Mechanical Coupling Between Myofibroblasts and Cardiomyocytes Slows Electric Conduction in Fibrotic Cell Monolayers

Susan A. Thompson, BS; Craig R. Copeland, BS; Daniel H. Reich, PhD; Leslie Tung, PhD

Background—After cardiac injury, activated cardiac myofibroblasts can influence tissue electrophysiology. Because mechanical coupling through adherens junctions provides a route for intercellular communication, we tested the hypothesis that myofibroblasts exert tonic contractile forces on the cardiomyocytes and affect electric propagation via a process of mechanoelectric feedback.

Methods and Results—The role of mechanoelectric feedback was examined in transforming growth factor-β–treated monolayers of cocultured myofibroblasts and neonatal rat ventricular cells by inhibiting myofibroblast contraction and blocking mechanosensitive channels. Untreated (control) and transforming growth factor-β–treated (fibrotic) anisotropic monolayers were optically mapped for electrophysiological comparison. Longitudinal conduction velocity, transverse conduction velocity, and normalized action potential upstroke velocity (dV/dt_{max}) significantly decreased in fibrotic monolayers (14.4±0.7 cm/s [mean±SEM], 4.1±0.3 cm/s [n=53], and 3.1±0.2% per ms [n=14], respectively) compared with control monolayers (27.2±0.8 cm/s, 8.5±0.4 cm/s [n=40], and 4.9±0.1% per ms [n=12], respectively). Application of the excitation-contraction uncoupler blebbistatin or the mechanosensitive channel blocker gadolinium or streptomycin dramatically increased longitudinal conduction velocity, transverse conduction velocity, and dV/dt_{max} in fibrotic monolayers (35.9±1.5 cm/s, 10.3±0.6 cm/s [n=17], and 4.5±0.1% per ms [n=14], respectively). Similar results were observed with connexin43–silenced cardiac myofibroblasts. Spiral-wave induction in fibrotic monolayers also decreased after the aforementioned treatments. Finally, traction force measurements of individual myofibroblasts showed a significant increase with transforming growth factor-β, a decrease with blebbistatin, and no change with mechanosensitive channel blockers.

Conclusions—These observations suggest that myofibroblast-cardiomyocyte mechanical interactions develop during cardiac injury, and that cardiac conduction may be impaired as a result of increased mechanosensitive channel activation owing to tension applied to the myocyte by the myofibroblast.

Key Words: arrhythmia • conduction • electrophysiology • myocardial infarction

Although cardiomyocytes are the undisputed functionally contractile cells in the myocardium, fibroblasts outnumber cardiomyocytes by nearly 2:1.1 Although it is well known that cardiac fibroblasts (CFs) produce and remodel the extracellular matrix in the heart,2 their involvement in cell signaling, heterocellular coupling, and mechanoelectric feedback, and their contribution to many pathological conditions, is becoming increasingly recognized.3 After injury, CFs contribute to wound healing by proliferating and differentiating into myofibroblasts, an α-smooth muscle actin (SMA)–expressing cell adept at contraction. Myofibroblasts use contraction and extracellular matrix remodeling to replace the necrotic myocardium with mature scar tissue after infarction;4–6 the formed scar tissue, consisting of extracellular matrix and persistent myofibroblasts, is electrically unexcitable and ultimately creates a substrate that is vulnerable to arrhythmias.

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A potent inducer of cardiac myofibroblast (CMF) differentiation both in vivo and in vitro is transforming growth factor-β (TGF-β).6 Expression of TGF-β remains low in the normal heart, but is markedly increased after cardiac injury.7 Sustained expression of TGF-β augments the conversion of fibroblasts to myofibroblasts and the contraction of myofibroblasts8 and ultimately contributes to myocardial fibrosis.9

Until recently, CFs were believed to act as passive electric insulators between myocytes, but new data suggest that fibroblasts play a more dynamic role in the electric activity of...
the heart. Fibroblast-myocyte electric coupling has been shown in vitro and in situ in regions of infarcted and healthy myocardium such as the sinoatrial node; this coupling enables fibroblasts to act as current sinks, short-range conductors, or even long-range conductors.\textsuperscript{10–12} Although electric coupling between myocytes and fibroblasts is suspected to be the culprit in slowing conduction velocity (CV) in fibroblast-supplemented models, a quantitative study on myocyte-fibroblast cell pairs showed that <10% of the 450 studied cell pairs expressed junctional connexin43 (Cx43).\textsuperscript{13} Furthermore, the limited amount of coupling found in situ suggests that fibroblasts may affect cardiomyocyte electrophysiology through a mechanism other than electric coupling.

Myofibroblast contraction is a crucial aspect of wound healing in injured tissues throughout the body,\textsuperscript{14} and contractile force permits cellular communication to be relayed through intercellular coupling.\textsuperscript{15} Therefore, we tested the hypothesis that mechanical coupling transmits the contractile force of myofibroblasts to myocytes, and that this interaction activates mechanosensitive channels (MSCs), which alter electrophysiological function and slow conduction. To conduct this study, we used an in vitro coculture model of neonatal rat cardiomyocytes and myofibroblasts stimulated by TGF-β, together with blockers of excitation-contraction coupling and MSCs.

Methods
An expanded Materials and Methods section is available in the online-only Data Supplement. In brief, 20-mm-diameter anisotropic monolayers of neonatal rat ventricular cells (NRVCs) were obtained by growing cells on parallel 20-μm-wide fibronectin lines formed by microcontact printing. Monolayers were treated with 5 ng/mL TGF-β for 48 to 72 hours to promote the CMF phenotype. Untreated (control) and TGF-β–treated (fibrotic) monolayers were compared by immunostaining for cardiac (troponin I; α-actinin) and fibroblast (prolyl-4-hydroxylase, SMA) markers and optically mapped with 10 μmol/L of the voltage-sensitive dye di-4-ANEPPS. Activation maps were obtained at 2-Hz pacing during constant superfusion (with bath volume exchange every 2 minutes); then, the excitation-contraction uncouplers blebbistatin or MSC blocker gadolinium or streptomycin was superfused over the monolayer to determine its impact on CV in the longitudinal (LCV) and transverse (TCV) directions, minimum cycle length before loss of 1:1 capture, incidence of spiral waves, pacing cycle length to initiate spiral waves, action potential duration, normalized upstroke velocity (dV/dt\textsubscript{max}), and conduction heterogeneity index in control and fibrotic monolayers. In subsequent experiments, a supplemented model was created in which CFs were separately pretreated with 5 ng/mL TGF-β for at least 48 hours and then added to patterned NRVC monolayers at a concentration of 300 000 to 400 000 cells per monolayer 24 hours before electrophysiological characterization. Fibroblasts were also transduced with Cx43 shRNA lentiviral particles containing a puromycin promoter. Two days later, cells stably expressing shRNA were isolated with puromycin, and Cx43 knockdown was confirmed with Western blots. As a negative control, fibroblasts were transduced with shRNA lentiviral particles encoding a scrambled shRNA sequence. The transduced myofibroblasts were treated with 5 ng/mL TGF-β for 48 hours, and 400 000 were added to control NRVC monolayers for subsequent electrophysiological analysis. Additionally, pure monolayers of untreated fibroblasts and TGF-β–treated myofibroblasts were characterized by immunostaining for actin, SMA, pan-cadherin, and Cx43; Cx43 levels were also quantified by Western blot. Traction forces of untreated fibroblasts and TGF-β–treated myofibroblasts were quantified by elastic micropost arrays of known stiffness.\textsuperscript{16} All data are expressed as mean±SEM. Two-tail Student t tests were performed for independent data such as fibrotic and control monolayers, and Wilcoxon signed-rank tests were performed for paired data such as fibrotic monolayers before and after treatment to determine statistically significant differences (P<0.05).
Figure 2. Characterization of electrical and mechanical coupling between cells. Magnified view of the heterocellular contact between either a cardiomyocyte and fibroblast (A) or a cardiomyocyte and myofibroblast (B) in a low-cell-density monolayer stained for α-smooth muscle actin (SMA; red), troponin I (blue), connexin43 (Cx43; white), and pan-cadherin (green). Even though the fibroblast in A has substantial Cx43 staining, there appear to be only 3 sites of junctional connexin between the 2 cells (red arrows) and little junctional cadherin. Gap junctions between myofibroblasts and cardiomyocytes are scarce, as indicated by the limited junctional Cx43 staining (white arrow), whereas adherens junctions are more prominent as visualized by the intense cadherin staining at the border between the 2 cells (B). Homocellular mechanical and electrical couplings between fibroblasts (C) and myofibroblasts (transforming growth factor-β [TGF-β] treated; D) were visualized with antibodies against SMA (red), actin (blue), Cx43 (white), and pan-cadherin (green). Monolayers of fibroblasts have an abundance of Cx43-containing gap junctions compared with cadherin-containing adherens junctions (C). Conversely, myofibroblasts treated with TGF-β have intense junctional cadherin expression and no apparent junctional Cx43 expression (D). A mixed population of fibroblasts and myofibroblasts showed preferential Cx43 and cadherin staining in localized regions of fibroblasts and myofibroblasts, respectively (E). Representative Western blots of the insoluble (F) and soluble (G) protein fractions show that control fibroblast monolayers have significantly higher insoluble Cx43 than TGF-β-treated myofibroblast monolayers, but significantly lower soluble Cx43, normalized to the loading control vimentin (n=10 and n=4, respectively; P<0.05).
The first goal of this project was to create an in vitro model that mimicked tissue-level aspects of cardiac fibrosis, including an excess of contractile myofibroblasts and slowed, heterogeneous conduction, compared with controls. Transforming growth factor-beta (TGF-β) was added to cocultured monolayers of cardiomyocytes and CFs. Several concentrations (2.5, 5, and 10 ng/mL) of TGF-β were tested to determine the dose-response effect on CV; 5 ng/mL was chosen for all subsequent experiments because it was the lowest dose that resulted in a significant and reproducible effect on CV. To mimic physiological conditions for all subsequent experiments, NRVCs were grown as anisotropic monolayers to evaluate their LCV and TCV. Anisotropic monolayers incubated with TGF-β are referred to as fibrotic monolayers, and untreated anisotropic monolayers are referred to as control monolayers.

Transforming growth factor-beta-induced fibroblast proliferation was signified by an increased number of cells positive for prolyl-4-hydroxylase (Figure 1A and 1D). Fibroblast density was much higher in fibrotic monolayers compared with control monolayers (70±6% versus 16.4±2%, respectively; n=6; P<0.05), and fibroblast size was much larger compared with that in control monolayers (620±46 μm² per cell versus 240±19 μm², respectively; n=17; P<0.05). Myofibroblast conversion was evaluated by SMA stress-fiber expression; untreated fibroblasts in control monolayers expressed limited amounts of SMA, whereas TGF-β induced a dramatic upregulation of SMA-expressing myofibroblasts (Figure 1). Z-stack images revealed that myofibroblasts were directly in contact with the cardiomyocytes, most often beneath the myocytes, which allowed the cells to interact in the z direction, and in the lateral spaces between the cardiomyocytes (Figure 1D through 1F). Furthermore, purified myofibroblasts treated with TGF-β had more organized SMA stress fibers (Figure 1B in the online-only Data Supplement) than control fibroblasts (Figure 1A in the online-only Data Supplement). Overall, TGF-β treatment increased the fraction of fibroblasts expressing SMA stress fibers from 49% in untreated fibroblasts to 81% (n=795 for control, n=350 for TGF-β treated; P<0.05).

Results

Treatment With Transforming Growth Factor-β Induces a Fibrotic Neonatal Rat Ventricular Cell Model

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Fibrotic monolayers (n=53) had significantly slower LCV and TCV (14.4±0.7 and 4.1±0.3 cm/s, respectively) compared with control monolayers (n=40; 27.2±0.8 and 8.5±0.4 cm/s, respectively; Figure 1G and 1H). Furthermore, fibrotic monolayers (n=17) had a significantly larger heterogeneity index of propagation (1.8±0.1) than control monolayers (n=14; 1.2±0.1; Figure 1I).

Mechanical Adherens Junctions Dominate Homocellular and Heterocellular Junctions Involving Myofibroblasts

Because coupling occurs between cardiomyocytes and fibroblasts in vitro,13 evidence for electric junctions (Cx43) and mechanical junctions (pan-cadherin) was examined between the 2 cell types. Heterocellular contacts between adjoining fibroblasts and myocytes in a control monolayer had some Cx43 expression, with a considerable amount of nonjunctional Cx43, and little cadherin expression (Figure 2A). However, contacts between adjoining cardiomyocytes and myofibroblasts in a fibrotic monolayer showed that cadherin was expressed more prominently than Cx43 (Figure 2B). In pure monolayers of CFs or CMFs, CFs expressed more junctional Cx43 (Figure 2C and 2E), whereas CMFs expressed more junctional cadherin (Figure 2D and 2E). Both control and fibrotic monolayers were also stained for Cx45, but little expression was observed (Figure II in the online-only Data Supplement). Connexin43 Western blots of the insoluble (Figure 2F) and soluble (Figure 2G) protein fractions revealed that TGF-β-treated myofibroblasts had significantly less insoluble Cx43 than untreated fibroblasts but significantly more soluble Cx43 (n=10 and n=4, respectively; P<0.05).

Inhibition of Contractile Force Restores Conduction

In fibrotic monolayers, the increased presence of myofibroblasts capable of exerting strong contractile forces had functional consequences. To test the hypothesis that myofibroblast contraction influences the conduction properties of the cardiomyocytes, blebbistatin was added to inhibit myosin II and to suppress contraction.17 Blebbistatin (5 to 10 μmol/L for 20 to 30 minutes) significantly increased CV in fibrotic monolayers but had no significant effect on control monolayers (Figure 3).

Mechanosensitive Channel Blockers Improve Conduction in Fibrotic Monolayers

Because contractile force has a significant effect on electric conduction, mechanoelectric coupling was investigated. After 20 to 30 minutes of treatment with an MSC blocker, gadolinium (20 to 50 μmol/L) or streptomycin (50 μmol/L), LCV and TCV increased significantly in fibrotic monolayers, but not in control monolayers (Figure 3). Conduction nonuniformity was common among fibrotic monolayers, as illustrated by the skewed elliptical pattern in Figure 3A and 3B, and was most likely the result of a heterogeneous density of myofibroblasts in culture, which ranged from 45% to 85%. Blebbistatin, gadolinium, and streptomycin produced more elliptical patterns of propagation and increased the uniformity of conduction in fibrotic monolayers (n=10), decreasing the heterogeneity index from 1.8±0.1 to 1.3±0.1 (P=10−3).

Inhibition of Contraction and Mechanosensitive Channels Increases Upstroke Velocity and Shortens Action Potential Duration in Fibrotic Monolayers

In addition to slowed, discontinuous conduction, fibrotic monolayers also have slowed dV/dt max compared with control monolayers (3.1±0.2 [n=14] versus 4.9±0.1 per ms [n=12], respectively; P=10−6), which increased after application of blebbistatin, gadolinium, or streptomycin (to 4.5±0.1% per ms [n=14]; Figure 4A and 4B). Calculations indicated that some but not all of the changes in dV/dt max could be accounted for by changes in CV. The action potential duration was also prolonged in fibrotic monolayers (186±4 [n=16] versus 148±7 [n=8] milliseconds; P=10−5 in control monolayers) and shortened significantly in response to the aforementioned drug treatments (to 161±4 milliseconds [n=10]; Figure 4A and 4C).

Supplemented Model Corroborates Results With Fibrotic Model

In the experiments described so far, TGF-β was added directly to a mixed population of fibroblasts and cardiomyocytes, which may affect the electrophysiology of the cardiomyocytes directly. To eliminate this potentially confounding effect, CMFs were separately treated with TGF-β and then added to untreated anisotropic NRVC monolayers (supplemented model). Untreated CFs were also added at similar numbers to untreated monolayers for comparison. The addition of CMFs or CFs decreased CV compared with control, although CMFs had a more dramatic effect than CFs, decreas-
CFs. 

$$dV/dt_{\text{max}}$$ decreased from 4.9 cm/s to 2.1 cm/s (n=26), compared with a decrease to only 1.3 cm/s (n=8; P<0.001; **significant difference from control and CF monolayers, P<0.001). C, Isochrone maps (10-millisecond spacing) comparing CMF-supplemented monolayers before and after treatment with gadolinium at 2-Hz pacing. Summary LCV and TCV line graphs of drug treatments for both CMF- (D) and CF- (E) supplemented monolayers. Blebbistatin (n=6) and mechanosensitive channel blockers (n=12) significantly increased LCV and TCV in CMF-supplemented monolayers (D) and, to a lesser extent, in CF-supplemented monolayers (n=3 and 10, respectively; E). *Significance after treatment, P<0.05.

**Connexin43 shRNA Myofibroblasts Retain Slowing Effects on Neonatal Rat Ventricular Cell Monolayers**

To ensure a minimal degree of electric coupling between myofibroblasts and myocytes, Cx43 was knocked down in myofibroblasts and transverse (TCV; B) conduction velocity (CV) summary dot plots show that both the addition of cardiac fibroblast (CFs; n=17) and cardiac myofibroblasts (CMFs; n=26) to neonatal rat ventricular cell monolayers decreased CV compared with control monolayers (n=26) and that CV was significantly lower in CMF-supplemented monolayers compared with CF-supplemented monolayers. *Significant difference from control monolayers, P<0.001; **significant difference from control and CF monolayers, P<0.001.

Figure 5. Drug effects on supplemented fibrotic model. Longitudinal (LCV; A) and transverse (TCV; B) conduction velocity (CV) summary dot plots show that both the addition of cardiac fibroblast (CFs; n=17) and cardiac myofibroblasts (CMFs; n=26) to neonatal rat ventricular cell monolayers decreased CV compared with control monolayers (n=26) and that CV was significantly lower in CMF-supplemented monolayers compared with CF-supplemented monolayers. *Significant difference from control monolayers, P<0.001; **significant difference from control and CF monolayers, P<0.001. C, Isochrone maps (10-millisecond spacing) comparing CMF-supplemented monolayers before and after treatment with gadolinium at 2-Hz pacing. Summary LCV and TCV line graphs of drug treatments for both CMF- (D) and CF- (E) supplemented monolayers. Blebbistatin (n=6) and mechanosensitive channel blockers (n=12) significantly increased LCV and TCV in CMF-supplemented monolayers (D) and, to a lesser extent, in CF-supplemented monolayers (n=3 and 10, respectively; E). *Significance after treatment, P<0.05.

Taking together, our results support the notion that the addition of TGF-β increases the contractile activity of CMFs, subsequently producing a greater impact on the electrophysiological function of NRVC monolayers.
Contractile Force of Myofibroblasts

Contractors of individual CFs were analyzed with elastic micropost arrays. Some of the CMFs treated with blebbistatin experienced actin destabilization (Figure 7C), which normally facilitates contraction compared with control CMFs (E = 251 ± 25 fJ [n = 17] and 228 ± 23 fJ [n = 20], respectively; P = 0.5; Figure 7C), confirming the presence of contractile myofibroblasts in the supplemented Cx43 knockout model.

Discussion

After an acute injury to the heart, as in myocardial infarction, fibroblasts participate in the wound healing process. Because of the limited capability of cardiomyocytes to regenerate, wound healing concludes with loss of ventricular muscle and formation of a stable scar, ultimately leading to a fibrotic,
arrhythmogenic substrate. The myofibroblast repairs the injured region through the formation of granulation tissue, using contraction to reduce the total scar volume. It is well known that mechanoelectric coupling among cardiac cells, the process in which mechanical perturbations alter electric activity, exists in the intact heart and in cardiac cell cultures. Cardiomyocytes also possess MSCs, which have an open probability that increases with stretch and a mechanical sensitivity that increases after myocardial infarction. Thus, we tested the hypothesis that increased vulnerability to arrhythmias after cardiac injury could be related to a tonic tugging force that myofibroblasts exert on the cardiomyocyte membrane through mechanical coupling and lead to conduction slowing and block through the action of MSCs.

We used TGF-β in our fibrotic models to induce fibroblast differentiation to contractile myofibroblasts. Although fibroblasts in culture transition to proto-myofibroblasts that express some stress fibers, incubation with TGF-β increases complete differentiation to myofibroblasts, expression of SMA, and generation of strong contractile forces. Thus, we tested the hypothesis that increased vulnerability to arrhythmias after cardiac injury could be related to a tonic tugging force that myofibroblasts exert on the cardiomyocyte membrane through mechanical coupling and lead to conduction slowing and block through the action of MSCs.

With TGF-β-treated myofibroblasts suggest that conduction slowing is exacerbated well beyond that obtained with supplementation with untreated fibroblasts (Figure 5A and 5B), without a concordant increase in Cx43 expression (Figure 2F and 2G).

The recent perspective that fibroblasts may have an active electric influence on cardiac electrophysiology has been bolstered by discoveries of in vitro and in situ gap junctional coupling between CFs and cardiomyocytes and by characterization of multiple fibroblast ion channels, including K_ATP channels, Na+/Ca2+ exchanger, and MSCs. Numerous studies have focused on the potential electrophysiological consequences of electric coupling between fibroblasts and cardiomyocytes, and the general dogma of these studies is that, through electric coupling, fibroblasts actively depolarize resting cardiomyocytes because of their more positive resting membrane potential.

However, in our studies, immunolabeling for Cx43 and pan-cadherin of fibroblast-only cultures suggests a different mechanism by which myofibroblasts and cardiomyocytes can interact. We found that SMA-negative fibroblasts expressed abundant Cx43, whereas SMA-positive myofibroblasts had little expression but enhanced pan-cadherin expression (Figure 2C through 2E), suggesting that myofibroblasts form strong mechanical rather than electrical junctions. However, TGF-β-treated myofibroblasts still had a significant amount

![Figure 7](http://circ.ahajournals.org/figs/2011/circ.11.1.2090.F7.large.jpg)

**Figure 7.** Drug effects on contraction in cardiac myofibroblasts. Vector plots of traction forces at each post generated from an untreated cardiac fibroblast (A) and a transforming growth factor-β (TGF-β)-treated cardiac myofibroblast (B). Cumulative distribution from the same culture of fibroblasts (C) shows that at any given strain energy per cell, TGF-β treatment increases the proportion of cells imparting at least that energy to the posts (n=36 for untreated fibroblasts and n=42 for TGF-β-induced myofibroblasts). Mechanosensitive channel (MSC) blockers, streptomycin (n=9) or gadolinium (n=10), had no significant effect on the average myofibroblast strain energy (D), whereas blebbistatin (n=10) significantly reduced the average strain energy by 74% (E). Cumulative distribution shows that at any given energy per cell (E), control myofibroblasts and connexin43 shRNA myofibroblasts have a similar fraction of cells imparting an energy $E_{CELL}$ that is at least E to the posts (F). *Significance after treatment, $P<0.05$. 

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of soluble Cx43 (Figure 2G), indicating that Cx43 is still present in the cytoplasm. This finding is consistent with recent evidence demonstrating that Cx43 is necessary for fibroblast differentiation into myofibroblasts. In heterocellular contacts between adjoining cardiomyocytes and myofibroblasts, we also found that adherens junction expression dominated over gap junction expression (Figure 2B). Our key finding that myofibroblast-induced slowing of conduction could be restored with contraction or MSC blockers (Figures 3 and 5) was unchanged with Cx43 silencing in the myofibroblasts (Figure 6). Furthermore, given that the changes in myofibroblast contractile force (Figure 7) paralleled the changes in CV brought about by both TGF-β and blebbistatin (Figures 1, 3, and 5), and considering the plethora of work demonstrating the significance of myofibroblast contraction in other tissues, we believe that mechanical coupling between myofibroblasts and cardiomyocytes is an important factor contributing to conduction slowing in cocultures of these 2 cell types. We suggest that this form of mechanical signaling can activate MSCs, which depolarize the cardiomyocyte membrane, thereby inactivating the Na⁺ channels that drive conduction. Furthermore, heterogeneity in the distribution of myofibroblasts can accentuate spatial gradients in conduction and action potential duration, leading to increased propensity to reentrant arrhythmias, which again may be suppressed by MSC or contraction blockers (Figure 8). As a caveat, we cannot rule out the possibility of paracrine signaling between myofibroblasts and cardiomyocytes as a means of modulating cardiac conduction, although the continuous exchange of solution in our experimental setup suggests that paracrine factors do not play a dominant role.

We believe that our in vitro model, consisting of longitudinally patterned myocytes and adjacent contractile SMA myofibroblasts, retains several key aspects of the environment in a healing infarct. Previous studies have shown that both SMA-positive myofibroblasts and TGF-β are markedly expressed during infarction and can remain elevated for several months to years in the infarct border zone. Furthermore, although numerous studies have shown that Cx43 is downregulated and redistributed after infarction, cadherin expression levels and distribution remain unaffected or are downregulated to a lesser degree compared with Cx43. On the other hand, our results are specific to in vitro conditions, and it remains to be seen whether they extrapolate to the in vivo heart in light of known differences between the 2 environments.

First, our cells are maintained under culture conditions (culture media, 2-dimensional rigid substrate, lack of hemodynamic loading), which can affect cell shape, protein expression, and cell function. Second, the spatial distribution of heterocellular gap junctions and adherens junctions in cell monolayers may
differ significantly from that found in vivo. Although myofibroblast-myocyte junctions have not been characterized in the intact heart, in normal myocardium, myocyte-myocyte junctions occur primarily within intercalated disks, whereas in cell culture they are distributed around the cell perimeter, although in infarcted myocardium the distribution also tends to occur around the cell perimeter. If future studies confirm our findings in infarcted regions of the heart in vivo, it is plausible that MSC blockers or fibroblast-specific contraction inhibitors may provide a means to reduce the incidence of arrhythmias.

Conclusions

Our data demonstrate that impaired conduction in an in vitro fibrotic model is mechanically dependent. Furthermore, our findings support the current view that myofibroblasts are capable of actively decreasing conduction among cardiomyocytes, and suggest that mechanical coupling between myofibroblasts and cardiomyocytes can play a more prominent role in this regard than electric coupling. Finally, we propose a novel mechanism in which myofibroblasts may impair cardiomyocyte electrophysiological function through the application of contractile force to the cardiomyocyte membrane and activation of MSCs.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Myocardial infarction engages a fibrotic process in which myofibroblasts secrete extracellular matrix proteins to replace the injured tissue. The contractile properties of myofibroblasts help to ensure a smaller and stronger scar area and to preserve mechanical function. However, in the infarct border zone, arrhythmias are prone to initiate owing to slowed and heterogeneous conduction. Although fibrosis has classically been considered arrhythmogenic because of the creation of an inexcitable region, together with zigzag conduction in the border zone, we tested the hypothesis that myofibroblasts can actively influence electrophysiological function through mechanoelectric coupling to the cardiomyocytes. In this study, impaired electric conduction in cocultured monolayers of myofibroblasts and cardiomyocytes can be dramatically improved by applying an excitation-contraction inhibitor or mechanosensitive channel blockers. Our findings advocate a novel mechanism whereby cardiac myofibroblasts exert tension on the myocyte membrane, which leads to slowed and heterogeneous electric conduction through the action of mechanosensitive ion channels. Provided that these in vitro results are corroborated in the intact heart, inhibition of this form of mechanoelectric interaction in the heart may be a way to decrease susceptibility to arrhythmias.
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Supplement Material

Expanded Methods

Microcontact printing. Photolithography was used to make a master pattern of 20\(\mu\)m wide lines separated by 10\(\mu\)m spacing on a silicon wafer.\(^1\) Polydimethylsiloxane (PDMS) stamps were made by combining the elastomer base and curing agent in a 10:1 ratio (Dow Corning, Midland, MI), pouring PDMS onto the master wafer, degassing to remove bubbles and baking overnight at 60°C. The PDMS was then peeled from the master wafer, and the individual patterns were punched into usable stamps. To prepare the cell substrate, 20-mm diameter glass coverslips (Bioscience Tools, San Diego, CA) were spin-coated with PDMS and sterilized by UV-exposure. Microcontact printing was accomplished by coating the PDMS stamps with 600\(\mu\)l of fibronectin dissolved in deionized water (50\(\mu\)g/ml) for at least 1 hour. Stamps were then rinsed lightly in deionized water, dried with nitrogen, and then gently pressed onto the prepared coverslips for at least an hour. Patterned coverslips were gently peeled away with tweezers and submersed in phosphate-buffered saline (PBS) until plating. The patterned surface allows the cells to grow as 20\(\mu\)m strands, which ultimately creates an anisotropic monolayer. Because the gap between strands is only 10\(\mu\)m, the cells are able to bridge the gaps periodically.

Cell culture. All animal experiments were performed in accordance with guidelines set by the Johns Hopkins Committee on Animal Care and Use and were in compliance with all federal and state laws and regulations. Neonatal rat ventricular cells (NRVCs) were enzymatically dissociated from the hearts of 2-day-old Sprague-Dawley rats (Harlan, Indianapolis, IN) with the use of trypsin (Amersham Biosciences, Piscataway, NJ) and collagenase (Worthington Biochemical Corporation, Freehold, NJ) as previously described.\(^2\) Freshly isolated NRVCs were
resuspended in M199 culture medium supplemented with 10% fetal bovine serum, glucose, L-
glutamine, penicillin, vitamin B12, HEPES buffer and MEM non-essential amino acids. Two 60-
minute pre-plating steps were performed to reduce fibroblasts and enrich cadriomyocyte content
in the culture, although the purified cell population still contained 15-25% fibroblasts by number,
but only approximately 5% by volume. One million NRVCs were added to each of the patterned
glass coverslips to form confluent monolayers. For some immunocytochemistry experiments,
only 200,000 cells were plated to improve visualization of the junctions between cells. Day 0 of
culture is defined to begin at the time of NRVC plating. After 24 hours, the monolayers were
washed with warm PBS, and fresh medium with 10% serum was added. Serum was reduced to
2% on Day 5 to inhibit non-cardiomyocyte proliferation.

Purified cardiac fibroblasts were obtained from the pre-plating steps and passaged 1-2
times to enrich fibroblast content. Fibroblasts were then trypsinized and plated on fibronectin
(25µg/ml) coated glass coverslips for subsequent analysis.

_Fibrosis Models._ On Day 3, 5 ng/mL of TGF-β (R&D systems, Minneapolis, MN), sometimes
supplemented with 100 µg/ml of sodium ascorbate (Sigma, St. Louis, MO), was added to NRVC
monolayers for 48 hours to increase fibroblast proliferation, conversion to myofibroblasts and
myofibroblast contraction.

In subsequent experiments, cardiac fibroblasts were pre-treated with TGF-β separately
for at least 48 hours to induce their transition to myofibroblasts. A subset of fibroblasts was left
untreated to serve as controls. Twenty-four to 48 hours prior to electrophysiological
characterization (Day 4/5), fibroblasts and TGF-β treated myofibroblasts were trypsinized,
counted and plated on top of patterned, non-TGF-β-treated monolayers at a concentration of
300,000 to 400,000 cells per monolayer. A subset of NRVCs were irradiated prior to plating to
prevent resident fibroblast proliferation before addition of exogenous fibroblasts and myofibroblasts. Similar results were obtained with and without irradiation.

**shRNA transduction.** Isolated fibroblasts were passaged once and plated at a density of 30,000 per well in a 12-well plate in antibiotic-free 10% FBS media. The next day Cx43 and control shRNA lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the media at an MOI of 1 and supplemented with 10 μg/mL of polybrene. The following day, the media was changed and 5 μg/mL puromycin was added 48 hours after transduction to select for fibroblasts infected with the virus. Selection continued for 3 days, and 48 hours before plating the fibroblasts, 5 ng/ml TGF-β was also added to the media to induce differentiation to myofibroblasts. Twenty-four hours before electrophysiological analysis, fibroblasts were trypsinized and either added to control NRVC monolayers at a concentration of 400,000 per monolayer or replated and lysed for western blot analysis.

**Immunocytochemistry.** Six-day-old control and TGF-β monolayers were analyzed for their expression of α-smooth muscle actin (Dako, Glostrup, Denmark or Abcam, Cambridge, MA), α-actinin (Abcam, Cambridge, MA), prolyl-4-hydroxylase (Millipore, Billerica, MA), troponin I (US Biological, Swampscott, MA), pan-cadherin (Abcam, Cambridge, MA), Cx43 (Sigma, St. Louis, MO), and Cx45 (Millipore, Billerica, MA) in cardiac fibroblasts and cardiomyocytes. Purified monolayers of fibroblasts were also stained with Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA). Cultured NRVCs were washed with PBS and fixed in methanol for 10 minutes at -20°C or 3.7% paraformaldehyde for 15 minutes at 25°C. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes and blocked using 10% goat serum in PBS for 25 minutes at room temperature. Primary antibodies were diluted 1:200 in blocking buffer, except antibodies against cadherin and SMA, which were diluted 1:100. Cells were incubated with primary antibodies for 1
hour at room temperature or overnight at 4°C and washed afterwards with TBS-T (10% TBS and 0.05% Tween 20 in deionized H₂O) 3 times for 10 minutes each. Cells were then incubated with Alexa Fluor-conjugated goat secondary antibodies (1:200, Invitrogen, Carlsbad, CA) for 1 hour at room temperature, followed by DAPI (30µM) for 15 minutes, and washed again 3 times with TBS-T. Finally, cells were mounted in Pro Long Gold Antifade (Invitrogen, Carlsbad, CA) on microscope slides and imaged with a confocal microscope (Zeiss LSM 510 Meta).

To quantify fibroblast proliferation and cell size, TGF-β treated and control NRVC monolayers were stained for nuclei (DAPI), cardiac (troponin I or α-actinin), and fibroblast/myofibroblast (prolyl-4-hydroxylase or SMA) markers. Images were analyzed in ImageJ by counting the nuclei of cells positive for prolyl-4-hydroxylase and measuring the area occupied by each fibroblast. Total fibroblast density was determined by taking the total fibroblast area and dividing it by the total area of the image. SMA expression was also analyzed in ImageJ by counting the nuclei positive for SMA stress fiber formation in both control and TGF-β treated fibroblast-only monolayers.

**Western blot.** To compare connexin43 levels between untreated fibroblasts and TGF-β treated myofibroblasts, the insoluble protein fraction was obtained from both cell types by first extracting soluble cytosolic proteins by incubating the monolayers in ice cold extraction buffer (50 mM Tris, 150 mM NaCl, 0.02% sodium azide, 1.0 mM PMSF, 1µg/ml Aprotinin, 0.2% Triton X-100, 1 mM Na₃VO₄, 50 mM NaF, and complete protease inhibitor (Roche, Basel, Switzerland)) twice for 5 minutes each time. The remaining insoluble proteins were then scraped from sample monolayers with 0.5% Triton X-100 extraction buffer, sonicated and incubated on ice for 30 minutes. The Bradford assay was completed to assess the amount of total protein in each sample, and equal amounts of protein were loaded with 5X loading buffer and 20X reducing agent (Fermentas, Ontario, Canada). Protein extracts were loaded into a 10% BioRad Ready
Gel with an XP ladder for size visualization and run at 50mA for 1 hour in running buffer (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred to a PVDF membrane at 300mA for 100 minutes in transfer buffer (Bio-Rad Laboratories, Hercules, CA) at 4°C. The membrane was dried overnight and blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour. Cx43 (Sigma, St. Louis, MO) primary antibody was diluted in Odyssey Blocking Buffer (1:1000), and loading control, vimentin (Dako, Glostrup, Denmark), was diluted 1:5000. The membrane was washed four times for 5 minutes each time in PBS with 0.1% Tween 20. The membrane was then incubated with the appropriate secondary antibodies in Odyssey Blocking Buffer (1:10,000). The membrane was washed 4 times for 5 minutes each time and imaged with Odyssey Infrared Imaging Station.

Mechanical contraction. Micropost array masters consisting of 4µm tall, 1.83µm diameter posts in a hexagonal close packed pattern with 4µm spacing were created in silicon wafers using deep reactive ion etching (gift from C. S. Chen, University of Pennsylvania). The master was cast in PDMS to create negative molds which were then used for fabrication of PDMS post arrays via replica molding. The post arrays were then chemically functionalized as previously described.4, 5

Untreated fibroblasts and TGF-β treated (5ng/ml for 48-72 hours) myofibroblasts were grown to confluence and then fluorescently labeled in a 5µM solution of CellTracker Green CMFDA (Invitrogen, Carlsbad, CA) for 45 minutes, followed by a 1 hour recovery period. Cells from each group were then trypsinized, plated onto post arrays, and left to attach and spread overnight (approximately 18 hours). Fluorescence images of single isolated cells and the underlying post array were taken on a heated stage using a Nikon Eclipse TE2000-E inverted microscope with epifluorescence attachment. Cellular traction forces on individual posts $F_j = k\delta_j$ were calculated from the measured deflections $\delta_j$ of the underlying post tops and the posts’
effective spring constant $k = 22.3 \text{ nN/µm}$ using image analysis software written in IgorPro (WaveMetrics, Lake Oswego, OR). The total strain energy per cell associated with these forces was computed as $E = \sum_{j=1}^{n} \frac{1}{2} k \delta_j^2$. In subsequent experiments, initial images of fibroblasts were gathered, and then fibroblasts were subjected to approximately 30 minutes of blebbistatin (10µM), gadolinium (50µM) or streptomycin (50µM) treatment. Before and after images were taken of the same fibroblasts to directly compare contractile changes with treatment.

**Optical Mapping.** Experiments were performed on Days 6-7 of culture. Cells were stained with 10 µM di-4-ANEPPS (Invitrogen, Carlsbad, CA) in Tyrode’s solution (135mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 0.33 mM NaH2PO4, 5.0 mM HEPES, and 5.0 mM glucose) for 5 minutes in a covered chamber and then washed with 37°C dye-free Tyrode’s solution. Monolayers were imaged as reported previously. Briefly, 100-beat drive trains of 10 ms monophasic pulses (1.2X diastolic threshold) at 2Hz pacing rate were applied by a point electrode and used for stimulation throughout the experiment. To determine the minimum cycle length before loss of 1:1 capture (MCL) and whether reentrant spiral waves could be initiated, stimulation began at 2 Hz and increased progressively in 1 Hz increments until 1:1 capture was no longer observed or reentry was initiated, and then again more slowly in 0.1 Hz increments starting from the previous MCL until 1:1 capture again was no longer observed or reentry was initiated.

**Experimental solutions.** Tyrode’s solution was used to superfuse cells continuously at 2.6 ml/min (which resulted in a volume exchange time of 2 min for the experimental chamber) under control conditions; drugs were added after initial recordings were taken. Three pharmacological agents were tested: 5-10µM blebbistatin (contraction inhibition), 20-50µM gadolinium, and 50µM streptomycin (both mechanosensitive channel blockers), all purchased from Sigma. The term
“drug” is used to apply to each intervention. Drug solutions were allowed to flow for 20 min for their effect to stabilize before any recordings were taken. Blebbistatin was prepared with DMSO.

**Data analysis.** Data were stored, displayed, and analyzed using software written in LabVIEW (Texas Instruments, Dallas, TX) and MATLAB (MathWorks, Natick, MA). Raw optical signals were detrended by subtracting a fitted third-order polynomial curve, smoothed with a five-point median filter and range normalized before generation of isopotential and isochrone maps, as previously described. Action potential duration at 80% repolarization (APD$_{80}$) was defined as the interval from the activation time to the instant during the repolarization phase when AP amplitude dropped to 20% of its maximum; APD$_{80}$ values were averaged across all channels and over 2 to 3 successive action potentials. Activation times were identified at the time of maximum positive slope (d$V$/d$V_{\text{max}}$) during action potential depolarization, and d$V$/d$V_{\text{max}}$ was normalized to the action potential amplitude. Conduction velocity (CV) was calculated from isochrone maps by measuring differences in activation times along a path perpendicular to the isochrone lines. To calculate heterogeneity index (HI), the differences in activation times between a specific recording site and its six surrounding sites were measured; the maximum value was chosen to represent the phase delay for that channel. A histogram of phase delays from all 253 sites was plotted, and HI was calculated by dividing P$_{95}$ - P$_{5}$ by P$_{50}$, where P$_{x}$ represents the value at the x$^{\text{th}}$ percentile.

**Statistics:** All data are expressed as mean ± standard error of mean. Standard, equal variance, two-tail Student’s t-tests were performed to determine statistically significant differences (p < 0.05) for independent data. For dependent data, in which measurements were taken on the same sample before and after treatment, Wilcoxon signed rank tests were performed to determine statistically significant differences. An F test was used to assess whether the variances in total fibroblast density and fibroblast area were equal, and since the null hypothesis
that the ratio of the variances of the populations is equal to 1 was rejected, unequal variance, two-tail Student’s t-tests were performed to determine significance. The rate of spiral wave incidence before and after treatment in fibrotic monolayers was statistically analyzed using a paired contingency table and Fisher’s exact test.

**Expanded Results**

*Connexin45 is not widely expressed in myofibroblasts.* Because some studies have shown that cardiac fibroblasts express connexin45,⁹ connexin45 junctional coupling was examined at hetero- and homocellular contacts. Immunostain images of DAPI (white), prolyl-4-hydroxylase (green), troponin I (blue) and connexin45 (red) show that connexin45 is not widely expressed between myocytes and fibroblasts in fibrotic monolayers (Supplemental Figure 2A-C). Further, immunostain images of a myofibroblast-only culture stained for connexin45 (green), SMA (red), and DAPI (blue) show that connexin45 is not widely expressed at cellular contacts (Supplemental Figure 2D-F).
References


Figure Legends

Supplemental Figure 1: Fibroblast morphology following drug treatments. Immunostain images of DAPI (blue), actin (green) and SMA (red) show little SMA expression in control fibroblasts, which is only evident in the slight yellow staining on the edges of the cells (A). TGF-β treatment (B) dramatically increased fibroblast size and development of organized SMA fibers. Blebbistatin disrupted SMA stress fiber organization in some TGF-β treated fibroblasts after 30 minutes, while other cells remained unaffected (C). Gadolinium had no visible effect on SMA stress fiber organization (D).

Supplemental Figure 2: Connexin45 expression. Immunostain images of DAPI (white), prolyl-4-hydroxylase (green), troponin I (blue) and connexin45 (red) show that connexin45 is not widely expressed between myocytes and fibroblasts in fibrotic monolayers (A-B). A magnified view of
the monolayer shows that connexin45 is not junctionally expressed between the myocyte and fibroblast (C). Immunostain images of a myofibroblast-only culture stained for connexin45, SMA, and DAPI also show that connexin45 is not widely expressed at cell contacts (D-F).
Supplemental Figure 1
Supplemental Figure 2