

EBIO Does Not Induce Cardiomyogenesis in Human Pluripotent Stem Cells but Modulates Cardiac Subtype Enrichment by Lineage-Selective Survival

Monica Jara-Avaca,^{1,5} Henning Kempf,^{1,5} Michael Rückert,¹ Diana Robles-Diaz,¹ Annika Franke,¹ Jeanne de la Roche,² Martin Fischer,² Daniela Malan,³ Philipp Sasse,³ Wladimir Solodenko,⁴ Gerald Dräger,⁴ Andreas Kirschning,⁴ Ulrich Martin,^{1,6} and Robert Zweigerdt^{1,6,*}

¹Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO), Department of Cardiothoracic, Transplantation and Vascular Surgery, REBIRTH-Cluster of Excellence, Hannover Medical School, Carl-Neuberg Straße 1, 30625 Hannover, Germany

²Institute for Neurophysiology, Hannover Medical School, Carl-Neuberg Straße, 30625 Hannover, Germany

³Institute of Physiology I, Life & Brain Center, University of Bonn, Sigmund-Freud-Straße 25, 53127 Bonn, Germany

⁴Center of Biomolecular Drug Research (BMWZ), Institute of Organic Chemistry, Leibniz University Hannover, Schneiderberg 38, 30167 Hannover, Germany

⁵Co-first author

⁶Co-senior author

*Correspondence: zweigerdt.robert@mh-hannover.de

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SUMMARY

Subtype-specific human cardiomyocytes (CMs) are valuable for basic and applied research. Induction of cardiomyogenesis and enrichment of nodal-like CMs was described for mouse pluripotent stem cells (mPSCs) in response to 1-ethyl-2-benzimidazolinone (EBIO), a chemical modulator of small-/intermediate-conductance Ca^{2+} -activated potassium channels (SKs 1–4). Investigating EBIO in human pluripotent stem cells (PSCs), we have applied three independent differentiation protocols of low to high cardiomyogenic efficiency. Equivalent to mPSCs, timed EBIO supplementation during hPSC differentiation resulted in dose-dependent enrichment of up to 80% CMs, including an increase in nodal- and atrial-like phenotypes. However, our study revealed extensive EBIO-triggered cell loss favoring cardiac progenitor preservation and, subsequently, CMs with shortened action potentials. Proliferative cells were generally more sensitive to EBIO, presumably via an SK-independent mechanism. Together, EBIO did not promote cardiogenic differentiation of PSCs, opposing previous findings, but triggered lineage-selective survival at a cardiac progenitor stage, which we propose as a pharmacological strategy to modulate CM subtype composition.

INTRODUCTION

Cardiomyocytes (CMs) from human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), possess a high potential to regenerate diseased hearts. They also provide a human cell source for more predictive drug discovery and safety platforms (Braam et al., 2010; Kempf et al., 2016).

Aiming at chemically defined conditions, we and others have improved cardiac differentiation by supplementing synthetic effectors such as the p38 MAPK inhibitor SB203580 (Graichen et al., 2008; Kempf et al., 2011) and WNT pathway inhibitors (Willems et al., 2011). Based on this work, recent progress demonstrated efficient induction of CMs by tightly controlling the temporal pattern of WNT pathway activity using chemical compounds (Lian et al., 2012), which subsequently enabled upscaling of CM generation directly from hPSC in suspension culture using instrumented, stirred tank bioreactors (Kempf et al., 2014, 2015; Kropp et al., 2016).

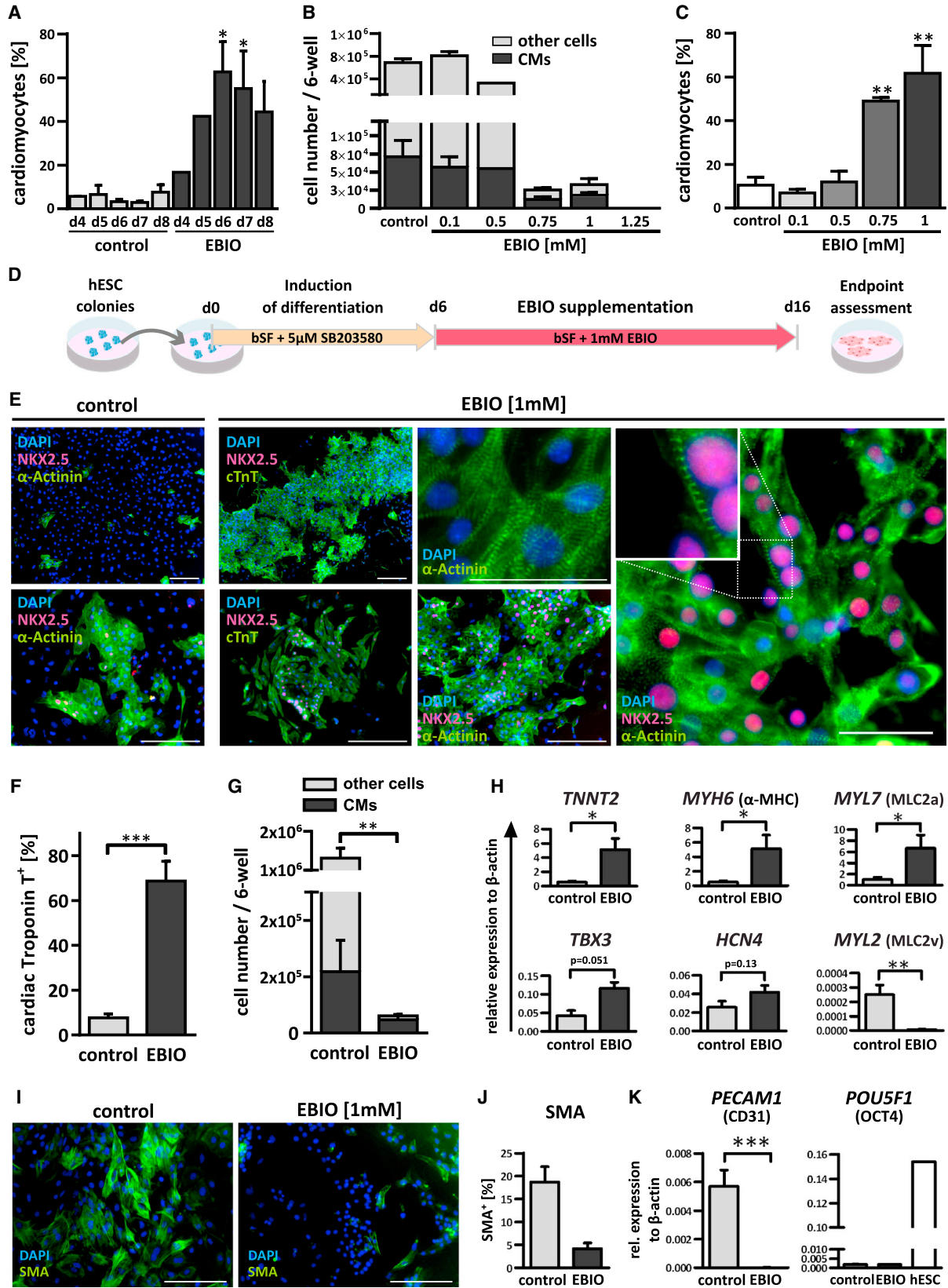
Another key requirement remains the targeted generation of specific CM subtypes. Successful differentiation into ventricular-, atrial-, or pacemaker-like CM subtypes was mainly investigated using mouse PSCs, but translation

to human cells remains a challenge (Birket et al., 2015; David and Franz, 2012).

Supplementation of the compound 1-ethyl-2-benzimidazolinone (EBIO), an established modulator of small- and intermediate-conductance Ca^{2+} -activated potassium channels (SKs), was described to induce the differentiation into highly pure CM populations from mouse ESCs. Interestingly, this was accompanied by the significant enrichment of pacemaker-like CMs (Kleger et al., 2010).

Small (SK1–3) and intermediate (IK or SK4) conductance channels are gated solely by $[\text{Ca}^{2+}]_i$ and exert hyperpolarizing effects that influence the activity of excitable and non-excitable cells (Kohler et al., 1996). Once activated, all SK channels can be kept in an open conformation by the prototype compound EBIO, causing a leftward shift in the Ca^{2+} -activation curves of SKs and thereby reducing the rate of deactivation upon Ca^{2+} removal (Devor et al., 1996; Hougaard et al., 2007). SKs regulate a wide range of physiological properties. However, their role(s) in developmental biology is not well understood, although differential expression of SKs in the cardiovascular system has been described (Tuteja et al., 2005).

Intrigued by the findings of Kleger et al. (2010) in mouse cells, we have investigated the effect of EBIO on human PSCs. We show that tightly controlled supplementation



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of EBIO during differentiation indeed results in high hPSC-CM purity, including a shift toward cell phenotypes with shorter duration of action potential (AP). In contrast to the proposed mechanism in mouse PSCs, our work highlights that CM subtype enrichment is not mediated by directing lineage-specific differentiation but rather by lineage-selective survival at the cardiac progenitor stage, demonstrating a pharmacological strategy for lineage purification.

RESULTS

CM Enrichment by EBIO Is Concentration and Time Dependent

HES3 cells were differentiated as embryoid bodies (EBs) in chemically defined conditions (bSF protocol; Xu et al., 2008) and supplemented with 1 mM EBIO (Kleger et al., 2010) or solvent controls (DMSO). Addition of EBIO from day 0 or day 3 onward led to almost entire cell loss and aberrant EBs lacking any contractility (Figure S1A). Treatment starting on days 4–8 allowed the formation of EBs that varied in size and microscopic appearance of beating foci and, strikingly, significantly reduced cell counts were observed in response to EBIO treatment compared with controls (Figure S1B). Quantification of cardiac troponin T (cTnT) and α -actinin staining revealed substantial CM enrichment of ~60% versus ~5%–10% in controls when initiating EBIO supplementation on day 6 (Figure 1A). CM enrichment was confirmed by increased gene expression levels of cTnT (TNNT2), myosin heavy chain 6 (MYH6), myosin light chain 2a (MLC2a; MYL7), and hyperpolarization-activated cation channel 4 (HCN4) (Figures S1C–S1F), whereas expression of the ventricular myocyte marker MYL2 (MLC2v) was reduced (Figure S1G). Only treatment at 0.75–1 mM EBIO resulted in increased CM purity (Figures 1B, 1C, and S1I) but reduced cell yields (Figure 1B) and

was accompanied by typical changes in EB morphology (Figure S1H; Movies S1 and S2).

In summary, 1 mM EBIO supplementation for 10 days starting on day 6 was most efficient in CM enrichment and was therefore applied in further experiments.

Optimized EBIO Treatment Leads to 9-Fold CM Enrichment

Endpoint analysis of EB-derived cells (Movie S3) revealed typical cross-striations of α -actinin/NKX2.5 double-positive cells, suggesting bona fide CM formation (Figure 1E) and significant ~9-fold enrichment from $7.7\% \pm 1.7\%$ to $68.7\% \pm 8.8\%$ cTnT⁺ cells, on average (Figure 1F). Importantly, we noted that the EBIO treatment was not only accompanied by an overall cell loss but also by ~80% reduced CM counts compared with controls (Figure 1G). A significant increase in expression of TNNT2, MYH6, and MYL7 was found, including modest elevation of the nodal-associated transcription factor TBX3 and the pacemaker channel HCN4, but MYL2 expression was reduced (Figure 1H).

Characterizing the remaining non-CMs showed ~4% smooth muscle actin (SMA)-positive cells compared with ~18% in controls (Figures 1I and 1J) and a substantial drop in endothelial cell marker CD31 expression (Figure 1K). Together, this indicates that the EBIO-mediated enrichment of CMs is accompanied by a loss of non-CM lineages including smooth muscle and endothelial cells, but also reduced overall CM counts.

EBIO-Dependent CM Enrichment and Cell Depletion Is PSC Line, Protocol, and Species Independent

To investigate the universal validity of EBIO-mediated CM enrichment, we tested an additional hES3 subclone as well as an entirely independent hPSC line by applying (1) a growth factor (GF)-directed differentiation protocol in serum-supplemented medium

Figure 1. Timed Treatment with 1 mM EBIO Results in 9-Fold Enrichment of CMs

(A) Quantification of CM purity based on cTnT staining for timepoint-dependent EBIO enrichment (n = 3 independent experiments; mean \pm SEM).

(B and C) EBIO concentration-dependent enrichment: total cell and CM numbers (B) and relative CM contents at the endpoint of differentiation (C) (n = 3 independent experiments; mean \pm SEM).

(D) Schematic outline of the EBIO-based, bSF differentiation protocol.

(E) Immunofluorescence analysis of replated control (left panel) and EBIO-treated (right panel) cells stained for α -actinin, cTnT, and NKX2.5 (scale bars, 200 μ m) showing typical cross-striations (far-right panel; scale bar, 50 μ m; inset: 4 \times magnification).

(F and G) Quantification of CMs at endpoint day 16 (n = 5 independent experiments of one to two replicates, six data points; mean \pm SEM) and (G) respective absolute cell numbers (n = 4 from three independent experiments; mean \pm SEM).

(H) qRT-PCR for TNNT2 (n = 3), MYH6, MLC2a, TBX3, MLC2v, and HCN4 (all n = 6 independent experiments; mean \pm SEM).

(I and J) Dissociated/replated cells stained for smooth muscle actin (SMA) on day 20 (I; scale bars, 200 μ m) and quantification thereof (J; n = 2 biological repeats; mean \pm SEM).

(K) qRT-PCR for the endothelial marker PECAM1 (CD31; n = 4 independent experiments; mean \pm SEM) and the pluripotency marker POU5F1 (OCT4; n = 6 independent experiments; n = 1 for hESC; mean \pm SEM).

*p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S1.

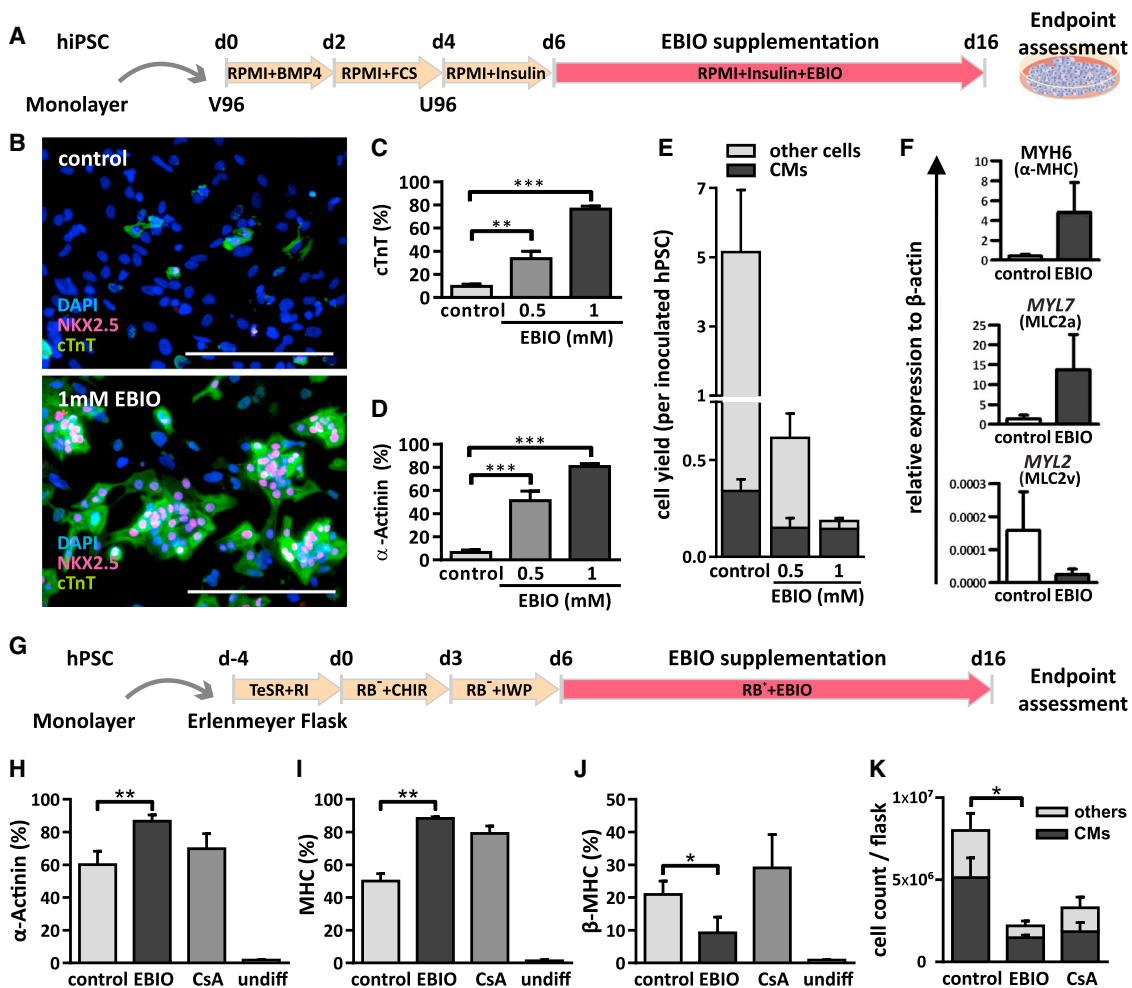


Figure 2. EBIO-Mediated CM Enrichment Is Independent of Applied Cell Lines and Differentiation Protocols

(A) Schematic of the GF differentiation protocol.

(B–F) Endpoint analysis on day 17 comparing EBIO treatment with controls (DMSO). (B) Representative pictures depicting dissociated and replated cells stained for cTnT and NKX2.5. Scale bars, 200 μ m. (C and D) Quantification of cells stained for cTnT (C) and α -actinin (D) ($n = 3$ independent experiments of three replicates, nine data points; mean \pm SEM). (E) Cell yields on day 17 revealed a \sim 5-fold expansion in controls (left bar) compared with a decrease in total cell counts and CMs (cTnT⁺ or α -actinin⁺ cells) in EBIO-treated conditions ($n = 2$ independent experiments; mean \pm SEM). EBIO (1 mM) was supplemented from day 6 to day 14. (F) EBIO-triggered CM enrichment was confirmed by qRT-PCR for MYH6 and MYL7 (MLC2a), but accompanied by reduction in MYL2 (MLC2v; all $n = 2$ independent experiments; mean \pm SEM).

(G) Schematic of the WNT protocol.

(H–J) Flow cytometric assessment on day 16 shows CM enrichment following EBIO treatment based on staining against α -actinin and pan-MHC, but is accompanied by a drop in expression of ventricular β -MHC ($n = 3$ independent experiments of one to two replicates, four data points). Cyclosporine A (CsA; 10 μ M) was applied as additional control substance ($n = 2$; mean \pm SEM).

(K) Total cell and cardiomyocyte yields per flask on day 16 ($n = 3$ independent experiments of one to two replicates for control and EBIO [four data points]; $n = 2$ independent experiments for CsA; mean \pm SEM).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Figure S2.

(intermediate cardiogenic efficiency [Burrige et al., 2011]) and (2) a small-molecule-based differentiation strategy using WNT pathway modulators (WNT protocol, high cardiogenic efficiency [Kempf et al., 2014]), respectively.

Using hCbiPSC2 cells in the GF protocol (Figure 2A), 1 mM EBIO resulted in CM enrichment of 76.4%–80.5% CMs (cTnT and α -actinin), whereas 0.5 mM EBIO resulted in modest CM enrichment of 33%–51% compared with 6%–9.5% CMs in controls (Figures 2B–2D and S2A).



Notably, only ~ 0.3 differentiated cells per inoculated hPSC were harvested following 1 mM EBIO treatment compared with ~ 5 cells in controls (Figure 2E). qRT-PCR for MYH6, MYL7, and MYL2 (Figure 2F) recapitulated expression patterns observed in the bSF protocol (Figure 1H).

Applying 1 mM EBIO to the HES3- α MHCneo line in the WNT protocol (Figure 2G) elevated the CM content from $60.2\% \pm 8.1\%$ (controls) to $86.7\% \pm 1.5\%$ for α -actinin and from $50.0\% \pm 2.6\%$ to $88.4\% \pm 0.95\%$ for pan-cardiac myosin heavy chain (MHC) staining (Figures 2H and 2I). Notably, specific analysis with β -MHC, the MHC isoform predominantly expressed in ventricular CMs (Gorza et al., 1984), showed a significant decrease from $20.9\% \pm 4.1\%$ in controls to $9.2\% \pm 2.4\%$ in treated cells (Figure 2J). Again, EBIO treatment was accompanied by $\sim 70\%$ overall cell loss (Figure 2K).

Cyclosporine A (CsA) was previously reported to modulate CM induction at a cardiac progenitor stage for murine and human PSCs (Cho et al., 2014; Fujiwara et al., 2011). We thus included this compound as an additional control. Interestingly, $10 \mu\text{M}$ CsA resulted in a substantial reduction of the overall cell counts similar to EBIO (Figure 2K), but without inducing significant CM enrichment and without reduction of β -MHC expression (Figures 2H–2J), suggesting distinct mechanisms between both substances.

Mouse ESC experiments equivalent to published procedures (Kleger et al., 2010) confirmed CM enrichment from $3.74\% \pm 0.8\%$ in controls to $71.1\% \pm 8.1\%$ in response to EBIO (Figures S2B–S2F). In line with our hPSC data, cell counts showed a drastic drop in overall and CM-specific numbers (Figure S2G) not reported for mouse PSCs in previous studies (Kleger et al., 2010; Muller et al., 2012).

To rule out batch-dependent effects of the drug, we obtained EBIO from two independent sources. High-performance liquid chromatography revealed $>99\%$ substance purity of three individual batches (Figure S2H), all of which showed undistinguishable biological activity (data not shown).

EBIO Treatment Results in Elevated Proportions of CMs with Short AP Duration

Whole-cell patch-clamp recordings revealed hPSC-CMs with ventricular-like, atrial-like, and nodal-like phenotype (Figure 3A). EBIO treatment caused a significant reduction of the overall AP duration (Figure 3B) by apparent depletion of CMs with long APs (ventricular-like), resulting in an enrichment of phenotypes with shorter APs (atrial-like, nodal-like, and spike-like), which tallies with our gene expression analyses. Kleger et al. (2010) reported an enrichment of pacemaker cells for murine CMs. Here, we tested this effect on human PSC-CMs. Indeed, we measured a number of bell-shaped APs in the EBIO-treated cells. At first glance, such AP shapes could be assigned as nodal-

like (Figure 3C, left traces) due to the low upstroke velocities, which is evoked by low expression of Nav1.5 sodium channels in bona fide pacemaker cells (Maier et al., 2003). However, spontaneously active CMs tested in our study displayed mean maximum diastolic potentials of -65 ± 3 mV ($n = 16$), whereby most Nav1.5 channels are inactivated. Therefore, we hyperpolarized respective cells to more physiological resting potentials around -80 mV and elicited APs by intracellular stimulation (Figure 3C, right traces). Under such conditions, most CMs produced APs with fast upstroke velocities (>30 mV/ms), considerable overshoot, and a clear plateau for ventricular-like cells (Figure 3C, upper traces) or triangular shapes typical of atrial-like cells (not shown). Only two CMs out of 56 EBIO-treated and 42 control cells tested (total 98 cells) maintained the bell-shaped AP appearance with low upstroke velocity, proving their nodal-like properties (Figure 3C, lower traces). Instead of the hypothesized pacemaker enrichment we frequently observed CMs with very short APs (AP duration at 50% of amplitude [APD₅₀] <20 ms) and fast upstroke velocities (82 ± 14 mV/ms, $n = 11$) in the EBIO-treated group (Figure 3D), designated spike-like in this study. Figure 3E summarizes the effect of EBIO on the distribution of CM phenotypes, demonstrating a decreased amount of ventricular-like cells and elevated proportions of CMs with short AP duration (atrial-like and spike-like). Notably, CsA supplementation tends to shift the subtype distribution in the opposite direction, i.e., supporting survival of CMs with long AP duration (Figures 3B and 3E), further suggesting a distinct mode of action compared with EBIO.

To further substantiate the electrophysiological data, we assessed gene expression patterns of established CM subtype-specific markers by microarray analysis applying cells derived by the WNT protocol (shown in Figures 2G–2K). EBIO supplementation resulted in reduced expression of ventricular markers MYL2, MYH7, IRX4, HEY2, and GJA1 (Figure S3A), consistent with (1) reduced MLC2v (MYL2) and β -MHC (MYH7) expression observed across all three differentiation platforms, and (2) the reduced fraction of CMs showing ventricular-like APs (Figure 3). In contrast, atrial-specific markers including Kir3.1, Kv1.5, and Cav1.3 (Devalla et al., 2015) were consistently upregulated (Figure S3B), while pacemaker-specific marker remained essentially unchanged (Figure S3C). Notably, upregulation of Kv1.5 increases the ultra-rapid potassium current (Kur) of atrial cardiomyocytes that tends to shorten APs (Christophersen et al., 2013; Grant, 2009). Moreover, our analysis unraveled an increased KCND3 and KCNA4 expression (Figure S3D), coding for Kv4.3 and Kv1.4 channels that define the plateau level of cardiac APs by transient potassium currents (I_{to}) in atrial and ventricular cardiomyocytes (Grant, 2009). It is tempting to speculate that increased expression of these channels might explain the

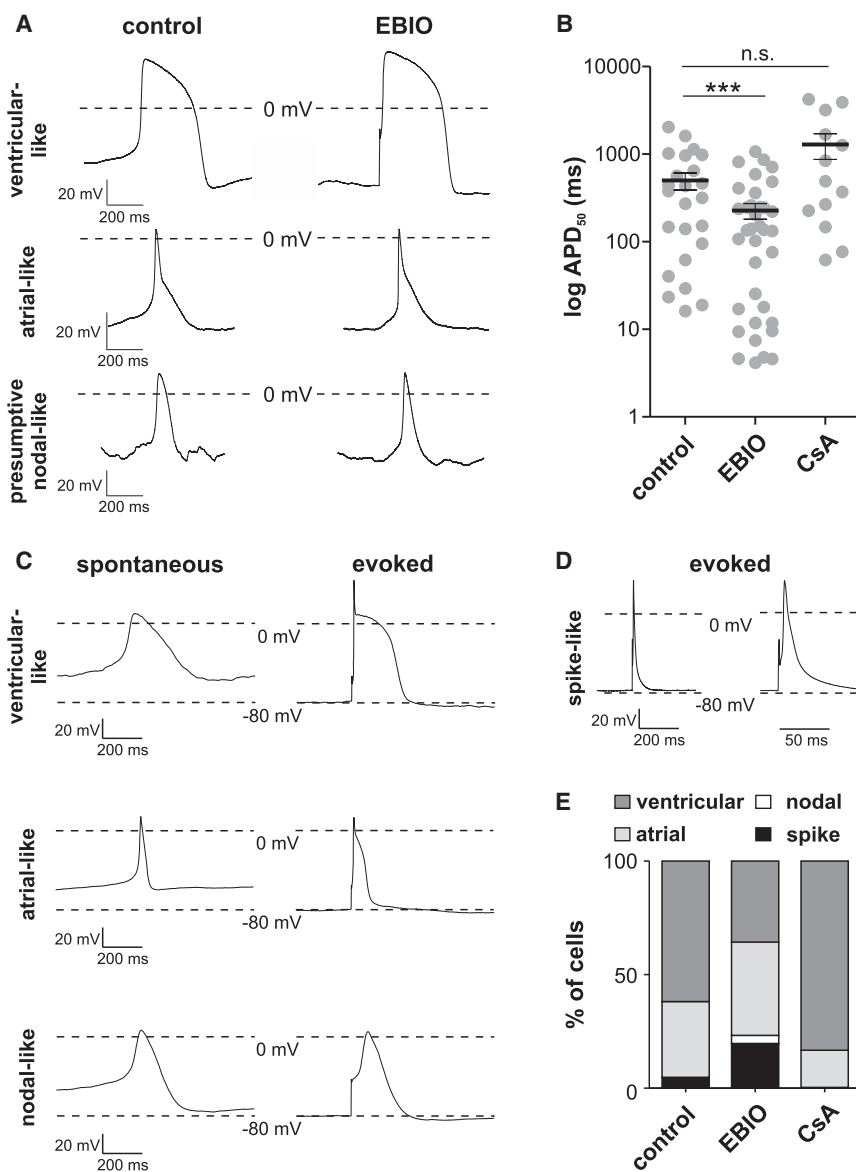


Figure 3. EBIO Treatment Alters CM Subtype Composition toward Shortened Action Potentials

(A) AP recordings from control (left) and EBIO-treated (right) hESC-derived CMs revealed differentiation into ventricular-like (upper panel), atrial-like (middle panel), and presumptive nodal-like (lower panel) cells.

(B) AP duration (APD_{50} , elicited at a resting potential of -80 mV) of control ($n = 24$) and EBIO ($n = 36$) or CsA ($n = 13$ cells from two independent differentiation experiments) treated CMs. Means \pm SEM are indicated in the dot plot. *** $p < 0.001$; n.s., not significant.

(C) Representative patterns of spontaneous and evoked (elicited at -80 mV) APs from the same cell of a ventricular-like cell (upper panel), atrial-like cell (middle panel), and nodal-like cell (lower panel), respectively.

(D) Representative recording of a spike-like CM with very short AP duration and fast upstroke velocity (note the expanded time scale in the right panel).

(E) CM subtype composition for control ($n = 42$), EBIO-treated ($n = 56$), and CsA-treated conditions ($n = 18$; cells from four independent differentiation experiments). Microarray analysis was conducted to confirm CM subtype shift and is shown in Figure S3.

occurrence of spike-like APs in EBIO-treated cells, when early outward currents exceed the calcium influx and impede the plateau generation in phase 2 of the AP.

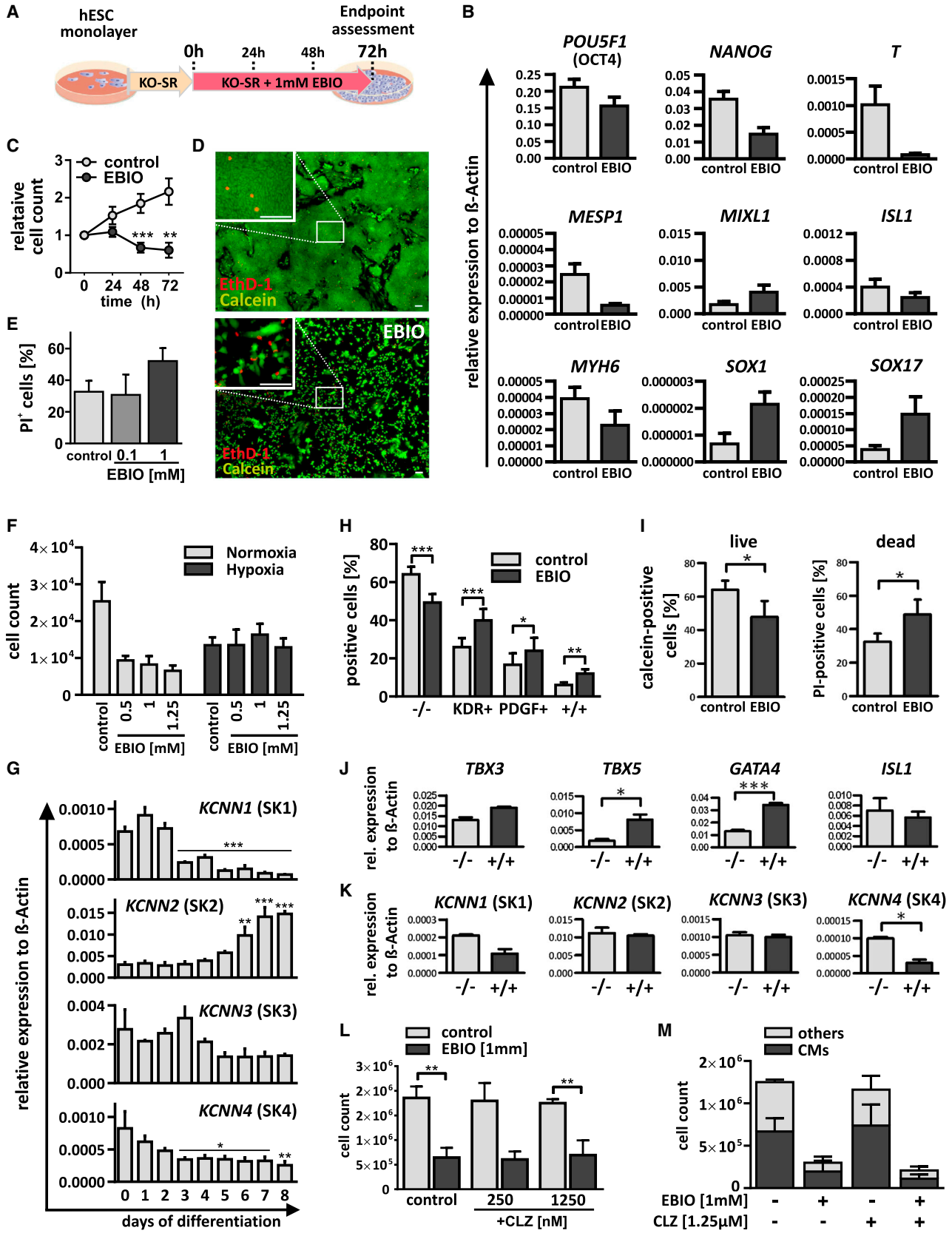
Together, these data suggest that EBIO triggers enrichment of cells with reduced AP duration, including higher content of atrial-like, some nodal-like, and appearance of spike-like identities, at the expense of ventricular-like cardiomyocytes.

EBIO Treatment Does Not Induce Expression of Mesodermal and Cardiac Lineage Markers but Interferes with the Proliferation and Viability of Undifferentiated hPSCs

Previous reports suggested that EBIO administration to human and mouse PSCs results in loss of pluripotency and

induction of mesodermal and the cardiac lineage (Kleger et al., 2010; Muller et al., 2012). In our hands, decreased expression of the pluripotency-associated markers OCT4 (POU5F1) and NANOG was indeed observed in hESCs treated with 1 mM EBIO (Figures 4A and 4B). However, mesodermal and cardiac markers (T, MESP1, MIXL1, ISL1, and MYH6) showed lower or similar expression levels compared with controls, whereas expression of SOX1 and SOX17, markers of early ectoderm and endoderm formation, respectively, were slightly upregulated, but at very low overall expression levels as expected for undifferentiated hPSC cultures (Figures 4A and 4B).

Growth kinetics revealed significantly reduced cell survival in response to EBIO compared with controls after 72 hr (Figure 4C). A large proportion of ethidium



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homodimer 1 (EthD-1) intercalating cells (Figure 4D) and the high rate of propidium iodide (PI)-positive cells (Figure 4E) underscored induction of cell death by 1 mM EBIO. Interestingly, shifting hPSC cultures from normoxic culture conditions (21% O₂) to low oxygen conditions (2.5% O₂) for 48 hr induced proliferation arrest and blocked the detrimental effect of EBIO in hESCs (Figure 4F) and hiPSCs (Figure S4D), suggesting that less proliferative cells are less susceptible to EBIO-mediated toxicity.

To test whether drug toxicity was restricted to highly proliferative hPSCs, we analyzed growth kinetics of additional human cell types. Foreskin fibroblasts and cord blood endothelial cells showed a complete block of cell proliferation but no apparent reduction in cell viability. However, highly proliferative HeLa cells mimicked the hPSC pattern regarding loss of cell viability (Figures S4A–S4C), underscoring that 1 mM EBIO induces cell death predominantly in highly proliferative cells.

SK4 Is Not Upregulated in Cardiac Progenitors during Early Cardiac Differentiation

EBIO is thought to mediate its effect specifically through positive modulation of SK channel activity (with SK4 being most sensitive to the drug) (Kleger et al., 2010). The time dependency of EBIO treatment prompted us to perform a detailed expression pattern analysis during the first 8 days of differentiation in the bSF protocol. The expression for cardiac differentiation markers resulted in expected patterns (Graichen et al., 2008) such as T and MESP1 expression peaking on days 2–3 followed by

upregulation of cardiac progenitor markers ISL1, TBX3, GATA4, TBX5, MEF2C, and NKX2.5 on days 3–5, respectively (Figure S4E). Regarding SK channel expression, we found substantial upregulation of SK2 from days 5–6 onward, whereas expression of other subtypes dropped or remained unchanged but were notably at a >10-fold lower expression level (Figure 4G).

We further explored this aspect via flow cytometry by analyzing SK expression in a cardiac progenitor population marked by platelet-derived growth factor receptor/kinase insert domain receptor positivity (PDGFR⁺/KDR⁺) (Kattman et al., 2011). A significantly higher amount of PDGFR⁺/KDR⁺ cells was observed after 48 hr of EBIO treatment on day 8 of differentiation (Figure 4H). Viability staining showed substantial EBIO-induced cell death (Figure 4I), suggesting that the increase in PDGFR⁺/KDR⁺ population was provoked by a loss of PDGFR⁻/KDR⁻ cells. We next analyzed SK channel expression in PDGFR⁺/KDR⁺-sorted cells. As expected, cardiac progenitor markers including TBX3, TBX5, and GATA4 (Figure 4J) were elevated in PDGFR⁺/KDR⁺, whereas almost no differential SK2 expression but notably a significantly lower expression of SK4 was detected (Figure 4K).

SK Channel Blockers Do Not Reverse EBIO-Mediated Effects

Aiming at specifically reversing the EBIO-mediated effects, several established SK channel blockers were supplemented at relevant concentrations in parallel to EBIO including clotrimazole (blocking SK4), apamin (blocking SK1–3),

Figure 4. EBIO Does Not Induce Mesodermal Differentiation and Mediates Its Cell-Selective Effects via an SK4-Independent Mechanism

- (A) Schematic outline of the supplementation procedure.
(B) qRT-PCR analysis for early differentiation markers shows no induction of mesodermal markers after 72 hr (n = 2 independent experiments of two replicates, four data points; mean ± SEM).
(C) Cell counts after 24–72 hr of treatment revealed decreased cell numbers in response to EBIO (n = 3 independent experiments; mean ± SEM).
(D) Live (calcein, green) and dead (EthD-1, red) staining after 72 hr. Scale bars, 100 μm.
(E) Flow cytometric quantification of propidium iodide (PI⁺; dead) cells after 72 hr (n = 2 independent experiments of two replicates, four data points; mean ± SEM).
(F) Culture of hPSCs under hypoxic conditions derogated the toxic effect of EBIO at all tested concentrations (n = 2 independent experiments of two replicates, four data points; mean ± SEM).
(G) Expression profile of SK1–4 (KCNN1–4) in the bSF differentiation (n = 3 independent experiments; mean ± SEM).
(H) Differentiating hESCs were treated on day 6 for 48 hr with 1 mM EBIO and analyzed for the expression of KDR⁺ and PDGFR⁺ by flow cytometry (n = 4 independent experiments; mean ± SEM).
(I) Cell viability was assessed after 48 hr of EBIO treatment on day 8 by flow cytometry for live (calcein) and dead (PI) cells (n = 4 independent experiments; mean ± SEM).
(J and K) qPCR analysis of cells sorted for KDR⁺/PDGFRα⁺ and KDR⁻/PDGFRα⁻ on day 6 of differentiation independent of EBIO addition (n = 3) for cardiac progenitor markers (TBX3, n = 2; TBX5, GATA4, ISL1, n = 3) and SK1–4 (n = 3; all independent experiments; mean ± SEM).
(L and M) Concomitant addition of clotrimazole (CLZ) does not block EBIO-mediated cell depletion in hPSCs after 48 hr of incubation (L; n = 3 for controls and 250 nM; n = 2 for 1,250 nM) and after treatment for 10 days during differentiation (M; n = 3, all independent experiments; mean ± SEM).
*p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.



and iberiotoxin (blocking large-conductance Ca^{2+} -activated potassium channels). None of these drugs derogated the EBIO-dependent cell depletion, either at the undifferentiated hPSC state (Figures 4L and S4F) or during differentiation (Figures 4M, S4G, and S4H). This suggests that the anti-proliferative and pro-cytotoxic effects of EBIO are independent of SK channel function.

Ultimately, we tested whether alternative positive SK modulators could mimic EBIO-mediated effects during hPSC differentiation. Interestingly, 15 μM CyPPA, a selective SK2/SK3 channel modulator (Hougaard et al., 2007), showed a similar response (Figures S4I, S4K, and S4L). In contrast, NS309, a highly potent EBIO analog (most potent on SK4 activity, $\text{EC}_{50} = 0.01 \mu\text{M}$) (Strobaek et al., 2004), resulted in neither CM enrichment nor apparent changes in overall cell counts (Figures S4J–S4L), as further validated in undifferentiated hPSCs (Figures S4M and S4N).

Together these data suggest that EBIO effects—proliferation arrest, cell depletion, CM enrichment, and CM subtype shift—are mostly (or entirely) independent of compounds' established modulation of SK4 activity but are likely mediated by other mechanisms.

DISCUSSION

Previous studies suggested the induction of cardiomyogenic differentiation with increased yields of nodal-like cells using 1 mM EBIO, a positive modulator of SK channel activity (SK4 $\text{EC}_{50} \sim 30 \mu\text{M}$; 10–20 times less potent for SK1-3 $\text{EC}_{50} \sim 600\text{--}1,000 \mu\text{M}$ [Pedarzani et al., 2001]). Here, we investigated the EBIO-mediated effect in four independent cardiac differentiation protocols, i.e., three for human and one for mouse PSC lines, with routine cardiac differentiation efficiencies ranging from 10% to 60% of CM induction. In striking contrast to previous reports, we could not attribute any mesoderm-inductive effects to EBIO (Figure 4B). Instead, EBIO induced significant cell loss accompanied by selective survival of CMs, ultimately leading to increased CM purity of >70% independent of the applied differentiation protocol (Figures 1 and 2). Likewise, we could not confirm the increased fraction of CMs with nodal-like APs, but observed an overall preponderance of CMs with significantly shortened AP duration following EBIO treatment (Figure 3).

During heart development, both conduction-system- and working-myocardium-forming CMs are derived from one common progenitor. Such primitive CM progenitors are hallmarked by TBX3 expression, proliferate slowly, soon become post-mitotic, and will mainly form nodal-like cells (Bakker et al., 2010). In our differentiation protocols applying hPSCs we showed that TBX3 as well as

the expression of other early cardiac progenitor markers (TBX5, GATA4, and ISL1) was upregulated on days 4–6, suggesting that primitive CM progenitors were formed within this time window under our in vitro conditions, representing a differentiation stage equivalent to early heart tube formation in vivo (McCulley and Black, 2012). These cardiac progenitors with their primary potential to form atrial cell types might be less sensitive to EBIO, possibly due to their specific ion channel expression pattern, early cell-cycle withdrawal, and/or metabolic features, resulting in their selective survival upon drug supplementation. In good agreement with our data, continuous application of the drug not only depleted most non-CM lineages but also interfered with the differentiation and/or proliferation of later-forming ventricular-like CMs, thereby inducing the observed CM subtype shift. Since our differentiation protocols, without drug administration, mainly yield ventricular-like cells (Kempf et al., 2014), inhibition and/or depletion of ventricular-like cell fates by EBIO may underlie the overall reduction of CM yields and depletion of cells with prolonged APs.

We functionally screened spontaneously active CMs for pacemaker properties, revealing that the majority of such cells had ventricular- or atrial-like but not nodal-like properties, when APs were elicited from physiological resting potentials (-80 mV). Nav1.5 is the main sodium channel specifying atrial and ventricular cardiomyocytes; this channel is semi-inactivated at -82 mV and nearly completely inactivated at -60 mV (Lei et al., 2004; Maier et al., 2003). Therefore, upstroke velocities are expected to be low whenever the membrane potential is shifted toward less negative values, which typically reflects conditions of immature atrial- and ventricular-like phenotypes at early stages of differentiation (Satin et al., 2004). In contrast, pacemaker cells of the sinoatrial node express Nav1.1. This channel activates and inactivates at approximately 25 mV more positive membrane potentials, and can therefore participate in the upstroke of pacemaker cells despite the slow diastolic depolarization of the heart (Lei et al., 2004). However, the Nav1.1 channel does not induce high upstroke velocities, since the upstroke of nodal cells is mainly dependent on voltage-activated calcium channels (Dan et al., 2014). Accordingly, our functional test is well suited to distinguish between APs representing immature atrial- or ventricular-like cardiomyocytes from bona fide pacemaker cells. Initiating measurements from -80 mV , the majority of CMs displayed APs with fast upstroke velocities whereby a broad range of AP duration patterns was observed. Recent reports indicate that the cell density as well as the cell source and/or culture conditions may influence AP shape variability and, subsequently, mean AP duration (Du et al., 2015; Hortigon-Vinagre et al.,



2016). To account for these issues, we applied equivalent culture conditions for drug treatment and controls and by ensuring harmonized conditions for patch-clamp analysis. This allowed unequivocal demonstration that EBIO treatment results in CM populations with significantly shorter APs. We interpret these results as enrichment of CMs with relatively short plateau phases (atrial-like) or even abolished plateaus (spike-like) resulting from ventricular-like phenotype reduction.

According to previous work, EBIO mediates its effects on mouse and human PSCs by compounds' established SK4-specific activity (Kleger and Liebau, 2011; Kleger et al., 2010). This is a critical notion, since the effect of EBIO is strictly dependent on drug concentrations above 500 μM in our study, which is actually in the range reported for EBIOs' EC_{50} for SK1–3. In line with this, we have observed a very low expression of SK4 in pluripotent cells, tallying with the lack of SK-specific currents demonstrated in hPSCs (Jiang et al., 2010). Moreover, we demonstrated further downregulation of SK4 during differentiation and more specifically in cardiac progenitors (Figures 4G and 4J). Furthermore, NS309, a >1,000-fold more potent EBIO analog (EC_{50} SK4 $\sim 0.01 \mu\text{M}$ [Strobaek et al., 2004]), did not mimic EBIO-mediated effects. In contrast, high concentrations of CyPPA, (i.e., 15 μM) an SK2/SK3-specific modulator, resulted in similar effects compared with EBIO. However, since the application of established SK blockers did not disrupt compound-dependent cell loss and CMs enrichment, a mechanism(s) mediated independently of SK channel modulation seems likely.

It is worth highlighting that a previously described method for CM purification by metabolic selection (Tohyama et al., 2013) results in a similar loss of 50%–90% of vital CM counts (Fuerstenau-Sharp et al., 2015). It is thus tempting to speculate that the EBIO effect is mediated through a metabolic mechanism, which is in agreement with the loss of cell sensitivity in less proliferative cells, i.e., irradiated fibroblasts and hPSCs switched to low oxygen conditions (Figures S4A–S4D, S4F, S4L, and S4M). However, more in-depth analysis beyond the scope of this study is required to further investigate these mechanisms.

In conclusion, we report pharmacological means for the efficient enrichment of CMs from human PSCs including a shift toward cells with shorter AP duration. Mechanistically, a yet unappreciated lineage selectivity of pharmacological SK modulators is described, which is likely independent of compounds' known modulation of SK channel activity. The resulting level of CM purity and the sensitivity of undifferentiated and proliferative hPSCs to cytotoxic drug effects are potentially valuable for the envisioned therapeutic applications of hPSC-derived CMs.

EXPERIMENTAL PROCEDURES

Human ESC and iPSC Culture

The human ESC line HES3 (ES Cell International) and the human iPSC clone hCBIpS2 (Haase et al., 2009) were cultured in 6-well plates (Nunc) on irradiated human foreskin fibroblasts (hFFs) (ATCC) or murine embryonic fibroblasts, respectively, in KO-SR medium consisting of KnockOut DMEM (Gibco) supplemented with 20% KnockOut Serum Replacement (Gibco), 1% non-essential amino acids (Gibco), 1 mmol/L L-glutamine (Gibco), and 0.1 mmol/L β -mercaptoethanol (Gibco). Basic fibroblast growth factor (bFGF) (Chen et al., 2012) was added, 50 ng/mL for HES3 and 10 ng/mL for hCBIpS2 cultivation. For passaging, cells were detached every 4–7 days using 0.2% collagenase IV (Gibco). Culture medium was changed every 2–3 days. For feeder-free cultivation, HES-3 cells were cultivated on Matrigel (BD Bioscience) in KO-SR medium with 100 ng/mL bFGF. For single culture, cells were seeded at 2.5×10^4 cells/cm² in the presence of 10 μM Rho-Kinase inhibitor (RI) Y-27632 (Paclecek et al., 2011). Medium was changed daily. For live/dead staining, plated cells were incubated for 30 min with 1 μM calcein and 4 μM ethidium homodimer 1 (Life Technologies) at 37°C.

bSF Protocol: Differentiation in Chemically Defined Conditions

Cells were mechanically detached from the conventional 6-well plate culture (Nunc) using a cell scraper and transferred to 6-well suspension plates (Greiner) in a well-per-well scheme for EB-based differentiation in basic serum-free (bSF) medium (Kempf et al., 2011; Xu et al., 2008) consisting of DMEM (Gibco) supplemented with 1% non-essential amino acids (Gibco), 2 mmol/L L-glutamine (Gibco), 0.1 mmol/L β -mercaptoethanol (Gibco), 5.6 mg/L transferrin (Sigma-Aldrich), and 37.2 $\mu\text{g/L}$ sodium selenite (Sigma-Aldrich). bSF medium was supplemented with 5 μM SB203580 (Graichen et al., 2008), a p38 MAPK inhibitor, from day 0 to day 6. The compounds 1-ethyl-2-benzimidazolinone (EBIO; 1 M stock solution; Tocris Bioscience or Institute of Organic Chemistry at Hannover University), CyPPA (15 mM stock solution, Tocris), and NS309 (3 mM stock solution, Tocris) were added at respective concentrations and timing as described in Results. DMSO (Sigma-Aldrich), the solvent of EBIO, CyPPA, and NS309, was applied in respective amounts in controls. Apamin was diluted in sterile H₂O (Tocris, 0.1 mM stock solution). Medium was changed every 2–3 days. Before EB dissociation for endpoint analysis, cells were cultured for 24 hr in bSF medium.

GF Protocol: Differentiation Based on Growth Factors

The iPSC line hCBIpS2 (Haase et al., 2009) was cultured on Gel-trex (Gibco) in a monolayer with feeder-conditioned medium for at least four passages (Burridge et al., 2011). EB-based differentiation was induced for 2 days cultivating 5,000 cell/V96 in RPMI medium (Gibco) containing 25 ng/mL BMP4 (R&D Systems), 10 ng/mL FGF2, insulin (Sigma-Aldrich), and polyvinyl alcohol (Sigma-Aldrich). After 2 days in medium supplemented with fetal calf serum, EBs were transferred to U96 plates in RPMI medium containing insulin (Sigma-Aldrich) on day 4. EBIO was added as indicated.



WNT Protocol: Differentiation Based on Small Molecules

The HES3- α MHCneo line (Schwanke et al., 2014) was differentiated as described previously (Kempf et al., 2014). In brief, cells were cultured under feeder-free conditions on Geltrex-coated flasks in mTeSR1 (STEMCELL Technologies) for at least four passages. Aggregates were generated from single-cell suspensions at 0.33×10^5 cells/mL in mTeSR1 supplemented with 10 μ M Y-27632 for 4 days. Differentiation was performed in agitated (orbital shaker at 75 rpm; Infors-HT) Erlenmeyer flasks (125 mL, VWR International) in 20-mL scale using RPMI supplemented with B27 without insulin (Life Technologies). For induction of differentiation, 7.5 μ M CHIR99021 (Institute of Organic Chemistry, Leibniz University Hannover) was supplemented during the first 24 hr, followed by 5 μ M IWP2 for 48 hr (Tocris). Medium was replaced every second day. The cells were maintained in RPMI supplemented with B27 (Life Technologies) from day 6 onward.

Mouse ESC Culture and Differentiation

The murine ESC line OG2 was cultured on irradiated mouse fibroblast or feeder-free as previously described (Mauritz et al., 2008). For differentiation, cells were detached from 6-well plates (Nunc) and dissociated with 0.025% trypsin (Sigma). EBs were formed with differentiation medium consisting of Iscove's modified Dulbecco's medium (Gibco) supplemented with 15% fetal calf serum, 1% non-essential amino acids (Gibco), 0.2 mmol/L L-glutamine (Gibco), and 0.1 mmol/L β -mercaptoethanol (Gibco), in hanging drops (600 cells/drop) for the first 3 days of differentiation. On day 3 EBs were transferred into suspension culture dishes (Nunc) and subsequently plated onto 0.1% gelatin-coated 6-well plates (Nunc, 10 EBs per 6 wells). Further differentiation was performed in 1 mM EBIO supplemented from day 1 to day 15 (Figure S3) as previously described (Kleger et al., 2010).

Statistical Analysis

Error bars represent mean \pm SEM unless otherwise noted. Statistical significance was calculated using an unpaired Student's t test. Multiplicity of testing was accounted for by one-way ANOVA followed by Bonferroni's post test. Significance is shown in the figures as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, and three movies and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2016.12.012>.

AUTHOR CONTRIBUTIONS

M.J.-A., R.Z., and U.M. designed the study; M.J.-A., H.K., M.R., D.R.-D., A.F., J.R., D.M., W.S., and G.D. performed the experiments; M.J.-A., H.K., M.R., J.R., M.F., D.M., P.S., U.M., and R.Z. analysed and interpreted the data; A.K. gave conceptual advice; M.J.-A., H.K., M.F., and R.Z. wrote the manuscript.

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