Heart Failure

Disruption of Striated Preferentially Expressed Gene Locus Leads to Dilated Cardiomyopathy in Mice

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Background—The striated preferentially expressed gene (Speg) generates 4 different isoforms through alternative promoter use and tissue-specific splicing. Depending on the cell type, Speg isoforms may serve as markers of striated muscle differentiation.

Methods and Results—To elucidate function of Speg gene isoforms, we disrupted the Speg gene locus in mice by replacing common exons 8, 9, and 10 with a lacZ gene. β-Galactosidase activity was detected in cardiomyocytes of the developing heart starting at day 11.5 days post coitum (dpc). β-Galactosidase activity in other cell types, including vascular smooth muscle cells, did not begin until 18.5 dpc. In the developing heart, protein expression of only Spegα and Spegβ isoforms was present in cardiomyocytes. Homozygous Speg mutant hearts began to enlarge by 16.5 dpc, and by 18.5 dpc, they demonstrated dilation of right and left atria and ventricles. These cardiac abnormalities in the absence of Speg were associated with a cellular hypertrophic response, myofibril degeneration, and a marked decrease in cardiac function. Moreover, Speg mutant mice exhibited significant neonatal mortality, with increased death occurring by 2 days after birth.

Conclusions—These findings demonstrate that mutation of the Speg locus leads to cardiac dysfunction and a phenotype consistent with a dilated cardiomyopathy. (Circulation. 2009;119:261-268.)

Key Words: cardiomyopathy ■ hypertrophy ■ myocytes ■ genes

Myosin light chain kinases (MLCK) are a family of proteins that are important for myocyte function, including structure and regulation of the actin-based cytoskeleton.1,2 One member of this family, striated preferentially expressed gene (Speg), has isoforms in both striated and smooth muscle cells.3 The Speg locus contains 2 transcriptional start sites, and through alternative promoter use and splicing in a tissue-specific manner, it generates 4 different isoforms.3 Spegα and Spegβ are expressed in striated muscle (cardiac and skeletal), aortic preferentially expressed gene (Apeg-1) is expressed in smooth muscle (predominantly vascular), and brain preferentially expressed gene (Bpeg) is expressed in the brain and aorta.3–5 Spegα and Spegβ share homology with MLCK family members. Specifically, the Speg isoforms, along with obscurin-MLCK, are unique members of the MLCK family, because they contain 2 tandemly arranged serine/threonine kinase (MLCK) domains.2 Beyond the MLCK domains, Spegα and Spegβ also contain immunoglobulin and fibronectin domains that are characteristic of the MLCK family of proteins. Prior investigations in our laboratory revealed that Spegα and Spegβ are sensitive markers of striated muscle differentiation and that Speg colocalizes with desmin in the sarcomeric Z disc; however, the functional significance of Speg isoforms is yet to be elucidated.

Editorial p 213
Clinical Perspective p 268

In contrast to smooth muscle cells, striated muscle cells of both cardiac and skeletal origin undergo terminal differenti-
ation shortly after birth in mammals, with the majority of these cells not retaining the ability to proliferate or regenerate after injury. It has been shown that myocardial regeneration occurs after injury because of the presence of cardiac progenitor cells, and this regeneration occurs predominantly in regions of the heart with a viable myocardium, outside of the infarcted area. Thus, the ability to regenerate myocytes at regions of injury, as well as the therapeutic potential of progenitor cells, has been an intense area of investigation in the field of cardiovascular medicine, because heart failure due to myocardial injury and death remains a difficult and deadly problem for millions of Americans.

For cardiomyocytes to function appropriately, contractile forces generated in the sarcomere are transmitted to the extracellular matrix. If this process does not occur appropriately, cardiac remodeling of either a hypertrophic or dilated phenotype takes place. Hypertrophic cardiomyopathy results in little or no increase in cardiac chamber volume but does cause thickening of the ventricular wall. In contrast, dilated cardiomyopathy (DCM) results in an increase in cardiac chamber volume and thinning of the ventricular wall. It has been suggested that familial forms of cardiomyopathy may result from alterations in different subsets of genes, for instance, sarcomeric gene mutations (hypertrophic) versus cytoskeletal, contractile, and calcium regulatory gene mutations (dilated). However, this is not totally inclusive, because ~10% of cases of familial DCM may be due to mutations in sarcomere protein genes.

To study the potential importance of the Speg gene locus in cardiovascular biology, we removed exons 8, 9, and 10 of the Speg locus by targeted deletion. This mutation disrupted all of the Speg isoforms. Mice homozygous for this mutation died in the immediate neonatal period and demonstrated a DCM with evidence of cellular hypertrophy, myofibril degeneration, and cardiac dysfunction. SpegA and SpegB proteins were the only isoforms detected during this developmental period in the heart. The present study demonstrates the importance of the Speg proteins for mouse cardiac development and survival.

**Methods**

Detailed methods are described in the expanded Methods section in the online-only Data Supplement.

**Generation of Speg Mutant Mice**

The cloning strategy for generation of the Speg targeting construct, Southern blotting, and genotyping are described in detail in the Data Supplement.

**Myofibril Extraction and Western Blotting**

The myofibril fraction from hearts at 18.5 days post coitum (dpc) was extracted as described previously. For more details, see the Data Supplement.

**Echocardiography**

A Vevo 770 high-resolution micro-ultrasound system and a 40-MHz probe (VisualSonics, Toronto, Canada) were used for transthoracic echocardiography on nonanesthetized 18.5-dpc animals, as described previously.

**Cardiac Weight Index**

At 18.5 dpc, the embryos were harvested and their hearts removed. Cardiac weight index was calculated as heart wet weight (in milligrams) divided by total body weight (in grams).

**Myocyte Size, Number, and Assessment of Apoptosis**

These cellular parameters were obtained as described previously. For more details, see the Data Supplement.

**2D Gel Electrophoresis and Western Blotting**

Protein from 18.5-dpc hearts was extracted as described previously. Electrophoresis and Western blotting were performed by Kendrick Laboratories Inc, Madison, Wis, as described in the Data Supplement.

**Isoelectric Focusing**

Heart tissue was harvested from 16.5-dpc hearts. Electrophoresis on an isoelectric focusing gel (pH 3–7, Invitrogen, Carlsbad, Calif) and Western blotting were performed as described in the Data Supplement.

**Statistical Analysis**

One-way ANOVA was used to determine differences between parameters of wild-type (+/+ ) and homozygous (−/−) embryos/neonates. Scheffé’s F test was used as a post hoc test, and differences were considered significant at P < 0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Targeted Disruption of Speg Gene Locus**

To generate Speg mutant mice, a targeting vector was constructed to replace exons 8, 9, and 10 of the Speg gene locus by a nuclear localized lacZ gene (Figure 1A, Data Supplement). Southern blot analyses (supplemental Figure IB) revealed that the 5′-probe hybridized to a 7-kb fragment in wild-type mice (+/+), a 4-kb fragment in homozygous mutant mice (−/−), or both fragments in heterozygous mice (+/−).

The Speg gene locus contains 2 transcription start sites (supplemental Figure IA), and through alternative splicing in a tissue-specific manner, it generates 4 different isoforms, namely, Spegβ, Spegα, Bpeg, and Apeg-1, with a transcript size of 11, 9, 4, and 1.4 kb, respectively. Spegβ and Bpeg are transcribed from the first promoter and Spegα and Apeg-1 from the second promoter. Northern blot analysis was performed on RNA extracted from 18.5-dpc hearts of +/+ , +/− , and −/− embryos. Figure 1A depicts the Speg isoforms and the location of specific probes for Northern analysis. Using a probe encompassing the Apeg-1 coding region (Figure 1B), we observed transcripts corresponding with Spegβ, Spegα, Bpeg, and Apeg-1 in +/+ and +/− embryos. Speg isoform levels were reduced in +/− compared with +/+ mice and were not detected in −/− embryos. Using the 5′ Speg probe, we detected transcripts that corresponded with Spegβ and Bpeg in +/+ and +/− embryos (Figure 1B). Interestingly, as the mRNA levels for Spegβ and Bpeg decreased in +/− and −/− embryos, deletion of exons 8 to 10 resulted in splicing of exon 7 into the lacZ splice acceptor site, which produced a mutant 6.4-kb message (Figure 1B). Using the 3′ Speg probe, we detected transcripts
corresponding with Spegα and Spegβ in +/+ embryos, and these transcript levels decreased in +/− and −/− embryos (Figure 1B). 18S hybridization verified equal RNA loading.

Western blot analysis was performed on myofibril fractions obtained from 18.5-dpc +/+ , +/−, and −/− hearts. Blotting with an affinity-purified Speg/Apeg-1 antibody revealed ~250-kDa and ~350-kDa bands consistent with Spegα and Spegβ in +/+ and +/− hearts but none in −/− hearts (Figure 1C). Even though mRNA for Apeg-1 and Bpeg was not detected in −/− hearts but none in −/− hearts, protein expression for Apeg-1 and Bpeg was present in heart homogenates of +/+ and +/− mice, protein expression for Apeg-1 and Bpeg was not detected in myofibril fractions of 18.5-dpc hearts. These data demonstrate that the Speg isoforms present in cardiac myofibrils at this developmental time point are of Spegα and Spegβ origin. The nonspecific band and reblotting with an antibody against desmin demonstrated equivalent protein loading.

LacZ Transgene Expression During Development in Heterozygous Speg Mutant Embryos

The gene-targeting strategy effectively knocked in the bacterial lacZ reporter gene under the transcriptional control of the Spegα/Apeg-1 promoter. We evaluated lacZ staining in the heterozygous embryos to determine the localization of Spegα/Apeg-1 promoter activity during development. At 11.5 dpc, lacZ staining was restricted to the heart (Figure 2A). Staining was evident in both right and left atria and future ventricles but not in the outflow tract (Figure 2B and 2C). At 16.5 dpc, lacZ staining remained predominantly in the heart (Figure 2D), and histological analysis revealed both atrial (Figure 2E) and ventricular (Figure 2F) staining in cardiomyocytes. At 16.5 dpc, there was no evidence of staining in the aortic arch or great vessels (Figure 2D). At 18.5 dpc, lacZ staining persisted in both right and left atria and ventricles of the heart (Figure 2G and 2H). Staining was apparent in the trachea (Figure 2G), but this staining was in the respiratory epithelium and not the airway smooth muscle cells (data not shown). At 18.5 dpc, staining was seen for the first time in elastic arteries, including the aorta, carotid arteries, and pulmonary arteries (Figure 2I). Within the vessel wall, staining was evident in smooth muscle cells but not endothelial cells, as seen in the pulmonary arteries (Figure 2J and 2K); however, there was evidence of coronary artery staining was present at 18.5 dpc (Figure 2L), which suggests a lack of β-galactosidase activity in muscular arteries. Taken together, these data demonstrate that Speg isoform expression was predominantly in cardiomyocytes of both atria and ventricles.
from 11.5 through 16.5 dpc (consistent with the Speg isoform), and expression outside the heart did not begin until 18.5 dpc in vascular smooth muscle cells of elastic arteries (consistent with the Apeg-1 isoform). Expression was not present in muscular arteries, including the coronary vessels.

**Neonatal Death in Homozygous Speg Mutant Mice**

Speg<sup>−/−</sup> mice on a 129Sv X C57BL/6 background were phenotypically normal and fertile and were bred to generate Speg<sup>−/−</sup> mutant mice. Among 122 embryos genotyped at 15.5 dpc or earlier, there was a genotype distribution of 26% +/-, 53% +/-, and 21% −/− (Table), which suggests no remarkable early embryonic death. Analysis of genotypes at 18.5 dpc revealed a mendelian distribution of 21% +/-, 58% +/-, and 21% −/− embryos; however, starting at day 2 postnatally, lethality increased dramatically in the Speg<sup>−/−</sup> neonates. By 21 days postpartum, of the 360 offspring from heterozygous breeding, there were only 2% viable Speg<sup>−/−</sup> mice (Table). These data indicate that Speg<sup>−/−</sup> mice survive embryonic development, but the majority of mutant mice die in the early postnatal period. After breeding the 129Sv X C57BL/6 Speg<sup>−/−</sup> mice to a pure C57BL/6 background (9 generations), no viable Speg<sup>−/−</sup> mice were observed postnatally. In contrast, the Speg<sup>−/−</sup> mice appeared normal, with no evidence of lethality in adult mice.

**Cardiac Abnormalities in Speg<sup>−/−</sup> Embryos**

Overall size and body weight were not different between Speg<sup>+/−</sup> (1.36±0.053 g), Speg<sup>−/−</sup> (1.30±0.038 g), and Speg<sup>+/−</sup> (1.21±0.025 g, P=0.1316; minimum of n=8 per group) pups at 18.5 dpc. The most obvious phenotype in the Speg<sup>−/−</sup> embryos was enlargement of the heart. By 16.5 dpc, the Speg<sup>−/−</sup> embryos began to show evidence of heart dilatation compared with Speg<sup>+/−</sup> embryos (Figure 3A); however, by 18.5 dpc, Speg<sup>−/−</sup> embryos revealed a more dramatic dilatation of the heart, with involvement of both right and left atrial and ventricular chambers (Figure 3A). The ventricular walls appeared thinner in Speg mutant hearts, and we found no evidence of septal defects between the right and left side of the heart, and no valvular abnormalities. The absolute cardiac weights were 7.7±0.4 mg for Speg<sup>++</sup> mice (n=15) and 10.3±0.6 mg for Speg<sup>−−</sup> mice (n=12, P<0.05). To further determine whether there was evidence of cardiac hypertrophy, cardiac weight index and morphological analysis of cardiomyocytes were assessed in Speg<sup>++</sup> and Speg<sup>−−</sup> embryos. Speg<sup>−−</sup> embryos revealed a significant increase in cardiac weight index compared with Speg<sup>++</sup> embryos (P<0.05; Figure 3B). In addition, differences were detected in the structural organization of the myocardium in Speg<sup>−−</sup> compared with Speg<sup>++</sup> mice at E18.5 (Figure 4A). The hearts of Speg<sup>−−</sup> mice were characterized by a reduced density of myocyte nuclei per unit area of tissue, which suggests that cellular hypertrophy and/or cell death contributed to the cardiac phenotype. However, measurements of apoptosis indicated that myocyte death was present in comparable proportions in both groups of animals (1.21±0.37 versus 1.18±0.39 apoptotic cells/1000 cells in Speg<sup>++</sup> and Speg<sup>−−</sup> hearts, n=5 and 6 mice, respectively). Therefore, the volume and number of maturing cardiomyocytes were determined quantitatively. Compared with Speg<sup>++</sup>, the myocardium of Speg<sup>−−</sup> mice was composed of myocytes that were 20% larger, whereas their number per mm<sup>2</sup> of tissue was reduced by 17% (Figure 4B). These observations suggest that mutation of the Speg gene locus affected the generation of parenchymal cells during development, evoking a cellular hypertrophic response.

Speg<sup>−−</sup> neonates allowed to breathe spontaneously after delivery at 18.5 dpc had significantly smaller lungs than Speg<sup>++</sup> neonates (Figure 3A), although lung weight was
comparable (data not shown). Speg<sup>−/−</sup> neonates breathing spontaneously on day 1 after birth, when comparably inflated, revealed lung architecture not remarkably different from Speg<sup>+/+</sup> neonates (supplemental Figure II). Reduction in lung size was directly related to an increase in heart size, which resulted in compression of the lungs. Examination of other organs did not show obvious abnormalities to account for the neonatal lethality, and no gross abnormalities in the vasculature (with normal anatomic positions of the great vessels) or skeletal muscle (including muscularization of the diaphragm, data not shown) were apparent at birth.

Further histological analysis of the Speg<sup>+/+</sup> and Speg<sup>−/−</sup> hearts was performed. Figure 5A depicts a section from the epicardial through the myocardial layers (not including the endocardium) of the left ventricular free wall of a Speg<sup>+/+</sup> pup, 1 day after birth. In contrast, an analogous section from a Speg<sup>−/−</sup> heart is shown in Figure 5B. The epicardial through myocardial layers of the left ventricular free wall were thinner in the Speg<sup>−/−</sup> heart, with the endocardial layer present in this section, as demarcated by the trabeculae (Figure 5B). In addition, there was evidence of dilated subepicardial vessels in the Speg<sup>−/−</sup> hearts compared with Speg<sup>+/+</sup> hearts (arrows). Higher-power images of cardiomyocytes from the myocardial layer of Speg<sup>−/−</sup> hearts revealed evidence of vacuoles and less well-developed striations (Figure 5D) than Speg<sup>+/+</sup> hearts (Figure 5C). To further assess for ultrastructural changes, hearts from C57BL/6 pure-background embryos (18.5 dpc) were harvested for transmission electron microscopy analyses. Speg<sup>+/+</sup> hearts showed a tightly packed sarcomere with normal alignment of myofibrils, intact Z discs, and clearly defined intercalated discs (Figure 5E); however, Speg<sup>−/−</sup> hearts appeared dramatically different, with evidence of myofibril degeneration and sarcomere fragmentation (Figure 5F). To assess the functional status of Speg mutant hearts, we performed echocardiograms on 18.5-dpc embryos. There was a marked reduction in cardiac function (left ventricular fractional shortening and ejection fraction) in Speg<sup>−/−</sup> hearts compared with Speg<sup>+/+</sup> hearts (P<0.05; Figure 5G).
Had fractional shortenings and ejection fractions of 10% and 23%/H11006 3%, respectively. These data suggest that the structural integrity of the heart is remarkably abnormal in the hearts. These spots were submitted for mass spectrometry analysis to identify the differentially phosphorylated proteins. The unique kinase domains develop DCM, we performed hearts of Speg mut mutant mice (with absence of the unique kinase domains) develop DCM, we performed 2D gel electrophoresis on protein extracts from 18.5-dpc Speg+/− and Speg−/− hearts, with anti-serine/anti-threonine antibodies. Molecular mass markers are along the vertical axis, and pH gradient (3.5 to 10) is along the horizontal axis. B, Protein extracted from 16.5-dpc hearts was subjected to isoelectric focusing electrophoresis followed by Western blotting with an anti-tropomyosin antibody. Protein from Speg−/− hearts was also incubated in the presence (+) or absence (−) of phosphatase (PPase), and then isoelectric focusing electrophoresis was performed. C, Conventional electrophoresis and Western blotting of protein from Speg+/− and Speg−/− hearts was performed with an anti-tropomyosin antibody.

mouse hearts had fractional shortenings of 40±2% and ejection fractions of 74±2%, whereas Speg−/− mouse hearts had fractional shortenings and ejection fractions of 10±1% and 23±3%, respectively. These data suggest that the structural integrity of the heart is remarkably abnormal in the Speg−/− embryos, with marked cardiac dysfunction.

Altered Phosphorylation of Tropomyosin in Speg Mutant Mice

To further understand why Speg mutant mice (with absence of the unique kinase domains) develop DCM, we performed 2D gel electrophoresis on protein extracts from 18.5-dpc hearts of Speg+/− and Speg−/− mice, followed by anti-phosphoserine/anti-phosphothreonine Western blotting. Differentially phosphorylated proteins were identified by mass spectrometry analysis. Figure 6A revealed 3 spots (identified by arrows) that were present in Speg+/− hearts but not Speg−/− hearts. These spots were submitted for mass spectrometry analysis to identify the differentially phosphorylated proteins. Tropomyosin (TM) proteins were in all of the spots, with TM-α-1 chain (α-TM or TM-1) predominating.

Tropomyosin-α-4 chain (TM-4) was also prominent in 1 of the spots, along with α-TM (supplemental Table 1), and there was less evidence for the contribution of TM-β chain (β-TM or TM-2). For further delineation of the phosphorylation status of tropomyosin, we ran protein extracts from 16.5-dpc Speg+/−, Speg−/−, and Speg−/− mouse hearts on an isoelectric focusing gel, followed by Western blotting of the transferred protein with an anti-TM antibody (Figure 6B). Protein extracts from the hearts of Speg−/− embryos demonstrated a different isoelectric focus (Figure 6B, lanes 3 and 7, lower bands) than hearts of Speg+/− and Speg−/− embryos. Moreover, when protein from Speg−/− hearts was treated with phosphatase, the migration pattern of TM shifted down, consistent with that of Speg−/− hearts. Although there was a difference in phosphorylation, the overall TM content by conventional Western blotting was not different between Speg+/− and Speg−/− hearts (Figure 6C). These data suggest a decreased level of phosphorylated TM in Speg−/− hearts.

Discussion

Heart growth during organogenesis occurs principally through the proliferation of cardiomyocytes. After birth, excluding progenitor cells, cardiomyocytes withdraw from the cell cycle, and further growth occurs by hypertrophy.18 A hypertrophic stimulus may progress to DCM in part by the activation of cell-death pathways, including apoptosis.19 However, in Speg−/− mice, there is no difference in myocyte apoptosis compared with Speg+/− mice, and mutation of the Speg gene locus affected the generation of parenchymal cells, which evoked a cellular hypertrophic response.

DCM is the most common form of primary cardiac muscle disease, with an estimated prevalence in the United States of 36.5 per 100 000 people.20 Furthermore, DCM is not only a disease of adults; it is one of the most common nonstructural fetal heart abnormalities.21,22 DCMs have been linked to gene mutations in cytoskeletal proteins, for example, dystrophin, desmin, and cardiac muscle LIM protein.13,23–28 However, gene mutations in sarcomere proteins (such as cardiac β-myosin heavy chain, troponin T, α-TM, cardiac actin, and cardiac myosin-binding protein C) have also been associated with dilated forms of cardiomyopathy and may account for a subset of these cases.13,28–31 Beyond the identification of specific genes, disease loci for familial DCM have been identified on various chromosomes, including regions on chromosomes 2q (2q14–q22, 2q31, and 2q35).12 These chromosomal loci harbor potential causative genes, and interestingly, the human Speg/Apeg-1 locus, mapped to chromosome 2, lies in close proximity to the 2q35 locus of desmin.24

In evaluating the Speg−/− embryos during development, the expression pattern of the lacZ transgene, driven by the Speg/Apeg-1 promoter, was localized exclusively in the heart (cardiomyocytes) throughout the first 16.5 dpc (Figure 2), a point in time when hearts in Speg−/− mice had already begun to enlarge (Figure 3A). At 18.5 dpc, cardiac expression remained robust, and vascular expression of the lacZ transgene first became evident in elastic arteries, although not in the muscular coronary arteries. Moreover, the structure of the pulmonary arteries and aorta did not appear abnormal in the...
mutant mice. These data suggest that developmental abnormalities in mutant embryos result from the loss of Spegα and Spegβ expression in cardiomyocytes. However, future studies will need to further assess any vascular contribution to the phenotype, particularly in the setting of a pathophysiological stress in Speg<sup>−/−</sup> mice.

The messages for all Speg isoforms are present in the mouse heart at day 18.5 (Figure 1); however, when assessing protein expression, only Spegα, and Spegβ are present in myofibrils. Northern blot analysis also demonstrated that as the mRNA levels for Spegβ decreased in heterozygous and homozygous mutant embryos, splicing of exon 7 into the lacZ splice acceptor site resulted in a mutant 6.4-kb message (Figure 1B). Because the heterozygous mice appear normal, with normal mendelian ratio at birth and normal survival and fertility, we do not expect the lethal cardiac phenotype to be related to this transcript encoding a dominant negative protein.

Spegα and Spegβ share homology with MLCK family members. MLCK itself is a calcium/calmodulin-dependent protein kinase that phosphorylates a serine on the regulatory light chain of myosin II. Vertebrates have 2 muscle-predominant MLCK genes: a skeletal/cardiac muscle MLCK gene, and a separate gene that through alternative promoter use gives rise to 2 smooth muscle MLCK genes and to telokin. Somlyo and colleagues reported a knockout of smooth muscle MLCK genes in mice, which led to death shortly after birth. Mutant hearts demonstrated irregular and dilated subepicardial vessels, as well as differences in the compact zone myocardial area compared with wild-type hearts. Spegα and Spegβ, along with obscurin-MLCK, are unique members of the striated muscle MLCK family, because they contain 2 tandemly arranged serine/threonine kinase (MLCK) domains. To the best of our knowledge, the generation of the Speg mutant locus in the present study is the first description of a tandem-kinase family member to be disrupted in mice.

In an effort to provide insight into a potential mechanism responsible for the DCM that develops in Speg mutant mice, we performed a proteomic screen using 2D gel electrophoresis on protein extracts from 18.5-dpc hearts of Speg<sup>−/−</sup> mice compared with Speg<sup>+/−</sup> mice. Furthermore, because of the unique tandemly arranged serine/threonine kinase domains in Spegα and Spegβ, we assessed differentially phosphorylated proteins by anti-phosphoserine/anti-phosphothreonine Western blotting and then performed mass spectrometry analysis to specifically identify the altered proteins. Our analysis, as shown in Figure 6A, revealed that phosphorylated proteins present in Speg<sup>+/−</sup> mice but absent in Speg<sup>−/−</sup> mice include TM isoforms. The predominant isoform identified was α-TM, which is known to be phosphorylated at its penultimate Ser283. α-TM is one of the regulatory proteins in the thin filament of striated muscle, and it binds with actin to form the backbone of the thin filament. Together with the troponin complex, α-TM and actin play a critical role in striated muscle contraction and relaxation. Phosphorylation of Ser283 is known to enhance actin-activated ATPase activity without altering calcium sensitivity. Of the TM isoforms, phosphorylation is higher in the α-TM subunit, and the phosphorylation status decreases after development and into adulthood. These data suggest a functional role for α-TM phosphorylation during muscle development. Interestingly, it has recently been shown that chronic activation of p38 mitogen-activated protein kinase in transgenic mouse hearts is able to depress sarcomeric function in association with an increase in dephosphorylation of α-TM. In the present study, phosphorylated isoforms of TM (particularly the α-isofrom) were absent in 18.5-dpc hearts of Speg<sup>−/−</sup> mice, in conjunction with a DCM that led to death. Although a TM kinase was originally isolated and partially purified in chicken embryos, the present study suggests that Speg plays a role in TM phosphorylation during development. This alteration in α-TM phosphorylation, in the setting of a mutated Speg locus, may contribute to the development of DCM in Speg<sup>−/−</sup> mice. However, we cannot exclude the possibility that decreased α-TM phosphorylation may be a consequence rather than a cause of cardiac dysfunction. The DCM may be more related to structural consequences of a disruption in Speg, a protein known to colocalize with desmin in the sarcomeric Z disc. The absence of Speg itself may alter the structural integrity and organization of the myofibril. Nevertheless, the present study demonstrates that Speg is important for cardiomyocyte development and that disruption of the gene locus leads to DCM and neonatal death in mice.

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Disclosures
None.

References
Striated muscle cells of cardiac origin undergo terminal differentiation shortly after birth in mammals, with the majority of these cells (other than cardiac progenitor cells) not retaining the ability to proliferate or regenerate after injury. For cardiomyocytes to function appropriately, contractile forces generated in the sarcomere are transmitted to the extracellular matrix. If this process does not occur appropriately, cardiac remodeling of either a hypertrophic or dilated phenotype takes place. Dilated cardiomyopathy is the most common form of primary cardiac muscle disease, with an estimated prevalence in the United States of 36.5 per 100,000 people. Furthermore, dilated cardiomyopathy is not only a disease of adults, it is also one of the most common nonstructural fetal heart abnormalities. Dilated cardiomyopathies have been linked to gene mutations in cytoskeletal and sarcomere proteins. Prior work in our laboratory has shown that Speg colocalizes with desmin in the sarcomeric Z disc. To study the potential importance of Speg in cardiovascular biology, we generated Speg mutant mice by disrupting the gene locus. This study demonstrated the importance of Speg proteins for mouse cardiac development, function, and survival.
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Generation of Speg Mutant Mice

To disrupt the Speg locus, we generated a targeting construct by deleting exons 8, 9, and 10 (Supplemental Figure IA). The Speg targeting vector was generated by first cloning a 6 kb 5’ fragment containing intron 6 and exon 7 into pBluescript vector (Stratagene), followed by a 3.1 kb 3’ fragment containing part of intron 10 and exons 11–14. To enable lacZ reporter gene expression under the control of the endogenous Speg/ Apeg-1 promoter in the mutant mice, we first engineered a synthetic splice acceptor (SA) site \(^1\) into the multiple cloning sites of pPD46.21 vector \(^2\). The SA-lacZ fragment was then inserted between 5’ and 3’ fragment of the targeting vector. A PGK-neo cassette was subsequently subcloned 3’ to the SA-lacZ for positive selection. A thymidine kinase cassette (PGK-TK) was ligated at the 5’-end to allow negative selection with gancyclovir. D3 ES cells were transfected, and correctly targeted ES clones were used to generate chimeric mice as described \(^3\). The resulting chimeras were then bred with wild type C57BL/6 mice to generate heterozygous (+/−) Speg mutant mice on a 129SvJ and C57BL/6 mixed genetic background. After breeding of the Speg\(^{+/−}\) mice, experiments were initially performed on homozygous (−/−) Speg mutant mice with a mixed 129SvJ and C57BL/6 genetic background, using littermates as controls. The mice on mixed genetic background were backcrossed to C57BL/6 wild type mice for 9 consecutive generations to yield Speg\(^{−/−}\) mice on a pure C57BL/6 background. The experiments initially performed on mice with a mixed genetic background were confirmed on mice with a pure C57BL/6 background. All animal
experiments were performed according to the National Institute of Health guidelines and all protocols were approved by Institutional Animal Care and Use Committee.

**Southern Blotting and Genotyping**

Genomic DNA was prepared from mouse-tail biopsies, digested with SacI, fractionated on an agarose gel, and transferred to nylon membranes as described previously \(^3\). The membranes were then hybridized with a \(^{32}\)P-labeled 5'-probe external to the targeting construct (Supplemental Figure IA).

**Total RNA Isolation and Northern Blotting**

Total RNA was isolated from 18.5 dpc hearts using Qiagen RNeasy kit (Qiagen, Valencia CA). Northern blot analysis was performed as described, using 10 \(\mu\)g of RNA \(^4\). The filters were hybridized with a \(^{32}\)P-labeled mouse Apeg-1 full-length cDNA probe, and with 5’ and 3’ Speg probes \(^4\). The Northern blots were also hybridized with an 18S probe to assess RNA loading.

**Myofibril Extraction and Western Blotting**

The myofibril fraction from 18.5 dpc hearts was extracted as described \(^5\). Myofibril protein (10 \(\mu\)g) was run on Novex 4-20% gradient gel (Invitrogen) and Western blotting was performed with affinity purified Speg/Apeg-1 antibody as previously described \(^4\). The same blot was stripped and reprobed with 1:3000 dilution of mouse monoclonal desmin antibody (Sigma) to check for equality of loading.
**Echocardiography**

A Vevo 770 high-resolution microultrasound system and a 40-MHz probe (Visualsonics, Toronto, ON) were used for transthoracic echocardiography on non-anesthetized 18.5 dpc animals. The hearts were imaged in the two-dimensional parasternal short-axis view, and an M-mode echocardiogram of the mid-ventricle was recorded at the level of papillary muscles as described previously. The end-diastolic and end-systolic internal dimensions of the left ventricle (LVID,d; LVID,s), and the end-diastolic and end-systolic volume of the left ventricle (LV vol,d; LV vol,s) were measured from the M-mode image.

FS % = (LVID,d - LVID,s)/LVID,d X 100; EF % = (LV vol,d - LV vol,s)/LV vol,d X 100.

**Cardiac Weight Index**

At 18.5 dpc, the embryos were harvested and their hearts removed. Cardiac weight index was calculated as heart wet weight (mg) divided by total body weight (g).

**Detection of β-galactosidase Activity**

Embryos at different gestational ages were harvested, fixed and stained for β-galactosidase activity as previously described. Formalin fixed 5 µm sections were counterstained with nuclear fast red according to standard procedures.

**Histology**

Embryos at different gestational ages were fixed, dehydrated and embedded in paraffin. Sections (5 µm) were stained with Hematoxylin and Eosin (H&E) for histopathological analysis.
**Myocyte size and number**

These cellular parameters were obtained from 18.5 dpc embryos as described\(^9,10\). Myocardial sections were stained with α-sarcomeric actin monoclonal antibody (Sigma, St. Louis, MO) and DAPI, and visualized by confocal microscopy. The number of myocyte nuclear profiles per unit area of tissue, \(N(n)_A\), was measured in sections in which cardiac muscle cells were cut perpendicularly. Nuclear length, \(D(n)\), was evaluated in longitudinally oriented myocytes to compute the number of myocytes per unit volume of myocardium, \(N(m)_V\):

\[
N(m)_V = \frac{N(n)_A}{D(n)}
\]

Myocyte cell volume, \(V(m)\), was assessed from the volume fraction of myocytes in the tissue, \(V(m)_V\), and the number of myocytes per unit volume of myocardium, \(N(m)_V\):

\[
V(m) = \frac{V(m)_V}{N(m)_V}.
\]

Five Speg\(^{+/+}\) and 6 Speg\(^{-/-}\) mice were employed in this analysis. A total of 220 and 3,407 myocyte nuclei were measured in longitudinal and transverse sections of the myocardium, respectively.

**Myocyte apoptosis**

Sections from 18.5 dpc embryos were incubated with cacodylate buffer containing terminal deoxynucleotidyl transferase (TdT), a mixture of FITC-labeled nucleotides, cobalt chloride and proteinase K (Clontech, Mountain View, CA)\(^10\). Five Speg\(^{+/+}\) and 6 Speg\(^{-/-}\) mice were employed in this analysis. A total of 20.8 mm\(^2\) of tissue were examined.
**Electron Microscopy**

Hearts from 18.5 dpc embryos were fixed in 0.1 M sodium cacodylate buffer containing 2% paraformaldehyde and 2.5% glutaraldehyde for 2 hours at room temperature and then overnight at 4°C. The embryos were postfixed in 1% osmium tetroxide/1.5% potassium ferrocyanide, dehydrated and embedded in Epon/Araldite resin.

**Two-dimensional Gel Electrophoresis and Western Blotting**

Protein from 18.5 dpc hearts was extracted as described, and the protein (400 µg) was run on a 10% acrylamide gel with a pH gradient of 3.5-10 ampholines by Kendrick Laboratories Inc., Madison, WI. After transfer, Western blotting was performed using anti-phosphoserine (Q5, Qiagen) and anti-phosphothreonine (Q7, Qiagen) antibodies, diluted to 1:4000. Spots present on the Speg+/+, but not the Speg−/−, gels were isolated and submitted for mass spectrometry at the Taplin Biological Mass Spectrometry Facility of Harvard Medical School.

**Isoelectric Focusing (IEF)**

Heart tissue harvested from 16.5 dpc hearts was lysed in Osmotic buffer (10mM Tris, pH 7.4, 0.3% SDS, 1x protease inhibitor, 1X phosphorylation inhibitor#1 and 1X phosphorylation inhibitor#2 (Sigma)), and protein fractions were electrophoresed on an IEF gel (pH 3~7, Invitrogen). The gel was transferred to nitrocellulose, and Western blotting was performed with a monoclonal anti-tropomyosin antibody (Sigma, Clone CH1), diluted to 1:1000.
### Supplemental Table I

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Sequence of tropomyosin (TM) isoforms identified by mass spectrometry, and the positions of each sequence. α-TM (TM 1 or TM α-1), β-TM (TM 2), and TM α-4 (TM 4).
Supplemental Figure I. Targeted disruption of the Speg gene locus in mice. (A) Schematic of the mouse Speg gene locus (top), targeting vector (middle), and predicted mutated allele (bottom). The targeting construct contains a neomycin (neo) positive selection cassette and a thymidine kinase negative selection cassette (PGK-TK). Homologous recombination resulted in replacement of exons 8, 9, and 10 of the Speg gene locus with a DNA fragment containing a splice acceptor (SA) site and a nuclear localized lacZ gene (NLSLacZ). A 5’ probe external to the targeting construct (as shown) was used to characterize the targeted locus after SacI (S) digestion. (B) Genotypes were determined by Southern blot analysis of SacI-digested genomic DNA from the offspring of Speg +/- mating. As predicted from the mutant allele diagram in (A), the 5’-probe hybridized to a 7 kb wild type (WT) fragment (left lane, +/+), a 4 kb mutant (Mut) fragment (right lane, –/–), or both fragments (middle lane, +/-).
Supplemental Figure II. Histology of lungs from wild type (+/+) and homozygous mutant (−/−) neonate mice day 1 after birth. Sections of lungs from Speg^+/+ (A, C) and Speg^−/− (B, D) mice were stained with H&E. Original magnification X40 (A, B), and X100 (C, D).
Supplemental References


