Reduced Cx43 Expression Triggers Increased Fibrosis Due to Enhanced Fibroblast Activity

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Background—Arrhythmogenic ventricular remodeling is hallmarked by both reduced gap junction expression and increased collagen deposition. We hypothesized that reduced connexin43 (Cx43) expression is responsible for enhanced fibrosis in the remodeled heart, resulting in an arrhythmogenic substrate. Therefore, we investigated the effect of normal or reduced Cx43 expression on the formation of fibrosis in a physiological (aging) and pathophysiological (transverse aortic constriction [TAC]) mouse model.

Methods and Results—The Cx43fl/fl and Cx43CreER(T)/fl mice were aged 18 to 21 months or, at the age of 3 months, either TAC or sham operated and euthanized after 16 weeks. Epicardial activation mapping of the right and left ventricles was performed on Langendorff perfused hearts. Sustained ventricular arrhythmias were induced in 0 of 11 aged Cx43fl/fl and 10 of 15 Cx43CreER(T)/fl mice (P<0.01). Cx43 expression was reduced by half in aged Cx43CreER(T)/fl compared with aged Cx43fl/fl mice, whereas collagen deposition was significantly increased from 1.1±0.2% to 7.4±1.3%. Aged Cx43CreER(T)/fl mice with arrhythmias had significantly higher levels of fibrosis and conduction heterogeneity than aged Cx43fl/fl mice without arrhythmias. The TAC operation significantly increased fibrosis in control compared with sham (4.0±1.2% versus 0.4±0.6%), but this increase was significantly higher in Cx43CreER(T)/fl mice (10.8±1.4%). Discoidin domain receptor 2 expression was unchanged, but procollagen peptide I and III expression and collagen type Iα2 mRNA levels were higher in TAC–operated Cx43HZ mice.

Conclusions—Reduced cellular coupling results in more excessive collagen deposition during aging or pressure overload in mice due to enhanced fibroblast activity, leading to increased conduction in homogeneity and proarrhythmia. (Circ Arrhythm Electrophysiol. 2012;5:380-390.)

Key Words: arrhythmia ■ collagen ■ electrophysiology mapping ■ connexin43 ■ fibroblast

In the heart, the highly orchestrated propagation of the electric impulse balances on the delicate interplay between excitability, cell-to-cell coupling, and architecture of myocardial tissue. An important aspect of the myocardial architecture is interstitial collagen (fibrosis), which, together with connexin43 (Cx43), determines cell-to-cell coupling in ventricular myocardium. Under normal physiological conditions, the amount of collagen between the cardiomyocytes is low (<1% of total tissue volume) but contributes to the structural organization of the heart and the anisotropic character of impulse propagation. We recently showed that, in senescent mouse hearts, collagen content was increased (200%) and Cx43 expression was decreased (50%), changes that were associated with increased inducibility of ventricular tachycardias.1 On the other hand, when the excessive deposition of fibrosis was prevented through long-term inhibition of the renin-angiotensin-aldosterone system, the normal pattern of Cx43 expression was preserved and arrhythmia vulnerability was strongly reduced.2

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In addition to those observations during physiological aging, a decreased Cx43 expression, in concert with increased collagen content (interstitial and reactive fibrosis), is also found under various pathophysiological conditions.3–5 These alterations have impaired conduction velocity (CV) of the cardiac impulse, by increasing the anisotropic ratio and heterogeneity of conduction. In concert, this predisposes the vulnerable heart to an increased risk for fatal arrhythmias that may partly account for the high incidence of sudden cardiac death in patients with remodeled hearts.9–11

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Up until now, it was unknown if, and if so how, increased collagen deposition and the accompanied decrease in Cx43 expression are related. In arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C), decreased Cx43 levels are found already in the early stages, whereas fibrosis becomes more abundant during the progression of the disease. This suggests a possible role of reduced Cx43 expression levels in the deposition of collagen in vulnerable hearts. A supportive recent in vitro study suggested increased proliferation of cardiac fibroblasts, the primary source of cells responsible for production of interstitial collagen, once the intercellular communication was inhibited.

Other studies suggest a possible role for the interplay between decreased Cx43 expression and increased collagen content in arrhythmia vulnerability. In adult mice (aged 12–17 months), interstitial fibrosis increased, but Cx43 expression was similar compared with young (aged 3–4 months) animals. Despite increased collagen content, arrhythmias could not be induced in the adult hearts. Another study showed that arrhythmias could not be induced in hearts without fibrosis but with reduced (50%) Cx43 expression. These data suggest that a combination of reduced Cx43 expression and increased collagen content is required to increase arrhythmogenicity.

In the present study, we hypothesized the following: (1) reduced Cx43 expression triggers enhanced fibrosis in the pathologically and physiologically remodeled heart and (2) the combination of excessive fibrosis and reduced Cx43 levels results in impaired cardiac impulse conduction and a high arrhythmia susceptibility. To investigate the potential role of reduced Cx43 expression in triggering collagen deposition in hearts under physiological and pathophysiological conditions, an aged (physiological model 1) and a transverse aortic constriction (TAC)–operated mouse model (pathophysiological model 2) were used. In model 1, mice with 100% (Cx43fl/fl) or 50% Cx43 expression (Cx43Cre-ER(T)fl) were aged 18 to 21 months. A previous study revealed that, at this age, interstitial fibrosis is highly increased. In model 2, 3-month-old mice of the same strain (Cx43fl/fl and Cx43Cre-ER(T)fl) underwent TAC (induced pressure overload) or sham surgery and were euthanized 16 weeks after surgery. Epicardial mapping was performed on Langendorff perfused hearts to assess impulse conduction and arrhythmia inducibility, and tissue was subsequently analyzed for Cx43 expression and fibrosis. Finally, fibroblast proliferation and activity were determined.

Our data showed that both physiological (aging) and pathophysiological (TAC) stress leads to more excessive fibrosis in mice with a 50% reduced expression of Cx43 (Cx43Cre-ER(T)fl) compared with controls (Cx43fl/fl) and that this was related to an increased activity of fibroblasts. Together with a heterogeneous reduced expression of Cx43, this resulted in dispersed conduction and proarrhythmia.

Methods

An expanded Methods section is available in the online-only Data Supplement. Brief descriptions are presented.

Animals

The Cx43fl/fl and Cx43Cre-ER(T)fl mice were generated as previously described. For the experiments on aged animals, 18- to 21-month-old Cx43fl/fl (n=11) and Cx43Cre-ER(T)fl (n=15) mice were used. No abnormalities in phenotype were found, and heart weight (HW)/body weight (BW) ratios, age, and sex distribution were similar in the 2 groups (online-only Supplement Table I).

Three-month-old Cx43fl/fl (n=11) and Cx43Cre-ER(T)fl (n=9) mice were TAC operated, as previously described. A gradient of ≈50% was confirmed by Doppler echocardiography. Sham–operated Cx43fl/fl (n=12) and Cx43Cre-ER(T)fl (n=10) mice were used as control. Animal experiments were performed in accordance with institutional guidelines for animal use in research.

Preparation of the Hearts and Ventricular Conduction

Mice were anesthetized by 2.5% isoflurane in oxygen. A 3-lead ECG was recorded and analyzed off-line, as previously described. Afterward, the heart was excised, prepared, and connected to a Langendorff perfusion setup, as previously described. Extracellular electrograms were recorded using a 208-point multitemporal electrode (16×13 grid, 0.5-mm spacing) of both the left ventricle (LV) and right ventricle (RV) of the heart, as previously described. Recordings were made during stimulation (1-ms pulse duration, 2×diastolic stimulation threshold) from the center of the grid at a basic cycle length of 150 ms. The effective refractory period (ERP) was determined by premature stimulation, and arrhythmia inducibility was tested by 1 to 3 premature stimuli and burst pacing (the online-only Data Supplement provides detailed protocols).

Statistical Analysis

The moment of maximal negative dV/dt in the unipolar electrograms was selected as the time of local activation and determined with custom-written software based on MatLab (2006, The MathWorks Inc; Natick, MA). Conduction velocities parallel and perpendicular (CVp, CVt) to fiber orientation were determined from activation maps generated from basic cycle length pacing. The anisotropic ratio was defined as CVp/CVt. Dispersion of conduction was assessed for both LV and RV using the method described by Lammers et al. Two-group comparisons were performed using an unpaired t test, and multiple-group comparisons were performed using a 1-way ANOVA with Bonferroni post hoc analysis. Arrhythmia vulnerability was compared by a Fisher exact test. Values are given as mean±SEM. P≤0.05 was considered statistically significant. Data were analyzed using SPSS 15.0 (2006, SPSS Inc; Chicago, IL) software.

Immunohistochemical and Histological Data

After electrophysiological measurements, hearts were rapidly frozen in liquid nitrogen and stored at −80°C. The online-only Data Supplement provides data on sectioning and (immuno)labeling. The amounts of fibrosis and Cx43, P1NP, and P3NP immunosignals were determined using at least 6 randomly chosen pictures of each heart at ×200 magnification. Blinded operators calculated Cx43, P1NP, and P3NP expression as percentage of the total tissue using Image J 1.40g (2008, National Institutes of Health; Bethesda, MD). Heterogeneity of Cx43 expression was determined using MatLab, as previously described.

Western Blot Analysis

Total cellular protein was isolated from 3 hearts of each group, as previously described. Equal amounts of protein (25 μg per lane) of each sample were separated on 10% SDS–polyacrylamide gels and transferred by electrophoresis to a nitrocellulose membrane (Biorad). The equality of protein transfer was assessed by Ponceau S staining. After first and second antibody incubation, immunoreactivity was detected using the enhanced chemiluminescence kit (Amersham). By using ImageQuant, we calculated the Cx43/Ponceau signal intensity ratio, which represents the actual Cx43 protein concentration of the different lanes.

Antibodies

All used antibodies are listed in the online-only Data Supplement.
Quantitative Polymerase Chain Reaction

Total RNA was isolated from frozen ventricular tissue using the Trizol procedure and subsequently treated with DNAse I, as previously described. After cDNA synthesis, expression of mouse collagen type 1a2 (COL1A2) was assessed by quantitative real-time polymerase chain reaction using TaqMan Gene Expression Assays with predesigned probe and primers (Applied Biosystems). The GAPDH was used as an internal reference.

Results

Aged Mice With Reduced Cx43 Expression

Most hearts of aged Cx43fl/fl mice were susceptible for monomorphic ventricular tachyarrhythmias (10 of 15 mice), in sharp contrast to aged Cx43Cre-ER(T)/fl mice, in which no arrhythmias were inducible (0 of 11 mice, P<0.01, Figure 1A). Most arrhythmias were provoked in the RV (9 of 10), indicating a higher arrhythmia vulnerability of the RV. Figure 1A shows a typical example of a sustained monomorphic ventricular tachycardia (VT) in an RV induced after burst pacing. The activation map of this VT (right) shows anisotropic reentry: impulse propagation occurred around a zone of conduction block, with slow conduction perpendicular to the fiber direction toward the apex of the heart in a localized area (open arrow).

The QRS duration was comparable between aged Cx43fl/fl and Cx43Cre-ER(T)/fl mice (11.20±0.29 ms versus 11.24±0.18 ms; Table 1). Representative epicardial activation maps of
Table 1. Electrophysiological and Tissue Characteristics of Aged Mice

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cx43fl/fl</th>
<th>Cx43Cre-ER(T)^R</th>
<th>VT^-</th>
<th>VT^+</th>
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<tr>
<td>Arhythmias, %</td>
<td>0</td>
<td>67</td>
<td>0</td>
<td>100</td>
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<tr>
<td>LV CVa, cm/s</td>
<td>58.8±5.0</td>
<td>57.9±3.1</td>
<td>59.6±4.9</td>
<td>56.2±4.3</td>
</tr>
<tr>
<td>RV CVa, cm/s</td>
<td>58.6±1.0</td>
<td>58.3±2.3</td>
<td>58.9±4.9</td>
<td>57.7±0.4</td>
</tr>
<tr>
<td>LV CVp, cm/s</td>
<td>35.8±4.0</td>
<td>32.2±0.8</td>
<td>32.9±0.4</td>
<td>31.6±1.5</td>
</tr>
<tr>
<td>RV CVp, cm/s</td>
<td>42.7±1.0</td>
<td>36.5±1.3</td>
<td>39.4±1.1</td>
<td>33.7±0.7</td>
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<tr>
<td>LV AR, CVa/CVp</td>
<td>1.6±0.13</td>
<td>1.79±0.08</td>
<td>1.81±0.15</td>
<td>1.77±0.07</td>
</tr>
<tr>
<td>RV AR, CVa/CVp</td>
<td>1.3±0.10</td>
<td>1.63±0.14</td>
<td>1.56±0.10</td>
<td>1.70±0.05</td>
</tr>
<tr>
<td>QRS duration, ms</td>
<td>11.2±0.29</td>
<td>11.2±0.18</td>
<td>11.1±0.37</td>
<td>11.3±0.20</td>
</tr>
<tr>
<td>LV CV Disp index</td>
<td>1.38±0.05</td>
<td>2.11±0.33</td>
<td>1.58±0.15</td>
<td>2.65±0.54</td>
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<tr>
<td>RV CV Disp index</td>
<td>1.48±0.02</td>
<td>1.81±0.13</td>
<td>1.50±0.00</td>
<td>2.13±0.13§</td>
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<tr>
<td>LV ERP, ms</td>
<td>82.7±5.9</td>
<td>64.7±5.5*</td>
<td>54.0±9.3*</td>
<td>70.0±6.5</td>
</tr>
<tr>
<td>RV ERP, ms</td>
<td>77.3±5.6</td>
<td>53.3±2.9†</td>
<td>52.0±6.6*</td>
<td>54.0±3.1*</td>
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<tr>
<td>Cx43 expression, %</td>
<td>2.98±0.80</td>
<td>1.54±0.27</td>
<td>1.50±0.36</td>
<td>1.56±0.40</td>
</tr>
<tr>
<td>Cx43 heterogeneity</td>
<td>23.7±1.49</td>
<td>31.6±2.26§</td>
<td>31.9±4.89</td>
<td>31.4±2.96</td>
</tr>
<tr>
<td>Fibrosis, %</td>
<td>1.10±0.21</td>
<td>7.38±1.27*</td>
<td>4.77±1.77</td>
<td>8.69±0.91*</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM unless otherwise specified. Cx43 indicates connexin43; VT-, Cx43Cre-ER(T)^R mice without arrhythmias; VT^+, Cx43Cre-ER(T)^R mice with arrhythmias; LV, left ventricle; CVa, longitudinal conduction velocity; RV, right ventricle; CVp, transversal conduction velocity; AR, anisotropic ratio; CV Disp, dispersion of conduction velocity; ERP, effective refractory period.

*P<0.05 vs Cx43fl/fl; †P<0.05 vs VT^+; §P<0.005 vs Cx43fl/fl; 6P<0.005 vs VT^+.

Aged Cx43fl/fl and Cx43Cre-ER(T)^R mice of both LV and RV during stimulation at a basic cycle length of 150 ms are shown in Figure 1B. Stimulation from the center of the grid resulted in anisotropic activation patterns determined by the fiber direction. Longitudinal CVs in both LV and RV were significantly slower in both aged Cx43fl/fl and aged Cx43Cre-ER(T)^R mice. In the transverse direction, conduction velocity (CVa) was significantly slower in RV of aged Cx43Cre-ER(T)^R mice compared with aged Cx43fl/fl mice (Table 1). Concomitantly, the anisotropic ratio was significantly higher in RV of aged Cx43Cre-ER(T)^R mice.

Interestingly, CVa in RV was significantly slower in Cx43Cre-ER(T)^R mice with arrhythmias (VT^+) compared with nonarrhythogenic aged Cx43Cre-ER(T)^R mice (VT^-), which can be appreciated in Figure 1B. The ERP was decreased in both LV and RV of Cx43Cre-ER(T)^R but was not different between VT^- and VT^+ (Table 1). Typically, activation maps of VT^- mice showed a higher heterogeneous activation pattern, with regions of local slow conduction. This was confirmed by using Western blot analysis (Figure 4B).

As expected, Cx43Cre-ER(T)^R hearts expressed approximately half of the amount of Cx43 compared with control, as determined by immunohistochemistry (52% of Cx43fl/fl, Figure 2A and Table 1) and Western blot (42% of Cx43fl/fl, Figure 2B). Cx43 expression levels were not different between VT^- and VT^+ (Figure 2A and 2B, Table 1). The expression pattern of Cx43 was heterogeneous in Cx43Cre-ER(T)^R hearts. Regions of low Cx43 were found adjacent to regions of normal Cx43 expression (Figure 2C, left), whereas the expression of N-cadherin remained homogeneous, indicating intact intercalated disks (Figure 2C, right). Cx43 heterogeneity, a relative value indicating variation in local expression of Cx43 over the sections, was determined. Aged Cx43Cre-ER(T)^R hearts had significantly higher heterogeneity values than aged Cx43fl/fl hearts. Heterogeneity in Cx43 expression was not different between VT^- and VT^+ (Table 1).

The presence of ventricular fibrosis was evaluated by histochemical analysis with picrosirius red staining. Age-related interstitial fibrosis was present in aged Cx43fl/fl hearts (Figure 2A). Interestingly, the amount of fibrosis was significantly higher in aged Cx43Cre-ER(T)^R hearts compared with Cx43fl/fl hearts (Figure 2D, Table 1). Moreover, the percentage of fibrosis in Cx43Cre-ER(T)^R VT^- hearts was almost 2-fold higher than in the VT^- group (Figure 2D), albeit statistically not significant (P=0.10).

Pressure Overload Mice With Reduced Cx43

In the model of physiological aging, the presence of reduced Cx43 resulted in excessive information of fibrosis and arrhythmias, suggestive that enhanced collagen deposition is caused by reduced Cx43 expression under circumstances of cardiac stress. Therefore, Cx43fl/fl and Cx43Cre-ER(T)^R mice were subjected to TAC or sham operation to quantify the effect of pathological stress and reduced Cx43 expression (in young animals) on the formation of fibrosis.

Sustained polymorphic ventricular tachyarhythmias were induced in 2 (18%) of 11 TAC-operated Cx43fl/fl hearts and 3 (33%) of 9 TAC-operated Cx43Cre-ER(T)^R hearts (Figure 3, NS). No arrhythmias were induced in sham-operated Cx43fl/fl and Cx43Cre-ER(T)^R hearts. An example of a sustained polymorphic VT is shown in Figure 3. All electrophysiological characteristics are summarized in Table 2.

Typical examples of Cx43- and N-cadherin-immunolabeled sections of sham- or TAC-operated Cx43fl/fl and Cx43Cre-ER(T)^R hearts are shown in Figure 4A. As expected, Cx43 expression was significantly lower (~50%) in sham-operated Cx43Cre-ER(T)^R hearts compared with sham-operated Cx43fl/fl hearts. The TAC operation did not affect Cx43 expression in Cx43fl/fl hearts but significantly decreased Cx43 expression in Cx43Cre-ER(T)^R hearts (Table 2), which was confirmed by using Western blot analysis (Figure 4B).

Hearts of sham-operated Cx43fl/fl and Cx43Cre-ER(T)^R mice displayed only low amounts of interstitial fibrosis (Figure 4A and 4C). Pressure overload by TAC surgery resulted in a significant increase in fibrosis in Cx43fl/fl hearts (Figure 4C). However, in TAC-operated Cx43Cre-ER(T)^R hearts, a significantly higher amount of fibrosis was detected (Figure 4A and 4C), indicating that pressure overload in the background of reduced Cx43 leads to enhanced fibrosis.

Fibroblast Proliferation and Activity

Both aged and TAC-operated Cx43Cre-ER(T)^R hearts showed more pronounced fibrosis compared with aged and TAC-operated Cx43fl/fl hearts. Enhanced fibrosis is due to enhanced collagen deposition, which may be caused by enhanced fibroblast proliferation, enhanced fibroblast activity, or both. There-
fore, we analyzed fibroblast proliferation and activity of TAC– and sham–operated Cx43fl/fl and Cx43Cre-ER(T)/fl hearts. Discoidin domain receptor 2 (DDR2) expression, specifically present at the cell membrane of fibroblasts in cardiac tissue, was evaluated by using Western blot analysis. Figure 5A shows that DDR2 expression was equal between Cx43fl/fl and Cx43Cre-ER(T)/fl hearts and that the TAC operation did not change DDR2 expression, suggesting that the number of fibroblasts remained unchanged.

Subsequently, the activity of fibroblasts was determined by expression of the procollagen peptide P1NP. Figure 5B shows low P1NP expression in sham–operated Cx43fl/fl and Cx43Cre-ER(T)/fl hearts. The TAC operation did not statistically alter P1NP expression in Cx43fl/fl hearts, although the expression tended to be somewhat more intense. However, P1NP expression was significantly increased in Cx43Cre-ER(T)/fl hearts after TAC surgery when compared with sham–operated hearts (Table 2). Comparable results were obtained for P3NP, another procollagen peptide (Figure 5C, Table 2).

Finally, we performed a quantitative polymerase chain reaction on COL1A2, the gene encoding for the α-2 chain of collagen type 1. Figure 5D shows that COL1A2 mRNA levels were comparable between sham–operated Cx43fl/fl and Cx43Cre-ER(T)/fl hearts. However, the TAC operation significantly increased COL1A2 mRNA levels in Cx43Cre-ER(T)/fl hearts.

Discussion
The main and novel finding of this study is that reduced expression of Cx43 leads to more excessive fibrosis during both physiological (aging) and pathophysiological (TAC) stress in mice because of increased activity of fibroblasts. Furthermore, heterogeneously reduced Cx43 expression, in concert with enhanced fibrosis, leads to an arrhythmogenic substrate as the result of dispersed conduction.

Fibroblast Proliferation and Activity
The increased collagen deposition during aging or after pressure overload is in agreement with previous results.\textsuperscript{15,19,27}
Interestingly, we showed that this increase in fibrosis is more prominent in Cx43Cre-ER(T)/fl compared with Cx43fl/fl hearts. As a possible mechanism for the enhanced fibrosis in stressed hearts with reduced Cx43 levels, we hypothesized that reduced Cx43 levels may alter cardiomyocyte/fibroblast and fibroblast/fibroblast communication, leading to increased fibroblast proliferation and/or activity. Previously, Zhang et al. showed that, in cultures of isolated adult murine ventricular fibroblasts, proliferation of fibroblasts was increased on decreasing levels of Cx43 expression. By using DDR2 as a selective marker for fibroblasts in the heart, our data showed that DDR2 expression was similar between Cx43Cre-ER(T)/fl and Cx43fl/fl hearts and that TAC surgery did not affect DDR2 levels, suggesting that the number of fibroblasts was equal among the groups. Apparently, the effect of decreased Cx43 expression on fibroblast proliferation in vivo differs from in vitro studies, possibly because of the presence of cardiomyocytes and/or contact inhibition.

To determine the activity of fibroblasts, we first measured expression of P1NP and P3NP, the propeptides of procollagen types I and III, respectively. The data showed a significant increase in P1NP and P3NP expression after the TAC operation, specifically in Cx43Cre-ER(T)/fl hearts. Furthermore, the COL1A2 mRNA level, encoding for the pro-α2 chain of procollagen type I, was significantly increased in Cx43Cre-ER(T)/fl hearts after TAC operation. These data indicate that an increased fibroblast activity, rather than an increased proliferation, accounts for the enhanced fibrosis in challenged Cx43Cre-ER(T)/fl hearts. Future research is required to unravel the exact mechanism by which reduced Cx43 levels can activate fibroblasts. A possible explanation could be found in a recent in vitro study by Bowers et al., showing that communication via Cx43 channels between cardiomyocytes and fibroblasts significantly influences cytokine production.

Although our data suggest that fibroblast activity (increased expression of procollagen), rather than proliferation (unchanged DDR2 levels), is related to the measured increase in fibrosis, we cannot exclude an altered proliferation of fibroblasts. More fibroblasts with less DDR2 per cell could still result in equal DDR2 levels and unchanged activity, but more fibroblasts could still produce the measured increase in procollagen. These data also do not answer the question of what triggers the increased deposition of collagen. This could equally well rely on a reduced gap junction coupling between fibroblasts, rather than a modified coupling between cardiomyocytes and fibroblasts.

Decreased Cx43 Expression Leads to Enhanced Fibrosis and Arrhythmias

Arrhythmogenic remodeled hearts are commonly hallmarkmed by a combined decrease in Cx43 and increase in collagen deposition. It is not clear, however, whether Cx43 expression and fibrosis are related and, if so, whether decreased Cx43 expression precedes the formation of fibrosis or is the resultant of fibrosis. In a previous study, we aged mice to 15 months. These mice had slightly increased levels of fibrosis, with normal levels of Cx43. The resultant was an electri-
junction remodeling precedes the process of collagen remod-
ifications regarding our current study can be drawn: (1) Gap
expression levels were normalized. Two important conclu-
resulted in reversal of gap junction expression, but not
enhanced interstitial fibrosis leads to separation of the myo-
mice31 prevents gap junction remodeling and leads to reduced
fibrosis after aging or aortic stenosis. These observations
strongly suggest that reduced levels of Cx43 trigger enhanced
fibrosis, not vice versa. This viewpoint is further supported
by the finding that our aged 22-month-old Cx43(fl/fl) control
mice have lower levels of Cx43 compared with 5-month-old
Cx43(fl/fl) mice, resulting in increased levels of fibrosis in the
aged mice (data not shown). Also, researchers have shown
that long-term inhibition of the renin-angiotensin-aldosterone
system of aging mice,2 cardiomyopathic hamsters,30 and TAC
mice13 prevents gap junction remodeling and leads to reduced
levels of fibrosis. The general mechanistic view is that
enhanced interstitial fibrosis leads to separation of the myo-
cardial fibers with subsequent reduction of gap junction
plaques. However, Qu and coworkers showed that treatment
of TAC mice with the aldosterone antagonist, spironolactone,
after gap junction remodeling and the formation of fibrosis
resulted in reversal of gap junction expression, but not
reversal of fibrosis. Furthermore, CV and gap junction
expression levels were normalized. Two important conclusions
regarding our current study can be drawn: (1) Gap
junction remodeling precedes the process of collagen remod-
eling and, in the case of reversal, gap junction remodeling
precedes the reduction of fibrosis. (2) Both reduced Cx43
expression and increased fibrosis are needed for conduction
slowing.

A strong argument supporting the first conclusion is found
in arrhythmogenic right ventricular dysplasia/cardiomyopa-
thy. The overt state of this disease is hallmarkd by reduced
Cx43 expression and prominent fibrosis, leading to arrhyth-
mias. However, the temporal evolution of the disease in the
concealed phase clearly starts with reduced expression of
Cx43, resulting from reduced desmosomal integrity, clearly
before the formation of fibrosis.12,13 The results of this study
imply that, potentially also in ARVD/C, reduced expression
of Cx43 triggers enhanced expression of collagen by the
fibroblasts and, thereby, furthers the course of ARVD/C.
Then, the combination of reduced Cx43 together with en-
hanced fibrosis will lead to the formation of the arrhythmo-
genic substrate (conclusion 2).

Several important remarks and considerations regarding
these conclusions should be made. First, although our general
viewpoint is that reduced Cx43 expression enhances the
formation of fibrosis, the finding that Cx43 expression is
further reduced in TAC–operated Cx43Cre-ER(T)/fl hearts, but
not in TAC–operated Cx43(fl/fl) hearts, indicates a reversed
effect of excessive levels of fibrosis on the expression of
Cx43. A plausible explanation for this finding, which requires
further investigation, might be that thick strains of collagen

Table 2. Electrophysiological and Tissue Characteristics of Sham– and
TAC–Operated Mice

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sham Group</th>
<th>TAC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cx43(fl/fl)</td>
<td>Cx43Cre-ER(T)/fl</td>
</tr>
<tr>
<td>Arrhythmias, %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LV CVt, cm/s</td>
<td>70.4±2.1</td>
<td>70.7±1.7</td>
</tr>
<tr>
<td>RV CVt, cm/s</td>
<td>71.5±2.3</td>
<td>70.2±3.0</td>
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<tr>
<td>LV CVt, cm/s</td>
<td>35.5±1.8</td>
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<tr>
<td>RV CVt, cm/s</td>
<td>42.2±2.1</td>
<td>42.3±3.2</td>
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<tr>
<td>LV AR, CVt/CVt</td>
<td>1.98±0.09</td>
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<td>RV AR, CVt/CVt</td>
<td>1.70±0.08</td>
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<tr>
<td>QRS duration, ms</td>
<td>9.9±0.14</td>
<td>10.2±0.15</td>
</tr>
<tr>
<td>LV CV Disp index</td>
<td>1.11±0.11</td>
<td>1.19±0.19</td>
</tr>
<tr>
<td>RV CV Disp index</td>
<td>1.01±0.01</td>
<td>1.06±0.06</td>
</tr>
<tr>
<td>LV ERP, ms</td>
<td>60.0±5.0</td>
<td>62.5±7.0</td>
</tr>
<tr>
<td>RV ERP, ms</td>
<td>57.3±6.6</td>
<td>57.8±6.2</td>
</tr>
<tr>
<td>Cx43 expression, %</td>
<td>4.34±0.60</td>
<td>2.14±0.25*</td>
</tr>
<tr>
<td>Cx43 heterogeneity</td>
<td>24.4±0.72</td>
<td>27.9±0.56</td>
</tr>
<tr>
<td>Fibrosis, %</td>
<td>0.42±0.06</td>
<td>0.41±0.04</td>
</tr>
<tr>
<td>PI NP, %</td>
<td>1.73±0.76</td>
<td>1.68±0.40</td>
</tr>
<tr>
<td>PNP, %</td>
<td>0.67±0.16</td>
<td>1.01±0.14</td>
</tr>
<tr>
<td>COL1A2</td>
<td>1.00±0.14</td>
<td>1.13±0.22</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM unless otherwise specified.
TAC indicates transverse aortic constriction; LV, left ventricle; CVt, longitudinal conduction velocity; RV, right ventricle; CVt, transversal conduction velocity; AR, anisotropic ratio; CV Disp, dispersion of conduction velocity; ERP, effective refractory period; Cx43, connexin43; COL1A2, collagen type 1 α2.

†P<0.05 vs Cx43(fl/fl) sham; †P<0.05 vs Cx43Cre-ER(T)/fl sham; †P<0.005 vs Cx43(fl/fl) sham; §P<0.005 vs Cx43Cre-ER(T)/fl sham; ‡P<0.005 vs Cx43(fl/fl) TAC; ¶P<0.05 vs Cx43(fl/fl) TAC.
disturb communication (and, thereby, the expression of communicating channels) between cardiomyocytes.

Second, there was a difference with previous studies using similar mice with a genetically induced 50% reduction in Cx43 expression or a combined 50% reduction in Cx43 and Nav1.5, in which impulse conduction was not (or hardly) disturbed. Interestingly, fibrosis was not found in these mice. In our study, aged and TAC–operated Cx43Cre-ER(T)/fl mice

Figure 4. Connexin43 (Cx43) expression and fibrosis in sham– and transverse aortic constriction (TAC)–operated mice. A, Typical pictures of Cx43 expression, N-cadherin staining, and overlay (top, 2nd and 3rd rows, respectively) and fibrosis (bottom row) in sham– and TAC–operated Cx43fl/fl and Cx43Cre-ER(T)/fl hearts. Bars represent 50 μm. B, Western blot analysis data on α-actinin and Cx43 of sham– and TAC–operated Cx43fl/fl and Cx43Cre-ER(T)/fl mice. C, The amount of collagen in sham– and TAC–operated hearts is quantified. *Statistically significant difference (P<0.05).
showed more fibrosis in the background of (physiological or pathological) stress only, in contrast to the previous studies (young animals, without stress). This strongly suggests that stress is a prerequisite for the excessive formation of fibrosis in Cx43Cre-ER(T)/fl mice. The lack of fibrosis in previous studies using heterozygous Cx43 mice probably accounts for the preserved conduction in these mice.

Another point that requires further attention is the actual contribution of a 50% reduction in Cx43 expression on the arrhythmogenesis in aged Cx43Cre-ER(T)/fl mice. The lack of fibrosis in previous studies using heterozygous Cx43 mice probably accounts for the preserved conduction in these mice.

The final remark is that our data do not exclude a possible role for other, unidentified changes in protein expression that might influence the susceptibility for arrhythmias. Potentially, the Cre-ER(T) system could directly alter protein expression, although secondary effects on conduction determinants of this inducible knockout system have, to our knowledge, not been described. On the other hand, previous studies with neonatal cardiomyocytes have suggested that changes in Cx43 expression can influence expression of other connexins and sodium current density. Although the indirect effects of a reduction in Cx43 may differ between neonatal and adult cardiomyocytes, possible changes in the expression of other conduction determinants could contribute to the arrhythmogenic phenotype of aged and TAC–operated Cx43Cre-ER(T)/fl mice.
Arrhythmogeneity

Aged Mice
In aged mice, ventricular tachycardias could only be induced in Cx43\textsuperscript{Cre-ERT\textsuperscript{T}/T} hearts. Tachycardias were monomorphic and based on anisotropic reentry. Several factors may be in favor of the high incidence of the reentry-based arrhythmias in the aged Cx43\textsuperscript{Cre-ERT\textsuperscript{T}/T} hearts: a heterogeneously reduced Cx43 expression with increased fibrosis, resulting in slower transverse conduction preferentially in RV; increased dispersed conduction; and a decreased ERP. This decreased ERP is in agreement with a previous study using senescent mice with enhanced fibrosis,	extsuperscript{1} which may be explained by a current-to-load mismatch due to uncoupling, supporting conduction at shorter coupling intervals, albeit at lower velocities.

TAC–Operated Mice
In TAC–operated mice, induced ventricular tachycardias were always polymorphic. The mechanism of the arrhythmias is not completely clear, but previous studies suggest that they are based on triggered activity.\textsuperscript{19} Increased ERP, indicative of prolonged action potential durations, and reduced cell-to-cell coupling are in favor of triggered activity. An increased ERP after TAC operation is in agreement with previous studies, and has been attributed to increased action potential duration because of a reduction in repolarizing potassium currents.\textsuperscript{19,35} Although a slowed and dispersed conduction, as the result of fibrosis and/or a heterogeneous reduction of Cx43, considerably increases the susceptibility for monomorphic ventricular tachycardias, based on anisotropic reentry, it hardly affects the vulnerability to triggered activity.\textsuperscript{36} This may explain why arrhythmia vulnerability was comparable between TAC–operated Cx43\textsuperscript{Cre-ERT\textsuperscript{T}/T} and Cx43\textsuperscript{fl/fl} hearts.

Clinical Relevance
This study shows that reduced levels of Cx43 promote the formation of fibrosis in stressed hearts. In several cardiac diseases, such as ARVD/C, gap junctional remodeling is already found in early stages, whereas fibrosis becomes more abundant during progression of the disease. Our data suggest that early normalization of Cx43 expression might, in part, prevent fibrosis formation, reducing the susceptibility to fatal arrhythmias.

Study Limitations
This study shows, in 2 different models, that, in the presence of stress, reduced levels of Cx43 result in more excessive fibrosis. Although our data suggest that an increased activity of fibroblasts underlies the enhanced fibrosis, technical limitations prevented us from unraveling the exact mechanism by which reduced Cx43 levels promote fibrosis. In addition, we have not determined whether the reduction of Cx43 in the cardiomyocytes is the actual cause for the observed phenotype or whether reduced Cx43 expression in the fibroblasts plays a major role. To unravel this issue, a mouse model with a cardiomyocyte-specific downregulation of Cx43 should be examined. Thorough analysis of the complete pathway of increased collagen deposition in both models may provide relevant insight in the formation of fibrosis in several cardiac disorders.

Acknowledgments
The P1NP antibody developed by Dr Heinz Furthmayr was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa, Iowa City.

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Disclosures
None.

References
Clinical Perspective

Collagen is an essential protein in the heart, which contributes to its structural organization and, together with gap junction proteins, determines the cell-to-cell coupling in the myocardium. Under normal conditions, the amount of collagen is <1% of the total tissue volume and there is a rigid balance between collagen synthesis and degradation. Cardiac structural remodeling, as occurs during physiological stress (aging) or pathophysiological stress (myocardial infarction), involves an increase in myocardial collagen deposition. This results in separation of cardiomyocytes, reduction in cell-to-cell communication, and impaired conduction of the electrical impulse, which potentially increases the vulnerability to tachyarrhythmias. This study shows that gap junction–mediated cell-to-cell coupling modulates the amount of collagen deposition in stressed hearts. Impaired cellular coupling results in greater collagen deposition, most likely because of enhanced fibroblast activity, which increases arrhythmia vulnerability in these hearts. These findings suggest that maintaining good cell-to-cell coupling in a stressed heart may help reduce collagen deposition and susceptibility to arrhythmias.
Reduced Cx43 Expression Triggers Increased Fibrosis Due to Enhanced Fibroblast Activity

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Data Supplement (unedited) at:
http://circep.ahajournals.org/content/suppl/2012/02/24/CIRCEP.111.966580.DC1
SUPPLEMENTAL MATERIAL

Materials and Methods

Animals

Cx43^{fl/fl} and Cx43^{Cre-ER(T)/fl} mice were generated as described previously\(^1\). For the experiments on aged animals, 18-21 months old Cx43^{fl/fl} (n=11) and Cx43^{Cre-ER(T)/fl} (n=15) mice were used. No abnormalities in phenotype were found, and HW/BW ratios, age and gender distribution were similar in the two groups (Supplemental Table S1).

Three months old Cx43^{fl/fl} (n=11) and Cx43^{Cre-ER(T)/fl} (n=9) mice were TAC-operated as described previously\(^2\). A gradient of \(~50\%\) was confirmed by Doppler echocardiography. Sham-operated Cx43^{fl/fl} (n=12) and Cx43^{Cre-ER(T)/fl} (n=10) mice were used as control. Animal experiments were performed in accordance with institutional guidelines for animal use in research.

Preparation of the Hearts and Ventricular Conduction

Mice were anesthetized by 2.5% isoflurane in oxygen. A 3-lead electrocardiogram was recorded and analyzed off-line as described previously\(^3\). Afterwards, the heart was excised, prepared and connected to a Langendorff perfusion setup as described previously\(^4, 5\). The hearts were continuously perfused with carbogen-gassed buffer of 37ºC, composed of (in mmol/L): NaCl 116, KCl 5, MgSO\(_4\) 1.1, Na\(_2\)HPO\(_4\) 0.35, NaHCO\(_3\) 27, glucose 10, mannitol 16 and CaCl\(_2\) 1.8.

Extracellular electrograms were recorded using a 208-point multiterminal electrode (16x13 grid, 0.5-mm spacing) of both the left and right ventricle of the heart as described previously\(^4\).

Recordings were made during stimulation (1 ms pulse duration, 2x diastolic stimulation threshold) from the center of the grid at a basic cycle length (BCL) of 150 ms. The effective refractory period (ERP), the longest coupling interval of the premature stimulus that failed to activate the entire heart,
was determined for each ventricle separately. Every sixteenth stimulus was followed by 1 premature stimulus. Starting at 140 ms, the coupling interval of the premature stimulus was reduced in steps of 10 ms until the ERP.

If spontaneous arrhythmias were absent, susceptibility for arrhythmias was provoked by programmed stimulation in the following sequence. First, 16 basic stimuli followed by 1 or 3 premature stimuli 5 ms longer than the locally determined ERP were applied. Next, if 1 or 3 premature stimuli failed to induce arrhythmias, 2-second burst pacing at the shortest possible cycle length was applied. Arrhythmias in mice were classified as sustained (>15 complexes followed stimulation), according to Lambeth Conventions 6.

Data Analysis

The moment of maximal negative dV/dt in the unipolar electrograms was selected as the time of local activation and determined with custom written software based on MatLab (2006, The MathWorks Inc., Natrick, MA) 7. Activation times were used to construct activation maps. Conduction velocities parallel (CV_L) and perpendicular (CV_T) to fiber orientation were determined from activation maps generated from BCL-pacing. Activation times of at least 4 consecutive electrode terminals along lines perpendicular to intersecting isochronal lines were used to calculate CVs. Anisotropic ratio was defined as CV_L/CV_T. Dispersion of conduction was assessed for both LV and RV using the method described by Lammers et al 8.

Immunohistochemistry and Histology

After electrophysiological measurements, hearts were rapidly frozen in liquid nitrogen and stored at -80°C. Coronal (4-chamber view) sections with a thickness of 10µm were taken from different levels of the hearts. After immunolabeling, sections were mounted in Vectashield (Vector Laboratories) and examined with a classic light microscope with epifluorescence equipment (Nikon Optiphot-2). To evaluate the presence of fibrosis, sections were fixed with 4% paraformaldehyde
(in PBS, 30 minutes at room temperature), stained with Picosirius Red and examined by light microscopy.

The amount of fibrosis and Cx43, P1NP and P3NP immuno-signals were determined using at least 6 randomly chosen pictures of each heart at 200x magnification. Blinded operators calculated Cx43, P1NP and P3NP expression as percentage of the total tissue using Image J 1.40g (2008, NIH, Bethesda, MD). Photomicrographs were transformed into RGB (i.e. Red Green Blue) stack, and true Cx43, P1NP or P3NP pixels were defined in the 256-leveled green channel using a minimal cut-off level. For fibrosis quantification, a comparable procedure was used, but now the range between 90 and 190 in the red channel was defined as true fibrotic tissue. Heterogeneity of Cx43 expression was determined using MatLab. Photomicrographs were transformed into 8-bit black (Cx43) and white (background) pictures (256 levels) with a cut-off level of 60. A custom written script in MatLab was used to assess for each black pixel the shortest distance to the next black pixel in a virtual circle around that pixel. The standard deviation of all shortest distances of all pixels was used as a measure of Cx43 heterogeneity.

**Western blotting**

Total cellular protein was isolated from 3 hearts of each group as described previously, and pooled. Equal amounts of protein (25µg/lane) of each sample were separated on 10% SDS-polyacrylamide gels and transferred by electrophoresis to nitrocellulose membrane (Biorad). Equality of protein transfer was assessed by Ponceau S staining. After first and second antibody incubation, immuno-reactivity was detected using the ECL chemiluminescence kit (Amersham). To quantify Cx43 protein expression, differences in specific protein concentrations were determined as follows. Processed films and Ponceau Red staining (gray scale scanned) were imported into ImageQuant software to measure separate protein band intensity. Unequal protein loading was corrected against total separated protein; the ratio Cx43/Ponceau Red signal intensity represents the actual Cx43 protein concentration of the different lanes.
**Antibodies**

The following antibodies were used: mouse monoclonal antibodies against Cx43 (1:250, Transduction Laboratories, used for Western blotting), N-cadherin (1:800, Sigma, Aldrich), α-actinin (1:1000, Sigma, Aldrich) and P1NP (1:250, DSHB, University of Iowa); rabbit polyclonal antibodies against Cx43 (1:250, Zymed, Invitrogen, used for immunohistochemistry) and P3NP (1:100, Millipore); a goat polyclonal antibody against DDR2 (1:100, Santa Cruz); Texas Red- and FITC-conjugated anti mouse or rabbit whole IgG (1:100, Jackson Laboratories) as secondary antibodies for immunohistochemistry; anti mouse or goat peroxidase (1:7000, Biorad) as secondary antibodies for Western blotting.

**Quantitative PCR**

Total RNA was isolated from frozen ventricular tissue using the Trizol procedure and subsequently treated with DNase I as described before \(^9\). First strand cDNA was prepared from 1 µg of RNA with Superscript II reverse transcriptase (Invitrogen) at 42 °C for 1h, using 6.7 µM oligo dT and 0.25 µg of random hexamers (Promeda) as starting primers.

After cDNA synthesis, expression of mouse collagen type 1α2 (COL1A2) was assessed by quantitative real-time PCR using TaqMan Gene Expression Assays with pre-designed probe and primers (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference.

**Statistics**

Two-group comparisons were performed using an unpaired t-test, multiple group comparisons using a 1-way analysis of variance (ANOVA) with Bonferroni post hoc analysis. Arrhythmia vulnerability was compared by a Fisher exact test. Values are given as mean ± SEM. \(P\leq0.05\) was considered as statistically significant. Data were analyzed using SPSS 15.0 (2006, SPSS Inc, Chicago, IL) software.
Supplemental Table S1

Statistics of heart and body weight, age and gender-distribution of aged and sham or TAC operated animals.

<table>
<thead>
<tr>
<th></th>
<th>BW (g)</th>
<th>HW (mg)</th>
<th>HW/BW (%)</th>
<th>age (months)</th>
<th>male (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged Cx43^{fl/fl}</td>
<td>33.9 ± 1.1</td>
<td>219 ± 13</td>
<td>0.68 ± 0.06</td>
<td>20.1 ± 0.7</td>
<td>55</td>
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<tr>
<td>Aged Cx43^{Cre-ER(T)/fl}</td>
<td>33.9 ± 1.4</td>
<td>202 ± 16</td>
<td>0.61 ± 0.03</td>
<td>20.7 ± 0.5</td>
<td>46</td>
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<td>Cx43^{fl/fl} sham</td>
<td>25.0 ± 1.1</td>
<td>142 ± 10</td>
<td>0.58 ± 0.02</td>
<td>6.6 ± 0.1</td>
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<td>Cx43^{Cre-ER(T)/fl} sham</td>
<td>26.0 ± 0.5</td>
<td>154 ± 6</td>
<td>0.59 ± 0.02</td>
<td>6.5 ± 0.2</td>
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<td>Cx43^{fl/fl} TAC</td>
<td>29.1 ± 1.4</td>
<td>262 ± 21*</td>
<td>0.90 ± 0.05*</td>
<td>6.7 ± 0.4</td>
<td>55</td>
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<tr>
<td>Cx43^{Cre-ER(T)/fl} TAC</td>
<td>25.8 ± 1.1</td>
<td>251 ± 21*</td>
<td>0.97 ± 0.08*</td>
<td>6.5 ± 0.2</td>
<td>63</td>
</tr>
</tbody>
</table>

BW, body weight; HW, heart weight. Data are mean ± SEM. * P<0.05 vs Cx43^{fl/fl} and Cx43^{Cre-ER(T)/fl} sham.
References Online Supplement


