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segmentournal of Cellular Biochemistry 112:2006–2014 (2011)

Journal of Cellular Biochemistry

Synthetic Sulfonyl-Hydrazone-1 Positively Regulates Cardiomyogenic MicroRNA Expression and Cardiomyocyte Differentiation of Induced Pluripotent Stem Cells

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are obtained from adult cells through overexpression of pluripotency factors. iPSCs share many features with embryonic stem cells (ESCs), circumventing ethical issues, and, noteworthy, match donor's genotype. iPSCs represent therefore a valuable tool for regenerative medicine. Cardiac differentiation of ESCs can be enhanced via microRNAs (miRNAs) and small chemical compounds, which probably act as chromatin remodelers. Cardiomyogenic potential of iPSCs is currently intensely investigated for cell therapy or in vitro drug screening and disease modeling. However, influences of small compounds on iPSC-related cardiomyogenesis have not yet been investigated in details. Here, we compared the effects of two small molecules, bis-peroxo-vanadium (bpV) and sulfonyl-hydrazone-1 (SHZ) at varying concentrations, during cardiac differentiation of murine iPSCs. SHZ ($5\,\mu M$) enhanced specific marker expression and cardiomyocyte yield, without loss of cell viability. In contrast, bpV showed negligible effects on cardiac differentiation rate and appeared to induce Casp3-dependent apoptosis in differentiating iPSCs. Furthermore, SHZ-treated iPSCs were able to increase beating foci rate and upregulate early and late cardiomyogenic miRNA expression (*miR-1*, *miR-133a*, and *miR-208a*). Thus, our results demonstrate that small chemical compounds, such as SHZ, can constitute a novel and clinically feasible strategy to improve iPSC-derived cardiac differentiation. J. Cell. Biochem. 112: 2006–2014, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: SULFONYL-HYDRAZONE; BIS-PEROXO-VANANADIUM; iPSCs; CARDIOMYOCYTES; microRNAs

Induced pluripotent stem cells (iPSCs) hold a great promise for in vivo cell therapy and in vitro drug screening [Kiskinis and Eggan, 2010]. iPSCs can be generated from adult cells (such as terminally differentiated fibroblasts or somatic progenitors) by exogenous overexpression of Oct4, Sox2, Klf4 and, eventually, cMyc [Takahashi and Yamanaka, 2006]. iPSCs have been generated in a wide variety of species, including mouse [Blelloch et al., 2007] and human [Park et al., 2008], and display many features of

pluripotency that are typical of embryonic stem cells (ESCs) [Marion et al., 2009]. The clinical potential of these cells resides in their patient-specific nature, given that iPSCs bear the same genotype of the adult donor body [Raya et al., 2009]. In principle, once generated from a patient, iPSCs can be genetically corrected and reinjected in vivo to regenerate a tissue [Hanna et al., 2007] or differentiated in vitro towards a specific lineage and used to screen drug functionality [Barbaric et al., 2010]. However, iPSCs still present major issues, such as recipient safety and differentiation efficiency [Miura et al., 2009].

Differentiation of ESCs or iPSCs towards cardiomyocytes or cardiac progenitors has gathered great attention, particularly as a potential reservoir of cells for in vivo heart regeneration and large-scale in vitro production of cardiomyocytes for cardiac drug screening [Yokoo et al., 2009]. Generally, ESC/iPSC cardiomyogenic differentiation results in contracting cardiac foci in vitro and the purified cardiomyocytes present in vitro and in vivo specific markers [Puceat, 2008], including *sarcomeric myosin heavy chain* (*MyHC*), cardiac troponins and *connexin43* (*Cx43*). In addition, several protocols have been developed to enhance differentiation efficiencies or cardiomyocyte purity, including cardiac body suspensions [Xu et al., 2006] and BMP4/activinA-mediated induction [Laflamme et al., 2007]. However, studies on differentiating cell survival rate and timings are still lacking.

Specific non-coding microRNAs (miRNAs) have been directly linked to cardiac differentiation, including *miR-1*, *miR-133a*, and *miR-208a* [Chen et al., 2009]. Forced expression of *miR-1* in ESCs promotes cardiomyocyte maturation, by *HDAC4* inhibition and *Mef2C* activation [Ivey et al., 2008]. *miR-133a* and *miR-208a* are located in *Mib1* and *Myh6* intronic regions, respectively [Chen et al., 2006]. *miR-133a* and *miR-208a* are expressed during early and late embryonic heart development and are altered during myocardial infarctions [Bostjancic et al., 2010].

Noteworthy, chromatin remodelers have a role in the induction of cardiac differentiation of embryonic tissues or iPSCs. Cardiac-specific *Baf60c*, in combination with *Gata4* and *Tbx5*, induces ectopic transdifferentiation of embryonic mesoderm to cardiomyocytes [Takeuchi and Bruneau, 2009]. Furthermore, trichostatin, a HDAC inhibitor, increases *Nkx2.5* expression and cardiac differentiation of murine iPSCs, in a cell line-dependent fashion [Kaichi et al., 2010].

Bis-peroxo-vanadate (bpV) can act as chromatin remodeler and is able to reprogram skeletal myoblasts into more immature cells with higher potential for proliferation, migration and differentiation both in vitro and in vivo [Castaldi et al., 2007]. However, the effects of bpV on cardiac myogenesis of iPSCs are as yet unknown.

Sulfonyl-hydrazones (SHZ) constitute a family of small chemical compounds that act as positive inducers of *Nkx2.5* and other cardiac mRNAs and proteins in murine ESCs. Furthermore, SHZ-treated human mobilized blood mononuclear cells engraft and recover injured rat hearts better than control cells [Sadek et al., 2008]. Thus, SHZ appears to be a promising chemical inducer for in vitro and in vivo cardiomyogenic stimulation, even though its effects on iPSCs have not yet been investigated.

Here we compare bpV and SHZ effects on iPSC-derived early and late cardiomyogenesis at different concentrations, monitoring the eventual effects on cell survival and apoptosis. We examine and quantify specific cardiac miRNA levels, beating foci percentages and cardiomyocyte isolation rates in order to verify whether small chemical compounds could effectively improve cardiomyogenesis of iPSCs.

MATERIALS AND METHODS

SMALL MOLECULES, iPSCs CULTURE, AND VIABILITY ASSAY

Sulphonyl-hydrazone-1 (SHZ) molecule was synthesized according to the procedure previously reported [Sadek et al., 2008], and resuspended in DMSO (Sigma, USA). Bis-peroxo-vanadium (bpV) was purchased (Alexis Biochemicals, USA) and resuspended in sterile PBS (Gibco, USA).

Murine fibroblast-derived iPSCs (clone O59 *Oct4::Neo*, kindly provided by R. Jaenisch's group) were cultured on a feeder layer of mytomicine-inactivated murine embryonic fibroblasts (iMEFs) in R1 medium, namely DMEM high glucose (Gibco), 15% knockout serum (Invitrogen, USA), 1% penicillin/streptomycin solution (Gibco), 1% L-glutamine (Gibco), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Gibco), 0.2% 2-mercaptoethanol (Gibco), and 1,000 U/ml of ESGRO® (Millipore, USA).

Cell viability was assessed using trypan blue (Lonza, USA) staining. After trypsin-induced detachment, cells were incubated for 5 min at room temperature (RT) with 0.4% trypan blue solution (diluted 1:1 in basal medium) and viable, non-stained cells were counted by Countess® automatic cell counter (Invitrogen).

CARDIAC DIFFERENTIATION PROTOCOL

To allow embryoid body (EB) formation, at day 0 iPSCs were resuspended in LIF-free R1 medium in presence of SHZ or bpV at several concentrations (5, 10, and 15 μM) and kept for 5 days in suspension drops (10^3 cells/20 μl). In positive control, 2.5 ng/ml BMP2 (Sigma) was added. At day 5, EBs were collected and seeded onto gelatin (Millipore)-coated six-well plates (NUNC, Denmark), as approximately 100 EBs/well. At day 6, medium was replaced by chemical-containing Maintenance medium, namely 70% DMEM high glucose, 17.5% Medium199 (Gibco), 5% Horse serum (Gibco), 1% penicillin/streptomycin solution, and 1% L-glutamine.

Cardiac foci started to beat at days 15–18 of differentiation and were filmed at brightfield light for 5 s. Percentages were calculated from at least three different random spots per treatment. Beating foci area was calculated using ImageJ software.

Flow cytometry analysis was performed at day 20 of differentiation. Differentiating cells were collected, washed twice in PBS and incubated 30 min at RT with rabbit IgG unconjugated (Epitomics, USA) in isotype control and rabbit polyclonal anti-Cx43 (SantaCruz, USA) in samples, diluted as 2 μl/10^5 cells in 200 μl. After PBS wash, isotype control and samples were stained with Alexafluor488-conjugated anti-rabbit donkey antibody (Invitrogen; 0.5 ml/10^5 cells in 200 ml) for 30 min at RT and then washed again twice and analyzed at FACSCanto machine (BD, USA).

Cardiomyocytes were isolated from differentiating foci at day 20, as previously reported [Puceat, 2008]. Briefly, after collection, cell pellets were digested three times for 15 min shaking at 37°C, with

3 ml of 0.06% Collagenase II (Sigma)/0.01% Pancreatin (Sigma) solution in ADS buffer 1× (ADS buffer 10×: 80 ml tissue culture-grade water (Gibco), 6.8 g NaCl (Sigma), 0.4 g KCl (Sigma), 0.12 g sodium phosphate (Sigma), 0.1 g magnesium sulphate (Sigma), 1 g glucose (Sigma), and 4.76 g HEPES (Gibco)). After collection of all single-cell suspensions, digestion was blocked with filtered FBS (Gibco). Cells were resuspended in 2 ml ADS buffer 1×, loaded onto a two layer-Percoll gradient (Sigma) and spun down at 3,000 rpm for 30 min at RT. Cardiomyocytes were collected as cells at the interface between Percoll gradients and plated on gelatin-coated dishes in Maintenance medium.

qPCR/TAQMAN ASSAYS

RNA was extracted with RNA Mini kit (Invitrogen) and 1 μg reverse transcribed by SuperscriptIII kit (Invitrogen). qPCR was performed on 1:5 diluted cDNA, via Syber Green Mix (Invitrogen), 15 s at 95°, 45 s at 60°, 40 cycles. Primers: Nkx2.5 FW AAGTGCTCTC-CTGCTTTCCCA, Nkx2.5 REV TTTGTCCAGCTCCACTGCCTTCT, Gata4 FW AGGGTGAGCCTGTATGTAATGCCT, Gata4 REV AGGA-CCTGCTGGCGTCTTAGATTT, Cx43 FW TACTTCAATGGCTGCTCCT-CACCA, Cx43 REV GCTCGCTGGCTTGCTTGTTGTAAT. Internal standard, Pgk FW CAAAATGTCGCTTTCCAACAAG, Pgk REV AACGTTGAAGTCCACCCTCATC.

Enriched small RNA fractions, containing miRNAs, were isolated using the mirVana™ miRNA Isolation Kit (Ambion, USA). Fifteen nanograms of miRNA-containing RNA were reverse transcribed with MultiScribe kit (Applied Biosystems, USA). qPCR was performed by Taqman probes and Universal Mix (Applied Biosystems), according to manufacturer's instructions. As internal standard, miR-16 was used.

WESTERN BLOTTING

For Western blotting (WB), cells were lysed in RIPA buffer (Sigma) supplemented with 10 mM NaF (Sigma), 0.5 mM sodium orthova-nadate (Sigma), 1:100 protease inhibitor cocktail (Sigma), and 1 mM PMSF (Sigma). Total protein mixes (20 μg) were separated on a 10% polyacrylamide gel and transferred on a nitrocellulose membrane (Whatman, Germany). After blocking for 1 h at RT (5% Skim-milk (Sigma) in TBS buffer supplemented with 0.2% Tween-20 (Bio-Rad, USA)), the membrane was incubated overnight at 4°C with 1% Skim-milk solution containing primary antibodies. Primary antibody dilutions: mouse monoclonal anti-MyHC (DSHB Hybridoma) 1:10; rabbit polyclonal anti-Cx43 (SantaCruz) 1:500; mouse monoclonal anti-cTnI (Abcam, UK) 1:500; rabbit polyclonal anti-cleaved caspase-3 antibody (Asp 175, Cell Signal, USA) 1:1,000. After incubation with donkey anti-mouse or anti-rabbit horseradish-peroxidase-coupled secondary antibody (diluted 1:5,000) for 1 h at RT, specific bands were detected with Chemiluminescent Peroxidase Reagent-3 (Sigma) and pictures were taken at GelDoc machine (Bio-Rad) with 60 s exposure. After stripping the membrane 15 min at 50°C, βTub bands were detected with 1:1,000-diluted mouse monoclonal anti-βTub antibody (Millipore). Quantification was performed via QuantityOne software (Bio-Rad), by comparing Casp3 bands to βTub bands, as internal standard.

IMMUNOFLUORESCENCE ASSAYS

Cells were fixed in 2% PFA (Polysciences, USA), permeabilized with 0.2% Triton (Sigma), and blocked with 1:10 donkey serum (Sigma). Primary antibody dilutions: mouse monoclonal anti-MyHC (DSHB Hybridoma) 1:1; rabbit polyclonal anti-Cx43 (SantaCruz) 1:300; mouse monoclonal anti-cTnI (Abcam) 1:100. Secondary Alexafluor-conjugated donkey antibodies (Invitrogen) anti-mouse and anti-rabbit were diluted 1:500. Nuclei were counterstained with 1:1,000 Hoechst (Sigma). Images were taken at Eclipse Ti microscope (Nikon, Japan) and merged by Image-Pro Plus 6.0 software (Nikon).

STATISTICAL ANALYSIS

Unpaired two-tailed Students' t-test was used as statistical tool. Data are shown as mean ± standard deviation and P-values <0.01 were considered significant.

RESULTS

COMPARISON BETWEEN bpV AND SHZ EFFECTS ON CARDIOMYOGENIC iPSC DIFFERENTIATION

According to our and other protocols [Puceat, 2008] of cardiomyogenic differentiation, murine iPSCs were grown as embryoid bodies (EBs) for 5 days (EB step), then plated on gelatin-coated dishes for subsequent maturation of EB-derived cardiac foci for another 10 days (maturation step). bpV and sulfonyl-hydrazone-1 (SHZ) were added at growing concentrations (5, 10, and 15 μM) during both EB and maturation steps. BMP2 treatment was also included as positive control, given that it enhances ESC-derived cardiac differentiation, as previously reported [Puceat, 2008].

At day 5 of differentiation, qPCR assay clearly showed that bpV and SHZ induce upregulation of early cardiomyogenic factors, such as *NK2 transcription factor related* (*Nkx2.5*) and *GATA binding protein 4* (*Gata4*), in a dose-dependent manner (Fig. 1A,B). bpV-induced upregulation was within the range of control and positive control values, whereas increasing concentrations of SHZ strikingly enhanced *Nkx2.5* and *Gata4* expression up to 10- and 3-fold, respectively, as compared to BMP2-treated cells (Fig. 1A,B). Furthermore, SHZ appeared to activate late cardiac markers in a dose-dependent manner, such as *connexin 43* (*Cx43*) at day 10 (Fig. 1C). Consistently, immunofluorescence stainings at day 15 showed that SHZ-treated (5 μM) iPSCs develop more organized patterns of cardiac myosin heavy chain (cMyHC), Cx43, and cardiac troponin I (cTnI) on cardiac foci than bpV-treated (5 μM) iPSCs and control iPSCs (Fig. 1D–L). Densitometric quantification confirmed that only SHZ (5 μM) increased significantly MyHC, Cx43, and cTni protein levels at day 15 of differentiation, whereas bpV (5 μM) induced no significant effects compared to control conditions (Fig. 1M,N). During cardiac foci differentiation, SHZ is a strong activator of early and late cardiac specific markers in murine iPSCs, whereas bpV shows modest effects.

bpV AND SHZ EFFECTS ON CARDIOMYOCYTE ISOLATION AND VIABILITY

To further assess bpV and SHZ effects on cardiac iPSC differentia-tion, we quantified the Cx43$^+$ cell fraction via flow cytometry at
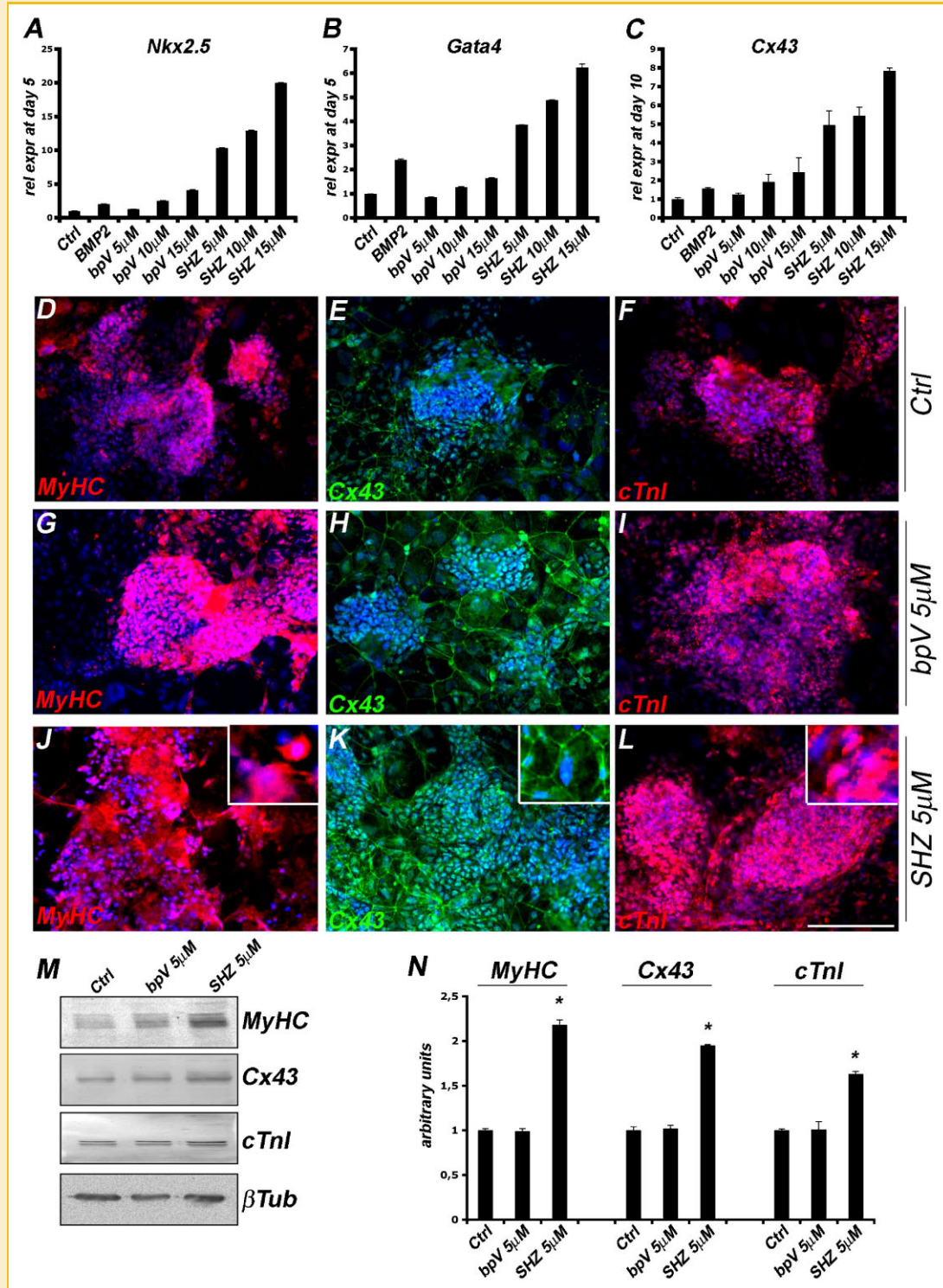
Fig. 1. bpV and SHZ effects on cardiac marker expression. A–C: qPCR charts show that bpV and SHZ upregulate *Nkx2.5* (A) and *Gata4* (B) at day 5 and *Cx43* (C) at day 10 in a dose-dependent fashion. SHZ-treated iPSCs display the highest levels of expression of all three markers (n = 3; values normalized vs. control iPSC; internal standard, *Pgk*; BMP2, positive control). D–L: Immunofluorescence stainings at day 15 of differentiation show expression patterns of cMyHC (D,G,J), Cx43 (E,H,K), and cTnI (F,I,L) in cardiac foci in control (D–F), bpV-treated (5 μM) (G–I), and SHZ-treated (5 μM) iPSC (J–L). SHZ-treated (5 μM) iPSCs show more organized patterns, both at large and small scales (J–L and insets) than bpV-treated (5 μM) or control iPSCs. Scale bar, 100 μm. M,N: Western blotting assays (M) and related densitometry charts (N) of control, bpV-treated (5 μM), and SHZ-treated iPSCs (5 μM) at day 15 are shown. Only in presence of SHZ (5 μM) did the differentiating iPSCs increase MyHC, Cx43, and cTnI protein levels. Protein levels were first related to βTub and background for each sample and then related to control (n = 3; βTub, internal control; $^*P < 0.01$ compared to control).

day 20 of differentiation. Cx43$^+$ fraction was discriminated as FITC$^+$ population compared to the isotype control. Unexpectedly, bpV 10 μM, bpV 15 μM, and SHZ 15 μM resulted in significantly lower Cx43$^+$ rates, whereas SHZ 5 μM significantly increased Cx43$^+$ percentage, compared to control (Fig. 2A). In addition, we isolated cardiomyocytes at the same time point by Percoll gradient [Puceat, 2008] from control and treated cells at increasing chemical concentrations. Consistently with flow cytometry data, only SHZ treatment (5 μM) resulted in a significantly higher yield, while bpV 10 μM, bpV 15 μM, and SHZ 15 μM conditions decreased the cardiomyocyte yield (Fig. 2B). Interestingly, higher concentrations of bpV and SHZ had also significant negative effects on cardiomyocyte viability 48 h after isolation, whereas SHZ 5 μM had beneficial effects (Fig. 2C). Immunostainings at 48 h after

isolation showed that cardiomyocytes derived from SHZ treatment (5 μM) presented 26 ± 2% of bi-nucleated cells (Fig. 2C, arrows) and a mature cardiac pattern of cTnI-positive fibers and Cx43-positive cytoplasm and membrane (Fig. 2D). To test the staining specificity, murine embryonic fibroblasts (MEFs) were stained with the same combination of antibodies, without any specific signal (Fig. 2E). Thus, SHZ (5 μM) appears to increase end-point cardiomyocyte yield and viability, which are, in contrast, decreased by higher concentrations of bpV and SHZ.

DETRIMENTAL EFFECTS OF bpV/SHZ AT HIGH CONCENTRATIONS

To assess potential detrimental effects of high bpV/SHZ concentrations during various differentiation steps, we assayed cell viability at days 5, 10, and 15. Interestingly, bpV treatment decreased cell
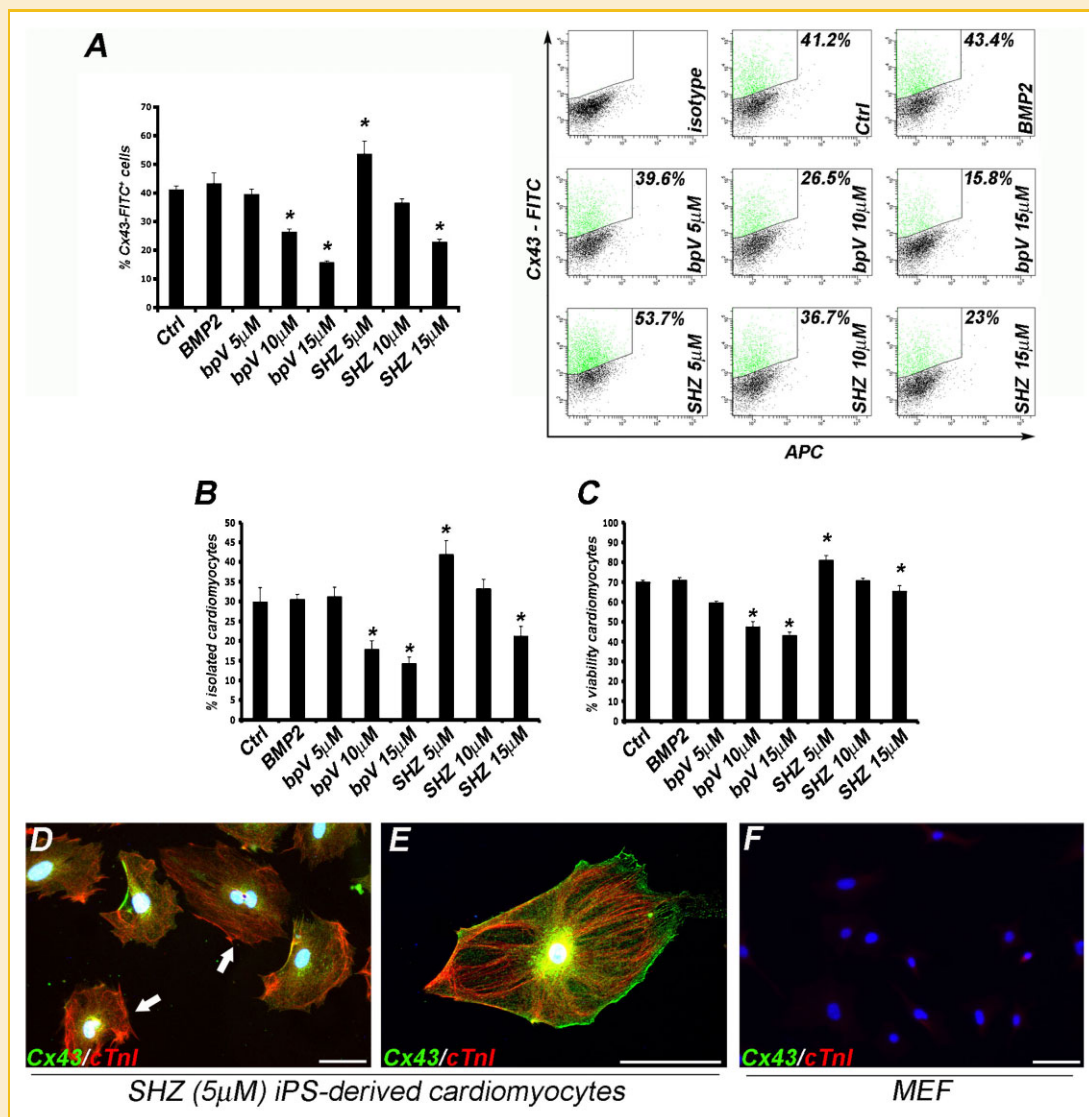

Fig. 2. Cardiomyocyte yield and viability. A: Flow cytometry chart (left) and dot plots (right) for Cx43–FITC in all iPSC differentiation conditions at day 20. Cx43$^+$ fraction is identifiable as FITC$^+$ population compared to isotype control (green populations in the dot plots; n = 3; *$P < 0.01$). B,C: SHZ–treated (5 μM) iPSCs present significantly higher yield (B) and viability (C) rates than controls, whereas high concentrations of bpV and SHZ induce significantly negative variations (n = 3; *$P < 0.01$). D–F: Immunofluorescence analysis for Cx43 (green) and cTnI (red) reveals that cardiomyocytes obtained from SHZ–treated (5 μM) iPSCs are enriched in binucleated cells (D) and, at higher magnification, show the typical staining of mature cardiomyocytes (E). MEFs were stained with the same antibody combination as a negative control (F). Scale bars, 100 μm.

viability in a dose-dependent fashion, starting from day 5 (Fig. 3A). SHZ 10 µM and SHZ 15 µM had similar negative effects from day 10, whereas SHZ- (5 µM) and BMP2-treated cells shared the highest viability scores (Fig. 3A). In order to find possible causes for this phenomenon, we tested activated apoptosis-effector caspase 3 (Casp3) protein levels at day 10 of differentiation. WB and densitometry showed that SHZ 15 µM, bpV 5 µM, and bpV 15 µM induced significantly higher activated Casp3 levels, than in control or SHZ-treated (5 µM) iPSCs (Fig. 3B,C). Thus, at higher concentrations, bpV and SHZ are able to induce a significant apoptosis-dependent loss of viability in differentiating iPSCs.

SHZ ENHANCES BEATING FOCI RATE AND CARDIOMYOGENIC miRNAs

Once EBs attach to gelatin-coated plastic and start to differentiate, resulting beating cardiac foci tend to flatten and to take contact with each other. This may constitute a hurdle in beating foci analyses. Therefore, we analyzed possible SHZ effects on beating activity, comparing both beating cardiac foci percentages and areas at day 20 of differentiation, between control and SHZ-treated (5 µM) iPSCs. As expected, SHZ treatment of iPSCs resulted in a significant increase in beating foci rate ($19.75 \pm 3.78\%$ vs. $10.5 \pm 1.29\%$, $P < 0.01$, n = 4) and area extension ($668 \pm 179 \, \mu m^2$ vs. $168 \pm 86 \mu m^2$, $P < 0.01$, n = 4), as compared to control (Fig. 4A,B and Supplementary Videos 1, 2).

To exclude the hypothesis that SHZ-dependent cardiomyogenic enhancement could rely on beneficial effects on cell proliferation, we compared growth curves of proliferating iPSCs and isolated cardiomyocytes, both derived from control and SHZ-treated (5 µM) iPSCs (as above described), in presence or absence of SHZ (5 µM). Neither proliferating iPSCs, nor control- or SHZ (5 µM)-derived cardiomyocytes showed any significant variation in proliferation
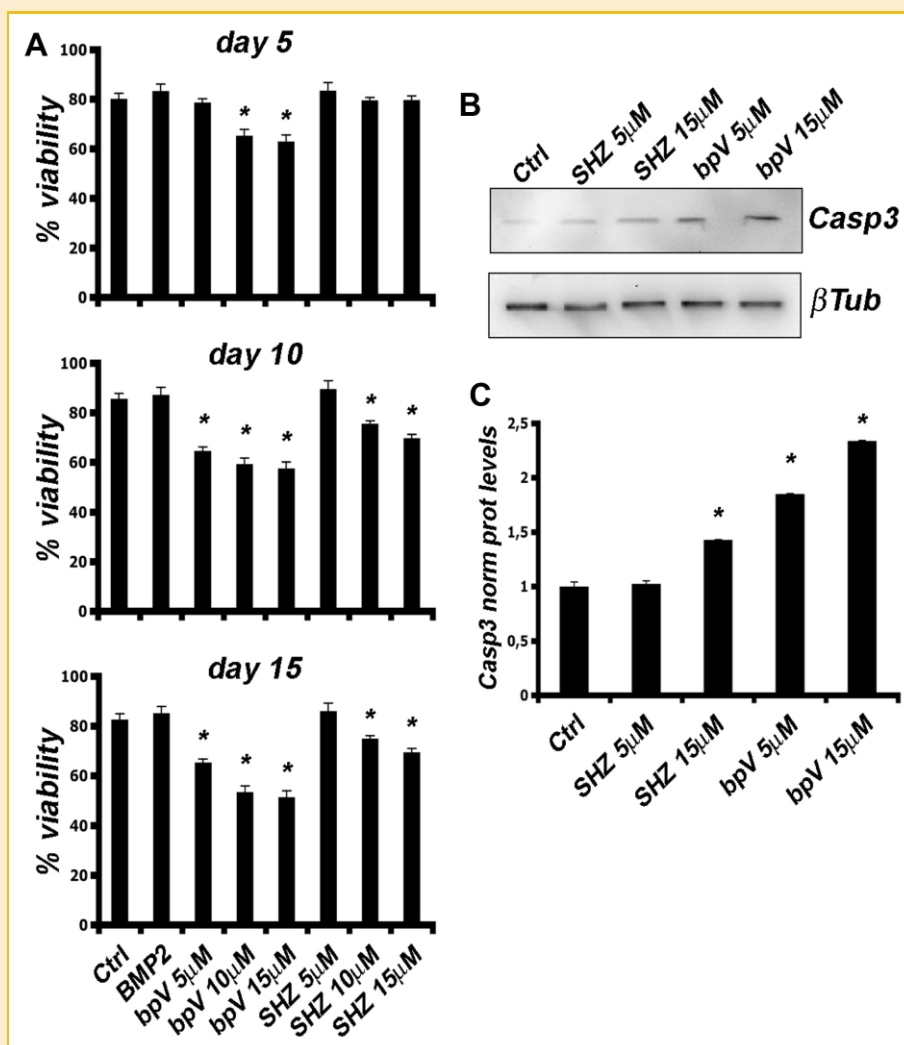

Fig. 3. High concentrations of bpV and SHZ have detrimental effects on cell viability. A: Cell viability charts show significant detrimental effects on differentiating iPSCs treated with bpV/SHZ high concentrations at day 5 (first row), day 10 (second row), and day 15 (third row) of differentiation, as compared to controls and SHZ 5 µM (n = 3; *$P < 0.01$). B,C: Western blot analysis show that bpV/SHZ high concentrations increase Casp3 activation at day 10 of differentiation (B). In (C), quantitative WB analysis is reported (n = 3; internal standard, βTub; *$P < 0.01$).
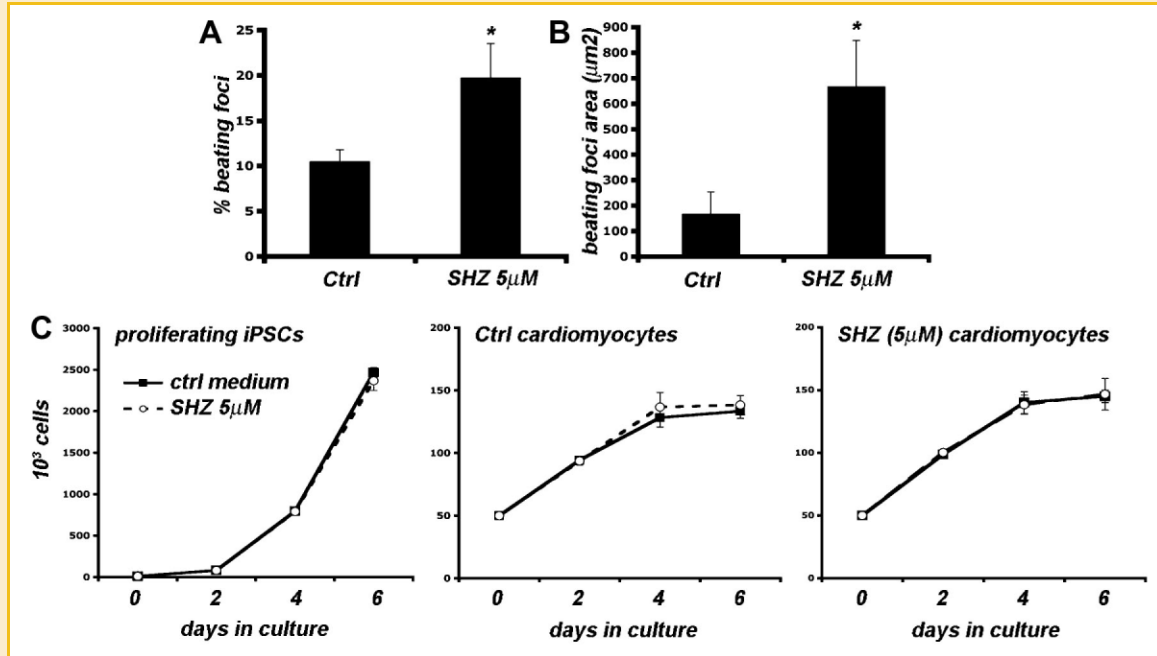
Fig. 4. SHZ enhances beating foci rate. A,B: At day 20, SHZ-treated (5 μM) iPSCs display approximately two-fold increase of beating foci percentage (A), as compared to control cells (n = 4; *P < 0.01). SHZ-treatment (5 μM), moreover, results into increased beating foci area (B), as compared to control iPSCs (n = 4; *P < 0.01). C: Proliferating iPSCs, cardiomyocytes derived from control iPSCs and cardiomyocytes derived from SHZ-treated (5 μM) iPSCs were cultured in control (straight line, closed dots) and in SHZ-supplemented (5 μM) growing media, without showing any significant variation in proliferation rate at any time point (n = 3 per population/condition).

rate in presence of SHZ-supplemented culture medium, as compared to control culture medium (Fig. 4C).

Moreover, we tested whether SHZ induced early (miR-1, miR-133a) and late (miR-208a) cardiomyogenic miRNAs. Taqman quantitative assays showed that both control and SHZ-treated (5 μM) iPSCs increased miR-1 and miR-133a expression at day 5 and decreased both miRNAs at day 10 (Fig. 5A). In contrast, miR-208a was downregulated at day 5 and upregulated at day 10 both in control and SHZ-treated (5 μM) iPSCs (Fig. 5B). However, SHZ-treated (5 μM) iPSCs significantly increased miR-1 and miR-133a expression at day 5 and miR-208a at day 10, as compared to control iPSCs at the same time point (Fig. 5A,B, asterisks).

Taken together, our data show that SHZ (5 μM) increases the expression of cardiac markers and cardiomyogenic miRNAs, cardiomyocyte yield and beating foci rate, without affecting iPSC viability or proliferation rate during differentiation.

DISCUSSION

Recently, cardiomyogenic commitment of iPSCs has gathered great attention as a potential patient-specific source of in vitro cardiomyocytes, disposable for drug screening, personalized analyses, and therapeutics. Nevertheless, cardiac differentiation of iPSCs still presents challenges and opportunities for optimization.

In this perspective, differentiation efficiency of multi- and pluripotent cells can increase through addition of small chemical compounds, acting as chromatin remodelers. These molecules are

generally not expensive, available in relatively large quantities and poorly immunogenic. Moreover, given that ESCs and iPSCs are intrinsically prone to differentiate into cardiomyocytes in vitro, chemical chromatin remodelers, capable of improving this natural commitment, may constitute a novel strategy for clinic-related cardiac cell differentiation. Noteworthy, SHZ (5 μM) was not able to increase proliferation rate of either proliferating iPSCs or cardiomyocytes and this is consistent with literature [Sadek et al., 2008].

However, the effectiveness and reliability of these compounds must be carefully addressed, in terms of cell viability and mechanisms of action. Our results show that both bpV and SHZ were able to increase early and late cardiac marker expression in a dose-dependent fashion, but high concentrations of both molecules induced an apoptosis-driven loss of viability during mid/late differentiation stages. This could explain, at least in part, the lower cardiomyocyte yields at higher concentrations of bpV and SHZ.

It may be possible that bpV-induced apoptosis in iPSCs could be related to the overactivating effects on NF-κB/IκBα pathway, given that bpV induces IκBα tyrosine phospshorylation and p65 nuclear translocation in adult progenitors [Castaldi et al., 2007]. However, further studies are required to elucidate the molecular mechanisms of iPSC apoptosis, driven by high concentrations of bpV, SHZ, and other chemical remodelers.

Furthermore, the effects on viability and differentiation of such molecules could vary depending on the cell sources of tested iPSC lines. Recently, it has been demonstrated that a residual epigenetic memory of reprogrammed cells can affect iPSC commitment and
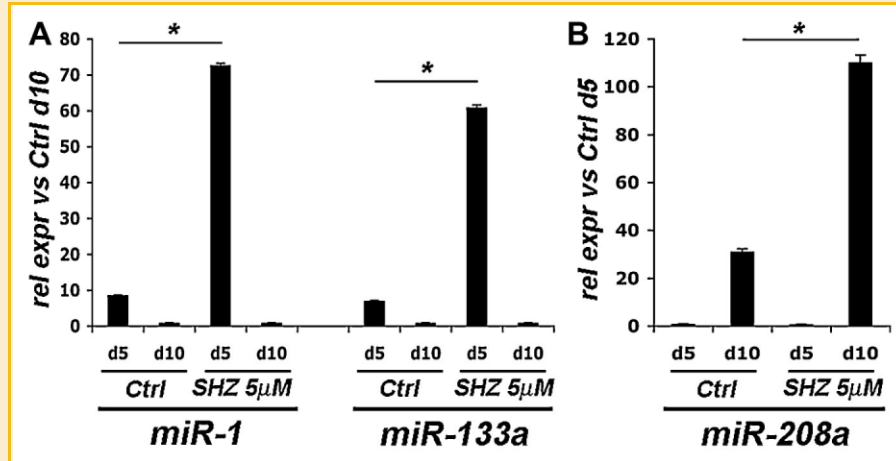
Fig. 5. SHZ induces cardiac miRNA expression. Taqman quantitative assay shows an approximately seven–fold upregulation of *miR–1* and *miR–133a* expression in SHZ–treated (5 μM) iPSCs at day 5, as compared to control iPSCs (A). In addition, *miR–208a* expression is approximately five–fold upregulated in SHZ–treated (5 μM) iPSC at day 10, as compared to control iPSCs (B) (n = 3; values normalized to control cells at day 10 in A and to control cells at day 5 in B; internal standard, miR–16; *P < 0.01).

differentiation rates [Kim et al., 2010]. It would be interesting to compare inducers of cardiac differentiation, such as SHZ, on iPSC lines generated from different cell sources.

A fine tuning of cardiac miRNAs would be an important step towards more refined iPSC differentiation protocols in vitro [Liu and Olson, 2010]. Our results indicate SHZ as a suitable molecule to increase in vitro iPSC cardiac differentiation at low concentration, especially in terms of cardiomyogenic miRNA activation. SHZ (5 μM) was able to increase *miR-1* and *miR-133a* at early differentiation stages and, later, *miR-208a*. Relations between SHZ and cardiac miRNAs should be specifically investigated in order to assess whether SHZ induces their overexpression per se or operates via an intrinsic cell feedback from *Nkx2.5/Gata4* activation. In fact, in ESCs, *miR-1* and *miR-133a* are able to promote cardiomyocyte specification by *Nkx2.5* suppression, following its upregulation during an earlier step [Takaya et al., 2009]. Our data are not sufficient to rule out one of the following hypotheses, that *miR-1*, *miR-133a*, and *miR-208a* are upregulated (i) by a direct SHZ-induced effect or, alternatively, (ii) by a cell-mediated effect that occurs downstream of a strong induction of *Nkx2.5* and *cardiac MyHC* (*Myh6*).

Recently, it has been showed that *miR-1* and *miR-133* act, respectively, as pro-apoptotic and anti-apoptotic factors in oxidative stress of terminally differentiated cardiomyocytes [Xu et al., 2007]. It is possible that, at high concentrations, SHZ could unbalance miR-1/miR133 ratio in differentiating iPSCs, leading to the observed increased apoptosis rate.

Although further molecular mechanisms are yet to be clarified, it is, however, interesting to notice that, at least in principle, SHZ and cardiac miRNAs may share similar mechanisms, namely to target endogenous chromatin-remodeling factors (such as HDACs) to shift expression patterns and promote subsequent differentiation.

In conclusion, our results shed a promising light on small chemical compounds, such as SHZ, in iPSC cardiac differentiation. Future optimizations on human iPSCs and on 3D-scaffolds or bioreactors could provide reliable, clinic- or industry-suitable protocols for patient-specific cardiomyocytes.

ACKNOWLEDGMENTS

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