Supplementary Data

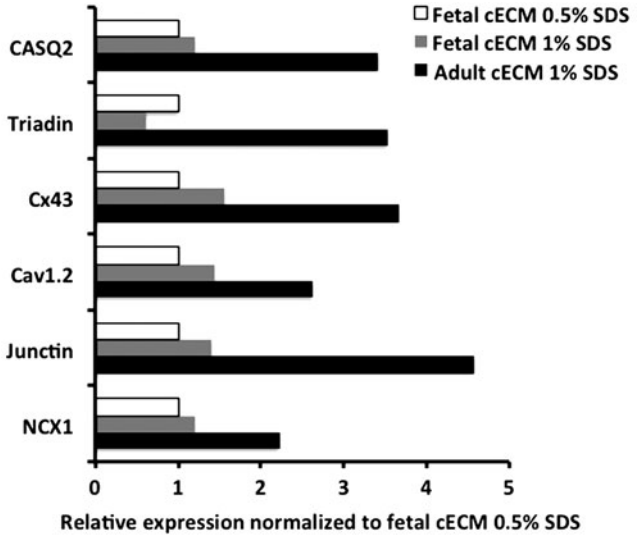
Supplementary Methods

Induced pluripotent stem cell-derived cardiomyocyte maturation

2D cultures. Before seeding the induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CMs) (Days 28–64 postinduction), tissue culture plates were coated with 1 mg/mL of fetal or adult bovine cardiac extracellular matrix (ECM) at 37°C overnight. The cells were harvested with 200 U/mL collagenase II (Life Technologies) for 1 h, followed by 3× washes with phosphate-buffered saline (PBS) (Ca/Mg free), 5 min of incubation in 10× TryplE (Life Technologies), and then seeded at 45,000 cells/cm.^{2}

3D cultures. The cardiac ECM was adjusted to pH 7.4 with 0.1 N NaOH and supplemented with 1/9 volume with 10× PBS (with Ca/Mg). The 3D scaffolds comprised 5.25 mg/mL cardiac ECM and 1.75 mg/mL fibrinogen (Sigma). The cells were seeded at 1.6×10^6 cells/mL. The 3D gels were polymerized by the addition of 1.3 U/mL thrombin (Sigma) and incubation at 37°C for 2 h before media were added. Each 3D tissue construct also contained 0.15 U/mL aprotinin (Sigma) in a total scaffold volume of 75 μL. Completed 3D gels were cultured in polydimethylsiloxane (PDMS; Dow Corning) retention rings, each with a diameter of 8 mm and an approximate height of 1 mm. Cells were harvested after 7 days by digesting the tissue constructs with 500 U/mL Collagenase II for 35–45 min.

Three independent biological experiments of the maturation studies were performed with each experiment using cardiac ECM from independent batch preparations and CM from independent cardiac differentiations.



SUPPLEMENTARY FIG. S1. Induction of iPSC-derived CM cardiac genes is indistinguishable on gels derived from fetal tissues decellularized with 0.5% and 1% SDS. Cardiac genes were measured using qRT-PCR and expression normalized to 2D fetal cardiac ECM decellularized with 0.5% SDS. The cardiac genes measured were Triadin, Junctin, *CACNA1C*/*CaV1.2* (L-type voltage-dependent calcium channel), *GJA1*/*Cx43* (connexin-43), *CASQ2* (calsequestrin 2), *NCX1* (sodium–calcium exchanger 1). Comparing expression from iPSC-derived CMs cultured for 7 days on 2D fetal cardiac ECM decellularized with 0.5% and 1% SDS with 2D adult cardiac ECM. CM, cardiomyocyte; ECM, extracellular matrix; iPSC, induced pluripotent stem cell; SDS, sodium dodecyl sulfate; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

Quantitative reverse transcription polymerase chain reaction

Total RNA was harvested from differentiated iPSCs using Trizol (Life Technologies) according to the manufacturer's recommended protocol and was treated with RQ1 DNAse (Promega) for 1 h. Three micrograms of RNA was used to generate cDNA using the iScript cDNA Synthesis Kit (BioRad). qRT-PCR was performed using SYBR green chemistry and an iQ5 BioRad icycler. mRNA levels were normalized to 18S expression and measured in triplicate. Primers were synthesized by Integrated DNA Technologies and their sequences can be found in Supplementary Table S4.

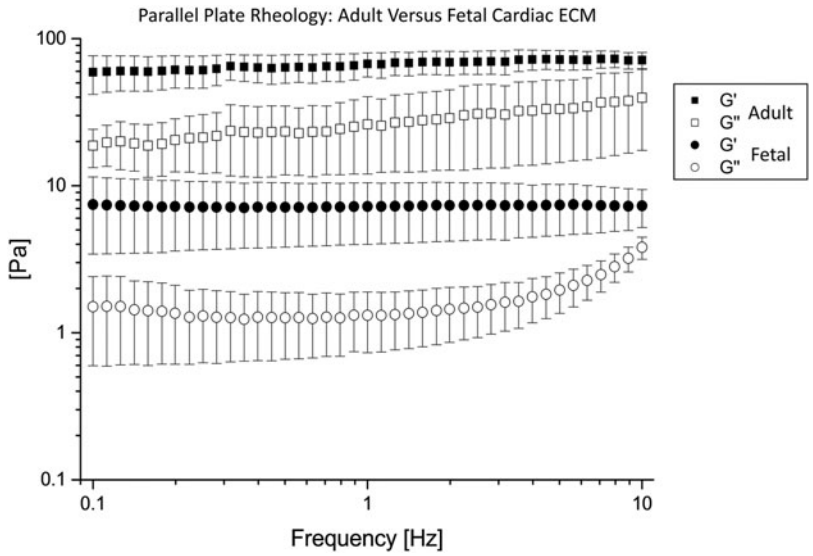
Calcium imaging and analysis

The iPSC-derived CMs have been engineered to express a calcium indicator (GCaMP6f reporter) that emits fluorescence in the presence of cytosolic calcium. Calcium transients were measured using an epifluorescent microscope with an inverted 10× objective (IX70 Olympus). Images were captured using an SPOT Idea 3.0 megapixel color fluorescent camera and SPOT acquisition software (SPOT Imaging Solutions). The cells were placed in a 37°C heated stage during imaging. The video was captured at 33 frames/s with 8×8 binning. Additionally, calcium transient data were reconfirmed using the Olympus FLUOVIEW FV1000 confocal laser scanning microscope to acquire line scans at the rate of 630 Hz. ImageJ was used to process and extract the temporal fluorescence intensity of the acquired video data.

Subsequent data were analyzed using MATLAB software (MathWorks). A customized algorithm determined the troughs and peaks of each calcium wave and measured the amplitude, maximum upslope, maximum downslope, time to peak, time to 50% decay, time to 75% decay, and beat rate. Additional measurements included the effect of caffeine on the slope of amplitude change.

Pharmacological treatment of CMs

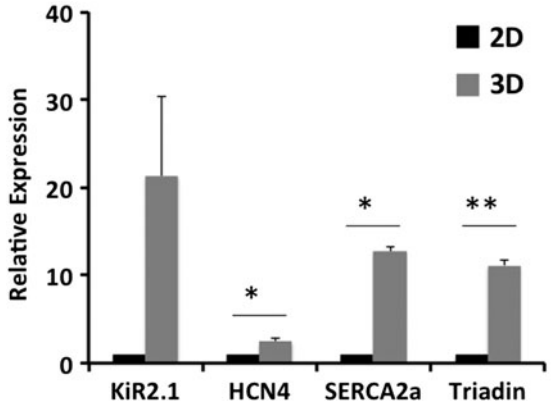
Baseline and drug recordings were taken before and after 10 min of incubation with the drug(s) on the 37°C stage. All recordings were taken over 1 min. The order of the β-adrenergic drug treatments was baseline, 1 μM isoproterenol (#I6504; Sigma), and 1 μM isoproterenol + 10 μM Propranolol (#P0884; Sigma). The effect of caffeine (#2793; Tocris) on calcium signaling was assessed at a final concentration of 20 mM. The video recordings were acquired before and during the addition of caffeine.



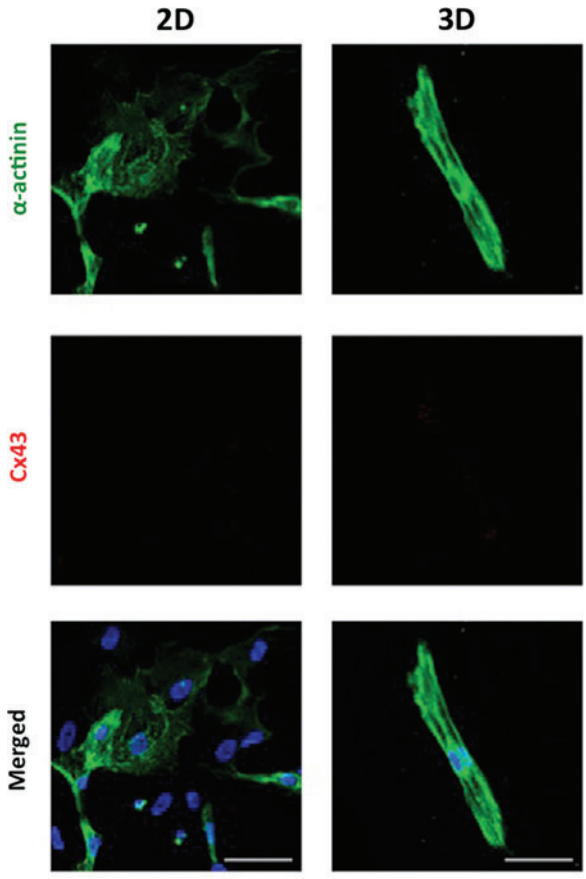
SUPPLEMENTARY FIG. S2. Adult cardiac ECM hydrogels have higher storage and loss moduli compared with fetal hydrogels. Parallel plate rheology was used to quantify the storage and loss moduli of each sample. A frequency sweep was performed at a strain amplitude of 1% from 0.1 to 10 Hz. Results are expressed as mean ± standard error for each frequency ($n = 3$).

nanoLC-MS/MS

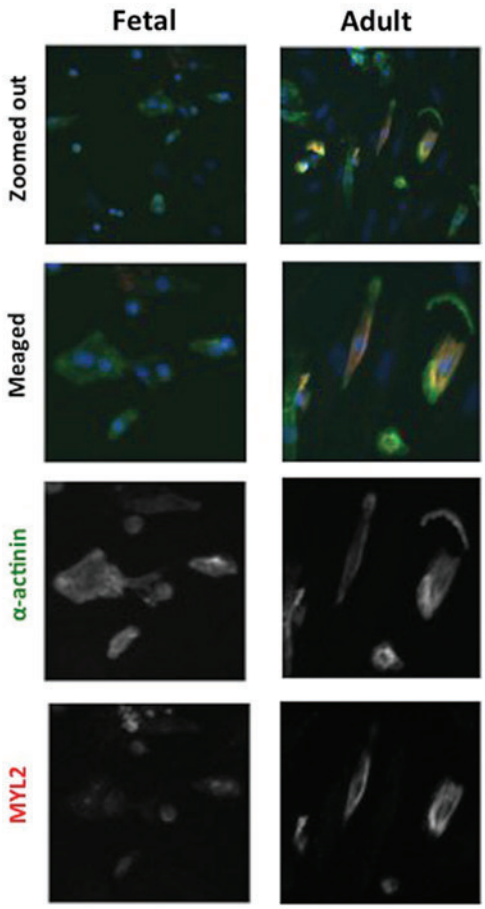
Decellularized fetal and adult tissue pellets were fully solubilized in a buffer comprising 4% sodium dodecyl sulfate, 10 mM TCEP, and 100 mM TEAB through repeated cycles of heating (95°C)/ultrasonication (40°C), followed by 120 min of ultrasonication. Protein levels were subsequently



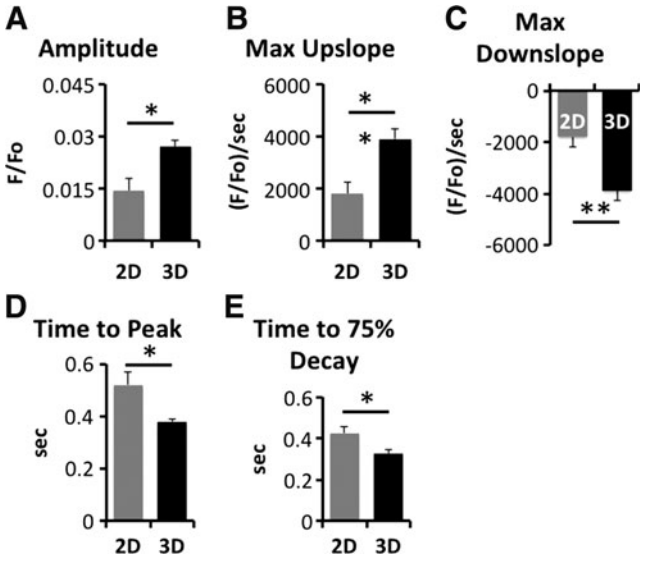
SUPPLEMENTARY FIG. S3. 3D adult cardiac ECM increases iPSC-derived CM gene expression of ion channels and calcium-handling proteins. Gene expression was measured using qRT-PCR and expression normalized to 2D adult cardiac ECM. Cardiac genes measured were *KCNJ2/Kir2.1* (inward rectifier potassium ion channel), *HCN4* (potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4), *SERCA2a* (sarco/endoplasmic reticulum calcium ATPase; $n = 2$), and Triadin. Gene expression in iPSC-derived CMs cultured in (3D) or on (2D) adult cardiac ECM. The *SERCA2a* values from four biological replicate experiments were 12, 13, 336, and 356. Results are expressed as mean ± standard error. ($n = 3$ unless otherwise stated, $*p > 0.05$, $**p > 0.01$, Student's *t*-test).



SUPPLEMENTARY FIG. S4. 3D adult cardiac ECM increases iPSC-derived CM protein expression of connexin 43 (Cx43). iPSC-derived CMs express sarcomere and Cx43 proteins. Immunofluorescent staining for Cx43 in iPSC-derived CMs cultured in 2D (*Left panels*) and 3D cardiac ECMs (*Right panels*). Scale bar is 50 μm.



SUPPLEMENTARY FIG. S5. iPSC-derived CMs cultured on adult cardiac ECM compared with fetal has increased protein expression of MYL2. Immunofluorescent staining of iPSC-derived CMs cultured in 2D cardiac ECM for MYL2**.**



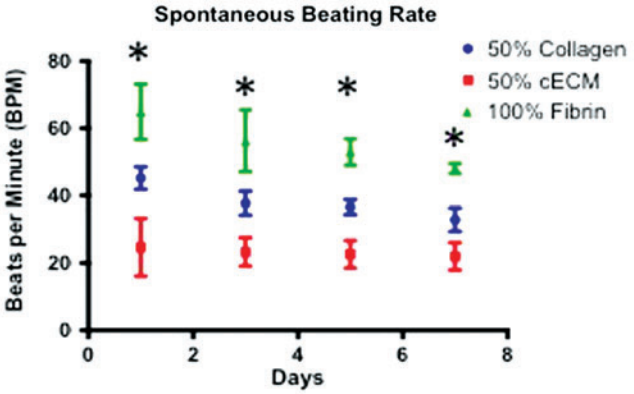
SUPPLEMENTARY FIG. S6. 3D adult cardiac ECM increases calcium signaling in iPSC-derived CMs. A GCaMP reporter was used to visualize calcium transients in iPSC-derived CMs after 7 days in 2D and 3D adult cardiac ECMs. The calcium transients were observed with confocal line scan imaging with line scan rates at 630 Hz. **(A–E)** iPSC-derived CMs cultured in 3D as aggregates displayed an increase in calcium signaling and kinetics compared with 2D. **(A)** Amplitude. **(B)** Maximum Upslope. **(C)** Maximum Downslope. **(D)** Time to peak. **(E)** Time to 75% decay. Results are expressed as mean ± standard error ($n = 6$–7; $^*p < 0.05$, $^{**}p < 0.01$, Student's t-test).

quantitated using the BCA assay. Equivalent mass amounts of the resulting samples were treated with iodoacetamide and trypsinized as described[55] with modifications,[56] and the resulting peptides desalted using stacked C18/SCX tips.[56,57] The resulting peptides from each sample individually were subjected to nanoLC-MS/MS using an LTQ Orbitrap Velos Pro mass analyzer (Thermo Fisher Scientific) connected to a nanoLC-Easy1000, with peptide separation in an in-house-packed 25 cm × 75 µm ID C18 nanospray tip. Peptides were resolved in segmented solvent gradients running from 6% to 35% CH_3CN in 0.1% formic acid over 135 min. FTMS precursor spectra were acquired at 60,000 resolution, and up to 20 of the most intense ions with charge states of +2 and higher in each precursor spectrum were subjected to rapid CID fragmentation and ion trap analysis.

Spectral data were recalculated using Mascot Distiller 2.5.0 (Matrix Science) and the resulting peak lists were searched against SwissProt (July 2014) with *Bos Taurus* taxonomy along with a database of common contaminants using Mascot Server 2.5.0 (Matrix Science), with Carbamidomethyl (C) and Oxidation (M) as fixed and variable modifications, respectively, and precursor and fragment mass tolerances of 10 ppm and 0.5 Da, respectively. Homology false discovery rates were either below or thresholded at 5%. Mascot protein score and emPAI values in Mascot exports were compared between samples using in-house software.

Mechanical analysis of 3D cardiac ECM hydrogels

Parallel plate rheology was conducted using an MCR 302 (Anton Paar) to determine the storage and loss moduli (reported as pascals; Pa) of fetal and adult 3D cardiac ECM



SUPPLEMENTARY FIG. S7. 3D adult cardiac ECM decreases iPSC-derived CM beat rate. **(A)** Spontaneous beating rate significantly decreased with respect to time in culture and matrix composition ($^*p < 0.0001$, two-way ANOVA).

Supplementary Table S1. SDS Concentration Used to Decellularize Cardiac Tissue Affects Polymerization of Fetal and Adult Bovine Cardiac ECMs

Fetal cardiac ECM						*Adult cardiac ECM*					
1% SDS					Day 3	1% SDS					Day 2–3
	1 h		2 h		100% Decellularized		1 h		2 h		100% Decellularized
	50 μL	75 μL	50 μL	75 μL			50 μL	75 μL	50 μL	75 μL	
16 mg/mL	—	—	Gel	Watery		16 mg/mL	Gel	Gel	Gel	Gel	
12 mg/mL	—	—	—	—		12 mg/mL	Gel	Gel	Gel	Gel	
10 mg/mL	—	—	—	—		10 mg/mL	Gel	Gel	Gel	Gel	
7 mg/mL	—	—	—	—		7 mg/mL	Gel	Gel	Gel	Gel	
0.5% SDS					Day 3						
	1 h		2 h		90% Decellularized						
	50 μL	75 μL	50 μL	75 μL							
16 mg/mL	Gel	Gel	Gel	Gel							
12 mg/mL	—	—	Gel	Gel							
10 mg/mL	—	—	Watery	Watery							
7 mg/mL	—	—	Watery	Watery							
0.25% SDS					Day 3						
	1 h		2 h		80% Decellularized						
	50 μL	75 μL	50 μL	75 μL							
16 mg/mL	Gel	Gel	Gel	Gel							
12 mg/mL	—	—	Gel	Gel							
10 mg/mL	—	—	Gel	Watery							
7 mg/mL	—	—	Watery	Watery							
0.1% and 0.05% SDS					Day 6						
	1 h		2 h		<70% Decellularized						
	50 μL	75 μL	50 μL	75 μL							
16 mg/mL	Gel	Gel	Gel	Gel							
12 mg/mL	Gel	Gel	Gel	Gel							
10 mg/mL	—	—	Gel	Gel							
7 mg/mL	—	—	Watery	Watery							

ECM, extracellular matrix; SDS, sodium dodecyl sulfate.

hydrogels comprising 5.25 mg/mL cardiac ECM and 1.75 mg/mL fibrinogen. Hydrogels were polymerized similar to the above experiments with measurements occurring after the 2-h incubation period at 37°C. Heating was conducted within a P-PTD200 Peltier temperature-controlled hood (Anton Paar). The storage modulus (G′) and loss modulus (G″) were measured at oscillation frequencies ranging from 0.1 to 10 Hz, with a constant strain amplitude of 1%. Experiments were conducted with a gap height of 200 μm using a 25-mm parallel plate. Each of the respective samples was measured in triplicate ($n = 3$).

Flow cytometry

Cells were fixed with 4% PFA for 10 min and then incubated in 0.75% saponin in PBS. Samples were stained with 1:100 anti-cTNT antibody (Thermo Scientific) or isotype control mouse IgG and subsequently incubated with goat anti-mouse FITC secondary antibody. Samples were run on a BD FACSCalibur flow cytometer and data were analyzed using FlowJo software.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% TritonX100, and then blocked with 0.1% TritonX100 and 4% bovine serum albumin (blocking buffer). Cells were incubated in primary antibodies: 1:100 MYL2 (ProteinTech), 1:100 CaV1.2 (Thermo Fisher), 1:100 Cx43 (Santa Cruz Biotechnology), and 1:800 α-actinin (Sigma-Aldrich) at 4°C overnight, followed by secondary Alexa Fluor 488/568 for 2 h in blocking buffer.

Cell nuclei were subsequently stained with DAPI for 30 min. The cells were imaged with either an epifluorescent microscope (Nikon Eclipse TE300) or a confocal laser microscope (Leica TCS SP8). Images were enhanced using ImageJ to improve visualization and all images within the experiment were adjusted equivalently.

Hydrogel encapsulation of human iPSC-CM spheroids

iPSC-derived CMs were seeded into AggreWell 400 (STEMCELL Technologies) at 720,000 cells per well. CM spheroids were allowed to aggregate and self-assembled for two days before encapsulation within the ECM.

ECM hydrogels consisted of 10 mg/mL bovine fibrinogen (Sigma-Aldrich) dissolved in Dulbecco's PBS (Life Technologies) and combined with 9 mg/mL rat tail collagen (BD Biosciences) and 10 mg/mL cardiac ECM. All matrices denoted as a partial percentage had the remaining volume completed with fibrin. Thrombin was mixed with the cell and extracellular matrix solution to yield a final concentration of 3 U/mL (0.3 U/mg fibrin). Spheroids resuspended in ECM mixture were cultured in three PDMS retention rings attached to a glass bottom dish (World Precision Instruments). The tissues were incubated for 30 min at 37°C to allow full polymerization, and then fed RPMI 1640 supplemented with B27. The medium was replaced every 2 days.

Supplementary Table S2. Key Proteins Identified in Decellularized Adult and Fetal Bovine Heart Tissue Preparations by NanoLC-MS/MS

	Bovine heart	
	Fetal	*Adult*
Fibrinogen	x	x
Collagen IV	x	x
Periostin	x	x
Fibrillin-1	x	x
Collagen III	x	x
Lumican	x	x
Fibronectin	x	x
Fibulin-5	x	x
Mimecan	x	
Collagen I	x	x
Decorin	x	x
Fibulin-1C	x	x
Elastin	x	x
Dermatopontin	x	x
Versican	x	

Data were thresholded at 4.4% and 4.8% FDR for adult and fetal samples, respectively.

Supplementary Table S3. Results of Split-Unit ANOVA of ECM Age and Geometry on iPSC-Derived CMs Cultured in Adult and Fetal Cardiac ECMs and 2D and 3D Geometries

p	*NCX1*	*Junctin*	*CaV1.2*	*Cx43*	*CASQ2*	*MYL2*
ECM age (fetal vs. adult)	0.603	0.937	0.645	0.876	0.511	0.150
ECM geometry (2D vs. 3D)	0.116	0.0103*	0.049*	0.015*	0.110	0.144
Geometry: age	0.816	0.9398	0.678	0.517	0.708	0.414

p Values are shown for each gene (*$p > 0.05$).
iPSC, induced pluripotent stem cell.

Supplementary Table S4. Quantitative RT-PCR Primer Sequences

Gene name	*Sequences 5′->3′*
Junctin FWD	ATT GCA TTG CTG GGC GTC TG
Junctin REV	GGC ATC ATC CAC ATC AAA ATC TCC
MYL2 FWD	AGC GGA CCC TGA GGA AAC CAT T
MYL REV	GGG AAG GCG GCG AAC ATC T
NCX1 FWD	GTC CAT CGC TGC CAT CTA CCA C
NCX1 REV	TAC AGC AGC ACC CCC ACA TTG A
Cx43 FWD	TCC CCT CTC GCC TAT GTC TCC TC
Cx43 REV	CTG CCC CAT TCG ATT TTG TTC TG
CaV1.2 FWD	ACA AGG GCC CCA TCT ACA ACT ACC
CaV1.2 REV	CGA TGA CGA AGC CCA CGA AGA T
18S FWD	CCC CGG CCG TCC CTC TTA
18S REV	CGC CCC CTC GAT GCT CTT AG
Triadin FWD	AGA GCC CCC AGG TTT TGA CAC A
Triadin REV	CGG GGG ATT TGG GCA CAG
CASQ2 FWD	GTG GCC CAG GTC CTT GAA CAT AAA
CASQ2 REV	GCT GCA AAC TCG CCA TCA AAC TCT

RT-PCR, reverse transcription polymerase chain reaction.