Desmosomal Dysfunction due to Mutations in Desmoplakin Causes Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy

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Abstract—Arrhythmogenic right ventricular dysplasia/cardiomypathy (ARVD/C) is characterized by progressive degeneration of the right ventricular myocardium, ventricular arrhythmias, fibrous-fatty replacement, and increased risk of sudden death. In mutations in genes, including 4 encoding desmosomal proteins (Junctional plakoglobin (JUP), Desmoplakin (DSP), Plakophilin 2, and Desmoglein 2), have been identified in patients with ARVD/C. Mutation analysis of 66 probands identified 4 variants in DSP; V30M, Q90R, W233X, and R2834H. To establish a cause and effect relationship between those DSP missense mutations and ARVD/C, we performed in vitro and in vivo analyses of the mutated proteins. Unlike wild-type (WT) DSP, the N-terminal mutants (V30M and Q90R) failed to localize to the cell membrane in desmosome-forming cell line and failed to bind to and communoprecipitate JUP. Multiple attempts to generate N-terminal DSP (V30M and Q90R) cardiac-specific transgenes have failed: analysis of embryos revealed evidence of profound ventricular dilation, which likely resulted in embryonic lethality. We were able to develop transgenic (Tg) mice with cardiac-restricted overexpression of the C-terminal mutant (R2834H) or WT DSP. Whereas mice overexpressing WT DSP had no detectable histologic, morphological, or functional cardiac changes, the R2834H-Tg mice had increased cardiomyocyte apoptosis, cardiac fibrosis, and lipid accumulation, along with ventricular enlargement and cardiac dysfunction in both ventricles. These mice also displayed interruption of DSP-desmin interaction at intercalated discs (IDs) and marked ultra-structural changes of IDs. These data suggest DSP expression in cardiomyocytes is crucial for maintaining cardiac tissue integrity, and DSP abnormalities result in ARVD/C by cardiomyocyte death, changes in lipid metabolism, and defects in cardiac development. (Circ Res. 2006;99:646-655.)

Key Words: arrhythmogenic right ventricular dysplasia/cardiomypathy ■ mechanical junctions ■ gene mutations

Arrhythmogenic right ventricular dysplasia (ARVD/C) is a complex myocardial disorder associated with right-sided cardiomyopathy and arrhythmia, and characterized by gradual loss of myocytes and replacement by fatty and fibrous tissue. It leads to dilation of the right ventricle (RV) and impaired cardiac function. In some patients, left ventricular (LV) involvement also occurs. The clinical course is characterized by ventricular arrhythmias (ventricular tachycardia), heart failure, syncope, and sudden death. In Italy, the prevalence has been reported to be 1:5000 population, accounting for 20% of sudden deaths in young adults and 25% of cardiac sudden deaths among athletes.

In familial cases of ARVD/C, autosomal dominant inheritance with reduced penetrance has been reported and accounts for ≈30% of cases. In the remaining sporadic cases it is believed to be due to an acquired etiology or to unidentified inheritance. To date, 10 genetic loci have been mapped for ARVD/C, and 6 genes have been identified. The first gene identified for autosomal dominant ARVD/C was the cardiac ryanodine receptor 2 (RyR2). In addition, 2 other genes responsible for Naxos Disease and Carvajal syndrome, complex autosomal recessive forms of ARVD/C with associated palmoplantar keratoderma and woolly hair have been identified as junctional plakoglobin (JUP) and desmoplakin (DSP), respectively.
respectively. JUP and DSP are major components of desmosomes. Mutations in DSP have also been shown to cause autosomal dominant ARVD/C. More recently mutations in plakophilin 2 (PKP2), desmoglein 2 (DSG2), and the 5′ untranslated region (UTR) of transforming growth factor beta-3 (TGF-β3) have been reported in patients with ARVD/C.

In this study, we investigated the frequency of DSP mutations in an unselected patient cohort from the North American ARVD/C Registry, and studied the effects of those ARVD/C-associated mutations on DSP function. We have identified multiple mutations in DSP in ARVD/C patients and have demonstrated that DSP expression in cardiomyocytes is crucial for maintaining cardiac tissue integrity. In addition, we have showed that abnormalities of DSP can result in loss of function in vitro and lead to defects in cardiac development, cardiomyocyte death, changes in lipid metabolism, and ultra-structural changes in intercalated discs (IDs) when overexpressed in mice.

Materials and Methods

Clinical Diagnostic Criteria for ARVD/C
All studies on human subjects were performed in accordance with local IRB regulations after informed consent, as described in the supplement, available online at http://circres.ahajournals.org.

Mutation Detection of DSP Gene
Mutations in DSP were detected by direct DNA sequencing, as described in the online supplement.

Desmosome-Forming Cell Line and Transient Transfection
The human tongue squamous cell carcinoma cell line SCC-9 was transfected using Effectene (Qiagen). Protein expression was investigated by immunohistochemistry, as described in the online supplement.

Generation of Transgenic Mice
Cardiac-restricted DSP transgenic (Tg) mice were generated using vectors containing the mouse alpha-myosin heavy chain (α-MyHC) promoter (Dr Jeffrey Robbins, University of Cincinnati, Ohio) and full-length wild-type (WT) and mutant DSP cDNA sequences, as described in the online supplement. Histologic and functional characterizations of those Tg mice are described in the online supplement.

Statistical Analyses
Statistical significance was analyzed by the unpaired two-tailed t test using the statistical software GraphPad Prism 4 to compare data sets between any two different groups. Values are shown as mean±SEM. Statistical significance was set at P<0.05.

Results

Mutation Analysis of the DSP Gene
Genetic variants in DSP were identified in 4 of the 66 probands (6%) enrolled in the North American ARVD/C Registry, comprising 1 nonsense and 3 missense substitutions. These substitutions included 88G>A (V30M), 269A>G (Q90R), 699G>A (W233X), and 8501G>A (R2834H) (Figure 1A), and none was identified in 200 ethnic matched control individuals (400 chromosomes).

The V30M and Q90R variations occur in the head region of the DSP protein, which is involved in binding to the linker proteins (JUP and PKPs) of desmosomes, and these abnormalities would be likely to affect these interactions and localization of DSP to desmosomes (Figure 1B). We could not detect the presence of the 699G>A (W233X) nucleotide change in the cDNA amplified from the lymphoblastoid cell line derived from patient blood. Therefore, we conclude that this nonsense substitution results in haploinsufficiency, probably due to increased decay of nonsense mRNA as previously described. R2834H would be likely to affect the DSP C terminus involved in the binding of DSP with intermediate filaments (Figure 1B). To test these hypotheses, we investi-
gated the effects of these missense substitutions in vitro and in vivo.

**Mutations in the N-Terminal DSP Result in Loss of Localization to Cell Membrane**

WT DSP NTP (N-terminal truncated DSP, first 584 aa, Flag tagged), V30M DSP NTP, Q90R DSP NTP, and W233X DSP (first 233 aa, GFP tagged) were expressed by transient transfection in the desmosome-forming cell line SSC-9 (Figure 2A). Whereas WT DSP NTP was localized at the cell membrane, both V30M and Q90R DSP NTP were mainly detected in the cytoplasm and W233X GFP formed perinuclear aggregates. Lower, Flag tagged WT, V30M, Q90R, or R2834H DSP FL (full length DSP, 2874 aa) were expressed in SCC-9 cells. Note WT and R2834H DSP FL were localized to the cell membrane, whereas V30M and Q90R DSP FL were present in the cytoplasm. (Flag tagged DSP NTP and DSP FL were detected using an anti-Flag antibody and stained green; GFP was shown in green; nuclei were stained blue; 400×).

**N-Terminal DSP Mutants Can No Longer Bind JUP In Vitro**

To investigate the effects of these mutations on the interactions between DSP and JUP, PKP-1, and PKP-2, coimmuno-
precipitation studies were performed. JUP was found to be immunoprecipitated by WT DSP NTP as previously reported,20 but not by either V30M or Q90R DSP NTP, suggesting that V30M and Q90R disrupt the binding ability of DSP N terminus (Figure 2B). No changes were observed in the immunoprecipitation of PKP-1 or -2 (data not shown).

Overexpression of N-Terminal DSP Mutants in the Myocardium Results in Early Embryonic Lethality

We attempted to generate cardiac restricted Tg mice expressing mutant or WT full-length DSP in the myocardium under the control of the α-MyHC promoter (Figure 3A). Although lines expressing R2834H or WT DSP were successfully generated, no founders for V30M- or Q90R-Tg mice were identified, despite repeated injections (supplemental Table I). We speculated that V30M and Q90R likely have adverse effects on embryonic development. Embryonic day (ED) 12.5 to 17.5 embryos were therefore isolated from newly injected pregnant mothers, and we found that there were no positive embryos after ED13.5. At ED13.5, hearts from V30M-Tg (from 4 founders) and Q90R-Tg (from 2 founders) showed severely reduced wall thickness (2 to 5 cell layers) and definitive ventricular dilation in comparison to R2834H-Tg, WT-Tg, and nontransgenic (NTG) littermates (10 to 12 cell layers; Figure 3B), suggesting cardiac development abnormalities with cardiac noncompaction and early embryonic lethality.

Mice Expressing R2834H DSP Display Cardiac Hypertrophy and Reduced Cardiac Function

We chose to compare the phenotypes of WT-Tg (from 2 founders) and R2834H-Tg lines (from 2 founders) with similar expression levels of DSP (2 to 3 times of the level of DSP in NTG littermates, determined by Western blot analysis of cardiac proteins). By 6 months of age, the R2834H-Tg mice (5.9 ± 0.2×10⁻³) had significantly increased heart weight/body weight ratios compared with NTG littermates (4.9±0.1×10⁻³) and WT-Tg mice (5.0±0.1×10⁻³) (n=7 per group; Figure 3C). In addition, histological analysis indicated that ventricular cardiomyocyte cross-section areas were 40% higher in R2834H-Tg mice (183.4±1.4 μm²) compared with WT-Tg (132.4±1.5 μm²) and NTG mice.

Figure 3. Generation of cardiac-specific WT and mutant DSP Tg mice. A, Schematic representation of construct to generate the DSP Tg mice. The α-MyHC promoter was used to drive the expression of human full-length DSP (WT or mutant) cDNA, tagged at the C terminus with Flag. B, Early embryonic lethality in Tg mice overexpressing N-terminal DSP mutants. ED13.5 embryonic hearts from V30M-Tg, Q90R-Tg, R2834H-Tg, WT-Tg, and NTG mice are shown. Note severely reduced ventricular wall thickness and ventricular dilation in V30M-Tg and Q90R-Tg mice compared with other groups. Boxed areas are shown at higher magnification, with the black bars illustrating the width of the ventricular wall thickness. L indicates the lumen of the heart. (Sections were stained with H&E; from the left to the right: 40× and 400×, respectively) C, Cardiac hypertrophy observed in R2834H-Tg mice vs controls. (*P<0.03, **P<0.01) D, Increased cardiomyocyte cross-section areas in R2834H-Tg mice. Left, Cardiac frozen sections were stained with C-dystrophin (green) to outline the sarcolemma. Bars, 50 μm. Right, Significant increase of ventricular cardiomyocyte cross-section areas in R2834H-Tg mice (quantified using Image-Pro Plus). (**P<0.001)
Increased Cardiac Fibrosis, Cardiomyocyte Apoptosis, and Lipid Accumulation in R2834H-Tg Mice

Histologic studies using H&E and Masson trichrome staining from multiple sections of the heart from at least 10 mice per group revealed focal myocyte loss and replacement by fibrous tissue in both ventricles (primarily in the LV) of R2834H-Tg mice, whereas WT-Tg mice had normal cardiac histology (Figure 5A through 5B). In addition, neutral lipid accumulation was focally observed in the cardiomyocytes of R2834H-Tg mice by Oil Red O staining (Figure 5C through 5D). Analysis for apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) indicated significantly increased cardiomyocyte apoptosis in R2834H-Tg mice with the proportion of positively stained nuclei estimated as 11.4±0.9‰ in these mice versus 1.4±0.9‰ in WT-Tg mice and 0.7±0.6‰ in NTG littermates (n=4 per group, >1000 nuclei per animal were counted; Figure 5E through 5F).

The R2834H DSP Mutation Results in Aberrant Intermediate Filament Localization

We hypothesized that the C-terminal mutation (R2834H) could affect the interaction between DSP and desmin filaments. In support of this hypothesis, there was less desmin detected at the IDs of R2834H-Tg mice, even though DSP and the intracellular distribution of desmin (at Z-disks) appeared normal (Figure 6A). Significantly reduced colocalization efficiency (Pearson correlation value) of DSP and desmin at IDs was noted in R2834H-Tg mice (0.23±0.02) in comparison to WT-Tg (0.69±0.01) and NTG mice.
The C-terminal DSP mutant interrupts the interaction between DSP and intermediate filaments, crucial for the correct localization of these filaments. Localization of other junctional proteins (JUP, PKP2, and connexin 43 (CX43)) at IDs were not changed (Figure 6C).

Ultra-Structural Changes of IDs in R2834H-Tg Mice

Electron microscopic evaluation of the myocardium from 9-month-old mice was performed, mainly focusing on the cell junctions. In R2834H-Tg mice, irregularly shaped IDs with markedly widened gaps between adjacent anchoring sarcomeres were noted, affecting both the adherens junctions and desmosomes. No lipid accumulation was identified in the sections examined, and no other cardiomyopathic changes were observed. In WT-Tg mice, electron dense materials were present in the IDs but not evident in Z-lines (Figure 7).

The R2834H DSP Mutation Results in Changes of Expression and Localization of Junctional Proteins

As previously reported, large proportions of junctional proteins (desmosomes and adherens junctions) are insoluble in extraction buffer containing nondenaturing detergents such as Triton X-100, because those proteins are associated with the cytoskeleton. Therefore, we performed Western blot analysis on total lysate, the Triton-X insoluble fraction (associated with the cytoskeleton), and the soluble fraction (not associated with cytoskeleton) of proteins isolated from Tg mouse hearts to assess the relative expression level and solubility of junctional proteins (Figure 8). As expected, overexpression of Flag-tagged DSP was noted in both WT-Tg and R2834H-Tg mice. Desmin expression was also increased in both R2834H-Tg and WT-Tg mice. Notably, large amount of JUP was present in the soluble fraction of R2834H-Tg mice by comparison with WT-Tg (small amounts) and NTG mice (almost undetectable). Furthermore, the expression of PKP2 and beta-catenin were moderately upregulated in R2834H-Tg mice, especially in the soluble fraction. Although the total amount of CX43 appeared unchanged in R2834H-Tg mice, there was a redistribution of CX43 into the soluble fraction. Those data suggest disassociation of a number of junctional proteins from cell-cell junctions in R2834H Tg mice (Figure 8).

Discussion

ARVD/C is a complex disorder with clinical manifestations including ventricular arrhythmias, and specific structural changes (fibrous-fatty tissue replacement) involving RV more often than LV, leading to heart failure and cardiac sudden death. Due to the clinical features of the disease, diagnosis is difficult and treatments are limited. Therefore, understanding the underlying mechanism of the development of ARVD/C would facilitate both diagnosis and treatment of the disease.

To date, mutations in 6 genes (RYR2, JUP, DSP, PKP2, DSG2, and TGF-β3) have been identified in patients with ARVD/C. The first gene identified was RYR2 for ARVD/C type 2. However, mutations in RYR2 have been shown to be
the major cause of catecholaminergic polymorphic ventricular tachycardia (CPVT), and it has been speculated that ARVD/C type 2 is a forme fruste of CPVT. Of the remaining 5 genes, 4 encode desmosomal proteins (JUP, DSP, PKP2, and DSG2). It has been reported that TGF-β3, the only one of these genes not encoding a desmosomal protein, regulates the expression of desmosomal genes including JUP and PKP2. Therefore, desmosomal protein dysfunction appears to be a “final common pathway” playing an important role in the development of ARVD/C.

Desmosomes are specialized cell–cell adhesion structures abundant in tissues (primarily skin and heart) undergoing high mechanical stress, connecting intermediate filaments to the cell membrane. There are 3 major groups of proteins in desmosomes: transmembrane proteins (or desmosomal cadherins) including desmocollins (DSCs) and DSGs; linker proteins (armadillo family proteins), JUP and PKPs; and DSP. JUP might serve dual roles as junctional and nuclear proteins (transcriptional cofactors of Tcf/Lef family in the canonical Wnt signaling pathway, with downstream targets involved in cell survival or adipogenesis). Deficiency of JUP, PKP2, or DSP in mice can lead to defects in heart morphogenesis and embryonic lethality due to disruption of desmosomes. DSP plays an important role in maintaining the integrity of desmosomes by binding to other desmosomal components through its N terminus and to intermediate filaments (desmin in the heart) through its C terminus.

In this study we have identified 4 novel mutations in DSP in 6% of the ARVD/C patients studied. We characterized the effects of these mutations on DSP function through a number of in vitro and in vivo studies. Our studies demonstrate that a nonsense mutation in the N terminus of DSP (W233X) can lead to haploinsufficiency, whereas two missense mutations in the N terminus of DSP (V30M and Q90R) affect the normal localization of DSP in vitro, probably due to loss of binding to JUP as shown by protein–protein interaction analysis. In addition, overexpression of the N-terminal mutants in embryonic mouse hearts can lead to embryonic lethality. The cardiac phenotypes of those embryos resemble that seen in JUP- and PKP2-deficient mice, which also have defects in cardiac morphogenesis. Based on these findings, we speculate that N-terminal mutants disrupt the normal function of desmosomes and lead to desmosomal instability. As a result, such defective desmosomes cannot sustain the constant mechanical stress seen in contracting cardiomyocytes, which in turn leads to cardiac dysfunction, cell death, and eventually embryonic lethality.
The overexpression of a C-terminal DSP mutation (R2834H) leads to cardiac defects in adult mice. This R2834H DSP mutant was still able to target to desmosomes both in vitro and in vivo, suggesting that these desmosomes can function sufficiently to allow cardiac development in the embryo. However, the R2834H-Tg mice developed cardiomyocyte apoptosis, cardiac fibrosis, and cardiac dysfunction during adulthood. Most of the cardiac dysfunction and histological changes observed in these animals were present in both ventricles, which is consistent with recent findings that ARVD/C patients carrying C-terminal DSP mutations had LV involvement. These mice also displayed significant lipid accumulation in myocytes, suggesting alteration of lipid metabolism. However, there was no myocyte replacement by adipose tissue. We speculate that this might be due to differences between the species or other epigenetic (environmental) factors. However, this is the first model in which lipid abnormalities have been notable and could suggest that this is the precursor to fatty replacement seen in ARVD/C.

In R2834H-tg mice, desmin failed to colocalize with DSP at the IDs by immunohistochemistry, which has also been noted in a human subject with Carvajal syndrome. Interruption of DSP–desmin interactions might lead to instability of desmosomes, resulting in reduced resistance to the constant mechanical stress seen in the cardiomyocyte. This was supported by ultra-structural evidence of ID remodeling (widened gaps of IDs) in R2834H-Tg mice. The ultra-structural changes seen in R2834H-Tg mice are similar to (but more severe than) that observed in human ARVD/C patients. As the fibrous tissue replacement and lipid accumulation were only detected focally in R2834H-Tg mice, we were unable to observe these changes in the electron microscopic evaluation, probably due to limitations of sampling. Although we observed ultra-structural changes (electron dense materials in the IDs) in WT-Tg mice, this was not due to the junctional proteins we examined (by immunohistochemistry and Western blot analysis) and not functionally related. We believe that changes of other desmosomal and junctional components (increased expression and redistribution of JUP, PKP2, and β-catenin), and changes of gap junction components (redistribution of CX43) in R2834H-Tg mice, are probably secondary to the interruption of DSP–desmin interactions due to the R2834H DSP mutation. The presence of excessive JUP and β-catenin in the Triton-X soluble fraction (mostly cytoplasmic) could potentially result in additional transcriptional changes important in cell survival or adipogenesis. These changes might further add to the development of the cardiac phenotypes we observed in the mutant DSP-Tg mice.

There have been several theories regarding the pathogenesis of ARVD/C. These include the “dysontogenetic theory,” which hypothesizes that ARVD/C might be a milder form of Uhl anomaly (paper thin RV with or without tricuspid valve anomalies) and that pathogenesis occurs due to the dysregulation of ventricular wall thickness either during development or postnatally. Another theory, the “apoptotic theory,” suggests that ARVD/C is due to increased apoptosis of cardiomyocytes. A third theory, the “transdifferentiation theory,” suggests that cardiomyocytes transdifferentiate into adipocytes in response to stress.
both the dysontogenetic and apoptotic theories. In addition, our data suggest that desmosomes are not only involved in maintenance of tissue integrity, but also participate in other cellular processes including cell death and lipid metabolism.

Based on previous studies and our current findings, we believe that disruption of desmosomal integrity (due to mutations of DSP in this case) is the key factor leading to the development of ARVD/C, which results in defective mechanical linkage and in turn leads to abnormal localization of other cell–cell adhesion junction proteins (with possible subsequent transcriptional changes of related genes), and changes in gap junction components. We suggest that these defects in the desmosomal “final common pathway” might lead to the ARVD/C phenotype including: fibrosis, adipocyte infiltration, and arrhythmias. Therefore, this supports the concept that ARVD/C is a disease of the desmosome. However, detailed studies are required to further elucidate the pathogenesis of ARVD/C and provide insight into improving diagnosis and treatment of this disease.

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### Disclosures

None.

### References


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METHODS

Clinical diagnostic criteria for ARVD/C

All studies on human subjects were performed in accordance with local IRB regulations after informed consent. Diagnostic criteria for entry into the study were based on those developed by McKenna et al.\textsuperscript{1} All patients were required to meet these criteria that are based on the presence of ventricular arrhythmias of left bundle branch morphology, the presence of functional and/or structural abnormalities of the right ventricle and fibrous-fatty infiltration of the right ventricle. Patients were evaluated by electrocardiography, Holter monitoring, electrophysiology testing, echocardiography, MRI, angiography, and either endomyocardial biopsy (EMB) autopsy, or explant histological analysis. After informed consent, blood was obtained for lymphoblastoid cell line immortalization and DNA extraction,\textsuperscript{2} as regulated by the Baylor College of Medicine Institutional Review Board (IRB).

Mutation detection of DSP gene

Patient genomic DNA samples were amplified by PCR using primers designed to amplify the coding exons of DSP (primer sequences available on request) and purified the PCR products using QIAquick PCR Purification Kits (Qiagen, Valencia, CA) or Montage PCR96 Filter Plate (Millipore, Billerica, MA). DNA sequence analysis was performed using Big Dye terminator chemistry (v3.1) and an ABI 3100 (Applied Biosystems, Foster City, CA) as previously described.\textsuperscript{3} In one case (non-sense mutation, W233X), cDNA was amplified from total RNA extracted from the lymphoblastoid cell lines derived from patient blood using Superscript III RT-PCR system (Invitrogen,
Carlsbad, CA). Specific cDNA primers were used to amplify the region containing the nonsense mutation.

**cDNA constructs encoding human desmosomal components**

Constructs encoding the N-terminus of DSP, DSPNTP (N-terminal truncated DSP, 584aa, C-terminus (CT) Flag tagged), full length human DSP vector (2871aa, CT Flag tagged), full length JUP (CT myc tagged), PKP1a (CT myc tagged) and PKP2a (CT myc tagged), have been described previously.4,5 Mutations were introduced using the Quickchange site-directed mutagenesis system (Stratagene, La Jolla, CA). W233X GFP DSP construct was obtained as subcloning the corresponding PCR product of first 699 nucleotide of DSP into CT-GFP fusion TOPO vector (Invitrogen).

**Antibodies**

Commercial primary antibodies against Flag (M2, Stratagene), C-myc (Santa Cruz Biotechnology, Santa Cruz, CA), C-dystrophin (Vector Laboratories, Burlingame, CA), DSP (DP2.15, Research Diagnostics Inc., Concord, MA), desmin (Abcam Inc, Cambridge, MA), PKP2 (Research Diagnostics Inc.), JUP (Invitrogen), β-catenin (Sigma-Aldrich, St. Louis, MO), connexin 43 (Sigma-Aldrich) and α-tubulin (Santa Cruz) for western blot and immunohistochemical analyses were employed, diluted as recommended by the manufacturer.

**Desmosome-forming cell line and transient transfection**
The human tongue squamous cell carcinoma cell line, SCC-9 was cultured in DMEM-F/12 containing 10% fetal bovine serum and transfected using Effectene (Qiagen). 24-48 hours post-transfection, the plated cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.5% Triton-X, and then incubated with primary antibodies for 1 hour followed by incubation with Alexa Fluor 488 conjugated secondary antibodies (Molecular Probes, Invitrogen) for 30 minutes. Subsequently, the sections were incubated with DAPI (4',6-Diamidine-2-phenylindole dihydrochloride, 2.5ug/ml, Roche Applied Science, Indianapolis, IN) for 5 minutes and mounted with prolonged gold antifade reagent (Molecular Probes, Invitrogen). Fluorescence was detected using an Olympus BX51 microscope.

Co-immunoprecipitation

HEK-293 cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum and transfected using Lipofectamine (Invitrogen). 48 hours post-transfection, total protein was extracted using Tris-buffered saline containing 1% Triton X-100\(^4\) and the FLAG-tagged proteins were immunoprecipitated using M2-Flag agarose beads (Sigma-Aldrich). The whole cell lysates and immunoprecipitated proteins were analyzed by western blot analysis, as previously described.\(^4,5\)

Generation of transgenic mice

The full-length wild-type and mutant DSP cDNA sequences were cloned into a vector containing the mouse alpha-myosin heavy chain (\(\alpha\)-MyHC) promoter (Dr. Jeffrey Robbins, University of Cincinnati). The linearized cDNAs were injected into fertilized
oocytes (2 cell blastocyst stage) derived from C57/BL(6J) mice, and then the oocytes were transferred into the oviducts of pseudopregnant FVB mice: these were performed by the Transgenic and Homologous Recombination Core Facility of Baylor College of Medicine. The genotype and copy number of offspring were determined by PCR and Southern blot analysis of genomic DNA. E12.5-17.5 embryos were isolated from pseudopregnant mothers after microinjection and genotypes were determined by analysis of DNA extracts from the yolk sac.

**Echocardiographic assessment of cardiac function**

The mice were anesthetized by intraperitoneal administration of the mixture of ketamine (47.8 mg/l), xylazine (8.6 mg/l) and acepromazine (1.4 mg/l) at a dose of 0.5-0.7 ml/kg of body weight. Echocardiographic measurements were obtained using a 15-MHz linear transducer (Sequoia C256 and 15L8, Acuson Sequoia Cardiac System, Siemens Medical Solutions, Malvern, PA), as previously described.6

**Magnetic resonance imaging (MRI) assessment of cardiac function**

MRI images were acquired using the Bruker Biospin 7T horizontal MR scanner (Ettlingen, Germany). All animals were sedated for the procedure with isoflurane. Heart rate, respiratory rate, and body temperature were monitored continuously throughout the exam. ECG and respiratory triggered cine gradient echo images were acquired in both the short axis and four chamber geometries. Fourteen to eighteen images were acquired for each cardiac cycle depending on the heart rate. Average heart rates varied from 450 to 540 bpm. The Images were acquired with a FOV of 3.4 cm², slice thickness of 1 mm
and an in-plane resolution of 135 micrometers. A typical volumetric image set covering both ventricles required 10-12 images. Images were analyzed off-line using custom designed software for standard planimetry techniques (ImageJ, NIH; Bethesda, MD).

Right and left ventricular endocardial and epicardial contours were drawn on each of the 10-12 short axis slices. The volumes were calculated directly from the contour data. The right ventricular thickening was calculated by first drawing 5 radial bisectors from the endocardial to the epicardial contour of the right ventricle. These were drawn in both diastole and systole. The thickening is calculated as the \([\text{thickness (systole)} - \text{thickness (diastole)}]/\text{thickness (diastole)}\). The thickening reported for each animal is the average thickening of the 5 radii.

**Histology and immunofluorescence microscopy**

Immediately after echocardiography or MRI, mouse hearts were isolated and continuously perfused with cardioplegic solution containing KCl 3.73 g/l, and 10% neutralized formalin (Sigma-Aldrich). Whole embryos were isolated from pseudo moms after microinjection. Perfused hearts and embryos were paraffin-embedded and 10µm sections were stained with hematoxylin and eosin (H&E) or Masson’s trichrome. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science). TUNEL positive control sections were obtained by treating cardiac sections of non-transgenic mice with TACS-nuclease (Trevigen, Gaithersburg, MD).

Fluorescence was detected using an Olympus BX51 microscope. For cryosections (5µm), mouse hearts or embryos were equilibrated in 30% sucrose solution and mounted in
Sections were fixed with acetone at -20°C for 10
minutes, followed by Oil Red O stain or immunofluorescent staining with primary
antibodies for 1 hour and incubation with Alexa Flour 488 or Texas Red conjugated
secondary antibodies (Molecular Probes, Invitrogen) or Alexa Flour 594 conjugated
Phalloidin for 30 minutes (Molecular Probes, Invitrogen). Sections were mounted with
prolonged gold antifade reagent (Molecular Probes, Invitrogen) and visualized using an
Olympus Fluoview confocal microscopy system. Multiple sections from at least 10 mice
per group were analyzed. To measure myocyte area, frozen cardiac sections were stained
with C-dystrophin to outline the sarcolemma, and suitable cross-sections with nearly
circular capillary profiles were quantified. The colocalization efficiency of DSP and
desmin was calculated using Pearson’s correlation value (between -1 to 1, with -1 being
no overlap, 1 being perfect registration). Quantitative image analysis was performed
using Image-Pro Plus software.

**Electron microscopy analysis**

Myocardial samples were fixed in 3% glutaraldehyde solution and processed
according to standard protocol in the Integrated Microscopy Core at Baylor College of
Medicine. Images were taken using Hitachi H-7500 Transmission Electron Microscope.

**Analysis of protein expression**

Mouse hearts were snap frozen in liquid nitrogen and homogenized in lysis buffer
containing 1% Triton X-100. The lysates were clarified by centrifugation at 20,000g for
30 minutes and the Triton-X insoluble fraction were solublized using the lysis buffer
containing 9M urea.\textsuperscript{8} Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL) and equal amounts of protein were subjected to SDS-PAGE (Invitrogen) followed by transfer to nitrocellulose membranes by electrotransfer, as previously described.\textsuperscript{9} Proteins were detected as previously described.\textsuperscript{9} Quantitative image analysis was performed using Personal Densitometer SI and ImageQuant 5.2 software.
References


### Online Table 1. Establishment of cardiac specific DSP transgenic (Tg) mice lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Total injection</th>
<th>Pups analyzed</th>
<th>Embryos analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Positive*</td>
</tr>
<tr>
<td>WT-Tg</td>
<td>4</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>V30M-Tg</td>
<td>8</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q90R-Tg</td>
<td>7</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2834H-Tg</td>
<td>4</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

ED: embryonic day.

* Positive founders are confirmed by PCR and western blot analysis using transgene specific primers or antibody.

** Positive embryos are confirmed by PCR and immunohistochemistry.
Online Table 2. Echocardiographic analysis of DSP Tg mice

<table>
<thead>
<tr>
<th></th>
<th>R2834H -Tg</th>
<th>WT-Tg</th>
<th>NTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD (cm)</td>
<td>0.41 ± 0.02</td>
<td>0.38 ± 0.01</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>LVESD (cm)</td>
<td>0.25 ± 0.02*</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>FS (%)</td>
<td>35.20 ± 1.68**</td>
<td>47.01 ± 1.97</td>
<td>46.75 ± 1.21</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.084 ± 0.008</td>
<td>0.070 ± 0.004</td>
<td>0.071 ± 0.006</td>
</tr>
<tr>
<td>LVPWs (cm)</td>
<td>0.13 ± 0.006</td>
<td>0.13 ± 0.003</td>
<td>0.13 ± 0.008</td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>0.14 ± 0.016*</td>
<td>0.099 ± 0.008</td>
<td>0.098 ± 0.009</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>29.01 ± 2.73</td>
<td>26.68 ± 2.56</td>
<td>31.87 ± 1.75</td>
</tr>
<tr>
<td>LV mass:Body weight</td>
<td>0.0050 ± 0.0003**</td>
<td>0.0035 ± 0.0002</td>
<td>0.0031 ± 0.0003</td>
</tr>
</tbody>
</table>

Values are shown as the mean ± SEM. n=5 for each group (6-month old). LVEDD: left ventricle end diastolic dimension; LVESD: left ventricle end systolic dimension; FS: fractional shortening; LVPWd: left ventricle posterior wall thickness at diastole; LVPWs: left ventricle posterior wall thickness at systole. * p<0.05 when compared with WT-Tg or NTG. ** p<0.01 compared with WT-Tg or NTG. No statistical difference was noted between WT-Tg and NTG.
Online Table 3. MRI analysis of DSP Tg mice

<table>
<thead>
<tr>
<th></th>
<th>R2834H-Tg</th>
<th>WT-Tg</th>
<th>NTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDV (µl)</td>
<td>72.33±4.62</td>
<td>54.50±4.85*</td>
<td>48.83±0.84**</td>
</tr>
<tr>
<td>RVEDV (µl)</td>
<td>69.90±4.39</td>
<td>56.30±2.62*</td>
<td>42.17±2.45**</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>49.35±2.75</td>
<td>68.53±2.54**</td>
<td>63.67±4.13*</td>
</tr>
<tr>
<td>RVEF (%)</td>
<td>50.08±2.89</td>
<td>65.93±1.15**</td>
<td>62.13±4.21*</td>
</tr>
<tr>
<td>RV Thickness Sys (cm)</td>
<td>0.057±0.0025</td>
<td>0.12±0.011**</td>
<td>0.11±0.0055***</td>
</tr>
<tr>
<td>RV Thickness Dia (cm)</td>
<td>0.038±0.0014</td>
<td>0.055±0.0044**</td>
<td>0.049±0.0036*</td>
</tr>
<tr>
<td>RV Thickening (%)</td>
<td>46.50±6.71</td>
<td>123.80±10.31***</td>
<td>115.70±6.89***</td>
</tr>
</tbody>
</table>

Values are shown as the mean ± SEM. n=4 for each group (9-month old). LVEDV: left ventricle end diastolic volume; RVEDV: right ventricle end diastolic volume; LVEF: left ventricle ejection fraction; RVEF: right ventricle ejection fraction; RV: right ventricle; Sys: systole; Dia: diastole. * p<0.05 when compared with R2834H-Tg. ** p<0.01 compared with R2834H-Tg. *** p<0.001 compared with R2834H-Tg. No statistical difference was noted between WT-Tg and NTG.