Desmoglein 2–Dependent Arrhythmogenic Cardiomyopathy Is Caused by a Loss of Adhesive Function

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Background—The desmosomal cadherin desmoglein 2 (Dsg2) localizes to the intercalated disc coupling adjacent cardiomyocytes. Desmoglein 2 gene (DSG2) mutations cause arrhythmogenic cardiomyopathy (AC) in human and transgenic mice. AC is characterized by arrhythmia, cardiodilation, cardiomyocyte necrosis with replacement fibrosis, interstitial fibrosis, and intercalated disc dissociation. The genetic DSG2 constellations encountered are compatible with loss of adhesion and altered signaling. To further elucidate pathomechanisms, we examined whether heart-specific Dsg2 depletion triggers cardiomyopathy.

Methods and Results—Because DSG2 knockouts die during early embryogenesis, mice were prepared with cardiomyocyte-specific DSG2 ablation. Healthy transgenic animals were born with a functional heart presenting intercalated discs with incorporated desmosomal proteins. Dsg2 protein expression was reduced below 3% in the heart. All animals developed AC during postnatal growth with pronounced chamber dilation, calcifying cardiomyocyte necrosis, aseptic inflammation, interstitial and focal replacement fibrosis, and conduction defects with altered connexin 43 distribution. Electron microscopy revealed absence of desmosome-like structures and regional loss of intercalated disc adhesion. Mice carrying 2 mutant DSG2 alleles coding for Dsg2 lacking part of the adhesive EC1-EC2 domains present an indistinguishable phenotype, which is similar to that observed in human AC patients.

Conclusions—The observations show that the presence of Dsg2 is not essential for late heart morphogenesis and for cardiac contractility to support postnatal life. On increasing mechanical demands, heart function is severely compromised as evidenced by the onset of cardiomyopathy with pronounced morphological alterations. We propose that loss of Dsg2 compromises adhesion, and that this is a major pathogenic mechanism in DSG2-related and probably other desmosome-related ACs. (Circ Cardiovasc Genet. 2015;8:553-563. DOI: 10.1161/CIRCGENETICS.114.000974.)

Key Words: arrhythmogenic right ventricular cardiomyopathy ■ calcification ■ desmosome ■ fibrosis ■ transgenic mice

Arrhythmogenic cardiomyopathy (AC), also referred to as arrhythmogenic right ventricular cardiomyopathy or arrhythmogenic right ventricular dysplasia, is a genetic heart disease. It affects primarily the right ventricle, but left ventricular involvement is also common. Ventricular tachyarrhythmia, chamber dilation, and fibrofatty replacement of cardiac tissue are key features of human AC. During its acute phase, the disease is a frequent cause of sudden cardiac death in young athletes and may lead to heart failure in the chronic phase.

Clinical Perspective on p 563

Mutations in desmosomal genes are the most common genetic alterations in AC, which has been referred to as a disease of the desmosome. Mutations in genes encoding phospholamban, desmin, transforming growth factor-β3, transmembrane protein 43, and the ryanodine receptor 2 have also been identified. Desmosome-like structures are part of the intercalated disc (ID), a composite adhesion site between adjacent cardiomyocytes. The ID provides mechanical, electrophysiological, and metabolic linkage between neighboring cells. Cardiac desmosomes are specialized multiprotein assemblies consisting of the calcium-dependent cell–cell adhesion transmembrane proteins desmoglein 2 (Dsg2) and desmocollin 2 (Dsc2) and attached linker molecules, that is, the armadillo repeat–containing plakophilin 2 (Pkp2) and plakoglobin (Pg), and the plakin domain–containing desmoplakin (Dsp), which in turn connects to cytoskeletal desmin intermediate filaments. Although all desmosomal proteins have been implicated in AC, the most commonly mutated genes are those encoding Pkp2 and Dsg2.

The observed autosomal dominant inheritance of desmoglein 2 gene (DSG2)–related AC cases suggests that reduction of the Dsg2 wild-type (WT) protein leads to a loss of cardiac function or that Dsg2 mutant (MT) protein exerts a dominant negative effect on the remaining Dsg2 WT

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protein. Two major types of nonexclusive pathomechanisms have been described to depend on Dsg2. As a cell–cell adhesion molecule, it provides adhesive force and thereby supports force transmission between contractile cardiomyocytes. Alternatively, Dsg2 regulates intracellular signaling. This function is expected to be linked to the cytoplasmic domain of Dsg2 and may involve associated signaling molecules, such as Pg and Pkp2/PKCα acting through downstream effectors implicating the Wnt or Hippo pathways.16,17

To examine mechanisms of DSG2-related cardiomyopathy, several murine models have been established. Complete constitutive deletion of Dsg2 was shown to be embryonic lethal18 as is the case for all other integral components of desmosomes.5 Heterozygous DSG2 knockouts (KO) are viable and do not develop overt AC under standard animal housing conditions.19 Subsequently, mice were generated overexpressing mutant Dsg2 N271S corresponding to the pathogenic human Dsg2 N266S mutation.11 The mutation is localized in the second extracellular calcium-binding pocket, presumably contributing to Dsg2’s adhesive function. These mice developed, in contrast to mice overexpressing the same amount of Dsg2 WT, symptoms of AC, including biventricular dilation, calcifying necrosis with replacement fibrosis, conduction defects, and arrhythmia, occasionally resulting in premature death.11 Later on, a knock-in mouse model was described producing mutant Dsg2 with a deletion in the extracellular domains EC1-EC2. Live-born homozygous DSG2MT carriers developed an AC-like phenotype during adolescence.19,20 The deleted part of Dsg2 includes the most amino-terminal calcium-binding site and is believed to be important for adhesion through homo- and heterophilic desmosomal cadherin interaction.21 Thus, peptides taken from this domain prevent Dsg2-dependent adhesion,22 and autoantibodies targeting the corresponding region in Dsg3 induce cell dissociation in pemphigus vulgaris.23

Taken together, neither overexpression nor ≈50% reduction of WT Dsg2 induces overt cardiomyopathy.11,18 But overexpression of mutant Dsg2 N271S in the presence of normal WT Dsg2 levels leads to dose-dependent cardiomyopathy.11,24 In contrast, heterozygous mice with only one DSG2WT and one DSG2MT allele seem to be healthy, although a slight upregulation in transcription of the stress-response genes ANF and BNF were detected at early disease stages.20 Furthermore, complete replacement of 2 DSG2WT by 2 DSG2MT alleles leads to an ≈75% reduction in Dsg2 protein expression and a pronounced AC-like phenotype.19,20 These observations may be explained either by loss of Dsg2-dependent adhesion or by altered cytoplasmic binding of the mutant Dsg2 to signaling molecules. To find out which mechanism is responsible for AC induction, we prepared cardiomyocyte-specific DSG2 KO mice and compared the phenotype to that observed in DSG2MT mice.

Materials and Methods

DNA Cloning and Generation of Knockout Mice
Standard methods were used for targeting construct preparation, transfection, selection, and characterization of embryonic stem cell clones, blastocyst injection, and mouse breeding (details in Data Supplement). The animal experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen under the reference number 8.87-50.10.37.09.114.

RNA Isolation, Reverse Transcription, and qRT-PCR
Total RNA was isolated from fresh tissue for preparation of cDNA and PCR amplification as specified in the Data Supplement.

Immunoblotting
Total protein extracts of fresh heart tissue were analyzed by immunoblotting as described in the Data Supplement.

Histological Stains and Immunohistology
Heart tissue was fixed in 4% formaldehyde/PBS for 12 h and subsequently embedded in paraffin. Sections were prepared and either Azan- or von Kossa-stained.

For immunofluorescence, sections were heated in citrate buffer (pH 6.0) in a pressure cooker for 3 minutes. Primary antibodies diluted in PBS/1% BSA were applied overnight at 4°C; polyclonal rabbit anti-Dsg2 (1:500; Dsg2 IC),20 guinea pig anti-desmoplakin (1:1000; DP-1, Progen), mouse anti-Pkp2 (1:12; 651101, Progen), goat anti-Pg (1:75; sc-30997, Santa Cruz), and rabbit anti-Cx43 (1:1000; C6219, Sigma). Three 5 minutes washings in modified Tris-buffered saline and Tween 20 (TBST;50 mmol/L, Tris- HCl [pH 7.5], 0.3 mol/L, NaCl, 0.05% Tween 20) were followed by secondary antibody incubation for 1 h at room temperature (goat anti-rabbit [A-11070], goat anti-mouse [A-11029], donkey anti-goat [A-21432] antibodies conjugated with Alexa488 and goat anti-guinea pig conjugated with Alexa555 [A-21435]; all from Invitrogen; diluted 1:500 in PBS with 1.5% BSA). Sections were washed 3× in modified TBST. Background reduction was achieved by 30 minutes incubation in 0.1% Sudan Black D/70% ethanol. Afterward slides were washed 3× in modified TBST and mounted in Mowiol 4–88 (Roth). Fluorescence micrographs were recorded with an Apotome (Zeiss).

Electron Microscopy
Before removal, the ventricular myocard was relaxed by retrograde perfusion with 4 to 5 mL relaxation buffer (30 mmol/L KCl, 300 mmol/L glucose). Heart samples were cut into cubes of ≈1 mm3 in fixative (3.7% formaldehyde, 1% glutaraldehyde, 11.6 g NaH2PO4·H2O, and 2.7 g NaOH per liter of ddH2O) and incubated for 2 h at room temperature. Incubations in 1% OsO4 for 1 h and in 0.5% uranylacetate/0.05 mol/L sodium maleate buffer (pH 5.2) for 2 h followed. Samples were dehydrated and embedded in araldite (48 h, 60°C) using acetone as intermediate. Ultrathin sections with a thickness of 65 to 75 nm were prepared, and contrast was enhanced by treatment with 3% uranylacetate for 4 minutes followed by 3 minutes incubation in 80 mmol/L lead citrate solution. Micrographs were recorded on an EM 10 (Zeiss) upgraded with a digital camera (Olympus) using iTEM software (Olympus).

Electrocardiography
Conscious mice were placed on an ECGenie unit (Mouse Specifics Inc.) and signals were recorded for 10 minutes. The best traces, each containing at least 10 cardiac cycles, were selected and analyzed using the ECGenie software.

Statistical Methods
All results are presented as mean±SD. The mean of data gathered from 1 animal was counted as 1 n. Data sets were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test. When data sets were not Gaussian distributed, nonparametric statistical tests were used. Statistical analyses comparing 2 groups with each other were performed with either 2-tailed Mann–Whitney test or 2-tailed t test using 95% confidence intervals. Statistical analyses comparing 3 groups were accomplished by either the Kruskal–Wallis test together with the Dunns post hoc test or the ANOVA analysis.
Results

Conditional Depletion of Desmoglein 2 in Cardiomyocytes

To prepare mice with a cardiomyocyte-specific DSG2 knock-out, we made use of a Myh6-driven Cre25 to delete exons 4 and 5 of a floxed DSG2 allele (Figure 1A). First, using a targeting construct, 6 recombinant AB2.2 embryonic stem cell clones were isolated. Correct recombination was detected in 2 clones by Southern blotting using 5′-external, internal and 3′-external probes (Figure 1A and 1B). They were injected into C57BL/6J morulae and blastocysts for transgenic line production. Transgenic animals were healthy, and the neomycin resistance gene was successfully removed by breeding with Flp−-deleter mice.26 For cardiomyocyte-specific DSG2 inactivation, mice with the floxed DSG2 alleles were mated with Myh6-Cre transgenic mice,23 which have been shown to initiate cardiac Cre-expression at embryonic day 7 (E7), resulting in efficient recombination of a reporter gene at E9 throughout the myocardium.27 PCR analyses of 4 animals confirmed correct recombination (Figure 1C). It was, however, difficult to deduce recombination efficiency because the cardiac tissue samples contain noncardiomyocytes, such as fibroblasts, endothelial cells, smooth muscle cells, and immune cells all of which are not targeted by the Cre recombinase and do not express Dsg2. RT-PCR was therefore performed to assess the efficiency of DSG2WT mRNA depletion. DSG2WT mRNA was reduced to <2% in DSG2cKO samples (Figure 1D). Instead, a shortened mRNA fragment was amplified. It encodes a nonfunctional aminoterminal polypeptide of only 80 amino acids.

Figure 1. Cardiomyocyte-specific inactivation of DSG2 by Myh6-Cre-mediated excision of floxed DSG2 exons 4–5 in transgenic mice. The scheme in (A) depicts part of the DSG2WT allele with exons 2–8 (E2 to E8) that was mutated by homologous recombination introducing loxP sites flanking exons 4 and 5 and an FRT-site bounded neomycin resistance cassette downstream of exon 5. The resulting allele DSG2 flox(E4-5-neo) was subsequently modified by Flpo-driven excision of the neomycin resistance cassette producing allele DSG2 flox(E4-5). Myh6-Cre-mediated recombination led to knockout allele DSG2cKO. Positions of EcoRV restriction sites (RV) and probes used for genotyping are demarcated. B, Autoradiographs of Southern blots after hybridization of gel electrophoretically separated and EcoRV-digested DNA from wild type (WT) and recombinant embryonic stem cells (cl 449) with either the 5′, internal or 3′ probes. Note that in addition to the 15.7 kb EcoRV fragment of the wild type allele other signals (5′ probe, 7.4 kb; internal probe, 2.3 kb; 3′ probe, 5.2 kb) are detected in the recombinant cell clone as predicted for allele DSG2 flox(E4-5-neo). C, Midori Green–stained, electrophoretically separated DNA fragments that were obtained by polymerase chain reaction (PCR) amplification of genomic regions surrounding the 5′ loxP site. Note that a single 167 bp fragment is amplified from DSG2WT mice and that an additional fragment is detectable in the heart but not liver of mice that also contain the Myh6-Cre transgene. The size (236 bp) of this fragment corresponds to that expected for the recombinated DSG2cKO allele. D, Left panel, PCR products amplified from cDNA derived from heart tissue of a DSG2floxE4-5 mice (WT), a mouse carrying an additional Myh6-Cre gene (cKO), and a DSG2floxE4-5WT mouse carrying a Myh6-Cre transgene (WT/cKO). Note that the DSG2WT mRNA fragment is undetectable in the homozygous DSG2cKO mouse, indicating efficient DSG2 recombination and altered mRNA production. In addition to the expected fragment reduction by 305 bp, an intermediate fragment was amplified (asterisk) because of incomplete splicing. D, Right panel, A histogram of qRT-PCR results detecting DSG2WT mRNA by amplification of a region encoded by E4–E5 from cardiac RNA (n=4 in each instance). Note that the expression is reduced to 46%±16% in heterozygous DSG2WT/cKO (WT/cKO) at 12 weeks and that it is almost undetectable (1.5%±0.6%) in homozygous DSG2cKO (cKO/cKO) mice at 2, 4, or 12 weeks.
acids consisting of the 29 amino acid signal peptide, the 25 amino acid prosequence, 24 amino acids of the extracellular domain EC1, and the 2 carboxyterminal amino acids histidine and leucine because of erroneous splicing of exon 3 onto exon 6. The processed carboxyterminal 26 amino acid peptide encompassing the W2 adhesion site, which is nonfunctional in Dsg2,25 may still be secreted. Of note, an additional unexpected mRNA species was amplified by RT-PCR from DSG2K0 samples (* in Figure 1D). On sequencing, it turned out to be a splice variant, in which 98 bp of DSG2 intron 3 (5′-ATT GGT TTA GTG GGG AAG CTT CAG GCT CGT GTG GAG GTC AGA GAA CAG CAG TGA GAG TGG CTG CTC CTC TGC CAT GTG TGT TCC AGG ACT GCA CTC AG-3′) remain between exons 3 and 6. This leads to an extension of exon 3 coding for the additional 16 amino acids GLVGKLIQARVEVREQQ before a stop codon prevents further translation. Taken together, both mRNA transcripts result in nonfunctional, presumably secreted peptides of 26 and 42 amino acids, respectively.

Next, Dsg2 protein expression was examined in DSG2K0 heart tissue. A ≈60% reduction was noted in heterozygous DSG2WT/K0 and ≈3% Dsg2 were detectable in homozygous DSG2K0/K0 (n=4) using 2 different antibodies directed against cytoplasmic epitopes of Dsg2 (Figure 2A). These results were confirmed by immunohistology (Figure 2C and 2D). Of note, expression and ID localization of other desmosomal proteins, such as Dsp, Pg, and Pkp2, were not visibly affected (Figures 2B and 2E–2H; n=4). Nuclear or nonjunctional plasma membrane staining was not seen for any of these antigens. Taken together, we conclude that Myh6-Cre–induced DSG2K0 results in efficient inhibition of Dsg2 protein expression in the hearts of transgenic mice without significantly affecting the expression level and localization of other major desmosomal proteins.

**Gross Morphological Manifestation of Cardiomyopathy**

Breeding heterozygous Myh6-Cre carriers with Myh6-Cre–negative mice in a homozygous DSG2WT background resulted in 40% DSG2K0/K0 progeny instead of the expected 50% at 4 weeks after birth (n=182). This situation is less dramatic than that encountered in DSG2MT mice expressing Dsg2 mutants lacking parts of their adhesive EC1-EC2 domains. In that instance, only 15% of the expected homozygous mutants were found at 4 weeks (n=104). Remarkably, most newborn mice appeared to be healthy in both situations. Myocardial alterations, however, became visible in all homozygous DSG2K0 and DSG2MT animals by the age of 4 to 6 weeks, that is, toward the end of postnatal heart growth (Figure ID in the Data Supplement). Most conspicuous were white, nontransparent, and differently sized areas on the cardiac surface occurring in the majority of DSG2MT and DSG2KO mice (Figure 3K and 3L; Figure 2C and 2D in the Data Supplement). Such lesions, which were never seen in DSG2WT animals, localized in 60% on the anterolateral surface of the left ventricle near the apex of DSG2MT hearts (n=79; see also Krusche et al.20). In addition, regions were noted, in which the convex and light red-colored even surface, which

![Figure 2.](https://example.com/fig2.png)

**Figure 2.** Dsg2 depletion and desmosomal protein expression in cardiomyocyte-specific DSG2K0 mice. A, Immunoblot detection of Dsg2 using rabbit (upper panel) and guinea pig antibodies (lower panel) in SDS-PAGE separated heart lysates of 12-week-old mice. Anti-β-actin antibodies (Actb) were used as controls after stripping. Genotypes were DSG2WT/WT (WT/WT); DSG2WT/flox(E4-5)+Myh6-Cre (WT/cKO); and DSG2flox(E4-5)/flox(E4-5)+Myh6-Cre (cKO/cKO). Note that Dsg2 levels are significantly reduced in heterozygotes (≈40%) and almost undetectable in homozygous conditional knockouts. B, Unchanged levels of plakoglobin (Pg) and desmoplakin (Dsp) in immunoblots (β-actin [Actb] as control). C–D′, Double fluorescence microscopy and corresponding interference contrast images of DSG2WT (C) and homozygous DSG2K0 (D; same genotypes as in A and B). Primary antibodies were rabbit anti-Dsg2 (C and D) and guinea pig antidesmoplakin (Dsp; C′ and D′). Note absence of Dsg2 immunoreactivity in the homozygous mutants (arrowheads in D) and ID colocalization of Dsg2 and Dsp in the wild type (arrowheads in C and C′). E–H, Despite loss of Dsg2 expression, DSG2K0 mice show normal ID localization (arrowheads) of Pg (F) and Pkp2 (H). The intensity of fluorescence signals is comparable to those of wild-type controls (E and G) and localization is identical to Dsp (E, F, G, H). Bars: 20 μm.
is typically encountered in the $DSG2^{WT}$ heart, was substituted by an uneven patchy dark red-colored surface (arrowheads in Figure 3I and 3J). Such alterations are characteristic features of wall thinning and aneurysms. Accordingly, enlargement of the right ventricle and both atria were observed (Figure I in the Data Supplement). Taken together, visual inspection of the dissected hearts sufficed to detect pathological anomalies in almost 100% of $DSG2^{MT}$ and $DSG2^{cKO}$ animals by 4 weeks (Figure ID in the Data Supplement).

**Histological Detection of Calcifying Replacement Fibrosis and Interstitial Fibrosis**

To further compare cardiac pathology between $DSG2^{MT}$ and $DSG2^{cKO}$ animals, detailed histological analyses were performed. Transmural fibrotic foci corresponding to the macroscopically visible surface lesions were detected by Azan staining (arrows in Figure 3C, 3C', 3F, and 3F'). Many of them contained calcifications as determined by von Kossa staining in both genetic situations (arrows in corresponding sections shown in Figure 4C, 4C', 4F, and 4F'). Careful examination revealed von Kossa–positive foci in up to a 100% of animals by 6 weeks. Ultrastructurally, remnants of cardiomyocytes were detected that were surrounded by calcified material (Figure 4G and 4H), providing evidence for cardiomyocyte necrosis, which has been identified as a key event in the pathogenesis of murine AC. Calcification, however, was not detectable in all lesions (arrowheads in Figure 3B, 3B', 3E, and 3E' and arrowheads in corresponding areas of adjacent sections shown in Figure 4B, 4B', 4E, and 4E'). These von Kossa–negative lesions likely contribute to the continuously increasing interstitial fibrosis observed in murine AC hearts.10 Taken together, the different types of fibrotic lesions are identical in $DSG2^{cKO}$ and $DSG2^{MT}$ hearts. Furthermore, comparable lesions have been identified in human AC patients with $DSG2$ mutations.8,10

**Disease Stages**

Gross morphological appearance and histology of most hearts was normal at 2 weeks (Figures I–III in the Data Supplement). Only 2 weeks later, visible changes, such as cardiomegaly and surface lesions with a preference for the right ventricle had developed (Figure I in the Data Supplement). At this time, the mRNA of the stress–response genes ANF (1.03±0.28 in $DSG2^{WT}$ versus 12.80±8.75 in $DSG2^{cKO}$), GDF15 (1.45±1.17 in $DSG2^{WT}$ versus 9.04±6.55 in $DSG2^{cKO}$), and CTGF (1.03±0.29 $DSG2^{WT}$ versus 2.58±1.20 in $DSG2^{cKO}$) was significantly elevated in $DSG2^{cKO}$ (for all genes: n=4, $P=0.0286$; for $DSG2^{MT}$, see Krusche et al20). The initial lesions contained necrotic cardiomyocytes and little collagen deposits (Figure II in the Data Supplement). At the same time, cell–rich infiltrates containing CD45-positive immune cells appeared in the lesions (Figure 5C and 5E). Furthermore, von Kossa–positive calcinosis was detectable in some lesions (Figure III in the Data Supplement). Increased collagen fiber deposition was noted by 6 to 12 weeks (Figure II in the Data Supplement).
which was accompanied by a reduction in CD45 cells (Figure 5D and 5F) in lesioned areas. In contrast, von Kossa–positive foci persisted (Figure III in the Data Supplement). These observations show that the timing and stages of disease progression of the newly established DSG2 cKO mice is indistinguishable from that observed in DSG2 MT animals.

Loss of Desmosome-Like Structures and Intercalated Disc Dissociation

To further work out the resemblance between DSG2−/− and DSG2 MT hearts at the ultrastructural level, electron microscopy was performed. For best evaluation, the myocardium was relaxed before fixation. Morphology and organization of sarcomeres were not visibly altered in longitudinal sections of intact cardiomyocytes. Cell–cell contact regions contained normal-appearing fasciae adhaerentes and adjacent gap junctions. Desmosome-like structures were frequently observed in IDs of WT control mice (arrows in Figure 6A and 6A′), but were not seen in IDs of DSG2 cKO and DSG2 MT mice (Figure 6B–6C′).

Although intercellular gap width was not altered in most instances, dissociation of IDs was occasionally noted in the mutant hearts (Figure 6D and 6E), resulting in remnant junctions with adhering actin filaments. Adjacent cytoplasmic regions appeared to be disorganized with multiple membranous structures and disrupted mitochondria. The disconnected intercellular gaps were usually filled with membranes and included large cytoplasmic organelles, such as mitochondria in some instances. In regions directly adjacent to fibrotic lesions, sarcomeres ended in membrane regions that were decorated by scarce plaque material and bordered directly to the collagen fiber–rich extracellular matrix (Figure 6F and 6G).

Altered Conduction and Connexin 43 Distribution

To examine electrophysiological alterations, electrocardiography was performed using the ECGenie system (Figure 7E and 7F). Despite normal heart rates (RR-intervals), highly significant prolonged PR and QRS intervals were detected in both the DSG2 MT (n=6) versus DSG2 WT (n=8) and DSG2−/− (n=8) versus DSG2control mice (n=10), indicating conduction defects of atria and ventricles. We therefore investigated the expression of the major myocardial gap junction protein connexin 43. Overall protein levels were similar in matched mutant and WT hearts (Figure 7D). Immunolocalization (n=5), however, revealed that connexin 43 staining was not restricted to IDs in DSG2−/− and DSG2 MT, but was also detectable in multiple puncta in the cytoplasm of peri-lesional cardiomyocytes (Figure 7A–7C).

Discussion

We report on the establishment and characterization of a mouse model, in which, for the first time, a desmosomal cadherin was tissue-specifically ablated in a specialized cell type, namely the cardiomyocyte. The birth of viable animals with inconspicuous gross heart morphology provides evidence that Dsg2 is not essential for growth and function of the

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heart during late embryogenesis and early postnatal life. This is remarkable because Dsg2 is by far the major Dsg isoform expressed in cardiomyocytes.3

All analyses performed in this study reveal a striking phenotypic resemblance between DSG2$^{cKO}$ and DSG2$^{WT}$ mice. This includes aspects of disease onset and progression, histological features, ultrastructural disturbances, and functional deficiencies. Furthermore, our observations are similar to those reported for mice overexpressing Dsg2 N271S.11 We therefore suggest that the same pathways induce cardiomyopathy in all 3 models.

The observed widening and frequent dissociation of intercalated discs in the 3 DSG2-mutant mouse strains (Kant et al19 and Rizzo et al24 and this study) presents strong in vivo evidence for compromised adhesion. Similar changes were noted in hearts of patients with Dsg2-related AC.10,12,29 In vitro experimentation further supports an adhesive function of Dsg2. Expression of different human AC-related Dsg2 mutants and inhibition of Dsg2 adhesion were reported to reduce adhesion upon expression in HL-1 cardiomyocytes32 (for contrasting results on another mutant see Gaertner et al30). The situation in other AC types is less clear. Although (for contrasting results on another mutant see Gaertner et al)11 We therefore conclude that altered Dsg2 lacking the EC1-EC2 domains does not. Reasons for this discrepancy may be that deletion of the EC1-EC2 segment is less severe than the single amino acid change in the DSG2$^{cKO}$ hearts, however, shows that Dsc2 is not able to fully compensate for the loss of Dsg2. Clearly, both cadherins are not interchangeable as is evident from the lack of overt cardiomyopathy in desmocollin 2 knockout mice.9 The clinical manifestation of an AC-like phenotype during the postnatal growth period of young DSG2$^{cKO}$ mice assigns an important function to Dsg2 in supporting the increasing workload of the growing heart. Yet, 50% reduction of Dsg2 in DSG2$^{WTcKO}$ and DSG2$^{WT/}$ $^{cKO}$ mice still supports overall normal heart function (this study and Eshkind et al38), although physical challenges may induce AC symptoms as has been observed in heterozygous Pg KO mice.30 In the situation of homozygous DSG2$^{WT}$ mice, both the adhesion defect of the mutant polypeptide and the reduction of the mutant Dsg2 to $\approx$25% likely contribute to AC-like pathogenesis.32 The new DSG2$^{cKO}$ mouse model provides proof that downregulation of Dsg2 below a critical level induces a cardiomyopathy that is similar to that observed in DSG2$^{WT}$ mice.

Of note, not only Dsg2 reduction but also overexpression of Dsg2 N271S leads to cardiomyopathy. Because overexpression of WT Dsg2 alone does not induce cardiomyopathy, one has to conclude that Dsg2 N271S interferes with WT Dsg2 function by a dominant negative mechanism.11 Interestingly, high overexpression results in a more severe phenotype than low overexpression.11 Given that Dsg2 N271S induces an AC-like phenotype in the presence of WT Dsg2, it is not clear why Dsg2 lacking the EC1-EC2 domains does not. Reasons for this discrepancy may be that deletion of the EC1-EC2 segment is less severe than the single amino acid change in the DSG2$^{WT}$ or that the level of mutant protein expression is relevant for disease development. In all instances, interference seems to occur at the ID because mutant Dsg2 is efficiently localized to the ID in the different murine models and human Dsg2–related AC.8,11,20,30,41

Multiple studies have assigned a crucial role of plakoglobin signaling to the initiation of AC in mouse and man.17,42–44 Comparing disease manifestation with the available binding sites of desmosomal cadherins for Pg or other desmosomal cadherin-dependent signaling molecules in the various murine models, however, shows that there is no clear correlation (Figure 8). We therefore conclude that altered Dsg2 cytoplasmic tail-dependent signaling, which relies on Pg displacement from the desmosome, is unlikely the major disease mechanism. This is also supported by AC development either in the absence of Pg16,37 or with unperturbed Pg expression in the ID.45 Instead, compromised desmosomal cadherin-mediated
adhesion may be at the core of disease initiation. This concept is strongly supported by the loss-of-function phenotype observed in the present study. It is indirectly supported by the observation that AC-associated Dsg2 mutations in the intracellular catenin-binding domain do not compromise Pg- or Pkp2-binding.8

It is of interest to note that Dsg2 reduction has also been observed in the hearts of PKP2\textsuperscript{ KO/KO} embryos,38 in heart-specific homozygous Pg knock-outs (JUP\textsuperscript{ KO/KO}),36,37 and in the presence of normal Pg ID expression in a heart-specific Dsp knock-out.43 Similar to the observations reported here, a selective loss of desmosome-like structures but not of adherens junctions and gap junctions was detected in the JUP\textsuperscript{ KO/KO}. In addition, Dsg2 downregulation has also been observed in human AC patients independent of the underlying mutations, even in the presence of normal Pg localization.

Figure 6. Ultrastructural comparison of DSG2\textsuperscript{ WT}, DSG2\textsuperscript{ MT}, and DSG2\textsuperscript{ cKO} hearts. A–C′, Electron micrographs of longitudinal sections showing sarcomeres next to IDs. Wild-type IDs contain typical desmosome-like structures (arrows), which are not seen in DSG2\textsuperscript{ MT} or DSG2\textsuperscript{ cKO} mice. In addition, dissociated IDs are frequent in mutant animals (D–F). The enlarged intercellular gap is filled with multilamellar bodies (arrowheads) and other cellular organelles, such as mitochondria (Mi; E). The remains of adhesion structures are still seen as dark accumulations at the borders of the disrupted myocytes (asterisks). F and G, Cardiomyocytes with normal mitochondria and sarcomeres (Sa), which are located next to collagen-rich fibrotic lesions (Co) and disrupted IDs. Scale bars: 1000 nm in A, D, and F (same magnifications in each row); 200 nm in A′, B′, and C′.
to IDs. A provocative conclusion is that downregulation of Pgc and Pkp2 may also lead to weakening of desmosomal cadherin–mediated adhesion and desmin intermediate filament anchorage.

DSP mutations31,45 and certain desmin mutations47,48 may contribute in a similar fashion to impair the stability and resilience of the desmosome-based transcellular scaffold. Thus, altered adhesion may be the primary dysfunction of desmosome-related cardiomyopathy that can be brought about in different ways as reflected by the different desmosomal gene mutations reported to date.

**Limitations**

Murine models of human AC present significant limitations given the differences in genetics, life span, immune response, epicardial fat, and secondary reactions, such as fibrotic scar formation with calcinosis typical in mice49 versus fibrotic replacement in human with variable amounts of adipose tissue.50 In addition, several aspects of our murine AC models remain to be explored. These include the analysis of embryonic lethality, detailed statistics of survival, examination of the effect of physical activity on modulating disease progression.

**Figure 7.** Comparison of connexin 43 expression and electrophysiological recordings of DSG2WT, DSG2MT, and DSG2cKO mice. A–C, Overlay of anticonnexin 43 immunofluorescence and interference contrast images of 12-week-old mice (n=6 for each genotype) demonstrating typical ID staining in all samples (arrowheads) and prominent cytoplasmic puncta (arrows) in cardiomyocytes next to fibrotic regions (fib) in DSG2WT and DSG2cKO samples. Bar: 50 μm in C (same magnification in A and B). D, Immunoblot detection of connexin 43 (Cx43) and actin (corresponding Ponceau-staining of PVDF membranes below) in total heart lysates (M, marker lane; WT, DSG2WT; MT, DSG2MT; cKO, DSG2cKO). E, Typical examples of averaged ECGenie recordings of ≈20-week-old mice that were used for further analysis. F, Histogram depicting RR, PR, and QRS intervals determined in wild-type (WT at left; n=6) versus DSG2MT (MT; n=8) and DSG2flox(E4-5) (WT at right; n=10) versus DSG2cKO (cKO; n=8) animals. Note the significant increase in PR and QRS intervals in both instances (**P<0.01; ***P<0.001).

**Figure 8.** Schematic representation of ID-localized desmosomal cadherins and associated signaling molecules in different mouse models with altered Dsg2 expression. The table also lists whether overt AC develops until the 12th week after birth. References are given for each animal model.
in homozygous mutants, and even more, the induction of disease symptoms in heterozygotes.

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Disclosures
None.

References


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**CLINICAL PERSPECTIVE**

The human disease arrhythmogenic cardiomyopathy (AC) has been linked to mutations in components of the intercalated disc, most notably to desmosomal proteins, including the desmosomal cadherin desmoglein 2. We have recently prepared desmoglein 2 knock-in mice with a targeted deletion of a part of its extracellular adhesive domain. These animals develop an AC-like phenotype. To find out whether loss of adhesive function or gain of malfunction determines pathogenesis, cardiomyocyte-specific desmoglein 2 knockout mice were prepared. We find a close resemblance of the knock-in and knockout mice: healthy transgenic animals were born with intercalated discs lacking typical desmosomes. During postnatal growth, both transgenic lines developed pronounced chamber dilation, calcifying cardiomyocyte necrosis, aseptic inflammation, interstitial and focal replacement fibrosis, and conduction defects with altered gap junction distribution. Electron microscopy revealed locally compromised intercalated disc adhesion. These findings show that the presence of intact desmoglein 2 is needed to maintain proper heart function in growing animals. They further suggest that loss of desmoglein 2 is linked to compromised adhesion, which may be a major pathogenic mechanism in AC. Thus, reduction of mechanical stress is expected to benefit affected individuals. In support, load-reducing therapies have been successful in ameliorating functional deficiencies in animal models of AC. Our observations provide a scientific rationale for the clinical recommendation of reduced physical activity and indicate that load reduction may benefit AC patients.
Desmoglein 2–Dependent Arrhythmogenic Cardiomyopathy Is Caused by a Loss of Adhesive Function

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Cloning of targeting construct

To prepare the targeting construct, the DSG2 3'-homology region was isolated from clone 3011 together with a neomycin resistance (NFL) cassette and the vector backbone as a 9046 bp XhoI-fragment and re-ligated resulting in clone 3060. The 2034 bp NFL-cassette was subsequently removed by EcoRI-restriction and re-ligation (clone 3062). To clone the 5'-homology region, the SpeI-restriction site of pBluescript KS (+) was removed by blunt end formation and re-ligation of SpeI-digested vector (clone 3061). The 5569 bp XhoI 5'-homology region was then excised from clone 3011 and inserted into clone 3061 resulting in clone 3063.7. The phosphoglycerate kinase-driven neomycin resistance cassette, which is flanked by two FRT-sites and flanked by a single loxP-site at its 3'-end, was amplified from clone 3060 with primers 07-24 (5'-GCC ACT AGT GCT GCG ATT ATA GGC CTG AG-3') / 07-25 (5'-GAT CAC TAG TGC GGG GAT AAT ACG ACT C-3') and was inserted blunt into the SpeI-linearized clone 3063.7 (clone 3064.1). Then, the 5'-homology region together with the NFL cassette was isolated as a 7620 bp XhoI-fragment from clone 3064.1 and the 7012 bp 3'-homology-vector fragment was isolated by linearization of clone 3062 with XhoI. Both fragments were then combined to yield targeting construct 3065.

Generation of knockout mice

Trypsinized AB2.2 embryonic stem (ES) cells (ATCC SCRC-1023) that had been grown in a 10 cm Petri dish were mixed with 200 µg of NotI-linearized targeting construct (clone 3065) for electroporation (800 V; 3 µF; 0.04 ms). Electroporated cells were seeded in ES-cell medium (GMEM [Gibco] supplemented with sodium pyruvate [PAA], non-essential amino acids [PAA], 5% fetal calf serum [Invitrogen], 5% newborn calf serum [Gibco], 0.1 mM β-mercaptoethanol, leukemia inhibitory factor [LIF] obtained from supernatants of LIF-expressing COS7 cells) and selected with 350 µg/ml G418 (Gibco). After 10 days G418-resistant clones were isolated and amplified. Genomic DNA was prepared and tested for homologous recombination by PCR to detect the recombined 2355 bp fragment with primers 08-21 (5'-TTT CAG ATT GGT TTA GTG GG-3') and 08-22 (5'-TAT GCT ATA CGA AGT TAT GAT ATC C-3'). PCR-positive ES-cell clones were further examined by Southern blot analysis. ES cell clones with correct DSG2 recombination were injected into blastocysts or morulae, which were implanted into pseudopregnant foster mothers. The resulting chimeras were bred with C57BL/6J mice and offspring with brown fur was analyzed for recombination by PCR. The transgenic animals were then bred with FLPo deleter mice (friendly gift from Dr. Anastasiadis, Dresden University)2 to remove the neomycin resistance cassette. To obtain cardiomyocyte-specific DSG2 recombination, mice were bred with αMyHC-Cre (now referred to as Myh6-Cre) transgenic mice (kindly provided by Dr. Schneider, Imperial College London).3
Southern blotting and PCR analyses

25 µg genomic DNA were digested with EcoRV and separated on a 0.7% agarose gel. DNA was depurinated in the gel by incubation in 0.25 M HCl for 20 min and was subsequently denatured by treatment with 0.4 M NaOH. DNA was blotted onto a neutral nylon membrane (Hybond-N, GE Healthcare) overnight in 0.4 M NaOH and the membrane was dried for 1 h at 80°C. 200 ng of each probe were labeled using Laddnerman Labeling Kit (TaKaRa) according to the instructions of the manufacturer. Easytides dCTP [α-32P] (Perkin Elmer) were used for probe labeling and labeled probes were purified using Probe Quant G50 Micro Columns (GE Healthcare).

Using primers 10-110 (5'-TCA AGC TTT AGC ACA AAC CAC CCA TGT C-3') and 10-111 (5'-TAG ATT TCG CAC ATT CAT GTG GGA AAC C-3') a 769 bp fragment was amplified from clone 3065 as 5'-probe and cloned into pUC18 after digestion with EcoRI and HindIII (clone 3067). To prepare a 3'-probe, a 1020 bp fragment was PCR-amplified with the help of primers 10-114 (5'-TAT CTA GAC ATT GAG CCA TCT CCT CAA C-3') and 10-115 (5'-ATG AGC TCA GTC ACC AGG GAT AGT C-3') and inserted after SacI/XbaI restriction into pUC18 (clone 3069). Labeled probes were prepared from purified plasmid inserts. Membrane with blotted genomic DNA was rehydrated and blocked at 64°C for 15 min in hybridization buffer (0.5 M phosphate buffer, 3.5% sodium dodecyl sulfate (SDS), pH 7.0, 100 µg/ml denatured salmon sperm DNA). 200 ng of denatured and labeled probe were added and hybridization was performed over night at 64°C. Membrane was shortly washed with wash buffer I (2x SSC [0.33 M NaCl and 30 mM trisodium citrate, adjusted to pH 7.0 with HCl], 0.1% SDS) at room temperature, followed by two washings for 20 min with wash buffer II (1x SSC, 0.1% SDS) at 64°C. Finally, the membrane was washed for 30 min with wash buffer III (0.1x SSC, 0.1% SDS) at room temperature.

A 406 bp internal probe was amplified from the targeting construct using primers 03-63 (5'-TCA GAC GAC TCA ACA AG-3') and 03-69 (5'-AAT CGA GGG AGT GGA GAA AC-3'). In this instance, the membrane-blotted DNA was rehydrated with Church buffer (40 mM phosphate buffer, 1% SDS, pH 6.8) at 65°C for 15 min. Blocking of unspecific probe hybridization was achieved by membrane incubation with pre-warmed QuikHyb Hybridisation Solution (Stratagene) containing 100 µg/ml denatured salmon sperm DNA. 200 ng of denatured and labeled probe were added and hybridization was performed over night at 65°C. Afterwards, membrane was washed 3 times with Church buffer at 62°C for 20 min.

Hybridization of probes was detected with the help of BioMax MR Film (Kodak) by autoradiography.

To determine whether Myh6-Cre driven recombination was successful a PCR using genomic DNA obtained from heart tissue and primers 12-7 (5'-GGT AAA TGC AGA CGG ATC AG-3'), 12-8 (5'-TGG GCT ACA CTC ATA GGA AG-3') and 12-99 (5'-TTG CAC AGG ACT CAG GAT TG-3') was performed.

RNA isolation, reverse transcription and qRT-PCR

Fresh tissue samples were homogenized in extraction buffer (PeqlabGold RNA kit, Peqlab) and proteins were removed using phenol/chloroform precipitation. Total RNA was then enriched using the PeqlabGold RNA kit in combination with on column DNase digestion according the instruction manual of the manufacturer (Peqlab).
To prepare cDNA, mRNA was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis kit with oligo-(dT)₁₈ oligonucleotides (Roche). qRT-PCR was performed with a LightCycler 96 (Roche), FastStart Essential DNA Green Master Kit or FastStart Essential DNA Probes Master (Roche) and the following forward, reverse primers and UPL-probes (Roche): wild type-specific DSG 2 (13-78 forward 5'-ACC GGG AAG AAA CAC CAT ATT-3'; 13-79 reverse 5'-AGG GCT TTT CCA GGT TGT TT-3'), ANF (09-50 forward 5'-CAC AGA TCT GAT GGA TTT CAA GA-3'; 09-51 reverse 5'-CCT CAT CTT CTA CCG GCA TC-3'; UPL-probe 25), CTGF (09-20 forward 5'-TGA CCT GGA GGA AAA CAT TAA GA-3'; 09-21 reverse 5'-TGA CCT GGA GGA AAA CAT TAA GA-3'; UPL-probe 71), GDF15 (09-78 forward 5'-GAG CTA CGG GGT CGC TTC-3'; 09-79 reverse 5'-GGG ACC CCA ATC TCA CCT-3'; UPL-probe 62), HMB5 housekeeping control (12-36 forward 5'-AAG TTC CCC CAC CTG GAA-3'; 12-37 reverse 5'-GAC GAT GGC ACT GAA TTC CT-3'; UPL-probe 42), HPRT housekeeping control (12-65 forward 5'-TAG ATC CAT TCC TAT GAC TGT AGA-3'; 12-66 reverse 5'-AAG ACA TTC TTT CCA GGT GAA GT-3') and TBP housekeeping control (11-25 forward 5'-GGG GAG CTG TGA GTG AA-3'; 11-26 reverse 5'-CCA GGA AAT AAT TCT GGC TCA-3').

**Generation of polyclonal Dsg2 antibody**

The antigen was obtained by conjugation of the synthetic peptide with sequence SVTGGQHELSEVDGRWEEHRSC to ovalbumine. The antibodies were generated in guinea pig by subcutaneous injection of 240 µg immunogenic polypeptide diluted in complete Freund's adjuvant followed by three boosting injections with 240 µg antigen diluted in incomplete Freund's adjuvant every two weeks. Serum was collected 14 days after the last immunization.

**Immunoblotting**

Fresh tissue samples were Dounce-homogenized in extraction buffer (10 mM Tris-HCl [pH 8.0], 2 mM MgCl₂, 10 mM KCl, 2% SDS supplemented with a complete mini protease inhibitor tablet per 10 ml [Roche]) and stored on ice. After preparation of all samples, they were heated at 94°C for 10 min and then directly placed on ice for 5 min. Lysates were cleared by centrifugation (4°C, 17000 g, 20 min) to obtain whole heart protein extract in the supernatant. Protein concentration was determined using DC Protein Assay (BioRad). 35 µg of protein per lane were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes using tank blotting. Membranes were afterwards blocked with 5% (w/v) low fat milk powder (Roth) in TBST (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% (v/v) Tween 20) for 2 h, washed 3 times for 5 min each with TBST and incubated with primary antibody diluted in TBST/1% low fat milk powder overnight at 4°C. Antibody dilutions were: 1:2500 for polyclonal rabbit anti-Dsg2 antibodies (Dsg2 IC)⁴ and polyclonal guinea pig anti-Dsg2 antibodies (see previous paragraph), 1:1000 goat anti-Pg (sc-30997, Santa Cruz), 1:200 mouse anti-Dsp (65146, Progen), 1:4000 rabbit anti Cx43 (C6219, Sigma), 1:4000 for polyclonal rabbit anti β-actin antibodies (A2066, Sigma). Membranes were washed 3 times in TBST and incubated for 1 h with horseradish peroxidase-coupled secondary antibodies (anti-rabbit and anti-goat antibodies from DAKO at 1:5000 and anti-guinea pig antibodies from Jackson at 1:5000, both diluted in TBST with 1% low fat milk powder). Signals were detected using ECL prime (GE healthcare) and a chemiluminescence imaging system (Fusion SL, Vilber Lourmat).
Figure S1. Gross morphological evaluation of dissected hearts taken from homozygous DSG2\textsuperscript{MT} and DSG2\textsuperscript{cKO} mice and DSG2\textsuperscript{WT} or DSG2\textsuperscript{flox(E4-5)} control animals. The graphs show the percentage of animals presenting dilation of the right ventricle (A) or visible surface lesions on the right (B) or left ventricle (C). The summary graph in (D) shows the percentage of animals with at least one phenotypic deviation. Note, that by 4 weeks 93% of the DSG2\textsuperscript{MT} and 100% of the DSG2\textsuperscript{cKO} mutant hearts are visibly abnormal. (E) represents the legend for A-D and (F) a listing of the number of animals examined for each phenotype at each time point.
Figure S2. Photomicrographs of Azan-stained myocardial sections obtained from DSG2\textsuperscript{WT} and DSG2\textsuperscript{cKO} animals highlighting the disease stages. Note the normal-appearing myocardium at 2 weeks and the formation of immune cell-rich lesions (*) in areas of cardiomyocyte death at 4 weeks (arrows) forming collagen-rich fibrotic foci. In addition, interstitial fibrosis increases over time (arrowheads). Scale bar: 1 mm for all survey views; 100 µm for all higher magnifications.
Figure S3. Pictures of von Kossa-stained myocardial sections obtained from DSG2\textsuperscript{WT} and DSG2\textsuperscript{cKO} animals depicting calcium deposition in lesions of mutant hearts. Note that the fibrotic lesions (arrows) present prominent black precipitates that are not yet detectable in fresh immune cell-rich lesions (*) and regions with interstitial fibrosis (arrowheads). Scale bar: 1 mm for all survey views; 100 µm for all higher magnifications.
References


