

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

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<https://doi.org/10.1016/j.stemcr.2019.06.004>

SUMMARY

Aiming at clinical translation, robust directed differentiation of human pluripotent stem cells (hPSCs), preferentially in chemically defined conditions, is a key requirement. Here, feasibility of suspension culture based hPSC-cardiomyocyte (hPSC-CM) production in low-cost, xeno-free media compatible with good manufacturing practice standards is shown. Applying stirred tank bioreactor systems at increasing dimensions, our advanced protocol enables routine production of about 1 million hPSC-CMs/mL, yielding $\sim 1.3 \times 10^8$ CM in 150 mL and $\sim 4.0 \times 10^8$ CMs in 350–500 mL process scale at >90% lineage purity. Process robustness and efficiency is ensured by uninterrupted chemical WNT pathway control at early stages of differentiation and results in the formation of almost exclusively ventricular-like CMs. Modulated WNT pathway regulation also revealed the previously unappreciated role of ROR1/CD13 as superior surrogate markers for predicting cardiac differentiation efficiency as soon as 72 h of differentiation. This monitoring strategy facilitates process upscaling and controlled mass production of hPSC derivatives.

INTRODUCTION

Progress in human pluripotent stem cell (hPSC) research has the potential to revolutionize regenerative medicine. Functional hPSC progenies may either fuel advanced *in vitro* assays for better drug development or replenish the loss of functional cells in diseased organs.

Given the high incidence of cardiac disorders, there have been substantial efforts in investigating cardiomyogenic differentiation of hPSCs. Stages of differentiation include early mesendoderm priming (Kempf et al., 2016), specification of cardiac progenitors (Soh et al., 2016), and directed differentiation into cardiomyocyte (CM) subtypes such as ventricular-, atrial- and nodal-like phenotypes (Devalla et al., 2015; Protze et al., 2017). Process specification was also accompanied by revealing more lineage-specific surface markers facilitating monitoring of differentiation stages and process optimization (reviewed in Skelton et al., 2017).

The field has also progressed from using recombinant factors toward chemical compounds for directing CM induction. These protocols typically aim at mimicking the biphasic pattern of WNT pathway upregulation and subse-

quent attenuation known from early heart development (Gonzalez et al., 2011; Lian et al., 2012; Tran et al., 2009; Ueno et al., 2007). Notably, chemical WNT pathway stimulators (particularly the GSK3 inhibitor CHIR99021 [CHIR]) or suppressors (including IWP2, IWR1, and Wnt-C59) have also been applied to specify other mesodermal lineages including hepatocytes (Siller et al., 2015) and skeletal muscle cells (Shelton et al., 2014). This highlights process complexity due to the multiple spatio-temporally dependent roles of the WNT pathway in development. Moreover, we have recently demonstrated that, in response to CHIR stimulation, a complex pattern of paracrine factors, whose feedback-controlled concentration depends on the applied cell density, substantially modulates early primitive streak (PS)-like priming (Gaspari et al., 2018; Kempf et al., 2016). Thus, in addition to the well-studied impact of the CHIR dose, the cell density and the exact process timing have a dominant impact on hPSC differentiation.

Cell production in suspension culture by the differentiation of matrix-free hPSC aggregates is more compatible with process upscaling. It facilitates transition to stirred tank bioreactors favored for process control and



optimization for conventional mammalian cell lines in the biotech industry. We and others demonstrated feasibility of suspension culture for both hPSC expansion (Abecasis et al., 2017; Kropp et al., 2016) and lineage differentiation, including successful CM, endothelial cell, and macrophage production (Ackermann et al., 2018; Chen et al., 2015; Fonoudi et al., 2015; Kempf et al., 2014; Olmer et al., 2018). However, whereas two-dimensional (2D) culture is restricted in their complexity, the number of process variables increases in 3D suspension culture. Besides the overall cell density, spherical aggregates (3D) increase in size over time (4D), thus constantly changing the physical and physiological parameters of the culture.

Multidimensional process parameters in combination with the known hPSC line-dependent properties often result into interexperimental variability. We have reported, for example, the average induction of ~80% CMs in stirred suspension, but noted process variability ranging from <60% to >90% CM content (Kempf et al., 2014).

We thus performed systematic modifications of process parameters in this study by applying several culture platforms and numerous hPSC lines. By focusing on the critical early steps particularly on hPSC aggregation and the timing of chemical WNT modulation, a more robust and efficient protocol was developed. This includes the systematic usage of chemically defined media compatible with large-scale cell production and transition to good manufacturing practice standards. Applying molecular cell analysis in response to process modifications a novel surface marker, ROR1, is revealed which, in combination with CD13, is superior for predictive monitoring of cardiac mesoderm formation.

RESULTS

WNT Pathway Inhibition Improves Priming toward Cardiac Mesoderm

After kick-starting cardiac differentiation by 24 h of CHIR supplementation, WNT pathway attenuation is typically initiated after a 48- to 72-h gap (Kempf et al., 2014; Lian et al., 2012). In contrast to CHIR treatment, temporal patterning of WNT inhibition was less systematically investigated, particularly in suspension culture. We thus compared the effect of early (days 1–3) with that of the commonly applied late (days 3–5) supplementation of WNT inhibitors (schematic in Figure 1A). Flow cytometry (FC)-based endpoint analysis for CM-specific markers including cardiac troponin T (cTNT), sarcomeric actinin (SA), and myosin heavy chain (MHC) on day 10 revealed only low CM induction of ~15%–18% (HES3) and <1.5%

(HSC-iPS2), respectively (Figures 1B and S1A), when the WNT antagonist IWP2 was added on day 3. In contrast, starting inhibition on day 1 (i.e., immediately after CHIR removal) resulted in >75% CM content for both cell lines and all markers tested (Figure 1B). This was confirmed by qRT-PCR (Figure S1D) and immune cytology-based analysis of suspension-derived, plated cells (Figure 1C).

Global gene expression analysis was conducted on day 0, day 1, and day 3 using HES3 (bright color code in Figure 1D) and HSC_iPS2 cells treated with (green dots) or without (gray dots) IWP2 on days 1–3 (Figure 1D). Principal component analysis showed expected clustering of day-0, -1, and -3 samples (Figures 1D and S1C). Notably, day-3 samples receiving no IWP2 (gray) appeared more distant to day 1 along the PC1 axis compared with IWP2-treated cells (green). This indicates that the (differentiation-induced) changes were less pronounced when IWP2 was supplemented, suggesting containment of differentiation progression by the inhibitor.

Interestingly, early WNT pathway inhibition appears to be essential to direct certain hPSC lines such as HSC_iPS2 toward the cardiomyogenic lineage, whereas the HES3 line seems to be less sensitive to external WNT inhibitors, in line with previous work (Kempf et al., 2014).

Gene ontology (GO) analysis of >10-fold regulated gene sets revealed that IWP2 treatment induced significant upregulation of genes related to the GO terms heart development and morphogenesis, while downregulated genes were associated with development and morphogenesis of the skeletal system, cartilage, bone, and cartilage, all resembling paraxial mesoderm derivatives (Figure S1B).

Patterning into cardiac versus presumptive presomitic mesoderm was confirmed by targeted heatmap analysis for associated GO terms, including osteoblast, skeletal muscle, mesenchyme, somite, and limb development. Remarkably, day-3 analysis of IWP-treated cells showed robust upregulation of key cardiac progenitor markers including *ISL1*, *GATA4*, *MEF2C*, *NXK2-6* (Biben et al., 1998), *FZD4* (Yoon et al., 2018), and *FOXC1/2*, as well as *TTN* and *TNNI1* (Figure 1E). Conversely, markers described to inhibit cardiac mesoderm but to promote presomitic specification including *CDX1/2* and *MSX1/2* (Greber et al., 2010; Mendjan et al., 2014) were significantly upregulated in the absence of early IWP2 treatment (confirmed for *CDX2* by FC; Figure S1E).

ROR1 Distinguishes Cardiac from Paraxial Mesoderm

Multiple surface markers were reported to identify cardiac mesoderm during hPSC differentiation. We evaluated expression of these markers in our 3D system regarding their ability to discriminate cardiac versus presomitic mesoderm on day 3 of differentiation on gene and protein levels. Most established markers showed only marginal or

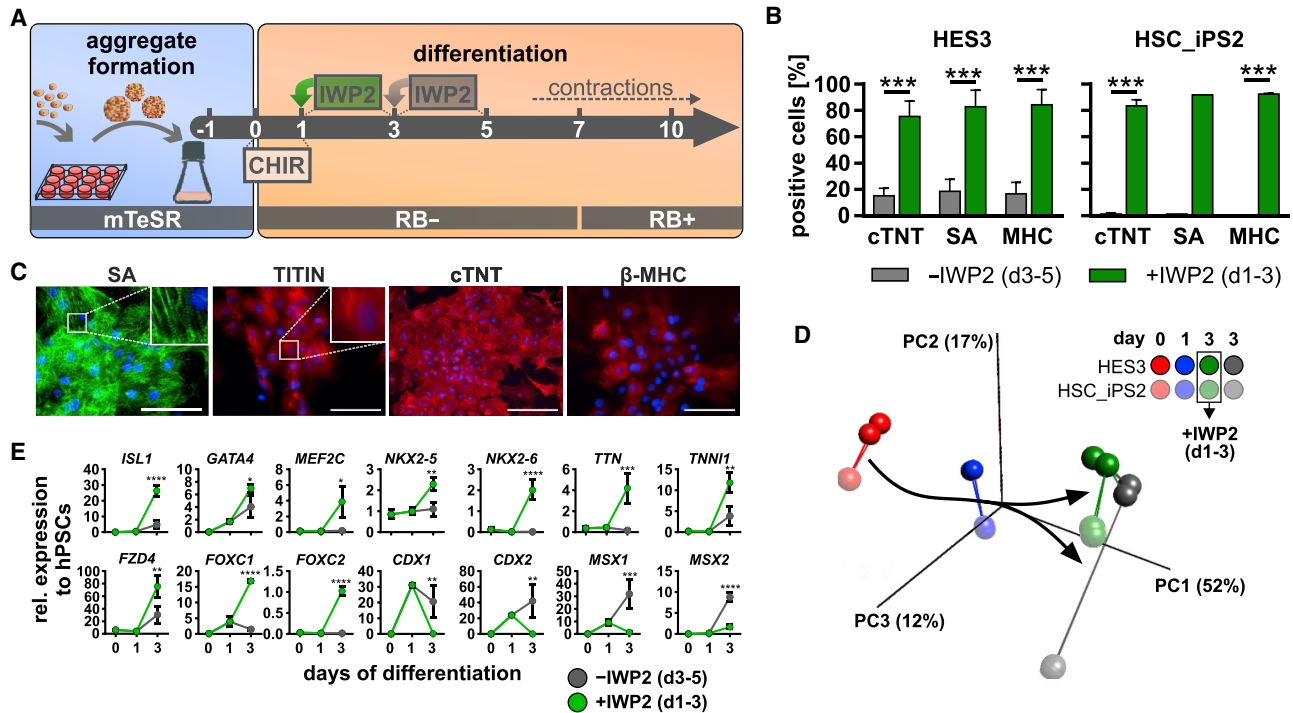


Figure 1. Timed WNT Pathway Inhibition Primes Mesendoderm toward Cardiac Mesoderm

(A) Differentiation schematic. Aggregates generated in 12-well plates were transferred to agitated Erlenmeyer flasks 1 day prior (−1) to differentiation induction by CHIR (0); WNT inhibition was performed on day 1 or day 3 by IWP2.

(B) CM content (%) measured by FC on day 10 following WNT inhibition on day 1 (■) and day 3 (■) for cardiac troponin T (cTNT), sarcomeric actinin (SA), and myosin heavy chain (MHC). 5 and 4 independent experiments were performed for hESCs and HSC_ips2 cells, respectively. $p < 0.001$.

(C) IF staining of plated cells (day 14 + 4) for structural cardiac markers SA, TITIN, cTNT, and β -MHC. Scale bars, 100 μ m.

(D) PCA ($\sigma/\sigma_{\max} = 0.184$) of microarray analysis shows clustering of samples over time and a distinct cell line-dependent pattern for IWP2 treatment. Two independent experiments were performed for each cell line.

(E) Time course of selected cardiac/presomitic mesoderm markers. $n = 4$ independent experiments based on microarray signal intensities. Error bars represent SEM. See also Figure S1.

low induction (*KDR*, *CXCR4*, and *GYP A*), while others were upregulated (*PDGFRA*, *NCAM*, *CD13*, and *ROR2*) but failed to discriminate between the two lineages (Figures 2A, 2B, S2A, and S2B). Interestingly, *ROR1*, in contrast to the structurally related protein *ROR2* (60% homology in protein sequence), depicted significantly higher levels in cardiac compared with presomitic mesoderm (Figures 2A, 2B, S2A, and S2C). Studies in mice reported *Ror2* expression throughout the entire PS (Matsuda et al., 2001) while recent data indicate distinct *Ror1* expression in the anterior-to-mid PS only (Peng et al., 2016) (Figure 2C) suggesting a distinct role of *Ror1* during mesendodermal patterning (note that T delineates the PS area of the epiblast of the gastrulating embryo; Figure 2C). Likewise, proteome data from human fetal tissues revealed prominent expression of *ROR1* but not other established surface markers in the developing heart (Kim et al., 2014) (Figure 2D). Indeed,

in contrast to the combination of $CD13^+/ROR2^+$ ($r = 0.73$), combining analysis of $CD13^+/ROR1^+$ by FC on day 3 and corresponding cardiomyocyte content on day 10 showed a more significant correlation ($r = 0.9$) (Figures 2E, 2F, and S2B), with $CD13^+$ serving as panmesoderm marker to exclude the potential presence of pluripotent cells that also express *ROR1* but lack *CD13* (Figure S2A).

Taken together, these data revealed *ROR1* as a marker for cardiac mesoderm which, in combination with *CD13*, enables early prediction of cardiac differentiation efficiency.

Cell-Line-Independent Cardiomyogenesis in Chemically Defined Media

Considering upscaling of CM production for clinical translation, the development of cost-efficient xeno-free processes is essential. Based on successful hPSC expansion

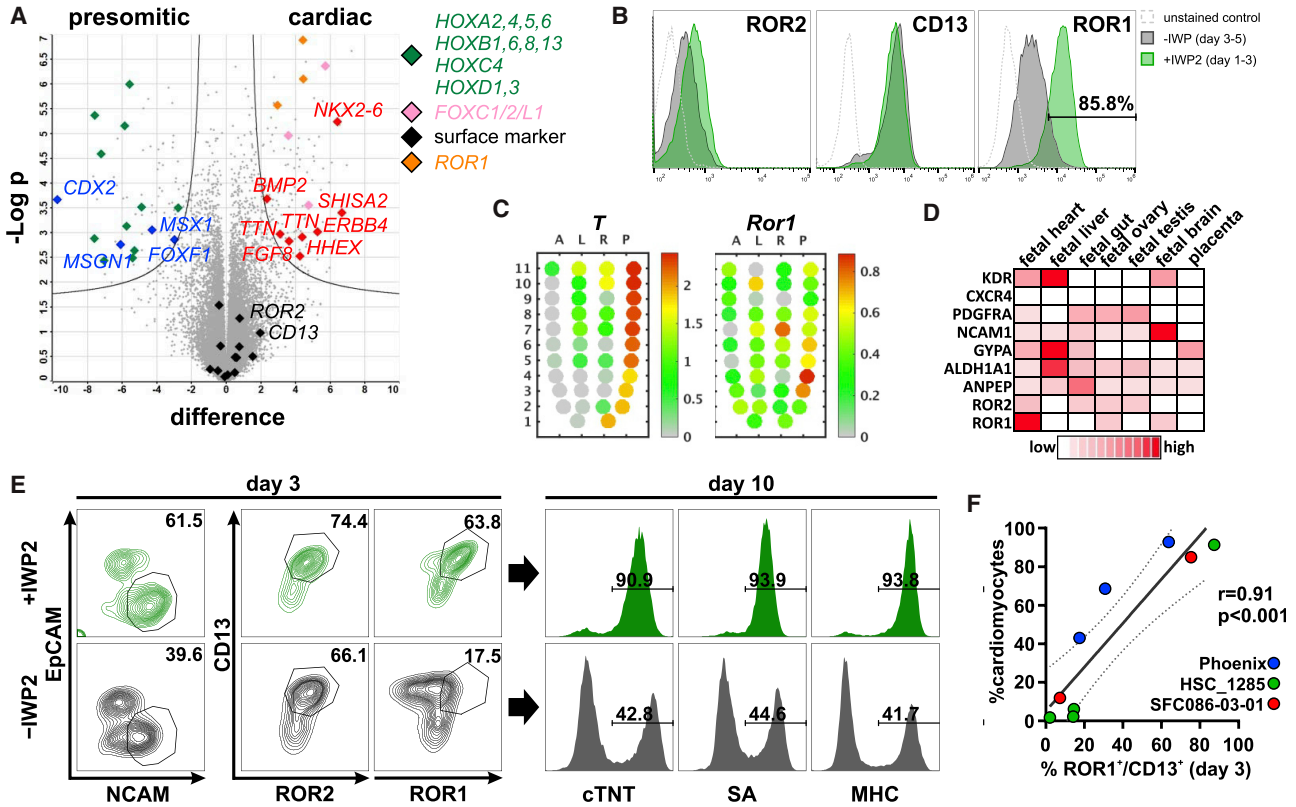


Figure 2. ROR1 Serves as Predictive Surrogate Marker for Cardiomyocyte Formation

(A) Volcano plot comparing cardiac and presomitic mesoderm on day 3 of differentiation. Key genes for each condition are highlighted in respective colors, either grouped in categories (■, ■, ■, ■) or individually (■, ■). Note the detection of *ROR1* (■) in the cardiac mesoderm by three independent probes and the minor difference for established surface markers (■), $n = 4$ independent experiments.

(B) Representative FC histograms for ROR2, CD13, and ROR1 on day 3 with (■) and without (■) IWP2 treatment on day 1.

(C) Gene expression in the gastrulating mouse embryo (Peng et al., 2016) indicates *Ror1* expression (right panel) in the anterior-to-mid PS region whereby T expression (left panel) increases toward the posterior PS.

(D) Pattern of mesodermal surface markers in human fetal tissues (Kim et al., 2014) indicates high *ROR1* levels in the heart.

(E) Representative FC on day 3 for NCAM/EpCAM, CD13/ROR2 and CD13/ROR1 with/without addition of IWP2 on day 1 and corresponding CM content (cTNT, SA, MHC) on day 10.

(F) Correlation of *ROR1*⁺/*CD13*⁺ on day 3 and respective CM content on day 10 based on three different cell lines; each dot represents an independent biological repeat. r denotes Pearson's correlation coefficient. Dotted lines denote 95% confidence interval.

See also Figure S2.

and maintenance of pluripotency in stirred suspension culture in chemically defined E8 (Chen et al., 2012; Kropp et al., 2016), it was applied to replace mTeSR used previously (Ludwig et al., 2006; Olmer et al., 2012; Silva et al., 2015) and in this work (Figures 1 and 2). For CM differentiation the chemically defined medium CDM3, recently established in 2D, was utilized (Burrige et al., 2014). Based on reports demonstrating the importance of the preculture strategy (Chen et al., 2015; Fonoudi et al., 2015; Kempf et al., 2014), we combined modified timing of aggregate generation in E8 with the improved WNT modulation described above. Aggregates were formed in E8 in stirred tank bioreactors for either 2 or 4 days before transition to

either CDM3 or RPMI/B27 (routinely used previously by Kempf et al., 2014; Lian et al., 2012), and for differentiation in rotated Erlenmeyer flasks (schematic in Figure 3A).

An increase in average aggregate diameter from $128.5 \pm 49.60 \mu\text{m}$ on day 2 to $203.2 \pm 79.3 \mu\text{m}$ on day 4 was observed (Figure 3B and picture inset in Figure 3A) while the cell density increased by ~ 4 -fold ($0.49 \pm 0.07 \times 10^6$ and $2.2 \pm 0.4 \times 10^6$ cells/mL, respectively; Figure 3C). Dissolved oxygen (DO) and pH (Figure 3D) values were substantially lower for day 4 than for day 2, demonstrating that, in addition to the mere change in aggregate diameters (suggested to impact on differentiation; Chen et al., 2015; Fonoudi et al., 2015), numerous other physical and

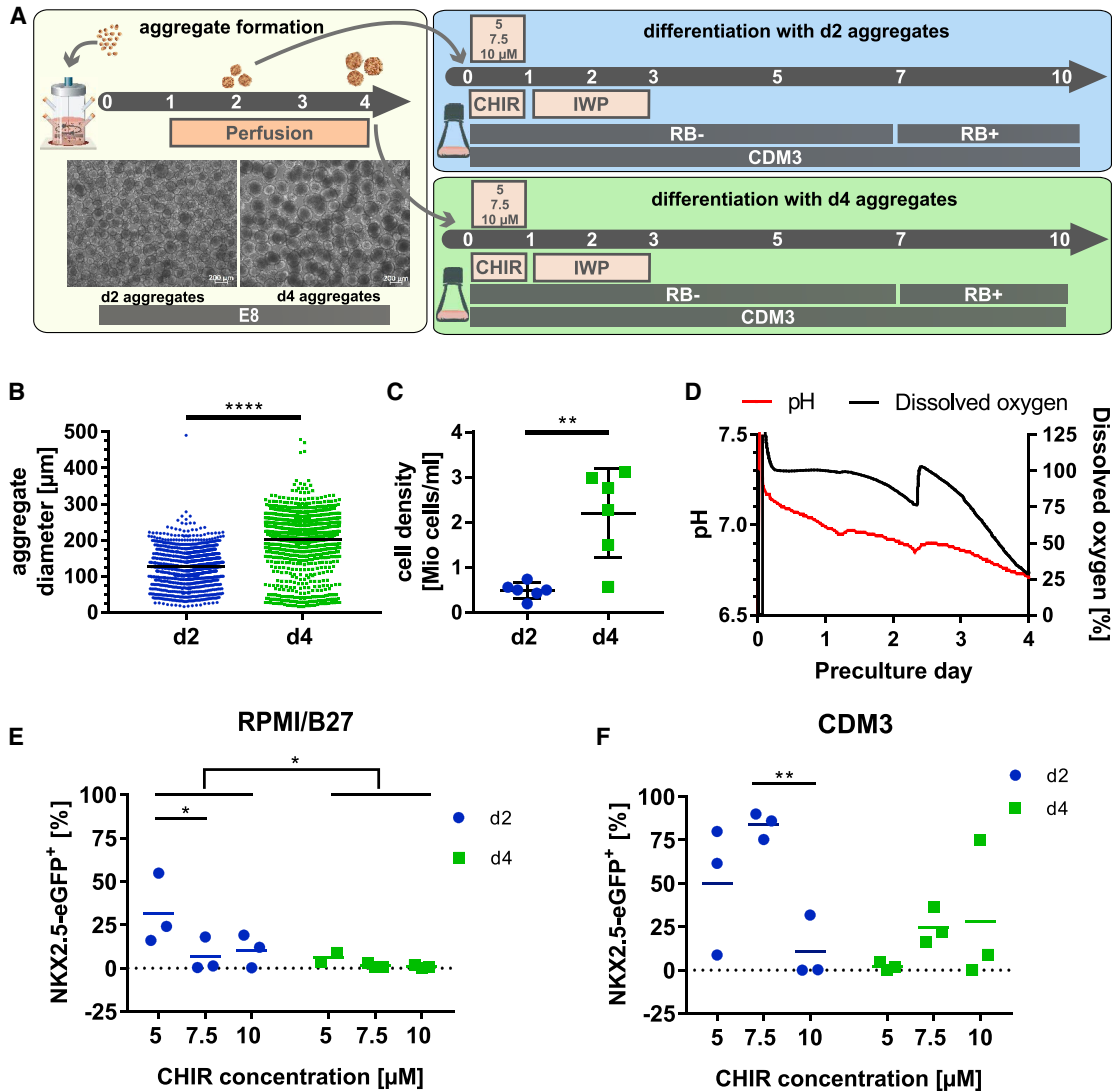


Figure 3. Day-2 Aggregates Derived in E8 Show Superior CM Formation in Chemically Defined Differentiation Medium

(A) Experimental scheme for optimizing expansion timing before differentiation. Microscopy of day 2/day 4 aggregates. (B) Diameter assessment: evaluating >100 aggregates on day 2/day 4 from three independent runs. $p < 0.0001$. (C) Cell density on day 2/day 4 of three independent runs in duplicates, respectively. $p = 0.0019$. (D) Representative pH/DO curves of preculture. (E and F) The impact of CHIR concentration and preculture duration. FC on day 10 revealed higher content of GFP⁺ CMs applying day-2 precultures in (E) RPMI/B27 ($n = 3$ independent experiments, preculture duration $p = 0.0262$ and 5 versus 7.5 μM CHIR $p = 0.0439$) or (F) CDM3 ($n = 3$ independent experiments, 7.5 versus 70 μM CHIR $p = 0.0095$). Error bars represent SEM. See also [Figure S3](#).

physiological parameters that may impact on differentiation vary as well.

Cells' sensitivity to WNT pathway stimulation may also depend on the applied media. The CHIR concentration previously optimized to 7.5 μM in mTeSR/RPMI/B27 was thus reinvestigated ([Kempf et al., 2014](#)). Twenty-four hours of CHIR treatment at 5 , 7.5 , or 10 μM , respectively was immediately replaced by IWP2 for 48 h (days 1–3), and

NKX2.5-eGFP expression was monitored as CM surrogate marker on day 10. For day-2 aggregates in RPMI/B27, $31.6\% \pm 11.8\%$ of NKX2.5-eGFP⁺ cells were observed at 5 μM CHIR and even lower values were found for 7.5 μM and 10 μM CHIR, respectively ([Figure 3E](#)). For day-4 aggregates, the amount of GFP-expressing cells was also highest at 5 μM CHIR but the overall values were lower compared with day-2 aggregates ([Figure 3E](#)). Differentiation in

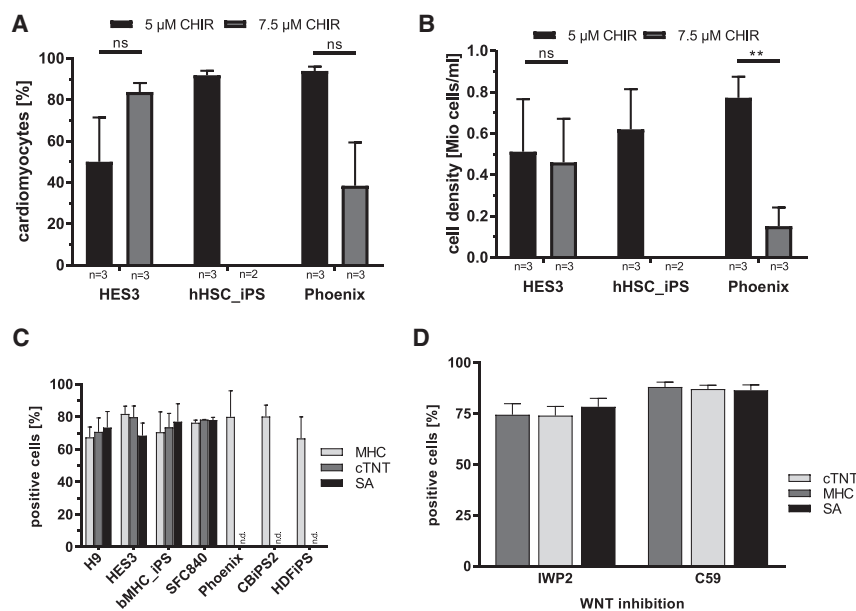


Figure 4. Robust Differentiation in Chemically Defined Conditions across Multiple hPSC Lines

(A and B) CM content (A) and respective cell numbers (B) on day 10 using bioreactor-derived aggregates from day-2 precultures of three cell lines differentiated in Erlenmeyer flasks in two or three independent experiments per cell line. (A) hESCs $p = 0.1972$, Phoenix cells $p = 0.0575$. (B) hESCs $p = 0.8835$, Phoenix cells $p = 0.0067$.

(C) Robustness of the chemically defined differentiation protocol was confirmed by FC for three markers with >70% CM found for seven cell lines tested in agitated 6-well plates in 2–5 independent experiments: hES3, bMHC_iPS, SFC840: $n = 2$; H9, CBiPS2, HDFiPS: $n = 3$; Phoenix: $n = 5$.

(D) Comparison of IWP2 versus Wnt-C59 added after 24 h of 5 μM CHIR treatment in three independent experiments revealed similar efficiencies in 6-well plates on day 10 for Phoenix. Error bars represent SEM.

CDM3 substantially improved NKX2.5-eGFP expression and was most robust combining E8-derived day-2 aggregates with 7.5 μM CHIR ($83.8\% \pm 4.4\%$; Figure 3F). Day-2 aggregates outperformed day-4 cells over the entire CHIR range tested (Figure 3F).

This chemically defined protocol (equivalent to schematic in Figure 3A) was tested with several lines (HES3-NKX2.5, HSC_iPS2, and Phoenix) with day-2 aggregates following differentiation in flasks at 5 μM and 7.5 μM CHIR.

In contrast to HES3-NKX2.5, 5 μM CHIR was optimal for HSC_iPS2 and Phoenix, inducing 91% and 93.7% \pm 3.6% CMs, respectively (Figure 4A). CHIR at 7.5 μM also resulted in substantially lower cell yield during subsequent differentiation (Figures 4B, S3A, and S3B), eventually preventing endpoint analysis of HSC_iPS2 progenies. On the other hand, at 5 μM CHIR treatment, robust CM yields were observed for all cell lines tested (Figure 4B) whereby HES3-NKX2.5 were, again, less sensitive to the applied CHIR dose. Taken together, these data revealed that 5 μM CHIR is most appropriate for our chemically defined cardiac differentiation protocol.

Additional human embryonic stem cell (hESC) and human iPSC (hiPSC) lines were assessed in a broadly available 6-well low-attachment platform revealing >70% CM induction across the seven cell lines tested (Figure 4C), supporting the general cell-line- and culture platform-independent utility of our protocol. Moreover, comparing the WNT pathway inhibitors IWP2 with Wnt-C59 using Phoenix showed comparable differentiation efficiencies (Figure 4D).

Process Upscaling in Stirred Tank Bioreactors Enables High Cell Yield at >90% CM Purity and Confirms Utility of CD13⁺/ROR1⁺ for Early Process Monitoring

Next, process upscaling in stirred tank bioreactors was performed, firstly in 150-mL (DASbox) and subsequently in up to 500-mL culture scale in a larger system (Bioblock). Applying the protocol in Figure 5A the cell density was adjusted to 0.5×10^6 cells/mL before induction of differentiation to consider the impact of the bulk cell density and interprocess reproducibility (Kempf et al., 2016).

Growth kinetics revealed that the cell density peaked at $>1.5 \times 10^6$ cells/mL on days 3–5 but declined thereafter, resulting in $1 \pm 0.1 \times 10^6$ total cells/mL on day 10 corresponding to $0.93 \pm 0.1 \times 10^6$ CMs/mL (Figures 5B and S4B), thus yielding $\sim 130 \times 10^6$ CMs per run in 150-mL scale.

On day 10, FC revealed a CM content of $93.5\% \pm 5.0\%$, $94.13\% \pm 2.8\%$, and $92.9\% \pm 3.64\%$ for cTNT, MHC, and SA, respectively (Figures 5C and S4A), demonstrating high process efficiency and reproducibility.

This endpoint analysis for CM markers was correlated to CD13⁺/ROR1⁺ on day 3 for a large number of experiments across the entire optimization phase, including preliminary conditions leading to low CM contents (Figure 5D). The observed correlation coefficient of $r = 0.63$ may not seem ideal but the following must be considered: (1) the biological variance inherent to such complex biological systems, in particular when correlating two time points being 7 days apart; and (2) the transient nature of the CD13⁺/ROR1⁺ expression whereby slight variations in

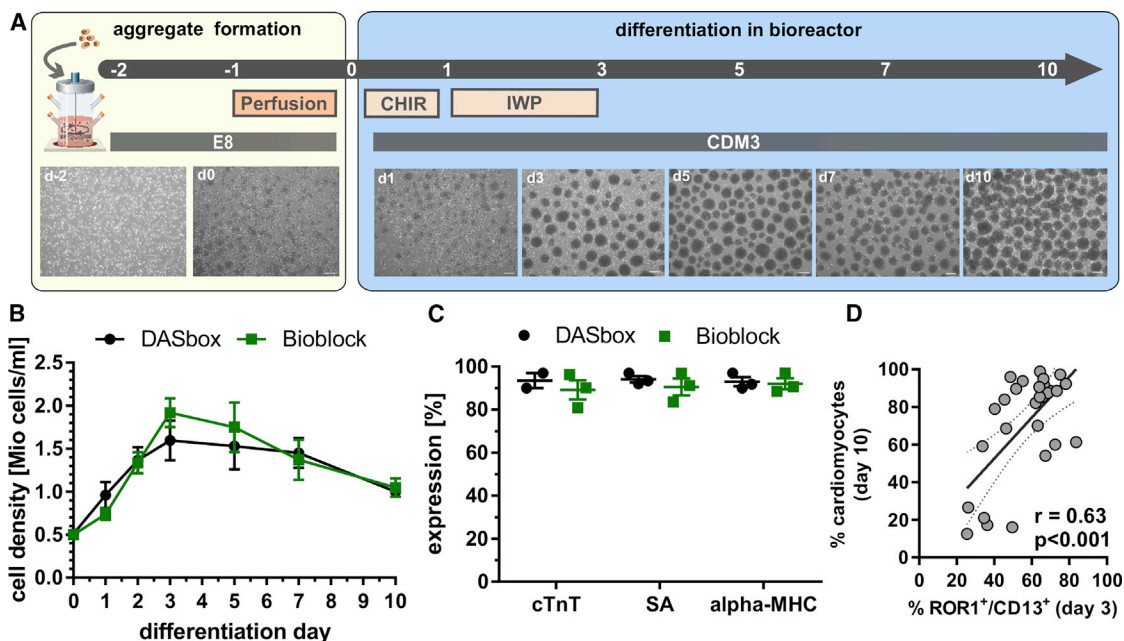


Figure 5. Process Upscaling in Stirred Tank Bioreactors Confirms Utility of CD13⁺/ROR1⁺ for Early Process Monitoring and Enables >90% CM Induction

(A) Scheme for differentiation in bioreactors and microscopic assessment of aggregates over time. Scale bars, 200 μm .

(B) Growth kinetics in DASbox ($n = 3$ independent experiments) and the Bioblock system ($n = 3$ independent experiments). Error bars represent SEM.

(C) Cardiac differentiation efficiency assessed by FC on day 10 for cTnT, SA, and MHC revealed >90% purity in DASbox and \sim 90% in Bioblock ($n = 3$ independent experiments). Error bars represent SEM.

(D) Correlation of ROR1⁺/CD13⁺ on day 3 and respective CM content on day 10 of Phoenix in specific differentiation conditions resulting from upscale experiments. r denotes Pearson's correlation coefficient ($n = 28$ in seven independent experiments). Dotted line denotes 95% confidence interval.

See also [Figure S4](#).

process timing and analysis may substantially impact on this correlation. With this in mind, the plot in [Figure 5D](#) highlights that the presence of low, i.e., \sim 20%–40% of CD13⁺/ROR1⁺ cells, on day 3 is a strong hint for low CM content, typically <30% on day 10. In contrast, measurement of \sim 60%–80% of CD13⁺/ROR1⁺ cells on day 3 typically resulted in 80%–98% CMs. As exemplarily depicted by inset pictures in the schematic ([Figure 5A](#)), the average cell aggregate diameter changed along bioreactor-based processes, rising from $112.8 \pm 34.5 \mu\text{m}$ (day 0) to $214.5 \pm 66.8 \mu\text{m}$ (day 10; [Figure S4D](#)). Moreover, online monitoring of pH and DO revealed typical “zigzag” curves between individual medium changes ([Figure S4C](#)). After day 3, the pH drop pattern was flatter in line with both decreasing cell numbers and the expected switch from a highly glycolytic metabolism established for the pluripotent state ([Cliff et al., 2017; Moussaieff et al., 2015](#)) toward more oxidative phosphorylation (and thus reduced lactate production) in differentiating cells. This was confirmed by glucose/lactate assessment ([Figure S4E](#)) for specific yield coefficient (Y)

calculation, which was highest during the first days of differentiation and dropped strongly between days 2 and 5 ([Figure S4F](#)).

We next performed a proof of concept for up to 3-fold process upscaling, i.e., from 150- toward 500-mL scale in a larger stirred tank reactor system (Bioblock) revealing high similarity for key parameters including growth kinetics, cell yields, and CM content, whereas a somewhat smaller mean aggregate diameter was observed at a larger scale ([Figure 5F](#)). The green bars in [Figure 5C](#) show that $89.13\% \pm 7.8\%$, $90.53\% \pm 6.7\%$, and $92.03\% \pm 4.4\%$ of cTnT-, SA-, and MHC-expressing CMs, respectively, were observed on day 10. The cell density was $1.05 \pm 0.19 \times 10^6$ cells/mL (green line in [Figure 5B](#)). Together these data demonstrate platform- and process-scale-independent robustness.

Qualitative Analysis Reveals High Purity of Functional Ventricular Cardiomyocytes

Assessing CMs' electrophysiological properties revealed 70 out of 73 action potentials (APs) with long plateau phase

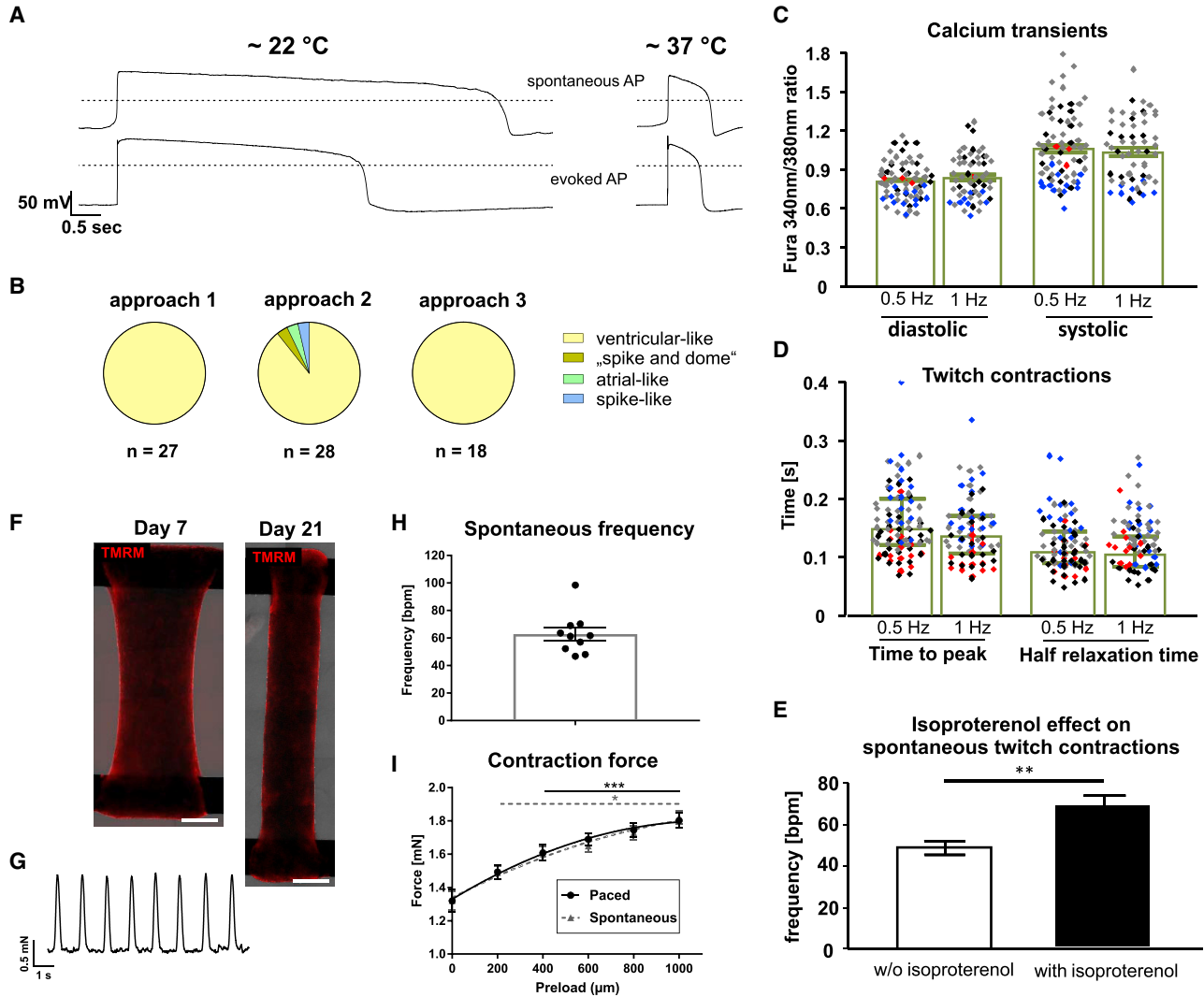


Figure 6. Characterization of CMs Generated by Chemically Defined Conditions

(A and B) Electrophysiology: (A) representative APs indicating ventricular-like CMs recorded at room temperature ($\sim 22^\circ\text{C}$) or $\sim 37^\circ\text{C}$. Spontaneous APs (upper) compared with evoked APs (lower) of the same cells, elicited by short stimuli from resting potentials of -80 mV ; dotted lines: 0 mV . CM phenotype distribution: (B) in three independent differentiations of Phoenix, 3 out of 73 measured cells deviated from the ventricular-like AP pattern.

(C–E) Single CM analysis: (C) systolic/diastolic calcium ratio at 0.5-/1-Hz stimulation; colors (■, ■, ■, ■) indicate four independent experiments ($n = 57\text{--}71$). (D) Time to peak of twitch contractions and half relaxation time at 0.5/1 Hz; four independent experiments, $n = 74\text{--}93$. Error bars denote median \pm IQR. (E) Isoproterenol-induced upregulation of beating frequency ($n = 5$ in one experiment).

(F–I) BCT analysis from two independent CM batches. (F) Tetramethylrhodamine methyl ester (TMRM) stain illustrates homogeneous CM distribution on day 7/day 21 of tissue formation. Scale bars, 1 mm . (G–I) Mechanical and electrophysiological properties of BCTs measured on day 21 ($n = 10$). (G) Example of spontaneous contraction pattern and (H) statistical analysis. (I) Force of spontaneous and paced contractions recorded over increasing preload.

Error bars represent SEM. See also Figures S5 and S6.

and steep phase-3 repolarization (physiological APs found at 37°C ; Figures 6A, 6B, S5A, and S5B), reflecting a ventricular-like phenotype with only three deviating APs noted in three independent differentiations (Figures 6A, 6B, S5A, and S5C–S5E).

For intracellular calcium transients and unloaded twitch contraction recordings (Figures 6C and 6D), plated CMs were electrically stimulated (Mutig et al., 2013). The averaged diastolic calcium ratio at 0.5 Hz (Figure 6C) was 0.8 ± 0.15 and thus higher than 0.6 ± 0.1 previously found



in hESC-CMs (Mutig et al., 2013; Weber et al., 2016), indicating a possibly lower activity of sarcoplasmic/endoplasmic calcium reticulum ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger in hiPSC-CMs in this study. This may be due to the shorter maturation time in culture (i.e., days 14–19 here versus day 35 in Weber et al., 2016). At both electrical stimulation frequencies, systolic calcium ratios (Figure 6C) were higher compared with (Weber et al., 2016) but the amplitude of calcium release (calculated as systolic peak/diastolic ratio) at 0.5 Hz was only slightly elevated (0.26 ± 0.18 versus 0.25 ± 0.1 in Weber et al., 2016). Time to peak and half relaxation times (Figure 6D) of twitch contractions were similar to historical data (Weber et al., 2016).

Notably, only 22% of all measured CMs were spontaneously contracting in the pacing-free interval of the acquisition protocol (Figure S6F). This is $\sim 16\%$ less than previously noted in hESC-CMs using the same analysis (Weber et al., 2016), indicating that (without applying explicit maturation) we here derived CMs exhibiting a less contractile, more ventricular-like phenotype, a property of working myocardium.

Moreover, prolonged cultivation of CM aggregates in 3D resulted in progressively increasing expressions of ventricular myosin light chain 2 (MLC2, Figures S6I and S6J) as indicated for prolonged cultivation of seeded CMs in 2D (Burridge et al., 2014; Lian et al., 2012).

Upon electrically evoked and spontaneous twitch contractions (Figures 6E and S6C–S6E), isoproterenol slightly increased the contraction amplitude and marked shortening of time to peak and half relaxation times (expected positive inotropic and lusitropic effects, Figures S6D and S6E), and a significant increase of the beating frequency of spontaneously contracting CMs from 48.8 ± 3.26 to 68.4 ± 4.4 beats/min (expected positive chronotropic adrenergic effects; Figures 6E and S6C). Moreover, endothelin-1 treatment for 24 h induced a typical increase in cell surface area of plated CMs (Carlson et al., 2013; Figures S6A and S6B).

Finally, our resulting CMs were used for bioartificial cardiac tissue (BCT) generation (Kempf et al., 2014; Kensah et al., 2013). After 21 days of cultivation, tissues displayed homogeneous distribution of aligned CMs and synchronized spontaneous beating at 62.8 ± 4.7 beats/min and contraction-relaxation times ($t_{C80\%}$) of 146.1 ± 6.0 and 181.8 ± 8.1 ms, respectively (Figures 6F–6I, S6G, and S6H). In response to increased preload, both spontaneous and electrically paced forces increased significantly to 1.82 ± 0.04 and 1.80 ± 0.04 mN, respectively (Figure 6I). We also observed frequency-dependent acceleration of relaxation: compared with the initial relaxation time at 1 Hz, $t_{R80\%}$ was reduced by 28% or 51% at 2 Hz or 3 Hz, respectively (Figures S6G and S6H).

Together, this highlights the typical physiological and mechanical properties of our hPSC-CMs indicating pronounced ventricular-like features.

DISCUSSION

Directed cardiac differentiation has been continuously improved over the past decade, progressing toward simpler, more robust, and scalable conditions (Kempf et al., 2014; Lian et al., 2012; Yang et al., 2008). In this study we introduce an integrated, chemically defined differentiation platform enabling the production of human cardiomyocytes in 3D aggregate culture suitable for upscaling in controlled stirred tank bioreactors.

Previous studies demonstrated a substantial impact of preculture conditions (Kempf et al., 2014) at the pluripotent hPSC aggregate stage (Kempf et al., 2014) and the early differentiation phase (Faial et al., 2015; Kempf et al., 2016; Mendjan et al., 2014) on cardiomyogenesis. Culture conditions were revealed that do support an accelerated differentiation progression including efficient PS formation and mesoderm induction, but ultimately failed to direct efficient cardiomyogenesis (Kempf et al., 2014, 2016; Laco et al., 2018; Mendjan et al., 2014).

We hypothesized here that, despite thoroughly directing hPSC priming toward precardiac mesoderm (i.e., by controlling cell aggregation and preculture timing, cell density adaptation ahead of differentiation, exact CHIR dosing, and incubation timing), the “48-h gap” of external WNT pathway control (which is inherent to the previous differentiation protocol, i.e., between CHIR deprivation after 24 h and before IWP addition at 72 h) is prone to perturbing lineage identity. Such perturbation is likely promoted by the complex paracrine environment and multiple signaling pathway activity in hPSCs downstream of CHIR addition (Gaspari et al., 2018; Kempf et al., 2016) and may lead to both a more broad induction of PS-like cell identities and less controlled progression to (posterior) mesoderm fates, i.e., paraxial mesoderm progenies.

We thus considered recent findings on mesoderm specification (Loh et al., 2016) to more stringently control hPSC priming to a defined transition point of lateral mesoderm specification (by meticulously defined preculture and CHIR treatment). This is followed by efficient transition of the entire progenitor population toward cardiac mesoderm (rather than undesired branching toward, e.g., limb bud mesoderm; Loh et al., 2016) by immediate chemical control of WNT pathway inhibition. The success of this immediate CHIR-to-IWP transition strategy is reflected in early gene expression data revealing suppression of CDX2 and MSX1, established inhibitors



of cardiac mesoderm formation (Mendjan et al., 2014; Rao et al., 2016).

Opposing the gene expression patterns specific to cardiac mesoderm (resulting from immediate IWP addition on day 1) versus presomitic mesoderm (achieved by the lack of IWP before day 3) allowed us to evaluate several known cardiac/mesodermal progenitor markers including KDR, PDGFR α , SSEA1, NCAM, ROR2, and CD13 (Ardehali et al., 2013; Evseenko et al., 2010; Kattman et al., 2011; Lee et al., 2017; Nelson et al., 2008; Yang et al., 2008; Yoon et al., 2018) regarding their suitability for early process monitoring and eventually predicting functional CM formation at later stages. Interestingly, none of the established markers was well suited to discriminate early cardiac from presomitic mesoderm. Instead, we identified ROR1 in combination with CD13 (to exclude presence of ROR1-expressing undifferentiated cells; see Figure S2A) as being more informative in distinguishing between cardiac and presomitic mesoderm. This marker combination on day 3 was indeed more prognostic for indicating CM content on day 10, as demonstrated for several hPSC lines and culture platforms (Figures 2F and 5D).

ROR1 and ROR2 are part of a WNT5A-ROR-DVL pathway in limb development, where both receptors are partially redundant (Ho et al., 2012). However, in line with our data, distinct ROR1 expression was reported in the presumptive cardiac PS region in birds and mammals (Alev et al., 2010; Kinder et al., 1999; Peng et al., 2016) and in embryonic heart tissue as well (Kim et al., 2014). This distinct pattern may hint toward a broader functional role of ROR1 during early heart development, potentially involving non-canonical WNT signaling via FZD4 during lateral plate mesoderm formation (Grumolato et al., 2010; Yoon et al., 2018). In summary, we show that ROR1 may be exploited to identify primed hPSC states and predict lineage commitment, complementing recent findings on cKIT, FZD4, and GARP (Bargaje et al., 2017; Loh et al., 2016; Yoon et al., 2018).

A number of recently published studies progress toward the mass production of hPSC-CMs in stirred suspension culture (Chen et al., 2015; Fonoudi et al., 2015). In notable accordance, we confirm the role of preculture duration; 2 days of aggregation in E8 was optimally equivalent in our setup to Chen et al., whereas Fonoudi et al. favored prolonged cultivation for 5 days (Chen et al., 2015; Fonoudi et al., 2015). However, both studies suggested that the time-dependent increase in aggregate diameter, rather than preculture duration itself, is a predominant factor. Our previous studies also indicate that the hPSC aggregate diameter and, in particular, the process-dependent pattern of aggregate formation and growth, is important (Kempf et al., 2014, 2015). However, whereas Chen et al. and Fonoudi et al. in unison support a median hPSC aggregate

diameter of ~ 175 – 220 μm as being optimal, we here found a median diameter of ~ 125 μm (preculture day 2) being advantageous for cardiomyogenesis as compared with ~ 200 μm (on day 4 or preculture; Figures 3B–3F). This comparison of different studies highlights that probably none of these process parameters (such as preculture duration, aggregate diameter, and inoculation cell density) represents an absolute requirement or a universal process-independent parameter for controlling directed lineage differentiation. These are rather correlative observations, which have to be interpreted within the context of the respective process. Interestingly, the group of Oh recently investigated the proliferative status of hPSCs regarding their impact on the cardiac differentiation efficiency (Laco et al., 2018). Results suggest that monitoring defined molecular markers of cell proliferation may indeed be a more universal, culture platform-independent approach to preassess hPSCs' compatibility with cardiac differentiation, although additional validation is required.

Regarding process yields, Chen et al. (2015) published ~ 0.2 million CMs/mL whereas Fonoudi et al. (2015) reported ~ 0.8 million CMs/mL in their approach (Chen et al., 2015; Fonoudi et al., 2015). We here show the induction of ~ 1 million CMs/mL in process dimensions from 150 to 500 mL across two bioreactor platforms at increasing dimension, ultimately resulting in the production of up to $\sim 450 \times 10^6$ CMs in a single process run. Using a non-directed differentiation approach followed by antibiotic selection, Correia et al. (2014) reported the production of 2.3×10^6 CMs/mL in WAVE bioreactors compared with 0.64×10^6 in stirred tank bioreactors (Correia et al., 2014). In another concept applying microcarriers, Ting et al. (2014) generated up to 1.6×10^6 CMs/mL. A meaningful cross-comparison of these studies is problematic, given the great heterogeneity of bioreactor platforms and the overall experimental environment. Importantly, our protocol described here is one of the first demonstrating chemically defined conditions in scalable suspension culture inducing a high CM content of $>90\%$.

Notably, functional characterization by numerous complementary methods revealed a predominantly ventricular-like CMs phenotype, which may support *in vitro* disease modeling and pharmacological assays as well as envisioned regenerative therapies of heart repair (Liu et al., 2018).

EXPERIMENTAL PROCEDURES

For standard methods and prepublished techniques, please refer to Supplemental Experimental Procedures.

Cell Culture

The cell lines HES3 (HES3-MIXL1^{wt/eGFP}; Davis et al., 2008, HES3-NKX2.5^{wt/eGFP}; Elliott et al., 2011), H9, HSC_iPS2



(hHSC_F1285_T-iPS2; MHHi006-A; Hartung et al., 2013), Phoenix (HSC_ADCF_SeV-iPS2; MHHi001-A; Haase et al., 2017; Kempf et al., 2016), CBiPS2 (hCBiPS2; Haase et al., 2009), bMHC_iPS (hF_HCMR723G_4F_iPS_Klon2_MOI1), SFC840 (SFC840-03-01, STBCi026-B; Christofferson et al., 2018; derived by the StemBANCC initiative <http://stembancc.org/>), HDFiPS (HDF-iPSC; Wattanapanitch et al., 2014), and CDX2_iPS (CDX2^{Venus} knockin reporter, MHHi007-A-1; Malysheva et al., 2018) were maintained in mTeSR (STEMCELL Technologies) or E8 medium as described previously (Kempf et al., 2015). In brief, cells were passaged every 3.5 days using Accutase (Life Technologies) and reseeded at $0.4\text{--}0.5 \times 10^4$ cells/mL on Geltrex-coated (Life Technologies) flasks in mTeSR1 (STEMCELL Technologies) or E8 medium including $10 \mu\text{M}$ Y-27632 (LU Hannover; Palecek et al., 2011). Cells were maintained at 37°C and 5% CO_2 and were regularly tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza).

Differentiation was conducted as illustrated in Figure 1A and as previously described (Kempf et al., 2014). At chemically defined conditions, cells were cultivated in E8 and differentiated in CDM3 (Burridge et al., 2014; for details see Supplemental Experimental Procedures).

Bioreactor Differentiation in Chemically Defined Conditions

Chemically defined differentiation in Erlenmeyer flasks (20 mL working volume) and in stirred tank bioreactors (working volume: 150 mL/DASbox Mini Bioreactor System or 350–500 mL/Bioblock DASGIP Parallel Bioreactor System) was performed as depicted in Figures 3A and 5A, respectively (Eppendorf). After established inoculation/preculture (Kropp et al., 2016) cell density was determined after 48 h and adjusted to 5×10^5 cells/mL for differentiation in CDM3 (Burridge et al., 2014) supplemented with $5 \mu\text{M}$ CHIR and $5 \mu\text{M}$ Y-27632. Precisely 24 h later the medium was replaced by CDM3 plus $5 \mu\text{M}$ IWP2, and pure CDM3 was added 48 h later and every 2–3 days thereafter. Aggregate diameter, cell density, and metabolites were analyzed as previously published (Kropp et al., 2016).

Statistics

Data are presented as mean \pm standard error of the mean. Unless otherwise noted, statistical significance was calculated using Student's *t* tests. To take account of multiplicity in group comparisons, one- or two-way ANOVA followed by Bonferroni's post test were conducted. Statistical significance is denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and ns (not significant) in the figures.

ACCESSION NUMBERS

The microarray data were deposited in the ArrayExpress database under accession number ArrayExpress: E-MTAB-6828.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.06.004>.

AUTHOR CONTRIBUTIONS

C.H., H.K., K.S., and R.Z. designed the experiments. C.H., A.F., W.L., M.S., N.W., F.O., J.d.I.R., F.P., S.B., S.W., S.M., K.S., and H.K. performed the experiments. C.H., K.S., M.S., N.W., J.d.I.R., I.G., M.F., H.K., and R.Z. analyzed and interpreted the data. T.K., I.G., M.F., K.W., U.M., and R.Z. provided conceptual advice and financial support. C.H., H.K., N.W., M.F., I.G., and R.Z. wrote the article.

ACKNOWLEDGMENTS

We would like to thank A. Kirschning and G. Dräger for providing Y-27632 and CHIR99021, A. Haase for providing various iPSC lines, S. Malysheva for providing the cell line CDX2_iPS, E. Stanley and A. Elefanty for providing the HES3-NKX2.5 and HES3-MIXL1 cell lines, A. Francino and F. Navarro-Lopez (Hospital Clinic, University of Barcelona, Spain) for providing skin fibroblasts from a patient with hypertrophic cardiomyopathy to generate the cell line bMHC_iPS, and T. Holler and A. Lingk for supporting and performing immunofluorescence. We also thank the RCU Transcriptomics at Hannover Medical School for performing the microarray experiments. R.Z. received funding from the German Research Foundation (DFG; Cluster of Excellence REBIRTH EXC 62/2, ZW64/4-1 as well as KFO311 and ZW64/7-1), German Ministry for Education and Science (BMBF, grants: 13N14086, 01EK1601A, 01EK1602A), StemBANCC (support from the Innovative Medicines Initiative joint undertaking under grant agreement no. 115439-2), resources of which are composed of financial contributions from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' kind contribution, and the European Union H2020 program to the project TECHNOBEAT (grant 66724). H.K. received funding from Joachim Herz Stiftung and MHH Hannover (HiLF grant). T.K. received funding from the German Research Foundation (DFG TK1187/21-1).

Received: December 20, 2018

Revised: June 21, 2019

Accepted: June 24, 2019

Published: July 25, 2019

REFERENCES

- Abecasis, B., Aguiar, T., Arnault, E., Costa, R., Gomes-Alves, P., Aspegren, A., Serra, M., and Alves, P.M. (2017). Expansion of 3D human induced pluripotent stem cell aggregates in bioreactors: bioprocess intensification and scaling-up approaches. *J. Biotechnol.* *246*, 81–93.
- Ackermann, M., Kempf, H., Hetzel, M., Hesse, C., Hashtchin, A.R., Brinkert, K., Schott, J.W., Haake, K., Kuhnel, M.P., Glage, S., et al. (2018). Bioreactor-based mass production of human iPSC-derived macrophages enables immunotherapies against bacterial airway infections. *Nat. Commun.* *9*, 5088.
- Alev, C., Wu, Y., Kasukawa, T., Jakt, L.M., Ueda, H.R., and Sheng, G. (2010). Transcriptomic landscape of the primitive streak. *Development* *137*, 2863–2874.
- Ardehali, R., Ali, S.R., Inlay, M.A., Abilez, O.J., Chen, M.Q., Blauwkamp, T.A., Yazawa, M., Gong, Y., Nusse, R., Drukker, M., et al. (2013). Prospective isolation of human embryonic stem cell-derived



- cardiovascular progenitors that integrate into human fetal heart tissue. *Proc. Natl. Acad. Sci. U S A* *110*, 3405–3410.
- Bargaje, R., Trachana, K., Shelton, M.N., McGinnis, C.S., Zhou, J.X., Chadick, C., Cook, S., Cavanaugh, C., Huang, S., and Hood, L. (2017). Cell population structure prior to bifurcation predicts efficiency of directed differentiation in human induced pluripotent cells. *Proc. Natl. Acad. Sci. U S A* *114*, 2271–2276.
- Biben, C., Hatzistavrou, T., and Harvey, R.P. (1998). Expression of NK-2 class homeobox gene *Nkx2-6* in foregut endoderm and heart. *Mech. Dev.* *73*, 125–127.
- Burridge, P.W., Matsa, E., Shukla, P., Lin, Z.C., Churko, J.M., Ebert, A.D., Lan, F., Diecke, S., Huber, B., Mordwinkin, N.M., et al. (2014). Chemically defined generation of human cardiomyocytes. *Nat. Methods* *11*, 855–860.
- Carlson, C., Koonce, C., Aoyama, N., Einhorn, S., Fiene, S., Thompson, A., Swanson, B., Anson, B., and Kattman, S. (2013). Phenotypic screening with human iPS cell-derived cardiomyocytes: HTS-compatible assays for interrogating cardiac hypertrophy. *J. Biomol. Screen.* *18*, 1203–1211.
- Chen, V.C., Couture, S.M., Ye, J., Lin, Z., Hua, G., Huang, H.I., Wu, J., Hsu, D., Carpenter, M.K., and Couture, L.A. (2012). Scalable GMP compliant suspension culture system for human ES cells. *Stem Cell Res.* *8*, 388–402.
- Chen, V.C., Ye, J., Shukla, P., Hua, G., Chen, D., Lin, Z., Liu, J.C., Chai, J., Gold, J., Wu, J., et al. (2015). Development of a scalable suspension culture for cardiac differentiation from human pluripotent stem cells. *Stem Cell Res.* *15*, 365–375.
- Christoffersson, J., Meier, F., Kempf, H., Schwanke, K., Coffee, M., Beilmann, M., Zweigerdt, R., and Mandenius, C.F. (2018). A cardiac cell outgrowth assay for evaluating drug compounds using a cardiac spheroid-on-a-chip device. *Bioengineering (Basel)* *5*. <https://doi.org/10.3390/bioengineering5020036>.
- Cliff, T.S., Wu, T., Boward, B.R., Yin, A., Yin, H., Glushka, J.N., Prestegard, J.H., and Dalton, S. (2017). MYC controls human pluripotent stem cell fate decisions through regulation of metabolic flux. *Cell Stem Cell* *21*, 502–516.e9.
- Correia, C., Serra, M., Espinha, N., Sousa, M., Brito, C., Burkert, K., Zheng, Y., Hescheler, J., Carrondo, M.J., Saric, T., et al. (2014). Combining hypoxia and bioreactor hydrodynamics boosts induced pluripotent stem cell differentiation towards cardiomyocytes. *Stem Cell Rev.* *10*, 786–801.
- Davis, R.P., Ng, E.S., Costa, M., Mossman, A.K., Sourris, K., Elefanty, A.G., and Stanley, E.G. (2008). Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. *Blood* *111*, 1876–1884.
- Devalla, H.D., Schwach, V., Ford, J.W., Milnes, J.T., El-Haou, S., Jackson, C., Gkatzis, K., Elliott, D.A., Chuva de Sousa Lopes, S.M., Mummery, C.L., et al. (2015). Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. *EMBO Mol. Med.* *7*, 394–410.
- Elliott, D.A., Braam, S.R., Koutsis, K., Ng, E.S., Jenny, R., Lagerqvist, E.L., Biben, C., Hatzistavrou, T., Hirst, C.E., Yu, Q.C., et al. (2011). NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nat. Methods* *8*, 1037–1040.
- Evseenko, D., Zhu, Y., Schenke-Layland, K., Kuo, J., Latour, B., Ge, S., Scholes, J., Dravid, G., Li, X., MacLellan, W.R., et al. (2010). Mapping the first stages of mesoderm commitment during differentiation of human embryonic stem cells. *Proc. Natl. Acad. Sci. U S A* *107*, 13742–13747.
- Faial, T., Bernardo, A.S., Mendjan, S., Diamanti, E., Ortmann, D., Gentsch, G.E., Mascetti, V.L., Trotter, M.W., Smith, J.C., and Pedersen, R.A. (2015). Brachyury and SMAD signalling collaboratively orchestrate distinct mesoderm and endoderm gene regulatory networks in differentiating human embryonic stem cells. *Development* *142*, 2121–2135.
- Fonoudi, H., Ansari, H., Abbasalizadeh, S., Larijani, M.R., Kiani, S., Hashemizadeh, S., Zarchi, A.S., Bosman, A., Blue, G.M., Pahlavan, S., et al. (2015). A universal and robust integrated platform for the scalable production of human cardiomyocytes from pluripotent stem cells. *Stem Cells Transl. Med.* *4*, 1482–1494.
- Gaspari, E., Franke, A., Robles-Diaz, D., Zweigerdt, R., Roeder, I., Zerjatke, T., and Kempf, H. (2018). Paracrine mechanisms in early differentiation of human pluripotent stem cells: insights from a mathematical model. *Stem Cell Res.* *32*, 1–7.
- Gonzalez, R., Lee, J.W., and Schultz, P.G. (2011). Stepwise chemically induced cardiomyocyte specification of human embryonic stem cells. *Angew. Chem.* *50*, 11181–11185.
- Greber, B., Wu, G., Bernemann, C., Joo, J.Y., Han, D.W., Ko, K., Tapia, N., Sabour, D., Sternecker, J., and Tesar, P. (2010). Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell* *6*, 215–226.
- Grumolato, L., Liu, G., Mong, P., Mudbhary, R., Biswas, R., Arroyave, R., Vijayakumar, S., Economides, A.N., and Aaronson, S.A. (2010). Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. *Genes Dev.* *24*, 2517–2530.
- Haase, A., Gohring, G., and Martin, U. (2017). Generation of non-transgenic iPS cells from human cord blood CD34(+) cells under animal component-free conditions. *Stem Cell Res.* *21*, 71–73.
- Haase, A., Olmer, R., Schwanke, K., Wunderlich, S., Merkert, S., Hess, C., Zweigerdt, R., Gruh, I., Meyer, J., Wagner, S., et al. (2009). Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell* *5*, 434–441.
- Hartung, S., Schwanke, K., Haase, A., David, R., Franz, W.M., Martin, U., and Zweigerdt, R. (2013). Directing cardiomyogenic differentiation of human pluripotent stem cells by plasmid-based transient overexpression of cardiac transcription factors. *Stem Cell Dev.* *22*, 1112–1125.
- Ho, H.Y., Susman, M.W., Bikoff, J.B., Ryu, Y.K., Jonas, A.M., Hu, L., Kuruvilla, R., and Greenberg, M.E. (2012). Wnt5a-Ror-Dishevelled signaling constitutes a core developmental pathway that controls tissue morphogenesis. *Proc. Natl. Acad. Sci. U S A* *109*, 4044–4051.
- Kattman, S.J., Witty, A.D., Gagliardi, M., Dubois, N.C., Niapour, M., Hotta, A., Ellis, J., and Keller, G. (2011). Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* *8*, 228–240.



- Kempf, H., Kropp, C., Olmer, R., Martin, U., and Zweigerdt, R. (2015). Cardiac differentiation of human pluripotent stem cells in scalable suspension culture. *Nat. Protoc.* *10*, 1345–1361.
- Kempf, H., Olmer, R., Haase, A., Franke, A., Bolesani, E., Schwanke, K., Robles-Diaz, D., Coffee, M., Gohring, G., Drager, G., et al. (2016). Bulk cell density and Wnt/TGFbeta signalling regulate mesendodermal patterning of human pluripotent stem cells. *Nat. Commun.* *7*, 13602.
- Kempf, H., Olmer, R., Kropp, C., Ruckert, M., Jara-Avaca, M., Robles-Diaz, D., Franke, A., Elliott, D.A., Wojciechowski, D., Fischer, M., et al. (2014). Controlling expansion and cardiomyogenic differentiation of human pluripotent stem cells in scalable suspension culture. *Stem Cell Rep.* *3*, 1132–1146.
- Kensah, G., Roa Lara, A., Dahlmann, J., Zweigerdt, R., Schwanke, K., Hegermann, J., Skvorc, D., Gawol, A., Azizian, A., Wagner, S., et al. (2013). Murine and human pluripotent stem cell-derived cardiac bodies form contractile myocardial tissue in vitro. *Eur. Heart J.* *34*, 1134–1146.
- Kim, M.S., Pinto, S.M., Getnet, D., Nirujogi, R.S., Manda, S.S., Chaerkady, R., Madugundu, A.K., Kelkar, D.S., Isserlin, R., Jain, S., et al. (2014). A draft map of the human proteome. *Nature* *509*, 575–581.
- Kinder, S.J., Tsang, T.E., Quinlan, G.A., Hadjantonakis, A.K., Nagy, A., and Tam, P.P. (1999). The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. *Development* *126*, 4691–4701.
- Kropp, C., Kempf, H., Halloin, C., Robles-Diaz, D., Franke, A., Scheper, T., Kinast, K., Knorpp, T., Joos, T.O., Haverich, A., et al. (2016). Impact of feeding strategies on the scalable expansion of human pluripotent stem cells in single-use stirred tank bioreactors. *Stem Cells Transl. Med.* *5*, 1289–1301.
- Laco, F., Woo, T.L., Zhong, Q., Szmyd, R., Ting, S., Khan, F.J., Chai, C.L.L., Reuveny, S., Chen, A., and Oh, S. (2018). Unraveling the inconsistencies of cardiac differentiation efficiency induced by the GSK3beta inhibitor CHIR99021 in human pluripotent stem cells. *Stem Cell Rep.* *10*, 1851–1866.
- Lee, J.H., Protze, S.I., Laksman, Z., Backx, P.H., and Keller, G.M. (2017). Human pluripotent stem cell-derived atrial and ventricular cardiomyocytes develop from distinct mesoderm populations. *Cell Stem Cell* *21*, 179–194.e4.
- Lian, X., Hsiao, C., Wilson, G., Zhu, K., Hazeltine, L.B., Azarin, S.M., Raval, K.K., Zhang, J., Kamp, T.J., and Palecek, S.P. (2012). Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc. Natl. Acad. Sci. U S A* *109*, E1848–E1857.
- Liu, Y.W., Chen, B., Yang, X., Fugate, J.A., Kalucki, F.A., Futakuchi-Tsuchida, A., Couture, L., Vogel, K.W., Astley, C.A., Baldessari, A., et al. (2018). Human embryonic stem cell-derived cardiomyocytes restore function in infarcted hearts of non-human primates. *Nat. Biotechnol.* *36*, 597–605.
- Loh, K.M., Chen, A., Koh, P.W., Deng, T.Z., Sinha, R., Tsai, J.M., Barkal, A.A., Shen, K.Y., Jain, R., Morganti, R.M., et al. (2016). Mapping the pairwise choices leading from pluripotency to human bone, heart, and other mesoderm cell types. *Cell* *166*, 451–467.
- Ludwig, T.E., Bergendahl, V., Levenstein, M.E., Yu, J., Probasco, M.D., and Thomson, J.A. (2006). Feeder-independent culture of human embryonic stem cells. *Nat. Methods* *3*, 637–646.
- Malysheva, S.V., Wunderlich, S., Haase, A., Gohring, G., Martin, U., and Merkert, S. (2018). Generation of a human CDX2 knock-in reporter iPSC line (MHHi007-A-1) to model human trophoblast differentiation. *Stem Cell Res.* *30*, 117–121.
- Matsuda, T., Nomi, M., Ikeya, M., Kani, S., Oishi, I., Terashima, T., Takada, S., and Minami, Y. (2001). Expression of the receptor tyrosine kinase genes, *Ror1* and *Ror2*, during mouse development. *Mech. Dev.* *105*, 153–156.
- Mendjan, S., Mascetti, V.L., Ortmann, D., Ortiz, M., Karjosukarso, D.W., Ng, Y., Moreau, T., and Pedersen, R.A. (2014). NANOG and CDX2 pattern distinct subtypes of human mesoderm during exit from pluripotency. *Cell Stem Cell* *15*, 310–325.
- Moussaieff, A., Rouleau, M., Kitsberg, D., Cohen, M., Levy, G., Barasch, D., Nemirowski, A., Shen-Orr, S., Laevsky, I., Amit, M., et al. (2015). Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab.* *21*, 392–402.
- Mutig, N., Geers-Knoerr, C., Piep, B., Pahuja, A., Vogt, P.M., Brenner, B., Niederbichler, A.D., and Kraft, T. (2013). Lipoteichoic acid from *Staphylococcus aureus* directly affects cardiomyocyte contractility and calcium transients. *Mol. Immunol.* *56*, 720–728.
- Nelson, T.J., Faustino, R.S., Chiriack, A., Crespo-Diaz, R., Behfar, A., and Terzic, A. (2008). CXCR4⁺/FLK-1⁺ biomarkers select a cardiopoietic lineage from embryonic stem cells. *Stem Cells* *26*, 1464–1473.
- Olmer, R., Engels, L., Usman, A., Menke, S., Malik, M.N.H., Pessler, F., Gohring, G., Bornhorst, D., Bolten, S., Abdelilah-Seyfried, S., et al. (2018). Differentiation of human pluripotent stem cells into functional endothelial cells in scalable suspension culture. *Stem Cell Rep.* *10*, 1657–1672.
- Olmer, R., Lange, A., Selzer, S., Kasper, C., Haverich, A., Martin, U., and Zweigerdt, R. (2012). Suspension culture of human pluripotent stem cells in controlled, stirred bioreactors. *Tissue engineering part C. Methods* *18*, 772–784.
- Palecek, J., Zweigerdt, R., Olmer, R., Martin, U., Kirschning, A., and Drager, G. (2011). A practical synthesis of Rho-Kinase inhibitor Y-27632 and fluoro derivatives and their evaluation in human pluripotent stem cells. *Org. Biomol. Chem.* *9*, 5503–5510.
- Peng, G., Suo, S., Chen, J., Chen, W., Liu, C., Yu, F., Wang, R., Chen, S., Sun, N., Cui, G., et al. (2016). Spatial transcriptome for the molecular annotation of lineage fates and cell identity in mid-gastrula mouse embryo. *Dev. Cell* *36*, 681–697.
- Protze, S.I., Liu, J., Nussinovitch, U., Ohana, L., Backx, P.H., Gepstein, L., and Keller, G.M. (2017). Sinoatrial node cardiomyocytes derived from human pluripotent cells function as a biological pacemaker. *Nat. Biotechnol.* *35*, 56–68.
- Rao, J., Pfeiffer, M.J., Frank, S., Adachi, K., Piccini, I., Quaranta, R., Arauzo-Bravo, M., Schwarz, J., Schade, D., Leidel, S., et al. (2016). Stepwise clearance of repressive roadblocks drives cardiac induction in human ESCs. *Cell Stem Cell* *18*, 341–353.
- Shelton, M., Metz, J., Liu, J., Carpenedo, R.L., Demers, S.P., Stanford, W.L., and Skerjanc, I.S. (2014). Derivation and expansion of



- PAX7-positive muscle progenitors from human and mouse embryonic stem cells. *Stem Cell Rep.* 3, 516–529.
- Siller, R., Greenhough, S., Naumovska, E., and Sullivan, G.J. (2015). Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. *Stem Cell Rep.* 4, 939–952.
- Silva, M.M., Rodrigues, A.F., Correia, C., Sousa, M.F., Brito, C., Co-roadinha, A.S., Serra, M., and Alves, P.M. (2015). Robust expansion of human pluripotent stem cells: integration of bioprocess design with transcriptomic and metabolomic characterization. *Stem Cells Transl. Med.* 4, 731–742.
- Skelton, R.J.P., Kamp, T.J., Elliott, D.A., and Ardehali, R. (2017). Biomarkers of human pluripotent stem cell-derived cardiac lineages. *Trends Mol. Med.* 23, 651–668.
- Soh, B.S., Ng, S.Y., Wu, H., Buac, K., Park, J.H., Lian, X., Xu, J., Foo, K.S., Felldin, U., He, X., et al. (2016). Endothelin-1 supports clonal derivation and expansion of cardiovascular progenitors derived from human embryonic stem cells. *Nat. Commun.* 7, 10774.
- Ting, S., Chen, A., Reuveny, S., and Oh, S. (2014). An intermittent rocking platform for integrated expansion and differentiation of human pluripotent stem cells to cardiomyocytes in suspended microcarrier cultures. *Stem Cell Res.* 13, 202–213.
- Tran, T.H., Wang, X., Browne, C., Zhang, Y., Schinke, M., Izumo, S., and Burcin, M. (2009). Wnt3a-induced mesoderm formation and cardiomyogenesis in human embryonic stem cells. *Stem Cells* 27, 1869–1878.
- Ueno, S., Weidinger, G., Osugi, T., Kohn, A.D., Golob, J.L., Pabon, L., Reinecke, H., Moon, R.T., and Murry, C.E. (2007). Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc. Natl. Acad. Sci. U S A* 104, 9685–9690.
- Wattanapanitch, M., Klincumhom, N., Potirat, P., Amornpisutt, R., Lorthongpanich, C., U-pratya, Y., Laowtammathron, C., Kheola-mai, P., Pongvarin, N., and Issaragrisil, S. (2014). Dual small-molecule targeting of SMAD signaling stimulates human induced pluripotent stem cells toward neural lineages. *PLoS One* 9, e106952.
- Weber, N., Schwanke, K., Greten, S., Wendland, M., Iorga, B., Fischer, M., Geers-Knorr, C., Hegermann, J., Wrede, C., Fiedler, J., et al. (2016). Stiff matrix induces switch to pure beta-cardiac myosin heavy chain expression in human ESC-derived cardiomyocytes. *Basic Res. Cardiol.* 111, 68.
- Yang, L., Soonpaa, M.H., Adler, E.D., Roepke, T.K., Kattman, S.J., Kennedy, M., Henckaerts, E., Bonham, K., Abbott, G.W., Linden, R.M., et al. (2008). Human cardiovascular progenitor cells develop from a KDR⁺ embryonic-stem-cell-derived population. *Nature* 453, 524–528.
- Yoon, C., Song, H., Yin, T., Bausch-Fluck, D., Frei, A.P., Kattman, S., Dubois, N., Witty, A.D., Hewel, J.A., Guo, H., et al. (2018). FZD4 marks lateral plate mesoderm and signals with NORRIN to increase cardiomyocyte induction from pluripotent stem cell-derived cardiac progenitors. *Stem Cell Rep.* 10, 87–100.

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Supplemental Information

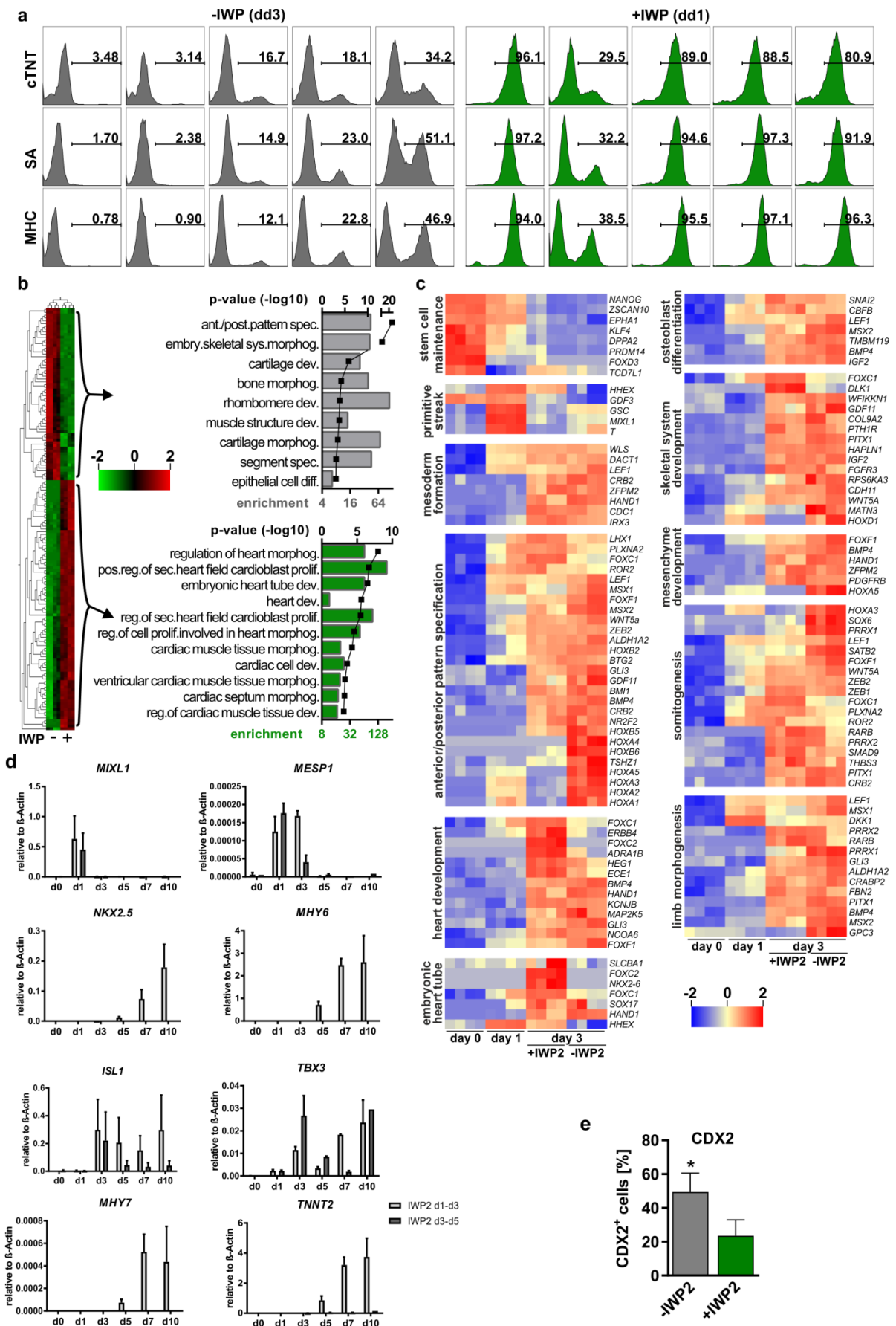
**Continuous WNT Control Enables Advanced hPSC Cardiac Processing
and Prognostic Surface Marker Identification in Chemically Defined
Suspension Culture**

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

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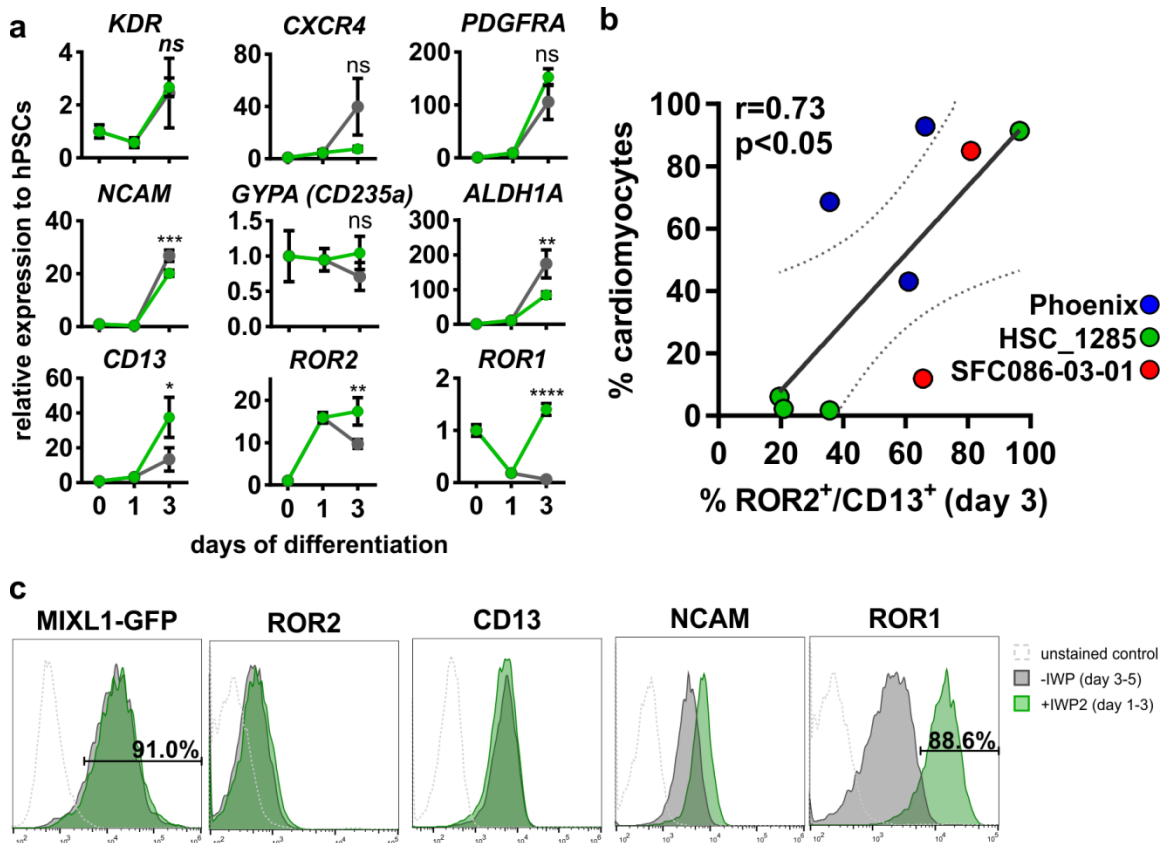
Figure S1. Related to Figure 1



(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 ($\sigma/\sigma_{\max} = 0.02$, $p < 0.01$) 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.

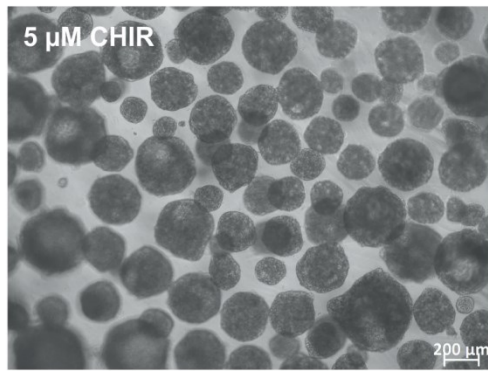
Figure S2. Related to Figure 2



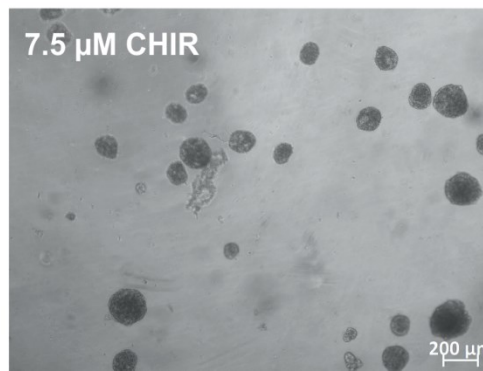
(a) Expression pattern of established mesodermal surface markers show only minor differences between cardiac (■) and paraxial (■) mesoderm on day 3 of *in vitro* differentiation from hPSCs. $n=4$ independent experiments based on microarray signal intensities normalized to hPSC levels. Error bars represent 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

a

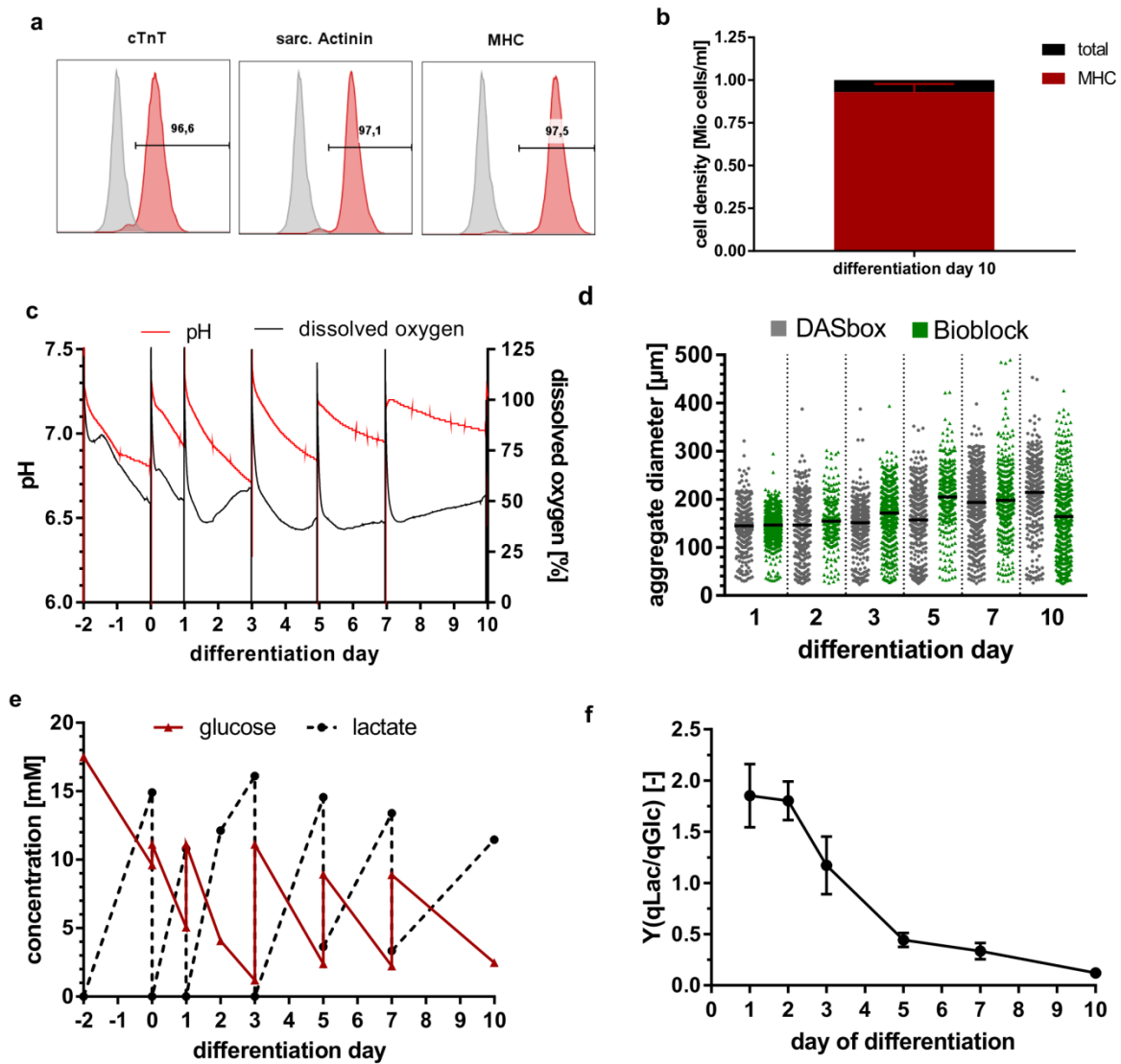


b



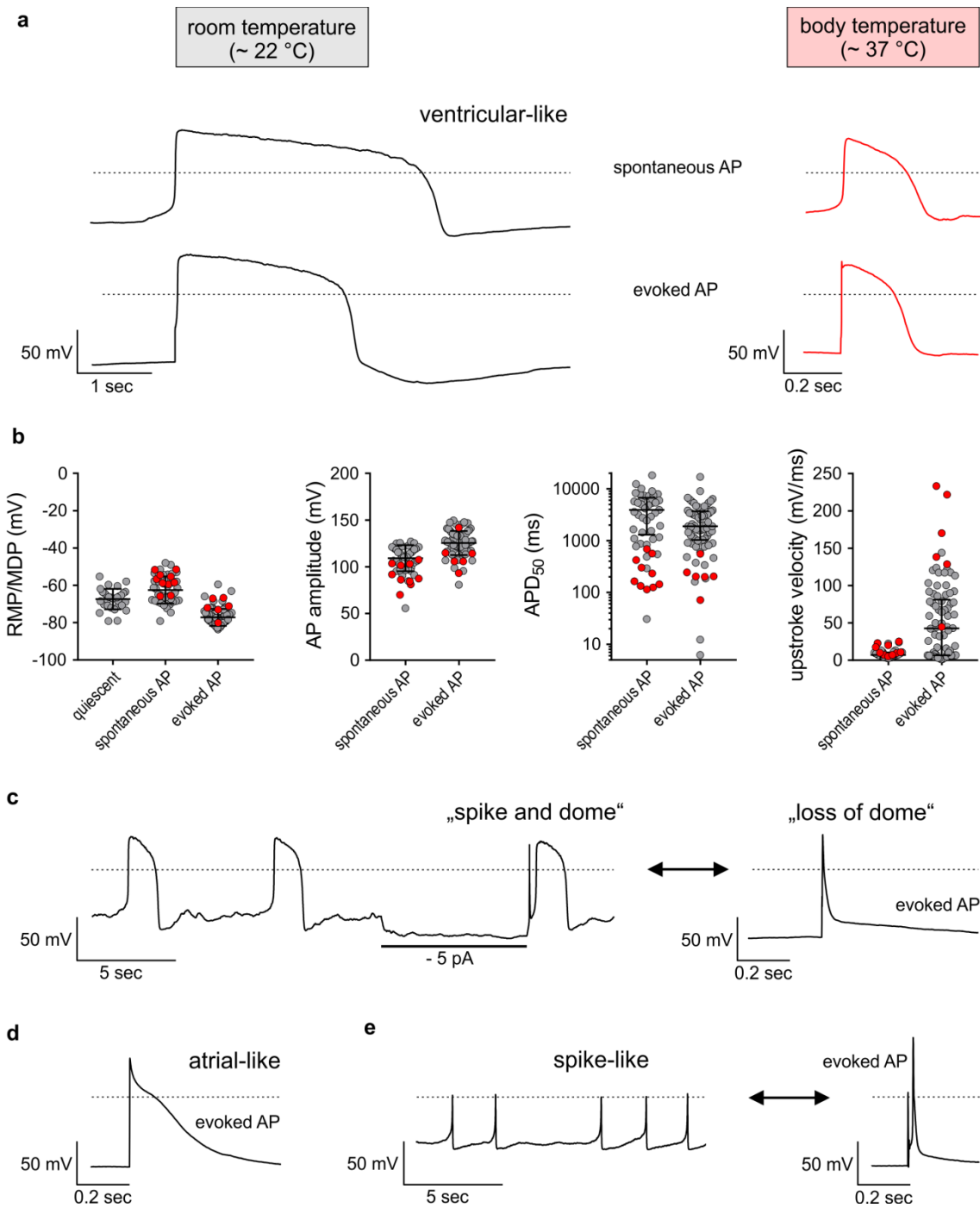
When applying **(a)** 7.5 μ M CHIR compared to **(b)** 5 μ M CHIR, cell death can be observed after 4 days of differentiation with the Phoenix line.

Figure S4. Related to Figure 5



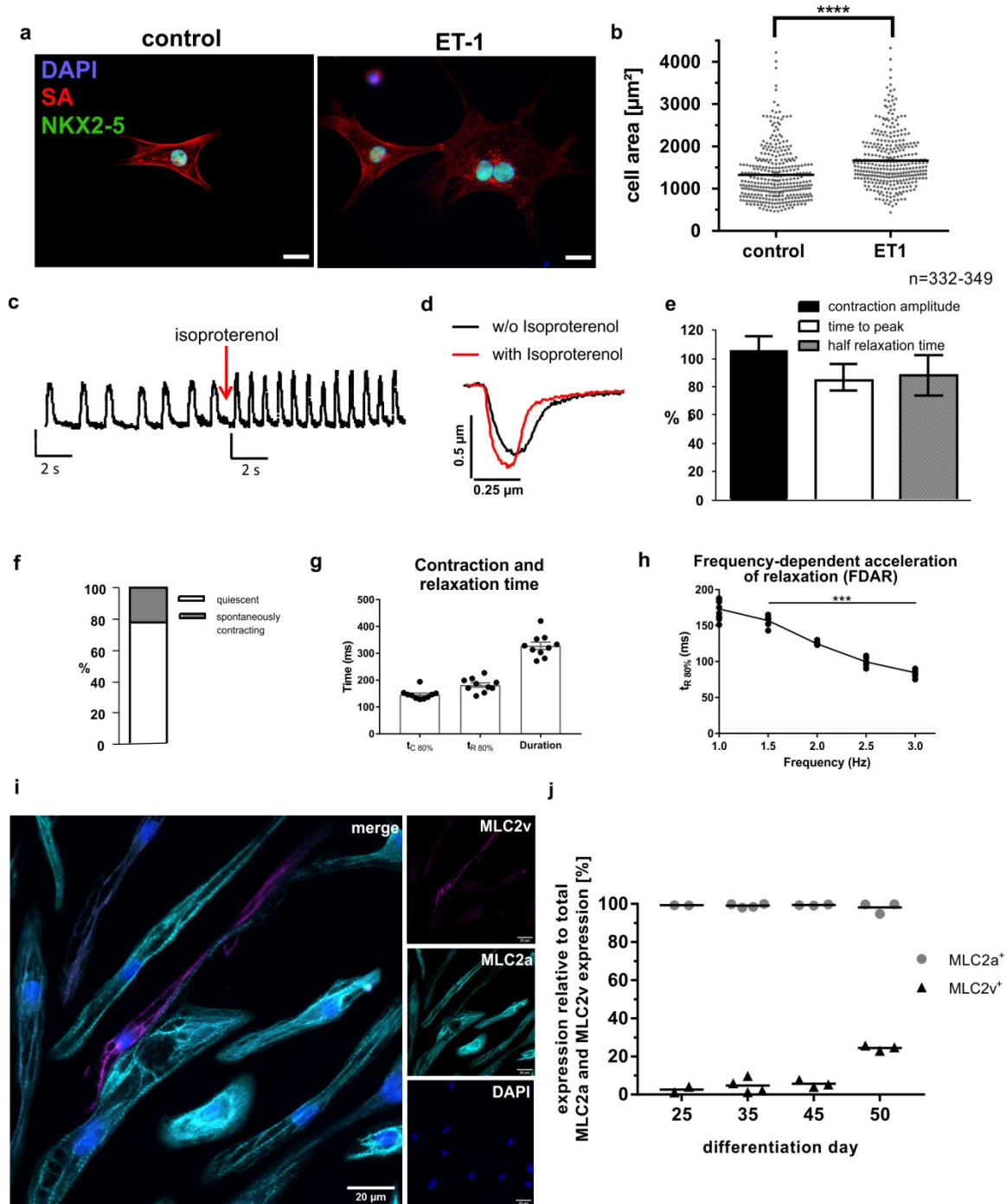
(a) FC of markers cTnT, sarcomeric actinin and myosin heavy chain for cardiomyocytes derived after 10 days of differentiation in the bioreactor (DASbox). (b) Cell density achieved in a bioreactor (n=3 independent experiments). (c) Representative process parameters (pH and DO) of the DASbox differentiation 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 $Y(q\text{Lac}/q\text{Glc})$ 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, APD₅₀, 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 (APD₅₀ 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 (1 μM) normalized to baseline levels. Error bars are mean \pm SEM for contraction amplitude and median \pm 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 \pm SEM, n=9 for g and h). (g) contraction duration at 80% peak height; t_c – contraction time, t_r - 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
<i>ACTB</i>	TGGAGAAAATCTGGCACC AC	GGTCTCAAACATGATCTG G	ACCGCGAGAAGATGACC
<i>MIXL1</i>	ATCCAGGTATGGTTCCAG	CCTAGCCAAAGGTTGGAA G	GTCAGAGTGGGAAATCC TTC
<i>MESPI</i>	CTCCGACAAGGACTGC	TGCCCCATGAGTCTGG	CTCCCTCGTCTCGTCCC
<i>ISL1</i>	GCTTTTCAGCAACTGGTCA A	TGCCTCAATAGGACTGGC TAC	GATACACCTAACAGCAT GG
<i>TBX3</i>	CCTGGAGGCTAAAGAACT TTG	TGGAGGAAACATTCGCCT TC	GTCATTACCAAGTCGGG
<i>MYH6</i>	AGAGCGAGTTCAAGCTG	CGAGACACTTTCTCCAGG TT	TCATCAAGGCCAAGGCA A
<i>MYH7</i>	TGTCCAAGTTCCGCAAGGT	GCTACTCCTCATTCAAG	ATTGGCACGAAGGGCTT G
<i>NKX2-5</i>	AAGGACCCTAGAGCCGAA A	GTTGTCCGCCTCTGT	CGGTGGAGCTGGAGAAG A
<i>TNNT2</i>	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 5×10^5 cells/well of a 12-well suspension plate (Greiner Bio One) in 1.5 ml mTeSR1 medium supplemented with 10 μ M 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 $3\text{--}6 \times 10^6$ 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 μ M CHIR99021 (LU Hannover) was added for 24h. Unless otherwise indicated, 5 μ M 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 $1.5\text{--}1.7 \times 10^5$ cells/ml in E8 supplemented with 10 μ M 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 μ M CHIR. After precisely 24 h, the medium was replaced by CDM3 supplemented with 5 μ M 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 μ g/ml human recombinant albumin (ScienCell, SC-OsrHSA) and 213 μ g/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, 1.5×10^5 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 β -mercaptoethanol (all Life Technologies), 10 μ M 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 μ g/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, monoclonal, 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 M Ω and 6 M Ω 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 CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.4 (adjusted with NaOH). The intracellular solution contained: 120 mM K-gluconate, 1 mM MgCl₂, 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 (APD₅₀) 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 \pm SD (RMP and AP amplitude) or medians and IQR (APD₅₀ 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 fibronectin 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 \pm 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 or spontaneous 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 μ M 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 2.5 μ M 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 IonWizzard® 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 μ l/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, 10⁶ dissociated CMs, and 10⁵ 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 μ M L-ascorbic acid, and 10 μ g/ml insulin) under standard cell culture conditions (37 °C, 5% CO₂) 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 μ m increments on the opposite side until 1000 μ m 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 μ m 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 resuspended 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 % β -Mercaptoethanol (Life Technologies) and 10 μ M 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.