Distinct Cellular Basis for Early Cardiac Arrhythmias, the Cardinal Manifestation of Arrhythmogenic Cardiomyopathy, and the Skin Phenotype of Cardiocutaneous Syndromes


Rationale: Arrhythmogenic cardiomyopathy is caused primarily by mutations in genes encoding desmosome proteins. Ventricular arrhythmias are the cardinal and typically early manifestations, whereas myocardial fibrofatty replacement is the pathological hallmark. Homozygous DSP (desmoplakin) and JUP (junction protein plakoglobin) mutations are responsible for a subset of patients with arrhythmogenic cardiomyopathy who exhibit cardiac arrhythmias and dysfunction, palmoplantar keratosis, and hair abnormalities (cardiocutaneous syndromes).

Objective: To determine phenotypic consequences of deletion of Dsp in a subset of cells common to the heart and skin.

Methods and Results: Expression of CSPG4 (chondroitin sulfate proteoglycan 4) was detected in epidermal keratinocytes and the cardiac conduction system. CSPG4<sup>−/−</sup> cells constituted ≈5.6±3.3% of the nonmyocyte cells in the mouse heart. Inducible postnatal deletion of Dsp under the transcriptional control of the Cspg4 locus led to ventricular arrhythmias, atrial fibrillation, atrioventricular conduction defects, and death by 4 months of age. Cardiac arrhythmias occurred early and in the absence of cardiac dysfunction and excess cardiac fibroadipocytes, as in human arrhythmogenic cardiomyopathy. The mice exhibited palmoplantar keratosis and progressive alopecia, leading to alopecia totalis, associated with accelerated proliferation and impaired terminal differentiation of keratinocytes. The phenotype is similar to human cardiocutaneous syndromes caused by homozygous mutations in Dsp.

Conclusions: Deletion of Dsp under the transcriptional regulation of the CSPG4 locus led to lethal cardiac arrhythmias in the absence of cardiac dysfunction or fibrofatty replacement, palmoplantar keratosis, and alopecia, resembling the human cardiocutaneous syndromes. The findings offer a cellular basis for early cardiac arrhythmias in patients with arrhythmogenic cardiomyopathy and cardiocutaneous syndromes. (Circ Res. 2017;121:1346-1359. DOI: 10.1161/CIRCRESAHA.117.311876.)

Key Words: arrhythmogenic right ventricular dysplasia ■ death, sudden ■ desmoplakins ■ desmosomes ■ neurofibromatosis

Arrhythmogenic cardiomyopathy (ACM) encompasses a group of myocardial diseases, whose cardinal manifestations are ventricular arrhythmias, occurring before and independent of systolic dysfunction. Ventricular arrhythmias, manifesting as palpitations, syncope, and sudden cardiac death, are the most common initial presentation of ACM. Atrial arrhythmias are also common and present in up to half of the patients with ACM, typically in those with atrial and right ventricular enlargement. Ventricular arrhythmias occur in the advanced stages of ACM in association with cardiac dysfunction.

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The classic form of ACM is arrhythmogenic right ventricular cardiomyopathy, which is characterized by progressive fibrofatty replacement of cardiac myocytes, predominantly but not exclusively in the right ventricle, myocyte atrophy, and apoptosis. Heart failure typically develops in those with long-standing disease, is usually refractory to therapy, and often requires heart transplantation.
Novelty and Significance

What Is Known?

- Arrhythogenic cardiomyopathy (ACM) is a primary disease of myocardium caused mostly by mutations in genes encoding desmosome proteins.
- Ventricular arrhythmias are the cardinal manifestation of ACM, occurring early and before cardiac dysfunction.
- Altered functions of ion channels in cardiac myocytes are implicated in the pathogenesis of ventricular arrhythmias in ACM.
- A subset of ACM manifests with skin abnormalities occurring in conjunction with cardiomyopathy and is referred to as cardiocutaneous syndrome.

What New Information Does This Article Contribute?

- CSPG4 (chondroitin sulfate proteoglycan 4) is a marker for cardiac conduction system, tagging a subset of cardiac conduction system cells.
- Cardiac cells expressing CSPG4 also express selected desmosome proteins and ion channels.
- Postnatal (day 21) inducible conditional deletion of Dsp gene, encoding desmoplakin, under the transcriptional regulation of the Csg4 locus, that is, in cells expressing CSPG4, leads to early ventricular and atrial arrhythmias and premature death in the presence of a preserved left ventricular systolic function, as observed in human ACM.
- Deletion of Dsp in the CSPG4pos cells also leads to progressive alopecia and severe palmoplantar keratosis, as observed in cardiocutaneous syndromes in humans.

The findings delineate a cellular basis for early ventricular arrhythmias in ACM—an important cause of sudden cardiac death in the young, particularly in athletes. The present findings along with the previous data point to participation of multiple cell types in the pathogenesis of ACM phenotypes, with CSPG4pos cells contributing to early cardiac arrhythmias, fibroadipocyte progenitor cells to fibroadiposis, and cardiac myocytes to cardiac dysfunction and heart failure. Delineation of the molecular and cellular basis of ACM could offer the opportunities to identify new diagnostic tools and novel therapeutic targets in ACM.

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>ACM</td>
<td>arrhythogenic cardiomyopathy</td>
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<tr>
<td>ACTN2</td>
<td>actinin α2</td>
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<tr>
<td>ADGRE4</td>
<td>adhesion G protein-coupled receptor F4</td>
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<td>CASP3</td>
<td>caspase 3</td>
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<td>CCS</td>
<td>cardiac conduction system</td>
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<td>CNNT2</td>
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<td>CSPG4</td>
<td>chondroitin sulfate proteoglycan 4</td>
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<tr>
<td>DSC2</td>
<td>desmocollin 2</td>
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<td>DSG2</td>
<td>desmoglienn 2</td>
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<td>DSP</td>
<td>desmoplakin</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<tr>
<td>GJA5</td>
<td>connexin 40</td>
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<td>ID</td>
<td>intercalated discs</td>
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<tr>
<td>JUP</td>
<td>junction protein plakoglobin</td>
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<tr>
<td>KCNE1</td>
<td>potassium voltage-gated channel subfamily E regulatory subunit 1</td>
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<td>KCNH2</td>
<td>potassium voltage-gated channel subfamily H member 2</td>
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<td>keratin 14</td>
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<tr>
<td>MKI67</td>
<td>proliferation marker KI67</td>
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<tr>
<td>PDGFRB</td>
<td>platelet-derived growth factor receptor beta</td>
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<tr>
<td>PKP2</td>
<td>plakophilin 2</td>
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<tr>
<td>PRDM1</td>
<td>P/SET domain 1</td>
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<tr>
<td>PTPRC</td>
<td>protein tyrosine phosphatase, receptor type C</td>
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<tr>
<td>SCN5A</td>
<td>sodium voltage-gated channel α subunit 5</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>TP53</td>
<td>tumor protein 53</td>
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<td>WT</td>
<td>wild-type</td>
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Mutations in 5 genes encoding desmosome proteins, namely PKP2 (plakophilin 2), JUP (junction protein plakoglobin), DSP (desmoplakin), DSC2 (desmocollin 2), and DSG2 (desmoglienn 2), are the most common known causes of ACM.7–12 Homozygous mutations in DSP and JUP cause ACM occurring in conjunction with palmoplantar keratosis, wooly hair, and alopecia, which are referred to as cardiocutaneous syndromes.7,8,12 Mutations in several other genes, including PLN and LMNA, encoding phospholamban and lamin A/C, respectively, also have been implicated in ACM.14,15

Desmosomes are components of the intercalated discs (IDs), which are primarily present in the epithelial cells and involved in cell-to-cell attachment, mechanotransduction, and mechanical integrity of the tissue.16–19 The mechanisms by which mutations in desmosome proteins lead to ACM and its typical phenotypes of ventricular arrhythmias, excess fibroadipocytes, or cardiocutaneous syndromes are not fully understood. Deletion of the Dsp gene exclusively in cardiac myocytes in mice leads to cardiac dysfunction, premature death, and mild fibroadipogenesis.19 However, cardiac arrhythmias in the myocyte-specific Dsp-deficient mice occur in the context of cardiac dysfunction but not independent of cardiac dysfunction.19 The arrhythmic phenotype in the cardiac myocyte-specific deletion of Dsp is in contrast to the human ACM, where cardiac arrhythmias are often the early manifestations, occurring before and independent of cardiac dysfunction.1,2,4

The dissociation of early cardiac arrhythmias and function might simply reflect potential differential effects of the desmosome protein mutations in stabilizing the ion channel at the IDs and maintaining mechanical integrity of the myocardium. Alternatively, it may indicate possible involvement of nonmyocyte cells in the pathogenesis of early cardiac arrhythmias in ACM.

Desmosome proteins are known to be expressed primarily in cardiac myocytes and epithelial cells. They are also expressed, albeit at lower levels, in the nonmyocyte cardiac cells, such as the mesenchymal cells and fibroadipocyte progenitors.20,21 Cells expressing the mutant desmosome proteins, whether myocytes or nonmyocyte cells, are expected to be involved in the pathogenesis of ACM.20,21

The publicly available RNA sequencing and microarray databases include Cspg4 (chondroitin sulfate proteoglycan 4
transcript) among those expressed in the cardiac conduction system (CCS), including the Purkinje fibers and the atrioventricular node.22,23 The CCS, sharing an embryonic origin with cardiac myocytes, also expresses desmosome proteins.24,25 CSPG4 is also expressed in the keratinocytes and hair follicle cells, which are known to express DSP.26,27 Therefore, CSPG4, along with desmosome proteins, might serve as a shared molecular link between early cardiac arrhythmias and the skin phenotype in the cardiocutaneous syndromes. Thus, we determined the phenotypic consequences of deletion of DSP under the transcriptional regulation of the Cspg4 locus.

**Methods**

An expanded Methods section is provided as Online Data Supplement.

Studies in the animal models conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee. Human tissue use was approved by the institutional review board.

**Histology (Hematoxylin and Eosin, Masson Trichrome, and Oil Red O Staining), Immunohistochemistry, and Immunofluorescence**

These techniques were performed as published.18,20,25 Custom-made and commercially available antibodies were used to detect expression of the proteins of interest, including CSPG4 and DSP in isolated cardiac myocytes, nonmyocyte cardiac cells, and myocardial sections (Online Table I).

**Isolation of Human and Mouse Adult Myocytes and Nonmyocyte Cardiac Cells**

Cardiac myocyte-depleted cell fraction was obtained by collagenase type 2 digestion as published.22,23 The tissue samples were digested with collagenase, filtered through a 40-μm cell strainer, and the cells were precipitated by centrifugation. The pelleted cells were resuspended in a complete medium in the presence of antibiotics and plated on plates coated with 0.1% gelatin.

Cells expressing CSPG4 were isolated from myocyte-depleted cardiac cells by flow cytometry and fluorescence-activated cell sorting (FACS), as described, except for using a custom-made rabbit anti-CSPG4 antibody.25

**Cspg4-DsRed.T1 Reporter Mice**

Cspg4-DsRed.T1 is a bacterial artificial chromosome transgenic reporter mouse line that expresses a red fluorescence protein variant, Ds-Red.T1, under the transcriptional regulation of the Cspg4 locus (stock number: 008241, Jackson Laboratory).29

**Cspg4-Cre/Esr1*:DspW/F and Cspg4-Cre/Esr1*:DspF/F Mice**

These mice were generated on crossing of Cspg4-Cre/Esr1*:Ds-Red.T1, under the transcriptional regulation of the Cspg4 locus, Dsp-W/F, and Esr1-F/F reporter mouse lines that express a red fluorescence protein variant, Ds-Red.T1, under the transcriptional regulation of the Cspg4 locus (stock number: 008241, Jackson Laboratory).29

**Quantitative Polymerase Chain Reaction**

Quantitative polymerase chain reaction was performed as published and the relative normalized values (2^(-ΔΔCt) method, shown as relative to the WT [wild-type] mice) were used to compare the transcript levels.19,28

**Immunoblotting**

Immunoblotting was performed as published.18,28

**Apoptosis**

Apoptosis was detected in thin myocardial section by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay, as published.19,33 The number of TUNEL-positive cells was quantified in ≥20000 cells per mouse and in 6 mice per group.

**Echocardiography**

Two-dimensional, M mode, and Doppler echocardiography was performed in 3- to 6-months-old Cspg4-Cre/Esr1*:DspF/F and Cspg4-Cre/Esr1*:DspW/F mice and their corresponding WT controls as published.11,20,24-27

**Electrocardiography and Electrophysiology**

Surface ECG and heart rate were recorded after insertion of 29-gauge needle electrodes subcutaneously into the forelimbs and connecting the leads to an Animal Bio Amp, PowerLab, and the LabChart7 software.

Electrophysiology studies were performed in isoflurane-anesthetized mice at temperature between 36 and 37°C. Intracardiac bipolar atrial and ventricular electrogroms were obtained using a 1.1F octopolar catheter (EPR-800; Millar Instruments), as described.28,34 Electrophysiology pacing protocols, including single, double, and burst ventricular pacing protocols, were used for ventricular tachycardia induction at baseline and after injection of isoproterenol (3 mg/kg IP). The atrial stimulation protocol to evaluate inducibility of atrial fibrillation was performed with incremental atrial pacing, as described.34 All pacing protocols were performed a maximum of 3x. Nonsustained and sustained ventricular tachycardia was defined as reproducible (at least twice) ectopic ventricular rhythms lasting 4 to 9 rapid consecutive ventricular beats and ≥10 beats, respectively. Atrial fibrillation was defined as reproducible rapid and fragmented atrial electrograms with irregular ventricular response for ≥1 second.

**Statistical Analysis**

Data that followed a Gaussian distribution pattern were presented as mean±SD and were compared between 2 groups by t test and among multiple groups by ANOVA followed by post hoc pairwise comparisons. Otherwise, data were presented as the median values and compared by Kruskall–Wallis test, as were the categorical data.

**Results**

**CSPG4 Is Expressed in CCS and Skin Keratinocytes**

To corroborate the transcriptomic data detecting the Cspg4 transcripts in the heart, nonmyocyte fraction of cardiac cells was isolated and sorted against a custom-made anti-CSPG4 antibody. FACS analysis showed that =5.6±3.3% of cardiac nonmyocyte cells expressed CSPG4 (Figure 1A; Online Figure I). To further corroborate detection of the Cspg4 transcripts in the CCS, coexpression of CSPG4 and CNTN2 (contactin 2)—an established CCS marker—in cardiac cells was analyzed by FACS and immunostaining. CSPG4 and CNTN2 were coexpressed in a subset of isolated cardiac nonmyocyte cells and about two third of cells expressing CSPG4 also expressed CNTN2 (Figure 1B and 1C; Online Figure I).

To detect expression and localization of CSPG4 in the heart, thin myocardial sections from adult mice were stained with a custom-made antibody against CSPG4. Expression of CSPG4 was detected in the atrioventricular node and the bundle branches, which were identified by the expression of CNTN2 or GJA5 (connexin 40)—2 well-established CCS markers (Figure 1D through 1G).22,35 To corroborate the immunofluorescence data, thin myocardial sections from the Cspg4-DsRed.T1 reporter mouse hearts were examined under fluorescence microscopy. DsRed.T1 expression,
reflecting *Cspg4* locus transcriptional activity and hence an antibody-independent surrogate for CSPG4, was detected in the atrioventricular node (Figure 1H). Ds-Red.T.1 expression and localization in the myocardial sections was largely consistent with the detection of CSPG4 expression in the CCS by immunofluorescence staining, albeit less pronounced.
Colocalization of CSPG4 and CNTN2 was partial, suggesting expression of CSPG4 in a subset of the CCS cells and cellular heterogeneity of the CCS. In addition to the CCS, CSPG4 was also expressed in a subset of cells within the interstitial tissue in the myocardium, likely representing capillary mural cells or pericytes (Figure 1I). Notably, expression of CSPG4 was not detected in the ventricular myocytes both by immunostaining and by examination of myocardial sections from the Cspg4-DsRed.T1 reporter mice (Figure 1H and 1I).

Given the detection of the CSPG4 in capillary mural cells and its known expression in a subset of pericytes, isolated cardiac nonmyocyte cells were costained for CSPG4 and PDGFRB (platelet-derived growth factor receptor beta) proteins, the latter a marker for pericytes. Expressions of the CSPG4 and PDGFRB proteins were detected in 8.1±10.6% and 2.6±3.3% of the isolated cardiac nonmyocyte cells, respectively (Online Figure IIA). Only ~10% of cells expressing PDGFRB also expressed CSPG4. In addition, CSPG4 was not expressed in the endothelial cells, marked by the expression of PECAM1 (platelet endothelial cell adhesion molecule 1; Online Figure IIB).

CSPG4 was also strongly expressed in murine back skin, as detected by immunoblotting and immunofluorescence staining (Figure 1J and 1K). Its expression was predominantly localized to epidermal keratinocytes in the interfollicular epidermis and hair follicles (Figure 1K). As expected, DSP was also expressed throughout the epidermis (Figure 1K). To further corroborate the findings, independent of the antibody performance, expression of CSPG4 was analyzed in the Cspg4-DsRed.T1 mice where DS-Red.T1 reporter protein serves as a surrogate for the expression of CSPG4 protein. DsRed.T1 expression was detected in the skin, predominantly in the interfollicular epidermis and hair follicles (Online Figure III).
**Selected Ion Channel and Desmosome Proteins Are Expressed in Cardiac CSPG4pos Cells**

Given that CSPG4 also tagged the CCS, expression of selected ion channel genes was analyzed in the CSPG4pos cells isolated from the heart by FACS. Immunofluorescence staining detected expression of SCN5A (sodium voltage-gated channel subfamily H member 2 or HERG), and KCNE1 (potassium voltage-gated channel subfamily H member 2 or HERG), and KCNE1 (potassium voltage-gated channel subfamily E regulatory subunit 1) in the cardiac CSPG4pos cells (Figure 2A).

To determine whether cardiac CSPG4pos cells express desmosome proteins and hence contribute to the pathogenesis of ACM caused by desmosome protein mutations, mouse cardiac nonmyocyte cell fraction was sorted against an anti-CSPG4 antibody, and the isolated cells were stained for the coexpression of selected desmosome proteins and CSPG4. Figure 2B illustrates expression of selected desmosome proteins, including DSP, JUP, and PKP2, along with the expression of CSPG4 in the FACS-isolated murine cardiac nonmyocyte cells.

To extend the findings to the CSPG4pos cells isolated from the human hearts, cardiac nonmyocyte cell fraction was isolated from the explanted human hearts not used for cardiac transplantation and stained with antibodies against CSPG4 and selected desmosome proteins. As observed in CSPG4pos cells isolated from the mouse heart, desmosome proteins were also coexpressed with CSPG4 in the isolated human cardiac nonmyocyte cells (Figure 2C).

To complement the results of immunofluorescence studies, RNA was extracted from isolated cardiac CSPG4pos cells and the transcript levels of selected genes encoding desmosome and ion channel proteins were quantified by quantitative polymerase chain reaction. The results verified expression of selected transcripts in the isolated cardiac myocytes, expression and localization of DSP protein in cardiac myocytes isolated from the isolated cardiac myocytes (Figure 2D).

Finally, to determine whether cardiac myocytes express CSPG4, isolated cardiac myocytes were stained for cardiac ACTN2 (actinin α2) and CSPG4. As shown (Online Figure IVA), CSPG4 was not expressed in cardiac myocytes. Likewise, transcript levels of Myh6 and Actc1 were quantified by quantitative polymerase chain reaction in isolated cardiac CSPG4pos cells. Myh6 and Actc1 transcript levels comprised approximately ≤0.01% and 0.04% of those in cardiac myocytes (Online Figure IVB).

**Postnatal In Vivo Deletion of Dsp in the CSPG4pos Cells (Cspg4-Cre/Esr1*:DspW/F and Cspg4-Cre/Esrl*:DspW/F Mice)**

To determine whether deletion of Dsp in CSPG4pos cells induces a cardiac or skin phenotype, one or both copies of the Dsp were deleted postnatally (day 21) under transcriptional regulation of the Cspg4 locus (Online Figure V). The approach of postnatal deletion reduced potential confounding effects, resulting from the transcriptional activity of the Cspg4 locus in other cell types during cardiac development. Recombination efficiency of the Dsp gene in the heart was low (1.63% in the Cspg4-Cre/Esr1*:DspW/F mice), which is consistent with the small number of cardiac CSPG4pos cells and the absence of transcriptional activity of the Cspg4 locus in other cardiac cells (Figure 3A). Recombination efficiency, however, was almost 100% in the skin in the Cspg4-Cre/Esr1*:DspW/F mice, in accord with the expression of CSPG4 in the keratinocytes and their abundance in the skin (Figure 3B).

To further confirm deletion of the Dsp gene in the CSPG4pos cells, expression of Dsp transcript and DSP protein were detected in the isolated cardiac CSPG4pos cells. Although DSP was expressed in the CSPG4pos cells isolated from the WT mouse hearts, it was not detectable in the CSPG4pos cells isolated from the Cspg4-Cre/Esr1*:DspW/F mouse hearts (Figure 3C). Likewise, Dsp transcript levels were significantly lower in Cspg4-Cre/Esr1*:DspW/F mice when compared with WT (46.3±10.0%; Figure 3D).

To exclude possible fortuitous deletion of Dsp gene in cardiac myocytes, expression and localization of DSP protein were analyzed in the whole heart and isolated cardiac myocytes, respectively. Dsp transcript levels were unchanged in cardiac myocytes isolated from the Cspg4-Cre/Esr1*:DspW/F mouse hearts, as compared with the WT myocytes (Figure 3E). Likewise, DSP localization to the IDs was unaltered in cardiac myocytes isolated from the Cspg4-Cre/Esr1*:DspW/F mouse hearts (Figure 3F). Finally, DSP protein levels in the isolated cardiac myocytes (Figure 3G) and in the whole heart (Figure 3H), where myocytes are the predominant cell type expressing DSP, were analyzed by immunoblotting and were not different among the WT, Cspg4-Cre/Esr1*:DspW/F, and Cspg4-Cre/Esr1*:DspW/F mice.

**Premature Death of Cspg4-Cre/Esr1*:DspW/F Mice**

Induced homozygous deletion of Dsp postnatally (day 21) led to a near total mortality within 2 to 3 months after induction (Figure 4A). The median survival age of the Cspg4-Cre/Esr1*:DspW/F mice was ~30 days post induced deletion of the Dsp gene. Dsp-deficient heterozygous mice had a normal survival ≤1 year of age (Figure 4A).

**Cardiac Arrhythmias and Conduction Defects in the Cspg4-Cre/Esr1*:DspW/F Mice**

To investigate the cause of premature death, cardiac rhythm was monitored for 4 hours per day for 3 consecutive days (a total of 12 hours) in each mouse. Implantable telemetric monitoring could not be performed because of a smaller body weight of the Cspg4-Cre/Esr1*:DspW/F mice, which hindered implantation of radio transmitter (Online Figure VI). During cardiac rhythm monitoring, the Cspg4-Cre/Esr1*:DspW/F mice exhibited spontaneous high-grade atrioventricular block, intermittent atrial fibrillation, and runs of nonsustained ventricular tachycardia (Figure 4B). ECG and rhythm phenotypes in the WT and Cspg4-Cre/Esr1*:DspW/F were unremarkable.

To further analyze the propensity of the Dsp-deficient mice to develop cardiac arrhythmias, 1- to 3-months-old WT (n=7) and Cspg4-Cre/Esr1*:DspW/F (n=10) mice underwent in vivo electrophysiology studies. Cspg4-Cre/Esr1*:DspW/F mice were not studied because they had normal survival and did not show rhythm abnormalities during cardiac rhythm monitoring. Heart rates at the baseline and conduction intervals were not significantly different between WT and Cspg4-Cre/
Figure 3. Fidelity of the approach to deleting the Dsp (desmoplakin) gene specifically in the CSPG4<sup>pos</sup> (chondroitin sulfate proteoglycan 4) cells. A and B, Recombination efficiency in the heart and skin tissues. The floxed, wild-type (WT), and recombined alleles are identified by size. Recombination efficiency was low in the heart and high in the skin in keeping with the small and high number of cells expressing CSPG4 in the heart and skin, respectively. C, Absence of DSP expression in the CSPG4<sup>pos</sup> cells isolated from the Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>F/F</sup> mouse hearts and the corresponding WT control. The upper immunofluorescence panel shows expression of CSPG4, the middle DSP, and the lower overlay of the 2 panels in the experimental groups. As shown, DSP was expressed in the CSPG4<sup>pos</sup> cells isolated from the WT but not from the Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>F/F</sup> mouse hearts. D, Quantitative polymerase chain reaction (PCR) data of the Dsp transcript levels in the CSPG4<sup>pos</sup> cells isolated from the WT and Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>F/F</sup> mouse hearts. Transcript levels of Dsp were reduced by >50% in the CSPG4<sup>pos</sup> cells isolated from Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>F/F</sup> mouse hearts, as compared with the WT cells (46.25±10.04%; n=3; P=0.006). E, Quantitative PCR data of the Dsp transcript levels in cardiac myocytes, which were unchanged in cardiac myocytes isolated from Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>F/F</sup> mouse hearts as compared with controls (n=6; P=0.858). F, Expression and localization of DSP in cardiac myocytes isolated from the WT and Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>F/F</sup> mouse hearts. DSP expression was detected and localized to myocyte ends in both genotypes. The panels also show absence of expression of the CSPG4 protein in cardiac myocytes. G, Expression of DSP protein, detected by immunoblotting, in the cardiac myocytes isolated from the WT, Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>W/F</sup> (heterozygous), and Cspg4-Cre/Esr1<sup>*</sup>:Dsp<sup>F/F</sup> (homozygous) mice. As shown, DSP levels were similar among the experimental groups, indicating intactness of DSP in cardiac myocytes. A corresponding blot for TUBA1A (tubulin α1), as a control for loading conditions, is also shown. H, Expression of DSP, CSPG4, and TUBA1A in the cardiac protein extracts from 2 mice per experimental group are shown. DSP protein levels were equal among the groups, in accord with the predominant expression of DSP in cardiac myocytes. TUBA1A indicates tubulin 1 alpha; and WT, molecular weight.
Esr1*;DspF/F mice, with the exception of the ventricular atrial Wenckebach cycle length at baseline, which was prolonged in the Cspg4-Cre/Esr1*;DspF/F mice (73.3±11.5 ms in the WT and 88.6±13.7 ms in Cspg4-Cre/Esr1*;DspF/F mice; Online Table II). Intraperitoneal injection of isoproterenol (3 mg/kg) increased the average heart rate equally in the WT and Cspg4-Cre/Esr1*;DspF/F mice.

Electrophysiology studies were completed in 8 Cspg4-Cre/Esr1*;DspF/F and 7 WT mice. Programmed electric stimulation evoked ventricular arrhythmias in 2 of 8 (25%) at the baseline and 5 of 8 (63%) after isoproterenol injection (3 mg/kg) in the Cspg4-Cre/Esr1*;DspF/F mice (Figure 4C). Likewise, atrial fibrillation was induced in 4 of 8 (50%) Cspg4-Cre/Esr1*;DspF/F mice. Thus, isoproterenol injection induced ventricular arrhythmias and atrial fibrillation in 9 of 10 (90%) of the Cspg4-Cre/Esr1*;DspF/F as compared with 1 of 7 (14%) in the WT mice (P=0.004). Cardiac rhythm was recorded in 2 Cspg4-Cre/Esr1*;DspF/F mice who died shortly after completion of the pacing protocols. The findings were remarkable for progressive bradycardia with marked
ST elevations and progressive heart block preceding death (Figure 4D).

**Normal Cardiac Function in the Cspg4-Cre/Esr1*:*DspF/F Mice**

To determine whether cardiac arrhythmias on deletion of Dsp in the CSPG4pos cells were associated with cardiac dysfunction or were independent of it, echocardiography was performed in 3-month-old Cspg4-Cre/Esr1*:*DspF/F mice and age- and sex-matched control WT mice. In addition, echocardiography was also performed in 6- to 10-month-old Cspg4-Cre/Esr1*:*DspW/W along with matching WT controls. The results, shown in Online Tables III and IV, are notable for a normal left ventricular chamber size (when corrected for body weight) and normal cardiac function.

**Increased Myocardial Apoptosis in the Cspg4-Cre/Esr1*:*DspF/F Mice**

Because apoptosis is a known phenotype of ACM, 19 ≈20 000 myocardial cells per mouse and 6 mice per group were examined by TUNEL assay. The number of cells staining positive in the TUNEL assay was increased by ≈5-fold in the Cspg4-Cre/Esr1*:*DspF/F as compared with WT mice (Figure 5).

**Absence of Excess Fibroadipocytes in the Heart in the Cspg4-Cre/Esr1*:*DspF/F Mice**

Given that CSPG4 is expressed in a subset of pericytes because pericytes might be a source of fibroadipocytes in ACM, and to determine whether cardiac arrhythmias were independent of fibroadipiosis, myocardial histology was examined on staining of thin myocardial sections with Masson trichrome and Oil Red O. Neither fibrosis, reflected in the collagen volume fraction, nor the number of Oil Red O-stained adipocytes was increased in the hearts of Cspg4-Cre/Esr1*:*DspW/W and Cspg4-Cre/Esr1*:*DspF/F mice as compared with the WT mice (Online Figure VII).

**Progressive Alopecia and Palmo/Plantar Keratosis in the Cspg4-Cre/Esr1*:*DspF/F Mice**

The Cspg4-Cre/Esr1*:*DspF/F mice exhibited progressive alopecia starting 2 weeks after induced deletion of the Dsp gene, which progressed to alopecia totalis by 4 to 6 months of age (Figure 6A). Likewise, the mice exhibited severe palmar and plantar keratosis (Figure 6B) resembling the skin phenotypes of human patients with Cavajal and Naxos syndromes, caused by homozygous deletion of DSP and JUP, respectively.7,8,38 Immunofluorescence staining of skin section showed effective ablation of DSP in the keratinocytes (Figure 6C), consistent with a high recombination efficiency (Figure 3B). The Cspg4-Cre/Esr1*:*DspW/W mice did not show similar skin phenotypes ≤1 year of age.

To delineate the mechanistic basis of alopecia and keratosis and given the role of CSPG4pos cells in renewal of the epidermal keratinocytes,26,27,39 skin tissue was stained for markers of cell proliferation, differentiation, inflammation,
Histological analysis of the skin of Cspg4-Cre/Esr1*:Dsp<sup>F/F</sup> mice revealed a defect in epidermal sheet formation and a hyperplastic interfollicular epidermis (Figure 6D). The Dsp-deficient mice displayed a hyperproliferative and thickened interfollicular epidermal basal layer, as demonstrated by immunohistochemical staining for the MKI67 (proliferation marker Ki67) and basal layer marker KRT14 (keratin 14), TP53 (tumor protein 53), and TP63 (tumor protein 63; Figure 6D). To determine whether epidermal differentiation was effected by Dsp deficiency, skin sections were stained for early differentiation markers KRT1 and KRT6 and terminal differentiation marker PRDM1. Staining for KRT1, KRT6, and PRDM1 showed strong suprabasal immunoreactivity (Figure 6D).

In line with the observed hyperproliferative phenotype, sebaceous glands were enlarged in the skin of Dsp-deficient mice, as assessed by staining for FAS (fatty acid synthase)—a sebocyte marker (Online Figure VIII).

Immunohistochemical staining of skin sections for the expression of apoptosis markers CASP3 (caspase 3) and BCL2 (B-cell lymphoma 2; apoptosis regulator) showed no detectable changes in BCL2 and CASP3 expressions (Online Figure VIII). Similarly, markers of inflammation, including ADGRE4 (adhesion G protein-coupled receptor F4), formerly known as F4/80—a macrophage marker—and TNF<sub>α</sub> (tumor necrosis factor-<sub>α</sub>)—a proinflammatory cytokine—were unchanged (Online Figure VIII). Likewise, the number of bone marrow-derived hematopoietic cells, identified by the expression of PTPRC (protein tyrosine phosphatase, receptor type C), formerly known as CD45, in the skin were unchanged.

Finally, to determine whether deficiency of DSP in skin keratinocytes impacted localization of JUP, thin skin sections from WT and Cspg4-Cre/Esr1*:Dsp<sup>F/F</sup> mice were costained for coexpression of KRT14 and JUP. The results showed unaltered localization of JUP in the skin keratinocytes, albeit JUP expression was markedly increased consistent...
with the increased thickness of epidermis in the Cspg4-Cre/Esr1*;DspF/F mice (Online Figure IX).

Discussion

Cardiac arrhythmias—the cardinal manifestations of ACM—are typically the first manifestations, occurring before and in the absence of cardiac dysfunction. Cardiac arrhythmias are considered to be the consequence of expression of the mutant or deficiency of the desmosome protein in cardiac myocytes. Experimentally, deletion of genes encoding desmosome proteins in cardiac myocytes in mice leads to cardiac dysfunction but not early cardiac arrhythmias. Cardiac arrhythmias in such models occur concurrently with cardiac dysfunction. The findings of the present study suggest a noncardiac myocyte origin of the early cardiac arrhythmias in ACM. The findings specifically implicate an origin from cells expressing CSPG4, likely the CCS, which are also known to express desmosome proteins and, hence, are expected to be affected in human ACM. Accordingly, deletion of the Dsp gene, specifically in the CSPG4 cells, which also tags the CCS in the heart, in addition to the capillary mural and neuroglial cells, leads to spontaneous atrial and ventricular arrhythmias and premature mortality. Notably, these arrhythmias occur in the presence of a normal cardiac function and absence of fibroadiposis. The phenotype simulates the early cardiac arrhythmias in humans with ACM and, hence, implicates a distinct cellular basis for early cardiac arrhythmias in ACM.

In accord with the expression of CSPG4 and DSP in skin progenitors and their role in keratinocyte renewal, deletion of Dsp specifically in the CSPG4 cells also led to enhanced proliferation and impaired terminal differentiation of keratinocytes and the clinical phenotype of alopecia totalis and severe keratosis. Strong suprabasal immunoreactivity against KRT1, KRT6, and BLIMP1 (beta-interferon gene positive-regulatory domain I binding factor) suggested initiation but delayed terminal differentiation of keratinocytes in the skin of Dsp-deficient mice. In line with earlier studies, Dsp deficiency in the epidermal basal layer caused defects in epidermal sheet formation on mechanical stress. However, no evidence for an inflammatory infiltrate or apoptosis was found. Increased expression of TP53, in the absence of apoptotic epidermal keratinocytes, is in accord with a hyperproliferative epidermal basal layer and impaired (or absent) terminal differentiation in the skin of Cspg4-Cre/Esr1*;DspF/F mice. The observed phenotype, resembling those found in human patients with Carvajal syndrome and Naxos disease, defines a cellular basis for the skin phenotype in cardiocutaneous syndromes.

Whereas ventricular and atrial arrhythmias are known phenotypic features of ACM, atioventricular block is not a recognized phenotype. Conduction defect in human ACM typically manifests as an epsilon wave, which also has been reported in PKP2 deficiency. Atrioventricular block was observed only in the Cspg4-Cre/Esr1*;DspF/F mice but not in heterozygous deletion of DSP, which is the common genotype in human ACM. Similarly, the relevance of progressive bradycardia and atioventricular block preceding death in the DSP-deficient mice to human ACM is unclear. These data along with the previous data point to the role of DSP in regulating cardiac rhythm and conduction. Studies in human patients with homozygous deficiency of DSP would be required to determine whether atioventricular block is also a phenotypic feature of human patients with ACM. Likewise, it is unclear whether atrial fibrillation—a known phenotype of ACM—also occurs before and in the absence of cardiac dysfunction in patients with ACM. In addition, the findings in the Cspg4-Cre/Esr1*;DspF/F might be pertinent only to a subset of human patients with the cardiocutaneous syndromes caused by DSP deficiency but not those caused by missense mutations or those caused by the heterozygous mutations. Finally, phenotypic differences between human ACM and the mouse model are inherent to the model systems, which provide valuable insight into the pathogenesis of the phenotype but typically do not recapitulate the human disease.

CSPG4 is also expressed in the mural capillary cells or pericytes and neuroglial cells, which might be a cell source of fibroadipocytes in ACM. The findings show that deletion of Dsp in the CSPG4 cells did not result in excess fibroadipocytes in the heart, hence excluding these cells, or at least the subset that express CSPG4, as the potential sources of the pathological hallmark of ACM.

The new findings, in conjunction with the previous data, suggest a potential multicellular origin of the ACM phenotypes. Accordingly, expression of the mutant proteins, or the deficiency thereof, in cardiac myocyte leads mainly to cardiac dysfunction, whereas the effect in cardiac fibroadipocyte progenitors is excess fibroadipocytes and that in CSPG4 cells cardiac arrhythmias. The multicellular basis of ACM is consistent with the molecular genetics of ACM because the causal mutations are germ line mutations affecting cell lineages that express the desmosome proteins. Accordingly, each cardiac cell type, expressing the mutant desmosome protein, mainly contributes to a specific phenotype; cardiac myocytes to cardiac dysfunction, CCS to early cardiac arrhythmias, and fibroadipocyte progenitors to excess fibroadipocytes.

Several lines of data support fidelity of the Cspg4-Cre mediated deletion of Dsp gene and intactness of expression of DSP in cardiac myocytes. First, by design, Dsp was deleted at postnatal day 21 to avoid potential deletion of Dsp in other cell types in which the Cspg4 locus might be transcriptionally active during the development. In support of the experimental approach, the Cspg4 locus, which drives expression of the Cre recombinase, is not transcriptionally active in adult cardiac myocytes—a prerequisite for the deletion of the Dsp gene. Accordingly, CSPG4 was not expressed in cardiac myocytes, and Dsp transcript levels were unchanged in the isolated adult cardiac myocytes. Likewise, DSP protein was expressed and localized to the IDs in the Cspg4-Cre/Esr1*;DspF/F mice. In addition, the DsRed.T1 reporter protein—a surrogate for the transcriptional activity of the Cspg4 locus in the Cspg4;DsRed.T1 reporter mice—was not detected in cardiac myocytes. The DsRed.T1 protein, however, seems to be insoluble and often difficult to detect. Therefore, it might not be the most reliable marker. Therefore, the data were complemented with immunofluorescence staining of isolated cardiac myocytes using custom-made anti-CSPG4 antibodies, which did not show expression of CSPG4 in cardiac myocytes. Finally, the Cspg4-Cre/Esr1*;DspF/F mice did not show evidence of cardiac dysfunction, which would be expected on deletion of DSP.
in cardiac myocytes, as reported previously.19 Consequently, the data indicate that cardiac arrhythmias and conduction defects in the Cspg4-Cre/Esr1*;DspF/F mice are independent of cardiac myocytes and cardiac dysfunction but rather reflective of deletion of the Dsp gene in nonmyocyte cells in the heart, specifically the CCS.

CSPG4 is also expressed in brain tissue and is a marker for neuralgic type II cells.47 However, neuralgic cells are not known to express desmosome proteins. Likewise, Dsp transcript was not detected by quantitative polymerase chain reaction and its protein by Western Blot in the brain tissue (data not shown). Other epithelial cells, such as the gastrointestinal epithelia cells, and vascular pericytes are expected to express CSPG4 and perhaps even the desmosome proteins. In addition, immunofluorescence staining of nonmyocyte cardiac cells showed heterogeneity of the CSPG4pos cells, a fraction of which also expressed the PDGFRB, a pericyte marker. Deletion of the Dsp gene in such cells and organs might cause concomitant phenotypes, which could confound the findings. However, concomitant phenotypes in organs other than the heart were not discernible at the clinical level, but such organs are not examined in great detail, based on the assumption that unanticipated phenotypes in noncardiac tissues would not directly relate to cardiac arrhythmias and conduction defects in the Cspg4-Cre/Esr1*;DspF/F mice.

The underpinning mechanism(s) of cardiac arrhythmias in ACM, in general, and early arrhythmias occurring in the presence of a normal left ventricular function, in particular, are not well understood. Desmosomes along with adherens junction are the main constituents of IDs responsible for cell–cell attachment structures and mechanotransduction. In the human heart, they assemble into a single structure, localized to the polar regions of cardiac myocytes, referred to as area composite.48 However, ID proteins are not exclusive to cardiac myocytes but are also expressed in the CCS, including His bundles and Purkinje fibers, which is in keeping with shared embryonic origin of CCS and cardiac myocytes.24 In contrast to cardiac myocytes, however, the ID proteins are not confined to the polar regions but are rather scattered in cell membrane throughout and involved in lateral cell–cell contacts.24 In addition to desmosome proteins, the ID protein constituents also include the ion channels, including SCN5A, which is responsible for the Brugada syndrome, long QT3, and familial atrioventricular block.49,50 Moreover, conductive proteins, such as GJA5 and Ankyrin G, also localize to the IDs.52,53 In accord with these data, CSPG4pos cells express several proteins, which are constituents of ion channels in the heart and are involved in cardiac arrhythmias, including SCN5A, KCNQ1, KCNH2, and KCNE1.54 Deficiency of DSP and other desmosome proteins could affect assembly and localization of proteins involved in cardiac conduction affecting the sodium current and conduction velocity and, therefore, predisposing to cardiac conduction defects and arrhythmias.55,56 In support of complex interactions between ID protein constituents, mutations in PKP2 have been shown to affect the sodium current and induce a phenotype resembling the Brugada syndrome, likely through interactions with SCN5A.44 Conversely, mutations in SCN5A have been associated with the arrhythmogenic right ventricular cardiomyopathy-like phenotype.57 Moreover, deficiency of desmosome proteins, including DSP, is associated with a reduced expression level of GJA5.58 Thus, the existing data point to intricate and complex interactions between the conduction system and the desmosome proteins in maintaining normal ion current and conduction in the heart.

In conclusion, the data suggest that deletion of Dsp gene in a subset of cardiac nonmyocyte cells and skin keratinocytes, marked by the expression of CSPG4, leads to cardiac arrhythmias, conduction defects, and premature death occurring in the absence of cardiac dysfunction and fibroadiposis, as in the early stages of human ACM. Likewise, it leads to severe palmar keratosis and alopecia, as observed in the cardiocutaneous syndromes in humans. The data also exclude CSPG4pos cells, which also include a subset of pericytes and neuralgic cells as a cell source of excess fibroadipocytes in ACM. Collectively, the findings indicate multicellular origin of cardiac phenotypes in ACM and provide insights into the pathogenesis of cardiocutaneous syndromes.

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Disclosures

None.

References


Distinct Cellular Basis for Early Cardiac Arrhythmias, the Cardinal Manifestation of Arrhythmogenic Cardiomyopathy, and the Skin Phenotype of Cardiocutaneous Syndromes


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A Distinct Cellular Basis for Early Cardiac Arrhythmias, the Cardinal Manifestation of Arrhythmogenic Cardiomyopathy, and the Skin Phenotype of Cardiocutaneous Syndromes


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Expanded Material and Methods
Studies in the animal models conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee. Human tissue use was approved by the Institutional Review Board.

**Generation of custom-made rabbit anti CSPG4 antibodies:** Three antibodies against the mouse CSPG4 protein (UniProt ID#: Q8VHY) were generated by immunization of rabbits with the antigen peptides, KELKLQTPADTVLSDSAPH-Cys, SSGSLDPYLGISRPLRGC, and RLSDGESFSQLQAGRVTYRAT-Cys.

**Detection of expression of CSPG4 in the ventricular myocardium:** To detect expression of CSPG4 in the heart, fresh frozen thin myocardial sections were co-immunostained with a custom-made rabbit anti CSPG4 antibody and an antibody against either α-actinin (ACTN1) to mark the myocytes, connexin 40 (GJA5 or CX40) to mark the CCS, and contactin 2 (CNTN2), also a marker for the CCS. Details of antibodies are provided in Online Table I.

**Isolation and culture of myocyte-depleted cells from the human hearts:** Cardiac myocyte depleted cell fraction was obtained by collagenase type 2 digestion of ~100 mg of fresh tissue. The tissue samples were minced and incubated in a 0.1% collagenase 2 solution (Worthington Biochemical Corp; Lakewood, NJ08701; cat# LS004176) for 45 min at 37 °C under gentle agitation. The collagenase activity was stopped by adding 10 mL of α Modification media (αMEM, Hyclone; cat# SH30265.01) supplemented with 10% stem cell certified Fetal Bovine Serum (ES-FBS, Hyclone; cat# SH30070.03-E) and 1% Antibiotic-Antimycotic solution (Gibco; cat # 15240). The solution was filtered through a 40 μm cell strainer (BD Bioscience cat# 352340) and centrifuged for 5 min at 300g. The cell pellet was washed one time with PBS and re-suspended in a complete medium, composed by α MEM supplemented with 20% ES-FBS, and 1% Antibiotic-Antimycotic solution, plated in 60-mm plates coated with 0.1% gelatin and placed in a 5% CO2 humidified incubator at 37 °C.

**Isolation of mouse cardiac CSPG4pos cells:** To isolate CSPG4pos cells, myocyte-depleted cardiac cells were subjected to flow cytometry and FACS using a custom-made rabbit anti CSPG4 antibody, as
described before with modifications. In brief, 1-3 months old mice were anesthetized by intraperitoneal (IP) injection of pentobarbital (62 mg/Kg) and anti-coagulated upon IP injection of 200 U of heparin. The heart was explanted and washed with cold sterile PBS. The ascending aorta was cannulated with a 22G blunt needle under a magnifying dissection microscope (Leica, S6D). The cannula was positioned above the aortic valve cusps and connected to a retrograde perfusion system. To remove excess blood, the heart was perfused with PBS at a constant rate of 4 mL/min at 42 °C and then the perfusate was switched to 275U/mL type 2 collagenase in α-MEM medium (Worthington, Lakewood, NJ). The digestion with collagenase was continued until the heart became pale and spongy. The heart was then disconnected from the cannula and minced into small pieces using fine scissors in a 60-mm dish containing 2 ml of the digestion buffer (collagenase). The mixture was pipetted up and down gently several times with a sterile plastic transfer pipet (2 mm opening). The digestion was stopped by adding 8 ml of 10% calf serum in the α-MEM medium. The cell suspension was filtered through a 100 µm nylon mesh, transferred to a 15-ml polypropylene conical tube, and centrifuged at 300 g for 5 min at 4 °C. The supernatant was removed, the cell pellet was washed twice in a MACS buffer (Miltenyi Biotec), and resuspended in 500 mL of MACS buffer. Cell were then incubated with the primary antibody for 1 hr at 4 °C in the dark, followed by two washes in the MACS buffer and incubation with the secondary antibody for 1 h. Cells suspensions were washed in the MACS buffer, filtered through a 35 µm nylon mesh and analyzed by FACS.

FACS isolated mouse CSPG4pos cells were plated onto 0.1% gelatin coated plates in a growth medium [α MEM supplemented with 10% ES-FBS, 10 ng/mL mouse basic Fibroblast Growth Factor (bFGF, R&D, Minneapolis, MN; cat #3139-FB), 1000 U/mL of mouse Leukemia Inhibitory Factor (mLIF, Millipore; cat # ESG1106) and 1% Antibiotic-Antimycotic solution] and incubated at 37 °C in a 5% CO2 humidified incubator overnight.

To detect and quantify expression of the genes of interest in cardiac CSPG4pos cells, these cells were collected directly into the TRIzol LS Reagent (Invitrogen; cat # 10296010 and 10296028) during FACS and used for RNA extraction. In brief, 20µg of glycogen was added to each TRIzol sample and RNA
was first extracted with a phenol/chloroform/isopropanol mixture, precipitated with 100% ethanol and used in the qPCR reactions.

**Detection of expression of desmosome proteins in isolated cardiac CSPG4pos cells:** To determine whether cardiac CSPG4pos cells expressed desmosome proteins, isolated cardiac CSPG4pos were stained with antibodies against desmosome proteins DSP, PKP2, and JUP (Online Table I).

**Isolation of mouse adult cardiac myocytes:** To verify specificity of Dsp deletion in CSPG4pos cells but not in cardiac myocytes, the latter cells were isolated, and co-stained with antibodies against CSPG4, ACTN2, and DSP. To isolate cardiac myocytes, the heart was harvested, as described above, and placed in a perfusion buffer [120 mM NaCl, 15 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4, 7H2O, 30 mM Taurine, 4.6 mM NaHCO3, 10 mM HEPES, 10 mM 2,3-Butanedione monoxime (BDM), and 5.5 mM Glucose; pH 7.0], as published. The ascending aorta was cannulated and the heart was perfused in a retrograde perfusion system, as described above, with the perfusion buffer at a constant rate of 4 mL/min at 42 °C. Upon washing out the blood, the perfusate was switched to a collagenase 2 digestion buffer (Worthington, Lakewood, NJ). Approximately 2-3 min after perfusion with the collagenase, 7.5 ul of 100 mM CaCl2 was added to the buffer and the digestion was continued until the heart became pale and felt spongy upon gentle pinching. The heart was removed from the cannula, minced into small pieces, and cells were dissociated, as described above. The digestion was stopped by adding stop buffer (10% calf serum and 12.5 µM CaCl2 in the perfusion buffer) and the cell suspension was filtered through a 100 µm nylon mesh into a polypropylene conical tube. An aliquot of 100 µl of 200 mM ATP was added to the tube and the myocytes were allowed to sediment by gravity for a few minutes at 4 °C, which was then followed by centrifugation at 20g for 3 min. Following removal of the supernatant, cells were subjected to a three-step calcium reintroduction by resuspending the cells in aliquots of 10 mL of stop buffer containing 2 mM ATP and increasing concentration of CaCl2 to 100 µM, 400 µM, and 900 µM for 3 minutes each. After each calcium reintroduction step, the suspension was centrifuged at 20g for 3 min and the pellet was resuspended in the buffer containing the corresponding incremental concentration of CaCl2. The final pellet was resuspended in a cardiac myocyte plating media (MEM media, 1% penicillin-streptomycin,
10% Calf serum, 10 mM BDM, and 2 mM ATP). Cardiac myocytes were plated on laminin coated cover slips for immunofluorescence or 6 well plates for RNA and protein extraction, and incubated immediately in a 2% CO2 incubator at 37°C. After 2 hours of incubation to allow the cells to attach to the substrate, the cells were collected for RNA extraction or fixed in 4% paraformaldehyde for immunostaining.

**Cspg4-DsRed.T1 reporter mice:** Cspg4-DsRed.T1 is a BAC transgenic reporter mouse line that expresses a red fluorescence protein variant under the transcriptional regulation of the Cspg4 locus (Stock No: 008241, Jackson Laboratory). In this mouse model, DsRed.T1 protein serves as a surrogate marker representing expression pattern of the CSPG4 protein.

**Cspg4-Cre/Esr1*:DspWF and Cspg4-Cre/Esr1*:DspWF mice:** To determine biological and functional significance of expression of DSP in CSPG4pos cells, Dsp gene was conditionally deleted using the inducible cre deleter BAC transgenic mice Cspg4-Cre/Esr1* (Stock No: 008538; Jackson Laboratory). In brief, Cspg4-Cre/Esr1* mice were crossed to DspF/F to generate Cspg4-Cre/Esr1*:DspWF mice and subsequently Cspg4-Cre/Esr1*:DspWF mice. Twenty-one day old mice carrying one or two floxed Dsp and the cre recombinase alleles were treated with I.P. injection of tamoxifen at 100 µg/g/d for 5 days. Activation of the cre recombinase by tamoxifen is expected to delete the floxed exon 2 in the Dsp gene specifically in cells that are transcriptionally regulated by the Cspg4 locus. Mice were genotyped by PCR of tail DNA. Oligonucleotide primers used in PCR reactions are listed in Online Table I. Age and sex-matched wild type (WT) were used as controls in all experiments.

**Quantitative polymerase chain reaction (qPCR):** qPCR was performed as published and the relative normalized values (2^-ΔΔ CT method, shown as relative to the WT mice) were used to compared the transcript levels. Oligonucleotide primers and probes are listed in online Table I.

**Immunoblotting:** Immunoblotting was performed as published. In brief, total proteins were extracted by homogenizing heart tissues or the collected cells in a RIPA lysis buffer [1X formulation: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% Sodium deoxycholate and 0.1% Sodium Dodecyl Sulphate (SDS); Pierce Biotechnology, Rockford, 8 IL; cat #89901] in the presence of protease inhibitors (complete protease inhibitor cocktail; Roche Diagnostics, GmbH, Mannheim, Germany;
Protein concentration was measured using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories; cat # 5000111). Aliquots of 30-50 µg of total protein were denatured in a Laemmli loading buffer at 95 °C for 5 min, loaded onto SDS-polyacrylamide gels (PAGE), subjected to electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 hr in 5% non-fat milk and incubated with the primary antibody of interest overnight at 4 °C. After 3 washes in TBS, the membranes were incubated with the corresponding horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour and the signal was detected by chemiluminescence. The membranes were stripped by incubation in the Restore PLUS Western Blot stripping Buffer (Thermo Scientific, Hudson, New Hampshire, cat #46430) for 10 min at room temperature, washed in TBS for 3 times, and reprobed with another antibody of interest. The primary and secondary antibodies and their titers are listed in Online Table I.

**Immunofluorescence:** Immunofluorescence staining to detect expression and localization of the proteins of interest was performed as published. In brief, the whole heart was embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc. Torrance, CA 90501; cat#4583) and frozen in isopentane (2-Methyl Butane, Sigma-Aldrich St Louis, MO, cat #320404). Thin myocardial sections (5 µm) were cut from the OCT blocks and fixed in 4% formaldehyde for 2.5 min. FACS isolated cells were cultured overnight and fixed in 4% formaldehyde for 10 min. The samples were washed 3 times in PBS and blocked in a 5% donkey serum diluted in PBS for 1 h at room temperature. After blocking, samples were incubated with antibodies against the target protein (list of antibodies is available in Online Table I) in 1% BSA in PBS overnight at 4 °C. After 3 washes, samples were incubated with fluorescence-labeled secondary antibodies in 1% BSA for 1 hour at room temperature. Samples were washed and stained with a 0.1 mg/mL of 4', 6 Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich St Louis, MO; cat# D8417) or DRAQ5 (Abcam, ab108410) for 2 min at room temperature, the latter two to identify the nuclei. After 3 washes in PBS wash, samples were mounted in fluorescent mounting medium (Dako North America Inc.6392 Via Real, Carpinteria CA 93013, cat# S3023), and examined under fluorescence microscopy (Zeiss, Axioplan Fluorescence Microscope).
**Histology and immunohistochemistry:** Whole heart histology was examined by H&E, Masson Trichrome, and Oil Red O staining of thin myocardial sections. For Oil Red O staining, thin myocardial sections prepared from OCT embedded hearts were washed one time with PBS and then fixed in 10% formalin for 15 min at room temperature. After 10 min washing under running water, samples were stained in a modified Mayer’s hematoxylin solution (Richard-Allan Scientific, Kalamazoo, MI; cat # 72804) for 2 min. Samples were then washed for 10 min in water, placed in 100% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264) for 2 min and then placed in Oil Red O 0.5% solution in propylene glycol (Poly Scientific, Bay Shore, NY; cat #s1848) overnight at room temperature. The Oil Red O solution was removed and the samples were dipped 2 times in 85% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264A). After 20 min washing the slides were mounted with aqueous mounting medium.

For H&E staining, the heart was formalin fixed by perfusion and incubated overnight in formalin at 4 °C. Tissue was dehydrated by ethanol xylene gradient and embedded in paraffin. Paraffin embedded hearts were sectioned into 5 µm horizontal or coronal sections, and dried overnight at 37 °C. Sections were deparaffinized by incubating twice in Xylene for 5 min each, 2 min each in 100%, 95%, and 70% ethanol for 2 min each, followed by washing with water for 1 min. Tissue was stained with Hematoxylin for 30 min at room temperature followed by 10 dips in water. Tissue was incubated in 70% ethanol for 1 min and immediately stained with Eosin Y solution for 30 seconds. Tissue was then dehydrated by ethanol xylene gradient. Slides were covered with coverslip using Cytoseal™ XYL (Thermo Scientific, 8312-4).

Masson Trichrome staining was performed per the manufacturer’s protocol (Sigma, HT15-1KT) using paraffin embedded heart sections. First, whole heart sections were deparaffinized as described above. Slides were incubated for 15 min in Bouin’s solution preheated to 56°C (Sigma, HT10-1). Slides were washed in running tap water for 10 min, followed by staining in Working Weigert’s Iron Hematoxylin Solution for 5 min. Slides were rinsed in running distilled water for 5 min and stained with Biebrich Scarlet-Acid Fuchsin (Sigma, HT15-1). Slides were dipped one time in distilled water and placed in Phosphotungstic/Phosphomolybdic Acid solution for 5 min, followed by analine blue solution (Sigma...
HT15-4) for 5 min and 1% acetic acid for 2 min. Finally, slides were rinsed in water, dehydrated, and mounted, as described above.

Five µm-sections were prepared from Paraffin wax-embedded back skin tissue and dewaxed using standard methods. Dewaxed sections were either stained with Hematoxylin and Eosin (H&E) or further processed for antibody staining. Antigen retrieval was performed using pepsin (1 mg/mL, Sigma-Aldrich) at pH 2, citrate buffer (10 mM, trisodium citrate dihydrate; 2.94 g/L) at pH 6 or Tris-EDTA (10 mM Tris base and 1 mM EDTA) at pH 9. Section were stained with primary antibody (see Table I) overnight at 4 °C then incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies. To reveal the signal, sections were incubated with 3,3’-diaminobenzidine (DAB). Sections were imaged using a Leica DM4000 optical microscope.

Detection and quantification of apoptosis: Apoptosis was analyzed in the heart by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, using the fragEL™ DNA Fragmentation Detection Kit (Millipore; cat # QIA-39). In brief, formalin fixed paraffin embedded thin myocardial sections were stained for fluorescein-labeled deoxynucleotides and counterstained with DAPI. The number of TUNEL positive cells was counted in 11 high magnification fields per section, 10 thin sections per heart, and in 5 mice per group (a total of ~20,000 cells per heart) and presented as percentage of the total cells, the latter identified by DAPI staining.

Echocardiography: 2D, M mode, and Doppler echocardiography was performed as published using a HP 5500 Sonos echocardiography unit equipped with a 15-MHz linear transducer. In brief, mice were anesthetized by IP injection of sodium pentobarbital (60 mg/Kg body weight) and chest hair were removed with hair removal cream. Wall thicknesses and left ventricular end diastolic and end systolic diameters were measured from M-mode images from the parasternal two-dimensional short-axis view at the tip of the mitral leaflet, using the leading-edge method. Left ventricular fractional shortening and mass were calculated as previously
described. 1, 5-7 All echocardiographic data represent the mean of 3 measurements on different cardiac cycles.

**Electrocardiography:** Mice were anesthetized as described above and kept on a heating plate set at 37°. 29-gauge needle electrodes were inserted subcutaneously into the forelimbs and ECG and heart rate were recorded and analyzed using the Animal Bio Amp, PowerLab, and the LabChart7 software (all from AD Instrument).

**Electrophysiology (EP):** Mice were anesthetized with isoflurane (average 1.75% in 100% oxygen), sufficient to maintain an adequate level of anesthesia, and 100% oxygen. Temperature was monitored continuously and maintained between 36-37 °C degrees. Surface electrocardiograms were monitored throughout. Intracardiac bipolar atrial and ventricular electrograms were obtained using a 1.1F octapolar catheter (EPR-800; Millar Instruments), as described. 8, 9 EP pacing protocols, including single, double, and burst ventricular pacing protocols were used for ventricular tachycardia (VT) induction at baseline and after injection of isoproterenol (3 mg/kg i.p.). Two drive trains were used at the baseline (90 msec and 70 msec) and after isoproterenol injection (80 msec and 70 msec). Ventricular doubles at baseline were performed at cycle lengths of 90/50/40 msec and 80/50/40 msec and on isoproterenol at 80/50/40 msec and 70/50/40 msec. Ventricular burst pacing was performed at a drive train of 60 msec. The atrial stimulation protocol to evaluate inducibility of atrial fibrillation was performed with incremental atrial pacing, as described. 9 All pacing protocols were performed a maximum of three times. Non-sustained and sustained VT were defined as reproducible (at least twice) ectopic ventricular rhythms lasting 4 to 9 beats and ≥10 beats, respectively. Atrial fibrillation was defined as reproducible rapid and fragmented atrial electrograms with irregular ventricular response for ≥1 second.

To evaluate heart rate variability, heart rates were measured for an average of 3 beats every 30 seconds during the electrophysiology study. Heart rates were not measured if within 10 seconds of a pacing drive train. The average change in heart rate during the EP study were compared between the WT and Cspg4-Cre/Esr1*:DspFF mice, who completed the EP studies.
Statistical analysis: Data that followed a Gaussian distribution pattern were presented as mean ± SD and were compared between two groups by t test and among multiple groups by ANOVA followed by post-hoc pairwise comparisons. Otherwise, data were presented as the median values and compared by Kruskall-Wallis test, as were the categorical data.
REFERENCES

### A. Information on antibodies used in the experiments

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<td>DPVM110HRP</td>
<td>DAB</td>
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<td>Anti-Mouse HRP</td>
<td>Immunologic</td>
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<td>DAB</td>
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B. TaqMan gene expression probes (from life technologies)

*Cspg4* Mm00507257_m1

*Dsp* Mm01351876_m1

*Gapdh* Mm99999915_g1

C. SYBR green oligonucleotide primers:

- **Gapdh:**
  
  Forward: AAC TTT GGC ATT GTG GAA GG
  Reverse: GGA TGC AGG GAT GAT GTT CT

- **Actc1:**
  
  Forward: TGT CAC CAC TGC TGA ACG TG
  Reverse: CTG GCC GTC AGG AAG TTC ATA

- **Myh6:**
  
  Forward: CTG TTC CTC TCT CCG TCC AG
  Reverse: ATT CTG TCA CTC AAA CTC TGGT

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D. PCR oligonucleotide primers for mouse genotyping:

- Floxed *Dsp*:
  
  Forward: TAAGCTCCCCTCACTTCTCCAG  
  Reverse: TTCTCTTTGTCTGTGTCATGT

- *Cspg4-Cre*:
  
  Forward: GCG GTC TGG CAG TAA AAA CTA TC  
  Reverse: GTG AAA CAG CAT TGC TGT CAC TT

  Internal Positive Control Forward: CTA GGC CAC AGA ATT GAA AGA TCT  
  Internal Positive Control Reverse: GTA GGT GGA AAT TCT AGC ATC ATC C

- *Ds-Red.T.1*:
  
  Forward: TTC CTT CGC CTT ACA AGT CC  
  Reverse: GAG CCG TAC TGG AAC TGG

  Internal Positive Control Forward: CTA GGC CAC AGA ATT GAA AGA TCT  
  Internal Positive Control Reverse: GTA GGT GGA AAT TCT AGC ATC ATC C
TABLE II
Electrophysiological Measurements in 2-3 Months Old Wild Type and Cspg4Cre/Esr1*:DspF/F Mice

| Abbreviations: | AVERP – atrioventricular effective refractory period when pacing at a drive train cycle length of 88msec; AVW – atrioventricular wenckebach cycle length; VAW – ventriculo-atrial wenckebach cycle length; VBCL – ventricular block cycle length; VERP – ventricular effective refractory period when pacing at a drive train of 88msec (baseline) and 78msec (isoproterenol). *Two mice did not complete the isoproterenol pacing protocols due to bradycardic death. |

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<td>Cspg4-Cre/Esr1*:DspF/F n = 10</td>
<td>p</td>
<td>WT n = 7</td>
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<tr>
<td>Heart Rate (bpm)</td>
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<tr>
<td>618 ± 49</td>
<td>545 ± 101</td>
<td>0.10</td>
<td>708 ± 40</td>
<td>645 ± 42</td>
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<tr>
<td>PR interval (ms)</td>
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<tr>
<td>30.0 ± 1.1</td>
<td>28.1 ± 2.9</td>
<td>0.20</td>
<td>26.9 ± 2.9</td>
<td>27.4 ± 2.4</td>
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<td>QRS duration (ms)</td>
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<td>9.4 ± 1.3</td>
<td>9.8 ± 1.0</td>
<td>0.52</td>
<td>9.9 ± 1.4</td>
<td>10.1 ± 0.9</td>
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<td>QTc (ms)</td>
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<td>42.3 ± 3.4</td>
<td>46.1 ± 5.1</td>
<td>0.10</td>
<td>44.7 ± 3.1</td>
<td>48.0 ± 4.8</td>
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<td>AVERP at 88msec</td>
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<tr>
<td>38.0 ± 10.9</td>
<td>46.9 ± 8.4</td>
<td>0.08</td>
<td>38.3 ± 6.9</td>
<td>38.7 ± 6.1</td>
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<td>AVW (ms)</td>
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<tr>
<td>59.1 ± 8.5</td>
<td>66.0 ± 8.0</td>
<td>0.11</td>
<td>56.7 ± 6.1</td>
<td>62.0 ± 6.0</td>
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<td>VAW (ms)</td>
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<td>73.3 ± 11.5</td>
<td>88.6 ± 13.7</td>
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<td>69.8 ± 8.3</td>
<td>77.3 ± 10.0</td>
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<td>VBCL (ms)</td>
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<td>39.4 ± 0.5</td>
<td>44.5 ± 7.2</td>
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<td>40.1 ± 1.8</td>
<td>43.1 ± 5.6</td>
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<td>VERP at 88/78 ms</td>
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<td>20.7 ± 6.6</td>
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<td>0.08</td>
<td>26.1 ± 11.6</td>
<td>25.3 ± 10.9</td>
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### TABLE III

**Echocardiographic Data in 3 Months Old Wild Type and Cspg4Cre/Esr1*:Dsp^{F/F} Mice**

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<tr>
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<th>Cspg4Cre/Esr1*:Dsp^{F/F}</th>
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<tr>
<td>N</td>
<td>14</td>
<td>14</td>
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<tr>
<td>M/F</td>
<td>8/6</td>
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<tr>
<td>Age (months)</td>
<td>2.95 ± 1.17</td>
<td>2.96 ± 1.1</td>
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<tr>
<td>Body weight (g)</td>
<td>20.4 ± 4.0</td>
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<tr>
<td>HR (bpm)</td>
<td>562 ± 60</td>
<td>532 ± 108</td>
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<td>IVST (mm)</td>
<td>0.83 ± 0.12</td>
<td>0.62 ± 0.07</td>
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<td>PWT (mm)</td>
<td>0.78 ± 0.18</td>
<td>0.60 ± 0.08</td>
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<td>LVEDD (mm)</td>
<td>2.83 ± 0.39</td>
<td>2.87 ± 0.3</td>
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<td>LVEDDi (mm/g)</td>
<td>0.11 ± 0.02</td>
<td>0.19 ± 0.028</td>
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<td>LVESD (mm)</td>
<td>0.90 ± 0.22</td>
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<td>FS (%)</td>
<td>68.14 ± 6.20</td>
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<td>LVM (mg)</td>
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<td>LVMi (mg/g)</td>
<td>2.57 ± 0.52</td>
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**Abbreviations:** HR, heart rate; bpm, beats per minutes; IVST, interventricular septal thickness; PWT, posterior wall thickness; LVEDD, left ventricular end diastolic diameter; LVEDDi, LVEDD divided by the body weight; LVESD, left ventricular end systolic diameter; LVESDi, LVESD divided for the body weight; FS, fractional shortening; LVM, left ventricular mass; LVMi, LVM divided by the body weight.
TABLE IV

Echocardiographic Data in 8 Months Old Wild Type and Cspg4Cre/Esr1*:\textit{Dsp}\textsubscript{WT} Mice

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<td>12</td>
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<td>M/F</td>
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<td>Age (months)</td>
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<td>Body weight (g)</td>
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<td>HR (bpm)</td>
<td>567.4 ± 68.35</td>
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<td>LVEDD (mm)</td>
<td>3.0 ± 0.22</td>
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<td>LVEDDi (mm/g)</td>
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<td>LVESD (mm)</td>
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<td>FS (%)</td>
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<td>LVM (mg)</td>
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<td>LVMi (mg/g)</td>
<td>2.6 ± 0.63</td>
<td>2.68 ±0.40</td>
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Abbreviations are as in Table III
Online Figure I: FACS analysis of cardiac CSPG4⁺ cells
A. Distribution plot of non-myocytes cardiac cells sorted against a custom-made anti CSPG4 antibody
B. Distribution plot of non-myocytes cardiac cells sorted against an established CNTN2 antibody
C. FACS plot with secondary antibodies only
D. FACS analysis of non-myocyte cardiac cells against CSPG4 and CNTN2 antibodies, showing the majority of cells
Online Figure II. Expression of CSPG4 in other cardiac cells and cardiac markers in CSPG4\textsuperscript{pos} cells:

A. Expression of CSPG4 in a subset of cardiac pericytes, identified by PDGRFB, the latter a marker for pericytes, in isolated non-myocyte cardiac cell fraction. Low (upper) and high (lower) magnification panels are shown. Approximately, 8.1±10.6% and 2.6±3.3% of cells stained positive for CSPG4 and PDGFRB, respectively. Only 9.8% of cells staining positive for CSPG4 also stained positive for PDGFRB.

B. Absence of expression of CSPG4 in cardiac endothelial cells, identified by PECAM1, a known marker for the endothelial cells, in Cspg4:Ds-Red.T.1 reporter mice. Ds-Red.T1 serves as a reporter for CSPG4\textsuperscript{pos} cells.
**Online Figure III.** This skin sections prepared from *Csg4:Ds-RedT.1* mouse showing expression of Ds-RedT.1 reporter protein, as a surrogate for the expression of CSPG4 protein, in epidermal skin and around hair follicles, the latter shown by an arrow. The upper and lower panels show low and high magnification fields, respectively. (hair shafts are autoflourescent)
Online Figure IV.

A. Co-staining of isolated cardiac myocytes from a wild type mouse heart showing absence of expression of CSPG4 in cardiac myocytes.

B. Extremely low levels of myosin heavy chain 6 (Myh6) and cardiac α actin (Actc1) transcripts, markers of cardiac myocytes in mouse cardiac CSPG4pos cells. Ct values of the amplifications plots are depicted relative to the corresponding Gapdh Ct values.
Online Figure V.
Design of the constructs for the specific deletion of Dsp gene under transcriptional regulation of the Cspg4 locus.
**Online Figure VI.** Growth retardation in the Csgp4-Cre/Esr1*:DspF/F mice. Body weight measured on a weekly basis was markedly less in the Csgp4-Cre/Esr1*:DspF/F as compared to WT or Csgp4-Cre/Esr1*:DspW/F mice.
Online Figure VII. Fibro-adipocytes in the heart

Panel A shows Masson trichrome stained thin myocardial section in the wild type (WT), Cspg4-Cre/Esr1*:*Dsp W/F and Cspg4-Cre/Esr1*:*Dsp F/F showing no discernible fibrosis.

Panel B shows Oil red O stained thin myocardial section, and Panel C shows quantiative data showing no increased in the number of Oil red O positive cells in the Dsp-deficient mouse hearts.
Online Figure VIII. Immunostaining of skin sections from wild type (WT) and desmoplakin-deficient (Cspg4-Cre/Esr1*::DspF/F) mice for selected markers of sebocytes, apoptosis and inflammation, showing no differences in the expression levels of selected markers of apoptosis or inflammation between the two groups. Increased expression of FAS, a marker for sebocytes, is consistent with the hyperproliferative skin phenotype.
Online Figure IX. Co-immunostaining for keratin 14 (KRT14) and junction protein plakoglobin (JUP) in wild type (top two panels) and Cspg4-Cre/Esr1*;DspF/F (bottom two panels) mice.

Low (top) and high (lower) magnification panels in each genotype group representing individually stained thin sections for JUP, KRT and DAPI are shown along with the merged panels. JUP is co-localized with KRT14 in epidermal keratinocytes in the wild type as well as DSP-deficient mice. JUP and KRT14 show increased staining reflective of increased thickness of the epidermis in the Cspg4-Cre/Esr1*;DspF/F mice. Scale bar: 50 μm.