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Rescuing Cardiovascular Disease Caused by Tropomyosin Mutations

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ABSTRACT

Cardiovascular diseases are a leading cause of death worldwide and health care expenditure. There are a variety of treatments for individuals diagnosed with cardiovascular disease depending upon the severity of symptoms, which include diet and exercise, medications, stents, and newly-developed gene therapies and drugs. The focus of this article will be on the molecular and biochemical mechanisms of how different genetic mutations in cardiac sarcomeric contractile proteins can lead to cardiomyopathic disease in mice. We will use the tropomyosin gene family and its associated proteins as a paradigm to illustrate the wide variety of clinical phenotypes that mutations in tropomyosin can impart. Potential therapeutic approaches for the rescue of cardiomyopathies, such as normalization of myofilament calcium sensitivity and introduction of chimeric genes/proteins, will be discussed as examples of "proof-of-concept" ideas. Researchers stand on the precipice of making exponential advances in the treatment of cardiovascular diseases – let us hope that we can soon take that forward step into this promising future.

Introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide, with atherosclerosis being a dominant cause of total CVD mortality and health care expenditure. Approximately 18 million lives each year succumb to these diseases. As a group, they include disorders of the heart and blood vessels, such as coronary heart disease, cerebrovascular disease, hypertrophic and dilated cardiomyopathies and other genetic and non-genetic conditions that can lead to heart failure. Risk assessment for individuals with CVD include measurements of inflammatory markers, lipoproteins, coronary artery calcium, exercise stress testing, imaging (including cardiac computed tomographic angiography) and family history. There are a variety of treatments for individuals diagnosed with CVD depending upon the severity of symptoms, but they include diet and exercise, medications (beta-blockers, aspirin and/or statins), stents, and newly-developed gene therapies and drugs, such as mavacamten which is used to treat obstructive hypertrophic cardiomyopathy.

Cardiomyopathies are a heterogenous group of diseases primarily defined by pathological alterations in the structure and physiological function of the heart. There are 5 different categories: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM), arrhythmogenic right ventricular cardiomyopathy (ARVC), and left ventricular noncompaction cardiomyopathy (LVNC). Common features are heart failure with reduced ejection fraction, peripheral edema, fatigue, dyspnea on exertion, syncope, and cardiac ischemia.[1,2] The focus of this article will be on the molecular and biochemical mechanisms of how genetic mutations can lead to cardiomyopathic conditions. Numerous cardiomyopathies are caused by mutations in the sarcomeric contractile proteins. Our approach has been to explore how mutations in tropomyosin (Tpm), a sarcomeric thin filament protein, cause various cardiomyopathic conditions.

Tropomyosin is an α-helical coiled-coil protein dimer that plays an essential role in the regulation of contraction and relaxation in the thin filament of the sarcomere. Tpm regulates this activity through its interactions with actin and the troponin complex. During muscle relaxation when cytoplasmic levels of calcium are low, Tpm blocks the myosin-binding site on the filamentous striated muscle actin. Upon stimulation, cytosolic calcium concentrations increase and bind to troponin C which, through its association with troponin T and I, mediates a conformation change in the Tpm position on actin. This repositioning of Tpm on actin exposes the myosin-binding site. Myosin binds to actin and triggers muscle contractile activity until the stimulation ceases, and calcium is resequestered into the sarcoplasmic reticulum.

There are 4 tropomyosin genes: α-, β-, γ-, and δ-Tpm, which all display a very high degree of nucleotide and amino acid conservation among themselves and among species. For example, between α- and β- striated muscle Tpm proteins, there is an 87% amino acid identity, and between α- and γ- striated muscle Tpm, there is a 91% amino acid identity.[3] Tpm proteins are 284 amino acids long and the differences in amino acid sequence between the different isoforms are scattered throughout the protein. For example, 25 of the 34 amino acid differences between α - and β-Tpm reside in the carboxyl half of the protein. In addition, there is a high degree of conservation with Tpm proteins among different species; for example, a 100% amino acid identity exists between human and murine striated muscle α-Tpm. This identity in amino acid and nucleotide sequences strongly suggests a significant overlap in physiological function of the respective Tpm proteins and allows investigations into these functions using various animal model systems.

Each of the four Tpm genes encodes numerous tissue and developmental specific isoforms. Through mechanisms of differential promoters, alternative splicing, and differential 3′ end processing/ polyadenylation, each of the Tpm genes generates striated muscle, smooth muscle, and non-muscle/ cytoskeletal isoforms.[4,5] The α-Tpm gene uses 15 exons to encode at least 10 distinct isoforms[4,6],

which includes the novel cardiac-specific Tpmκ isoform that is expressed in humans, rats, and chickens, but not in mouse.[7,8] In this paper, we specifically examine the Tpm gene family and their associated proteins as a paradigm to illustrate the clinical cardiomyopathic manifestations of Tpm mutations, along with potential therapeutic remedies.

Methodologies Employed in Studying Effects of Tropomyosin Mutations

There are a significant number of mutations in tropomyosin that are documented to result in cardiovascular disease. To examine the effects of these mutations, we determined tropomyosin mRNA and protein expression levels in human hearts. We also developed an antibody to the human Tpmκ cardiac-specific isoform and quantified its expression in control and DCM patient hearts.[7] Hearts were excised from the patients and control groups in accordance with the Declaration of Helsinki as adopted and promulgated by the US National Institutes of Health and with the rules and regulations of the University of Cincinnati's Institutional Ethics Committee.

In order to gain a greater understanding of the physiological function of the Tpm mutations, we developed transgenic and knockout mouse models. Transgenic mice (FVB/N background) were generated with cDNAs encoding Tpm isoforms (α-, β- or γ-Tpm), chimeric α-/ β-Tpm, human Tpmκ isoform, HCM or DCM Tpm mutations (Glu180Gly and Glu54Lys, respectively), and alterations in the Tpm phosphorylation site (Ser283Asp and Ser283Ala). The cardiac-specific α-myosin heavy-chain promoter was used in these expression vectors. This approach is warranted because as the exogenous expression of the various Tpm proteins occurs, there is a reciprocal and concomitant decrease in expression of the endogenous wild-type Tpm protein so that the total level of Tpm remains unchanged in the hearts of these transgenic mice. Generation of the α-Tpm knockout mice was accomplished by replacing a 4.4-kb α-Tpm genomic fragment containing the straited muscle-specific exons 12 and 13 with a HPRT minigene cassette.[9] The linearized construct was electroporated into murine embryonic stem (ES) cells, followed by drug selection and cellular blastocyst injection which resulted in germ-line transmission; chimeric males were bred to establish the colony.

A functional analysis of the murine hearts was ascertained by using the work-performing heart and anterograde perfused heart preparations.[10-12] Heart rate, left ventricular pressure, rates of pressure development and decline, time to peak pressure and time to half-relaxation were calculated. Skinned fiber bundle preparations and force measurements were obtained from detergent-extracted fibers dissected from the papillary muscle.[10, 11, 13] *In vivo* echocardiographic measurements were conducted using two-dimensionally targeted M-mode studies with a 9 mHz imaging transducer.[14] Measurements included end diastolic dimension, end systolic dimension, end diastolic thickness of the septum, posterior wall thickness, and fractional shortening.

Histological analyses were performed on heart tissue fixed in 10% neutral buffered formalin. Following embedding in paraffin, sections (5μm) were prepared and stained with hematoxylin and eosin, or trichrome stain to ascertain fibrosis (Prabhakar et al., 2001).

Animal experiments were approved by the University of Cincinnati's Institutional Animal Care and Use Committee.

Expression of Tropomyosin Isoforms in the Human Heart

Initial studies were focused on identifying and quantifying the Tpm isoforms expressed in the human heart. Analysis of mRNA levels found that there is ~ 50% striated muscle α-Tpm mRNA, with the remainder being the straited muscle α-Tpmκ mRNA, a human cardiac-specific Tpm isoform.[7] Although there is a 3-fold increase in expression of cardiac Tpm total transcripts between fetal and adult human stages, the ratio of Tpmκ and α-Tpm remains constant at 50:50. Additional studies

showed that there are lower, but equivalent levels of β-Tpm mRNA during both the fetal and adult stages.

To determine Tpm protein composition in human hearts, Western blot analyses revealed that in the adult human heart, 90 – 94% is α-Tpm, 3 – 5% is Tpmκ, and 3 – 5% is β-Tpm. Although the TPMκ and α-Tpm mRNAs are expressed in equivalent amounts, 90 – 94% of the total translated Tpm protein is α-Tpm, with the remaining be Tpmκ and β-Tpm. Similar discordant mRNA and protein levels are also found in α-myosin heavy chain expression in human ventricles.[9,10] These variations in mRNA vs protein levels illustrates that post-translational mechanisms play a dominant role in the regulation of contractile protein levels in the heart, a feature that is further substantiated in gene knockout experiments (see below).

We developed and employed a Tpmκ isoform-specific antibody to determine levels of this isoform in the left ventricular free wall of control hearts, along with samples from DCM patients and heart failure patients.[7] Results demonstrate there is differential expression of Tpmκ protein in the cardiomyopathic patient samples, with a 2-fold increase in both DCM and end stage heart failure patients for Tpmκ expression over control patients. It also appears that β-Tpm protein levels decrease in the patients; subtle changes in α-Tpm protein levels are not detected. Whether the increased Tpmκ expression is a cause or a consequence of the cardiomyopathic disease process is unknown, but it may serve as a potential biomarker indicating the onset of the DCM disease process. These results are significant in that Tpmκ protein may replace the α-Tpm isoform in the cardiac sarcomere and alter its performance.

To address the physiological function of Tpmκ in cardiac muscle, we generated transgenic mice that overexpress this isoform in the heart.[7] Incorporation of increased levels of Tpmκ protein in cardiac myofilaments leads to a murine DCM phenotype (Table I). Physiological alterations in the hearts of these mice include decreased fractional shortening, systolic and diastolic dysfunction, and decreased myofilament calcium sensitivity with no change in maximum developed tension. Additional biophysical studies demonstrate there is less protein structural stability and weaker actin-binding affinity of Tpmκ when compared with α-Tpm protein. The fact that human patients with chronic DCM and heart failure symptoms exhibit a cardiac pathology and physiology similar to the Tpmκ transgenic mice is striking because of the small but significant increase in Tpmκ protein levels in these patients. During normal cardiac function, the low levels of Tpmκ may be offset by the low levels of β-Tpm (which has the opposite effect on cardiac myofilament calcium sensitivity). In DCM and heart failure patients, there is decreased cardiac performance which correlates with increased Tpmκ levels. Additional studies demonstrate that normalization of myofilament calcium sensitivity can improve cardiac performance in heart failure patients.[10,17,18]

Table I: Genetic Models of Tropomyosin Cardiomyopathies

Model	Genetic Change	Phenotype	Phenotypic Rescue
α-Tpm -/-	Gene Deletion	Embryonic Lethal	No
α-Tpm +/-	Single Allele Deletion	Wildtype	Translational Compensation
α-Tpmκ	Increased Transcription	DCM	--------
β-Tpm Overexpression	Increased Transcription	DCM	Calcineurin Inhibitors
γ-Tpm Overexpression	Increased Transcription	Wildtype	No
α-Tpm Asp175Asn	Missense Mutation	Mild HCM	--------
α-Tpm Glu180Gly	Missense Mutation	Severe HCM	Yes
α-Tpm Glu54Lys	Missense Mutation	Severe DCM	--------
α-Tpm Ser283Asp	Missense Mutation	DCM	--------
α-Tpm Ser283Ala	Missense Mutation	Physiological Hypertrophy	--------
α-Tpm Glu180Gly + Ser283Ala	Two Missense Mutations in the Same Gene	Wildtype	Yes
α-Tpm Glu180Gly / Chimera 1	Modifier Loci	Wildtype	Yes
α-Tpm Glu180Gly / TnI peptide	Modifier Loci	Wildtype	Yes
α-Tpm Glu180Gly + SERCA2	Modifier Loci	Wildtype	Yes

Expression of Tropomyosin during Development

To understand the significance of Tpm in the heart, we utilized the murine animal model system because of the identity between human and murine Tpm protein sequence, along with the ability to generated knockout and transgenic mice. In addition, by utilizing these *in vivo* mouse models, we can conduct extensive and detailed molecular, biochemical, morphological and physiological analyses. Initially, we conducted a profile of Tpm isoform expression during cardiac development by focusing the analyses on murine embryogenesis, using both *in vivo* and *in vitro* conditions. Embryonic stem (ES) cells were employed which mimic different stages of murine embryonic development, including the differentiation of primitive organ systems including the myocardium. Results demonstrate that in the mouse embryo, the smooth muscle isoforms of α-Tpm and β-Tpm, along with the striated muscle isoform of β-Tpm are constitutively expressed from 4.5 day post coitus (p.c.) through adulthood.[19] These same isoforms are also expressed constitutively in both undifferentiated

embryonic stem cells and differentiated stem cells at all stages (day 1.5 – 22 days *in vitro*). Additional results show the γ-Tpm and δ-Tpm non-muscle isoforms are also constitutively expressed during these time points in both embryonic and differentiated stem cells. Interestingly, the α-Tpm striated muscle isoform does not initiate expression until later in development: embryonic day 7.5 p.c. and day 6 in differentiating ES cells.[19] This expression correlates with the formation of the primitive cardiac tube in the 7.5 day mouse embryos.[20,21] The expression of Tpm striated muscle-specific transcripts in these stages of cardiac development has also been reported for cardiac myosin heavy chain.[22] These results demonstrated that striated muscle Tpm isoforms are present during the earliest functional stages of the heart, and that these isoforms are identical to those present throughout cardiac development. Further analyses show that both striated muscle α- and β-Tpm isoforms are expressed during cardiogenesis (day 11 – 19 embryonic hearts), with the α-Tpm transcripts and proteins becoming the predominant Tpm isoform in the adult heart; the ratio of striated muscle α- to β-Tpm mRNAs changes from 5:1 to 60:1 during the embryonic to adult transition.

Deletion of Tropomyosin and Other Contractile Protein Genes

From the developmental study of Tpm expression, it was found that all 4 Tpm genes are expressed from embryogenesis through adulthood, with α-Tpm being the predominant tropomyosin isoform in the heart. To assess the functional and developmental significance of α-Tpm, this gene was disrupted by homologous recombination in the murine system. Homozygous α-Tpm gene deletions (knockouts) are embryonic lethal, dying between 8 – 11.5 days post coitum.[9,23] Heterozygous mice for α-Tpm show a 50% reduction in cardiac muscle α-Tpm mRNA, with no compensatory increase in transcript levels of striated muscle β-Tpm or γ-Tpm isoforms. However, the reduction in α-Tpm mRNA levels is not reflected at the Tpm protein level where normal amounts are produced and integrated into the cardiac myofibrils through an increased loading of polysomes on the α-Tpm transcripts, resulting in protein levels similar to those found in wild-type mice (Table I).[9] This data demonstrates that a change in steady-state level of α-Tpm mRNA does not affect the relative amount of translated mRNA and the amount of synthesized protein. Also, physiological analyses of the myocardial and myofilament function show no differences between heterozygous α-Tpm and control mice. These studies strongly suggest that nonsense mutations or deletions to a single α-Tpm allele that abrogate its expression can result in a morphological and physiologically normal functioning heart through translational regulation that plays a major role in the control of Tpm expression.

The fact that the homozygous α-Tpm knockout mice were embryonic lethal indicates that compensation by other Tpm isoforms (β-Tpm, γ-Tpm, δ-Tpm) does not occur or is not sufficient to rescue the phenotype. Preliminary studies show that a homozygous gene knockout of the β-Tpm gene results in embryonic lethality with a failure of the fertilized eggs to implant (data not shown). To address the significance of other Tpm genes, studies were initiated to address whether homozygous deletion of the γ-Tpm gene would also result in lethality. Results show that expression of the γ-Tpm gene is required for embryonic development; a knockout of a constitutive exon in this alternatively spliced gene led to ES cell and embryonic lethality.[24] Interestingly, knocking out an alternatively spliced C-terminal exon in the γ-Tpm gene was compatible with healthy and viable mice presumably due to compensation in producing other γ-Tpm isoforms that utilize alternative 3′ processing exons.[25] These results suggest that although functional redundancy does not exist among the Tpm genes, there can be significant redundancy between isoforms from the same gene.

The essential function of Tpm is also illustrated when examining mutations and ablations in other systems. In yeast, disruption of the α-Tpm gene leads to disappearance of actin cables supporting cytoskeletal architecture and disrupting cytokinesis.[26,27]

Haploinsufficiency of Tpm in Drosophila leads to disruption of myofibrillar thin filament assembly.[28,29] Also, deficiency of Tpm in axolotl results in severe cardiac abnormalities, which includes disorganized myofibrillar structures with an associated lack of heartbeat.[30] These studies emphasize the importance of Tpm in the viability and physiology of both the cardiac and body systems during early development.

Additional contractile genes have also been found to be essential for viability. Studies by Kumar et al. ablated the cardiac α-actin gene in the mouse and found that most of the homozygous targeted mice do not survive till birth, and the remainder die within two weeks postpartum.[31] Interestingly, mice lacking cardiac α-actin can be rescued to adulthood by the ectopic expression in the heart of enteric smooth muscle γ-actin; however, these rescued mice exhibit an enlarged, hypertrophied cardiac phenotype with reduced contractility and disorganized myofilaments in isolated cardiomyocytes. In the heterozygous mice, there is a reduction in cardiac α-actin transcript and protein levels that is intermediate between wild type and homozygous knock-out mice; these heterozygous mice also express compensatory increased levels of skeletal and vascular smooth muscle α-actin.[31] These heterozygotes also exhibit reduced rates of contraction and relaxation with a slight cardiac hypertrophy. An examination of knockout mice for murine α-myosin heavy chain showed dosage effects and functional deficits in the heart.[32] Homozygous null animals die between 11 and 12 days *in utero* of gross heart defects, while heterozygous mice survive, but exhibit fibrosis and alterations in sarcomeric structure. There are also severe impairments of both cardiac contractility and relaxation in these heterozygotes. In summary, α-Tpm mutations associated with haploinsufficiency do not lead to cardiac abnormalities, as occurs with cardiac α-actin and α-myosin heavy chain gene ablations.

Increased Beta-Tropomyosin Expression Causes Cardiac Dysfunction

Since both straited muscle α- and β-Tpm isoforms play key roles in cardiac muscle, we examined whether we could elucidate the functional differences between them. Our approach was to generate transgenic mice that overexpress the straited muscle β-Tpm isoform in the murine mouse heart.[12] These transgenic mice showed a 150-fold increase in β-Tpm mRNA expression, along with a 34-fold increase in the associated protein. These mice also exhibited a concomitant decrease in α-Tpm transcripts and protein. There were no detectable changes in other contractile protein isoforms or levels. Morphological examination showed no detectable changes in the structure of the transgenic hearts or associated sarcomeres. A physiological analysis revealed normal myocardial contractility; however, there are significant alterations in diastolic function associated with a delay in the time of relaxation and a decrease in the maximum rate of relaxation in the left ventricle. In addition, myofilaments isolated from the β-Tpm transgenic mice exhibit a significant increase in their calcium sensitivity.[11,13,33]

To explore further the significance of altering the α- to β-Tpm isoform ratio in the developing murine myocardium, we generated transgenic mice that express high levels of β-Tpm in the heart.[34] Decreasing the α/ β-Tpm ratio in murine cardiac muscle leads to severe atrial and ventricular dysfunction with death ensuing between 10-14 days postnatally. Pathological abnormalities included thrombus formation in the lumen of both atria and in the subendocardium of the left ventricle. Other morphological changes include atrial enlargement, fibrosis, and diffuse myocytolysis. These results firmly demonstrate an essential difference in Tpm isoform function in physiologically regulating cardiac performance. These studies also exemplify that alterations or mutations in the transcriptional rate of α- versus β-Tpm expression can dramatically affect cardiac development and function, resulting in severe pathological consequences (Table I). Confirmatory studies demonstrating the linkage between transcription of α- and β-Tpm is shown through the rescue of the high expression β-Tpm transgenic mice where shutting off expression of β-Tpm with 5-propyl-2-thiouracil leads to a reciprocal increase in

α-Tpm expression.[35] Additional studies by Sussman et al also show that the cardiac hypertrophy demonstrated in the high expression β-Tpm transgenic mice can be prevented by calcineurin inhibitors such as cyclosporin and FK506.[36] These calcineurin inhibitors can also rescue cardiac abnormalities in other cardiomyopathic models associated with expression of genetically-modified tropomodulin, ventricular light chain 2, and retinoic acid receptor. Collectively, these studies show that alterations in calcium handling may play a critical role in the development of cardiomyopathic conditions.

With the discovery that overexpression of β-Tpm results in altered cardiac performance and potentially, severe cardiac disease, an examination was conducted to determine which regions of the Tpm protein are responsible for differences between α-Tpm and β-Tpm. The approach was to utilize transgenic mice that exchanged regions within α-Tpm for their β-Tpm counterpart. In a series of papers, we examined two regions of Tpm that correspond to the areas that bind the troponin T complex (TnT): (1) the carboxyl-terminal amino acids 258 – 284; and (2) the internal TnT binding amino acids 175 – 190; and (3) both TnT binding regions within Tpm (amino acids 175 – 190 and 258 – 294).[33,37,38] Results show that in all three of these models, there were no morphological or pathological alterations in the heart. However, there were significant changes exhibited in sarcomeric performance in the whole heart and in isolated cardiac myofilaments. Exchanging the carboxyl-terminal region of α-Tpm for β-Tpm (using the α-Tpm as the backbone for the transgene) significantly decreased their rates of contraction and relaxation. The cardiac myofilaments containing this chimeric protein induced a decrease in the maximum tension and ATPase rate, together with a significant decrease in the sensitivity to calcium.[36] When the internal TnT domain was exchanged (Tpm amino acids 175 – 190), the transgenic hearts showed decreased rates of both contraction and relaxation, but with no effect on tension development. Surprisingly, there was an increase in the myofilament calcium sensitivity, which is the opposite response observed in the chimeric Tpm protein with the exchange of the 158 – 180 amino acids.[38] When both TnT binding regions are exchanged in Tpm, both decreased systolic and diastolic performance occurs. However, there are no differences in calcium sensitivity, but do exhibit a decrease in maximum developed tension.[33] Collectively, these results demonstrate that the two TnT binding regions in Tpm play a crucial role in systolic and diastolic function, work synergistically to regulate myofilament calcium sensitivity, but with the C-terminus playing a dominant role in determining force development.[3]

Although there is significant amino acid homology among the α-, β-, and γ-Tpm striated muscle isoforms, the previous studies demonstrated significant physiological differences in contractile and relaxation performance, along with differences in myofilament calcium sensitivity between α-Tpm and β-Tpm hearts. As previous reported, there is a 91% amino acid identity between α- and γ-Tpm striated muscle isoforms.[3] Also, previous work shows there is no endogenous expression of the γ-Tpm isoform in human or murine hearts.[7,39] To address whether the few amino acid differences that exist between α- and γ-Tpm might contribute to physiological changes in various cardiac parameters, we generated transgenic mice that overexpress the γ-Tpm striated muscle isoform in the heart.[40] Results show that these mice have normal life spans, and there are no morphological abnormalities in their sarcomeres or hearts (Table I). Physiological assessment of these mouse hearts reveal a hyperdynamic effect on systolic and diastolic function, coupled with a decreased sensitivity in calcium force generation in cardiac myofiber bundles. These results indicate that compensatory expression of either β-Tpm or γ-Tpm in the absence of α-Tpm would result in hearts displaying abnormal cardiac function.

Tropomyosin Mutations and Hypertrophic Cardiomyopathy

The significance of sarcomeric contractile proteins in human cardiac disease was first demonstrated by

Drs. J. and C. Seidman who found that missense mutations in myosin heavy chain correlate with familial hypertrophic cardiomyopathy (HCM).[41] Since then, over 1500 mutations in contractile protein genes have been associated with HCM which include β-myosin heavy chain, myosin-binding protein C, the regulatory and essential light chains, α-tropomyosin, troponin T, I, and C, and cardiac actin.[42] In addition, there are mutations in other cardiac genes that lead to HCM, such as phospholamban and calsequestrin. Most of these HCM mutations are due to missense mutations causing in a single amino acid change that results in a severe cardiac disease. The Seidman group first reported the association of Tpm with HCM.[43,44] There are at least 17 mutations in α-Tpm that have been found to cause HCM and 11 mutations that give rise to dilated cardiomyopathy (DCM).[42] No mutations have been identified in β-Tpm or γ-Tpm that result in either HCM or DCM. There appears to be variability in the severity of the pathological phenotype associated with HCM caused by Tpm mutations depending upon allelic variants, modifier genes, founder effects, and environmental influences.[44]

The first two HCM mutations identified in α-Tpm were Asp175Asn and Glu180Gly. Our laboratory developed two mouse models for these mutations which were the first *in vivo* transgenic mouse models to examine mutations in thin filament proteins that cause HCM.[14,45,46] Since there is 100% amino acid identity in α-Tpm between mouse and human sequences, the mutations incorporated in these transgenic mice reflect mutations and expression found in HCM patients. A consistent finding in these studies is that as exogenous Tpm expression is increased in these transgenic mice, there is a reciprocal decrease in endogenous wild-type Tpm protein expression so that the total amount of Tpm protein remains unchanged in the heart.[12,14,40,45] The Asp175Asn HCM mice showed a mild hypertrophic response with diminished contractile and relaxation rates (Table I).[14] In contrast, the Glu180Gly mice demonstrated a severe cardiac hypertrophy with significant fibrosis and atrial enlargement, resulting in lethality between 4.5 – 6 months of age (Table I).[45,46] Physiological analyses show significant impairments in both contractility and relaxation in the hearts and enhanced myofilament calcium sensitivity. These studies further demonstrate that single missense mutations in a contractile protein gene, namely α-Tpm, can result in severe cardiomyopathic conditions. Interestingly, although α-Tpm protein with these HCM mutations are expressed in both cardiac and skeletal musculature, pathological and physiological alterations appear to occur only in the heart. A possible explanation for this is that Tpm expression in the heart is ~ 92% α-Tpm, whereas in skeletal muscle, there is much more expression of diverse Tpm isoforms.[39]

Rescue of Hypertrophic Cardiomyopathy Phenotype

The development of mouse models that mimic human HCM diseases provide researchers with the opportunity to examine various methods for rescuing these mice from cardiomyopathy. As previously mentioned, cardiac thin filaments with HCM mutations exhibit an increased sensitivity to calcium. We hypothesized that by normalizing myofilament calcium sensitivity, we could phenotypically rescue the HCM phenotype exhibited by our Glu180Gly mice. To test this hypothesis, we generated a new transgenic mouse that expressed both the Glu180Gly protein along with a α-Tpm/ β-Tpm chimeric protein; these new mice displayed a normal cardiac morphology with no pathological abnormalities, improved cardiac function and normal myofilament calcium sensitivity (Table I).[10,11,47] These results demonstrate that calcium desensitization of myofibrils is a viable therapeutic option for treatment of HCM. This idea was extended and validated by cross-breeding the Glu180Gly mice with phospholamban knock-out mice.[48] Phospholamban is a calcium-handling protein that regulates calcium uptake into the sarcoplasmic reticulum. Results show that phospholamban ablation in the Glu180Gly mice rescues cardiac function and morphological abnormalities; there was a reversal of cardiac hypertrophy, fibrosis, and abnormal physiological

function in these rescued mice.[48] Additional microarray analysis found that 62 transcripts are highly involved in the suppression of the HCM phenotype.[49] These studies show that by modulating sarcoplasmic reticulum calcium cycling, many of the deleterious aspects of HCM caused by mutations in α-Tpm can be reversed.

In an extension of the work showing that a knock-out of phospholamban could rescue the Glu180Gly, we increased the uptake of calcium into the sarcoplasmic reticulum by increasing sarco(endo)plasmic reticulum calcium ATPase (SERCA2a) expression using an adenoviral vector.[47,50] Results show that administration of this SERCA2a expression vector improved heart pathology, cardiac function, fibrosis, and cardiac hypertrophy (Table I). This work was extended to human studies by administration of an adeno-associated virus expressing SERCA2a in phase 1 and 2 clinical trials for heart failure patients.[51,52] Results showed most patients exhibited marked improvements within 6 months, which included improved ejection fraction and end-systolic volume measurements. The success of these human trials demonstrates that regulation of calcium uptake in the sarcoplasm can be a potential treatment modality for heart failure patients.

Additional studies were initiated to examine whether there were other mechanisms that could be employed to rescue the HCM phenotype. We investigated whether oxidative myofilament modifications can reverse the diastolic dysfunction associated with HCM.[53] The Glu180Gly hearts display early signs of oxidative stress in the form of increased oxidative modifications of myosin binding protein C and activation of the MAPK signaling cascade. After N-acetylcysteine (NAC) administration, the morphology and diastolic function of the Glu180Gly hearts was similar to controls, indicating that NAC treatment rescued the diastolic dysfunction and hypertrophy. It was also found that there was increased SERCA2a expression, normal levels of phospholamban phosphorylation, and normal myofilament calcium sensitivity.[53] These results indicate that oxidative myofilament modifications are an important mediator in diastolic function which can be of potential use in the treatment of HCM.

Oxidative stress may be a principal component in triggering cardiac hypertrophy, fibrosis, and diastolic dysfunction.[7,54,55] Studies show that sphingosine-1-phosphate (S1P) and its receptor regulate and modulate oxidative signaling. One modulator of the S1P receptor is FTY720 which can prevent the progression of hypertrophy and fibrosis in a mouse model of pressure-overload hypertrophy.[56] To examine whether this oxidative signaling modulator could rescue the Glu180Gly HCM mice, FTY720 treatment was initiated, and results show that there was a partial reversal of diastolic dysfunction, a reduction in left atrial enlargement, and a decrease in oxidative modification of myosin binding protein C.[57] These results support a potentially translatable therapeutic approach for diastolic dysfunction that is observed in HCM.

Another potential treatment modality extends from studies showing that the C-terminal peptide of Troponin I (TnI) can act as a myofilament calcium desensitizer.[58] Using this short peptide, studies addressed whether it would be of therapeutic value to treat the Glu180Gly HCM mice.[59] When the TnI peptide was administered to these mice, there was a normalized decrease in calcium sensitivity in cardiac myofilaments, thereby demonstrating a potential therapeutic potential for the treatment of diastolic dysfunction of the heart (Table I).

Tropomyosin Mutations and Dilated Cardiomyopathy

Dilated cardiomyopathy (DCM) is a significant cause of morbidity and mortality in humans. DCM is characterized by chamber dilation, systolic and/or diastolic dysfunction, arrhythmias, and sudden cardiac death. There are both non-genetic and genetic causes that lead to this cardiomyopathic condition: non-genetic etiologies include viruses, cardiotoxicity, recreational drugs, and chemotherapeutic medications; genes associated with DCM are often

associated with the sarcomere and the cytoskeleton.[60,61] The most common sarcomeric genes have mutations in α- and β-myosin heavy chain, myosin binding protein C, actin, Tpm, troponin T, I, and C, desmin, vinculin, and muscle LIM protein.[42]

Tropomyosin mutations associated with DCM occur throughout the length of the 284 amino acid protein.[42] These mutations are all missense mutations which may disrupt the coiled-coil dimer by changing the electrostatic charge interactions between the amino acids to alter the Tpm dimerization, binding to actin or the troponin complex, and/or disrupt force transmission through the sarcomere.[62]

At least 11 distinct α-Tpm mutations lead to DCM. To investigate the structural and physiological consequences of known DCM mutations in Tpm, we generated the first mouse model of a sarcomeric thin filament protein that leads to this cardiomyopathic condition.[63] The mutation is the substitution of a glutamic acid for lysine at amino acid 54 which increases the positive charges in an otherwise negatively charged region of the molecule;[62] this alteration may disrupt protein stability or surface electrostatic charge characteristics, leading to defective force transmission which has been attributed to the cause of DCM.[62] The Glu54Lys transgenic mice exhibited histological and morphological changes in their hearts which are associated with the DCM phenotype with the progression to heart failure and death often ensuing by 6 months (Table I).[63] Decreases in left ventricular fractional shortening and impaired systolic and diastolic function was evident, along with a decreased sensitivity to calcium. Also, structural analysis showed the Glu54Lys mutation impaired Tpm flexibility, which may influence actin binding and myofilament calcium sensitivity. In addition, the Glu54Lys transgenic hearts exhibited a significant decrease in Tpm phosphorylation which indicated that altered phosphorylation may be a factor in the linkage of the mutation to DCM.[64] This work on the transgenic Glu54Lys mice illustrates how these animal model systems can provide information that is essential to understanding the disease state and development of therapeutic approaches in the treatment of DCM.

Effects of Tropomyosin Phosphorylation on Cardiomyopathy

Phosphorylation of cardiac proteins is a major post-translation mechanism that regulates physiological performance of the heart. Protein kinase A (PKA), protein kinase C (PKC), protein kinase G, calmodulin kinase II, myosin light chain kinase, casein kinase II, and other kinases regulate contractile protein phosphorylation and sarcomeric performance during both normal and stress-associated situations. The primary site of Tpm phosphorylation is at serine 283, the penultimate amino acid of Tpm.[65] Tpm phosphorylation appears to be controlled by casein kinase II.[66] During rat development, 70% of cardiac α-Tpm is phosphorylated, which decreases to 30% postnatally;[67] in murine hearts, we found there are high levels of Tpm phosphorylation at 1.5 months (35%), which decreased to 23% by 15 months; however, in the adult, levels are much higher.[66,68] This work indicates that Tpm phosphorylation may play a crucial role in myofibrillogenesis. To address the state of Tpm phosphorylation in human hearts, we probed myofibrillar proteins from adult left and right ventricles, and the interventricular septum. Results show that phosphorylated Tpm is present in all ventricular regions of the human heart.[68]

To address the role of phosphorylation in the heart, we generated transgenic mice which replaced the sole Tpm phosphorylation site (serine) with a phosphorylation mimetic (aspartic acid) at amino acid 283. High expression of this transgene resulted in a severe DCM phenotype with death occurring within 1 month of birth (Table I). Moderate expression of this S283D transgene led to a mild myocyte hypertrophy and fibrosis, did not affect lifespan, and was coupled with impaired diastolic function, but no alterations in calcium sensitivity of the myofibers.[66,69] These results demonstrate the functional significance of Tpm phosphorylation in the heart, but also illustrates that drug targets

associated with Tpm phosphorylation and/or dephosphorylation may prove useful as therapeutic agents in the treatment of cardiac disease.

To determine what potential effects would occur if Tpm phosphorylation was restricted, we generated transgenic mice where the amino acid 283 serine was replaced with alanine (Ser283Ala). When Tpm is dephosphorylated, the transgenic mice exhibit a compensated cardiac hypertrophy (Table I).[70] This hypertrophic phenotype occurs without alterations in cardiac function, myofilament calcium sensitivity, cooperativity, or response to β-adrenergic stimulus. Interestingly, if these Ser283Ala transgenic mice are stressed with transaortic constriction to induce pressure overload, there are greater functional defects than in control mice.[69,70] Collectively, these results suggest that modification of the Tpm phosphorylation status in the heart, depending upon the cardiac state/condition, may modulate the development of cardiac hypertrophy.

To ascertain Tpm phosphorylation in HCM, we determined the levels of Tpm phosphorylation in the HCM Glu180Gly transgenic mouse hearts. We examined these levels for a period of 1.5 – 5 months. Results show that the levels of Tpm phosphorylation depend upon the age of the mice; unlike control mice whose Tpm levels in hearts gradually decrease from 37% to 22% between 1.5 – 5 months, the HCM Glu180Gly hearts show a relatively constant level of 28% Tpm phosphorylation.[68] This level of Tpm phosphorylation at 4 – 5 months in the HCM hearts is higher than levels found in the control mice. The increased levels of Tpm phosphorylation in the aging HCM Glu180Gly hearts begs the question of whether the increased Tpm phosphorylation levels are a cause or effect of the cardiomyopathic disease process. With these results, we hypothesized that decreasing Tpm phosphorylation may be beneficial in the context of a chronic cardiomyopathic condition. To address this, we generated transgenic mice that simultaneously expressed both the HCM Glu180Gly and the Ser283Ala missense mutations. Our results showed that mice expressing both mutations exhibited no signs of cardiac hypertrophy with improved cardiac function (Table I).[68,69,71] Additional experiments show that there is an increase in the levels of phospholamban phosphorylation which results in an increased uptake of calcium into the sarcoplasmic reticulum. The increased flexibility of Tpm bearing the Glu180Gly mutation may be offset by the loss of phosphorylation in the Tpm overlap region of adjacent Tpm molecules. This was the first study to demonstrate that decreasing Tpm phosphorylation could rescue a HCM phenotype. Interestingly, another study has shown that pseudo-phosphorylation in Tpm with the Ser283Asp or Ser61Asp (a phosphorylation site in cytoskeletal non-muscle Tpm) can sometimes prevent/negate biochemical alterations induced by cardiomyopathic mutations.[72] Collectively, these studies suggest that alterations in Tpm phosphorylation may play a key role in future treatments of cardiomyopathic conditions.

Therapeutic Approaches to Muscle Diseases

In recent years, gene therapy has made significant progress in the treatment of numerous disease conditions, including striated muscle diseases. Many of these treatments were first developed in animal models; recently, more have advanced to human clinical trials with their subsequent approval for patients. As previously mentioned, studies demonstrated that removal of cytosolic calcium by delivery of Serca2a in the failing hearts of mice, sheep, and swine using adeno-associated viral (AAV) transfer systems can largely reverse cardiac dysfunction and improve systolic and diastolic performance.[47,73,74] These studies were extended and demonstrated that heart failure patients who were administered an AAV expressing Serca2a exhibited marked improvements within 6 months in their cardiovascular function.[18,51,52] Other treatments for cardiovascular conditions are drug therapies, such as beta-blockers and anticoagulants, and using a recently-developed medication called mavacamten, which is used to treat obstructive HCM by decreasing hypercontractility through impairment of myosin-

actin cross bridges. Antisense nucleotides have also been shown to work in failing heart patients by decreasing phospholamban expression and inhibiting phospholamban-Serca2a interactions, resulting in decreased contractility.[75-77]

The use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a gene-editing technique that has revolutionized therapeutic approaches in the treatment of diseases. This tool allows the targeting of specific genes to precisely modify DNA sequences in living organisms, including human cells. The practicality of CRISPR usage in humans has been demonstrated in the treatment of sickle cell disease where patient's bone marrow cells are removed, correction of the β-globin chain is conducted using CRISPR, followed by the re-introduction of the patient's gene-corrected bone marrow cells. The use of this gene editing technique in the correction of cardiovascular disease is more limited, but it is the ultimate goal of numerous investigations. Some of the limitations in utilizing this technique are the ability to specifically target cardiac cells, along with issues of long-term expression.[78]

Initial studies which examined the usage of CRISPR strategies in the cardiac field were conducted on both early-stage human zygotes and mouse models. Human ova were fertilized *in vitro* with sperm which had been injected with CRISPR- Cas9 reagents containing targeting sequences to repair an endogenous HCM mutation in the myosin binding protein C gene.[79] Results showed that the HCM mutation was corrected without evidence of off-target effects, thus illustrating the potential of this approach in addressing cardiovascular disease conditions, although serious ethical concerns need to be addressed with this approach.

The usage of the CRISPR strategy was employed to correct mutations in the dystrophin gene in a mouse model which illustrated its potential in addressing Duchenne Muscular Dystrophy. In this work, investigators used CRISPR to correct a specific nonsense mutation or to skip an exon endocing a premature stop codon in generating the primary mRNA transcript.[80] Results show that injection of mouse zygotes are successful in the production of dystrophin in both fast and slow skeletal muscles in the mouse line MDX which normally does not express dystrophin.[80] Additional work demonstrated that an intraperitoneal injection of the CRISPR reagents could result in producing dystrophin in the MDX striated muscles. These studies demonstrate the therapeutic potential for using CRISPR to address corrections in inherited diseases in terminally differentiated cellular systems.

Another approach which has significant potential in addressing diseases associated with skeletal muscle, such as muscular dystrophies, has been developed by the Millay lab. In this work, AAV expression viruses can fuse with skeletal myoblasts to deliver dystrophin minigene constructs for expression.[81] This novel approach may have the potential to treat multiple types of skeletal muscle myopathies, including oculomotor and myotonic dystrophies. Furthermore, use of these expression AAV viruses that fuse with skeletal myoblasts could also be used to deliver antisense oligonucleotides as therapeutic agents specifically to striated muscle tissue.

Conclusions

Although the diagnosis and treatment of cardiovascular diseases is still in its infancy, significant advances in the understanding of genetic causes and molecular pathways have been accomplished through the usage of animal model systems. These animal models demonstrate that specific mutations within the same gene can result in vastly different diseases, such as HCM versus DCM. Further, these models allow for the development of "proof-of-concept ideas" and testing various treatment modalities in the correction of these cardiomyopathic conditions. With respect to HCM and DCM, studies strongly implicate regulation of cytosolic calcium levels and the sarcomeric response to calcium as key mechanisms to control aberrant systolic and diastolic function and the development of a cardiomyopathic condition. As exemplified in the Tpm cited studies, increasing or decreasing the

myofilament sensitivity to calcium through chimeric Tpm proteins, Tpm phosphorylation, and TnI peptides can rescue the DCM or HCM phenotype. The usage of antisense nucleotides to decrease phospholamban levels can increase calcium uptake into the sarcoplasmic reticulum to treat HCM. Additionally, CRISPR-mediated genome editing, coupled with AAV-delivery systems may hold the future correction of cardiovascular diseases. At this point in time, researchers stand on the precipice of making exponential advances in the treatment of these diseases – let us hope that we can soon take that forward step into this promising future.

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