Supplement Material

Expanded Methods

Microcontact printing. Photolithography was used to make a master pattern of 20μm wide lines separated by 10μm 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 600μl of fibronectin dissolved in deionized water (50μg/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 20μm strands, which ultimately creates an anisotropic monolayer. Because the gap between strands is only 10μm, 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.

Immunocytochemsitry. 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 H_2O) 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 Na_3VO_4, 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.[3, 4]

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 were calculated from the measured deflections of underlying post tops using image analysis software written in IgorPro

(WaveMetrics, Lake Oswego, OR). 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.[5] 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.[6] 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 (dV/dt_{max}) during action potential depolarization, and dV/dt_{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^{th} percentile.[7]

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

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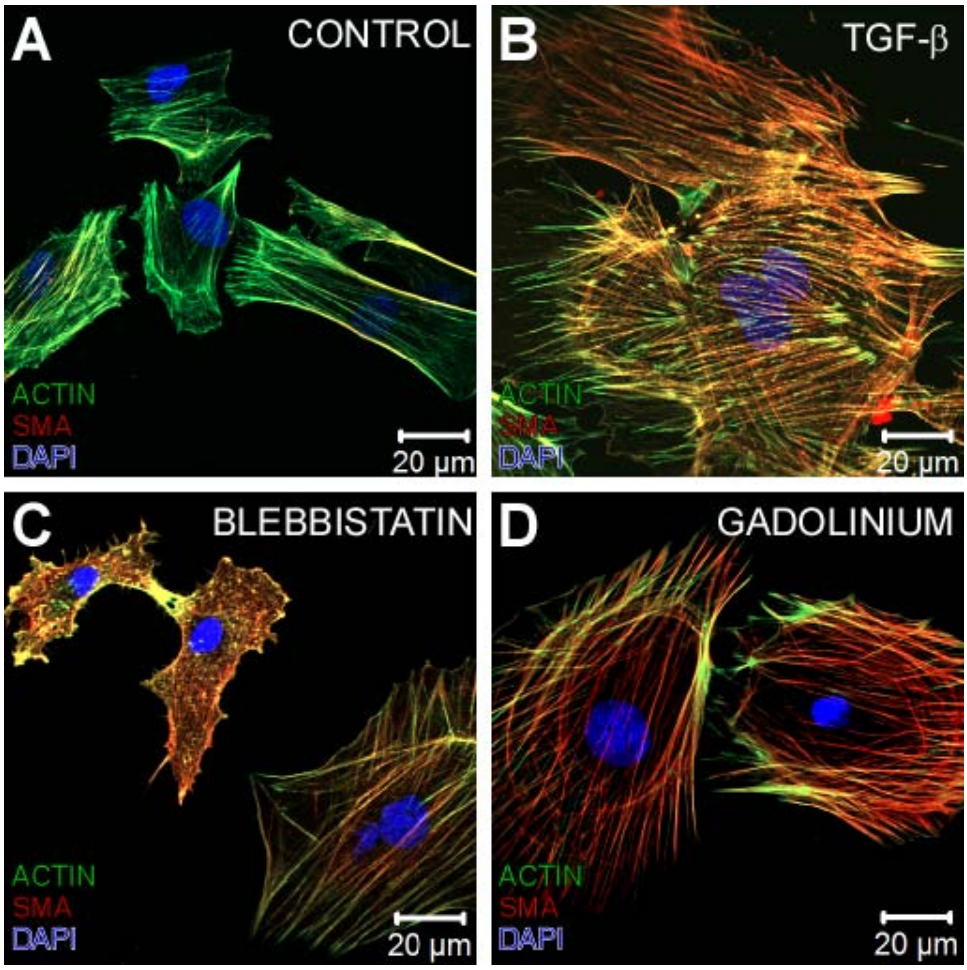
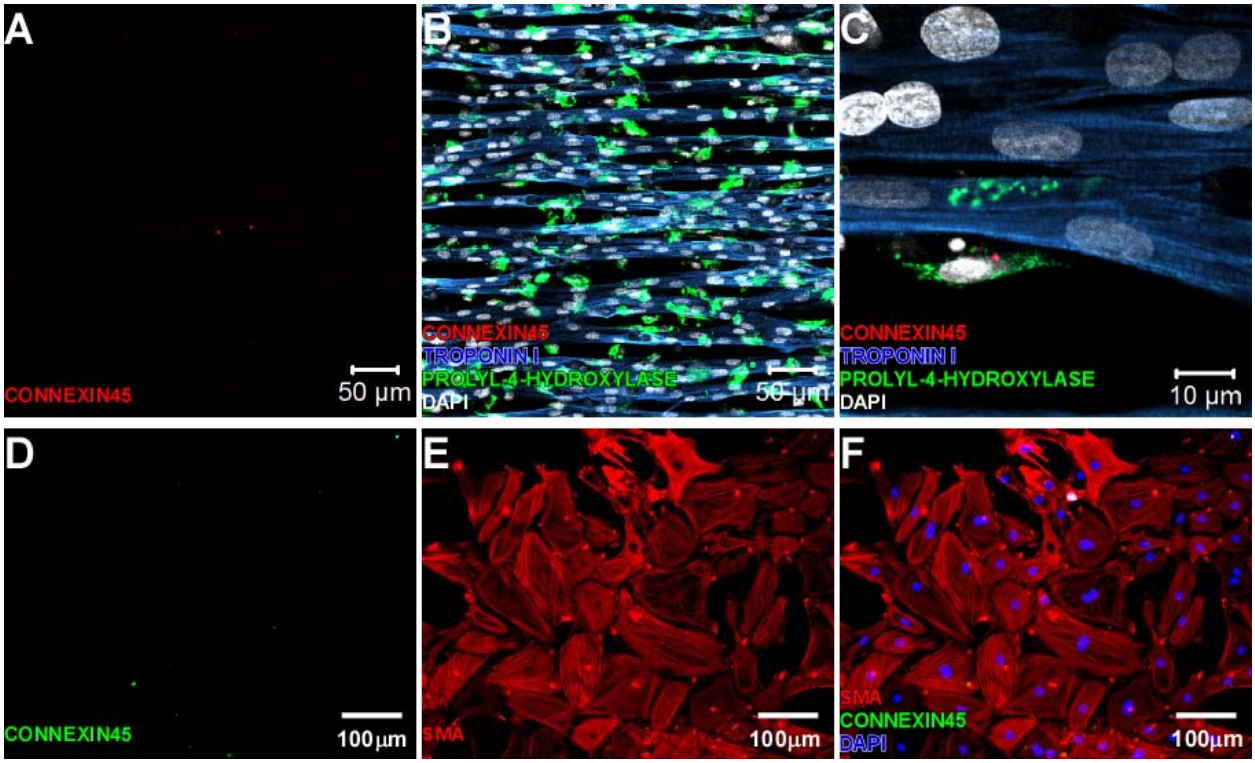
Figure Legends

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