TGF-β₁ (Transforming Growth Factor-β₁) Plays a Pivotal Role in Cardiac Myofibroblast Arrhythmogenicity

Nicolò Salvarani, PhD*; Ange Maguy, PhD*; Stefano A. De Simone, MSc; Michele Miragoli, PhD; Florian Jousset, PhD; Stephan Rohr, MD

Background—TGF-β₁ (transforming growth factor-β₁) importantly contributes to cardiac fibrosis by controlling differentiation, migration, and collagen secretion of cardiac myofibroblasts. It is still elusive, however, to which extent TGF-β₁ alters the electrophysiological phenotype of myofibroblasts and cardiomyocytes and whether it affects proarrhythmic myofibroblast–cardiomyocyte crosstalk observed in vitro.

Methods and Results—Patch-clamp recordings of cultured neonatal rat ventricular myofibroblasts revealed that TGF-β₁, applied for 24 to 48 hours at clinically relevant concentrations (≤2.5 ng/mL), causes substantial membrane depolarization concomitant with a several-fold increase of transmembrane currents. Transcriptome analysis revealed TGF-β₁-dependent changes in 29 of 63 ion channel/pump/connexin transcripts, indicating a pleiotropic effect on the electrical phenotype of myofibroblasts. Whereas not affecting cardiomyocyte membrane potentials and cardiomyocyte–cardiomyocyte gap junctional coupling, TGF-β₁ depolarized cardiomyocytes coupled to myofibroblasts by ≥20 mV and increased gap junctional coupling between myofibroblasts and cardiomyocytes >5-fold as reflected by elevated connexin 43 and consortin transcripts. TGF-β₁-dependent cardiomyocyte depolarization resulted from electrotonic crosstalk with myofibroblasts as demonstrated by immediate normalization of cardiomyocyte electrophysiology after targeted disruption of coupled myofibroblasts and by cessation of ectopic activity of cardiomyocytes coupled to myofibroblasts during pharmacological gap junctional uncoupling. In cardiac fibrosis models exhibiting slow conduction and ectopic activity, block of TGF-β₁ signaling completely abolished both arrhythmogenic conditions.

Conclusions—TGF-β₁ profoundly alters the electrophysiological phenotype of cardiac myofibroblasts. Apart from possibly contributing to the control of cell function in general, the changes proved to be pivotal for proarrhythmic myofibroblast–cardiomyocyte crosstalk in vitro, which suggests that TGF-β₁ may play a potentially important role in arrhythmogenesis of the fibrotic heart. (Circ Arrhythm Electrophysiol. 2017;10:e004567. DOI: 10.1161/CIRCEP.116.004567.)

Key Words: fibroblasts • gap junctions • ion channel • myocytes, cardiac • myofibroblast • transforming growth factors

Myofibroblasts are not normally found in the healthy working myocardium but appear in old age and in the context of injuries to the heart, such as mechanical overload and infarction, where they contribute importantly to fibrotic tissue remodeling by excess secretion of extracellular matrix proteins. Fibrotic remodeling compromises pump function and promotes cardiac arrhythmias by the formation of a non-uniform substrate for electric activation. Apart from this indirect role in arrhythmogenesis, myofibroblasts were shown in vitro to contribute directly to arrhythmias by causing partial depolarization of electrotonically coupled cardiomyocytes, which results in proarrhythmic slow conduction and precipitation of ectopic activity. The fibrotic response to tissue injury and the appearance of myofibroblasts is orchestrated by several growth factors, cytokines, and hormones. Among these, TGF-β₁ (transforming growth factor-β₁) plays a central role by initiating the phenotype switch from fibroblasts to myofibroblasts and by affecting myofibroblast motility, contractility, and secretion of extracellular matrix proteins. Because previous studies with cell types different from myofibroblasts showed that mitotic activity, progression through the cell cycle, cellular differentiation, migration, and contractility depend on the membrane potential, we hypothesized that control of these processes in cardiac myofibroblasts by TGF-β₁ may similarly be associated with a change in the electrophysiological phenotype of myofibroblasts and that these changes may affect their proarrhythmic potential.
WHAT IS KNOWN

- TGF-β1 (transforming growth factor-β1) contributes importantly to cardiac fibrosis by inducing a phenotype switch of fibroblasts to hypersecretory myofibroblasts that are responsible for excessive collagen deposition within the myocardium.
- In vitro, myofibroblasts are known to induce proarrhythmic slow conduction and ectopic activity after establishment of heterocellular gap junctional coupling with cardiomyocytes.

WHAT THE STUDY ADDS

- Presence of TGF-β1 at physiologically relevant concentrations is pre-requisite for myofibroblasts to exert proarrhythmic effects on electrotonically coupled cardiomyocytes.
- Mechanistically, the proarrhythmic effect of TGF-β1 results from profound alterations of the electrical phenotype of myofibroblasts that are associated with changes in gene expression of multiple ion channels, ion pumps, and connexins.
- Apart from revealing new aspects of TGF-β1 signaling in cardiac cellular electrophysiology, the findings suggest that this cytokine may possibly serve as a biomarker for hearts at risk for proarrhythmic fibrotic remodeling.

Our results demonstrate that TGF-β1 significantly alters the electrical phenotype of cardiac myofibroblasts and accentuates their arrhythmogenic crosstalk with cardiomyocytes. The finding that block of canonical TGF-β1 signaling causes complete suppression of slow conduction and ectopic activity in models of cardiac fibrosis assigns this cytokine a central role in proarrhythmic myofibroblast–cardiomyocyte interactions in vitro.

Materials and Methods
An expanded Method section is available in the Data Supplement.

Cell Culture Models
Experiments were conducted in agreement with relevant Swiss Federal guidelines for animal experimentation. Primary cultures of 1-day-old Wistar neonatal rat ventricular cardiomyocytes and myofibroblasts were established as described before. In short, single-cell suspensions obtained from trypsin-digested ventricular tissue were subjected to differential pre-plating to separate fast adhering cell suspensions obtained from trypsin-digested ventricular tissue according to previously described methods.13 Cardiac fibroblasts obtained photolithographically structured as to form dense cardiomyocyte monolayers for optical recordings (1500 cells/mm²). Cardiomyocyte monolayers were photographed and the bottom of the coverslip was engraved with a glass marking diamond. This permitted the unequivocal retrieval of recording sites after immunostaining for comparative structure–function analysis.

Solutions and Drugs
During single-cell patch-clamp experiments and optical recordings, preparations were superfused with supplemented Hanks’ balanced salt solution. Long-term measurements of spontaneous activity were conducted in supplemented culture medium under incubating conditions. Drugs added to the cell cultures for 24 to 48 hours included TGF-β1 (R&D Systems) and blockers of the TGF-β receptor type I, SB431542 and LY364947 (Tocris). The time window for drug exposure (24–48 hours) was chosen based on the results of pilot experiments showing that the effects of TGF-β1 on the frequency of ectopic activity reached a plateau after ≈24 hours.

Immunocytochemistry
The myofibroblast phenotype was confirmed by staining the cells for α-SMA using standard immunocytochemistry protocols. Lengths of junctional contacts in cell pairs were measured using an image analysis program (ImagePro, Media Cybernetics).

Deep RNA Sequencing
A detailed description of the RNA-Seq protocols and the respective data analysis is given in the Data Supplement.

Cell Proliferation Assay
The effects of TGF-β1 on myofibroblast proliferation were assessed in myofibroblast cultures using a cell proliferation kit (XTT; Roche; details are available in the Data Supplement.

Patch-Clamp Recordings
Patch-clamp experiments were performed at room temperature using an EPC10 dual channel amplifier (HEKA). Whole-cell currents were normalized to cell capacitance and are reported as pA/pF. Gap junctional conductance was determined using established procedures (Data Supplement). For correlating electrophysiological results with cellular morphology, patch-clamp recording sites were photographed and the bottom of the coverslip was engraved with a glass marking diamond. This permitted the unequivocal retrieval of recording sites after immunostaining for comparative structure–function analysis.

Optical Measurement of Impulse Conduction
Impulse propagation characteristics along strand preparations were assessed at 36°C using the voltage sensitive dye di-8-ANEPPS. Optically recorded action potential amplitudes were normalized (%APA), and maximal upstroke velocities (dV/dtmax) were calculated in units of %APA/ms. Conduction velocity (0) was computed by linear regression of activation times recorded along the preparations.

Long-Term Optical Recording of Ectopic Activity
Ectopic activity was assessed with a custom-made videomicroscopy system installed in an incubator. Preparations consisted of patterned growth cell discs (10-mm diameter) having a cellular composition identical to the strand preparations.

Statistical Analysis
Values are reported as mean±SD. Number of samples refer to independent experiments. In box plots, horizontal bars indicate medians.
and the Tukey whiskers extend to data points that are less than 1.5x of the interquartile range away from the first/third quartile. Summary statistics (mean±SD) are depicted adjacent to each box plot. Normal distribution of the data sets was verified using the Shapiro–Wilk test. Data were compared using a 2-tailed Student t test (heteroscedastic), and differences between data sets were considered significant at P<0.05. Differential gene expression was calculated using DESeq2, and the Benjamini–Hochberg–adjusted P value (Padj)<0.05.18

Results

TGF-β1 Alters the Electrical Phenotype of Cardiac Myofibroblasts

Current-to-voltage relationships differed significantly between myofibroblasts treated for 24 to 48 hours with either 2.5 ng/mL TGF-β1 or 10 µmol/L SB431542, a specific inhibitor of the TGF-β receptor type 1 (Figure 2A). Whereas, compared with controls, TGF-β1 shifted the reversal potential by 6.8±3.1 mV in depolarizing direction and increased transmembrane currents, the reverse was observed with SB431542 alone, indicating that the blocker efficiently suppressed TGF-β1 signaling. Compared with SB431542 conditions, TGF-β1 induced a 3.7-fold increase of inward currents at −80 mV to −1.32±0.54 pA/pF and a 1.8-fold increase of outward currents at +40 mV to 3.65±1.90 pA/pF (n=7). The respective net currents induced by TGF-β1 amounted, as shown by the difference currents in Figure 2C (right), to −1.0 pA/pF at −80 mV and +1.6 pA/pF at +40 mV. As reflected by the changes in current-to-voltage curves, TGF-β1 induced, compared with SB431542 conditions, a significant depolarization of the membrane potential ($V_m$) of myofibroblasts from −48.1±7.9 to −19.8±5.0 mV and a 6-fold reduction of their membrane resistance ($r_m$: 2.4±0.9 to 0.4±0.3 GΩ; n=7; Figure 2D). Cell capacitance was not affected by treatment, indicating that myofibroblast cell size was not altered by TGF-β1. In regard to the structural phenotype of myofibroblasts, TGF-β1 and SB431542 exerted no noticeable effects on the organization and abundance of α-SMA–decorated stress fibers (Figure 2E), which concurs with the finding that TGF-β1 only moderately affected α-SMA mRNA (+16.1%; data from Table) and α-SMA protein (+21%; Figure I in the Data Supplement).

Of note, the finding that SB431542 significantly affected current-to-voltage characteristics of myofibroblasts indicates presence of substantial TGF-β1 activity under control condition. This background activity was likely due to TGF-β1 originating from the serum in the culture medium or the secretion of TGF-β1 by myofibroblasts and cardiomyocytes, or both.28,29 Background activity was present in all experiments shown below and is characterized by control values being consistently intermediate to values obtained in preparations treated with TGF-β1 and SB431542, respectively. According to these findings, the effects of TGF-β1 described below are consistently referred to SB431542 conditions unless stated otherwise.

TGF-β1 Depolarizes Cardiomyocytes Only When They Are Coupled to Myofibroblasts

Exposing single cardiomyocytes to TGF-β1 or SB431542 did neither affect their general morphology nor their $V_m$ ($≈−75$ mV), $r_m$ (≈280 MΩ), or cell capacitance (≈20 pF; Figure 3A and 3B). When coupled to single myofibroblasts, however, $V_m$ of single cardiomyocytes was significantly depolarized to −62.0±11.5 mV (control conditions) and −5.5±8.4 mV after TGF-β1 treatment (n=11; Figure 3C). This depolarization was prevented by TGF-β1 receptor blockade as illustrated by the finding that...
Figure 2. Effects of TGF-β1 (transforming growth factor-β1) on the cellular electrophysiology and morphology of cardiac myofibroblasts. **A**, Current-to-voltage (I–V) relationships obtained under the 4 conditions indicated (2.5 ng/mL TGF-β1; 10 µmol/L SB431542; 2.5 ng/mL TGF-β1 plus 10 µmol/L SB431542; mean±SD; number of independent experiments are listed in the panels). Inset in leftmost panel illustrates the voltage clamp protocol used. **B**, Overlay of mean I–V relationships. **C**, Difference in currents obtained under the conditions indicated. **D**, Effects of TGF-β1 signaling on the membrane potential, membrane resistance, and cell capacitance of cardiac myofibroblasts. Number of independent experiments are listed in the panels. Means±SD are depicted in blue. **E**, Immunofluorescence images (green, α-smooth muscle actin; blue, 4′,6-diamidino-2-phenylindole) of single cardiac myofibroblasts subjected to the 4 conditions indicated.
cardiomyocytes coupled to myofibroblasts retained a $V_m$ not different from that of single cardiomyocytes in presence of SB431542 ($-71.0\pm6.1$ versus $-75.4\pm2.2$ mV; n=13 and 10).

These results demonstrate that cardiomyocytes are depolarized by coupled myofibroblasts only if stimulated by TGF-\(\beta_1\). Moreover, the hyperpolarization of control preparations by SB431542...
lends further support to the notion that functionally relevant concentrations of TGF-β₁ are present in the control culture medium.

Heterocellular Gap Junctional Coupling Is Increased by TGF-β₁

Previous immunofluorescence studies demonstrated presence of Cx43 and Cx45 at both homo- and heterocellular cell junctions of cardiomyocytes and cardiac myofibroblasts. To quantify the effect of TGF-β₁ on intercellular communication, we assessed gap junctional conductances (g_j) in cardiomyocyte–cardiomyocyte and myofibroblast–cardiomyocyte cell pairs. The lengths of individual cell–cell contacts were determined and, for myofibroblast–cardiomyocyte pairs, the myofibroblast phenotype was confirmed by α-SMA staining following each patch clamp experiment (Figure 4A). Junctional conductances exhibited, as shown in Figure 4B, a close to linear dependence on junctional contact lengths that permitted the scaling of g_j to the length of contact (g_j,s) for comparison purposes. Under control conditions, g_j,s of cardiomyocyte–cardiomyocyte and myofibroblast–cardiomyocyte cell pairs amounted to 7.7±2.4 nS/µm (n=10) and 1.4±0.6 nS/µm (n=9), respectively. Whereas g_j,s of myofibroblast–cardiomyocyte cell pairs was not affected by TGF-β₁ or SB431542 (8.8±3.4 versus 8.3±1.5 nS/µm; n=7 and 6; Figure 4C), TGF-β₁ caused, compared with SB431542 conditions, a 5.7-fold increase of g_j,s of myofibroblast–cardiomyocyte cell pairs (2.8±0.8 versus 0.5±0.3 nS/µm; n=7 and 11), which is expected to contribute to cardiomyocyte depolarization by coupled myofibroblasts.

TGF-β₁–Induced Depolarization of Cardiomyocytes Coupled to Myofibroblasts: Paracrine, Molecular, or Electrotonic Effect?

Although cardiomyocyte depolarization by coupled myofibroblasts is likely based on transjunctional current flow from depolarized myofibroblasts, it cannot be excluded that it additionally depends on paracrine effects or on direct molecular signaling through gap junctions that may cause persistent changes in cardiomyocyte electrophysiology secondary to alterations in gene expression. To differentiate among these possibilities, we developed a killing protocol where we continuously monitored V_m of cardiomyocytes in heterologous cell pairs while mechanically disrupting the myofibroblast cell membrane using a second patch pipette. This approach allowed for the electric isolation of cardiomyocytes without using chemical uncouplers that are notorious for their side effects on membrane currents. Before myofibroblast killing, cardiomyocytes of control and TGF-β₁–treated cell pairs exhibited a depolarized phenotype and fired spontaneous action potentials that developed on the basis of phase 4 depolarizations (Figure 5A, left 2 panels). At the moment of myofibroblast killing, V_m of the now uncoupled cardiomyocytes showed a transient overshoot followed by hyperpolarization to resting potentials typical for single cardiomyocytes. This process was
associated with loss of spontaneous activity and restitution of normal cardiomyocyte excitability as demonstrated by regularly shaped action potentials in response to suprathreshold current injections (duration, 3 ms; amplitude, 50–150 pA; bottom panels). Membrane potentials of cardiomyocytes in SB431542-treated preparations (Figure 5A, rightmost panel) showed a similar overall response with the exception that cardiomyocytes lacked spontaneous activity and displayed a normal Vm both before and after killing of the myofibroblast, thereby confirming that block of TGF-β1 signaling prevents cardiomyocyte depolarization by coupled myofibroblasts. As summarized in Figure 5B, healing over of cardiomyocytes (ie, time needed for Vm to reach 90% of its steady state value after killing of the MFB) was not dependent on treatment and lasted, on average, between 62 and 89 seconds. Killing of myofibroblasts resulted in a significant hyperpolarization of cardiomyocytes by −11.6±7.7 mV (control; n=12) and by −21.0±5.9 mV (TGF-β1–treated preparations; n=8), whereas Vm of cardiomyocytes treated with SB431542 remained unchanged (−73.1±3.0 versus −75.3±3.1 mV; n=10; Figure 5C). Of note and as shown in Figure 5D, steady state Vm of cardiomyocytes after destruction of coupled myofibroblasts was not different from Vm measured in single cardiomyocytes undergoing identical treatments. Collectively, these findings demonstrate that neither paracrine nor direct molecular signaling contributed to the depolarized phenotype of cardiomyocytes coupled to TGF-β1–stimulated myofibroblasts, thereby confirming the central role played by heterocellular electrotonic coupling in cardiomyocyte depolarization. This conclusion was furthermore supported by the finding that the gap junctional uncoupler palmitoleic acid reversibly suppressed spontaneous activity in 21 of 22 cardiomyocytes coupled to myofibroblasts at 40 µmol/L (Movie I in the Data Supplement).

Effects of TGF-β1 on the Transcriptome of Cardiac Myofibroblasts

Clues as to the molecular basis of TGF-β1–induced changes in myofibroblast electrophysiology were obtained from transcriptome analysis. The approach was validated by confirming that TGF-β1 stimulation reproduced the well-established upregulation of collagen gene expression (Table I in the Data Supplement).
Table. Regulation of Ion Channel, Ion Pump, and Connexin Gene Expression by TGF-β1

<table>
<thead>
<tr>
<th>Gene, Protein</th>
<th>TGF-β1 FPKM, Mean±SD</th>
<th>SB431542 FPKM, Mean±SD</th>
<th>TGF-β1 vs SB431542 (Fold Change)</th>
<th>DESeq2 Comparison (Log2-Fold Change±SE)</th>
<th>DESeq2 Comparison (FDR)</th>
<th>References</th>
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<td>Na+ channels (3/6)</td>
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<td>Nalcn, Na leak channel</td>
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<td>Cnst, Consortin</td>
<td>23.98±2.04</td>
<td>8.70±0.16</td>
<td>2.75</td>
<td>1.44±0.10</td>
<td>39.2E-44</td>
<td>…</td>
</tr>
<tr>
<td>Structural proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acta2, α-SMA</td>
<td>7027±174</td>
<td>604±777</td>
<td>1.16</td>
<td>0.22±0.07</td>
<td>3.00E-3</td>
<td>2</td>
</tr>
</tbody>
</table>

Numbers in parentheses in the subtitles indicate significantly affected genes vs totally detected genes for each category. Absence of references in the rightmost column indicates that the respective transcripts were not detected in cardiac myofibroblasts before. α-SMA indicates α-smooth muscle actin; FPKM, fragments per kilobase of transcript per million mapped reads; Nalcn, sodium leak channel; TGF-β1, transforming growth factor-β1; and TRP, transient receptor potential.
In regard to the electrical phenotype of myofibroblasts, TGF-β, significantly affected transcription of 29 of 63 ion channel, ion pump, and connexin genes detected in myofibroblasts. Results are presented in the Table and are limited to transcripts that were differentially regulated by TGF-β, versus SB431542 and displayed an FPKM (fragments per kilobase of transcript per million mapped reads) ≥0.5.

### Ion Channels
TGF-β, reduced transcription of the voltage-independent sodium channel Nav2.1 (−62%) and the β subunit Scn1b (−33%) while upregulating the sodium leak channel (380%).

Transcription of Cav1.2 (L-type) and Cav2.1 (R-type) was reduced in presence of TGF-β, by −43% and −56%, respectively. Among the 12 differentially regulated potassium channels/subunits, TGF-β, caused downregulation of 8 transcripts (Kir2.1, Kir.6/SUR2, K2P3.1, Kv2.1, Kv4.3, Kv9.3, Kv11.2: −33 to −96%) while upregulating K2P6.1, K2P12.1, Kv1.6, and Kv2.2 (170%–480%). Among 7 detected transient receptor potential (TRP) channels, TRPA1 and TRPM7 were upregulated, whereas TRPV2 was downregulated by TGF-β, Chloride channels were not affected by TGF-β.

### Ion Pumps
TGF-β, had variable effects on Na/KATPase subunits with transcription of the α1 isoform being upregulated, whereas α2, β2, and β3 were downregulated. The Ca<sup>2+</sup>ATPase PMCA1 was downregulated.

### Connexins
TGF-β, caused upregulation of Cx43 and Cx46 and downregulation of Cx45 and Cx31. Of note, the analysis revealed substantial upregulation (275%) of consortin transcripts that encode for a recently discovered protein thought to be involved in the efficient membrane targeting of connexins.

### TGF-β<sub>1</sub> Slows Impulse Conduction in Models of Fibrotic Cardiac Tissue

Effects of TGF-β<sub>1</sub> on action potential propagation in multicellular tissues were assessed in strands of cardiomyocytes (control) and cardiomyocytes coated with myofibroblasts (fibrosis model) using voltage sensitive dyes. For TGF-β<sub>1</sub> experiments, the coating density of myofibroblasts was reduced from 500 to 300 cells/mm<sup>2</sup> because at 500 myofibroblasts/mm<sup>2</sup>, preparations became nonresponsive to electric stimulation at high myofibroblast density. In this case, the fibrosis model from 290±53 to 133±48 mm/s and caused a decrease of dV/dt<sub>max</sub> from 64.4±8.7 to 30.2±10.3 %APA/ms (n=79 and 48, respectively). A slight reduction of both parameters was also observed in control cardiomyocyte strands exposed to maximal TGF-β<sub>1</sub> concentrations, which likely reflects the effect of TGF-β<sub>1</sub> on residual myofibroblasts in the preparations. In absence of exogenously added TGF-β<sub>1</sub>, block of TGF-β<sub>1</sub> signaling with SB431542 increased θ and dV/dt<sub>max</sub> dose dependently (IC<sub>50</sub> = 0.3 µmol/L). At 10 µmol/L, SB431542 increased θ from 206±39 (n=60) to 368±32 mm/s (n=56) and dV/dt<sub>max</sub> from 44.9±9.3 to 73.3±5.2 %APA/ms (Figure 6C). Results obtained with SB431542 were fully reproduced by another selective inhibitor of the TGF-β receptor type 1, LY364947, thereby confirming that the effects of TGF-β<sub>1</sub> on θ and dV/dt<sub>max</sub> in the fibrosis model were mediated by this specific receptor that was stimulated by background TGF-β<sub>1</sub> activity present under control conditions (Figure III in the Data Supplement). This conclusion was further supported by the finding that culturing strand preparations for 24 hours under conditions of low levels of endogenous/background TGF-β<sub>1</sub>, (10x increased extracellular volume, no serum) caused θ and dV/dt<sub>max</sub> of the fibrosis models to converge toward values recorded in control cardiomyocyte strand preparations kept under identical conditions (Figure IV in the Data Supplement).

### TGF-β<sub>1</sub> Affects Ectopic Activity in Models of Cardiac Fibrosis

Myofibroblasts precipitate ectopic activity in dense networks of cardiomyocytes secondary to the establishment of heterocellular gap junctional coupling to cardiomyocytes. As reported before for unstructured cardiomyocyte–myofibroblast cell monolayers, discs of cardiomyocytes coated with myofibroblasts showed a characteristic transient increase of myofibroblast-dependent ectopic activity in response to the medium exchange (Figure 7A). In preparations kept under control conditions, peak frequencies (+27%) were reached 1.7 hours following the medium exchange. Thereafter, frequencies decreased steadily to −38% after a further 36 hours. Addition of TGF-β<sub>1</sub> (2.5 ng/mL) did not affect peak frequencies (+22%) but caused persistently increased levels of ectopic activity thereafter. In presence of SB431542, peak frequencies (+18%) were followed by a steady decline of ectopic activity between 15 hours and transient bursts of activity being interrupted by periods of quiescence, which accounts for the noisy appearance of the traces. The comparison of all 3 conditions shown in Figure 7B demonstrates that, relative to controls, TGF-β<sub>1</sub> caused an increase of the frequency of ectopic activity that reached a stable plateau (+21%) after 20 hours. By contrast, SB431542 led to a progressive decline of ectopic activity with preparations being intermittently completely quiescent and average frequencies being depressed by >60% after 36 hours. Uncoated cardiomyocyte discs showed no spontaneous activity under any condition during the entire observation period, indicating that TGF-β, is unable to elicit ectopic activity in dense networks of cardiomyocytes lacking a myofibroblast coat (data not shown).
Figure 6. Effect of TGF-β₁ (transforming growth factor-β₁) signaling on impulse conduction in patterned growth cardiomyocyte (CMC) and myofibroblast–cardiomyocyte (MFB–CMC) cell strands. A, Top 2 panels, phase contrast and corresponding immunofluorescence image (α-smooth muscle actin green; nuclei blue) of strand preparations with optical detector positions indicated by red circles. Bottom 2 panels, optically recorded action potential upstrokes during propagated activity from left to right and plot of activation times vs distance from which conduction velocities (θ) were calculated by linear regression. B, Dependence of θ (left) and dV/dt max (right) on TGF-β₁ concentration (red, CMC cell strands; green, MFB–CMC cell strands). Stippled lines with colored bands refer to mean±SD values obtained under control conditions. C, Same for preparations subjected to TGF-β₁ receptor block with SB431542.
Discussion

The results of this study demonstrate that TGF-β1 profoundly alters the electrical phenotype of cardiac myofibroblasts. Results further reveal that these alterations are pre-requisite for myofibroblasts to depolarize electrotonically coupled cardiomyocytes and that, accordingly, TGF-β1 stimulation is mandatory for myofibroblasts to induce slow conduction and ectopic activity in models of cardiac fibrosis. Overall and pending proof of electrotonic interactions of myofibroblasts with cardiomyocytes in vivo,34 the findings suggest that TGF-β1 may contribute to cardiac arrhythmias in ways going beyond the mere stimulation of extracellular matrix secretion.

Effects of TGF-β1 on Myofibroblast Electrophysiology and Heterocellular Gap Junctional Coupling

Overall, TGF-β1 caused a substantial increase of transmembrane currents in myofibroblasts that resulted in significant reductions of their $r_m$ and $V_m$. Consistent with the observed changes in transmembrane currents, RNA-Seq revealed TGF-β1–dependent upregulation of nonspecific cation channels (TRPs and sodium leak current) paired with a reduction of hyperpolarizing ion channels and ion pumps (Kir2.1, K2P3.1, Na/KATPase [α2 and β2,3 subunits], and PMCA1). The steep transition from inward to outward currents underlying the sigmoidal shape of

Figure 7. Effect of TGF-β1 (transforming growth factor-β1) on ectopic activity in myofibroblast–cardiomyocyte disc preparations. A, Frequency of spontaneous activity under control conditions followed by a medium exchange (dotted line) without (control, n=6) and with TGF-β1 (2.5 ng/mL; n=7) or SB431542 (10 µmol/L; n=6). Left, original recordings of individual preparations. Right, normalized frequencies of spontaneous activity (mean±SD). B, Left, superposition of the normalized frequencies (mean±SD) obtained under the 3 different experimental conditions (black, control; green, TGF-β1; red, SB431542). Right, normalized differences in ectopic activity of treated vs control preparation with bars indicating statistically different values (green, TGF-β1 vs control: 0.0006<P<0.05; red, SB431542 vs control: 5.4×10⁻⁵<P<0.05; brown, TGF-β1 vs SB431542: 1.2×10⁻⁷<P<0.05).
TGF-β1-induced currents may be explained by upregulation of KvL.6 and Kv2.2 in combination with downregulation of CaV channels. Not a priori consistent with electrophysiological observations were TGF-β1-dependent upregulations of K2P6.1 K2P12.1, Na/KATPase (α1 subunit) and downregulations of TRPV2, Nav2.1, Kv2.1, Kv4.3, Kv9.3, and Kv11.1, all of which would tend to offset the effects described above. Further to identifying molecular targets of TGF-β1 signaling, RNA-Seq revealed presence of 11 transcripts of ion channels/pumps not yet reported to be present in cardiac myofibroblasts. Among these, the recently discovered sodium leak channel is of particular interest because it was strongly upregulated by TGF-β1. This channel was shown to depolarize neurons by up to 10 mV and may therefore contribute substantially to TGF-β1-induced myofibroblast depolarization.31 Extensive future studies are needed to untangle to which extent the various changes in single-gene expression translate into changes in functional ion channels/pumps and to unravel the relative contribution of each gene product to the electrophysiological phenotype of TGF-β1-stimulated myofibroblasts.

TGF-β1 has been reported before to increase Cx43 protein expression in cultured mouse cardiac fibroblasts and was suggested to underlie increased levels of Cx43 in fibroblasts isolated from infarcted myocardium.35,36 Functionally, TGF-β1 caused a several-fold increase of gap junctional coupling between myofibroblasts and cardiomyocytes in this study. Whereas coupling of myofibroblast–cardiomyocyte cell pairs was modest in absence of TGF-β1, this channel was shown to depolarize neurons by up to 10 mV and may therefore contribute substantially to TGF-β1-induced myofibroblast depolarization.31

Mechanism of TGF-β1–Induced Depolarization of Cardiomyocytes Coupled to Myofibroblasts

By contrast to its pronounced effects on the cellular electrophysiology of myofibroblasts, TGF-β1 failed to affect r∞ and V∞ of single cardiomyocytes. The latter finding concurs with the results of a previous in vivo study showing that cardiomyocyte overexpression of TGF-β1 has no effect on V∞ of atrial cardiomyocytes.37 Furthermore, we found that TGF-β1 does not affect intercellular conductance of cardiomyocyte–cardiomyocyte cell pairs that amounted, on average, to ≈300 nS. This value is in the range of conductances measured previously in cardiomyocyte cell pairs isolated from intact hearts, thereby validating our model in respect to cardiomyocyte–cardiomyocyte coupling.38–40

From the above and based on the finding that V∞ of cardiomyocytes coupled to myofibroblasts remained unchanged in presence of SB431542, it follows that proarrhythmic depolarization of cardiomyocytes coupled to TGF-β1-stimulated myofibroblasts was the exclusive result of the cytokine-induced changes in myofibroblast electrophysiology and coupling. The central role of heterocellular electroneutral coupling was confirmed by the finding of immediate normalization of cardiomyocyte electrophysiology after killing of the TGF-β1-stimulated coupled myofibroblasts and by the finding that spontaneous activity of cardiomyocytes coupled to myofibroblasts was abolished during chemical uncoupling. The results of the killing protocol furthermore excluded that paracrine or direct molecular signaling through gap junctions may have caused a persistently depolarized cardiomyocyte phenotype secondary to altered gene expression.41 The question of whether the TGF-β1-dependent changes in V∞, r∞, and g∞ of myofibroblasts were of sufficient magnitude to explain cardiomyocyte depolarization on a quantitative basis was addressed with a simulation study where we used a theoretical framework that predicts V∞ of cardiomyocytes coupled to myofibroblasts based on actual V∞ and r∞ of myofibroblasts and g∞ recorded in cell pairs subjected to TGF-β1 and SB431542, respectively (Table II in the Data Supplement). The calculations showed that theoretically predicted values for V∞ of cardiomyocytes coupled to myofibroblasts are virtually identical to measured values, thereby demonstrating that the combined effects of TGF-β1 on V∞, r∞, and g∞ of myofibroblasts fully account for the extent by which myofibroblasts depolarize coupled cardiomyocytes and that other mechanisms, such as activation of mechanosensitive channels of cardiomyocytes by mechanically coupled myofibroblasts, play a minor, if any, role.42

Essential Role of TGF-β1 in Myofibroblast-Dependent Slow Conduction and Ectopic Activity

The TGF-β1–induced changes in the electrical phenotype of myofibroblasts predict that the well-established phenomena of myofibroblast-induced proarrhythmic slow conduction and ectopic activity in multicellular models of cardiac fibrosis should show a strong dependence on TGF-β1 signaling. This was the case as TGF-β1 slowed θ and dV/dtmax in a dose-dependent manner and increased ectopic activity. Vice versa, selective block of canonical TGF-β signaling caused complete normalization of conduction and suppressed ectopic activity, despite the continued presence of functional heterocellular gap junctional coupling. This demonstrates that coupling of nonexcitable cells to cardiomyocytes does not necessarily disturb cardiac electrophysiology. In this context, it is tempting to speculate that myofibroblasts deprived of TGF-β1 stimulation reassert a fibroblast-like electrical phenotype and that conduction observed under these conditions may reflect the situation of normal fibroblasts being electrotonically coupled to cardiomyocytes in intact healthy myocardium.

Relevance for the Intact Heart

Although a direct validation of the findings of this study in vivo is not feasible with presently available experimental methodologies, a comparison of TGF-β1 concentrations measured in vivo to TGF-β1 concentrations exerting adverse...
effects in our model is insightful. In the fibrosis model, \( \theta \) and \( dV/dt\_\text{max} \) showed a steep dependence on TGF-\( \beta_1 \) concentrations between 80 and 250 pg/mL. In vivo, conservative values for total TGF-\( \beta_1 \) levels obtained from healthy subjects were reported to be \( \leq 500 \) pg/mL.\(^{4,14}\) When assuming a ratio of 10 for total versus active TGF-\( \beta_1 \),\(^{45}\) this corresponds to concentrations of active TGF-\( \beta_1 \) \( \leq 50 \) pg/mL (i.e., to concentrations that did not yet affect conduction and automaticity in the fibrosis models of this study). However, slight elevations of TGF-\( \beta_1 \) above this value slowed conduction and increased ectopic activity, indicating a narrow margin of safety that may be readily overcome in vivo in situations of increased TGF-\( \beta_1 \). secretion as is generally the case during cardiac fibrotic remodeling and in patients with coronary heart disease. Does this make TGF-\( \beta_1 \) a useful biomarker for fibrotic/arrhythmogenic remodeling? Presently available evidence in regard to this issue is inconclusive.\(^{46}\) This may be due, at least in part, to problems associated with the reliable determination of TGF-\( \beta_1 \) plasma levels.\(^{43,44}\) If these difficulties can be overcome and if methodologies are conceived which permit investigations of electrotonic interactions between myofibroblasts and cardiomyocytes in vivo, plasma levels of TGF-\( \beta_1 \) may eventually turn into a meaningful biomarker for hearts at risk for arrhythmias. Moreover, TGF-\( \beta_1 \)-regulated ion channels may emerge as new targets in antiarrhythmic therapies that circumvent known problems associated with general suppression of TGF-\( \beta_1 \) signaling.

Conclusions
Overall, the results of this study reveal that TGF-\( \beta_1 \) causes profound alterations in the electrophysiological phenotype of cardiac myofibroblasts that are reflected by complex changes in gene expression of ion channels and pumps. Going beyond the possibility that these changes may modulate myofibroblast function in terms of contractility, migration, and secretion of extracellular matrix components, the data presented demonstrate that TGF-\( \beta_1 \)-dependent alterations in the electrical phenotype of myofibroblasts are conditio sine qua non for the establishment of proarrhythmic heterocellular interactions with cardiomyocytes, such as slow conduction, conduction blocks, ectopy, and reentrant activity. According to these findings and pending proof of electrotonic interaction between stromal and parenchymal cells in vivo, targeted interference with TGF-\( \beta_1 \) signaling may therefore not only alleviate detrimental effects of cardiac fibrosis in general but may also have direct antiarrhythmic effects by abolishing adverse electrotonic crosstalk between cardiomyocytes and myofibroblasts.

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Disclosures
None.

References


TGF-β₁ (Transforming Growth Factor-β₁) Plays a Pivotal Role in Cardiac Myofibroblast Arrhythmogenicity

Nicolò Salvarani, Ange Maguy, Stefano A. De Simone, Michele Miragoli, Florian Jousset and Stephan Rohr

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell culture

Primary cultures of neonatal rat ventricular cardiomyocytes (CMCs) and myofibroblasts (MFBs) were obtained using methods described in detail before.1 Experimental protocols complied with relevant institutional guidelines for animal experimentation and were approved by the State Veterinary Department. In short, the apical two thirds of neonatal rat ventricles of 1-day old rats (Wistar) were cut into small pieces and dissociated in Hank’s balanced salt solution (HBSS without Ca²⁺ and Mg²⁺; Bioconcept) containing trypsin (0.1%, Sigma) and pancreatin (120 µg/ml, Sigma). Complete tissue disintegration took 4 to 5 dissociation cycles lasting 15min each. After each cycle, tissue pieces were left to sediment by gravitation and the supernatant containing the dissociated cells was removed and stored on ice. The dissociated cells were centrifuged and re-suspended in culture medium consisting of M199 with Hank’s salts (Sigma), penicillin (20000 U/L; Sigma), streptomycin (34 µmol/L; Biochrom; Bioswisstec), vitamin B12 (1.5 µmol/L; Sigma) and 10% neonatal calf serum (NCS; Biochrom; Bioswisstec). In order to separate fast adhering fibroblasts from slowly adhering CMCs, the cell suspension was pre-plated for 2h 15min in 75 cm² cell culture flasks. The supernatant containing mostly cardiomyocytes was collected, passed through a cell strainer and supplemented with vitamin C (18 µmol/L; Sigma), epinephrine (10 µmol/L; Sigma) and bromodeoxyuridine (100 µmol/L; Sigma). After cell counting and appropriate dilution of the suspension, cells were plated onto different types of substrates (cf. below). Medium exchanges were performed 24h after plating with supplemented M199 containing a reduced amount of NCS (5%) and every 48h thereafter. Fibroblasts obtained from the pre-plating procedure were thoroughly washed to remove non-attached / weakly attached cells and were kept in supplemented medium without bromodeoxyuridine. Cultured fibroblasts expressed α-smooth muscle actin (α-SMA) within 24 to 48h indicating a rapid phenotype switch to MFBs.2 MFBs were kept in culture for 8d before being used in the experiments. Cell cultures were maintained in an incubator (Jouan) at 35 °C in a humidified atmosphere containing 1.2% CO₂.

Single cell – cell pair cultures: For patch-clamp experiments, CMCs or MFBs were seeded on collagen (Type I or Type IV, Sigma) coated glass coverslips at 40 to 80 cells/mm² which resulted in low density cultures with single cells and homologous cell pairs suitable for assessing intercellular conductances. CMC-MFB cell pairs were obtained by producing low-density CMC cultures to which MFBs were added 24h later (cell densities as for single cell cultures). Cell culture media and medium exchange protocols were identical to those described above. Pharmacological interventions were started 48h after initiation of the culture with drugs being added during a medium exchange.

Patterned growth cell cultures: Strands of CMCs measuring 0.6 x 5 mm were obtained by seeding CMCs at a density of 1500 cells/mm² onto photolithographically generated strips of collagen type IV (Sigma) as described in detail before.1 The cell density used resulted in uniform dense monolayers of CMCs after 24h. For models of cardiac fibrosis, 24h old CMC strands were coated with MFBs at a density of 500 cells/mm² which resulted in an MFB coat covering ~70% of the CMC monolayers. Pharmacological interventions were started 24h after addition of the MFBs.

Cell culture and sample preparation for western blotting and deep RNA-Seq: After 8d in culture, MFBs were detached and plated at a density of 240 cells/mm² in 25 cm² culture flasks. Culture
medium was renewed after 24h at which time drugs were added (2.5 ng/ml TGF-β1 or 10 µmol/L SB431542). After 48h of treatment, cells were detached, centrifuged and the pellet was snap-frozen for subsequent total RNA isolation or western blotting. Three distinct sets of cultures per intervention were included in the RNA-Seq study, 5 distinct sets in the western blot experiments.

Solutions and drugs
During optical and single cell patch-clamp experiments, preparations were superfused with Hanks’ balanced salt solution (HBSS, Sigma) containing 1% NCS and (mmol/L) NaCl 137, KCl 5.4, CaCl2 1.3, MgSO4 1.2, NaHCO3 4.0, KH2PO4 0.5, NaH2PO4 0.3, D-glucose 5.5 and HEPES 10 (pH 7.40). For the determination of $g_j$ using dual whole cell patch-clamp experiments, preparations were superfused with a solution containing (in mmol/L): NaCl 140, TEA-Cl 5.4, CaCl2 2, MgCl2 1, D-Glucose 1, 1% NCS and HEPES 10 (pH 7.40).

Drugs added to the cell cultures for 24 - 48h included TGF-β1 (R&D Systems), SB431542 (Tocris) and LY364947 (Tocris) at concentrations indicated in the manuscript. The time window for drug exposure (24 – 48 h) was chosen based on the results of pilot experiments showing that the effects of TGF-β1 on the frequency of ectopic activity reached a plateau after ~ 24 h. TGF-β1 stock solutions (1 µg/ml) were prepared by dissolving the growth factor in 4 mmol/L HCl containing 1 mg/ml BSA (Sigma). SB431542 and LY364947 were dissolved in DMSO as to obtain 10 mmol/L stock solutions that were further diluted in experimental media to reach the concentrations indicated in the manuscript.

Immunocytochemistry
Preparations were washed with phosphate buffered saline (PBS, Invitrogen) followed by fixation with 2% paraformaldehyde for 5 min. After another wash step, they were incubated for 20min with blocking buffer (PBS containing 20% goat serum) before being exposed for 2h to the α-SMA antibody (mouse monoclonal, Thermo Fisher) dissolved in PBS containing 1% goat serum and 0.15% triton X-100. After washing, preparations were incubated for 20min with secondary antibodies (AlexaFluor 488 or AlexaFluor 546, goat anti-mouse, Molecular Probes) containing DAPI (Molecular Probes). This step was followed by washing and mounting. All steps were performed at room temperature. The preparations were imaged on an inverted microscope equipped for epifluorescence (Zeiss, Axiovert 200) using a high sensitivity camera (Spot RT, Diagnostic Instruments). Image analysis was performed with ImagePro (Media Cybernetics). Images used for comparing the effects of different drugs on cell abundance/phenotype were obtained using identical imaging conditions/parameters and underwent the same level adjustments during subsequent image analysis.

Total RNA isolation, RNA-sequencing and post-analysis
Total RNA was obtained from cell pellets (cf. above) using mirVana™ (ThermoFisher Scientific). The RNA integrity was assessed using the Agilent 2100 bioanalyzer. The RNA Integrity Number (RIN) of all the samples included in this study was > 9.0. Libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit (Illumina). RNA sequencing was performed on an Illumina HiSeq™ 2500 system (2x125bp).

Between 39 and 49 mio read pairs were obtained per sample and the quality of the reads was assessed using fastqc v.0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads were mapped to the rat reference genome (Rnor 5.0, ensembl release 78) with Tophat v.2.0.13. We used htseq-count v.0.6.1 to count the number of reads overlapping with each gene, as specified in the
ensembl annotation. The Bioconductor package DESeq2 v. 1.6.3 was used to test for differential gene expression between the two conditions. FPKM values were calculated with DESeq2, with the total exon lengths of each gene based on the ensembl annotation file.

**Western blotting**

Total protein extraction from the snap-frozen cell pellets (cf. above) was performed by homogenizing the pellets in TNE-buffer containing (mmol/L): Tris 25, NaCl 150, EDTA 5, EGTA 5 and 1% Triton x-100 (pH 7.34). A cocktail of protease (cOmplete Mini EDTA-Free; Roche) and phosphatase (PhosStop; Roche) inhibitors was added to prevent protein degradation. Homogenized samples were incubated on ice for 30min and then centrifuged at 1,000 g for 10min. The supernatant was collected and protein concentration determined using a Pierce protein assay (ThermoFisher Sc.). Protein samples (20 µg) were separated on 8% poly-acrylamide SDS-PAGE and transferred electrophoretically onto PVDF membranes. The PVDF membranes were blocked in a PBS-solution containing 0.05% (v/v) Tween-20 and 5% (w/v) nonfat dried milk (NDM) and incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.05% Tween-20 and 1% NDM. After washing with PBS-Tween solution/1% NDM, membranes were hybridized with HRP-conjugated secondary antibody. Immunoreactive bands were detected by enhanced chemiluminescence using BioMax MS/MR films. Protein quantification was performed with the Quantity One® software (Biorad). All of the expression data are normalized to GAPDH staining for the same samples on the same gels.

**Antibodies:** Rabbit polyclonal anti α-SMA (Rb-9010-P; 1/2000; ThermoFisher) and mouse monoclonal anti-GAPDH (10R-G109a; 1/10000; Fitzgerald) were used as primary antibodies. Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (111-035-144) and AffiniPure donkey anti-mouse IgG (715-035-151) were used as secondary antibodies (1/10,000; both from Jackson).

**Cell proliferation measurements**

After 8d in culture, MFBs were detached and plated at a density of 500 cells/mm² in a 24 well cell culture treated plate (Costar 3524). Culture medium was renewed after 24h at which time drugs were added (2.5 ng/ml TGF-β1, 10 µmol/L SB431542 or a combination of both). MFB proliferation was evaluated after 48h treatment using the cell proliferation kit II (XTT) from Roche. Absorbance was measured at 405nm with a reference wavelength at 595 nm following 4.5 hours incubation with the XTT solution (0.3mg/mL) at 37°C. Results are expressed as percentage of control.

**Patch-clamp recordings**

Single or dual whole cell recordings were performed using an EPC-10 patch-clamp amplifier (HEKA) controlled by PatchMaster software (HEKA). Signals were filtered (2.9 kHz), digitized (5 kHz) and stored for off-line analysis with PatchMaster or FitMaster software (both HEKA). Low density cell cultures on glass coverslips were transferred to a custom-made experimental chamber that was fixed to the stage of an inverted microscope (Eclipse TE2000, Nikon). During the entire experiment, cells were superfused with experimental solution at 2-3 ml/min (room temperature). Patch pipettes were pulled from borosilicate glass capillaries (GC150F-10, Harvard Apparatus) with a horizontal puller (DMZ; Zeitz Instruments). The pipette filling solution for single cell experiments contained (in mmol/L): K-aspartate 120, NaCl 10, MgATP 3, CaCl₂ 1, EGTA 10, HEPES 5 (pH 7.2). For the determination of gap junctional conductance (gj) using dual whole cell patch-clamp recordings, the pipette solution contained (in mmol/L): CsCl₂ 120, NaCl 10, MgATP 3, CsOH 16, EGTA 10, HEPES
(pH 7.2). Pipette resistances ranged from 2 to 6 MΩ and pipette potentials were zeroed before cell contact. After seal formation (2-10 GΩ) and rupturing of the patch, cell capacitance, series resistance ($r_s$: 3–9 MΩ) and liquid junction potentials (12.4 mV as calculated by pCLAMP software; Axon Instruments) were compensated. Membrane resistances of cells ($r_m$) were calculated from voltage changes in response to hyperpolarizing current steps (5 pA, 500 ms). Cell capacitance ($c_m$) was determined by using the LockIn extension feature of Patchmaster. Whole cell currents were normalized to $c_m$ and are reported as pA/pF. Experiments were performed with 1 to 3d old preparations. Data were obtained from > 3 different cultures for each experimental condition.

Specific voltage or current clamp protocols described below were used for the determination of current-to-voltage (I-V) relationships, membrane potentials ($V_m$), $r_m$, $c_m$ and $g_j$.

**Whole cell recordings, single cells:** $V_m$ of single CMCs and single MFBs were determined in current clamp mode. I-V relationships of single MFBs were obtained using voltage ramp protocols where, starting from a holding potential of -72 mV, $V_m$ was stepped from -90 to 65 mV during 1.6s (speed: 97 mV s$^{-1}$; overall sweep length: 2s). Ramp protocols were repeated 3 times at inter-sweep intervals of 3 s.

**Determination of $g_j$:** Intercellular conductance of cell pairs was assessed using established dual whole cell patch-clamp protocols. Experiments were carried out on homologous CMC-CMC and heterologous CMC-MFB cell pairs. Patch pipettes were mounted on separate micromanipulators (MP-225, Sutter Instrument Company) and signals were recorded with a dual patch-clamp amplifier (EPC-10, HEKA). After establishing successful whole cell recording conditions, the membrane potential of both cells was clamped to -42 mV. While keeping the voltage of cell 1 constant, cell 2 was stepped to ±50 mV in 10 mV increments lasting 200 ms. Values for the resulting transjunctional currents ($I_j$) in cell 2 were determined at steady state ($I_{j,ss}$). The protocol was then reversed (stepping of cell 1, recording of $I_{j,ss}$ in cell 2). Absence of significant differences between $I_{j,ss-1}$ and $I_{j,ss-2}$ indicated that $g_j$ was not affected by asymmetric leak currents. Because membrane resistances were high compared to series resistances ($r_s$), non-junctional membrane currents were negligible and, accordingly, a simplified electrical circuit with three resistances in series ($r_s$, $r_{gap}$, $r_s$) was used to calculate gap junctional resistance. The length of cell-cell junctions of each cell pair investigated was assessed in microscopic phase contrast images using routines implemented in ImagePro software (ImagePro, Media Cybernetics, USA).

**Whole cell recordings, killing protocol:** To investigate the possibility that MFBs coupled to CMCs may affect CMC electrophysiology in ways different from electrotonic coupling, we developed a protocol where $V_m$ of CMCs was continuously monitored in current clamp mode while the adjacent MFB was mechanically disrupted (‘killing protocol’) with a second pipette. This protocol permitted the monitoring of the electrical activity of CMCs under control conditions, during the ‘killing’ process and the recovery period. Full recovery of CMC excitability was confirmed by eliciting action potentials by application of 3 ms long depolarizing current pulses.

**Correlation of patch-clamp recordings with the morphological phenotype of cells investigated:** In experiments where patch-clamp data were correlated with the structure and phenotype of cells or cell pairs under investigation, the phenotype of MFBs was routinely confirmed by immunocytochemistry (presence of α-SMA) following each experiment. For this purpose, patched cells/cell pairs were photographed and the site of the recording marked with a fine diamond point marker during the experiment. After fixation and staining of the preparations, the marks in the glass in combination with the microscopic image permitted an unequivocal retrieval of the cell(s) that had been electrophysiologically characterized for further morphological analysis.
Optical measurements of impulse conduction

Impulse propagation characteristics along strand preparations were assessed optically following staining for 4 min with the voltage sensitive dye di-8-ANEPPS (135 µmol/L; Biotium). Stained preparations were mounted in a temperature controlled chamber placed on the stage of an inverted microscope (Zeiss 135M) and were superfused at 36°C with HBSS containing 1% NCS. Individual cell strands were stimulated with a bipolar electrode consisting of a glass micropipette filled with HBSS and a silver wire coiled around the shank. The electrode was placed ≥ 1 mm from the measurement site in order to permit propagation to reach steady-state conditions at the recording site. Individual strands were pre-stimulated for 10 s by a stimulator (SD9, Grass Instruments Co., Quincy, MA) at a basic cycle length of 500 ms prior to recording of a single propagating action potential using a custom made fiber optic recording system described in detail elsewhere. Recordings were made at 20x magnification (Apochromat 20x, 0.8 N.A., Zeiss) resulting in a spatial resolution of 50 µm. The amplitude of optically recorded action potential upstrokes was scaled to 100% (%APA). Maximal upstroke velocities (dV/dt_{max}) were calculated from %APA values and are given in %APA/ms. With an assumed action potential amplitude of 100 mV, %APA/ms values correspond to V/s. Activation profiles of the preparations were assessed from the time when depolarization reached 50% of the entire action potential amplitude (activation time). Conduction velocities were computed by linear regression of activation times determined at 18 sequential recording sites that covered a distance of 900 µm.

Longterm assessment of ectopic activity

Continuous recordings of spontaneous activity of discs of cardiomyocytes coated with MFBs were obtained with a video camera (Mikrotron, EoSens; 1280x1024 pixels; 500 frames per second) coupled to an inverted microscope (Zeiss, Axiovert S100) equipped with phase contrast optics. The entire system was placed in an incubator to ensure stable environmental conditions during the entire recording period (up to 48h). Preparations placed in multiwell dishes were imaged at 10x magnification and positions were changed by means of a motorized x-y table. Contraction signals were derived from the video data by realtime subtraction of consecutive images followed by thresholding and by summing intensity differences as recorded at each pixel. This algorithm permitted the unequivocal identification of synchronized contractions with high signal-to-noise ratios. A detection algorithm identified the contractions and, after assessing beat-to-beat periods, calculated average beat rates for recording periods of 20s. The software used to acquire and analyze the data was custom-developed.

Videomicroscopy of ectopic activity in heterologous cell pairs

Acute effects of gap junctional uncoupling on spontaneous activity of single CMCs coupled to MFBs were assessed in low density cultures (cf. above). Preparations were mounted in a temperature controlled chamber placed on the stage of an inverted microscope (Zeiss 135M) and were superfused at 36°C with HBSS containing 1% NCS. Preparations were scanned for MFB-CMC cell pairs with CMCs typically showing spontaneous activity (20x phase contrast objective). After acquiring a 20s lasting movie under control conditions with a CMOS camera (XIMEA xiQ), superfusion was switched to HBSS containing 1% NCS and palmitoleic acid (40 µmol/L; Sigma) with a second movie being recorded 10-15min after the beginning of exposure to this gap junctional uncoupler. Thereafter, washout with control solution was initiated and a final movie was acquired after a further ~20 min.
Statistics

Values are reported as means ± SD in the text. Number of samples refer to independent experiments. In box plots with Tukey whiskers, mean values are marked by a cross. The whiskers extend to data points that are less than 1.5 x IQR (interquartile range) away from 1st/3rd quartile. Summary statistics (mean±SD) are depicted adjacent to each box plot. Normal distribution of the datasets was verified using the Shapiro-Wilk test. Unless indicated otherwise, data were compared using a 2-tailed Student $t$ test (heteroscedastic except for the data in Figure S1B of this supplement that were analyzed with a paired t-test) and differences between data sets were considered significant at $p<0.05$. Differential gene expression was calculated using DESeq2 v.1.6.3 (cf. paragraph on ‘Total RNA isolation, RNA-sequencing and post-analysis’ above). A gene was considered to be differentially expressed if it had a Benjamini-Hochberg adjusted $p$-value ($p_{adj}$) < 0.05.$^{15}$
Supplemental Figures, Tables and Movie

Effect of TGF-β₁ on α-SMA protein expression in myofibroblasts

Effects of TGF-β₁ signaling on α-SMA expression were assessed in 5 paired myofibroblast (MFB) cell cultures exposed to 2.5 ng/ml or 10 μmol/L SB431542 for 4h. As shown in Figure S1 by a representative western blot and data obtained from 5 MFB cultures, TGF-β₁ caused a significant increase of α-SMA protein by 21% when compared to SB431542 conditions (1.15±0.22 to 1.39±0.25; p<0.005; n=5; data normalized to GAPDH expression).

Block of ectopic activity in heterologous cell pairs during gap junctional uncoupling

Movie S1 shows an example of a myofibroblast-cardiomyocyte (MFB-CMC) cell pair with the coupled CMC showing spontaneous activity. This typical feature of heterologous cell pairs is the result of CMCs being depolarized by coupled MFBs into a range of membrane potentials supporting spontaneous electrical activity.¹⁶ As illustrated by the movie, superfusion of the preparation with the gap junctional uncoupler palmitoleic acid (40 μmol/L) caused reversible suppression of spontaneous activity, thereby demonstrating that gap junctions are the substrate of arrhythmogenic MFB-CMC interactions and that other forms of intercellular communication like nanotubes or mechanical coupling are unlikely to play a role. The single frame excerpt from the movie depicted below provides cell type definitions and a length scale.

Movie S1: Image of a single frame with labeled cells and scale bar.
Regulation of collagen and lysyl oxidase genes by TGF-β1

Transcriptome analysis was validated by assessing changes in collagen transcripts following TGF-β1 stimulation, which is firmly established to increase collagen production in fibroblasts at the transcript and protein level. Among 38 detected collagen transcripts (Table S1), 23 were differentially regulated by TGF-β1 with 15 genes being upregulated 1.4 to 92.7-fold as opposed to 8 transcripts that were downregulated 1.5 to 3.5-fold. Upregulated transcripts concerned the 7 most abundant collagens including collagen type I. Upregulation of collagen genes was paralleled by a 1.9 to 6.7-fold upregulation of the ECM cross-linking lysyl oxidase genes LOX and LOXL1 – 4.

<table>
<thead>
<tr>
<th>Gene</th>
<th>TGF-β1 FPKM (mean±SD)</th>
<th>SB431542 FPKM (mean±SD)</th>
<th>TGF-β1 vs. SB431542 DESeq2 comparison (Log2FoldChange ± SE)</th>
<th>DESeq2 comparison (Padj)</th>
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<td></td>
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</tr>
<tr>
<td>Col1a1</td>
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<td>Col1a2</td>
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<td>Col8a2</td>
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<td>Col9a1</td>
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<tr>
<td>Col11a1</td>
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<td>22.2 ± 3.0</td>
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<tr>
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<td>69.5 ± 5.8</td>
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<tr>
<td>Col22a1</td>
<td>0.60 ± 0.24</td>
<td>0.98 ± 0.12</td>
<td>0.62</td>
<td>-0.65±0.23</td>
</tr>
</tbody>
</table>

Table S1: Regulation of collagen and lysyl oxidase transcripts by TGF-β1.
Effect of TGF-β1 and SB431542 on MFB proliferation

To investigate whether TGF-β1 signaling affects MFB proliferation, MFB monolayer cultures seeded at densities identical to those used in the fibrosis models (500 cells/mm²) were exposed for 48h to 2.5 ng/ml TGF-β1, 10 µmol/L SB431542 or a combination thereof before being analyzed with a cell proliferation assay (XTT). Compared to control preparations, neither of the interventions had an effect on cell proliferation (Figure S2), thereby excluding the possibility that TGF-β1 dependent changes in MFB density may have contributed to changes in impulse conduction and ectopic activity in the fibrosis model of this study.

![Figure S2. TGF-β1, signaling and MFB proliferation](image)

Exposing MFBs to TGF-β1, SB431542 or a combination thereof for 48h had no effect on MFB proliferation rates (n=5 for each condition).

Effects of block of TGF-β1 signaling with LY364947 on impulse conduction

To assure that the effects observed with SB431542 were specific for the TGF-β receptor type I, we investigated whether an alternate small molecule blocker of this receptor, LY364947, was similarly able to rescue conduction in the cardiac fibrosis model (strands of CMCs coated with MFBs). Indeed, and as is shown in Figure S3, LY364947 caused a dose-dependent increase of conduction velocities

![Figure S3. Effect of block of TGF-β1 signaling with LY364947 on impulse conduction in the cardiac fibrosis model. A, LY364947 caused a dose-dependent increase of conduction velocities in strands of CMCs coated with MFBs (green) to values not different from CMC only cell strands (red). B, Same as A for maximal upstroke velocities. Stippled lines and bands: mean±SD of control values. n= 40-85.](image)
(θ) and maximal upstroke velocities (dV/dtmax). When applied at concentrations ≥ 10 µmol/L for 24h, LY364947 led to complete recovery of θ and dV/dtmax to values not different from CMC control strands indicating that Vm of CMCs in the fibrosis model returned to normal values in presence of this blocker of the TGF-β receptor type I. As for SB431542, LY364947 (at 10 µmol/L) did not affect θ and dV/dtmax of control CMC cell strands therefore lending further support to the conclusion that TGF-β1 signaling is mandatory for MFBs to induce slow conduction in electrotonically coupled CMCs.

**Effects of no-serum / large-volume culture conditions on background TGF-β1 activity**

The finding that block of TGF-β receptors with SB431542 or LY364947 reverts slow conduction in fibrosis preparations kept under control conditions suggests that there is background activity of TGF-β1, present in the culture medium. If this activity is due to TGF-β1 originating from the serum in the culture medium and/or the secretion of TGF-β1 by MFBs and CMCs, omitting the serum in the medium and simultaneously increasing the cell culture volume to dilute TGF-β1 secreted by the cells should increase θ and dV/dtmax in a manner similar to TGF-β receptor blockade. This hypothesis was investigated by culturing strands of CMCs coated with MFBs (fibrosis model) for 24h in 20 ml of culture medium (10x increased volume) that was devoid of serum. In the incubator, preparations were placed on an orbital shaker (10 RPM) to reduce the thickness of the unstirred layer. Preparations kept under normal conditions served as controls. As shown in Figure S4, both θ and dV/dtmax of fibrosis models subjected to no-serum/large-volume conditions increased significantly compared to controls (152.0±21.2 mm/s to 286.6±35.0 mm/s; 55.5±4.7 % to 71.1±2.6 %APA/ms). While not quite reaching values of CMC strands kept under identical conditions (315.1±17.7 mm/s; 74.7±1.6 %APA/ms), the results illustrate that components of the serum and/or components secreted by the cells are essential for slow conduction present in the fibrosis model under control conditions. Because block of TGF-β receptors with SB431542 and LY364947 has highly similar effects, the component responsible is likely TGF-β1.

**Figure S4.** Effects of no-serum/large-volume culture conditions on conduction- and action potential upstroke velocities in cardiac fibrosis models. A, Comparison of conduction velocities measured in control preparations (CMC strands, red; CMC-MFB strands, green) vs. preparations exposed for 24h to no-serum/large volume conditions. B, Same as A for maximal upstroke velocities. Mean ±SD; number of preparations indicated at the bottom of the graphs.
Membrane potentials in heterologous cell pairs: Theoretically predicted vs. measured values

Since $V_m$ of CMCs and MFBs differ significantly, resistive coupling between the two cell types will result in intercellular current flow leading to convergence of the two membrane potentials. The degree to which this occurs depends on the relative size of the membrane resistances of the two cell types and the gap junctional conductance, $g_j$. Having determined all relevant parameters in individual cells ($V_{m, CMC}$, $r_{m, CMC}$, $V_{m, MFB}$, $r_{m, MFB}$), we asked what the resultant $V_m$ would be if these cells were joined by values of $g_j$ as determined in cell pairs. Apart from being of interest from a theoretical point of view, deviations of the predicted $V_m$ CMC from actually measured $V_m$ CMC in heterotypic cell pairs would suggest that factors different from electrotonic coupling, like exertion of strain of MFBs on adjacent CMCs followed by activation of stretch sensitive ion channels, would contribute to the depolarized CMC phenotype. Accordingly, we applied equations developed by Xie and Qu modified as to include standard deviations obtained in the experiments to our single cell data as follows:

At steady state, the resting potentials of coupled cells are

$$V_{C MC} = E_{CMC} + \frac{1}{g_{CMC}} (E_{M FB} - E_{CMC}) / \left( \frac{1}{g_j} + \frac{1}{g_{M FB}} + \frac{1}{g_{CMC}} \right) \tag{1}$$

$$V_{M FB} = E_{M FB} - \frac{1}{g_{M FB}} (E_{M FB} - E_{CMC}) / \left( \frac{1}{g_j} + \frac{1}{g_{M FB}} + \frac{1}{g_{CMC}} \right) \tag{2}$$

where $g$ are conductances (1/r) and $E$ is the resting potential of uncoupled cells. The propagation of uncertainty of $V_m$ of each cell is computed as:

$$\sigma^2_{V_{CMC}} = \left( \frac{\partial V_{CMC}}{\partial E_{CMC}} \right)^2 \sigma^2_{E_{CMC}} + \left( \frac{\partial V_{CMC}}{\partial E_{M FB}} \right)^2 \sigma^2_{E_{M FB}} + \left( \frac{\partial V_{CMC}}{\partial g_j} \right)^2 \sigma^2_{g_j} + \left( \frac{\partial V_{CMC}}{\partial g_{M FB}} \right)^2 \sigma^2_{g_{M FB}} \tag{3}$$

$$\sigma^2_{V_{M FB}} = \left( \frac{\partial V_{M FB}}{\partial E_{CMC}} \right)^2 \sigma^2_{E_{CMC}} + \left( \frac{\partial V_{M FB}}{\partial E_{M FB}} \right)^2 \sigma^2_{E_{M FB}} + \left( \frac{\partial V_{M FB}}{\partial g_j} \right)^2 \sigma^2_{g_j} + \left( \frac{\partial V_{M FB}}{\partial g_{M FB}} \right)^2 \sigma^2_{g_{M FB}} \tag{4}$$

When inserting $V_m$ and $g$ obtained in single cell measurements and $g_j$ determined in dual whole cell patch-clamp experiments in the equations above, theoretical estimates of $V_m$ of CMCs coupled to MFBs are, as shown in Table S2 (green columns), virtually identical to actually measured values which indicates that electrotonic coupling alone fully accounts for the partial depolarization of CMCs by coupled MFBs. This finding is in accordance with the results of the ‘killing protocol’ and indicates that additional depolarizing mechanisms such as activation of stretch sensitive channels or paracrine interactions play a minor, if any, role in MFB induced CMC depolarization.
Table S2: Prediction of $V_m$ of CMCs coupled to MFBs based on electrophysiological parameters recorded in single cells. Left block of data: $V_m$ and membrane resistance ($r_m$) measured in single CMCs and MFBs, $g_j$ measured in CMC-MFB cell pairs and $V_m$ measured in CMCs coupled to MFBs (green background). Right block: theoretically predicted $V_m$ of CMCs (green background) and MFBs (white background) when coupled to each other. Measured and theoretically predicted $V_m$ of CMCs coupled to MFBs were compared with the z-test.
Supplemental References


