Interplay Between Heart and Skeletal Muscle Disease in Heart Failure
The 2011 George E. Brown Memorial Lecture
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Abstract: The study of single gene disorders often provides insight for more complex human disease. Mutations in the genes encoding the dystrophin protein complex cause muscular dystrophy and cardiomyopathy by destabilizing the plasma membrane of skeletal myofibers and cardiomyocytes. In these diseases, progressive skeletal muscle degeneration and weakness contribute to cardiac dysfunction. Moreover, the pace and pattern of muscle weakness, along with onset of cardiomyopathy, is highly variable even when associated with the same identical mutation. Using a mouse model of muscular dystrophy and cardiomyopathy, we identified genetic loci that modify muscle pathology and cardiac fibrosis. Distinct genetic modifiers were identified for diaphragm and abdominal musculature, and these genetic intervals differ from those that regulate pathology in the skeletal muscle of the limbs and the heart. One modifier gene was identified and highlights the importance of the transforming growth factor-β pathway in the pathogenesis of muscular dystrophy and cardiomyopathy. We determined that canonical transforming growth factor-β signaling contributes to heart and muscle dysfunction using a Drosophila model. Together, these studies demonstrate the value of using a genetically sensitized model to uncover pathways that regulate heart failure and muscle weakness. (Circ Res. 2012;110:749-754.)

Key Words: cardiomyopathy ■ heart failure ■ muscular dystrophy ■ modifiers

The George E. Brown Lecture at the annual Scientific Sessions, hosted by the Basic Cardiovascular Sciences Council, honors Dr Brown’s work as a pioneering physician scientist. His early investigation into human cardiovascular physiology focused on the peripheral circulation.1,2 Dr Brown was the first chair of the Section on Circulation and emphasized the importance of basic investigation for the American Heart Association’s mission. In the current era, the toolbox for investigation is considerably more complex and the ability to carefully delineate molecular pathways responsible for pathophysiology grows each day. The work discussed herein focuses on rare genetic disorders that affect both heart and skeletal muscle as cardiomyopathy and muscular dystrophy. Although these diseases are not common in the population, the pathways of myocyte injury and degeneration are broadly shared in many types of disease. By using animal models that mirror the human pathophysiology, we are positioned to delineate mechanisms that can be extrapolated more broadly to heart failure and its management.

The Architecture of Striated Muscle
Cardiomyocytes and skeletal myofibers share a similar basic cellular architecture. Striated muscle cells are composed of sarcomeres arranged longitudinally in series, as well as in parallel, to optimize force generation and transmission. Electron-dense Z bands, or Z disks, span the diameter of striated muscle cells and attach to the plasma membrane by way of specialized units known as costameres.3,4 Costameres are rib-like structures distributed along the surface of the sarcolemma that link the cytoskeleton to the extracellular matrix. The dystrophin glycoprotein complex is enriched at costameres and is positioned to transmit force laterally with respect to the myocyte’s long axis. Dystrophin, the protein product of the Duchenne Muscular Dystrophy gene, is a cytoskeletal protein that binds actin at its amino terminus and along its rod region. The carboxyl terminus of dystrophin links directly to transmembrane proteins that then bind components in the extracellular matrix (Figure 1). The primary link between the dystrophin complex and the matrix is through the heavily glycosylated protein, dystroglycan. The sarcoglycan complex, composed of α, β, γ, and δ subunits, stabilizes the interaction between α and β dystroglycan, thereby reinforcing the connection from the cytoskeleton through the membrane into the extracellular matrix. Autosomal recessive mutations in the sarcoglycan genes cause the phenotype of muscular dystrophy and cardiomyopathy simi-
lar to what arises from dystrophin mutations. Disruption of the dystrophin complex renders heart and muscle cells susceptible to contraction-induced damage and leads to progressive weakness and cardiomyopathy.5,6 Nearly all muscle groups are adversely affected by dystrophin and sarcoglycan mutations including the muscles of the limbs and trunk. Respiratory muscle dysfunction leads to hypoventilation and chronic hypoxia. The combination of respiratory and cardiac muscle is often lethal for patients with dystrophin and sarcoglycan gene mutations. Overall, the phenotypic constellation associated with mutations in dystrophin or β-,-, or δ-sarcoglycan is similar, although there is variation in age of onset and progression.

Although the internal cellular architecture of cardiomyocytes and skeletal myofibers shares similarity, the positioning of cells within the heart or muscle group differs. Skeletal myofibers are elongated syncytial cells; a single cell may span the length of a muscle. Myofibers within normal muscle have a consistent cross sectional area. With muscular dystrophy, there is degeneration of myofibers accompanied by ongoing regeneration. This combination of necrotic myofibers alongside newly regenerated myofibers results in fiber size variation within the muscle. Myofibers with the largest diameter are thought to be more susceptible to contraction-induced damage. In muscular dystrophy, the robust regeneration that typifies skeletal muscle is exhausted over time, and the muscle is replaced by fibrosis leading to scar that can be readily visualized on MRI. Fatty infiltration of muscle also occurs, and this replacement of normal muscle by fibrosis and fat defines muscular dystrophy. In contrast to skeletal myofibers, normal cardiomyocytes are small and binucleate. The intercalated disks that link the short axis of cardiomyocytes are enriched with desmosomal proteins. Lateral attachments between neighboring cardiomyocytes are less uniform, owing to the circumferential arrangement of cells within the ventricles. Dystrophin and sarcoglycan gene mutations are associated with progressive loss of cardiomyocytes and replacement of the heart by fibrosis. Therefore, although cardiomyocytes and skeletal myofibers differ in morphology and alignment, both are susceptible to contraction-induced disruption in the absence of dystrophin.5,6

**Evidence for Genetic Modifiers in Human Genetic Disease**

Genetic modifiers are thought to be important for many single gene mendelian disorders, and also for more complex genetic diseases.7,8 Modifiers enhance or suppress the disease process through pathways unrelated to the primary defect. There is a single common SGCG mutation responsible for Limb Girdle Muscle Dystrophy (LGMD) 2C.9 This mutation, 521ΔT, has been described in LGMD patients from nearly every continent, and it is notable is that both mild and severe forms of muscular dystrophy are associated with this same single mutation.10,11 Individuals with the frame-shifting 521ΔT mutation may lose ambulation by the early second decade and have accompanying cardiomyopathy. Alternatively, individuals with the 521ΔT mutation may remain ambulatory beyond age 30 and the heart may be spared. Skeletal and cardiac muscle involvement is not always tightly correlated since cardiomyopathy may develop independent from skeletal muscle weakness. The identification of the single common γ-sarcoglycan gene mutation underscores the presence of modifier features since the primary mutation, on its own, does not entirely predict clinical outcome.

Clinical outcome from genetic disease is modified by environmental factors, including exercise and diet, and these influences have been documented for other inherited forms of cardiomyopathy.12–14 Genetic modifiers are those variants which when coinherited either enhance or suppress disease. We developed an animal model of LGMD 2C by deleting the γ-sarcoglycan gene, Sgcg, to use mice to search for genetic modifiers.15 Animal models, particularly mice, are useful for mapping modifiers given the consistency from inbred strains and uniform housing conditions that minimize environmental influences. It is hoped that genetic modifiers identified in mice will translate to human disease. It is possible that genetic modifiers for one form of disease will also modify other genetic forms of cardiomyopathy and muscular dystrophy, or even nongenetic forms of heart failure and muscle weakness.

**Mapping Genetic Modifiers Using Mouse Models of Human Disease**

Quantitative trait locus (QTL) mapping is a method to map genetic modifiers.8 QTL mapping requires quantitative mea-
ures of phenotype, and the genetic analysis need not specify the mode of inheritance. To map QTLs associated with muscular dystrophy and cardiomyopathy, we used 2 independent measures. The first assay quantifies membrane fragility by measuring Evans blue dye uptake within dystrophic muscle. With disruption of the dystrophin complex, as occurs in genetic forms of muscular dystrophy and cardiomyopathy, the sarcolemma becomes fragile. The patchy nature of dye uptake in dystrophic muscle reflects a nonuniform injury process. Measuring dye content in the entire muscle eliminates sample bias that can arise from studying only sections of muscles. Additionally, this method is sufficiently high throughput and reliable to allow for QTL mapping. The second quantitative assay used for QTL mapping determined the amount of hydroxyproline per given gram of tissue as a reflection of collagen deposition and fibrosis. This determination of fibrosis, like the measurement of dye uptake, samples the entire muscle group. Mapping genetic modifiers in mice often relies on inbred strains such as C57Bl6/J and 129/SVemst/J(129) suppressed both dye uptake and fibrosis of the skeletal muscle as well as the lung. Mice deleted for the gene responsible for Marfan syndrome, FBN1, the gene responsible for Marfan syndrome. LTBP4 is highly expressed in heart and skeletal muscle as well as the lung. Mice deleted for LTBP4 develop pulmonary fibrosis and cardiomyopathy. All members of this superfamily are characterized by the presence of multiple epidermal growth factor (EGF) repeats distributed along the length of the protein. Interspersed are a smaller number of 8-cystine domains, some of which directly bind TGFβ. The amino terminus of LTBP4 directly binds to the extracellular matrix. LTBP proteins, together with the latent TGFβ, form the large latent complex in the extracellular matrix. With injury, locally acting proteases cleave LTBP4 resulting in the release of TGFβ to bind neighboring receptors. TGFβ engagement of its receptor activates bothcanonical and noncanonical TGFβ signaling. The Ltbp4 gene contains 39 exons distributed over 34 KB. The proline-rich region of the protein is encoded by exons 11, 12, and 13. An insertion/deletion polymorphism within exon 12 of Ltbp4 gene was identified that encoded an LTBP4 protein with altered proteolytic susceptibility. This insertion/deletion polymorphism showed the tightest correlation with membrane leakage and fibrosis in quadriceps muscle. We replicated this finding in an independent cohort of animals finding that the Ltbp4 insertion/deletion polymorphism is a modifier of muscle disease and muscular dystrophy.

**TGFβ Signaling as a Modifier**

Canonical TGFβ signaling relies on SMAD phosphorylation and SMAD-driven gene transcription. To assess whether the insertion/deletion Ltbp4 polymorphism resulted in differential SMAD signaling, fibroblasts from the 129 and D2 backgrounds were treated with the small latent complex. D2 fibroblasts were found to produce more pSMAD2/3 in response to TGFβ exposure than those from the 129 background. This difference was detected in both the presence and absence of the Sgcg allele, indicating that this is an inherent feature of the DBA background and not a strict consequence of the dystrophic process. Together, these data demonstrate the mechanism of action of the insertion/deletion polymorphism in Ltbp4 and its capacity to modify muscular dystrophy.

LTBP proteins and TGFβ are coreleased from secreting cells and then deposited in the matrix. In the matrix, the LTBP-TGFβ complex lies dormant as the large latent complex affixed to the matrix though microfibrils. The deletion allele of Ltbp4 was associated with increased nuclear pSMAD2/3 compared with Sgcg mice with the insertion Ltbp4 allele. Specifically, pSMAD2/3 was seen in myonuclear clusters in skeletal and cardiac muscle. Interestingly, these clusters were often seen within the right ventricle. This model suggests that levels of TGFβ signaling are critical for determining severity of disease. We hypothesize that “hyper-TGFβ” signaling may be deleterious, particularly in disorders where there is chronic injury and hence chronic and continuous activation of TGFβ signaling.

TGFβ receptors activate both canonical and noncanonical TGFβ signaling, and both signaling pathways are positioned to contribute to pathogenesis. Cohn et al used a neutralizing anti-TGFβ antibody or the angiotensin receptor blocker losartan to treat the mdx mouse model of Duchenne Muscular Dystrophy and found reduced pSMAD, improved fibrosis, and muscle regeneration. This work parallels what had been shown using losartan to prevent aortic dilation in a mouse model of Marfan syndrome. More recent studies have suggested that noncanonical TGFβ pathways may be particularly important for the vascular disease in Marfan syndrome, specifically ERK1/2 signaling. Interestingly, the same anti-TGFβ antibody did not protect against the dilation and fibrosis in the murine heart after transaortic constriction in the pressure-overload model of hypertrophy. In contrast, cardio-

**Ltbp4 Is a Modifier of Muscular Dystrophy**

Latent TGFβ-binding protein (LTBP4) is a member of the fibrillin superfamily. The fibrillin superfamily includes 4 LTBP5s and 3 fibrillins, including FBN1, the gene responsible for Marfan syndrome. LTBP4 is highly expressed in heart and skeletal muscle as well as the lungs. Mice deleted for Ltbp4 develop pulmonary fibrosis and cardiomyopathy. All members of this superfamily are characterized by the presence of multiple epidermal growth factor (EGF) repeats distributed along the length of the protein. Interspersed are a smaller number of 8-cystine domains, some of which directly bind TGFβ. The amino terminus of LTBP4 directly binds to the extracellular matrix. LTBP proteins, together with the latent TGFβ, form the large latent complex in the extracel-

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The above document contains a detailed description of the role of Ltbp4 in muscular dystrophy and cardiomyopathy, with a focus on the genetic and molecular mechanisms involved. It highlights the importance of latent TGFβ-binding protein (LTBP4) as a modifier of muscular dystrophy, emphasizing the role of polymorphisms in disease progression and the potential therapeutic interventions targeting TGFβ signaling pathways.
myocyte-specific deletion of Tgbr2 was associated with reduced fibrosis and preservation of normal cardiac size after transaortic constriction.29 The authors attributed this difference in rescue to noncanonical TGFβ signaling, specifically to TGFβ-associated kinase (TAK)-1 and observed that bone morphogenetic protein (BMP)7 signaling may be important. However, the authors also note that the anti-TGFβ antibody may have insufficient access to myocytes and found that genetic ablation of Tgbr2 more effectively reduced elevated pSMAD2/3 levels than the anti-TGFβ antibody. Therefore, pathogenesis is likely mediated by multiple signaling pathways as well as critical thresholds of activation.

Reducing Canonical TGFβ Signaling Improves Both Heart and Muscle Function

To investigate whether TGFβ signaling is pathogenic, we relied on a Drosophila model of heart and muscle disease. This model was previously generated by deleting the Drosophila Sgcd gene using imprecise P element excision.30 Degenerating fly muscle does not become infiltrated by fibroblasts or inflammatory cells, and therefore the features of this model may reflect muscle-intrinsic properties. The indirect flight muscle in adult Drosophila is composed of 6 longitudinally arranged muscles; this muscle displays tears within Sgcd null muscle, and such tears are rarely seen in wild-type muscle.30 Sgcd mutant flies allowed ad libitum exercise had larger and more obvious tears within the muscle. Therefore, contraction-induced damage is a feature of the Sgcd null muscle similar to what has been noted for mammalian dystrophic muscle.31 Sgcd mutant flies displayed little upward walking ability in a 10-second walk test. Sgcd mutants were crossed with an allele in which the indifference containing Smox is expressed under the control of a SMAD responsive enhancer,32 revealing both quantitative and qualitative evidence for increased TGFβ signaling.31 Myonuclei immediately adjacent to muscle tears had visible β-galactosidase activity. When mechanically injured, normal fly muscle showed increased β-galactosidase activity in those nuclei immediately adjacent to muscle disruption (Figure 2), indicating that hyper-TGFβ signaling normally occurs within myofibers as a response to injury.

To test whether canonical TGFβ signaling was pathogenic, we introduced allele designed to reduce but not eliminate components of the SMAD pathway. We crossed heterozygous alleles for Medea (homologous to SMAD4), MAD (SMAD1/5/8), or Smox (SMAD2/3) each to Sgcd flies. Introducing heterozygous alleles allowed partial reduction of TGFβ signaling.31 We found that reducing the dose of TGFβ signaling using a Medea allele improved walking in Sgcd null flies to normal baseline levels. To ask whether reducing TGFβ signaling also improved cardiac function, we analyzed cardiac function in flies. The Drosophila heart tube is a dorsal structure in the adult fly that beats rhythmically to supply the open circulatory system. This rhythmic beating and heart tube dimensions can be visualized using optical coherence tomography. Sgcd flies had a dilated and poorly contractile heart tube characterized by increased end-diastolic and end-systolic diameters. Reducing SMAD signaling normalized both end-diastolic and end-systolic diameters in mutant flies. Heart tube function was restored by haploinsufficiency of Medea or Smox but not MAD.31 Interestingly the MAD allele, which is more important for BMP signaling, did not restore normal cardiac diameters but did restore walking function. Since genetic reduction using these alleles is constitutive in all cells, improved walking occurred independent of correcting heart function. In this model, improving skeletal muscle function is sufficient to improve performance in the walking assay in the absence of correcting heart tube dysfunction.

Additional Genetic Modifiers for Cardiomyopathy and Muscular Dystrophy

The initial search for genetic modifiers in Sgcd mice relied on a subset of 80 animals that showed the most extreme phenotypes.18 A genomewide scan was extended to a larger number of Sgcd null animals (n=280) to search for additional modifiers. This analysis confirmed the chromosome 7 modifier containing Ltbp4 as a strong determinant of membrane fragility and fibrosis. Nearly 40% of the phenotypic variance of Evans blue dye uptake was explained by this locus.33 Notably, all limb-based skeletal muscles demonstrated a highly significant contribution of this locus including the quadriceps, gastrocnemius/soleus, triceps, and gluteus/hamstring muscles. With this same analysis, we identified a region on chromosome 9 that correlated with cardiac fibro-
Ongoing efforts are aimed at identifying this gene. A region on chromosome 18 correlated with abdominal muscle membrane damage. We also identified a region on chromosome 3 that correlated with both diaphragm muscle fibrosis and abdominal muscle fibrosis, providing genetic evidence that the abdominal muscles contribute to respiratory function.

Genetic Modifiers Integrate Physiology

During the normal cycle of inspiration and expiration, the diaphragm and abdomen move in concert with each other, and respiratory muscle activity can both directly and indirectly affect cardiac function. Epaxial muscles (the intercostal and paraspinal muscles) have a distinct developmental origin compared with the hypaxial (limb and abdominal) muscles. The diaphragm muscle has a mixed developmental origin because of its crucial and costal aspects. The abdominal muscles can be viewed as accessory respiratory muscles. In muscular dystrophy, abdominal muscle dysfunction occurs early and accompanies decreased respiratory function.

Respiratory dysfunction in muscular dystrophy derives from primary pathology in the diaphragm, intercostal and abdominal muscles. Hypoventilation and hypoxia trigger increased pulmonary artery pressure, thereby increasing the workload of the right ventricle. Thus, chronic hypoxia adversely affects right ventricular function by elevating pulmonary artery pressures and increasing contraction-induced damaged in the right ventricle. In the Sgcg null mouse model, right ventricular cardiomyocyte nuclei were observed with increased pSMAD2/3 staining, consistent with a model where there are two primary cardiomyocyte defects that are further exacerbated by reduced respiratory function.

Heart failure itself, especially in late stages, is associated with cachexia. Ablation of the cardiomyocyte myostatin gene was found to prevent skeletal muscle wasting in a pressure overload model of heart failure. These data suggest that the endocrine function of the heart, mediated by myostatin, has a significant effect on skeletal muscle growth. Other muscle growth factors such as insulin-like growth factor may also be important for cardiac-mediated effects on skeletal muscle growth. Respiratory function is impeded in a number of states such as obesity and chronic lung disease, and therefore in these settings hypoventilation may contribute significantly to cardiac dysfunction. The similar modifiers affecting diaphragm and abdominal muscle fibrosis integrate the underlying pathophysiology. The role of respiratory muscle function should be more carefully considered in other forms of cardiomyopathy, especially in patients in whom there are often comorbidities. Together, these findings provide genetic evidence that reinforces what is known from physiological studies. The identification of the genes within the identified QTLs will shed further light on the interplay between muscle and heart function and will lead to new pathways to exploit for diagnostics and therapeutics.

Sources of Funding

This work was supported by the NIH through NHLBI, NIAMS, and NINDS, the Muscular Dystrophy Association and the American Heart Association.

Disclosures

None.

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doi: 10.1161/CIRCRESAHA.111.256776

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