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Supplemental Information

Functional Properties of Engineered Heart Slices Incorporating Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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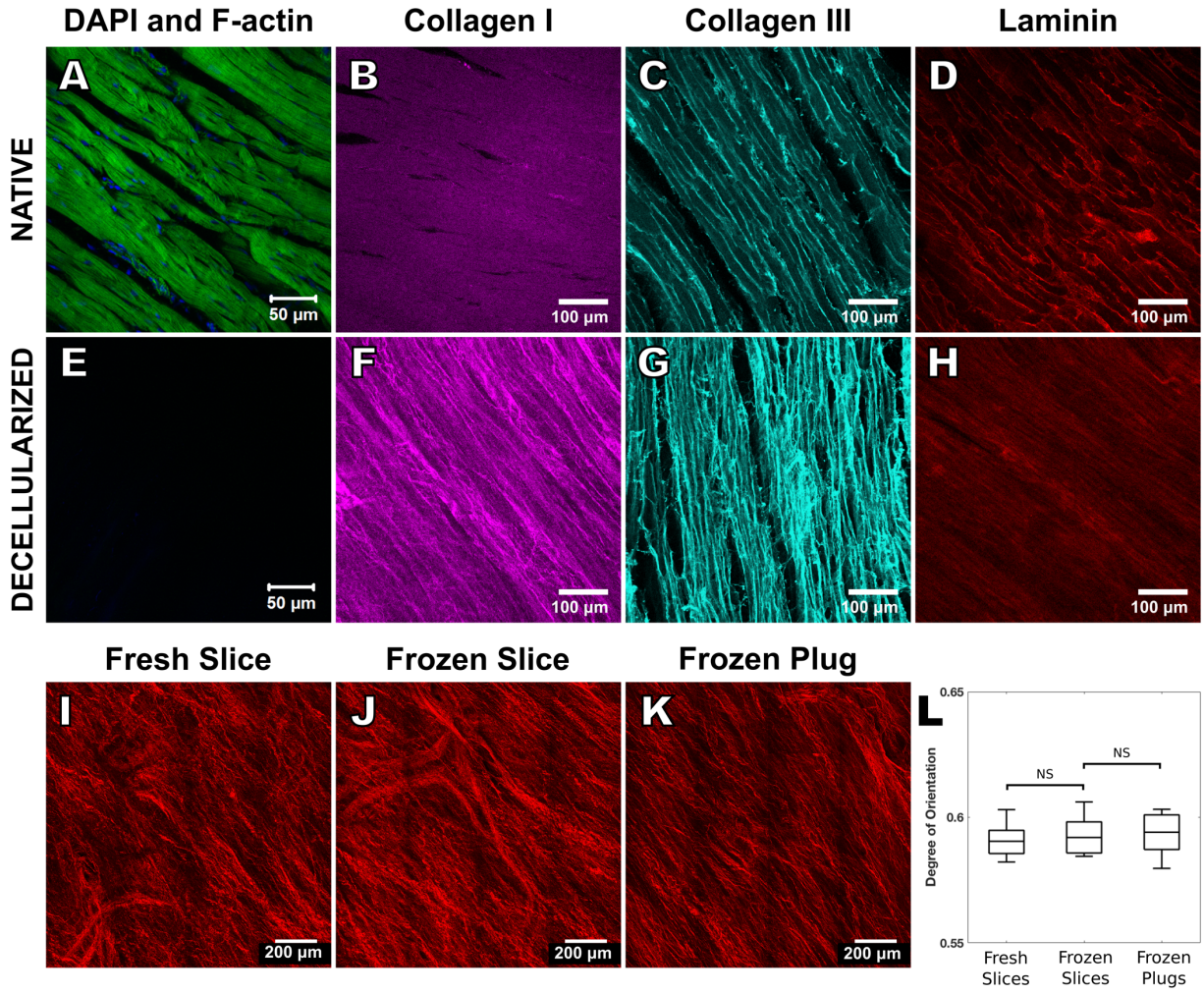

Figure S1. *Characterization of dECM slices.* Related to Figure 1. Immunostaining of slices for F-actin (green) and DAPI (blue) indicates the presence of (A) cellular content before but (E) not after decellularization. The extracellular matrix components for (B,F) collagen I, (C,G) collagen III, and (D,H) laminin in the slice both before (top row) and after (bottom row) decellularization. Second harmonic generation imaging showing the collagen fibers in (I) a slice sectioned and decellularized from a fresh porcine heart, (J) a slice sectioned from a porcine heart frozen for > 48 hours at -80°C and subsequently decellularized, and (K) a slice originating from a plug frozen for > 48 hours at -80°C and subsequently sectioned and decellularized. (L) The degree of orientation of the collagen fibers was similar in all three groups, n = 8 for each group. Error bars denote standard deviation. NS indicates means are not significantly different.

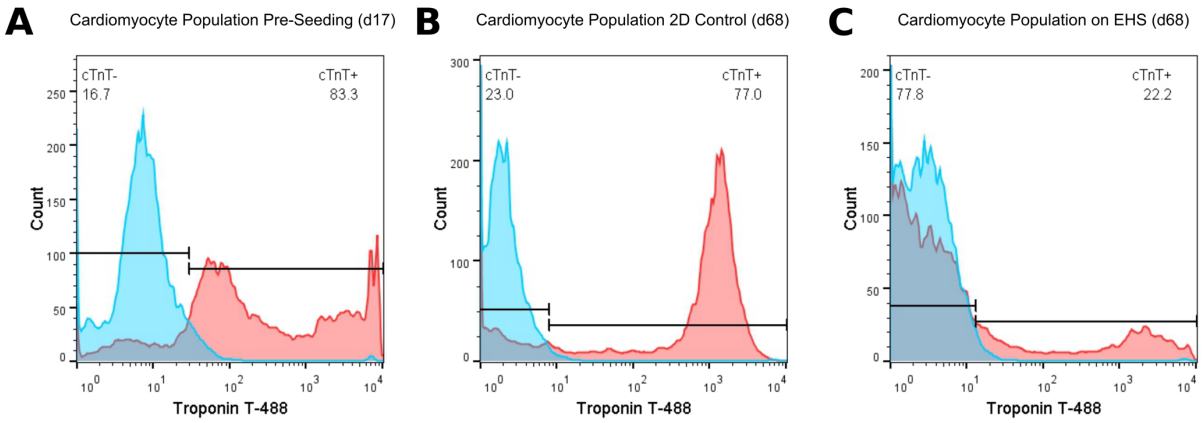

Figure S2. *Characterization of cell populations.* Related to Figure 2. Cardiomyocytes were analyzed via flow cytometry at several stages: on day 17 (d17) after initiating cardiomyocyte differentiation according to (A) standard protocols, (B) after re-plating into 2D tissue culture plates at d17 and long-term maintenance in culture for an additional 61 days, and (C) after re-plating onto dECM slices at d17 to form EHS and maintained in culture for an additional 61 days. Data shows percentage of non-cardiomyocytes (cTnT-, blue) cell and percentage of cardiomyocytes (cTnT+, red) in each panel. Histogram counts were extracted by gating on unstained cells and cells stained only with a secondary antibody control (to remove autofluorescence and background nonspecific secondary staining).

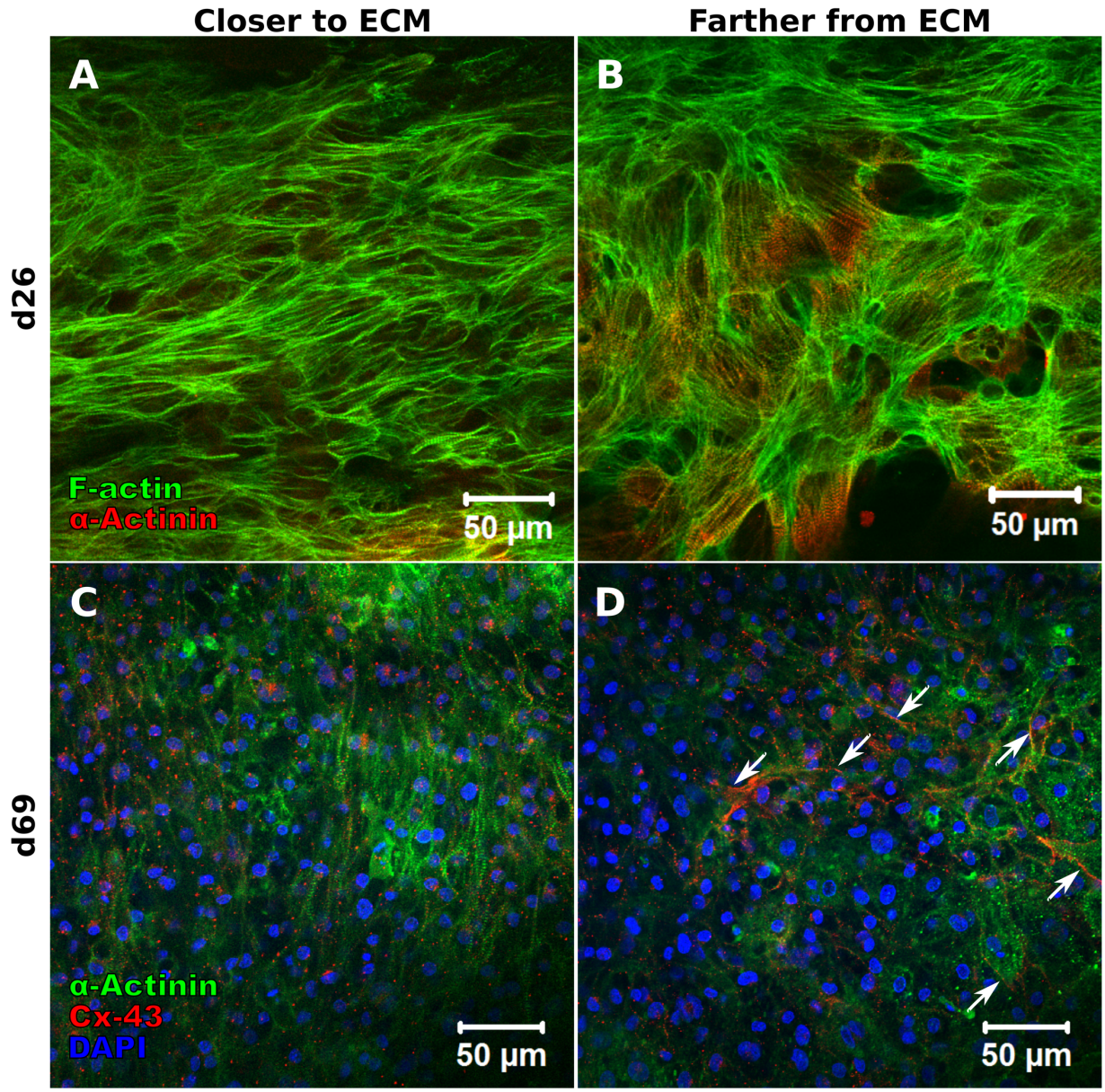

Figure S3. *Morphology of hiPSC-CMs on EHS.* Related to Figure 2. EHS seeded with hiPSC-CMs had multiple layers of cells, with (A,C) CMs in contact with the ECM of the slice exhibiting greater alignment than (B,D) CMs in a second layer sitting on top of the first CM layer, farther from the slice and forming the surface of the EHS. (D) EHS also exhibited localization of Cx43 around the perimeters of the cells (white arrows). Top row images are from d26 EHS and bottom row images are from d69 EHS.

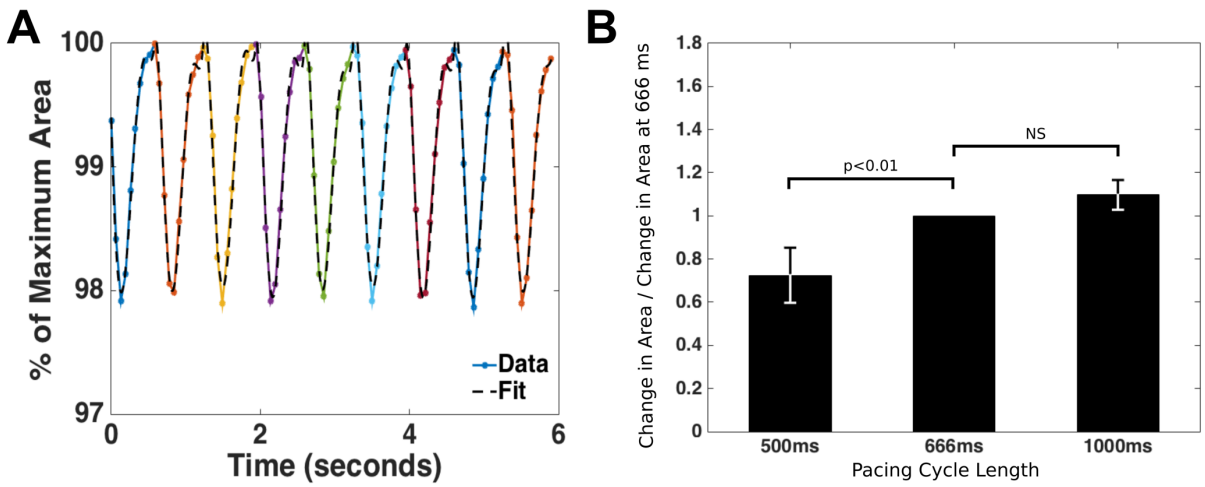

Figure S4. *Contraction of EHS in response to different pacing rates*. Related to Figure 3. (A) Contraction traces of EHS exposed to 1μM isoproterenol plotted in Fig. 3C. Each cycle of contraction and relaxation was fit by a polynomial of degree 5 using a least squares fitting method. The data for each cycle are plotted in a different color, and the fitted curve for that cycle plotted on top using a dashed black line. The difference in the minimum (representing maximum EHS contraction during one cycle) calculated by our data compared to that calculated by the best fit line was 0.02±0.01% for the control, 0.06± 0.04% for the isoproterenol, and 0.05±0.03% for the washout traces plotted in Fig. 3C. (B) Contraction amplitudes at 500 ms and 1000 ms pacing rates were normalized by the contraction amplitudes at a pacing rate of 666 ms. Error bars denote standard deviation. NS indicates mean is not significantly different from 1. $n \geq 6$ for each group.

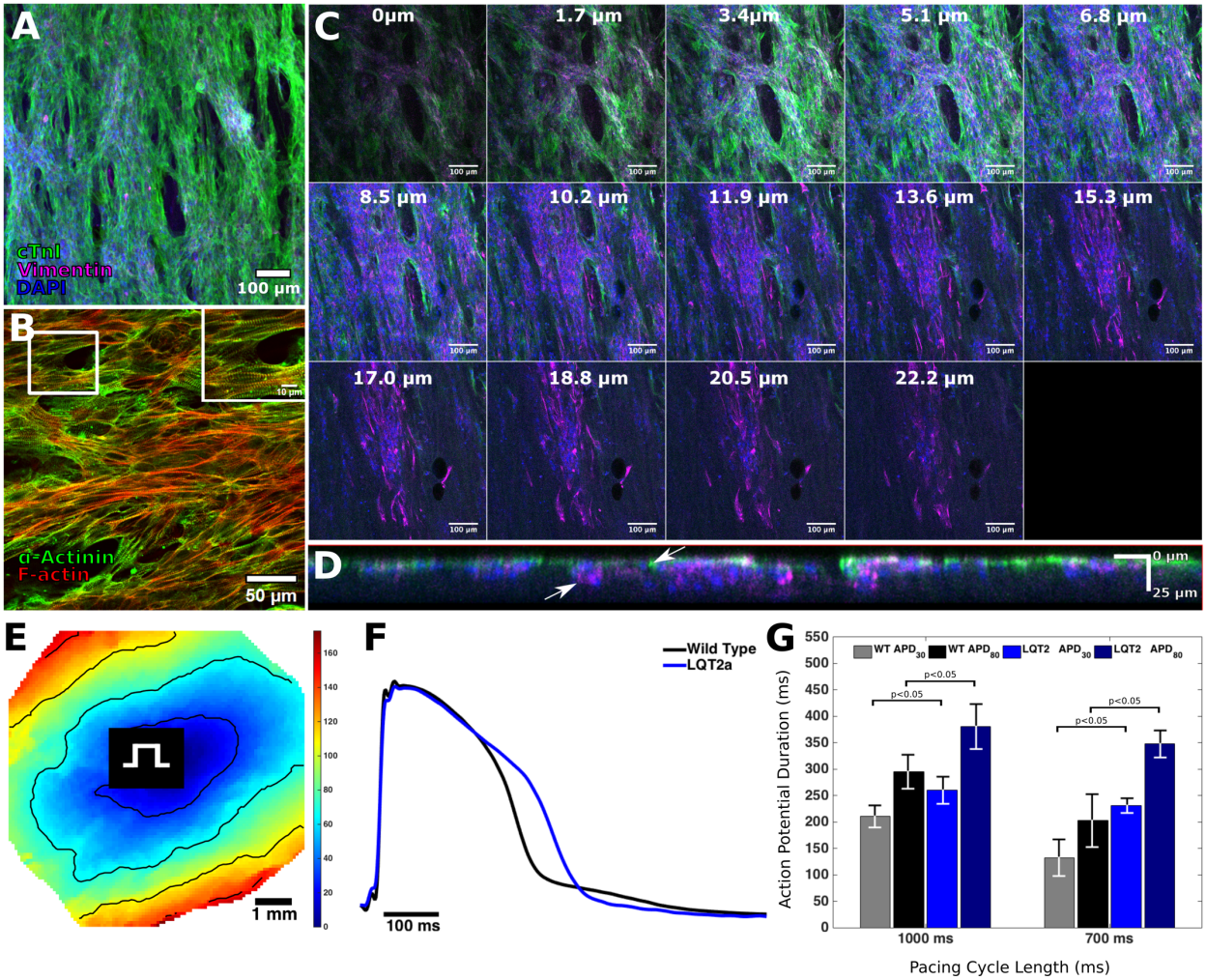

Figure S5. *EHS comprised of LQT2 hiPSC-CMs.* Related to Figure 4. EHS were seeded with LQT2 hiPSC-CMs 10 days after the start of differentiation and maintained in culture for 13 days before evaluation (at d23). (A) Immunostaining for cardiac troponin I (green), DAPI (blue) and vimentin (magenta) reveals alignment of hiPSC-CMs. (B) Cardiomyocytes on LQT2 EHS stained with α-actinin (green) and F-actin (red) had striations typical of sarcomeric structures (inset scale bar = 10 µm). (C) Confocal images of the EHS in (A) display the distribution of cardiomyocytes (stained for cardiac troponin I, green) and non-myocytes (stained for vimentin, magenta). Images were taken at intervals of 1.7µm from the surface of the EHS. Cell nuclei are indicated by DAPI staining in blue. (D) An orthogonal slice of the entire volume reveals multiple layers of cells, with cardiomyocytes on the surface (top arrow) and non-myocytes underneath, closest to the ECM of the slice (bottom arrow). (E) Activation map of EHS with LQT2 hiPSC-CMs paced at 1000 ms cycle length shows anisotropic conduction. Black lines are isochrones at 40 ms intervals. Rectangular symbol indicates pacing site. (F) Average traces for wild type (WT, black) and LQT2 (blue) EHS paced at 1000 ms cycle length reveal differences in action potential morphology. (G) APD_{80} and APD_{30} were greater in LQT2 EHS versus WT EHS at 1000 ms and 700 ms paced cycle lengths. Error bars denote standard deviation. n = 5 for WT EHS and n = 3 for LQT2 EHS.

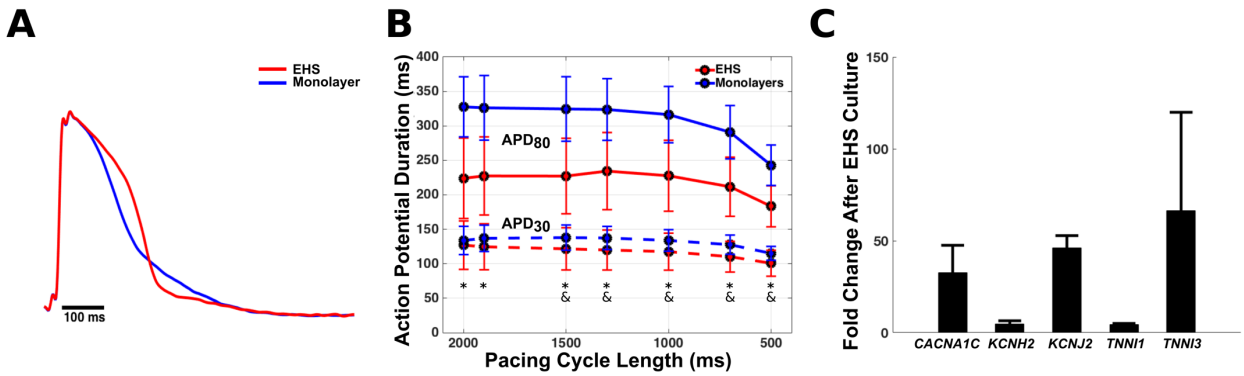

Figure S6. *Action potential morphologies and qRT-PCR analysis of ion channel transcripts.* Related to Figure 6. Sample action potential traces (A) illustrate the differences in action potential shape of EHS and monolayers (d40-70). Action potential durations (B, APD_{80} values are connected using solid lines and APD_{30} values are connected by dashed lines) plotted for pacing cycle lengths ranging from 2000 ms to 500 ms. Error bars denote standard deviation. * indicates $p<0.05$ when comparing mean APD_{80} for monolayers and mean APD_{80} for EHS at the specified cycle length. & indicates $p<0.05$ when comparing mean APD_{30} for monolayers and mean APD_{30} for EHS at the specified cycle length. n = 28-31 for EHS and n = 15-16 for monolayers. (C) Expressions of ion channel transcripts were altered in d69 EHS compared to control d22 hiPSC-CMs prior to seeding on EHS. n = 3 for EHS and n = 4 for controls. Error bars indicate standard deviation.

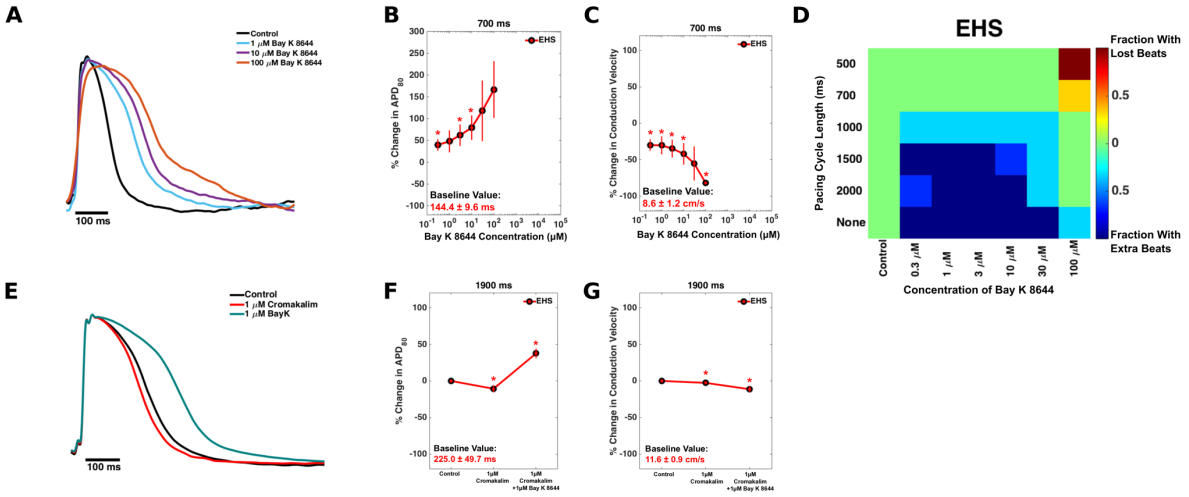

Figure S7. *Application of cardioactive drugs to EHS after prolonged culture.* Related to Figures 6 and 7. (A) Sample traces of EHS paced at 700 ms indicate the changes in action potential shape in the presence of increasing concentrations of Bay K 8644. (B) Bay K 8644 prolonged APD_{80}, and (C) decreased conduction velocity in a concentration-dependent manner. (D) Bay K 8644 also decreased the maximum capture rate. (E) Sample traces of d201 EHS paced at 1900 ms at baseline (black trace), after the application of 1 μM cromakalim (red trace), and after the additional application of 1 μM Bay K 8644 (green trace) indicate changes in action potential shape. (F) Cromakalim and Bay K 8644 both decreased APD_{80} and (G) slowed conduction velocity compared to baseline values. Baseline values of APD_{80} and conduction velocity before the application of drug are indicated in (B,C,F,G). Error bars denote standard deviation. n = 3 in (B,C,D) and n = 4 in (F,G). * indicates $p<0.05$ when comparing the percent change to 0 using paired, unequal variance t-test.

Video S1. *Contracting EHS.* Related to Figure 3A-C. (Top) Free edge of EHS shortens in response to pacing at a cycle length of 666ms. (Bottom) The degree of contraction is indicated by the continuous plot of EHS area, which decreases from 100% (the maximum area at rest) with each paced beat.

Video S2. *Optical mapping video of EHS.* Related to Figure 4A. The EHS was paced in the center at a cycle length of 500ms, and the sample action potential recordings are from the site denoted by the magenta dot on the optical map. The bottom trace indicates the pacing pulses, and the vertical dashed line spanning both the upper and lower traces indicates the time point in the action potential corresponding to the displayed optical map.

Paced Cycle Length (ms)	n	APD_{30} (ms)	APD_{80} (ms)	Longitudinal Conduction Velocity (cm/s)	Transverse Conduction Velocity (cm/s)	Anisotropy Ratio
400	4	129±3	183±6	8.9±1.5	7.4±1.0	1.2±0.1
500	6	152±15	214±20	11.6±2.1	8.3±1.7	1.4±0.2
600	6	165±19	235±23	13.6±2.1	9.7±1.7	1.4±0.1
700	6	178±24	253±29	15.4±2.4	10.9±1.8	1.4±0.1
800	6	186±28	267±32	16.6±1.8	11.4±0.9	1.5±0.1
900	6	198±24	283±34	18.9±2.3	13.0±1.3	1.5±0.2
1000	6	211±21	295±32	18.8±1.3	13.4±1.4	1.4±0.1

Table S1. *Electrophysiological measurements of d54-58 EHS.* APD_{30}, APD_{80}, longitudinal and transverse conduction velocities, and anisotropy ratios correspond to plots in Figure 4. Data represent mean±SD.

Paced Cycle Length (ms)	APD_{30} (ms) d62-82 (n=3)	APD_{30} (ms) d201 (n=5)	APD_{80} (ms) d62-82 (n=3)	APD_{80} (ms) d201 (n=5)	Conduction Velocity (cm/s) d62-82 (n=3)	Conduction Velocity (cm/s) d201 (n=5)
400	---	93±14	---	139±19	---	9.5±1.5
500	---	111±11	---	162±16	---	11.1±1.5
700	172±15	128±16	250±17	184±21	13.9±1.9	12.3±1.7
900	187±19	137±15	271±21	194±20	15.9±1.7	13.2±1.8
1000	196±22	143±15	286±25	202±21	16.6±1.8	13.8±1.9
1300	209±25	149±19	301±30	209±23	17.8±1.6	14.4±2.0
1500	220±30	153±18	312±31	213±23	18.1±1.5	14.6±2.0
1700	---	155±18	---	215±22	---	15.1±1.7
1900	231±33	153±18	323±37	212±22	18.4±1.8	15.4±1.3
2000	---	160±19	---	220±21	---	16.0±1.3

Table S2. *Electrophysiological measurements of d62-82 and d201 EHS.* APD_{30}, APD_{80}, and conduction velocities correspond to plots in Figure 5. Data represent mean±SD.

Supplemental Experimental Procedures

Unless otherwise stated, reagents were acquired from Thermo Fisher Scientific, Waltham, MA.

1. *Preparation of EHS*

Hearts obtained from slaughterhouse pigs were rinsed in distilled and deionized water to remove blood and stored overnight at -20°C. The following day, the hearts were allowed to thaw at room temperature for 1 hour. A metal 12 mm diameter punch was sterilized using 70% ethanol and used to punch out plugs of tissue from the left ventricle. Plugs were trimmed in order to fit into 35 mm culture dishes and stored at -80°C until slicing, a minimum of 16 hours. Plugs were allowed to thaw in room temperature distilled water supplemented with 100 U/mL Penicillin-Streptomycin and 0.1X antibiotic-antimycotic. After thawing, plugs were blotted dry, placed in a 35 mm culture dish with the epicardium pressed against the bottom of the dish, and embedded in 4% w/v low gelling temperature agarose (Sigma-Aldrich Corp., St. Louis, MO) dissolved into distilled water with penicillin-streptomycin and antibiotic-antimycotic. The agarose was allowed to solidify at 4°C for 15 minutes, and then the agarose disc containing the plug was removed from the culture dish and attached to the cutting stage of a vibratome (7000smz, Campden Instruments, Lafayette, IN) using cyanoacrylate glue (3M, Maplewood, MN) with the epicardium positioned at the top of the plug. The plug was sectioned into 300μm-thick slices parallel to the epicardium using a ceramic blade oscillating at a frequency of 100 Hz with an amplitude of 1 mm and advancing at a speed of 0.01 to 0.03 mm/second. The cutting solution in which the plug was immersed (phosphate buffered saline (PBS) supplemented with antibiotics) was kept at 4-8°C during slicing. Slices were stored in PBS supplemented with antibiotics at 4°C overnight.

Slices were decellularized using a procedure modified from Ott et al. (Ott et al., 2008). All detergents for decellularization were diluted in distilled water supplemented with antibiotics. PBS was also supplemented with the same antibiotics. Slices were each placed in a well of a 12-well plate, and 1 mL of each of the decellularization solutions was added to each well. The plate was placed on a rotator (Orbit 1000 Digital Shaker, Labnet International Inc., Edison, NJ) and agitated 180 rpm in the presence of the following solutions: 1% sodium dodecyl sulfate (SDS) for 3 hours (replaced with fresh solution after 1.5 hours), water for 15 minutes, 1% Triton-X 100 (Sigma-Aldrich Corp.) for 7 minutes, and PBS for 45 minutes (replaced with fresh solution every 15 minutes). Samples were left in PBS on rotator at 160 rpm overnight to rinse out any remaining detergents.

Plastic 12 mm coverslips were immersed in 70% ethanol and wiped dry. After an overnight wash in PBS, slices were carefully handled with forceps, spread over the coverslips and wrapped around the edges of the coverslips. Slices attached to the coverslips were placed into wells of a 24 well plate, immersed in PBS with antibiotics, and stored at 4°C until seeding (up to 2 weeks).

2. *hiPSC Differentiation and Culture*

Wild type and LQT2 hiPSCs were plated into wells of 6-well plates coated with 1:200 Geltrex:DMEM/F-12. For the first 22 hours, hiPSCs were maintained in Essential 8 medium (E8) with 10 μM Y-27632 dihydrochloride (Tocris Bioscience, Bristol, UK). Afterwards, hiPSCs were rinsed with DMEM/F-12 and fed with E8 medium every day. On the fourth day, when cells had reached about 80% confluence, the medium was replaced with RPMI 1640 supplemented with B-27, minus insulin and 6 μM CHIR-99021 (Selleck Chemicals, Houston, TX) to initiate differentiation (d0 of differentiation). Over the course of the next week, medium was changed as follows: RPMI 1640 with B-27 without insulin (B-27 minus) on d2, B-27 minus and 5 μM IWR-1 (Sigma-Aldrich Corp.) on d3, B-27 minus on d5 and d7, and RPMI 1640 with B-27 with insulin (B-27 plus) on d9 and every other day afterwards. Spontaneous beating in the monolayers was observed starting at d7 to d10. On d10 to d12, the hiPSC-CM monolayers were washed with 0.5 mM EDTA (Mediatech, Inc., Manassas, VA) and then incubated in EDTA for 5 minutes at 37°C. Afterwards, the EDTA was aspirated off, and 0.05% Trypsin-EDTA was added for 3 minutes at 37°C. Cells were triturated before Defined Trypsin Inhibitor was added to stop the digestion. The resultant suspension was centrifuged at 200 g for 5 minutes (Centrifuge 5702, Eppendorf AG, Hamburg, Germany). After aspirating off the supernatant, the cell pellet was resuspended in B-27 plus. The PBS was removed from dECM slices, and the suspension of hiPSC-CMs was pipetted on top of the slices at a density of 0.8 to 1.3 million cells per cm^2. Slices were maintained in culture for 16 to 190 days, and the B-27 plus medium was replaced every other day for the duration of culture.

3. *Imaging of Extracellular Matrix and EHS*

dECM slices or EHS were fixed in 4% paraformaldehyde solution (Affymetrix, Inc., Cleveland, OH) for 10 minutes and rinsed twice with PBS. Samples were stored in PBS at 4°C until immunostaining. To immunostain for ECM proteins, slices were immersed in Target Retrieval Solution (Dako North America, Inc., Carpinteria, CA) for

20 minutes in a steamer. Samples were subsequently rinsed in distilled, deionized water for 5 minutes, blocked with 10% peroxide solution in water (Sigma-Aldrich Corp.) for 10 minutes, and rinsed twice for 5 minutes each time with Dulbecco's Phosphate Buffered Saline (DPBS). Samples were incubated in primary antibodies against collagen I (C2456, Sigma-Aldrich Corp.), collagen III (ab7778, Abcam, Cambridge, MA), and laminin (L9393, Sigma-Aldrich Corp.). The next day, slices were washed three times for 5 minutes each with TBS-T (0.05% Tween 20, Sigma-Aldrich Corp.) in Tris Buffered Saline (TBS, Quality Biological, Gaithersburg, MD) and incubated in secondary antibodies (Invitrogen, Waltham, MA) for 45 minutes at room temperature. EHS were shielded from exposure to light and washed in three rounds of TBS-T for 5 minutes each round. Afterwards, samples were mounted onto slides, a drop of Prolong Gold Antifade Mountant was added onto the slices, and a glass slide was placed on top. Samples were left to dry for a minimum of 24 hours before acquiring images using a confocal microscope (LSM 510 Meta, Zeiss, Oberkochen, Germany). Fibrillar collagen in unstained dECM slices was also imaged by second harmonic generation (SHG) using a multiphoton microscope (710NLO, Zeiss) with excitation at 880 nm, and emission acceptance at 415-450 nm.

To immunostain for cellular proteins, EHS were permeabilized with cold 0.5% Triton-X 100 (Sigma-Aldrich Corp.) in PBS for 20 minutes, followed by blocking with 10% goat serum (Life Technologies, Carlsbad, CA) for 1 hour at room temperature. Primary antibodies against cardiac troponin I (T8665-13F, United States Biological, Pittsburg, PA), α-actinin (A7811, Sigma-Aldrich Corp.), connexin 43 (C6219, Sigma-Aldrich Corp.), or vimentin (M0725, Dako North America, Inc.) in antibody diluent (Dako North America, Inc.) were added overnight at 4°C. The next day, EHS were washed with TBS-T as described above. Afterwards, samples were stained with 4',6-Diamidino-2 Phenylindole, Dihydrochloride (DAPI) for 25 minutes at room temperature. Samples were subjected to three more rounds of TBS-T washing before the addition of a drop of Prolong Gold Antifade Mountant and a glass slide on top of each sample. Alternatively, some samples were permeabilized as previously described and stained with Alexa Fluor 488 Phalloidin and DAPI at room temperature for 25 minutes. Samples were washed in TBS-T and mounted for imaging as described above. Images of stained samples were acquired using a confocal microscope (LSM 510 Meta, Zeiss).

To prepare samples for transmission electron microscopy, EHS were fixed in 2.5% glutaraldehyde and 3 mM $MgCl_2$ in 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 4°C. Samples were subsequently rinsed three times (15 minutes per rinse) in a 0.1 M sodium cacodylate buffer supplemented with 3 mM $MgCl_2$ and 3% sucrose, shielded from light, and left for one hour in a solution of 1% osmium tetroxide in 3 mM $MgCl_2$ and 0.1 M sodium cacodylate buffer. After undergoing two rounds of rinsing with water for 5 minutes each, the samples were stained in 2% aqueous uranyl acetate in the dark for 1 hour. Samples were then dehydrated in a graded series of ethanol washes (30%, 50%, 70%, 90%, and three washes of 100% ethanol), followed by two washes with propylene oxide (for 5 minutes each) and an overnight incubation in 1:1 propylene oxide:epon. The next day, samples were incubated in epon with catalyst three times for 2 hours each at room temperature and for 2 days at 60°C. Samples were examined using a transmission electron microscope (Phillips/FEI BioTwin CM120 TEM, Hillsboro, OR).

4. Calculation of Collagen Fiber Orientation

SHG images were acquired of three types of decellularized slices (dECM slices) after decellularizing: fresh slices (native slices from sections of fresh plugs prepared right after the hearts were acquired that were decellularized), frozen slices (fresh slices that were stored at -80°C for over 48 hours), and frozen plus (fresh plugs that were stored at -80°C for over 48 hours, thawed, and subsequently sectioned). Both fresh and frozen plugs were acquired from the same hearts, and fresh and frozen slices were derived from adjacent sections of the same plug. SHG z-stack images had an area of 1.875 mm^2 and were processed in MATLAB to quantify fiber orientation and fiber degree of alignment. After compressing z-stack images in ImageJ using a maximum intensity z-axis projection, each compressed image was converted to an RGB image using ImageJ and segmented in MATLAB. SHG images were then segmented into 6889 subregions. The sectioning frame originated at the top left corner of the image and continuously shifted to the right or downwards by 0.0133 mm to create a new subregion with an area of 0.071 mm^2.

The absolute logarithmic magnitude of the 2D Fast-Fourier transform (2D-FFT) was calculated for each subregion in the segmented image. The direct current frequency was shifted to the center of the 2D-FFT plot, and the magnitude of the 2D-FFT was normalized so that it had a magnitude of 1. Since the 2D-FFT is symmetric, only half of it was used in the analysis. The angle of orientation from the horizontal axis of each coordinate in the 2D-FFT was calculated by converting from Cartesian to polar coordinates.

The radial sum of the 2D-FFT, normalized to the number of pixels along each radius, was plotted as a function of the orientation for each subregion. The orientation of the maximum 2D-FFT sum determined the angle perpendicular to the fiber orientation in that subregion. The mean and standard deviation of the fiber orientation were calculated for all subregions in each image. Mean values of fiber orientation were plotted for 8 slices within

each group. The fiber degree of alignment was calculated as the maximum 2D-FFT sum divided by the total sum of the 2D-FFTs of all subregions within an image.

5. Calculation of Nuclear Elongation and Alignment

Time-matched EHS and monolayers were immunostained with DAPI and imaged using a confocal microscope, as described above. Images of nuclei were thresholded above a baseline noise level and segmented, removing overlapping areas as necessary, by using a previously described method (Plissiti et al., 2014) with some modifications. A recursive search for concavity points was performed; when a concavity point was found, the outline between it and the convex hull was searched for more concavities. Further, the case where non-adjoining sections of an outline are part of the same nucleus was permitted. An ellipse was fitted to each nucleus using a method previously described (Fitzgibbon et al., 1999). The nuclear elongation ratio, mean angular orientation, and standard deviation of the angular orientations were calculated, as previously described (Bray et al., 2010).

6. Flow Cytometry Analysis

Flow cytometry analysis was performed on d17 (prior to monolayer seeding), and on monolayers and EHS that had been seeded at d17 and cultured for 61 days (d68). Monolayers and d17 cells were washed with 5 mM EDTA and subsequently incubated in 5 mM EDTA at 37°C for 5 minutes. The EDTA solution was aspirated off and replaced with 0.05% Trypsin-EDTA for 4-6 minutes at 37°C, until the cells easily detached from the bottom of the wells when the culture dish was agitated. The Trypsin-EDTA solution containing cells was neutralized using Defined Trypsin Inhibitor, and the cells were centrifuged at 200 g for 5 minutes before fixation. EHS were unhooked from their underlying plastic coverslip, transferred to a 12-well plate and rinsed with DPBS containing calcium and magnesium (DPBS +/+). After removing the DPBS, EHS were incubated in 10 mg/mL of collagenase IV with 10% fetal bovine serum in DPBS +/+ and 50 ug/mL of DNAse I in 0.15 M NaCl (Sigma-Aldrich Corp.) for 30 minutes on a shaker at 37°C. The collagenase and DNAse solution were subsequently removed, and the EHS were rinsed twice with PBS (without calcium or magnesium). After removing the PBS, EHS were incubated in 0.05% Trypsin-EDTA for 5 minutes on a shaker at 37°C and broken up by pipetting up and down. The Trypsin-EDTA solution containing the recovered cells was neutralized and centrifuged as described above prior to fixation.

Dissociated cells were fixed in 4% paraformaldehyde (Affymetrix) for 10 min at room temperature. Cells were then simultaneously and permeabilized and blocked in PBS containing 0.1% BSA (Sigma-Aldrich Corp.), 5% goat serum, and 0.1% Triton-X 100 (Sigma-Aldrich Corp.) for 30 min. Cells were incubated with mouse anti-cTnT antibody diluted 1:200 in FACS buffer (PBS with 0.1% BSA and 0.1% Triton-X 100) for 1 hr at 4 °C, washed three times with FACS buffer, and incubated with anti-mouse Alexa Fluor® 488 antibody in FACS buffer (1:200, Invitrogen) for 30 min at 22 °C in the dark. After washing and re-suspending in PBS with 0.1% BSA, cells were strained through a 30 μm filter and run on a FACSCalibur cytometer (BD Biosciences, Woburn, MA). Secondary controls consisted of cells incubated only with anti-mouse Alexa Fluor® 488 secondary antibody. Single cells were identified and gated based on their forward and side scatter, and cardiomyocytes were gated based on their cTnT expression. Data were analyzed using FlowJo X Software. Histogram counts were extracted by gating on unstained cells and cells stained only with secondary antibody control (to remove autofluorescence and background nonspecific secondary staining).

7. Contraction Measurements

Tyrode's solution was prepared by combining 1.8 mM $CaCl_2$, 5 mM glucose, 5 mM HEPES, 1 mM $MgCl_2$, 5.4 mM KCl, 135 mM NaCl, and 0.33 mM NaH_2PO_4 in ddH_2O and adding NaOH to raise the pH to 7.4 (all chemicals from Sigma-Aldrich Corp.). EHS were placed in a 35 mm dish filled with Tyrode's solution and set on a stage heated to 31±0.1°C (Warner Instruments, Hamden, CT). A section of each EHS was unhooked and allowed to move freely throughout the duration of the experiment. The EHS were allowed to equilibrate in Tyrode's for 5 minutes before the start of pacing with a point electrode. Each sample was paced for 1 minute at each of 3 cycle lengths (1000 ms, 666 ms, 500 ms), while the freely moving region was kept approximately vertical in the field of view and imaged at 4x magnification using a CCD camera (Swiftcam, Swift, Schertz, TX) at a rate of 14-17 fps with 320x256 pixel resolution. Samples were also imaged during pacing at 666 ms after the application of 1 μM isoproterenol (Sigma-Aldrich Corp.) for 2 minutes and after washout of the drug for 2 minutes. We applied a pacing cycle length shorter than 1000 ms in the presence of isoproterenol to overcome the increase in spontaneous rate in response to the drug. Custom MATLAB scripts were used to segment the images by applying a user-defined threshold to assign each pixel in the image as either belonging to the EHS, which was darker, or the background, which was lighter. From this, the area of EHS was calculated in each image. The change in area from a fully relaxed state (reference frame, designated as 100% EHS area) was determined for each frame in the time series. The minimum area (maximum change in area) was averaged over time (multiple cycles of contraction) and relaxation for

each pacing condition. The change in area in the presence of isoproterenol and after washout was compared to the change in area prior to the application of drug for each EHS.

8. Electrophysiological studies

EHS were stained with 10 μM of the voltage-sensitive dye di-4-ANEPPS (Sigma-Aldrich Corp.) in Tyrode's solution for 10 minutes at 37°C. Afterwards, EHS were rinsed twice with Tyrode's and placed in a 35 mm dish filled with Tyrode's and 10 μM of the contraction inhibitor blebbistatin (Sigma-Aldrich Corp.). This dish was set on a stage heated to 37°C and allowed to equilibrate for at least 5 minutes. Samples were point paced with at least 30 stimulus pulses at a range of cycle lengths, starting from 2000 ms and decreasing until the they lost capture. EHS were optically mapped during pacing using a 100x100 pixel CMOS camera (MiCAM Ultima-L, SciMedia, Costa Mesa, CA).

During drug studies, recordings at a range of pacing rates were taken at baseline (prior to the addition of drug) before replacing the solution in the dish with Tyrode's supplemented with blebbistatin and the lowest concentration of drug studied. The sample was paced at the same range of rates and mapped 7 minutes after the addition of drug. Afterwards, the bath solution was replaced with Tyrode's with blebbistatin and the next lowest drug concentration and the procedure repeated. This method was applied for all concentrations of each drug and, except where noted in the figures, only one drug was tested per sample. In these studies, we superfused EHS or monolayers with the following drugs: E-4031 (Tocris Bioscience), $BaCl_2$ (Sigma-Aldrich Corp.), Chromanol 293B (Tocris Bioscience), Nifedipine (Tocris Bioscience), Bay K 8644 (Tocris Bioscience), and Cromakalim (Sigma-Aldrich Corp.).

Optical mapping data was analyzed using custom MATLAB scripts. Recordings at each pixel were de-noised using a previously described method (Little and Jones, 2010) to regulate total signal variance and convolved with a 5x5 spatial Gaussian filter. Activation times were defined as the maximum of the derivative of membrane potential (dV/dt), which was calculated as previously described (Chartrand, 2011). Histograms of local conduction velocities for each EHS were fitted to a Gaussian curve and the mean of the curve was defined as the average conduction velocity (CV). To determine longitudinal and transverse CVs, a bimodal Gaussian curve was fitted to the local CVs. Action potential durations at 30 and 80 percent repolarization (APD_{30} and APD_{80}) were determined for all local traces over the recording region for each EHS and fit with Gaussian curves to determine the mean value for each EHS, as described for CV measurements. For drug studies, average APD and CV measurements for each concentration were normalized by average APD and CV measurements at baseline (without the drug).

9. Quantitative RT-PCR

mRNA was isolated from EHS (d69) and monolayers (d22) using the following procedure: incubation in TRIzol Reagent for 5 minutes at room temperature, incubation in chloroform for 3 minutes, centrifugation at 12,000g for 15 minutes at 4°C, collection of colorless phase that separated at the top of the centrifuged sample, addition of isopropyl alcohol and incubation at room temperature for 10 minutes, centrifugation at 12,000g for 10 minutes at 4°C, solubilization of RNA pellet in 75% ethanol, centrifugation at 7,500g for 5 minutes at 4°C, air drying of sample, and resuspension of the RNA pellet in DEPC-treated water. mRNA from two EHS were combined for each EHS replicate.

Reverse transcription was performed to create cDNA with the PCR Master Mix kit, using the MyGo Mini PCR system (IT-IS Life Science Ltd., Republic of Ireland). RT-PCR was performed on each target in triplicate, using the following primers:

Gene Target	**Associated Protein**	**Primer**	
CACNA1C	L-type calcium channel	Forward	CACGGCTTCCTCGAATCTTG
		Reverse	CTGTGGAGATGGTCGCATTG
KCNH2	Voltage-gated potassium channel	Forward	AGGAGCGAACCCACAATGTC
		Reverse	AGGTGGTGCGGAAGTTGATG
TNNI1	Troponin I, slow skeletal type	Forward	GGTGGATGAGGAGCGATACG
		Reverse	CAGGCTGGAGGGAAGAAGTG
TNNI3	Troponin I, cardiac	Forward	CCAACTACCGCGCTTATGCC
		Reverse	CCTTCTCGGTGTCCTCCTTC

The PCR program run for each sample consisted of 120 seconds hold at 95°C, 40 cycles of amplification that alternated between 90°C and 65°C, 10 seconds pre-melt hold at 95°C, and a melting step that increased from 60°C to 97°C at 0.1°C/second. CT values were obtained using MyGo Mini PCR Software (IT-IS Life Science Ltd.)

and CT was calculated for each gene of interest (GOI) in EHS to determine fold change over transcript levels expressed monolayers according to the formula:

$$\Delta\Delta C_T = C^{EHS}{}_{T,GOI} - C^{EHS}{}_{T,ACTN2} - C^{Mn}{}_{T,GOI} + C^{Mn}{}_{T,ACTN2}$$

where $C^{EHS}{}_{T,GOI}$ refers to C_T of EHS for the GOI, $C^{EHS}{}_{T,ACTN2}$ refers to C_T of EHS for ACTN2 (α-actinin, which was used as a normalizing gene), $C^{Mn}{}_{T,GOI}$ refers to C_T of monolayers for the GOI, and $C^{Mn}{}_{T,ACTN2}$ refers to C_T of monolayers for ACTN2.

10. Statistics

All data are presented as mean±SD. Measurements of nuclear elongation were log transformed and reported as the interval of the log-transformed mean ± SD after inverse transformation into linear space (Bland and Altman, 1996). For contraction experiments, a one-tailed Wilcoxon test was used to determine statistically significant differences from 1 for isoproterenol-treated and washout groups. For studies on rate-dependence of contraction, a two-tailed Wilcoxon test was used to determine statistically significant differences from 1 for groups paced at 500 ms and 1000 ms cycle lengths. A two-tailed Wilcoxon test was also used for statistical significance between APD measurements for WT and LQT2 EHS. Paired, unequal variance, two-tailed t-tests were performed for all other drug studies and unpaired, unequal variance, two-tailed t-tests were performed for optical mapping studies not involving drugs, studies comparing d62-82 EHS to d201 EHS, and for orientation analysis of fresh slices, frozen slices, and frozen plugs.

11. Supplemental References

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