Stem Cell Reports, Volume *13*

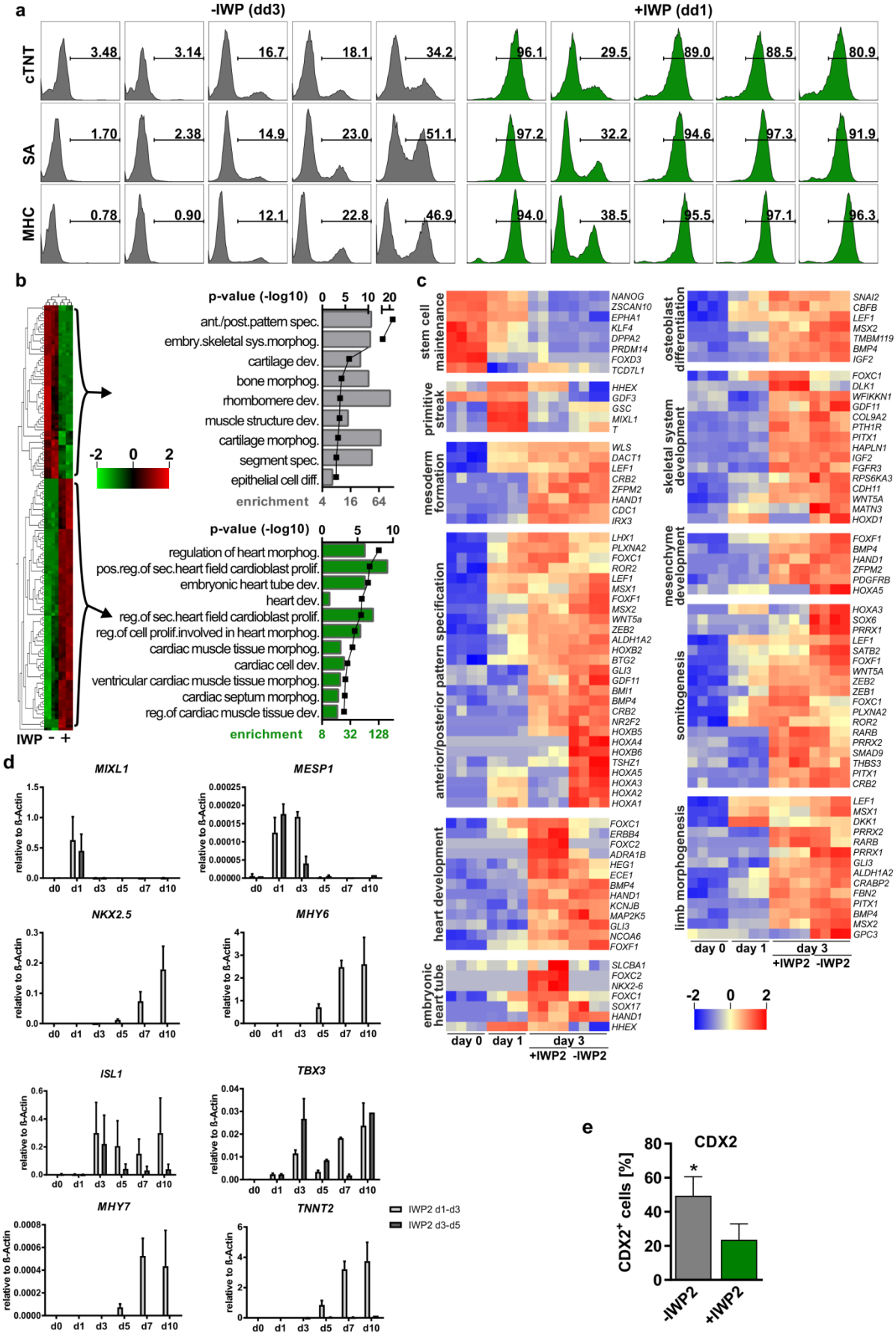
Supplemental Information

Continuous WNT Control Enables Advanced hPSC Cardiac Processing and Prognostic Surface Marker Identification in Chemically Defined Suspension Culture

Caroline Halloin, Kristin Schwanke, Wiebke Löbel, Annika Franke, Monika Szepes, Santoshi Biswanath, Stephanie Wunderlich, Sylvia Merkert, Natalie Weber, Felix Osten, Jeanne de la Roche, Felix Polten, Kai Wollert, Theresia Kraft, Martin Fischer, Ulrich Martin, Ina Gruh, Henning Kempf, and Robert Zweigerdt

Inventory Supplemental Information

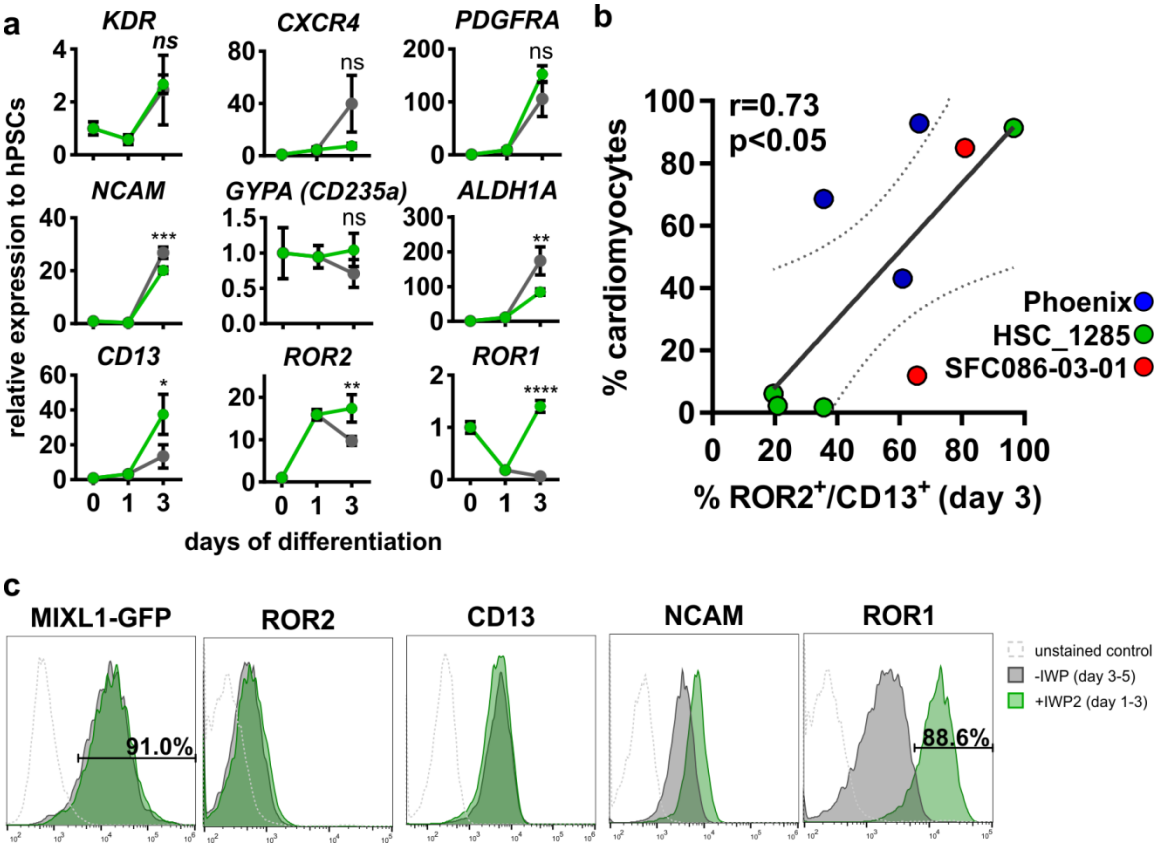
Figure S1. Related to Figure 1



(a) Representative FC data of hES3 showing individual flasks for cardiac quantification based on staining against cTNT, SA and MHC on day 10 of differentiation in 5 independent experiments. **(b)** Heatmap of >10-fold regulated genes on day 3 following IWP2 treatment on day 1 (left panel; σ/σmax =0.02, p<0.01) and associated gene ontology analysis of upregulated (lower panel) and downregulated (upper panel) genes of 4 independent experiments. **(c)** Heatmap of gene ontologies associated with early development show predominant expression of

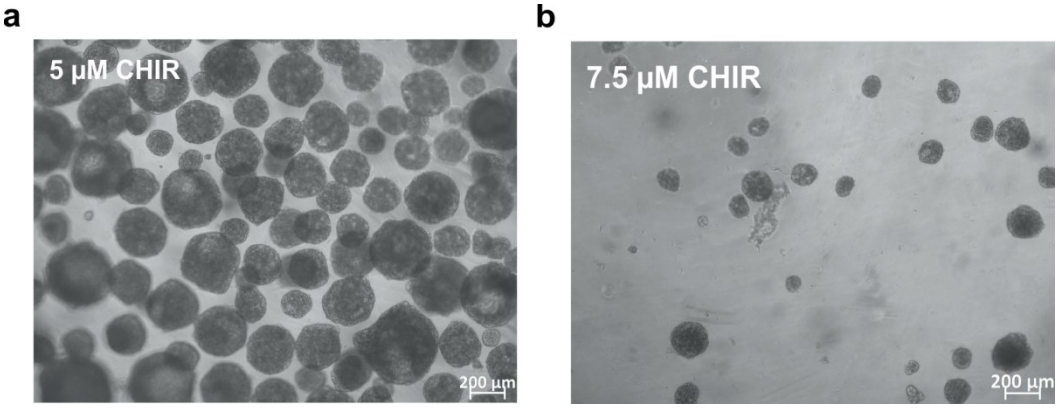
genes associated with stem cell maintenance on day 0, primitive streak formation on day 1, mesoderm formation and anterior/posterior pattern specification on day 3. Addition of IWP2 on day 1 results in increased gene expression of genes associated with heart development and heart field specification on day 3, while absence of IWP2 tends to increase genes of the paraxial mesoderm including genes associated with osteoblast differentiation, skeletal system development, mesenchyme development, somitogenesis and limb morphogenesis (4 independent experiments). (**d**) Quantitative real-time analysis to compare the effect of WNT inactivation via IWP2 addition on d1-d3, or IWP2 addition on d3-5, on the expression of cardiac genes using the HES3 cell line (n=3 independent experiments). **(e)** Pooled FC for CDX2-Venus expression of day 2 and 3 samples conducted in 2 independent experiments in triplicates using the CDX2_iPS cell line (n = 6 repeats independent experiments). Error bars represent SEM.

Figure S2. Related to Figure 2



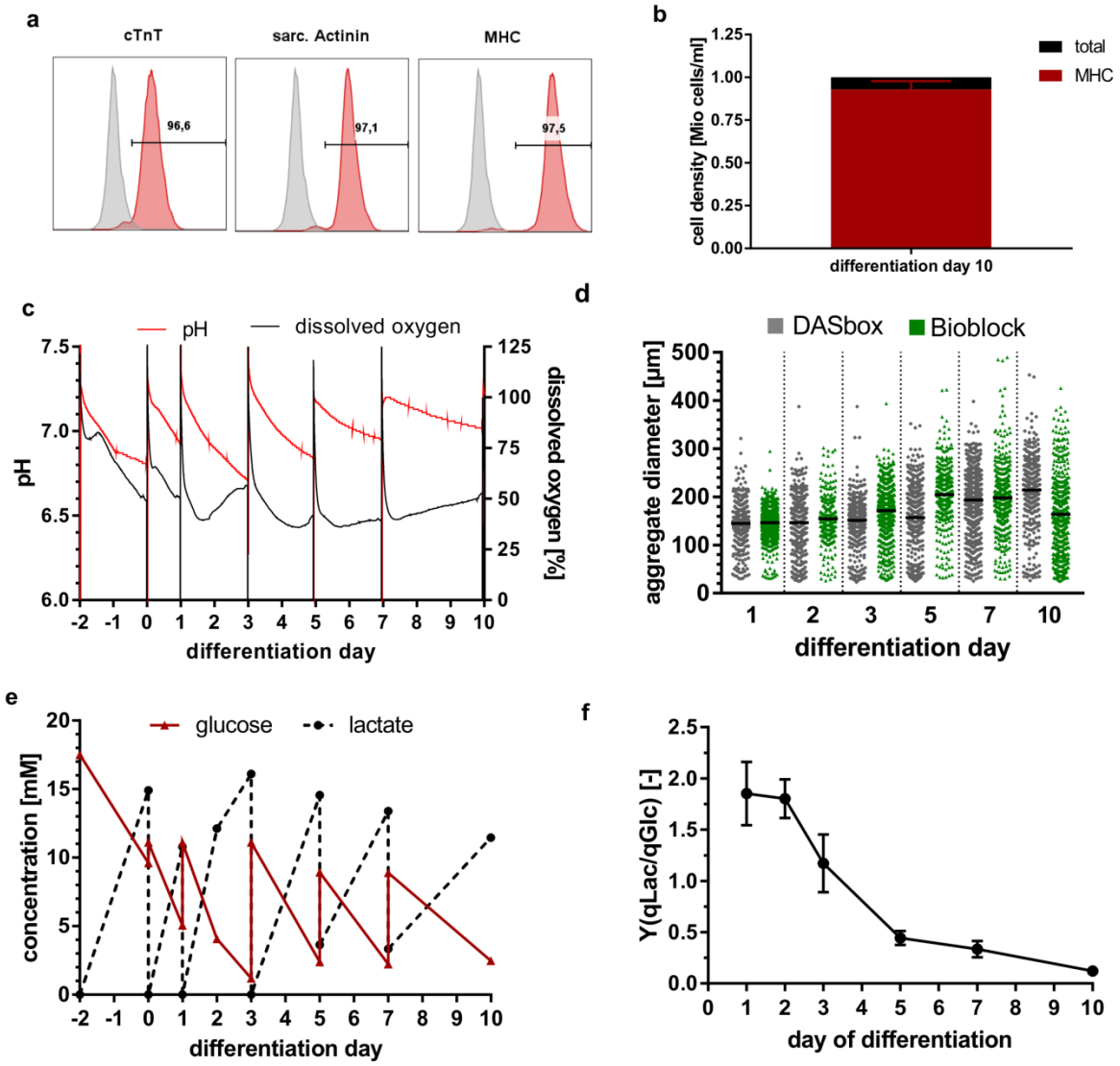
(a) Expression pattern of established mesodermal surface markers show only minor differences between cardiac (■) and paraxial (■) mesoderm on day 3 of *in vitro* differentiation from hPSCs. n=4 independent experiments based on microarray signal intensities normalized to hPSC levels. Error bars represent SEM. (**b**) Correlation of $ROR2^{+}/CD13^{+}$ on day 3 and respective cardiomyocyte efficiency on day 10 based on 3 different cell lines; each dot represents an independent biological repeat. r = pearson's correlation coefficient. 95% confidence interval is indicated by dotted lines. **(c)** Representative FC on d3 using the MIXL1-GFP reporter line for ROR2, CD13, NCAM and ROR1 with (■) and without (■) IWP2 treatment on day 1. Unstained control is shown in dashed lines.

Figure S3. Related to Figure 4



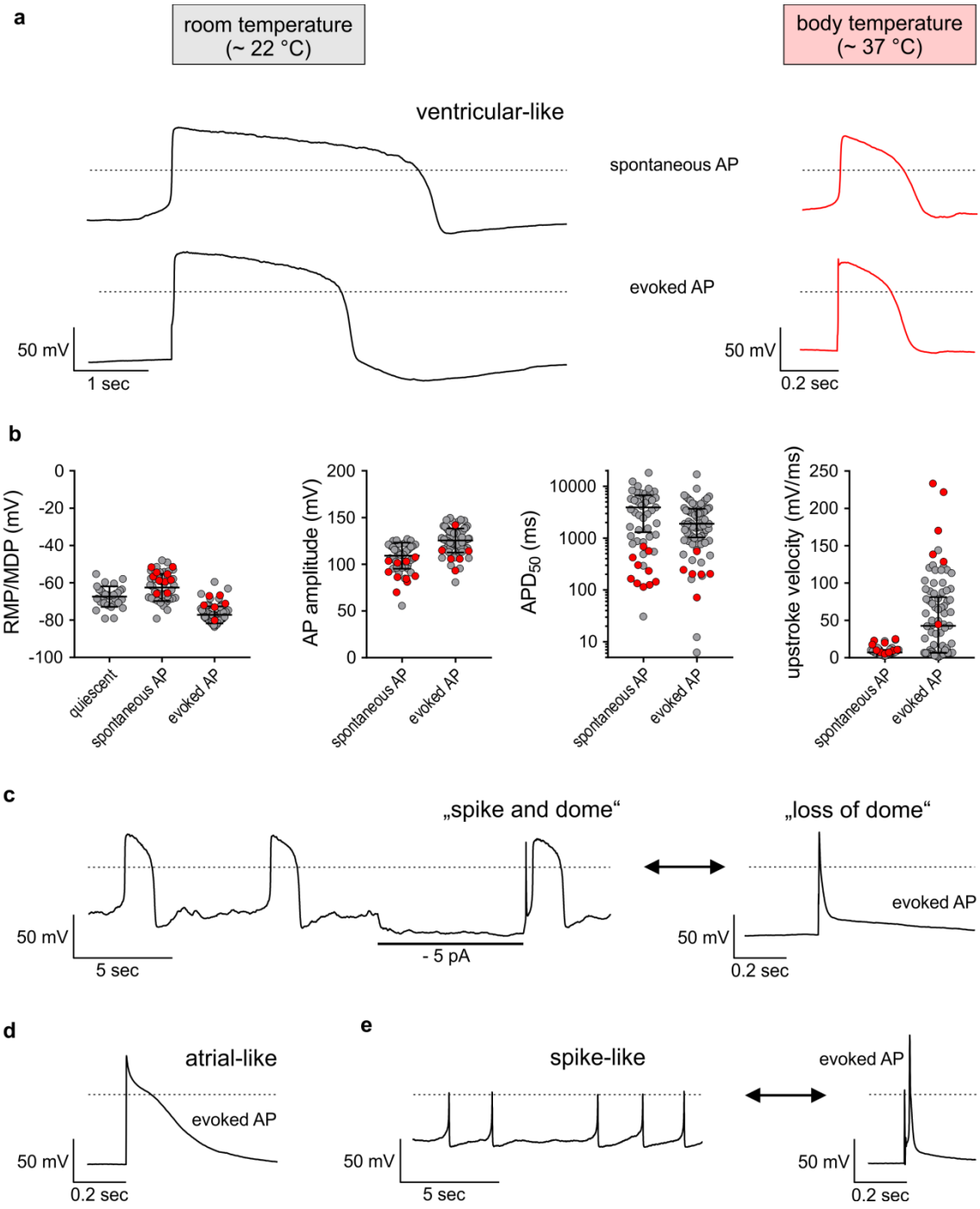
When applying **(a)** 7.5 µM CHIR compared to **(b)** 5 µM CHIR, cell death can be observed after 4 days of differentiation with the Pheonix line.

Figure S4. Related to Figure 5



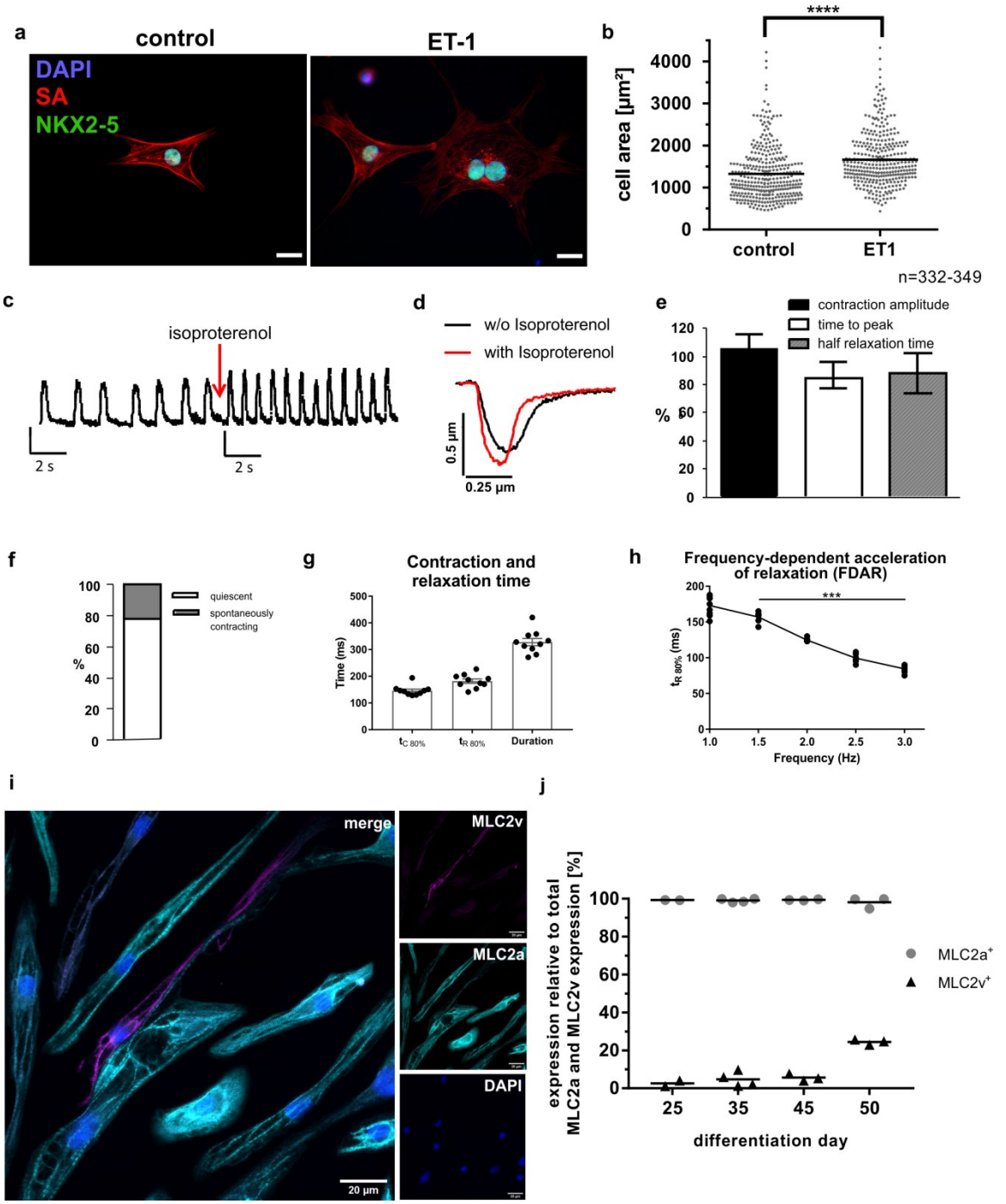
(a) FC of markers cTnT, sarcomeric actinin and myosin heavy chain for cardiomyocytes derived after 10 days of differentiation in the bioreactor (DASbox). **(b)** Cell density achieved in a bioreactor (n=3 independent experiments). **(c)** Representative process parameters (pH and DO) of the DASbox differentiation show oscillation of DO independently of medium exchanges. **(d)** Quantification of aggregate diameters of 2 – 3 independent runs over the 10-day differentiation process in DASbox and Bioblock bioreactors (n=3 DASbox and n=2 in Bioblock bioreactor). **(e)** Glucose and lactate levels of a representative bioreactor differentiation (150 ml scale). **(f)** The specific yield coefficient of lactate from glucose Y(qLac/qGlc) strongly reduces over the course of the differentiation in bioreactors at both process scales (n=5-6 independent differentiations). Error bars represent SEM.

Figure S5. Related to Figure 6.



(a) Representative action potentials from ventricular-like CMs recorded at room temperature (~ 22°C) or at body temperature (~ 37°C). Spontaneous action potentials (upper traces) are compared to evoked action potentials of the same cells (lower traces), when the membrane potential was artificially hyperpolarized to resting conditions around -80 mV. - Note the different scale bars at 22 °C and 37 °C. **(b)** RMP (MDP for regularly beating CMs) and AP parameters (AP amplitude, APD_{50}, and upstroke velocity) obtained from spontaneous and evoked APs to characterize the CM phenotype in three independent experiments. Evoked APs were elicited by short current pulses from resting membrane potentials adjusted to about -80 mV. Scatter plots include the mean ± SD (RMP/MDP and AP amplitude) or median and IQR (APD_{50} and upstroke velocity) for room temperature only (grey symbols; quiescent: n = 31; spontaneous AP: n = 42; evoked AP: n = 70). Values for body temperature (red symbols) are overlaid but not included in calculations. **(c-e)** Recordings from three CMs that deviate from the consistent ventricular-like phenotype. - Dotted lines in A and C-E represent 0 mV.

Figure S6. Related to Figure 6.



(a,b) Effect of 24 h of Endothelin-1 treatment on the surface area of plated single CMs combining three independent experiments. Error bars represent s.d. **(c)** Representative sample recordings of isoproterenol effects onto the frequency of the spontaneous contractions. Isoproterenol application is indicated by the red arrow. **(d)** Effects of isoproterenol onto electrically evoked contractions. Representative sample recordings of averaged cell length changes of electrically evoked twitch contraction of the same cardiomyocyte, before (black trace) and after isoproterenol application (red trace).**(e)** Twitch parameters in % (contraction amplitude, time to peak and half relaxation time) after isoproterenol application (1µM) normalized to baseline levels. Error bars are mean±SEM for contraction amplitude and median±IQR for time to peak and half relaxation time of twitch. n=14 cells from two independent batches. **(f)** Fractions of quiescent and spontaneously contracting single CMs from four independent batches. (**g,h**) Mechanical and electrophysiological properties of BCTs from two indep. CM batches. BCTs measured on day 21 (mean±SEM, n=9 for g and h). **(g)** contraction duration at 80% peak height; tC – contraction time, tR - relaxation time. **(h)** Relaxation time/frequency relationship. **(i)** hiPSC-CMs were immunostained against atrial (MLC-2A) and ventricular (MLC-2V) isoforms of myosin light chain 2 to show the distribution of both isoforms. Immunofluorescence microscopy revealed a heterogeneous expression with cells either purely expressing MLC-2A (cyan) or MLC-2V (magenta), or mixed isoforms. The hiPS-CMs also exhibit a characteristic sarcomeric pattern. Nuclei were counterstained with DAPI (blue). **(j)** FC of MLC2a and MLC2v expression relative to total MLC2 expression over the course of prolonged cultivation (n=2-3 independent experiments).

Supplemental tables

Supplemental Table. Sequences of the PCR primers:

Gen	Forward	Reverse	Probe
ACTB	TGGAGAAAATCTGGCACCAC	GGTCTCAAACATGATCTGG	ACCGCGAGAAGATGACC
MIXL1	ATCCAGGTATGGTTCCAG	CCTAGCCAAAGGTTGGAAG	GTCAGAGTGGGAAATCCTTC
MESP1	CTCCGACAAGGACTGC	TGCCCCATGAGTCTGG	CTCCCTCGTCTCGTCCC
ISL1	GCTTTTCAGCAACTGGTCAA	TGCCTCAATAGGACTGGCTAC	GATACACCTAACAGCATGG
TBX3	CCTGGAGGCTAAAGAACTTTG	TGGAGGAAACATTCGCCTTC	GTCATTACCAAGTCGGG
MYH6	AGAGCGAGTTCAAGCTG	CGAGACACTTTCTCCAGGTT	TCATCAAGGCCAAGGCAA
MYH7	TGTCCAAGTTCCGCAAGGT	GCTACTCCTCATTCAAG	ATTGGCACGAAGGGCTTG
NKX2-5	AAGGACCCTAGAGCCGAAA	GTTGTCCGCCTCTGT	CGGTGGAGCTGGAGAAGA
TNNT2	TGACCACCTGAATGAAG	GATCCTGTTTCGGAGAAC	GAAATATGAGATCAATG

Supplemental Table. media composition

E8	DMEM/F12 (Life Technologies) supplemented with 64 mg/L ascorbic acid 2-phosphate, 14 µg/L sodium selenite, 543 mg/L NaHCO3, 20 mg/L insulin, 10.7 mg/L human recombinant transferrin (all Sigma-Aldrich), 100 µg/L bFGF (LU Hannover(Chen et al., 2012)) and 2 µg/L TGFβ1 (Peprotech).
CDM3	RPMI1640 (with 2 mM glutamine) supplemented with 495 µg/ml human recombinant albumin (ScienCell, SC-OsrHSA) and 213 µg/ml ascorbic acid (Sigma).

Supplemental experimental procedures

Differentiation in defined conditions

Differentiation was conducted as illustrated in Figure 1a. hPSC cultures were harvested using Accutase and inoculated as single cells at 5x105 cells/well of a 12-well suspension plate (Greiner Bio One) in 1.5 ml mTeSR1 medium supplemented with 10 µM Y-27632 (day -4). After 72 hours (day -1), formed aggregates of 3 individual wells were harvested, dissociated using Accutase and counted. Aggregates corresponding to 3-6x106 cells were pooled in 20 ml mTeSR medium and transferred to 125 ml Erlenmeyer flask (Corning). After 24h, medium was changed to RPMI1640 (with 2 mM glutamine) supplemented with B27 without insulin (Life Technologies, referred to as RB-). To induce mesendodermal differentiation, 7.5 µM CHIR99021 (LU Hannover) was added for 24h. Unless otherwise indicated, 5 µM IWP2 (Tocris or LU Hannover) was added after 24h (day 1) for 48h. Medium was refreshed every 2-3 days. Insulin was added from day 7 onwards (referred as RB+). Erlenmeyer flasks were agitated on an orbital shaker (Celltron) at 70 revolutions per minute (rpm) and incubated at 37°C and 5% CO2.

Differentiation in chemically defined conditions in suspension culture

hPSC cultures were harvested using accutase and seeded at 1.5-1.7x105 cells/ml in E8 supplemented with 10 µM Y27632 in either low-attachment six well plates (Greiner Bio One, 3 ml working volume) or single use 125 ml Erlenmeyer flasks (Corning, 20 ml working volume) placed on orbital shakers (Celltron, 70 rpm). 48h after inoculation, the medium is replaced by CDM3 supplemented with 5 µM CHIR. After precisely 24 h, the medium was replaced by CDM3 supplemented with 5 µM IWP2 unless indicated otherwise. After another 48h, the medium was replaced by CDM3 and medium was exchanged every 2-3 days. CDM3 (Burridge et al., 2014) is comprised of RPMI1640 (with 2 mM glutamine) supplemented with 495 µg/ml human recombinant albumin (ScienCell, SC-OsrHSA) and 213 µg/ml ascorbic acid (Sigma).

Aggregate diameter, cell density and metabolite analysis

Sampling of the bioreactor was performed as previously described (Kempf et al., 2014; Kropp et al., 2016). Microscopic images were taken of 2 ml samples using Axiovert A1 (Zeiss) and a minimum of 100 aggregates were analysed using ImageJ to evaluate the mean aggregate diameter throughout the bioreactor cultivation and differentiation. Samples were then dissociated through incubation of Accutase for 3 min at 37 °C (for hPSCs between day 0 and day 5 of differentiation) and collagenase B incubation at 37 °C for 10-20 min (from differentiation day 7 onwards (1 mg/ml, Roche). Dissociated cells were counted in 1 ml PBS using the Vi-CELL XR (Beckman Coulter). Supernatant of the samples are used to measure glucose and lactate concentrations (BIOSEN C-line, EKF Diagnostic) and specific Yield coefficient of lactate from glucose is calculated as described previously (Kropp et al., 2016). Data were processed and visualized using GraphPad Prism.

Microarray

Microarrays were conducted as described previously(Kempf et al., 2014). In brief, a customized whole human genome oligo microarray 4x180K-054261On1M (design ID 066335, Agilent Technologies, A-GEOD-20606) covering roughly 26000 human transcripts was hybridized with Cy3-labeled cRNA and scanned on the Agilent Micro Array Scanner G2565CA. Data were analyzed using Qlucore Omics Explorer 3.3 (Qlucore AB, Lund, Sweden) for PCA, heatmap generation and identification of >10-fold regulated genes. The RCUTAS filter tool (Research Core Unit Transcriptomics of Hannover Medical School) was used to visualize individual gene expression patterns. Volcano plot (FDR=0.1, S0=0.5) was generated using Perseus (v.1.5.6.0)(Tyanova et al., 2016). GO analysis of >5-fold regulated genes was performed using webgestalt software (http://bioinfo.vanderbilt.edu/)(Wang et al., 2013). The microarray data were deposited under accession number E-MTAB-6828 in the ArrayExpress database (www.ebi.ac.uk/arrayexpress).

Analysis of fetal tissue proteome

Data on 30 histologically normal patients are available under http://humanproteomemap.org/query.php, based on the publication of (Kim et al., 2014). All 7 fetal tissues were analyzed for KDR, CXCR4, PDGFRA, NCAM1, GYPA, ALDH1A1, ANPEP, ROR2, ROR1.

Flow cytometry

Aggregates were dissociated into single cells using collagenase B (1 mg/ml, Roche) for 15-30 minutes at 37°C. For staining of intracellular cardiac markers, 1.5x105 cells were fixed and permeabilized according to manufacturer's instructions (Fix & Perm, An der Grub) or ice-cold methanol (90%) for 15 minutes. Anti-cardiac Troponin T (1:200, clone 13-11, Thermo Scientific), anti-sarcomeric α-actinin (1:800, EA53, Sigma-Aldrich or 1:20, REA402, Miltenyi Biotech), anti-myosin heavy chain (1:20, MF20, Hybridoma Bank), anti-MLC2a (1:100, Synaptic Systems) and respective isotype controls (DAKO) were detected using appropriate Cy5-conjugated antibodies (1:200, Jackson ImmunoResearch). Anti-MLC2v (1:100, abcam) was detected using Cy3-conjugated antibody (1:200, Jackson ImmunoResearch). Anti-ROR1-VioBright FITC, anti-ROR1-PE (1:50, clone 2A2, Miltenyi Biotech), anti-EpCAM-FITC (1:100, EBA-1), anti-NCAM-PE-CF594 (1:50, B159, both

BD biosciences), anti-ROR2 (1:50, #231509, R&D Systems), anti-CD13 (1:50, WM15, BD pharmingen), anti-CXCR4-APC (1:100, 12G5, eBioscience) were incubated for 25 minutes at 4°C. Data were acquired on an Accuri C6 flow cytometer (BD Biosciences) and analyzed using FlowJo software (Flowjo, LLC).

Immunofluorescence

Aggregates were dissociated using collagenase B or using the STEMdiff™ Cardiomyocyte Dissociation Kit (STEMCELL Technologies) on day 14 of differentiation and plated in IMDM+GlutaMAX™ (LifeTechnologies) supplemented with 20 % FCS (HyClone), 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM ß-mercaptoethanol (all Life Technologies), 10 µM Y-27632 and 1% penicillin/streptomycin (Biochrom) on 6-well Geltrex® coated plates or on glass slides coated with 0.1% gelatin (Sigma) and 4 µg/ml fibronectin (Corning) in 12-well culture plates. Cells were fixed after 2-4 days with 4% paraformaldehyde for 15min at RT. After blocking using Tris-buffered saline (5% donkey serum, 0.25% Triton X-100) cells were incubated with anti-cardiac Troponin T (1:200, clone 13-11, Thermo Scientific), anti-cardiac Troponin T (1:200, polyclonal, abcam), anti-sarcomeric α-actinin (1:800, EA53, Sigma-Aldrich), anti-myosin heavy chain beta (1:2000, NOQ7.5.4D, Sigma), anti-titin (1:30, 9D10, Hybridoma Banks), anti- NKX2.5 (1:800, E1Y8H, Cell Signalling Technology), IgM and IgG isotype controls (DAKO), respectively, and detected using appropriate Cy2, Cy3-conjugated antibodies (1:200, Jackson ImmunoResearch). Nuclei were counterstained with DAPI and samples were analyzed using the Axio Observer A1 (Zeiss).

For staining of MLC2a and MLC2v, dd14-old cells were dissociated and plated onto fibronectin covered glassed coverslips as described above. Cells were cultivated for another 35 days, then fixed with 4% PFA at RT for 60min and permeabilized in 0.2% Triton X-100 (Roche, Basel, Switzerland) and subsequently blocked using 5% bovine serum albumin (Gerbu Biotechnik GmbH, Heidelberg, Germany). Cells were incubated overnight (4°C) with specific antibodies against myosin light chain 2a (MLC-2A, 1:100, mouse, monocolonal, Synaptic Systems, SY 311 011) and myosin light chain 2v (MLC-2V, 1:100, rabbit, polyclonal, Proteintech 10906-1-AP). Alexa 555 (donkey anti-mouse, 1:400, A31570, Life Technologies, Bleiswijk, NL) and Alexa 680 (donkey anti-rabbit, 1:400, A10043, Thermo Fisher Scientific GmbH, Dreieich, Germany) were used as secondary antibodies. 4′,6-diamidino-2-phenylindole (DAPI, 1:12500, D9542, Sigma-Aldrich, Deisenhofen, Germany) was used as a nuclear counterstain. Coverslips were embedded in Fluoroshield (Sigma-Aldrich, Deisenhofen, Germany). Immunofluorescence images were recorded with an IX83 inverted fluorescence microscope (Olympus, Tokyo, Japan).

Patch clamp analysis

Standard whole cell patch clamp recordings were performed on single hiPSC-derived cardiomyocytes using an Axopatch 200B amplifier in the current clamp mode (Molecular Devices, Sunnyvale, CA, USA). Cells were singularized (using the STEMdiff™ Cardiomyocyte Dissociation Kit, Stemcell technologies) on glass cover slips at differentiation day 17 or 19 and measured 4 - 16 days after plating. Pipettes with resistances between 2 MΩ and 6 MΩ were pulled from borosilicate glass (Harvard Apparatus, Holliston, MA, USA). The extracellular Tyrode solution contained: 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4 (adjusted with NaOH). The intracellular solution contained: 120 mM K-gluconate, 1 mM $MgCl_2$, 3 mM Mg-ATP, 10 mM EGTA, and 10 mM HEPES, pH 7.2 (adjusted with KOH). Junction potential was calculated and corrected using the JPCalc software (Dr. P. Barry, University of South Wales, Sydney, Australia (Barry, 1994)). Experiments were performed at room temperature or at 37 °C (by heating the bath solution with a Peltier temperature control system PTC-20, npi electronic GmbH, Tamm, Germany). Resting membrane potential (RMP) and the shape of action potential (AP), either spontaneously elicited or evoked by short intracellular current pulses (0.4 - 6 nA, 1 ms), were analyzed to characterize the CM phenotype. AP amplitudes were measured from peak to resting membrane potential or from peak and maximum diastolic potential, if membrane potential was not stable in regularly beating CMs. AP duration at 50% repolarization (APD_{50}) and upstroke velocity were also determined. We furthermore analyzed the AP parameters after hyperpolarization of CMs to physiological resting potentials (close to -80 mV) by injection of small holding currents (-2 pA to -30 pA). Data were analyzed using Clampfit software (Molecular Devices, Sunnyvale, CA, USA) and are presented as scatter plots including means ± SD (RMP and AP amplitude) or medians and IQR (APD_{50} and upstroke velocity).

Single cell contraction analysis

Measurements of twitch contractions and intracellular calcium transients were performed at d14-19+6-15 (first number: days after differentiation of the CMs, second number: days after plating onto fibronection coated coverslips in a density of 30 000 cells/ well on cardiomyocytes) from four independently generated batches.

For measurement of twitch contractions a coverslip with plated hiPSC-CMs was placed into a homemade perfusion chamber and perfused with Hepes buffer containing in mmol/L: NaCl 117, KCl 5.7, NaH2PO4 1.2, MgSO4 0.66, glucose 10, sodium pyruvate 5, creatine 10, Hepes 20, EGTA 0.01, CaCl2 1.25, pH 7.4 as described previously (Mutig et al., 2013; Weber et al., 2016). Cardiomyocytes were electrically stimulated to contract at 0.5 and 1Hz, 25V, 4 ms impulse duration at 37±0.5°C via two platinum electrodes and MyoPacer® EP Cell Stimulator (IonOptix Corp., Milton, MA, USA). During the acquisition protocol electrical stimulation

was paused for 30 seconds to register spontaneous contraction activity of the CM. Contractions were recorded using MyoCam edge detection acquisition module (IonOptix Corp., Milton, MA, USA).

For analysis of each cardiomyocyte 20-30 single twitches were averaged and time to peak (ttp), half relaxation time (hrt), phases of thereof, velocities and rates of contractions and relaxation and contraction amplitude were calculated using IonWizzard software (IonOptix Corp., Milton, MA, USA). Fraction of the spontaneously contracting CMs from all measured CMs was calculated. Each cell, which showed any spontaneous activity in the pacing-free interval was determined as spontaneously contracting CM.

Adrenergic stimulation of single cardiomyocytes with Isoproterenol hydrochloride

To detect effects of adrenergic stimulation, electrically evoked orspontaneous contractions of the single plated CM were recorded for one minute using MyoCam edge detection acquisition module (IonOptix Corp., Milton, MA, USA). After that isoproterenol hydrochloride (Sigma, I6504) diluted to 1 µM in the perfusion solution was influted into the perfusion chamber. Effects of isoproterenol hydrochloride onto the beating frequency of the spontaneously contracting single CMs and onto the time to peak, half relaxation time and contraction amplitude of the electrically evoked twitch contractions were recorded and stored using IonWizzard software (IonOptix Corp., Milton, MA, USA). Effects of isoproterenol onto the beating rate of the CMs was analyzed by manually counting the frequency of contractions in a time period of 30 s before and after isoproterenol application. Effects of isoproterenol onto the parameters of the evoked contractions were analyzed using IonWizzard software (IonOptix Corp., Milton, MA, USA).

Analysis of intracellular Ca2+-transients

Intracellular Ca2+-transients of single cardiomyocytes were recorded using a dual excitation fluorescence photomultiplier system (IonOptix Corp., Milton, MA,USA) as described previously (Mutig et al, 2013; Weber et al., 2016). Briefly, cardiomyocytes were loaded with 2.5µM fura-2 AM (Invitrogen™ Corp., Molecular probes®, Eugene, OR, USA) and incubated for 25-30 min at 37°C, 5% CO2. Then, cardiomyocytes were rinsed twice for 15 min. and fluorescence measurements were performed. Ratio transients were analyzed and diastolic and systolic ratios, ratio amplitude, time to peak and half decay times of calcium transients were calculated using IonWizard® software.

BCT generation and cultivation

Bioartificial cardiac tissues (BCT) were generated and cultivated as described earlier (Kensah et al., 2013) with some modifications. Briefly, differentiated CMs were dissociated using STEMdiff™ Cardiomyocyte Dissociation Kit (STEMCELL Technologies, Vancouver, CA) according to manufacturer's instructions. The liquid cell-matrix mixture (250 µl/BCT) composed of Matrigel (10%; BD Biosciences, Heidelberg, DE), rat collagen type I (1.35 mg/ml; Trevigen, Gaithersburg, US), 3.6% 0.4 M NaOH, 10^6 dissociated CMs, and 10^5 irradiated human fibroblasts (ATCC, Manassas, US) was poured into silicon molds containing 2 titanium rods for anchorage. After solidification (30 min) the tissues were cultivated in BCT medium (DMEM with 12% horse serum, 1 mM L-glutamine, 1% Penicillin/Streptomycin, 30 µM L-ascorbic acid, and 10 µg/ml insulin) under standard cell culture conditions (37 ◦C, 5% CO_2) for 21 days. Following day 7 (6 mm initial slack length) a growing static stretch protocol of 0.4 mm/4 days was applied until 7.6 mm final slack length was reached. Tissue maturation and CM viability was monitored during cultivation using an AxioObserver Z1 fluorescence microscope and ZEN software (both Zeiss, Jena, DE). To visualize the distribution of viable CMs, the mitochondria-specific dye tetramethylrhodamine methyl ester (TMRM; Thermo Fisher Scientific, Waltham, US) was added to the culture medium in a 25 nM concentration.

Force measurement of BCTs and evaluation

Active contraction forces and passive forces were measured on D21 of tissue cultivation as described earlier (Kensah et al., 2011) using a custom-made bioreactor system (Central Research Devices Service Unit, MHH, Hannover, DE). BCTs were placed into a culture vessel (BCT medium, 37°C) allowing for contraction force recording on one side, and precise application of increasing preload in 200 µm increments on the opposite side until 1000 µm is reached. After every preload step, spontaneous contraction as well as paced contraction (mean of 5 biphasic pulses, 10 ms, 25 V) values were measured, and stress relaxation was allowed until a stable baseline level was reached. At 1000 µm preload, contractions were recorded while the pacing frequency was stepwise increased from 1 Hz to 3Hz in 0.5 Hz steps. The spontaneous beating frequency, contraction time, relaxation time, and contraction duration at 80% peak height were calculated using self-developed MATLAB (version R2018b) scripts.

Analysis of mRNA expression by quantitative real-time PCR (RT-qPCR)

Total RNA was prepared using the RNeasy Kit (Macherey-Nagel, Düren, Germany) and reverse transcribed with Superscript II (Invitrogen) using oligo dT primers according to manufacturer's instructions. Primer sequences are given in Supplemental Table 1 RT-qPCR was performed in duplicate using a Mastercycler® ep realplex² (Eppendorf, Hamburg, Germany) and the Solaris™ qPCR Assays (Dharmacon™, GE Healthcare, England). The

size of amplicons and absence of nonspecific products was controlled by melting curves. Relative changes in gene expression were analysed using the Mastercycler® ep realplex Software Version 2.0 (Eppendorf). Expression levels of the target genes Mixl1, Mesp1, ISL1, Tbx3, Nkx2.5, α-myosin heavy chain (MYH6), β-myosin heavy chain (MYH7), and cardiac Troponin T (cTnT, TNNT2) were normalized to β-actin (Supplemental Fig.7).

Cellular hypertrophy induction with Endothelin-1

Generated hPSC- CMs were dissociated around dd15-dd18 using the STEMdiff™ Cardiomyocyte Dissociation Kit (STEMCELL Technologies) and resusupended in IMDM+GlutaMAX™ (Life Technologies) supplemented with 20% Foetal Calf Serum (HyClone), 1% non-essential amino acids (Life Technologies) 1 % Penicillin/Streptomycin(Biochrom), 0.5% L-glutamine (Life Technologies), 0.2 % ß-Mercaptoethanol (Life Technologies) and 10 µM Y-27632. Dissociated cells were plated at 60,000 cells/well in 6-well Geltrex® coated plates and cultivated for 24 h at 37 °C, 5% CO2 before replacing the medium with maturation medium (Drawnel et al., 2014) supplemented with/without. 100 nM Endothelin-1 (ET1) (Sigma-Aldrich) to induce hypertrophy. Brightfield images were acquired 24 h post stimulation and the cell size analysis was performed using the Axiovision software (Zeiss). Thereafter the samples were fixed and immunofluorescence staining was performed.

References

Barry, P.H. (1994). JPCalc, a software package for calculating liquid junction potential corrections in patch-clamp, intracellular, epithelial and bilayer measurements and for correcting junction potential measurements. J Neurosci Methods *51*, 107-116.

Drawnel, F.M., Boccardo, S., Prummer, M., Delobel, F., Graff, A., Weber, M., Gerard, R., Badi, L., Kam-Thong, T., Bu, L.*, et al.* (2014). Disease modeling and phenotypic drug screening for diabetic cardiomyopathy using human induced pluripotent stem cells. Cell Rep *9*, 810-821.

Kensah, G., Gruh, I., Viering, J., Schumann, H., Dahlmann, J., Meyer, H., Skvorc, D., Bar, A., Akhyari, P., Heisterkamp, A.*, et al.* (2011). A novel miniaturized multimodal bioreactor for continuous in situ assessment of bioartificial cardiac tissue during stimulation and maturation. Tissue engineering Part C, Methods *17*, 463-473.