SUPPLEMENTARY MATERIAL

Detailed Methods

Preparation of Tissue Culture Molds and Frames

A custom negative mold designed to produce two troughs (2 mm diameter x 7 mm length) was machined from Teflon (Figure S1). Degassed PDMS was poured over the negative molds and cured at 80 °C for ≥ 4 hours to create re-usable tissue molds. Tissue molds were sterilized, coated for ≥ 1 hour in 0.2% (w/v) pluronic F-127, and rinsed once with water immediately prior to each use. Frames were laser-cut from spunbound nylon sheets (Cerex Advanced Fabrics), sterilized by soaking in 70% ethanol, and allowed to dry for ≥ 1 hour prior to use.

Neonatal Rat Ventricular Myocyte Isolation

NRVMs were isolated from 2-day-old Sprague Dawley rats as previously described [1]. Ventricles were excised and minced into 10-15 pieces in ice-cold digestion buffer (HBSS with 1.2 mM $MgCl_2$). After 6 washes in ice-cold buffer, minced hearts were transferred to buffer containing 1 mg/mL trypsin (US Biological) and incubated overnight (16-18 hours) at 4 °C with gentle agitation. Minced hearts were washed once in ice-cold buffer, then pre-digested in buffer containing 250 U/mL Collagenase Type 2 (Worthington) for 1 minute (37 °C, orbital rotation @ 90 rpm). After discarding cells from the pre-digest, 4 serial digestions in 250 U/mL Collagenase were applied to complete the cell dissociation (3-4 minutes each, 37 °C, 90 rpm). Cells from each digest were collected in ice-cold buffer, centrifuged, resuspended in buffer, and passed through 100 μm filter. Filtered cells were centrifuged again and resuspended in 37 °C pre-plate media (formulation in Table S1). Cell suspension was pre-plated on a non-coated tissue culture flask for 45 minutes to enrich the fraction of cardiomyocytes in the cell suspension. Non-adherent cells were collected, counted, and used for cardiobundle construction. Typical yield from 1 litter of 10 neonates was 50-55 x 10^6 cells.

NRVM Cardiobundle Fabrication and Culture

The complete hydrogel formulation is listed in Table S2. Pre-plated cell suspension was centrifuged and resuspended to a concentration of 20.8 x 10^6 cells/mL in 3D NRVM Media (media formulation in Table S1). To prepare the gels, cell suspension was added to pre-mixed fibrinogen + Matrigel®. Thrombin was mixed with the 2x cardiac medium, and the 2x medium + thrombin solution was added to complete the gel mixture. Complete gel was mixed and quickly pipetted into tissue molds with frames (70 μL per mold). The gel was incubated for 45 minutes at 37 °C to allow polymerization and attachment to the frame, then submerged in 3D cardiac medium (1 mold per well of a 12 well plate). The next day, frames with gels attached were carefully removed from the molds by grasping the frame with fine-tip forceps. Full media changes of 1.5 mL per well were completed every other day. Cardiobundles were cultured at 37 °C on a static (non-moving) platform or a dynamic (rocking) platform (± 30 degree tilt, 0.4 Hz) for a period of 14 days. For certain experiments, cardiobundles were switched from static to dynamic culture and vice versa on day 7, and cultured for an additional 7 days (Figure S10). Protein isolation for western blot analysis of kinase phosphorylation was conducted 24 hours after the switch. In selected studies, rapamycin (500 nM), phenylephrine (20 μM), gadolinium (20 μM), or streptomycin (50 μM) were added to culture medium on day 7 and replenished with each media change.

hPSC-CM Differentiation

All studies were carried out with H9 hESCs (WiCell), HES2 hESCs (differentiated to CMs by Vistagen Therapeutics) or a normal iPSC line (gift from Thomas Ribar, Duke iPSC Core Facility). Undifferentiated

hPSCs were maintained in either TeSR-E8 or mTeSR-1 medium as previously described [2, 3]. Cardiac differentiation was carried out using two different protocols, both of which utilized small molecule Wnt agonists and inhibitors [4, 5]. For the Palecek protocol, hPSCs were cultured in RPMI 1640 medium containing B27 (minus insulin) supplement for 7 days, including CHIR (10-14 μM) from day 0-1 and IWP2 (5 μM) from day 3-5. Cells were cultured in RPMP 1640 medium containing B27 supplement beyond day 7. For S. Wu lab (JOVE) protocol, hPSCs were cultured in RPMI 1640 medium containing B27 (minus insulin) supplement for 8 days with daily media changes, including CHIR (6 μM) from day 0-2 and IWR1 (5 μM) from day 4-6. Cells were cultured in RPMI 1640 medium containing B27 supplement beyond day 8. In some studies, metabolic selection using no glucose CDM3 medium was carried out from day 10-12 [6, 7]. CDM3 medium consists of RPMI 1640 medium (lacking glucose) supplemented with 0.5 mg/ml recombinant human serum albumin and 213μg/ml L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate [7]. In a small subset of experiments, the J. Wu lab protocol (CDM3)[7] for differentiation was used. In addition, commercially available, HES2-derived CMs were provided by Vistagen Therapeutics for a small study. For all experiments, hPSC-CM cardiobundles were constructed between day 12 and 22 of culture with cells that were 83±3.7% positive for cardiac troponin T.

hPSC-CM Cardiobundle Culture

hPSC-CM cardiobundles were constructed similarly to NRVM cardiobundles. hPSC-CM were dissociated with 0.25% trypsin and resuspended in fibrin-based hydrogel according to Table S2. Complete gel mix was pipetted into PDMS molds with nylon frames and polymerized at 37 °C. Cardiobundles were removed from molds 1-2 days after fabrication and cultured free-floating on a rocker or static platform. Cardiobundles were cultured in 3DRB+ medium for 7 days, then 5% FBS DMEM medium for 14 days prior to functional testing and immunostaining (for culture media formulations, see Table S5). In a portion of experiments, cardiobundles were switched between static and dynamic culture after 7 days in 5% FBS medium.

Immunostaining and Image Analysis

Cardiobundles were fixed in 2% paraformaldehyde (PFA) overnight at 4 °C. Fixed cardiobundles were cut in half, and one half of the cardiobundle was incubated for 20-25 minutes in optimal cutting temperature (OCT) medium then frozen. Ten μm cross sections were cut using a cryostat microtome. Intact and sectioned samples were permeabilized with Triton-X for 30 minutes at room temperature (0.5 % v/v Triton-X for intact samples, 0.1 % for sectioned samples). Blocking solution consisting of 1% bovine serum albumin (BSA) and 10% chick serum in PBS was applied at 4 °C (24 hours for intact samples, 4 hours for sectioned samples). Primary antibodies (listed in Table S3) were diluted in blocking solution and applied overnight at 4 °C, followed by secondary antibodies at room temperature for 2 hours. Samples were imaged using a Zeiss 510 inverted confocal microscope. Image analysis of cross-sections was performed using custom ImageJ programs. F-actin$^+$ area was determined by subtracting background from the F-actin channel and applying Li's minimum cross entropy threshold. Total area was determined by applying Li's threshold to the merged (F-actin, Vimentin, DAPI) image, processing with the built-in "Fill Holes" function, and using the wand tool to select the entire area within the cross-section's outer boundary. Nuclei were counted by previously described methods [8]. F-actin$^+$ nuclei numbers were determined by counting the nuclei within the region of interest (ROI) defined by thresholding the F-actin channel.

DNA Content Assay

Total DNA content in cardiobundles was quantified using Hoechst assay[9] in order to estimate cardiobundle cell number. Specifically, single cardiobundles were digested using animal tissue lysis

buffer (ATL) and proteinase K from Qiagen DNeasy Blood & Tissue Kit for 3-4 hours at 56 °C in a working volume of 100 µL. Lysed sample was first diluted 1:1 with ATL buffer, then mixed 1:1 with Hoechst 33258 solution (Thermo, 0.2 µg/mL in Tris-buffered saline with 1 mM EDTA). Two replicates of each sample were read on a 96-well fluorescent microplate reader (Ex/Em of 350/460). Cell number in cardiobundle was derived using standard curve that related measured DNA content and known cell number in serial dilutions of lysed cells used for making cardiobundles.

Western Blot

To isolate protein, 3-4 cardiobundles were pooled together and minced in ice-cold RIPA buffer with protease inhibitor (Sigma), agitated intermittently for 2-3 hours, and centrifuged at 15000g for 30 minutes to remove insoluble matter. Protein concentration was measured using BCA assay (Pierce). 15 µg of protein sample were run in each lane of a 4%-15% gradient polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked in TBS with 0.1% Tween 20 and 5% milk, carefully cut at the appropriate molecular weight markers, and probed for antibodies listed in Table S3 (4 °C, overnight). Per manufacturer instructions, blocking solution for phosphorylated and total mTOR, S6K, AMPK, Akt, and ERK1/2 contained 5% (w/v) BSA instead of 5% milk. HRP-conjugated secondary antibodies were applied for 1 hour at room temperature. Chemiluminescence was imaged using a Bio-Rad ChemiDoc system, and protein bands were quantified by densitometry in ImageJ.

qPCR

Total RNA was isolated according to instructions of RNeasy fibrous tissue kit (Qiagen). The RNA of 2 cardiobundles was pooled together, and concentration was measured using a Nanodrop 1000. Reverse transcription was run on equal amounts of RNA using iScript cDNA Synthesis kit (BioRad). qPCR was performed using iTaq Universal SYBR Green Supermix kit (BioRad). Relative expression of indicated genes was quantified by the ΔΔCT method, using β-2 microglobulin (B2M) as the housekeeping gene. Primers sequences are listed in Table S4.

Optical Mapping

Action potential propagation was measured by optical mapping, similar to previously described methods [10, 11]. Live cardiobundles were stained with the potentiometric membrane dye di-4 ANEPPS (10 µM, 5 minutes, room temperature). Stained cardiobundles were immersed in 37 °C Tyrode's solution with 10 µM blebbistatin to inhibit contractions, and imaged with a 4x microscope objective. Action potentials were initiated by a platinum point stimulus electrode at the position where one end of the cardiobundle is connected to the frame. After 10 s of pre-pacing, signals were recorded with a photo diode array (channel diameter 750 µm, magnified channel diameter 187.5 µm) at a sampling rate of 2.4 kHz. Activation times of individual channels were calculated as the time of maximum voltage upstroke, and average conduction velocity (CV) was calculated by a linear fit of distance vs activation time (Figure S8). Action potential duration was calculated as the difference between activation time and 80% repolarization. For NRVM cardiobundles, pacing rate was increased from a basal rate of 2 Hz in increments of 1 Hz, with maximum capture rate recorded as highest rate at which each stimulus elicited an action potential for a duration of 2 s (after 10 s of pre-pacing). For hPSC-CM cardiobundles, pacing began at 1 Hz and increased in 0.5 Hz increments.

Isometric Force Testing

Contractile force was measured using a custom force measurement apparatus under field stimulation, using a previously described custom system [11, 12]. Cardiobundle pairs were separated into single cardiobundles and immersed in 37 °C Tyrode's solution with 1.8 mM $CaCl_2$. One end of the cardiobundle

frame was pinned to a fixed PDMS block, and the other end was pinned to a PDMS block connected to a force transducer. With the bundle held at culture length, the frame edges parallel to the cardiobundle were cut, and contractions elicited by field stimulus from platinum electrodes. Rat cardiobundles were paced at a basal rate of 2 Hz (1.5 Hz for human cardiobundles), with 10 s of pre-pacing applied during constant mixing of the Tyrode's bath. After pre-pacing, 2 s of data were recorded and analyzed for active twitch force using a custom MATLAB program. Total twitch duration was measured by defining the beginning and end of a contraction at 10% of peak amplitude; rise time and decay time were measured from 10% - 90% amplitude and from 90% - 10% amplitude, respectively. Cardiobundles were stretched in increments of 4% elongation, with recordings taken at each length until maximum active force was achieved (typically 4% or 8% elongation). For studies of passive tension, cardiobundles were stretched to 12% elongation.

Calcium Imaging

For NRVM, calcium imaging was performed in cardiobundles transduced with the genetically encoded calcium indicator GCaMP6 under the control of MHCK7 promoter [13] at the time of cardiobundle construction. For hPSC-CM, cardiobundles were loaded with Rhod-2 AM (according to manufacturer instructions) immediately prior to calcium imaging. After 2 weeks of culture, cardiobundles were paced at 1 Hz by field electrodes in 37 °C Tyrode's solution and imaged with a high speed EMCCD camera (Andor iXon 897 Ultra). Calcium amplitude was measured by normalizing the change in fluorescence during calcium transients to the baseline fluorescence ($\Delta F/F$ method[14]).

Legends for Video Files

Supplemental Video 1

Rocking platform was used for dynamic culture of cardiobundles in 12-well tissue culture plates. The plates on the incubator shelf were used for static culture.

Supplemental Video 2

Spontaneous contractions of day 7 NRVM cardiobundles cultured in static or dynamic conditions. The cardiobundles are anchored to a 1-mm wide nylon frame.

Supplemental Video 3

Action potential propagation in 2-week, dynamically cultured NRVM cardiobundle visualized by optical mapping using di-4-ANEPPS. Point stimulus is applied to the cardiobundle at the bottom of the field of view, at rates of 2, 4, and 8 Hz. The length of cardiobundle shown is 4 mm.

Supplemental Video 4

Action potential propagation in 2-week, dynamically cultured hPSC-CM cardiobundle visualized by optical mapping using di-4-ANEPPS. Point stimulus is applied to the cardiobundle at the bottom of the field of view, at rates of 1 and 2 Hz. The length of cardiobundle shown is 4 mm.

Supplemental Video 5

NRVM cardiobundles cultured dynamically for 2 weeks exhibit macroscopic contractions in response to electric field stimulus at a rate of 1 Hz. Four (4) pairs of cardiobundles are displayed, with each pair anchored within a porous nylon frame. Outer frame dimensions are 10 x 10 mm.

Supplemental Tables

Table S1: Culture media for rat cardiomyocytes.

	NRVM Pre-plate media	3D NRVM media	2x cardiac media
Basal media	DMEM / F12 (Thermo 11330)	Low glucose DMEM (Thermo 11885 or Sigma D6046)	2x concentrated low glucose DMEM (Thermo 31600-034)*
FBS	10%	--	--
HS	10%	10 %	20%
ACA	--	1 mg/mL	2 mg/mL
AA 2-P SSH	--	50 µg/mL	--
CEE	--	1 %	--
Penicillin	5 U/mL	5 U/mL	10 U/mL
Vitamin B12	2 µg/mL	2 µg/mL	4 µg/mL

FBS: Fetal Bovine Serum
HS: Horse Serum
ACA: Aminocaproic Acid
AA 2-P SSH: Ascorbic Acid 2-phosphate sesquimagnesium salt hydrate
CEE: Chick embryo extract
* prepared following supplier instructions, but with half of the recommended final volume (i.e., 1 packet into 500 mL instead of 1000 mL)

Table S2: Formulation of hydrogel.

Component	Starting Concentration	Final Concentration in Gel	% of Final Gel Volume	Volume for 450 µL (6 cardiobundle pairs)
Cell Suspension	20.8 x 10^6 per mL	10 x 10^6 per mL	48 %	216 µL
Fibrinogen	10 mg/mL	2 mg/mL	20 %	90 µL
Matrigel	--	10 % (v/v)	10 %	45 µL
2x cardiac media	--	--	20 %	90 µL
Thrombin	50 U/mL	1 U/mL	2 %	9 µL

Table S3: List of primary antibodies.

Antigen	Supplier	Cat. #	Application	Dilution
Sarcomeric α-actinin (SAA)	Sigma	A7811	IF WB	1:200 1:1000
Cardiac Troponin T (cTnT)	Abcam	Ab45932	WB	1:1000
Connexin 43 (Cx43)	Abcam	Ab11370	IF	1:200
N-cadherin (Ncad)	Abcam	Ab12221	IF	1:400
α Myosin Heavy Chain (Myh6)	Santa Cruz	SC-32732	WB	1:200
Vimentin (Vim)	Abcam	Ab89996	IF	1:500
Collagen 1 (Col 1)	Abcam	Ab34710	IF	1:400
Caveolin 3 (Cav3)	Abcam	Ab2912	IF	1:200
Lamin B1	Abcam	Ab16048	WB	1:2000
GAPDH	Santa Cruz	SC-32233	WB	1:1000
Phospho-mTOR (Ser2448)	Cell Signaling Technology	5536	WB	1:2000
Total mTOR	Cell Signaling Technology	2983	WB	1:2000
Phospho-S6K (Thr389)	Cell Signaling Technology	9205	WB	1:1000
Total S6K	Cell Signaling Technology	9202	WB	1:1000
Phospho-Akt (Ser473)	Cell Signaling Technology	4060	WB	1:2000

Antigen	Supplier	Cat. #	Application	Dilution
Total Akt	Cell Signaling Technology	4691	WB	1:2000
Phospho-AMPK (Thr172)	Cell Signaling Technology	2535	WB	1:1000
Total AMPK	Cell Signaling Technology	5832	WB	1:1000
Phospho-ERK1/2 (Thr202/Tyr204)	Cell Signaling Technology	9101	WB	1:1000
Total ERK1/2	Cell Signaling Technology	4695	WB	1:1000

IF: Immunofluorescence
WB: Western Blot

Table S4: List of qPCR primers.

Gene	Description	Forward Primer	Reverse Primer
Atp2a2	SERCA-2a	TGTGCTCTGTGTAATGACTCTG	CTCCGTGTCGAATACATTCATCT
Cacna1c	L-type Ca Channel ($Ca_v1.2$)	ACTTCATCATCCTCTTCATCTGTG	CCAGCTTCTTTCTCTCCTTCTC
Myh6	Myosin heavy chain, α	CAGAAGAAACTGAAGGAAAACCAG	TCTAGCCTCTCACTGATCTCC
Myh7	Myosin heavy chain, β	TATCAGTACATGCTGACAGATCG	CTCTTGGTGTTGACGGTCTTAC
Gja1	Gap junction protein α-1 (Connexin 43)	GAGCTGTCGATTATGGAGGA	AGGTTCAGTTGGGGGATG
Scn5a	V_m-gated Na channel ($Na_v1.5$)	CGTAACTTCACCGAGCTCAA	CCCACATAGTAACACATCCGT
B2M	β-2 Microglobulin	GCTTGCAGAGTTAAACACGTC	CCAGATGATTCAGAGCTCCAT
Acta1	α-skeletal Actin	CTCTCTCTCCTCAGGACGACAATC	CAGAATGGCTGGCTTTAATGCTTC
Nppa	Atrial natriuretic peptide (ANP)	GCGAGCAGACCGATGAA	CCCGAGAGCACCTCCA
Phd2	Prolyl hydroxylase domain 2	CTGGGACGCCAAGGTGA	CAATGTCAGCAAACTGG
Phd3	Prolyl hydroxylase domain 3	GTTCAGCCCTCCTATGC	ACCACCGTCAGTCTTTA

Table S5: Culture media for hPSC-CM cardiobundles.

Component	3DRB+ media	5% FBS media
Basal media	RPMI 1640	Low glucose DMEM (Thermo 11885 or Sigma D6046)
FBS	--	5 %
ACA	2 mg/mL	2 mg/mL
AA 2-P SSH	50 μg/mL	50 μg/mL
1-thioglycerol	0.45 μM	0.45 μM
Sodium Pyruvate (100x)	1%	-- *
NEAA (100x)	1%	1%
Pen Strep (100x)	1%	1%
B27 supplement (50x)	2%	--

FBS: Fetal Bovine Serum
ACA: Aminocaproic Acid
AA 2-P SSH: Ascorbic Acid 2-phosphate sesquimagnesium salt hydrate
NEAA: Non-essential amino acids
* included in basal media

Table S6: Effects of dynamic culture and phenylephrine on NRVM cardiobundles, compared with changes during postnatal cardiac development and pathological hypertrophy of the adult heart.

Parameter	Dynamic Culture	Phenylephrine	Postnatal Development	Pathological Hypertrophy
Active force	↑	↔	↑ [15]	↓ [16, 17]
Passive force	↔ or ↑	↑	↑ [18, 19]	↑ [20]
Twitch kinetics	↔	↓	↑ [21]	↓ [16, 22]
CV	↑	↓	↑ [1, 23]	↓, ↔, or ↑ [24]
APD	↔	↑	↓ [25, 26]	↑ [27, 28]
CM size	↑	↑	↑ [29, 30]	↑ [16, 17]
α-skeletal actin	↑	↑	↑ then ↓ [31, 32]	↑ [16, 17]
$Na_v1.5$	↑	↔	↑ [33, 34]	↔ or ↓ [35, 36]
SERCA-2a	↑	↓	↑ [37]	↓ [17, 38]
α-MHC	↑	↓	↑ [39]	↔ or ↓ [16, 17]
β-MHC / α-MHC ratio	↔	↑	↓ [39, 40]	↑ [16, 17]

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