., 2008]. In addition, transthoracic echocardiography was used to gain insight into the effects of thyroid hormone-induced changes in heart structure and contractility.

3.2 Materials and Methods

3.2.1 Animals and Tissues

Adult (older than 4 months, either sex) Sprague-Dawley rats were segregated based on genotype: wild type (Wt) and homozygote mutant (Hm) rats, which lack a functional Rbm20 gene, have been described previously [Greaser et al., 2008; Guo et al., 2012]. Wt and Hm rats were then split into control (n=9; WtC, HmC), hyperthyroid (WtT, HmT), and hypothyroid (WtP, HmP) treatment groups. Hyperthyroid rats were injected intraperitoneally with 0.1 mg/kg body weight of L-3,3’,5’-triiodothyronine (T3) daily for 21 days [Li et al., 2011]. Hypothyroid rats were gavaged orally with 0.1% 6-propyl-2-thiouracil (PTU) daily for four months [Wu et al., 2007]. All experiments and procedures involving animal care and handling were conducted in accordance with protocols approved by the University of Wisconsin-Madison Animal Care and Use Committee.

3.2.2 Thyroid Characterization and Echocardiography

At the end of the treatment period, rats were anesthetized with isofluorane and subjected to echocardiographic analysis described previously [Guo et al., 2012]. Briefly, cardiac function and dimensions were analyzed using a Sonos 5500 ultrasonograph with a 15-MHz transducer in M-mode (Philips, Andover, MA). Following the echocardiograms, the rats were weighed (body weight) and blood was drawn from the rats to quantify the
levels of T3 (hyperthyroid group) and thyroxine (T4; hypothyroid group) by ELISA (GenWay Biotech, San Diego, CA). The rats were then euthanized and the heart and skeletal muscles were harvested. Skeletal muscle was immediately frozen in liquid nitrogen and finally stored at -80°C until use for protein analysis, whereas left ventricles were dissected, blotted and weighed prior to freezing in liquid nitrogen and storing at -80°C.

3.2.3 Electrophoresis

A small piece (~100 mg) of left ventricle and skeletal muscle was homogenized in SDS sample buffer (8 M urea, 2 M thiourea, 3% SDS, 75 mM dithiothreitol, 50 mM TrisHCl, pH 6.8) using a small Dounce homogenizer as described previously [Warren et al., 2003]. To examine titin isoform expression, the samples were electrophoresed using a large-format 1% agarose gel and then silver-stained to detect titin isoforms [Warren et al., 2003]. The expression level of titin isoforms was quantified using NIH Image software (Version 1.63, National Institutes of Health, Bethesda, MD). Isoform band densities were compared to each other and against total density of all titin isoforms. For determination of the N2BA to total titin isoform ratio, intensities of all bands were summed and the N2BA band was divided by the total densities of all titin isoforms present. To examine the cardiac myosin heavy chain (MHC) isoform expression, the samples were electrophoresed using large-format 8% SDS-PAGE gels (30% acrylamide, 50:1 acrylamide:bisacrylamide ratio) modified from [Talmadge and Roy, 1993] and stained with Coomassie brilliant blue to detect MHC isoforms. The MHC isoform expression was quantified using the computer program GelBandFitter [Mitov et al.,
Skeletal muscle MHC was separated using the method previously described [Talmadge and Roy, 1993], stained with Coomassie blue and quantified using NIH Image.

### 3.2.4 Western Blotting

Left ventricles were homogenized in SDS sample buffer and electrophoresed on 8% small-format SDS-PAGE gels and transferred onto PVDF membranes using a Trans-Blot Turbo Blotting System (Bio-Rad, Hercules, CA). Important proteins of the PI3K/Akt signaling pathways were selected to study expression level. Antibodies directed against the pleckstrin homology (PH) domain of the Akt protein (1:750 dilution), phosphorylated Ser473 residue on Akt (1:1000), phosphorylated Thr308 residue on Akt (1:750), and phosphorylated Ser2448 residue on mammalian target of rapamycin (mTOR, 1:750) were used (all antibodies were from Millipore, Temecula, CA). The PH domain antibody was utilized to detect total Akt protein expression, while the phospho-specific antibodies measured the phosphorylation level of their respective residues. Higher phosphorylation levels demonstrate increased activity of the PI3K/Akt signaling pathway [Kuzman et al., 2005a]. Membranes were exposed to either rabbit or mouse-directed secondary antibodies (1:4000, GE Healthcare, Buckinghamshire, UK) and developed on CL-Xposure blue film (Thermo Scientific, Rockford, IL). Protein levels were normalized with a GAPDH antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and WtC was set equal to 1.

### 3.2.5 Statistical Analysis
Data was analyzed using a Proc GLM method in SAS (Version 9.3) to determine statistical significance, and p-values of less than 0.05 were considered significant. All numbers are expressed as means ± SE.

3.3 Results

3.3.1 Hyperthyroid Characteristics

Circulating serum levels of \( L-3,3',5' \)-triiodothyronine (T3) were tested using ELISA on control (WtC and HmC) and hyperthyroid (WtT and HmT) rats. Compared to WtC, HmC rats’ T3 serum level (ng/mL) was slightly lower \((p < 0.05)\). Circulating T3 was significantly higher in hyperthyroid groups relative to control groups. WtT was significantly higher than WtC (WtC: 0.78 vs. WtT: 1.18), and HmT was significantly higher than HmC (HmC: 0.65 vs. HmT: 1.30, Fig. 3.1A). This confirms earlier observations that a 21-day T3 injection was quite efficient at inducing hyperthyroidism [Li et al., 2011], probably to the same degree in WtT and HmT rats.

Left ventricular weight and left ventricular weight:body weight ratio of WtC and HmC rats was similar (Fig. 3.2), showing that Rbm20 status is not likely to have an effect on ventricular and body weights. WtT and HmT left ventricular weights were significantly higher \((p < 0.01)\) than their control counterparts (Fig. 3.2A), suggesting cardiac hypertrophy has occurred. The left ventricle:body weight ratio was higher \((p < 0.01)\) in WtT (3.23) and HmT (3.24) compared to WtC (2.46) and HmC (2.01) rats (Fig. 3.2B). T3 treatment (hyperthyroidism) has a profound impact on left ventricle weight:body weight in both Wt and Hm rats. This effect of T3 treatment most likely
results from T3-induced left ventricular hypertrophy and elevated metabolic rate lowering the body weight as reported previously [Morkin et al., 1983].

### 3.3.2 Hypothyroid Characteristics

Thyroxine (T4) serum levels were analyzed with ELISA on control (WtC and HmC) and hypothyroid (WtP and HmP) rats. PTU-treated hypothyroid rats demonstrated significantly lower ($p < 0.0001$) T4 levels (mg/mL) compared to control groups (Fig. 3.1B). PTU was shown to be quite efficient at decreasing T4 levels in both WtP (WtC: 2.36 vs. WtP: 0.28) and HmP (HmC: 2.64 vs. HmP: 0.35). This supports previous observation that a four-month PTU treatment is successful at inducing hypothyroidism [Wu et al., 2007], most likely to the same degree in WtP and HmP rats.

Left ventricle weights were similar across WtC, WtP, and HmC (Fig. 3.2A). HmC and HmP were also similar. The left ventricle:body weight ratio was also similar between these four groups. There was no corresponding increase in the left ventricle:body weight ratio as observed in hyperthyroid rats, and both Wt (WtC: 2.21 vs. WtP: 2.64) and Hm (HmC: 2.75 vs. HmP: 2.15) were similar (Fig. 3.2B).

### 3.3.3 Hyperthyroid Echocardiography

To assess in vivo left ventricular structure and function, echocardiographic analysis was performed on anesthetized control and T3-treated rats. Compared to WtC, fractional shortening (FS; Fig. 3.3A) was significantly lower in HmC rats, but ejection fraction (EF) was higher in HmT compared to HmC and was similar between WtC and WtT (Fig. 3.3B). Time intervals for isovolumic relaxation time (IVRT; Fig. 3.3C) and ejection time (ET; Fig. 3.3F) were significantly longer in HmC. Isovolumic contraction
time (IVCT; Fig. 3.3D) and heart rate (HR; Fig. 3.3E) were similar between WtC and HmC rats. The Rbm20 deletion was found to have a profound effect on FS and IVRT, but not on EF, IVCT, HR, and ET. The T3 treatment effects were qualitatively similar in both Wt and Hm rats. Genotype differences between WtT and HmT were observed. HmT reduced IVRT to a shorter time than did WtT. T3 treatment caused a significant increase in FS, EF, and HR and a decrease in IVRT, IVCT, and ET in HmT rats. There were more significant quantitative differences found in HmT vs. HmC rats than in wild type groups. Hyperthyroidism in Wt rats was only successful in changing HR and ET to a significant degree.

3.3.4 Hypothyroid Echocardiography

PTU treatment altered left ventricular cardiac function as analyzed through echocardiography (Fig. 3.3). FS and EF were similar in WtP and HmP relative to their control groups. However, IVRT, IVCT, and ET intervals were significantly longer in PTU-treated rats; WtP was longer than WtC, and HmP was longer than HmC. HR was also depressed in hypothyroid groups, comparing both WtP and HmP to their control counterparts. Some genotype-related changes were noted. PTU treatment had a similar effects on IVRT and IVCT (HmP > WtP). Other hypothyroid characteristics did not appear affected by genotype, including FS, EF, HR, and ET, since WtP and HmP were similar in these traits. PTU-induced hypothyroidism did alter some cardiac characteristics.

3.3.5 Myofilament Protein Profile in Hyperthyroid Rats
A typical agarose gel depicting left ventricle titin isoforms is shown in Figure 3.4. Wt ventricles expressed three titin isoforms, N2BA-N1 (3.39 MDa), N2BA-N2 (3.22 MDa) and N2B (2.97 MDa), whereas Hm ventricles expressed a single slow migrating titin isoform, N2BA-G (3.83 MDa), an observation consistent with previous studies [Greaser et al., 2008; Guo et al., 2012]. In Wt rats, T3 treatment had no apparent effect on the size of N2BA-N1, N2BA-N2, or N2B. Wt rats’ N2BA-1 and N2BA-2 proportions were measured. T3 caused an increase the expression of N2BA-N1 and reduced the expression of N2BA-N2 (Fig. 3.4B). Further gel densitometric analysis indicated that T3 treatment increased the overall expression of N2BA isoforms and reduced N2B expression (Fig. 3.4C). T3-induced hyperthyroidism caused a shift in titin isoform expression proportion from the shorter and stiffer N2B to the longer and more compliant N2BA isoform in Wt rats. This effect was absent in Hm rats, which did not undergo any changes to their N2BA-G isoform expression pattern.

Large-format SDS-PAGE was used to analyze cardiac MHC expression of left ventricles (Fig. 3.5A). The MHC isoform expression was similar in WtC (55.4 ± 2.1% α-MHC and 44.6 ± 2.1% β-MHC) and HmC (51.6 ± 2.1% α-MHC and 48.4 ± 2.1% β-MHC) ventricles (Fig. 3.5B), confirming earlier observation [Patel et al., 2012]. Thus, Rbm20 deletion has no significant effect on cardiac MHC isoform expression. T3 treatment totally eliminated β-MHC in Wt and Hm rats. This hyperthyroid-induced increase of the faster α-MHC expression is similar to that reported previously [Klein and Danzi, 2007].
Skeletal muscle MHC was analyzed using a small-format SDS-PAGE system [Talmadge and Roy, 1993]. Rat slow-twitch soleus and fast-twitch tibialis anterior skeletal muscle was selected for MHC isoform expression profile analysis. The expression levels of Type I and IIA MHC isoforms in soleus (Fig. 3.6A) and Type IIX and IIB MHC isoforms in tibialis anterior (Fig. 3.7A) were similar in WtC and HmC, suggesting no significant effect of Rbm20 deletion on skeletal muscle MHC expression. In soleus muscle, T3 treatment altered the MHC expression profile, shifting it to the fast MHC isoform (Fig. 3.6B). T3 treatment increased Type IIA isoform expression at the expense of Type I MHC, to the extent that WtT and HmT were significantly different than their control groups. Conversely, T3 did not affect MHC isoform expression in tibialis anterior, which expressed similar levels across WtC, WtT, HmC, and HmT (Fig. 3.7B). Only the slow-twitch soleus muscle was affected by thyroid treatment.

3.3.6 Myofilament Protein Profile in Hypothyroid Rats

Rat left ventricle was analyzed for titin isoform expression, and PTU-treated rats’ titin isoforms were examined. WtP continued to express N2BA-N1, N2BA-N2, and N2B, but the proportions of each were altered in comparison to WtC. WtP rats increased the relative expression of the larger N2BA-N1 at the expense of the N2BA-N2 isoform (Fig. 3.4B). Next, the ratio of N2BA isoforms to N2B was analyzed, and WtP showed a significant increase ($p < 0.0001$) in this ratio (i.e., an increased proportion of N2BA isoforms) relative to WtC. Interestingly, WtP also showed a significantly higher N2BA:N2B ratio compared to WtT (Fig. 3.4C). Just as in WtT, PTU treatment was
successful in shifting the titin profile to a more compliant phenotype. No changes to the HmP’s N2BA-G isoform were observed (Fig. 3.4A).

Cardiac MHC expression demonstrated a similar, but opposite, change in PTU-treated rats (Fig. 3.5A). PTU treatment dramatically shifted the MHC expression profile toward an increased proportion of β-MHC. Wt β-MHC proportion increased from 44.6 ± 2.1% to 94.4 ± 2.1% following PTU treatment, and Hm β-MHC levels increased from 48.4 ± 2.1% to 91.5 ± 2.1% after PTU administration (Fig. 3.5B). The shift in hypothyroid rats was not complete, but PTU did significantly alter the MHC profile.

In a similar manner, PTU altered the skeletal muscle MHC profile as well. PTU treatment changed the MHC expression in the soleus (Fig. 3.6A) but not the tibialis anterior (Fig. 3.7A). WtP and HmP both experienced an increased proportion of Type I MHC in the soleus at the expense of Type IIA (Fig. 3.6B). The change in WtC vs. WtP was significant, but HmP was not significantly different from HmC. The tibialis expression profile was similar across genotypes and treatment groups, and it appears that thyroid hormone levels specifically affect slow-twitch skeletal muscle fiber types (Fig. 3.7B).

3.3.7 Hyperthyroid PI3K/Akt Signaling Pathway

The effects of Rbm20 deletion and T3 treatment on several proteins of the PI3K/Akt signaling pathway in Wt and Hm left ventricle tissue were investigated by Western blotting (Fig. 3.8A). This was determined using antibodies directed against the PH domain of Akt, Akt phosphorylation sites using antibodies against Ser473 and Thr308, and mTOR phosphorylation state using an antibody against Ser2448. The
intensities of target proteins were expressed relative to GAPDH, and normalized to WtC values. The relative expression of PH domain and Ser473 was significantly higher ($p < 0.01$), Thr308 was lower, but not significant, and Ser2448 was higher, but not significant, in HmC compared to WtC rats (Fig. 3.8B-E). This suggests that Rbm20 deletion has a more pronounced effect on expression level of Akt and phosphorylated Ser473 Akt and a marginal effect, if any, on phosphorylated Thr308 Akt and Ser2448 mTOR. T3 treatment significantly increased the relative expression of PH domain in both Wt and Hm rats, of Ser473 in Hm but not in Wt rats, and of Ser2448 in both Wt and Hm rats.

3.3.8 Hypothyroid PI3K/Akt Signaling Pathway

PTU-induced hypothyroidism did not demonstrate the drastic changes that were evident in hyperthyroid rats. There were no differences in PH domain, Akt Ser473, and Akt Thr308 expression in WtC vs. WtP or HmC vs. HmP (Fig. 3.8B-D). Also, there was no difference in mTOR Ser2448 between WtC and WtP, but HmC was significantly higher than HmP (Fig. 3.8E). PTU did not affect PI3K/Akt signaling to the extent that T3-induced hyperthyroidism did, as only Hm rats’ mTOR phosphorylation state was affected.

3.4 Discussion

In the present study, we examined the effects of hyperthyroidism and hypothyroidism on titin and MHC expression, the phosphorylation state of proteins in the PI3K/Akt signaling pathway and ventricular function using Wt rats and rats lacking a functional Rbm20 gene (Hm). Multiple protein and cardiovascular changes were
observed, demonstrating the effect of thyroid hormone levels and Rbm20 on titin isoform expression.

HmC did not show any left ventricular hypertrophy relative to WtC, as left ventricle weights and left ventricle weight:body weight were similar between Wt and Hm. Echocardiography results did, however, demonstrate differences between WtC and HmC rats. Fractional shortening decreased in HmC, and isovolumic relaxation time and ejection time intervals were increased without a functional Rbm20 gene. These differences were established in a larger set (n=9) of younger animals (4-5 months of age) than reported previously [Guo et al., 2012]. These changes are similar to those of diastolic dysfunction [Rajan et al., 2007], supporting the observation that Rbm20 deletion can induce human dilated cardiomyopathy [Brauch et al., 2009; Li et al., 2010].

It is recognized that alternative splicing of a single titin gene facilitates the transition from N2BA titin isoform in neonates to ~10% N2BA and ~90% N2B titin isoform in adult rat ventricles [Warren et al., 2004]. Here we report that deletion of Rbm20 not only prevents such a shift in titin isoform expression with age but also causes the expression of a giant N2BA-G titin isoform, confirming earlier results [Greaser et al., 2008]. Cardiac MHC was unchanged in Hm rats, which indicates that Rbm20 does not dictate MHC content. Despite elevated expression levels of proteins in the PI3K/Akt signaling pathway involved in alternate splicing of titin, the absence of a titin isoform shift suggests that alternative splicing of titin via this pathway requires the presence of Rbm20 as reported earlier [Guo et al., 2012]. The mechanism by which Rbm20 activates PI3K/Akt signaling pathway is not entirely clear. We have previously shown that the
maximum Ca\textsuperscript{2+} activated force generated by skinned trabeculae lacking Rbm20 is ~35% lower than in trabeculae expressing Rbm20 [Patel et al., 2012].

### 3.4.1 T3-Induced Hyperthyroidism

Wt and Hm rats treated with T3 for 21 days had significantly higher serum levels of T3, showing that treatment was effective at inducing hyperthyroidism. Left ventricle weights were significantly higher in hyperthyroid groups, and the ratio of left ventricle weight:body weight was also significantly higher in hyperthyroid rats. This hyperthyroid-induced cardiac hypertrophy is a common effect of elevated thyroid hormone levels [Dorr et al., 2005; Morkin et al., 1983], which occurred regardless of Rbm20 status.

The thyroid hormone-stimulated cardiovascular activity was also manifest in the cardiovascular function results, which were increased following T3 treatment. Fractional shortening was decreased in Hm, while isovolumic relaxation and contraction time were increased in Hm rats. Changes in Wt rats were limited to heart rate and ejection time. This confirms previous data regarding effects of hyperthyroidism [Klein and Ojamaa, 2001]. The dilation inherent to the Hm phenotype did not impact the effects of hyperthyroidism, as cardiac output was enhanced to a greater degree than in Wt.

### 3.4.2 PTU-Induced Hypothyroidism

ELISA results demonstrated that T4 had significantly decreased following 4-month PTU administration, showing an approximately 10-fold decrease in serum T4 levels. Therefore, the quantity and length of PTU treatment was sufficient to induce hypothyroidism. In left ventricle weights, there was no difference between neither WtC
and WtP nor HmC and HmP. Further, the left ventricle weight:body weight ratio also showed no difference between control and hypothyroid groups. These results were consistent with those reported previously [Wu et al., 2007].

Changes occurred in cardiac functionality as analyzed through echocardiography, with decreased cardiac output inherent to hypothyroidism being demonstrated. No changes in fractional shortening and ejection fraction were observed between groups. The depressive effects of hypothyroidism did not affect the Rbm20-deleted phenotype, which had already demonstrated decreased cardiovascular function. Isovolumic relaxation and contraction time and ejection time intervals were significantly longer in PTU-treated animals (both genotypes) compared to their control groups. The significantly decreased heart rate of both PTU groups also mimics the brachycardia that is typically observed in hypothyroid patients [Klein and Ojamaa, 2001].

**3.4.3 Left Ventricular Titin Expression**

Prior research has shown the effect of altered thyroid status on titin isoform expression. Cardiomyocytes treated with T3 showed an increase in the N2B proportion [Krueger et al., 2008], and PTU administration to adult rats resulted in a larger titin isoform being expressed [Wu et al., 2007]. Here, we show changes in the titin isoform profile of rats that have been treated with T3 or PTU to induce hyperthyroidism or hypothyroidism, respectively. Of both N2BA isoforms present in adult Wt rats, the WtC animals expressed 36.6% of the larger N2BA-N1 isoform and 63.4% of the smaller N2BA-N2 titin isoform. T3 and PTU both significantly increased the proportion of the larger N2BA isoform. WtT rats expressed 68.4% N2BA-N1, and WtP rats expressed
59.4% N2BA-N1 following their respective treatments. While our data is not in strict accordance with that in the literature, we show that regardless of thyroid hormone levels, deviation from a euthyroid status will cause an upregulation of the compliant titin isoforms. We speculate that the increased N2BA-N1 of the WtT group is due to a compensatory mechanism that prevents excessive hypertrophy caused by hyperthyroidism. WtP rat’s increased N2BA-N1 may aid in reducing diastolic heart stiffness. Despite identical treatment to the Hm rats, no changes in titin isoform expression were observed. HmC expresses a single N2BA-G isoform band, as did HmT and HmP animals following treatment. This strongly suggests and supports previous observations [Guo et al., 2012] that Rbm20 is primarily responsible for titin alternative isoform expression.

**3.4.4 Left Ventricular Myosin Heavy Chain Expression**

Much is known regarding the influence of thyroid hormone on MHC expression and its ability to shift the isoform profile [Klein and Danzi, 2007]. Hyperthyroidism increases the proportion of α-MHC in rodent cardiac tissue, and hypothyroidism will shift the MHC profile toward a predominantly β-MHC phenotype. There was no significant difference between WtC and HmC rats, but hyper- and hypothyroid treatment groups exhibited significantly altered MHC isoform profiles. WtT and HmT shifted completely to 100% α-MHC, while WtP and HmP expressed 94.4 and 91.5% β-MHC, respectively. The hypothyroid groups did not experience a total conversion to β-MHC, because the thyroid gland was still partially active and produced reduced amounts of T4 (Fig.3.1B). However, it is known that a thyroidectomized rat will possess a complete β-MHC profile,
and this can be reversed with T3 treatment [Danzi et al., 2005]. Despite Rbm20’s absence, cardiac MHC will be affected by thyroid hormone levels.

### 3.4.5 Skeletal Muscle Myosin Heavy Chain Expression

Our results are consistent with previous observations that hyperthyroidism has its largest impact on slow muscle but is incapable of influencing change in fast-twitch skeletal muscle [Caiozzo et al., 1991; Fitzsimons et al., 1990]. The soleus demonstrated MHC changes to type I and IIA based on thyroid levels. WtC and HmC expressed similar proportions, and WtT and HmT increased the proportion of type IIA. However, thyroid hormone (and genotype) was unable to shift MHC content in tibialis anterior (TA). This limb muscle is comprised primarily of type IIX and IIB MHC, and all groups expressed similar levels of both isoforms. Hypothyroid rats showed similar results. Soleus muscle demonstrated changes in PTU-induced hypothyroidism. WtP and HmP increased the proportion of type I MHC relative to control groups. TA analysis did not show any difference in MHC proportion, regardless of thyroid hormone levels. Again, regardless of the genotype, thyroid hormone was able to alter MHC content in slow-twitch, but not fast-type, skeletal muscle. Our rat mutation seems to primarily affect titin and its related systems.

### 3.4.6 PI3K/Akt Signaling Pathway Proteins

Examination of activated proteins of the PI3K/Akt signaling pathway via Western blotting yielded interesting results. Thyroid hormone has been known to activate the PI3K/Akt signaling pathway in the heart [Kuzman et al., 2005b], and this pathway can also regulate serine/arginine-rich (SR) proteins [Blaustein et al., 2005], the protein family
to which Rbm20 belongs. SR proteins can regulate mRNA alternative splicing through a variety of mechanisms, including the PI3K/Akt pathway. This study shows an increase in Akt-PH domain expression as well as changes in the phosphorylation state of Ser473 in Hm compared to Wt. PH domain expression was also higher in WtT and HmT versus control counterparts, which may suggest an increased activation of this pathway.

PI3K/Akt signaling pathway proteins, for the most part, were not changed when PTU brought about hypothyroidism. The Akt PH domain expression in HmC and HmP was greater than WtC and WtP, respectively, but no other protein’s expression was altered by blockage of thyroid hormone production. Regardless of the PI3K/Akt signaling pathway protein expression levels, Rbm20 appears to be vastly more important to titin alternative splicing than thyroid hormone and its associated pathways. Further investigation is required to fully comprehend the effects of hyperthyroidism in our mutant rat model and its PI3K/Akt signaling pathway.

3.4.7 Conclusions

At this study’s onset, we were curious about thyroid hormone’s noted effects on titin expression. Our mutant rat model provided us with an opportunity to study the necessity of Rbm20 following the manipulation of thyroid hormone levels. Previous studies describing thyroid hormone’s effects on titin demonstrated changes [Krueger et al., 2008; Wu et al., 2007] have been conducted. However, the Hm rats presented a unique angle: that is, how are thyroid hormone, Rbm20, and titin expression related? From these results, we show that while thyroid hormone impacts cardiovascular and myofibrillar characteristics, titin expression and its related systems are relatively
unaltered. Thyroid hormone was incapable of rescuing normal titin isoforms, but led to other changes in MHC and cardiovascular function. Hyperthyroid groups showed increased cardiac output relative to control counterparts, and hypothyroid rats showed depressed function. Titin isoform levels were only altered in Wt animals. Wt titin expression was also shifted to a more extensible isoform profile with T3 and PTU treatment. The changes observed in echocardiography are characterized by the giant titin isoform of the Hm phenotype, as well as thyroid hormone levels. Thyroid hormone played a crucial role in our findings, but not in those regarding titin. It is possible that thyroid hormone will interact, directly or indirectly, with Rbm20 to bring about changes in the titin message. Ultimately, though, it appears that Hm rats require Rbm20’s presence in order to induce an appropriate titin expression profile; thyroid hormone alone is insufficient to play this role.

References


Figure 3.1. Rat serum thyroid hormone ELISA. A. Triiodothyronine (T3) serum levels of wild type (Wt) and homozygote mutant (Hm) control (WtC and HmC) and T3-treated (WtT and HmT) adult rats. Treatment groups had significantly higher \( (p < 0.0001) \) T3 levels compared to control groups. B. Thyroxine (T4) serum levels of control and PTU-treated (WtP and HmP) adult rats. Treatment groups had significantly lower \( (p < 0.0001) \) serum levels of T4 compared to control groups. Asterisks indicate significant difference relative to control groups.
Figure 3.2. Left ventricle mass and left ventricle to body weight ratio. A. Left ventricles were weighed immediately following sacrifice. Hyperthyroid groups were significantly higher ($p < 0.01$) than control groups. Hypothyroid groups were similar to their control groups. B. Left ventricle weights were divided by body weights to determine the ratio. WtT and HmT were significantly higher than other treatment groups. There was no difference between control groups and hypothyroid groups. Treatment groups with different letters are significantly different ($p < 0.05$).
Figure 3.3. Echocardiography results. A. Percent fractional shortening. WtC and WtT were similar, and HmT was significantly higher ($p < 0.05$) compared to HmC. WtT and HmT were similar to their control groups. B. Percent ejection fraction. WtC and WtT were similar, but HmT had higher percent ejection fraction relative to HmC. WtC and WtP were similar, as were HmC and HmP. C. Isovolumic relaxation time. WtC and WtT were similar, and HmC had a longer IVRT compared to all other groups. HmC was significantly higher ($p < 0.001$) compared to HmT. WtP was significantly longer than WtC, and HmP was significantly longer than HmC. D. Isovolumic contraction time. HmC was significantly longer than HmT, and WtP and HmP were significantly longer than WtC and HmC, respectively. E. Heart rate. WtT and HmT had the highest heart rates, significantly higher than WtC and HmC. HmP had a lower heart rate than HmC. F. Ejection time. WtC and HmC were significantly longer than WtT and HmT. WtP and HmP were significantly longer than WtC and HmC. Treatment groups with different letters of significance are different ($p < 0.05$).
**Figure 3.4. Cardiac titin expression.** A. Representative titin gel. Wt rats express primarily a 2.97 MDa N2B titin along with a combination of N2BA-1 and N2BA-2 isoforms. Hm rats express a 3.83 MDa N2BA-G isoform. B. N2BA proportions in Wt rats. WtT and WtP both express significantly higher levels of N2BA-1 ($p < 0.0001$) compared to WtC. C. N2BA:total titin ratio. WtT and WtP expressed significantly higher ratios ($p < 0.0001$) compared to WtC. Asterisks indicate significant difference relative to control groups.
Figure 3.5. Cardiac myosin heavy chain isoform expression. A. Representative cardiac myosin heavy chain SDS-PAGE. B. Cardiac MHC results. WtC and HmC had similar MHC content. WtT and HmT expressed exclusively α-MHC, and WtP and HmP expressed high levels of β-MHC. Control groups had significantly different ($p < 0.0001$) levels of MHC content versus treatment groups.
Figure 3.6. Soleus myosin heavy chain isoform expression. A. Representative soleus myosin heavy chain profile on SDS-PAGE. B. T3 and PTU treatment were successful in shifting myosin heavy chain content. WtC and HmC were similar. Results were compared within fiber type and across treatment groups. Treatment groups with different letters of significance are different.
Figure 3.7. Tibialis anterior myosin heavy chain isoform expression. A. Representative tibialis anterior myosin heavy chain profile. B. There were no differences across genotype or treatment. Results were compared within fiber type and across treatment groups. Bars indicate means ± SE.
**Figure 3.8. Western blot results.** A. Representative western blot results from Akt signaling pathway proteins. Akt pathway proteins levels were compared with GAPDH levels to determine relative expression to WtC. B. Akt PH domain. Hm groups expressed higher levels of general Akt protein relative to Wt groups. C. Phospho-Ser473 residue on Akt. HmT had the highest expression level, and Wt groups were similar. D. Phospho-Thr308 residue on Akt. There were no differences across genotype or treatment. E. mTOR phospho-Ser2448. HmP had the lowest expression levels, and T3 treatment increased phosphorylation levels. Treatment groups with different letters of significance are different.
CHAPTER FOUR: TITIN ISOFORM CHANGES IN A FAMILIAL HYPERCHOLESTEROLEMIC SWINE MODEL

Abstract

Familial hypercholesterolemic (FH) pigs develop atherosclerosis, but the effects on the myocardium are unknown. Here, we report changes in the isoform expression of the giant protein titin following dietary restrictions to FH pigs. Titin is responsible for maintenance of passive tension and structural support within the heart, and changes to its isoform expression have been found in human coronary artery disease. Porcine titin normally expresses a small N2B titin and two larger N2BA titin isoforms. However, when FH swine were limit-fed to 80% (80) or 60% (60) of ad libitum, the presence of a novel, larger N2BA titin molecule was discovered. This isoform was not present in conventional animals. The novel N2BA isoform (NE) comprised between 5-10% of the total titin and was found to be approximately 3.58 MDa in size in the limit-fed pigs. Myosin heavy chain isoform content and Rbm20 levels, the protein responsible for titin alternative splicing event, were unchanged. Crosses of FH pigs with guinea swine (FG) also showed the presence of NE titin. Intriguingly, FG left ventricles and atria were comprised of two or three N2BA titin isoforms, while right ventricles always contained three N2BAs. This is the first study that demonstrates that titin isoform expression can be altered by dietary intervention.
4.1 Introduction

Coronary artery disease (CAD) remains a leading cause of death in the developed world [Breslow, 1997], and is attributed to approximately 40% of U.S. deaths [Berneis et al., 2005]. Atherosclerosis, the thickening of the arteries, is one of the most common CADs [Ross, 1999]. Hypercholesterolemia is a risk factor for atherogenesis and is characterized by elevated levels of plasma low-density lipoprotein (LDL) cholesterol [Kannel et al., 1979]. The deposition of lipid and plaque build-up on the arterial wall can lead to cardiac ischemia and infarction, and patients suffering from hypercholesterolemia will have increased quantities of macrophages and lipids [Napoli et al., 1997; Simionescu et al., 1986]. Often, patients demonstrating hypercholesterolemia show high lipid and cholesterol levels in the blood due to diet or lifestyle [Int Task Force Prevent Coron Heart, 1998]. However, genetics can also induce CAD; familial hypercholesterolemia (FH) has been discovered in individuals with a mutation in the LDL receptor (LDLR) gene. LDLR removes LDL from the bloodstream to be processed [Jansen et al., 2005]. Because of the malfunctioning receptor, LDL cannot be cleared from the arteries. The FH phenotype is characterized by normal levels of high-density lipoprotein (HDL) and significantly higher levels of LDL, leading to an increased risk of CAD [Goldstein and Brown, 1979].

The Rapacz FH swine model has been studied extensively for several decades, since its characteristics closely resemble the CAD of FH patients [Rapacz et al., 1986]. FH pigs will develop elevated LDL levels and atherosclerotic lesions even when provided a low-fat, low-cholesterol diet [Checovich et al., 1984]. It was determined that FH
animals are prone to CAD, myocardial infarction, and ischemia, rendering them very similar to human patients afflicted with the same conditions [Hasler-Rapacz et al., 1995; Prescott et al., 1995; Prescott et al., 1991]. Specifically, it was revealed that HDL levels are reduced in FH pigs, but the ratio of total cholesterol to HDL is elevated [Hasler-Rapacz et al., 1994], which correlates closely with human familial hyperlipidemias [Prescott et al., 1991; Rapacz et al., 1986; Rosenfeld et al., 1988]. The presentation of the FH phenotype was found in pigs homozygous for an LDLR mutation [Hasler-Rapacz et al., 1998], leading to a depressed ability to clear the bloodstream of LDL. Indeed, FH swine provided with a high-cholesterol diet experienced heightened total cholesterol [Hasler-Rapacz et al., 1994], and animals given access to ad libitum feed also presented aggravated features. Recent efforts have been directed toward the introduction of the FH mutation to a colony of guinea swine. The characterization of the FH-guinea swine crossbreds is still under investigation.

This FH animal model has been the subject of multiple studies, but it was the intent of this report to elucidate the FH phenotype’s effect on the myocardium, in particular the isoform expression of the giant protein titin. It is well-established that heart failure and ischemia will lead to rampant changes within the myocardium and myofibril [Jin et al., 2008], and pathophysiologies induced by titin dysfunction has been studied [LeWinter et al., 2007], but there remains much to be learned about the link between titin and CAD.

Titin, or connectin [Maruyama, 1976; Wang et al., 1979], is the largest known protein, and in cardiac tissue it maintains passive force and modulates the sarcomere’s
role in ventricular filling [LeWinter et al., 2007; Tskhovrebova and Trinick, 2003]. This is accomplished through titin isoform expression modulation, since titin size will dictate the degree of cardiac flexibility and resting tension [Neagoe et al., 2003]. Cardiac titin contains mixtures of a stiffer N2B isoform and a larger, more compliant N2BA isoform. These isoforms will be expressed in varying proportions, dependent upon species and developmental stage. Expressed at high levels in prenatal and perinatal mammals, N2BA decreases in proportion during development, being replaced by more N2B. The final ratio varies widely between species [Cazorla et al., 2000; Freiburg et al., 2000; Labeit and Kolmerer, 1995]. This developmental isoform switch is imperative for proper cardiac function, and its failure will lead to pathology. Large mature mammals, including humans and pigs, express higher proportions of N2BA titins [LeWinter and Granzier, 2010] and demonstrate increased cardiac compliance [Chung et al., 2011] compared to rodent hearts. A recent study demonstrated that guinea swine possess high amounts of N2BA titin, resulting in a corresponding decreased passive tension [LeWinter et al., 2010]. However, a perturbation to this isoform profile is also a source of several cardiomyopathies. For example, abnormal titin expression has been implicated as a root cause of ischemic heart failure [Neagoe et al., 2002].

Since hypercholesterolemia leads to arterial blockage, ischemia, and CAD, it is worth exploring these effects on titin. The FH swine model allowed us to study the effects of diet-induced hypercholesterolemia on cardiac composition, especially that of the extensible protein titin. We report that all conventional swine expressed two N2BA titin isoforms, and most ad libitum fed FH pigs expressed two N2BAs, while limit-fed FH
animals showed a novel, larger N2BA titin. FH animals limited to 80% or 60% ad
libitum caused a reversion to a more extensible titin isoform. The limit-fed FH swine
expressed three N2BA titins in total; all pigs expressed N2B in conjunction with these
N2BA isoforms. Therefore, it is shown that the titin isoform ratios can be altered through
dietary intervention. This finding holds great promise in the treatment and prevention of
ischemia and atherosclerosis.

4.2 Materials and Methods

4.2.1 Animal characterization

In the first portion of this study, adult swine used were either conventional,
mixed-breed commercial animals (C) or familial hypercholesterolemic pigs (FH) that
have been described previously [Prescott et al., 1995; Rapacz et al., 1986]. The FH
model exhibits spontaneous cardiovascular disease and can demonstrate ischemic heart
disease. A total of 18 female FH pigs from 4 litters were group-housed post-weaning for
one week, after which time they were housed individually. Following dietary treatment,
FH pigs were sacrificed at approximately 9 months of age via stunning and
exsanguination following standard industry procedure. Mixed sex C pigs were group-
housed for the entire treatment duration. Both groups of animals were fed a standard
corn-soybean diet, and FH pigs were divided into three groups. One group was fed ad
libitum (AL), another group 80% ad libitum (80), and the last group was fed 60% of ad
libitum (60). C animals were also provided 100% ad libitum feed. Feed amounts for
60% and 80% ad libitum were calculated using mean weight and feed intake of the ad
libitum group. It is important to note that conventional pigs have a different genetic
background than that of FH pigs and were therefore unable to serve as a true control group. However, titin is not believed to be a related genetic condition in FH pigs.

Next, FH X guinea (G) swine crosses were produced. The FH pigs described above were crossed with guinea swine to produce an F1 generation of heterozygotes (FG). F1 crossbreds were crossed with each other to create a mixed F2 generation (normal, heterozygote, and homozygote for the FH mutation). Mixed sex F2 crosses were provided ad libitum feed and sacrificed at 6 months of age. The F2 generation of animals was used for analysis in this study, although there was no method so far to distinguish the genotype of an individual pig. Titin agarose gel electrophoresis [Warren et al., 2003] was performed to segregate the F2 pigs into two separate groups. Of the 23 pigs analyzed via gel electrophoresis, 14 showed 3 N2BA titins (G3), and 9 showed 2 N2BA titins (G2). For analysis, G3 pigs were compared against G2 pigs since we were unable to determine the genotype. A minimum of four pigs per group were utilized in this study. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

4.2.2 Tissue samples

Immediately following exsanguination and evisceration, whole hearts were collected and a small piece (~100 mg) of left ventricle (LV), right ventricle (RV), and left atrium (LA) was excised, flash-frozen in liquid nitrogen, and then stored at -80°C until processing. For the FG portion of this study, pigs labeled “G3” possessed three N2BAs and those labeled “G2” possessed two N2BA titin isoforms. Care was taken to obtain heart tissue at identical locations from all animals. Cardiac tissue was removed from the
freezer and homogenized in a small handheld homogenizer with SDS sample buffer (8 M urea, 2 M thiourea, 3% SDS, 75 mM dithiothreitol, 50 mM TrisHCl, pH 6.8) according to the previous method [Warren et al., 2003].

4.2.3 Electrophoresis

In this study, cardiac titin isoforms and myosin heavy chain (MHC) isoforms were analyzed using specialized electrophoretic methods. For titin isoform expression, electrophoresis was performed on samples in a large-format 1% agarose gel and then silver-stained to determine the relative levels of titin isoform [Warren et al., 2003]. The expression proportions of titin isoforms were quantified with NIH Image (Version 1.63, National Institutes of Health, Bethesda, MD). Titin isoform band densities were summed, and individual isoforms were calculated as a percentage of total titin expressed. All pigs expressed, in decreasing size: N2BA-N1 (N1), N2BA-N2 (N2), and N2B isoforms. Depending upon the treatment group of FH pigs, some animals demonstrated the presence of a novel, larger, third N2BA isoform (NE). Titin molecular weights were also calculated on silver-stained gels using the previously described method [Warren et al., 2003]. For cardiac MHC isoform analysis, large-format SDS-PAGE was used to separate α-MHC (upper band) and β-MHC (lower band). MHC gels were 8% acrylamide (50:1 acrylamide:bisacrylamide ratio) modified from the previous report [Talmadge and Roy, 1993], stained with Coomassie brilliant blue, and destained in a 10% methanol and 7.5% acetic acid aqueous solution. Gels were dried between sheets of cellophane and scanned into Adobe Photoshop.

4.2.4 Western blotting
Since it is known that the splicing factor Rbm20 is responsible for titin isoform expression [Guo et al., 2012], FH pig LV tissue samples were split into those expressing two (2N2BA) or three (3N2BA) N2BA bands. Small-format 8% polyacrylamide gels were electrophoresed, proteins were transferred onto a PVDF membrane using a Trans-Blot Turbo Blotting System (Bio-Rad, Hercules, CA), and membranes were blocked in 5% non-fat dry milk overnight at 4°C. A rabbit Rbm20 antibody (1:1000 dilution) was exposed to the membrane for 3 hours, and a secondary anti-rabbit antibody (1:4000, GE Healthcare, Buckinghamshire, UK) was used. Rbm20 expression levels were normalized with a GAPDH antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). For the FG tissue, two antibodies were employed to discern the presence of specific exons of the titin protein. Large-format 1% agarose gel electrophoresis was used to separate the titin isoforms. Following electrophoresis, the proteins from the agarose gels were transferred as above onto PVDF membranes. The membranes were blocked in 5% non-fat dry milk at 4°C overnight. An H4 titin antibody [Trombitas et al., 1999] was used at a 1:4000 dilution and exposed for 3 hours, followed by anti-mouse antibody incubation (1:8000 GE Healthcare) for 1 hour. H4 antibody recognizes several epitopes in the C-zone region of the A-band, exons that are constitutively expressed in all full sized titins. FG pig tissue was also immunoblotted using an antibody directed against exons 87-88 (87-88) of the titin, found within the middle Ig domain. After agarose gel electrophoresis, these tissues were manipulated in the same manner as the H4 antibody, although 87-88 was diluted 1:750 and anti-rabbit antibody (GE Healthcare) was diluted 1:4000. All
immunoblots were developed on CL-Xposure blue film (Thermo Scientific, Rockford, IL).

4.2.5 Quantitative real-time RT-PCR

TRIzol (Invitrogen, Carlsbad, CA) was used to extract total RNA, according to the manufacturer’s instructions. Approximately 60 ng of RNA was reverse-transcribed with 5 µM random hexamers, 1 mM dNTP, 7.5 mM MgCl₂, 40 U RNasin (Promega, Madison, WI), 5X ImProm-II Reaction Buffer, and 250 U of ImProm-II Reverse Transcriptase (Promega, Madison WI). The reaction tubes were incubated at 25°C for 15 min, 42°C for 60 min, and 70°C for 15 min, and cooled to 4°C. The produced cDNA was stored at -80°C until use. Four primer pairs were created and were confirmed with gel analysis to produce a single PCR product. The primer sets amplified titin exons 60, 75, and 326; GAPDH primers were used to normalize the relative amount of target mRNA. The primer sets used are shown in Table 4.2. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) with a 20 µl reaction. 96-well reaction plates were run on an Opticon 2 real-time PCR instrument (MJ Research (St. Bruno, Canada) with a denaturation step at 95°C for 15 sec, an annealing and extension step at 60°C for 1 min, for a total of 40 cycles. A minimum of two independent readings were used for analysis. The relative amount of target mRNA was normalized to GAPDH mRNA amount according to previous methods [Pfaffl, 2001]. FG pigs used here were separated into G3 and G2 groups, but since RV always contained three N2BA isoforms, only G3 was analyzed in RV tissue.

4.2.6 Statistical analysis
Data was analyzed using a Proc GLM method in SAS (Version 9.3) to determine statistical significance, and p-values of less than 0.05 were considered significant. All numbers are expressed as means ± SE. Bars with different letters of significance were different ($p < 0.05$). Bars with asterisks in Fig. 4.4 were different than other groups ($p < 0.05$).

4.3 Results

4.3.1 FH myosin heavy chain expression

Previous studies indicate that healthy, adult swine, cardiac tissue will only demonstrate one MHC isoform, depending on the location. Myocardial tissue from the atria will show only α-MHC, while ventricular tissue expresses β-MHC [Reiser and Kline, 1998]. Humans show a similar MHC profile in contrast to small rodents, which express both isoforms in a given cardiac region to varying degrees. However, in the diseased human heart, this balance can be disrupted, leading to a shift in the MHC expression and a change in the contractile profile [Gupta, 2007]. A representative FH MHC gel is shown in Figure 4.1. Adult rat left ventricle expresses both MHC types, while control (C) pig left ventricle only shows β-MHC. Pigs were segregated into those groups permitted ad libitum feed (AL), 80% of ad libitum (80), and 60% of ad libitum (60). Similarly, there was no change across any of the FH left ventricle (LV) treatment groups, as all pigs expressed 100% β-MHC. Right ventricular (RV) samples also showed exclusively β-MHC, while left atria (LA) only expressed α-MHC. There was no MHC isoform shift regardless of dietary effect and treatment in the FH pig model. Thus it appears that the FH mutation does not directly impact cardiac MHC expression.
4.3.2 *FH titin expression*

In the conventional pig, cardiac titin is made up of several isoforms: a smaller N2B and two larger N2BAs. Previous human studies investigating ischemia-induced cardiomyopathy showed an increased proportion of N2BA titin at the expense of N2B [Neagoe et al., 2002]. Here, we show the appearance of a novel N2BA titin band (NE, Fig. 4.2) that is larger than the known N2BA isoforms in particular treatment groups. Both conventional pigs (C) and ad libitum fed (AL) FH pigs only showed two N2BA titins in most cases, while 80% and 60% limit fed pigs both displayed a new titin isoform band. None of the C pigs showed a third N2BA band, and of the five AL pigs analyzed, only one showed NE titin. Conversely, five out of six 80 pigs and four out of five 60 pigs showed the N2BA-NE titin at the expense of N2BA-N2 (Fig. 4.3A). One from each of the 80 and 60 groups did not show NE titin. The animal that did not show the NE band was the same across all tissues. In LV tissue, C pigs had higher proportions of N2B, while not demonstrating any N2BA-NE. Trends were similar in RV (Fig. 4.3B); C did not have any N2BA-NE, but had much higher ($p < 0.0001$) proportions of N2BA-N2 relative to 80 and 60. AL pigs also had significantly higher levels of N2BA-N1 titin compared to C ($p < 0.001$), suggesting that hypercholesterolemia may demonstrate some ischemia-induced changes. Limit-feeding the 60 pigs led to a higher proportion ($p < 0.05$) of N1 relative to 80% ad libitum. LA samples (Fig. 4.3C) showed similar trends. NE titin was observed mostly in 80 and 60 animals, and these pigs’ N1 proportion was significantly higher ($p < 0.01$) than C and AL pigs. This shift is at the expense of the
N2B proportion, which is lower in 80 and 60. Overall, changes in titin isoform proportions were similar across the three tissue types analyzed.

Next, isoform ratios were calculated. FH left ventricles (Fig. 4.4A) all had higher percentages of N2BA titin relative to total titin compared to C ($p < 0.05$). Similar results were found in RV, and AL, 80, and 60 were all similar (Fig. 4.4B). However, left atria showed a similar N2BA percentage in C, AL, and 60 pigs, but 80 was significantly higher ($p < 0.05$) compared to other groups (Fig. 4.4C). The ratio of N2BA:N2B titin showed similar trends (Fig. 4.5). C had lower proportions of N2BA in LV and RV (Fig. 4.5A-B), but 80 LA had a significantly higher proportion compared to C LA ($p < 0.05$, Fig. 4.5C).

### 4.3.3 FH Rbm20 expression

To quantify the levels of Rbm20, the splicing factor responsible for titin isoform differences, pig LV samples were split into two groups: one group containing C and AL pigs, and the other comprised of 80 and 60 pigs. Because the former contained two N2BA bands, it was designated 2N2BA, and the latter contained three N2BA bands, it was designated 3N2BA. AL pigs with two N2BAs were used for analysis, and 80 and 60 pigs with NE titin were used. A representative western blot of these samples is shown in figure 4.6A. Using GAPDH to normalize the Rbm20 expression level, it was determined that there was no difference between those left ventricles containing three or two N2BA isoforms (Fig. 4.6B). 2N2BA pigs expressed similar Rbm20 levels relative to 3N2BA pigs (1.00 vs. 1.16, respectively).

### 4.3.4 FG myosin heavy chain expression
Following the crossbreeding of the FH swine model with a guinea swine breed, cardiac tissue was analyzed. Cardiac MHC was analyzed using large-format SDS-PAGE. As in FH swine, FG pigs also showed no discernible difference in MHC isoform expression, regardless of genotype (Fig. 4.7). Since FG pigs were of mixed genotype, this information indicates that regardless of the mutation’s presence, MHC content is unaffected. Both types of ventricular tissue were found to express 100% β-MHC, and left atrium expressed 100% α-MHC. This data shows that FH genotype does not alter MHC content in the heart.

4.3.5 FG titin expression

FG pigs’ titin was also analyzed to determine the titin expression profile. FG pigs appeared as two distinct groups, one expressing three N2BA bands (G3), and the other expresses two N2BAs (G2). The representative gel image shows these different groups (Fig. 4.8). Wild type rats express predominantly N2B titin at 2.97 MDa, and human soleus exhibits only an N2A titin at 3.70 MDa. FG left ventricle and left atria showed two different groups (G3 and G2), while all right ventricular tissue exhibited three N2BA bands (G3). In LV, G3 animals had significantly higher ($p < 0.05$) proportions of NE and N1, and G2 had higher levels of N2B titin (Fig. 4.9A). For RV analysis, G3 and G2 pigs (based on LV gel classification) were studied separately, but both groups demonstrate similar proportions of all titin isoforms (Fig. 4.9B). Left atrial tissue followed a similar trend as left ventricle, with the presence of NE in G3 animals and a significantly lower ($p < 0.001$) proportion of N2B relative to G2 pigs (Fig. 4.9C). FG titin molecular weights were also determined using the method described [Warren et al., 2003], and isoform
molecular weights appear in Table 4.1. G3 and G2 titin sizes were analyzed separately, but there was no significant size difference within or across tissues. NE molecular weights were between 3.571 and 3.588 MDa and were similar across all analyzed tissues. Regardless of whether FG pigs showed two or three N2BA isoforms, N1, N2, and N2B isoform size was unchanged across groups. This would suggest that despite their genetic background, FG pigs will express titins with highly similar exonic profiles, and the presence of the NE isoform in LV, RV, and LA is comparable in identity as well.

FG titin isoform ratios were also calculated. As a percent of total titin, G3 N2BA levels were significantly higher than G2 in LV (Fig. 4.10A) and LA (Fig. 4.10C, p < 0.05), but percent N2BA of total titin in RV were similar between groups (Fig. 4.10B). The ratio of N2BA:N2B showed a similar trend: LV and LA (Fig. 4.11A and 4.11C) had significantly increased (p < 0.01) proportions of N2BA in G3 animals relative to G2. RV (Fig. 4.11B) did not show a difference between groups. G3 animals, which expressed NE titin, were successful in shifting titin isoform expression to increased compliancy.

4.3.6 FG titin protein exon expression

FG pigs’ titin exonic expression was also studied through immunoblotting. The H4 anti-titin antibody was used to test if the different isoform bands between G3 and G2 pigs all contained titin (Fig. 4.12A). This was successfully demonstrated, with G2 and G3 samples showing 2 and 3 N2BA isoform bands respectively plus the one for N2B. An antibody directed against exons 87-88 (Fig. 4.12B) targeted the N2BA isoforms, the only isoforms previously known to contain exons 87 and 88. Therefore, G3 animals still showed three N2BA isoforms, while G2 pigs showed the presence of only two N2BA
isoforms. As in the silver-stained titin gels in Figure 4.8, all right ventricles showed three N2BA isoforms, while left ventricles and left atria were split into two groups.

### 4.3.7 FG swine titin gene expression levels

Previous studies have demonstrated that the exon expression changes responsible for variation in titin isoform size are mainly localized in the middle Ig region (exons 50 to 90) [Freiburg et al., 2000]. Thus it was suspected that the NE titin might have additional middle Ig domains due to inclusion of more exons in the corresponding mRNA. We therefore compared the levels of several middle Ig exons with the level of a constitutively expressed exon from the titin A-band region. Quantitative, real-time PCR was utilized to determine the expression levels of specific titin exons. Primers targeting exon 60, 75, and 326 were used to analyze exonic expression of the titin sequence. The primer sets used for this portion of the study are listed in Table 4.2. At this time the entire porcine titin sequence is incomplete, but a single band was obtained following normal PCR with each of these primer sets. Conventional and FG swine were analyzed in this portion of the study. The gene of interest was expressed relative to GAPDH expression, and FG pigs were compared to a conventional pig’s expression level (Table 4.3). The large variability between samples somewhat limits the confidence in the conclusions. However, the studied titin exons were found to be expressed at significantly higher levels than their conventional counterparts. Exon 60 levels were elevated relative to C pigs in all animals studied except for one G3 left ventricle (Table 4.3A). There did not appear to be a difference between exon 60 in G3 and G2 LV, but G3 LA showed much higher levels compared to G2 left atrial tissue. Similarly, exon 75 expression was elevated in all FG
pigs analyzed relative to C (Table 4.3B), and there was no difference between G3 and G2 animals. Lastly, exon 326 expression also demonstrated the same trend as the prior two analyzed exons; all FG pigs’ 326 expressions were much higher relative to C pigs (Table 4.3C). These results suggest that FG swine maintain high titin expression despite their novel genetic status, and that the hypercholesterolemic effects of the mutation accompany increased message expression levels of the giant protein titin.

4.4 Discussion

In the present study, the cardiovascular effects of a genetic strain of pigs with high circulating cholesterol levels were investigated. This swine group has been the subject of much study [Hasler-Rapacz et al., 1995; Rapacz et al., 1986], as they are an excellent familial hypercholesterolemic model. Since myocardial proteins can be altered in a diseased state, the main emphasis of this report will be directed toward the effect of hypercholesterolemia on heart function. Of particular note are the adjustments to the giant protein titin, which is known to change in the diseased heart [LeWinter et al., 2007]. In the first set of experiments, FH pigs were limit fed, bringing about the presence of a third N2BA (NE). The FG swine used in the second portion of this study showed expression of the NE titin isoform as well.

4.4.1 FH myosin heavy chain expression

As one of the most critical proteins in muscle, myosin must be uniquely attenuated to properly function in a specific location. Myosin heavy chain isoforms are precisely expressed and mammalian cardiac muscle is comprised of either α-MHC or β-MHC, dependent chiefly upon the developmental timeframe and localization. In large
mammals, left and right ventricles are composed almost entirely of β-MHC, the proportion of which increases as development occurs. Conversely, α-MHC is the predominating isoform found in the atrial tissue of swine and humans [Weiss and Leinwand, 1996]. However, MHC isoform shifts will occur if the cardiac system transitions to a state of heart failure. Human patients suffering from ischemic or dilated cardiomyopathy were discovered to have left atria with significantly higher levels of β-MHC, while ventricular tissues still showed high levels of β-MHC [Reiser et al., 2001]. All FH pig groups, regardless of dietary treatment, did not experience a shift in MHC proportions despite suffering from apparent atherosclerosis and ischemia. Left and right ventricles expressed 100% β-MHC, and left atria demonstrated the exclusive presence of α-MHC (Fig. 4.1). FH swine were sacrificed at roughly 9 months of age following 8 months of dietary treatment. While this time period should have been sufficient for MHC transition, providing al libitum feed to the AL group did not change their MHC profile. It is understandable that 80 and 60 animals were unaltered, since limit-feeding should have prevented a pathology-induced MHC transition.

4.4.2 FH titin isoform expression

In much the same way as MHC content, titin undergoes isoform changes throughout the developmental process. Cardiac titin exists as the smaller N2B and the larger N2BA isoforms. Fetal stage titin is exclusively compliant N2BA, with the transition to stiffer isoforms occurring postnatally [Opitz et al., 2004]. Currently, porcine titin isoform expression warrants further investigation, but larger mammals generally have higher proportions of N2BA compared to smaller mammals [Neagoe et al., 2003].
Recent studies examining swine cardiac titin functionality found that pig titin has a similar physiological stretch profile and isoform expression to human tissue [Chung et al., 2011], and that miniswine left ventricle also expresses higher levels of extensible N2BA [LeWinter et al., 2010]. LeWinter and colleagues have reported that miniswine can achieve longer sarcomere lengths without heightened tension development, primarily through higher levels of N2BA. It has also been established that ischemic human patients will undergo a titin isoform shift toward the direction of increased N2BA and decreased myofibrillar stiffness [Neagoe et al., 2002].

An understanding of these two factors led us to examine the relationship between familial hypercholesterolemia and titin in an FH swine model. A novel, third N2BA isoform (NE) was observed in FH pigs that were limit-fed to 80% and 60% of ab libitum and one FH pig fed ad libitum (Fig. 4.2). Across left and right ventricles and left atria, restricting the diet over an 8 month period led to NE expression. The presence of this new N2BA was at the expense of N2BA-N2 titin, the proportion of which dropped in 80 and 60 FH pigs, and C and AL pigs had higher levels of N2B. These findings suggest that the extent of ischemia is not total in FH pigs, since AL should have expressed higher levels of the compliant N2BAs. Human CAD shows increased expression of N2BA relative to N2B; thus, 80 and 60 pigs should have demonstrated increased N2B at the expense of N2BA. However, based on the gel analysis in figures 4.3-4.5, we found that limit fed animals increased their compliant titin. The expression of the N2BA-NE titin in limit-fed animals invites the idea that, through the presence of increased compliancy, cardiac titin can restructure the heart to compensate for the onset of ischemic heart
disease. Conventional swine used in this study also bears an explanation, since the genetic background between C and FH pigs are different. All C swine showed two N2BA titins, but we are sensitive to the fact that another factor may be at play within the FH mutant swine which results in NE titin expression.

The FH pig mutation is unable to remove LDL from circulation, increasing the risk of cardiovascular disease [Jansen et al., 2005]. It may be that the titin isoform changes to the limit-fed pigs are compensatory to relax the tension on the heart that would otherwise build up in hypercholesterolemic subjects. It is unclear at this time why limit-fed swine would increase their compliant titin levels, including the presence of a large, novel N2BA. The manifestation of NE titin is a unique finding that bears further study into the mechanism behind its appearance. This is the first study to demonstrate a difference in titin isoform expression following dietary intervention.

4.4.3 FH Rbm20 expression

Rbm20 is regarded as the factor responsible for titin alternative splicing [Guo et al., 2012], and its malfunction leads to dilated cardiomyopathy and significantly larger titin isoforms. A rabbit polyclonal antibody and western blotting was used to determine Rbm20 levels in FH pigs. Samples were split into two groups: one composed of conventional and ad libitum-fed pigs (2N2BA); and one made up of 80% and 60% limit-fed pigs (3N2BA, Fig. 4.6A). The levels were normalized to GAPDH protein expression, and although 3N pigs expressed more relative Rbm20, no statistical difference was shown in the analysis (Fig. 4.6B). This data may indicate that some other mechanism is responsible for the increased and novel N2BA expression, or that perhaps Rbm20 acts in
a more indirect manner in titin alternative splicing. The presence of Rbm20 in swine has not yet been fully characterized, so this remains a possibility. More study is required to elucidate how Rbm20 functions in pig titin alternative splicing.

4.4.4 FG myosin heavy chain expression

Showing a similar expression pattern as FH pigs, the FG swine, sacrificed at 6 months, also did not demonstrate a change in MHC levels. Both ventricles expressed exclusively β-MHC, and left atrium expressed only α-MHC (Fig. 4.7). This data strongly suggests that the FH swine model does not impact MHC expression. While studies examining other species’ MHC alterations in both the healthy and diseased state, conventional and FH swine did not undergo any change in their myosin content.

4.4.5 FG titin isoform expression

FG pigs’ titin expression remains to be further investigated, although it is shown that left ventricles and atria can express two or three types of N2BA titin molecules, while right ventricles will always show three N2BAs (Fig. 4.8). At present there is not a way to determine the genotype of the FG pigs used in the current study. To produce FG pigs, normal FH pigs were crossed with guinea swine, and the animals used in this study were some mixture of normal, heterozygote, or homozygote for the mutation. Following titin gel analysis, pigs were segregated into two groups, depending upon their N2BA number. In figure 4.9A, G3 left ventricles had N2BA-NE and a higher proportion of N2BA-N1, at the expense of the smaller isoforms. G3 left atria expressed NE, with a corresponding decreased proportion of N2B (Fig. 4.9C). The isoforms were similar in size to those of conventional pigs (Table 4.1), and it can be supposed that FG pigs
express a similar exon profile compared to their normal counterparts. It appears that a new N2BA isoform is apparent, based on their genetic background. These results are intriguing, in that the FH mutation may not exhibit simple autosomal dominance, but that a heterozygote individual may demonstrate hypercholesterolemic symptoms. Indeed, in the 23 pigs analyzed in this study, 14 expressed NE titin in their left ventricles and atria (61%), and 9 expressed only two N2BA titins (39%). While this is not indicative of a typical dominant mutation, the number of pigs in the first set of litters here may have been too low to replicate a 25:50:25 wild type: heterozygote: homozygote ratio. It is possible that if the G3 FG pigs had been subjected to dietary restriction, they may have increased their NE expression or perhaps shown another novel N2BA titin isoform. Particularly in the case of N2BA-NE, questions arise regarding the characterization and identity of the new exons present. It has been thus far assumed that because titin mis-splicing is evident in some diseases, those events are occurring within alternative splicing hotspots. These hotspots are within the titin I-band and include the Ig domains and the PEVK region [Bang et al., 2001; Freiburg et al., 2000]. Our data is in support of the prior titin ischemia study [Neagoe et al., 2002], but it is possible that the methods employed here allowed for greater resolution of the titin profile. The SDS-agarose gel used by our lab has permitted cleaner band detection, while previous gel electrophoresis used for titin migration was insufficient to provide a complete picture of the titin isoforms present, including that of the novel N2BA-NE isoform. We also observed a higher proportion of N2BA (approximately 50-60% in left ventricles and atria; roughly 70% in right ventricles) in swine myocardium compared to 30% seen in human patients. The
increased level of swine N2BA may make the transition to another N2BA isoform easier. The information presented here demonstrates the plasticity of titin expression, but further study is required to determine which exons are present in the novel N2BA-NE.

4.4.6 FG titin protein exon expression

FG pigs showed the presence of a novel N2BA isoform, so western blotting of titin exons was performed. H4 antibody [Trombitas et al., 1999] targets the titin molecule at several epitopes in the middle of the A-band, and showed a robust signal for all tissues studied (Fig. 4.3C). G3 and G2 pigs showed three and two N2BA bands, respectively. The H4 immunoblots resembled a normal silver-stained agarose gel, strongly suggesting that all three bands are indeed titin isoforms. Likewise, an antibody directed against exons 87-88 demonstrated similar expression as that of a silver-stained gel, except without the presence of an N2B isoform band (Fig. 4.3D). G3 tissues showed three N2BA bands and G2 showed two N2BA bands, just as in previous analyses. Since there was no difference between G3 and G2 animals, it may be that the novel N2BA-NE isoform is due to the presence of another group of exons located elsewhere on the titin mRNA message. Exons 87-88 lie within the middle-Ig domain, so another region may be impacted instead. It has been reported that the PEVK domain can vary wildly in sequence and splicing [Bang et al., 2001; Greaser et al., 2005; Warren et al., 2004], so additional PEVK exons being included in G3 animals is quite feasible. A more complete protein and cDNA analysis is necessary to determine exactly which exons are expressed in these pigs.

4.4.7 FG swine titin gene expression levels
Quantitative RT-PCR was performed on FG pigs to examine the expression levels of specific titin exons. The porcine titin sequence is still incomplete (accession number: M97767), but a high degree of conservation among mammalian species is known to exist [Fritz et al., 1993]. A BLAST search was performed to find similar exonic sequences to that of human titin. Exons 60, 75, and 326 were studied here, and the former two are found within the middle Ig region, while the latter is within the A-band end. The primer set specificity was confirmed with gel analysis, and correct amplicons were obtained before proceeding with the qPCR analysis. A conventional pig’s expression levels were normalized to one with GAPDH primers, and FG were compared relative to the conventional pig. All FG expression levels were higher than those of the conventional pig, except for one G3 LV exon 60 (Table 4.3). The remaining results showed much higher levels in the FG pigs, regardless of their N2BA pattern. Since exons 60 and 75 are both within the middle Ig region, this supports the above immunoblotting data, and exon 326 should be constitutively expressed in cardiac tissue, regardless of the N2BA isoform profile. The high levels can be explained by the fact that both G3 and G2 exon levels are heightened, so the I-band may be impacted differentially in this mutation state.

Therefore, the genetic analysis provided by the qPCR results supports the presumption that the change to the G3 pigs is not within the Ig domain, but possibly the PEVK region or another splicing hotspot. The genetic analyses described must be taken as preliminary results, since expression quantities were variable. Further genetic study must be performed to examine the specific exons which are affected by the FG animal strain.

4.4.8 Conclusions
FH swine have been studied for several decades as a model for human familial hypercholesterolemia as well as cardiovascular disease. However, the relationship of atherosclerosis and ischemia to titin remains to be established. While it has been reported that titin isoform shifts occur during ischemia, a full complement of its effects was unclear. Utilizing a more sensitive gel analysis method allowed us to detect a new N2BA titin isoform, although the specific exons which comprise this isoform have yet to be identified. Further, the increased quantity of compliant isoforms in FH pigs does not fit the current human model of ischemic disease and titin changes. The dietary restrictions imposed on the FH pigs also led to making the pig titin more dissimilar from the conventional group. At this time it is difficult to ascertain the exact implications for these findings, but the ability of dietary intervention to alter titin expression, at least in hypercholesterolemic swine, is a result that may lead to more important discoveries.

References


Figure 4.1. SDS-PAGE analysis of cardiac myosin heavy chain isoforms. Dietary effects of FH pigs on cardiac MHC. Regardless of treatment, all pigs expressed the same predominant MHC isoform as the conventional pig. Ventricles demonstrated only β-MHC, and left atrium only showed α-MHC. LV: left ventricle; RV: right ventricle; LA: left atrium; C: conventional pig; AL: ad libitum fed pig; 80: 80% ad libitum fed pig; 60: 60% ad libitum fed pig
Figure 4.2. Agarose gel electrophoresis of titin isoforms. FH dietary effects on cardiac titin isoform. C and AL pigs express N2BA-N1, N2BA-N2, and N2B titin isoforms, while 80 and 60 pigs express N2BA-NE in addition to the previous three isoforms. The NE isoform is larger than the known pig titin isoforms.
Figure 4.3. Titin isoform proportions. A. Left ventricle titin proportions. AL, 80, and 60 showed the presence of NE titin and decreased levels of N2B. B. Right ventricle titin proportions. AL, 80, and 60 showed NE titin and decreased N2B proportions, while C and AL had significantly higher proportions of N2. C. Left atria titin proportions. AL, 80, and 60 showed NE titin presence and decreased levels of N2BA-N2. Diet-restricted 80 and 60 had increased N2BA-N1 levels.
Figure 4.4. Percent N2BA of total titin. A. Left ventricle N2BA percentages. C pigs had significantly less \( (p < 0.05) \) N2BA percentage of total titin relative to FH groups. B. Right ventricle N2BA percentages. C pigs had significantly less \( (p < 0.05) \) N2BA percentage of total titin relative to FH groups. C. Left atrium N2BA percentages. 80 pigs had a significantly higher \( (p < 0.05) \) level of N2BA relative to C, but was similar to other groups. Asterisks indicate significant difference relative to control groups.
Figure 4.5. Ratios of N2BA to N2B. 

**A.** Left ventricle ratios. All FH groups had significantly higher ratios of N2BA relative to C. 

**B.** Right ventricle ratios. All FH groups had significantly higher ratios of N2BA relative to C. 

**C.** Left atrium ratios. Only 80 pigs had a significantly higher ratio of N2BA:N2B relative to C. AL and 60 were similar to C.
Figure 4.6. Rbm20 western blot. **A.** Representative western blot of Rbm20 and GAPDH protein in C and FH swine left ventricle. **B.** Rbm20 expression levels of 2N2BA and 3N2BA pigs relative to GAPDH. No difference was found between the two groups. Bars represent means ± SE.
Figure 4.7. FG swine cardiac myosin heavy chain isoform expression. No changes were observed between pigs expressing 3 N2BA titins and those expressing 2 N2BA titins. All ventricles possessed β-MHC, and left atrium showed α-MHC. G3: three N2BA titin; G2: two N2BA titin.
Figure 4.8. FG swine titin isoforms. Representative titin gel of FG swine. n=6 for each group. Animals were segregated based on their N2BA expression. Pigs showed three (G3) or two (G2) N2BAs. LV tissues consisted of either two or three N2BA bands, all RV samples contained three N2BAs, and LA was composed of two or three N2BA bands. NE: N2BA-NE; N1: N2BA-N1; N2: N2BA-N2; N2BA-G: N2BA giant isoform; G3: three N2BA titin; G2: two N2BA titin; rat LV: wild type rat left ventricle; Hu Sol: human soleus. Values denoted by different letters are significantly different (p < 0.05).
Figure 4.9. FG titin isoform proportions. A. LV titin proportions. G3 had an NE titin band, and G2 had a significantly higher N2B proportion. B. RV titin proportions. All titin isoforms were unchanged across groups. C. LA titin proportions. G3 showed an N3 band, and G2 had a significantly higher proportion of N2B titin. Bar represent means ± SE. Bars with different letters of significance are different ($p < 0.01$).
Figure 4.10. FG percent N2BA of total titin. A. LV results. G3 had a significantly ($p < 0.001$) higher percentage of N2BA relative to total titin. B. RV results. There was no difference between G3 and G2 RV. C. LA results. G3 had a significantly higher ($p < 0.001$) percentage of N2BA relative to total titin. Asterisks indicate significant differences ($p < 0.01$).
**Figure 4.11. FG proportion of N2BA to N2B.** **A.** LV results. G3 had a significantly higher ($p < 0.001$) proportion of N2BA to N2B. **B.** RV results. There was no difference between G3 and G2 RV. **C.** LA results. G3 had a significantly higher ($p < 0.001$) proportion of N2BA:N2B. Asterisks indicate significant differences ($p < 0.01$).
Figure 4.12. FG titin exon western blot. A. H4 antibody western blot of FG pigs. Bands that appeared were the same as those in silver-stained titin agarose gels. G3 pigs had three N2BA bands, while G2 pigs had only two N2BA isoforms. N2B bands also appeared. B. Titin exon 87-88 antibody of FG pigs western blot. G3 pigs had three N2BA isoforms, and G2 pigs had two N2BA isoforms. N2B isoform bands did not appear.
## Titin Sizes of FH Guinea Swine (KDa)

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Table 4.1. Titin isoform sizes of adult FG swine. The novel N2BA band molecular mass is calculated. Left ventricle and left atria samples were split into two groups, depending on their expression (3 different N2BA bands vs 2 N2BA bands), and all right ventricle samples showed three N2BA isoform bands. Numbers are means ± SE.
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Table 4.2. PCR primers used for quantitative real-time PCR. Titin primers targeted specific exons within the titin gene, and GAPDH was used to normalize the expression level of the target genes.
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Table 4.3. Quantitative real-time PCR. A. Titin exon 60 expression. Values were converted to ratios with the conventional pig numbers set to 1. G3 and G2 pigs’ exon 60 expression was higher compared to conventional pigs in all cases except for one G3 LV. B. Titin exon 75 expression. All G3 and G2 pigs had a higher exon 75 expression pattern compared to C. C. Titin exon 326 expression. All G3 and G2 had higher exon 326 expression relative to C. All results provided were determined using the previous method described [Pfaffl, 2001]. LV: left ventricle; RV: right ventricle; LA: left atrium.
CHAPTER FIVE: SUMMARY

In chapter two, the sarcomeric protein profiles of normal and mutant rats (Hm) with a non-functional Rbm20 gene were described. As a recently characterized disease model, Hm rats mimic dilated cardiomyopathy through ventricular dilation and the ablation of titin alternative splicing, resulting in a new, giant titin isoform (N2BA-G). To date, it is known that this Rbm20 mutation affects over 30 genes, so we were curious to see if other proteins in the Hm rat’s sarcomere were altered. In this portion of the study, we observed the normal N2B titin isoform in wild type animals, and N2BA-G in Hm. However, following phosphoprotein gel analysis of both genotypes, it was determined that this mutation does not change the phosphorylation state of titin, despite the presence of more exons. Titin phosphorylation has been shown to influence the physiological effects by decreasing passive tension in the heart, but the decreased stiffness in Hm rat hearts must be due to the increased size of the protein. Additionally, cardiac and skeletal muscle myosin heavy chain (MHC) isoform content was also similar; Rbm20 does not appear to impact the expression pattern of MHC, even though cardiomyopathies will often induce MHC isoform changes. Other major proteins of the skeletal muscle sarcomere were analyzed as well, and no differences between genotypes were found. These results indicate that while Rbm20 may influence the splicing events of titin and multiple other proteins, its effects within the sarcomere are limited primarily to titin. Cardiac and skeletal muscle seems to be impacted in the same manner by this mutation, since both tissues demonstrate a single, large N2BA-G titin molecule.
Thyroid hormone is an essential factor necessary for metabolic regulation, and if its levels are disrupted, cardiovascular pathologies will result. Previous study has revealed that raising or lowering thyroid hormone levels will cause a shift in titin isoform expression, in addition to that of MHC. Thyroid hormone injections induced hyperthyroidism in normal and Hm rats, and propylthiouracil severely blunted circulating thyroid levels to present a hypothyroid state. Hyperthyroid animals of both genotypes demonstrated increased cardiac functionality relative to controls, but Hm ventricular dilatation effects were observed in the lengthened isovolumic relaxation time and ejection time. Of the hyperthyroid treatment groups, differences between normal and Hm were seen, dependent upon the physiological changes that both groups had undergone. Generally speaking, echocardiographic results of hypothyroid animals showed depressed cardiac function, including decreased heart rate and longer contraction and relaxation intervals. MHC content was dependent on thyroid status, not genotype. Hyperthyroid animals expressed exclusively α-MHC, and hypothyroid animals expressed significantly higher levels of β-MHC in left ventricles. A fast-twitch (tibialis anterior, TA) and slow-twitch (soleus, SOL) muscles were analyzed for MHC expression, as well. SOL shifted toward a faster phenotype in hyperthyroid and a slower phenotype in hypothyroid conditions, but TA was unaffected by thyroid state. The most revelatory results, though, concerned titin expression. Wild type rats expressed significantly higher levels of N2BA titin relative to total titin, as well as higher levels of N2BA-1, the larger of the N2BA isoforms. Hm rats, regardless of thyroid status, continued to express only N2BA-G titin.
This finding further supports the conclusion that Rbm20 is primarily responsible for the titin alternative splicing event. Prior studies have investigated titin isoform shifts following thyroid treatment, but this study is the first to demonstrate that in order for this shift to occur, Rbm20 must be present. Thyroid hormone acts upon the PI3K/Akt signaling pathway to induce downstream changes, and Western blotting was employed to analyze this pathway in our mutant rats. Akt’s PH domain and its phosphorylated Ser473 residue were expressed at higher levels in Hm rats, especially in hyperthyroid mutants. The mTOR phosphorylated Ser2448 was also highest in hyperthyroid wild type and Hm animals. In this portion of the report, modulation of thyroid hormone levels was successful at stimulating changes in the cardiovascular system. While it appears that ultimately Rbm20 is the principal driver of titin alternative splicing, changes in the Hm rat’s physiology can be made to occur, enabling the possibility of lessening the symptoms of dilated cardiomyopathy and other ventricular disorders.

Familial hypercholesterolemia (FH) is a genetic disease which disables the body’s ability to clear lipid from the bloodstream, leading to blockage and infarct. A sedentary lifestyle and unhealthy diet is another root cause of hypercholesterolemia. The titin profile of an FH swine model was analyzed following dietary treatments: pigs were either given ad libitum access to feed or limit-fed. We found that limit-fed FH pigs resulted in the expression of a novel, larger N2BA titin (N2BA-NE), but ad libitum-fed pigs did not manifest this third N2BA isoform. NE titin is 3.58 MDa in size, one of the largest titin isoforms discovered to date. It appears that limit feeding shifted titin expression to a
more compliant profile. MHC content was unchanged across all groups, and interestingly, so was Rbm20. This data suggests that, in swine, Rbm20 may not be the only regulator of titin isoform expression, and that other factors may be necessary for the splicing events. FH guinea swine (FG) were generated for this study, and they too presented NE titin. In left ventricle and left atria, FG pigs either expressed two or three N2BAs, but all right ventricles analyzed contained NE titin. Immunoblotting showed that all pigs expressed exons 87-88, and genetic analysis with qPCR demonstrated the presence of exons commonly found in the middle Ig domain. This data suggests that the FH mutation in this genetic strain of swine must alter the titin splicing events in another location than those analyzed. It is possible that there are multiple controls for titin alternative splicing, or that FH pigs will express NE titin as a compensatory mechanism to prevent a more severe phenotype upon high lipid build-up within the blood vessels. At this juncture, it is unclear exactly what is causing NE to appear or what process is involved to bring about this new isoform. We speculate that additional PEVK exons may be included for NE presence, but further information is needed to elucidate how FH pigs are experiencing the high levels of cholesterol in the bloodstream and what sort of changes are occurring within the animal’s physiology.

In conclusion, this report reiterates the critical role which titin plays on the striated muscle ultrastructure, in addition to the numerous ways in which its expression pattern can be manipulated. The Hm rat mutation has proved to be an excellent model for studying the effects of compliant titin in the cardiovascular system, and Rbm20
appears to be the most important factor in titin isoform expression. This mutation does not alter titin phosphorylation, nor does it change any other sarcomeric proteins studied here. In the rat, the data shows that Rbm20 is the protein responsible for the titin profile, despite any extrinsic interventions. Thyroid hormone is unable to influence titin in Hm rats, even though it must act in conjunction with Rbm20 in normal rats to induce a changed titin pattern. The hypercholesterolemic swine model used here presents us with further questions, since it appears that Rbm20 is not the sole manipulator of titin expression in the pig. Data in chapter four shows that while Rbm20 levels were unchanged between free feed access and limit-fed treatment groups, the latter showed a novel titin isoform. This information suggests that Rbm20 may not be required in swine to induce a titin isoform shift, but rather, dietary changes may be sufficient in maintaining a healthy physiology. Further investigations into this pig model are necessary to fully elucidate the mechanism through which this change is occurring. Lastly, FG pigs can also express the novel NE titin, proving that the FH effects are consistent with those from a conventional background. FG animals should be utilized in future studies to fully understand how NE titin is arising, and the answer may not include Rbm20.

The totality of this work has answered some questions regarding the secondary effects of the Rbm20-deleted Hm rat. It seems that removing functional Rbm20 from the rat will lead to N2BA-G titin’s appearance, but other myofibrillar characteristics are unaffected. Thyroid hormone cannot induce change if Rbm20 is not present. FH swine
manifest a novel titin isoform, but the precise steps involved in porcine titin splicing bears further investigation.


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