SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell cultures

Cardiomyocytes

Ventricular cardiomyocytes were dissociated from 2 day old neonatal Sprague-Dawley rats (Charles River Laboratories) and preplated to remove faster adhering non-myocytes, as previously described[1]. Circular coverslips (22mm diameter, Aclar®, Electron Microscopy Sciences) were coated with 25µg/ml fibronectin (Becton Dickinson) in PBS for 2 hrs at 37°C, and then cardiomyocytes obtained after the preplating were seeded at a density of 2300cells/mm^2 in DMEM/F12 medium (Gibco) supplemented with 10% calf serum (Colorado Serum Company) and 10% horse serum (Hyclone). Cells were allowed to adhere overnight, and unattached cells were discarded after a wash with phosphate buffered saline. Forty-eight hours after seeding, the seeding media was switched with a defined, serum-free maintenance media[2] (control media) which was used for the remainder of the culture time. Serum-free media was composed of DMEM/F-12 media (GIBCO) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 µg/ml L-thyroxine, 0.1 µg/ml insulin, 0.5 µg/ml transferrin, 2.5 µg/ml ascorbic acid, 1 nM lithium chloride, and 1 nM sodium selenite (Sigma)[2].

Cardiac fibroblasts

Neonatal rat cardiac fibroblasts (CFs) were recovered from the first preplating step of the cardiomyocyte isolation. CFs were cultured in M199 (Gibco) supplemented with 10% fetal bovine serum (Hyclone), HEPES (10mM, Gibco), non-essential amino acids solution (1X, Gibco), L-glutamine (2mM, Gibco), dextrose (3.5mg/ml, Sigma), cobalamin (4µg/ml, Sigma), and Penicillin G (100units/ml, Sigma). Upon reaching confluence at day 7, the fibroblasts were

split and passaged at a 1:2 ratio and used at passage 1 after 3 days of culture. At passage 1, a high purity of cells (>99%) and fibroblast phenotype was confirmed by positive staining for vimentin and negative staining for sarcomeric α-actinin, smooth muscle actin, and von Willebrand factor[2].

Co-Culture Studies

The noncontact co-culture studies (Treatment 1, Fig. 6A1) were performed in modified 60mm tissue culture treated Petri dishes (Falcon). A sterile 21mm Aclar® coverslip was coated with cell-repelent Pluronic solution (0.2%, Molecular Probes) to prevent cell attachment and was then secured in the center of a 60mm Petri dish using three sterile minute pins (Fine Science Tools) as depicted in Fig. 6A1. Cardiac fibroblasts were seeded (and attached only around the Aclar coverslip) at an initial density of 100/mm^2 to yield the same final cell number per area as the CFs used for the other conditioned media studies. After seven days of fibroblast culture (as in the other conditioning studies), the blank Aclar coverslip was removed and replaced with a 6 day old cardiac monolayer. The surrounding fibroblasts and cardiac monolayer were then cultured in 2.8ml of control media for 24h. This exact volume was chosen to yield fibroblast conditioning with the same cell number per media volume (and thus the same concentration of paracrine factors) as in other conditioned media studies. However, in this particular setting, the CFs conditioning took place in the presence of cardiomyocytes (shared media) but without direct contact of the CFs and cardiomyocytes. In addition, 6 day old control monolayers were introduced in the same setting but without the fibroblast layer and incubated for 24 hours either in control media (Control 1, Fig. 6A1) or media conditioned as in other studies by CFs alone

(Conditioned 1, Fig. 6A1). After 24h, the cardiac monolayers were removed from the Petri dishes and assessed for electrophysiological function by optical mapping.

Optical mapping

Optical recordings of membrane voltage were performed in confluent monolayer cultures using previously described methods[1]. Specifically, monolayers were stained with the voltage-sensitive dye di-4-ANNEPS (16μM, Molecular Probes) for five minutes at room temperature and then placed in a custom-designed temperature-controlled chamber (Dagan Corp.) and gravity perfused with 37°C oxygenated Tyrode's solution. Cultures were illuminated by a 250W Tungsten lamp (Oriel). Electrical stimuli were delivered at a basic pacing rate of 2Hz by a bipolar platinum point electrode positioned ~1mm above the monolayer surface at the center of the monolayer. The pacing rate was increased every minute in steps of 1 Hz until partial conduction block occurred. Fluorescence maps were recorded using a fiberoptic bundle with 504 hexagonally arranged optical fibers (Red Shirt Imaging), converted to voltage with photodiodes, sampled at 2.4 kHz, and stored on a computer. Data acquisition, shutter and stimulus control were synchronized using custom-designed LabView 7.1 software (National Instruments). For data analysis, signals from the recording sites were detrended by fitting and subtracting a 3rd order polynomial and then filtered using a combination of Savitzky-Golay and low-pass temporal filters to maintain action potential upstroke integrity while removing the noise from the repolarization phase of the action potential. Local activation times at basic pacing rate were determined as times of the maximal action potential upstroke and used to construct isochrone maps and calculate conduction velocities. Action potential duration, APD80, was defined as the interval from activation time to 80% repolarization of the action potential. Maximum capture rate

was defined as a maximum pacing rate at which each stimulus was followed by successful action potential propagation over a period of at least 30s.

Immunocytochemistry

Immunostaining was performed as previously described[2,3]. Briefly, cell cultures were fixed in a 1:1 v/v solution of methanol and acetone, permeabilized with 0.05% Triton X-100 (Sigma), blocked, and incubated with primary antibody overnight [anti-sarcomeric α-actinin, 1:100, Sigma; anti-connexin-43 and anti-connexin-43 non-phosphorylated, 1:100, Zymed; and anti-vimentin, 1:1000, Sigma]. Secondary antibody [anti-rabbit and anti-mouse Alexa Fluor IgGs, 1:200, Molecular Probes] was then applied for 2 hours. DAPI (Sigma) was used for visualization of the nuclei. Immunostained cultures were imaged using a fluorescent microscope (Nikon TE2000U), a CCD camera (Scion), and IPLab image acquisition software (Scanalytics).

Cardiac cultures were immunostained for sarcomeric α-actinin, to assess general cell appearance, vimentin, to assess for the presence of fibroblasts, and both phosphorylated and unphosphorylated connexin 43, to assess cellular connectivity. For quantification of the immunostaining, ten non-overlapping images were randomly acquired per each coverslip at a magnification of 20X and used for analysis. Custom Matlab software was used to assess the area ratio of phosphorylated (PO4) and unphosphorylated (UnPO4) connexin-43 (Cx43) staining. The number of positively stained image pixels with an intensity greater than 10% of the maximum image pixel intensity were counted, their area calculated, and divided by the number of nuclei per image, indicating the expression of total Cx43 and UnPO4-Cx43 per cell. Similarly, segmentation analysis was used to select and measure vimentin positive areas (used to label

fibroblasts) in each image, and the obtained results were normalized by the total number of nuclei per image.

As a positive control for the $UnPO_4$-Cx43 antibodies, we immunostained cardiac monolayers exposed to hypoxia, as previously described[4]. Briefly, an 18mm round glass coverslip was gently placed on top of a 22mm cardiac monolayer for 1h. The glass coverslip greatly reduced the media volume accessible to the myocytes underneath, yielding hypoxic conditions[4]. Dephosphorylation of connexin 43 has been shown to occur as a result of cellular changes produced during ischemia and hypoxia[5,6]. As expected, extensive positive staining for $UnPO_4$-Cx43 was found in the area of the cardiac monolayer covered by the glass coverslip (Fig. 4B1 inset) with negligible levels (not shown) in the area of the cardiac monolayer that had not been covered.

Fluorescence recovery after photobleaching (FRAP)

FRAP was used to evaluate functional connectivity between cardiomyocytes as previously described[7,8]. The speed of recovery of fluorescence in a photobleached cell, by the passage of dye from surrounding coupled cells through functional gap junctions, was assessed using confocal microscopy. Control and CF-conditioned monolayers were labeled with calcein AM (Molecular Probes, 2 μM, 30 minutes at 37°C), washed with PBS, and imaged using an upright confocal microscope (Zeiss LSM 510). An individual cardiomyocyte, in confluent contact with other cardiomyocytes, was photobleached with a 488 nm Argon laser to remove the calcein fluorescence. Fluorescence recovery in the target cells was monitored by acquiring an image every 10 seconds for at least 4 minutes after photobleaching. Custom MATLAB software was used to analyze the images. Recorded fluorescence values were normalized to those

immediately before and after bleaching and then corrected for the gradual bleaching of the entire field during the recovery[7]. The percent fluorescence recovery was plotted versus time (Fig. 4D1,2) and fitted using an exponential function:

$$F(t) = F_\infty + (F_0 - F_\infty)e^{-t/\tau}$$

where t is the time after photobleaching, F(t) is the normalized fluorescence intensity, F_0 is the fluorescence intensity immediately post-bleaching at t=0, F_∞ is the fluorescence intensity at the end of recovery, and τ is the time constant of recovery, as previously described[7]. Then, the transfer constant k (min^{-1}) was calculated as $k=1/\tau$ divided by the number of cells in contact with the target cell.

Apoptosis detection

The presence of apoptotic cells was quantified using a commercially available TUNEL fluorescence kit (ApopTag Plus, Chemicon), per manufacturer's instructions. Briefly, apoptotic nuclei were labeled with fluorescein-conjugated antibodies, and cell nuclei were counter-stained with DAPI. Images were captured with a CCD camera and IPLab software, and the number of apoptotic nuclei was automatically counted using the segmentation analysis and expressed relative to total number of nuclei stained by DAPI.

qRT-PCR

Total RNA was isolated from cardiac monolayers using an RNeasy Mini Kit (Qiagen) per the manufacturer's instructions. Briefly, 3 monolayer cultures per experimental group were lysed, pooled, homogenized using a QIAshredder spin column (Qiagen) and applied to an RNeasy mini column. The total RNA bound to the column was then eluted with RNase-free

water. The quantity of total RNA and A260/A280 ratio for nucleic acid purity was measured with a spectrophotometer (Smartspec Plus; BioRad). All samples used had an A260/A280 ratio of 1.9 or greater.

RT-PCR primers and probes were custom designed and validated by Dr. Hyung-Suk Kim of the Gene Expression Laboratory at the University of North Carolina, Chapel Hill. The sequences of the primers and probes are listed in Supplemental Table 1. One-step RT-PCR was performed for each sample and for each gene in triplicate in 96-well optical plates (PE Applied Biosystems) on an ABI Prism 7300 Sequence Detection system (PE Applied Biosystems). All TaqMan PCR data were captured using Sequence Detection Software (SDS version 1.6; PE Applied Biosystems). For every sample, an amplification plot was generated and from this plot a threshold cycle (Ct), value was calculated. The data was quantified using the $2^{-\Delta\Delta Ct}$ method, as previously described[9], by comparing the signal of the target transcript of the treated group with that of the control group, both relative to an internal control, 18S.

Sharp microelectrode recordings

Sharp microelectrodes were fabricated from standard wall borosilicate glass capillary tubes (Sutter) using a P-97 Sutter micropipette puller to generate electrodes with tip resistances between 50 and 80 MΩ when backfilled with 3M KCl. The electrodes were connected to the headstage of a Multiclamp 700B amplifier (Axon Instruments, Inc.) using a silver chloride wire embedded within the micropipette holder. A reference silver chloride wire was connected to the bath chamber through an agar bridge. Cultures were placed into a temperature controlled chamber mounted on an inverted microscope (Nikon Eclipse TE2000) and perfused with warm (35ºC) Tyrode's solution.

Electrode potential offset and capacitance were neutralized prior to cell impalement. Upon the impalement and after the resting potential has stabilized, a bipolar point electrode was used to elicit electrical propagation at the periphery of a monolayer at a 2 Hz pacing rate using a Grass stimulator (Astro-Med). Action potentials were recorded in cells positioned at least 3 mm away from the stimulus site using the current clamp mode of the Multiclamp 700B amplifier. Recordings were sampled at 20 kHz, digitized using a BNC-2090 adaptor and NIDAQ-MX computer interface (National Instruments Corporation, Austin, TX), and low-pass filtered at 4.5 KHz using a 4-pole Butterworth filter. Data was analyzed using WinWCP software (Dr. John Dempster, University of Strathclyde, Glasgow, Scotland) to derive membrane resting potential, maximum depolarization slope of action potential, and action potential duration at 80% of repolarization.

Supplemental References

1. Bursac N, Parker KK, Iravanian S, Tung L. Cardiomyocyte cultures with controlled macroscopic anisotropy: a model for functional electrophysiological studies of cardiac muscle. *Circ Res*. 2002;**91**:e45-54.
2. Pedrotty DM, Klinger RY, Badie N, Hinds S, Kardashian A, Bursac N. Structural coupling of cardiomyocytes and noncardiomyocytes: quantitative comparisons using a novel micropatterned cell pair assay. *Am J Physiol Heart Circ Physiol*. 2008;**295**:H390-H400.
3. Klinger R, Bursac N. Cardiac cell therapy in vitro: reproducible assays for comparing the efficacy of different donor cells. *IEEE Eng Med Biol Mag*. 2008;**27**:72-80.
4. Pitts K, Toombs C. Coverslip hypoxia: a novel method for studying cardiac myocyte hypoxia and ischemia in vitro. *Am J Physiol Heart Circ Physiol*. 2004;**287**:1801-1812.
5. Beardslee M, Lerner D, Tadros P, Laing J, Beyer E, Yamada K *et al*. Dephosphorylation and intracellular redistribution of ventricular connexin 43 during electrical uncoupling induced by ischemia. *Circ Res*. 2000;**87**:656-662.
6. Turner M, Haywood G, Andreka P, You L, Martin P, Evans W *et al*. Reversible connexin 43 dephosphorylation during hypoxia and reoxygenation is linked to celluar ATP levels. *Circ Res*. 2004;**95**:726-733.
7. Abbaci M, Barberi-Heyob M, Stines J, Blondel W, Dumas D, Guillemin F *et al*. Gap junctional intercellular communication capacity by gap-FRAP technique: a comparative study. *Biotechnol J*. 2007;**2**:50-61.

8. Wade M, Trosko J, Schindler M. Fluorescence photobleaching assay of gap junction-mediated communication between human cells. *Science*. 1986;**232**:525-528.
9. Livak K, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2-delta deltaCT method. *Methods*. 2001;**25**:402-408.

Supplemental Table

Table 1. TaqMan primers and probes for rat genes.

Gene	Type	Sequence (5'-3')
18S	Forward	AGA AAC GGC TAC CAC ATC CA
	Reverse	CTC GAA AGA GTC CTG TAT TGT
	Probe	f AG GCA GCA GGC GCG CAA ATT AC q
Connexin43	Forward	AAG TGA AAG AGA GGT GCC CA
	Reverse	GTG GAG TAG GCT TGG ACC T
	Probe	f TCC AGA AGC TTC CCC AAG GCA CTC q
Connexin45	Forward	CCT TTC ACT TGG AAT ACA CCA T
	Reverse	CAG TGT ACT GGA TTT GAT CTG G
	Probe	f CTC CCC CAG GCT ATA ACA TTG CTG q
KIR2.1	Forward	GTG TCT GAG GTC AAC AGC TT
	Reverse	GAA ACC ATA GCC GAT GGT TG
	Probe	f CGG CTG CCT TCC TCT TCT CCA TTG A q
KCND3	Forward	CTC AGC ACC ATC CAC ATC CA
	Reverse	CAG TCC GAC GTC TGC TTT CA
	Probe	f AGT GAG CAG CCC TCC CTC ACC AC q
SCN5A	Forward	GTG GTC ATC CTC TCC ATT GT
	Reverse	AGA CGG ATG ACC CGG AAG A
	Probe	f CAC TGT CCT CTC CGA CAT CAT CCA G q
αMHC	Forward	AGC TCA GTC AGG CCA ATA GA
	Reverse	TGG GTG TCC TTC AAG TGG GC
	Probe	f CGT TCT TCA AGT GCT TCT GGG CCT CT q
βMHC	Forward	AGC TCA GTC ATG CCA ACC GC
	Reverse	TGA GTG TCC TTC AGC AAA CT
	Probe	f AGG CTC TTC ACT TGT TTC TGG GCC TC

f, Reporter dye(FAM:6-carboxyfluorescein); q, Quencher dye(TAMRA:6-carboxytetramethyl1-rhodamine)