Spring 2020

Potential Role of the Large Protein Titin in the Development of Heart Failure

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POTENTIAL ROLE OF THE LARGE PROTEIN TITIN IN THE DEVELOPMENT OF HEART FAILURE

By

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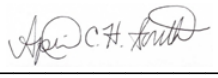
Submitted in Partial Fulfillment
of the Requirements for
Graduation with Honors from the
South Carolina Honors College

April 2020

Approved:



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Thesis Summary

I will be exploring the impact of the large protein Titin on heart failure with a preserved ejection fraction (HFpEF). It was hypothesized that myocardial Titin plays a significant role in the progression of HFpEF through isoform, N2BA and N2B, changes. It was hypothesized that an increase in the quantity of N2B Titin, less compliant, and a decrease in N2BA Titin, more compliant, will be observed as HF progresses, contributing to an increase in passive stiffness involved in heart contraction. I will cover a detailed report on the role of Titin in HFpEF and propose research methods to explore further.

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Introduction

The rate and incidence of heart failure (HF) continue to increase as the leading cause of death across the globe ("Heart Disease Statistics and Map," 2019). HF arises from several distinct etiologies, which, in turn, have demonstrated distinctly different therapeutic responses and clinical outcomes. One of the more common causes of HF is that which arises from left ventricular (LV) pressure overload (LVPO). LVPO is characterized by an impairment in LV filling due to cardiomyocyte stiffness and thus a reduced compliance in contraction and relaxation. Cardiomyocyte stiffness, the stiffness in heart muscle cells, is linked to the elastic protein titin. This stiffness been identified as an important contributor to high myocardial diastolic stiffness (Hamdani & Paulus, 2013).When LVPO impairment has a stable ejection fraction (EF) range (> 45%), the disease is classified as heart failure with a preserved EF (HFpEF). Recent studies have shown that up to half of HF is classified as HFpEF, however there is still an insurmountable need for a greater understanding of the biological factors which constitute the disease (Komajda et al. 2011). There has been limited progress in developing diagnostics and therapeutics for HFpEF as this requires a greater understanding of factors which contribute to increased LV myocardial stiffness. The incidence of HFpEF has significantly increased since 1987, also contributing to the urgency of finding better treatment options (Owen et al. 2006). Past studies have suggested that shifts in large protein structures within the cardiac cells change in expression and structure with LVPO, and one such protein is termed titin.

Over the past 20 years, Dr. Frank Spinale has directed a research effort on "the thematic area of cardiovascular remodeling with a particular focus upon heart failure". Dr. Spinale and his colleague Dr. Shayne Barlow, a research veterinarian, have been researching HFpEF by observing changes and patterns of hearts in pigs. They perform this research by implanting an

ascending aortic cuff into pigs and inflating the cuff over a period of 5 weeks to simulate hypertension, which eventually results in heart failure. Before the implantation of the cuff and over the course of the 5 weeks with the programmed LVPO, several serial echocardiograms are performed to examine the changes in chamber stiffness, the left atrial area, regional myocardial strain, and the increase in collagen fibers. Samples of left ventricular myocardium were also obtained in both the control and PO (5 weeks post-implantation) pigs to examine changes over time.

The central hypothesis of the study was that early measurements of diastolic myocardial stiffness would be predictive of the progression of HFpEF. Several research efforts were and are still being conducted to assess the biological factors that contribute to the myocardial stiffness. The focus of this research paper is on titin's contribution to the stiffness and how that affects the progression of HFpEF. My plan was to utilize the LV myocardium samples to examine the titin isoforms and assess the changes between the control and PO pigs. The ultimate goal is to increase the scientific scope of knowledge of HFpEF and to what extent the giant protein titin plays a role in the progression of the disease.

Role and Structure of Titin

In the sarcomere, the large protein titin spans from the Z disc to the M line. It is embedded in the Z disc at its N-terminus, spans the I band and thick-filament-binding A band region, and the C-terminus is embedded at the M-line (LeWinter & Granzier, 2015). Titin aids in contraction by assisting myosin to pull on actin, drawing the thin filaments towards the M-line, shortening the sarcomere. Titin's position can be seen in both a relaxed sarcomere and **Figure 1** and a contracted sarcomere in **Figure 2**.

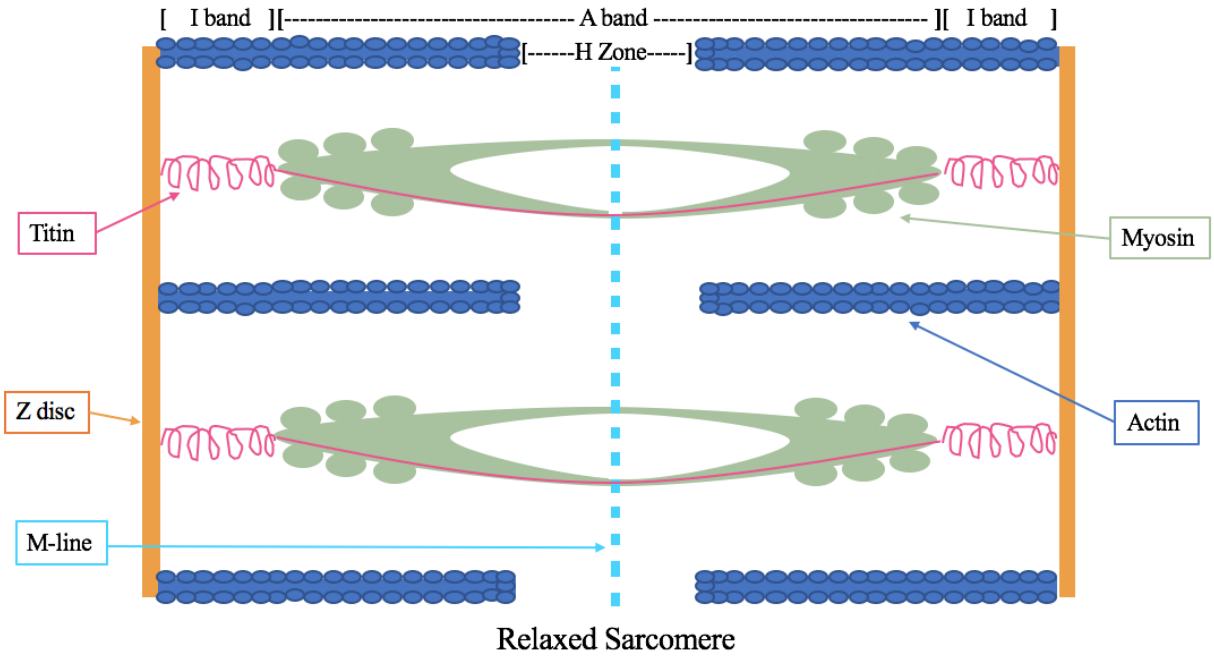

Figure 1: In a relaxed sarcomere, the muscle is not contracted, generating a passive force (adapted from Cammarato, 2005).

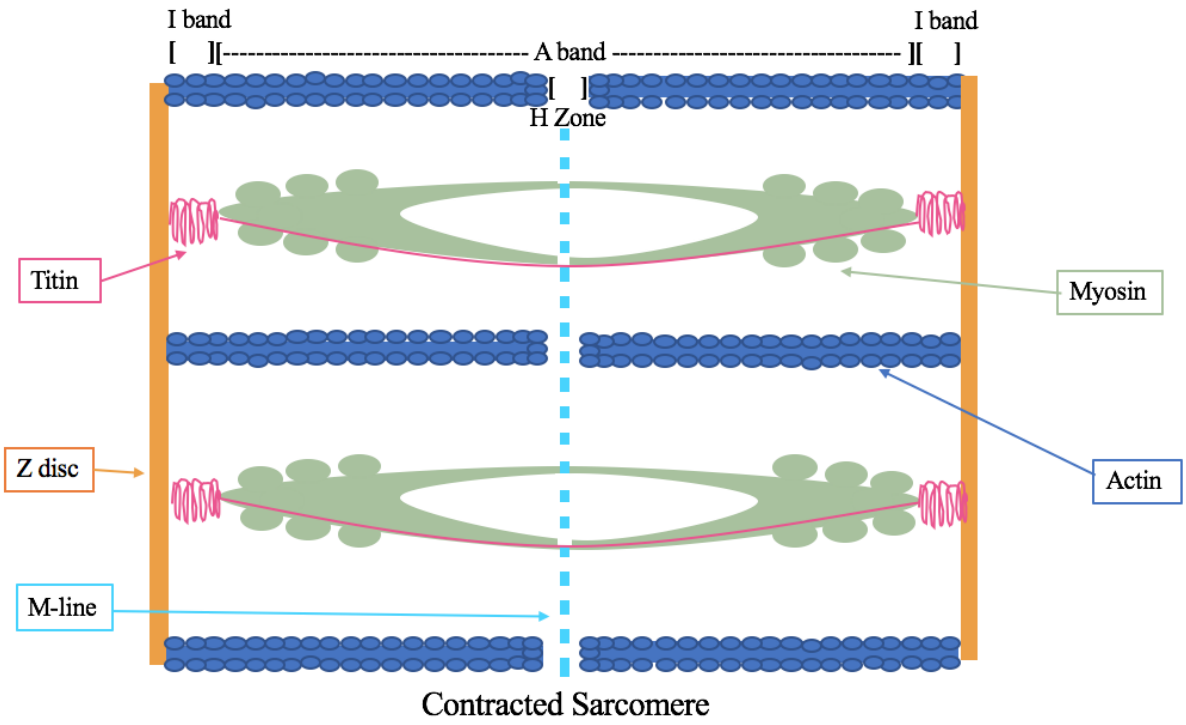

Figure 2: The binding of myosin molecules with actin results in actin being pulled towards the myosin with the help of titin recoiling, resulting in a shortening of the I band and the H zone. The A band does not shorten in length during contraction. (adapted from Cammarato, 2005).

Cardiomyocyte stiffness is directly related to the giant protein titin. Titin is an elastic protein in the sarcomere that extends and recoils to allow for heart contraction (Tskhobrebova & Trinick, 2010). Titin's elastic properties allow the sarcomere to stretch and shrink, regulating muscle contraction and sarcomere length (Castro-Ferreira et al., 2010). Titin is a bidirectional spring that is "responsible for early diastolic recoil and late diastolic distensibility of cardiomyocytes" (Hamdani & Paulus, 2013). During diastole when the heart muscle relaxes and the sarcomere is stretched, a passive force is generated (Granzier & Labeit, 2004). This extensibility characteristic of the I-band region of titin generates passive stiffness and is an essential factor in diastolic filling (Granzier & Labeit, 2004). It was shown that when titin's I band region was removed, no passive force develops at all, indicating the extent of titin's contribution to passive force (LeWinter & Granzier, 2010). When the sarcomeres shorten below their slack length, the matrix is pulled and the heart contracts, generating a restoring force (RF) (Granzier & Labeit, 2004). There is a direct correlation between titin stiffness and the restoring force (RF) magnitude, suggesting that changes in titin stiffness can drastically affect the diastolic function of the ventricle (Savarese et al., 2018). Titin's elastic recoil also contributes to diastolic suction (LeWinter & Granzier, 2015). Additionally, Titin facilitates length-dependent activation, a key factor in the Frank-Starling relation (LeWinter & Granzier, 2015).

The large protein Titin, roughly 3700 kDa, is encoded by the TTN gene on chromosome 2 in the 2q31 region (Castro-Ferreira et al., 2010). The TTN gene is composed of 363 exons which code for 38 separate 138 amino acid residues (Granzier & Labeit, 2004). Exons 1 to 251 encode for the Z-dic and I-band region of the protein (Granzier & Labeit, 2004). The extensible I-band region, the portion of the protein that expands and recoils to allow for contraction, includes tandem immunoglobulin (Ig) residues, a PEVK domain, and the N2B unique sequence

(N2B-Us) (Granzier & Labeit, 2004). The PEVK exons, rich in proline, glutamine, valine, and lysine residues, comprise a PEVK domain which functions to regulate the conformation of Titin, contributing to the elasticity of the extensible region (Hidalgo et al., 2009). The cardiac-specific N2B-Us is a phosphorylation site for several kinases and functions to decrease passive force levels. Exons 252 to 353 encode for the Titin that spans the A-band region of the sarcomere (Granzier & Labeit, 2004). The portion of the protein that spans A-band may function to regulate assembly of the thick filament (LeWinter & Granzier, 2010). Exon 358 encodes for a serine-threonine-kinase domain in which the function remains unknown. The carboxy terminus integrated in the M-band contains a kinase domain (LeWinter & Granzier, 2010).

The TTN gene can be spliced in two distinct ways in the myocardium, creating two major classes of cardiac isoforms - the N2B and the N2BA isoforms (Warren et al., 2003). The N2B Titin isoform is the smaller isoform, roughly 0.3MDa shorter than N2BA (Cazorla et al., 2000). This N2B isoform is created by skipping exons 120 to 175 and splicing together the coding exons 119-176, resulting in shorter PEVK and Ig domains (Cazorla et al., 2000). The N2BA Titin isoform includes an N2A element, exons 102-109, that N2B isoform does not have. Additionally, the N2BA isoform also has longer PEVK segments and additional Ig domains in the I-band region (Savarese et al., 2018). Because both of the isoforms are found in the heart, both have the cardiac-specific N2B-US. See **Figure 3** for a diagram of the N2B and N2BA isoforms.

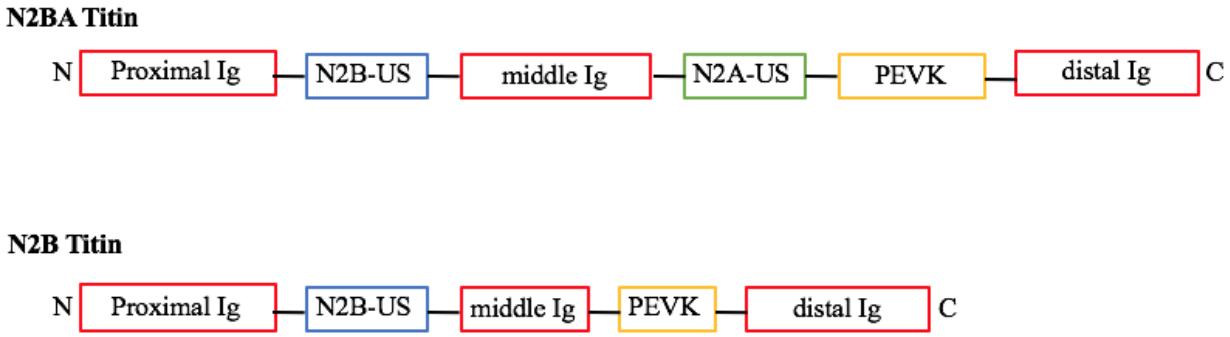

Figure 3: N2BA Titin (top) has a unique N2A domain and longer PEVK and Ig domains in the I-band region, compared to N2B Titin (bottom) (adapted from LeWinter & Granzier, 2010).

Titin-Dependent Passive Tension

Titin's elastic properties, and thus stiffness, are associated with different isoform expressions because splicing differences cause changes in the functionality of the protein. Because N2B Titin has a shorter extensible region, the PEVK domain and smaller Ig segments, the isoform is less compliant compared to the more compliant N2BA isoform of titin (Savarese et al., 2018). Sarcomeres with a higher portion of N2B titin, the smaller of the two cardiac isoforms, are not able to stretch to the same degree as those with N2BA titin. This is due to a significantly lower fractional extension of the extensible region of the protein and results in a higher passive stiffness (Cazorla et al., 2000).

Cazorla et al. studied titin-based stiffness by analyzing the tension from several myocardial samples, including pig and mouse myocytes (Cazorla et al., 2000). They discovered that mouse ventricular cells were significantly stiffer than pig myocytes (Cazorla et al., 2000). Further, Cazorla et al. investigated the cause of the difference in stiffness, including differences in the fractional area of myofibrils, differences in the cellular content of titin, and differences in the titin/MHC ratios, which all were determined to not be significant factors of stiffness (Cazorla et al., 2000). Cazorla et al. found that coexpression of the titin isoform on the

myocardial level is a result of coexpression at the level of a single cardiac myocyte (Cazorla et al., 2000).

It is important to note that different organisms have variation in titin isoform expression. The human myocardium expresses both N2B and N2BA, and thus the N2BA/N2B ratio can be a measure of passive stiffness. A higher ratio of N2BA/N2B would show decreased passive stiffness and a lower ratio of N2BA/N2B would show increased passive stiffness. In smaller mammals, such as adult rodents, the N2B isoform predominates in the LV (Savarese et al., 2018). The N2BA/N2B ratio would therefore be lower, indicating a higher passive stiffness (Savarese et al., 2018). Humans have a higher quantity of the N2BA isoform in the LV, resulting in a higher ratio than the rodents and thus a lower passive stiffness. Recent studies report that the N2BA/N2B ratio in the adult human LV is roughly 0.6 (Savarese et al., 2018). Studies by Warren et al. have shown that the N2BA/N2B ratio decreases as the cardiomyocytes become increasingly hypertrophic (Warren et al., 2003).

While other biological factors, including collagen and myosin, may contribute to passive stiffness, extensive studies have been done to show the impact of titin to cardiac passive stiffness. Hamdani and Walter utilized KO mice that had a significant amount of Ig domains removed (Hamdani & Paulus, 2013). They left the collagen content unaltered as a constant to see the effects of only titin on the stiffness. The results showed that deleted segments resulted in a shorter sarcomere and thus increased stiffness, and that the mice developed HFpEF (Hamdani & Paulus, 2013). These results demonstrate the significant effects of titin in myocardial stiffness and the development of HFpEF without involvement of the extracellular matrix (ECM) (Hamdani & Paulus, 2013).

It can be expected that an increase in the quantity of N2BA titin will result in a decrease in both the sarcomere stiffness and myocardial stiffness (Cazorla et al., 2000). Lower myocardial stiffness in the LV will allow for faster filling of the LV and a larger end-diastolic volume (Cazorla et al., 2000).

Regulating Titin-Dependent Passive Tension

Titin-dependent passive tension can be regulated by utilizing several biological factors, including phosphorylation through kinases, the formation of disulfide bridges, and isoform variation.

Several different protein kinases can directly affect titin-dependent passive tension. Phosphorylation of phosphosites in N2B-Us by both protein kinase A (PKA) and protein kinase G (PKG) results in decreased passive tension (Warren et al., 2003). Yamasaki et al. showed that B-adrenergic stimulation of rat cardiac myocytes activates PKA to phosphorylate N2B-USs (Yamasaki et al., 2002). Krüger et al. discovered that protein kinase G (PKG) has been shown to phosphorylate serine 469 of the N2B-Us of human titin through a cGMP/PKG signaling cascade, also reducing passive stiffness (Krüger et al., 2009).

Protein kinase C alpha (PKC-α) also phosphorylates titin at serines in the PEVK residues (Warren et al., 2003). However, unlike PKG, experiments by Hidalgo et al. showed that PKC-α increases passive stiffness (Hidalgo et al., 2009). They showed that in mouse and pig cardiomyocytes, phosphorylation of the PEVK domain by PKC-α increases stiffness by up to 30% (Tharp et al., 2019). Experiments with knockout mice revealed that when PEVK sites were genetically removed, PKC-α did not affect passive tension, proving a significant link between the PEVK site, PKC-α, and passive tension (LeWinter & Granzier, 2015).

It was recently discovered that extracellular-signal-related-kinase-2 (ERK2) phosphorylates the N2B-Us at three serine residues (LeWinter & Granzier, 2014). It was assumed that phosphorylation by ERK2 will lower passive tension, but experiments are required for confirmation (LeWinter & Granzier, 2014). Hidalgo et al. also discovered that the CaMKIIδ, a cardiac specific isoform of the CaMKII Calcium dependent protein, phosphorylates titin at both the PEVK and N2B-Us domains (LeWinter & Granzier, 2014). While phosphorylation of the PEVK domain increases passive tension, it was suggested that phosphorylation of the N2B-Us will reduce passive tension, but further experiments are needed to prove this (LeWinter & Granzier, 2014).

Due to the presence of six cysteine residues in the human N2B-Us, there is a possibility for another form of posttranslational modifications - disulfide bridges (LeWinter & Granzier, 2014). Depending on the oxidation state, the cysteine residues have the potential to interact with each other, forming these bridges (LeWinter & Granzier, 2014). Under oxidative conditions, which is an expected in HF, there is an increased passive tension as a result of the disulfide bridges.

Research by Granzier et al. shows that the interaction between titin's PEVK region and F-actin also contribute to an increase in tension (Granzier & Labeit, 2004). Granzier finds that the dynamic interaction between F-actin and the region can retard sliding during contraction, thus contributing to an increase in passive stiffness (Granzier & Labeit, 2004). Further research was done by Campbell et al. to explore this interaction in both titin cardiac isoforms and it was discovered that this dynamic interaction is more pronounced in the less compliant N2B isoform, contributing to the increase in passive stiffness (Campbell et al., 2003).

Differential splicing resulting in different isoforms is also utilized to offset increased tension. Granzier suggests that titin's elasticity can be altered in a matter of weeks (Granzier & Labeit, 2004). Studies of patients with HFpEF show altered isoform expression ratios. Studies show an upregulation of more compliant isoforms, N2BA, to compensate for the increased stiffness of the ECM and the increased demand on the LV during PO (LeWinter & Granzier, 2015). Makarenko et al. discovered that while increasing the expression of N2BA isoform may initially improve diastolic function, as the myocardium continues to fail, systolic function may worsen due to the decrease of passive stiffness (Tharp et al., 2019).

While the mechanisms for these isoform changes are not fully known, a recent discovery of the RBM20 protein splice factor is promising. The RBM20 protein is specific to muscle cells, and it contributes to the formation of the spliceosome (Tharp et al., 2019). Through experiments utilizing KO rats that have had RBM20 removed, it has been shown that RMB20 contributes to isoform variation because there is a change in the N2BA/N2B ratio (Tharp et al., 2019). LeWinter and Granzier state that more research should be done to understand the mechanisms of titin isoform expression (LeWinter & Granzier, 2015).

Proposed Research and Therapeutic Possibilities

One proposal to potentially treat HFpEF would be utilizing methods to decrease cardiomyocyte stiffness. The studies by Yamasaki et al. and Krüger et al. provide promising results that an increase in the phosphorylation of the extensible region of titin by protein kinases A (PKA) and G (PKG) result in a decrease stiffness. An increase of PKA phosphorylation through B-adrenergic stimulation is not clinically sound, however, because of an increased risk of arrhythmia (Hamdani & Paulus, 2013). Hidalgo et al. showed that phosphorylation of the extensible region of titin by protein kinase C alpha (PKC-α), alternatively, increases passive

stiffness. Several studies have shown upregulation of PKC-α in human HF (Hidalgo et al., 2009). More research should be done on the kinases, the phosphorylation site, and the phosphorylation pathway. After this is known, research should be conducted to explore how to stimulate an increase of phosphorylation of titin by PKG at (see figure 4) and how to decrease phosphorylation of titin by PKC-α (see figure 5) as a potential HFpEF treatment option. Ideally, altering the phosphorylation levels would significantly reduce the passive stiffness and thus increase diastolic function in HFpEF patients.

One limitation in downregulating PKC- α is its potential adverse effects on other parts of the body. PKC- α is an extremely complex kinase that plays a part in a large amount of other cellular functions, including apoptosis, motility, differentiation, proliferation, cellular transformation, and more (Singh et al., 2017). If there is a way to specifically target the myocardium PKC- α to decrease passive tension without it affecting any other cellular functions, it would be ideal. However, this is not realistic until the complex PKC- α protein, its phosphorylation pathway, and other biological factors that affect the protein are fully understood.

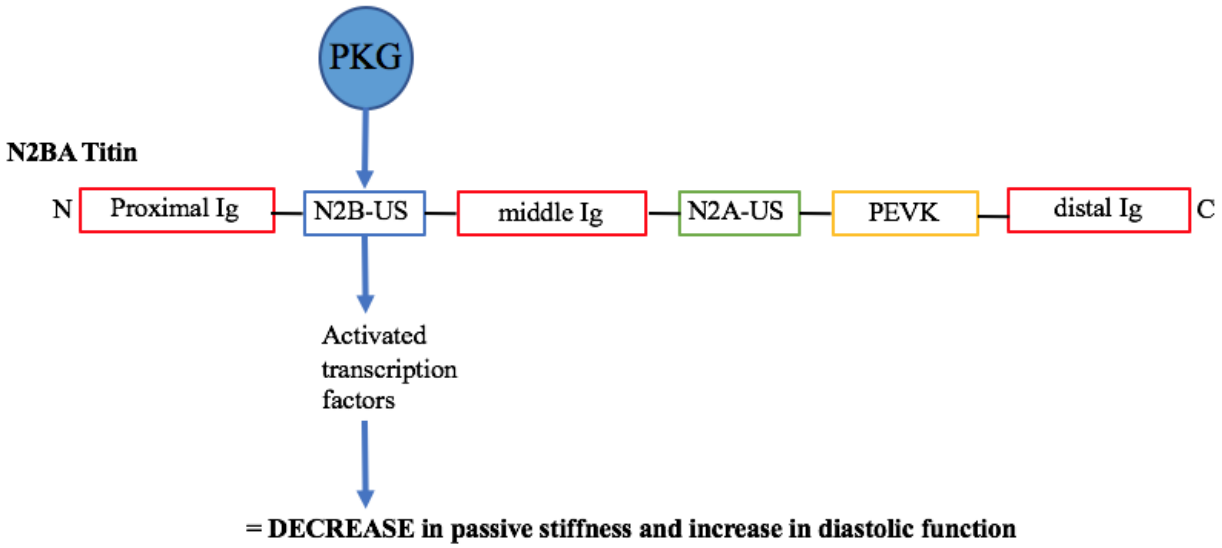

Figure 4: A potential therapeutic strategy for treatment of HFpEF is to stimulate the phosphorylation pathway of PKG, phosphorylating S469, resulting in the activation of

transcription factors to decrease passive stiffness, thus improving diastolic function (adapted from LeWinter & Granzier, 2010).

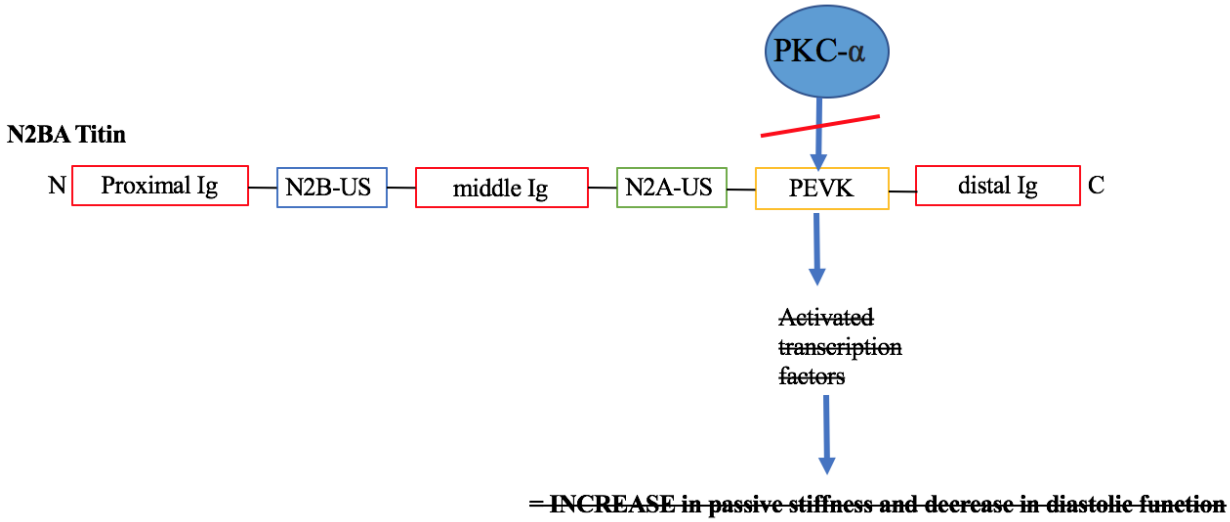

Figure 5: The normal phosphorylation pathway of PKC-α at the PEVK domain results in the activation of transcription factors, thus increasing the passive stiffness and worsening diastolic function. By inhibiting PKC-α (shown with red line), the phosphorylation pathway would not occur so myocardial stiffness and diastolic function would not be affected (adapted from LeWinter & Granzier, 2010).

Another research proposal for the treatment of HFpEF is to control the RBM20 splice factor. Manipulating the RMB20 splice factor to adjust titin isoform expression levels and improve diastolic function without worsening systolic function should be explored (Tharp et al., 2019). Because patients with diastolic dysfunction have lower N2BA/N2B ratios when compared to healthy patients, it would be ideal to manipulate the splice factor to increase N2BA levels for a more compliant sarcomere, thus improving diastolic function. A research study by Tharp et al. used a mouse model to explore HFpEF (Tharp et al., 2019). It was discovered that inhibiting RBM20 and increasing N2BA/N2B levels led to an improvement in HFpEF (Tharp et al., 2019).

By exploring these promising breakthroughs further, I believe that the scientific community will be closer to the treatment of HFpEF. It is my hope that we can work together to find a cure for the biggest killer on the planet, heart failure.

Acknowledgements

I greatly appreciate the guidance by both my thesis director, Dr. April South for her help guiding me through the thesis process and motivating me along the way. I would also like to thank Dr. Spinale, my second reader and lab mentor, for sparking my interest in HFpEF and narrowing down my research to the large protein titin. Additionally, a huge thank you to Lisa Freeburg who taught me so much about several research techniques and guided me through my research process. Lastly, thank you to my friends and family who motivate me, allow me to vent to them on hard days in the lab, and support me in everything I do. Thank you all!

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