Elmadbouh
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Generation of muscle progenitors from human-induced pluripotent stem cells

Ibrahim Elmadbouh*

Abstract

Background: Small molecules have a role in the differentiation of human-induced pluripotent stem cells (hiPSCs) into different cell linages. The aim of this study was to evaluate the differentiation of hiPSCs into cardiac or skeletal myogenic progenitors with a single small molecule.

Methods: hiPSCs were treated with three different small molecules such as Isoxazole-9, Danazol and Givinostat in serum-free medium for 7 days. Cell viability, qRT-PCR, western blots, and immunostaining were assessed after treatment of hiPSCs with small molecules.

Results: Higher hiPSC viability was observed in hiPSCs treated with Isoxazole-9 (25 μM), Danazol (25 μM) and Givinostat (150 nM) versus control ($P < 0.05$). Givinostat had dual effect by generating both skeletal and cardiac progenitor cells versus Isoxazole-9 and Danazol after 7 days. Givinostat treatment induced upregulation of skeletal myogenic genes and their protein expression levels on day 4 and further increased on day 8 ($P < 0.05$) versus control. Furthermore,positive stained cells for Pax3, Myf5, MyoD1, dystrophin, desmin, myogenin, and β-catenin at 1 month. Givinostat increased upregulation of cardiac gene expression levels versus control after day 4 ($P < 0.05$), with positive stained cells for Nkx2.5, GATA4, TnT, TnI, connexin 43 and α-sarcomeric actinin at 1 month.

Conclusions: Pretreatment of hiPSCs with Givinostat represents a viable strategy for producing both cardiac/skeletal myogenic progenitors in vitro for cell therapies against myocardial infarction and Duchenne muscular dystrophy.

Keywords: Givinostat, Pharmacological preconditioning, Differentiation of hiPSCs, Cell signaling

Introduction

Most tissues have endogenous stem/progenitor cells which, upon injury or disease to the organ, can proliferate and differentiate at the damaged site [1]. The adult heart is composed mainly of postmitotic and terminally differentiated cells [2], with a limited intrinsic capacity to regenerate lost or damaged myocardium that causes heart failure [3]. Also, mature skeletal myocytes cannot be expanded in large quantity in vitro culture, and the self-renewing stem cell population in muscle; satellite stem cells become activated and differentiate when introduced to in vitro culture [4], with a limited capacity to regenerate lost or damaged myofibers in muscle injury or muscular dystrophy [5, 6]. Duchenne muscular dystrophy (DMD) is X-linked genetic disease, caused by mutations in dystrophin gene that leads to a complete absence of the dystrophin-associated protein complex (DAPC) [4], loss of myofibers which are ultimately replaced by fibrotic scars and fat deposition [5, 6] and reduced nitric oxide (NO)-mediated NO-dependent S-nitrosylation in myofibers [4].

Gene and cell therapy approaches are currently being used for treating muscular dystrophy or myocardial ischemia/infarction but are still far from clinical translation [7, 8]. Currently, various myogenic progenitors have been isolated from pre- or postnatal skeletal/cardiac muscles as well as nonmuscle somatic tissues [9]. Human induced pluripotent stem cells (hiPSCs) are available alternative to human embryonic stem cells (hESCs)

*Correspondence: ibrahim.elmadbouh@gmail.com

Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Menoufia University, Shebin Elkom, Egypt

as an autologous cell source (fibroblast or nonmuscle somatic tissue) for therapeutic and research applications [10]. Generation of iPSCs from patients with DMD and their induction to cardiomyocytes [5]. Cardiomyocytes or skeletal myoblasts derived from hESCs or hiPSCs are a potential source for cell replacement therapy [3] and useful tool in the investigation of cardiac/skeletal development, disease modeling, and drug testing [8]. Despite considerable progress, the efficiency of differentiating stem cells toward neomyogenesis has been very challenging [11].

Chemical biology (small molecules or combination of signaling molecules) approaches using readily available and inexpensive synthetic bioactive molecules that regulate stem cell fate could potentially rectify these problems and mimic the developmental cues that can induce cardiogenesis/ myogenesis in vitro [3, 12–17]. However, the generation of a homogeneous population of cardiomyocytes/skeletal remains an important limitation of the current methodologies. Since hiPSCs or hESCs are pluripotent cells with the ability to differentiate into specialized cells in response to appropriate signals using small molecules [9].

We selected 3 small molecules such as Isoxazole 9 (a synthetic promotor of adult neurogenesis) [14, 18], Danazol (an androgen similar to testosterone) [19] or Givinostat (a histone deacetylase inhibitor, HDACi) [4] to induce differentiation of hiPSCs to myogenic progenitors for muscle regeneration in myocardial infarction or DMD. Preclinically, HDACi (Givinostat) have been shown to potentiate myogenesis and the gene expression profile selectively in proliferating myoblasts and not in terminally differentiated myotubes in DMD [4], and cardioprotective effect in myocardial repair during preconditioning of donor stem cell with Givinostat [20]. An oral administration of Givinostat in the treatment of DMD has an epigenetic drug tested in a Phase I/II clinical trial on DMD boys between 8 and 10 years of age [21].

Therefore, the main objective of this study was to differentiate hiPSCs into cardiac/skeletal myogenic progenitors with a single small molecule for cell therapy.

Material and methods

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), University of Illinois at Chicago and The Ohio State University, USA.

Human iPSCs culture

ATCCCYS0105 human iPSCs were purchased from American Type Culture Collection (ATCC® ACS-1021™, Manassas, USA). This cell line derived from primary cardiac fibroblasts obtained from a healthy donor. Fibroblasts were reprogrammed by the expression of *OCT4, SOX2, KLF4* and *MYC* gene sequences using Sendai viral transduction. hiPSCs were identified by staining with anti-human TRA-1-60, SSEA4 and Oct4 antibody-positive cells (Fig. 1A, B).

Human iPSCs culture with 3 small molecules

Human iPSCs were cultured on vitronectin-coated plates with mTeSR™1 medium (Stemcell™ Technologies Inc, USA) at 37 °C with humidified air with 5% CO2. To induce differentiation of hiPSCs with 3 small molecules: Isoxazole 9 (ISX9, 10–50 μM, Stemcell™, Technologies Inc, USA), Danazol (DAN, 10–50 μM, Sigma-Aldrich, USA) or Givinostat (GIV, ITF2357, 10–200 nM, Stelleckchem, USA) were cultured in mTeSR™1 medium. Cell viability (CCK8 Assay) was assessed after 2 days with 3 small molecules treatment. The nontoxic concentration of pretreatment hiPSCs was ISX9 (25 μM), DAN (25 μM) and GIV (150 nM) for subsequent experiments and was assessed after 7 days for western blots and Real-time qPCR as below.

Skeletal myoblast differentiation

To induce differentiation of hiPSCs with small molecule, Givinostat (150 nM) was added in mTeSR™1 medium containing Rho-associated kinase (ROCK) inhibitors as 5 μM Thiazovivin (Stelleckchem, USA) for skeletal muscle specification [22]; and was assessed after 4–8 days for western blots and Real-time PCR. To induce terminal differentiation of iPSCs to skeletal myoblast after treatment with small molecule, medium was changed to STEMdiff™ APEL™ medium (StemCell Technology, USA) with 2% vol/vol horse serum (Life Technologies, USA) and insulin transferrin selenium ethanolamine (ITSE animal free, InVitria, USA). Cells were fed every 2 days. The hiPSCs-differentiated skeletal progenitor cells were followed up with PCR, western blots, and immunostaining to detect the mature cells.

Cardiomyocyte differentiation

To induce cardiac differentiation of iPSCs with small molecule, Givinostat (150 nM) was added in RPMI medium (Gibco, Life Technology) containing B27 minus insulin supplement (B27C; Life Technologies) with ALK4/5/7 (TGFβ type-I receptor) inhibitors as 2 μM SB431542 (Stelleckchem, USA) for cardiac progenitor specification [22]; and was assessed after 4–8 days. After 7 days from treatment, the medium was changed to RPMI medium (containing B27 complete supplement (B27C; Life Technologies). Thereafter, cells were fed every 2 days with RPMI medium containing B27 complete supplement.

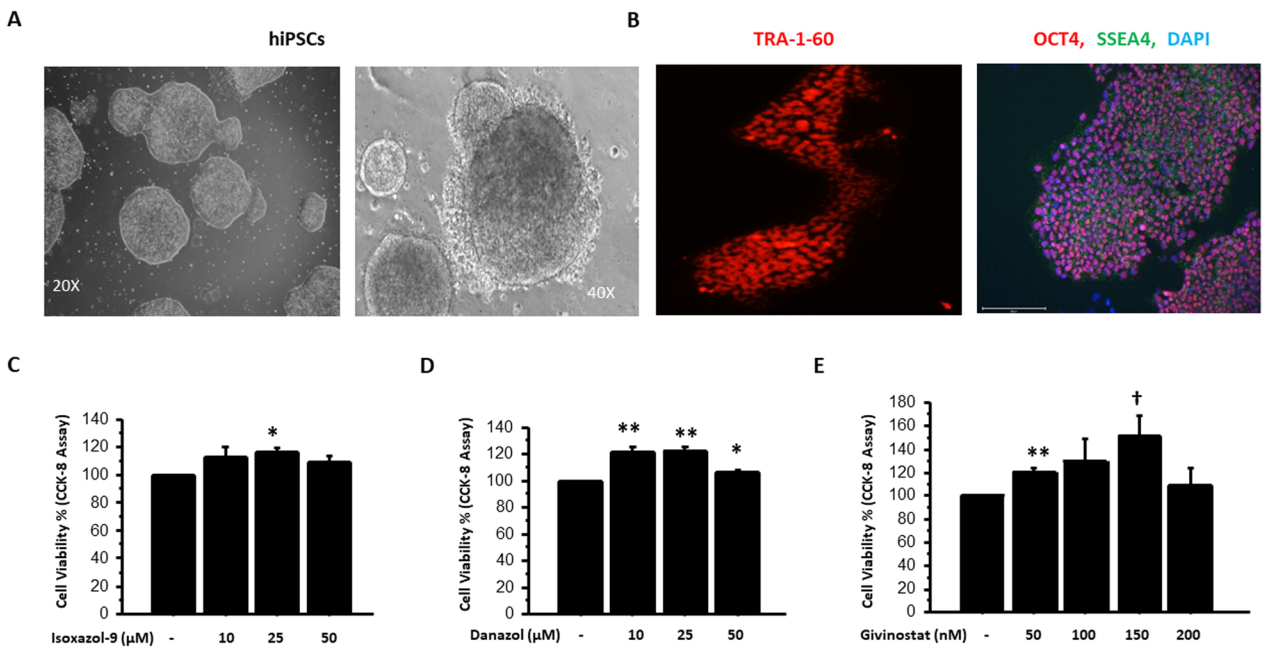

Fig. 1 Identification of human induced pluripotent stem cells (hiPSCs) and the effect of small molecules treatment on in vitro hiPSCs culture: Representative microscopic picture of hiPSCs (20X and 40X, **A**) were identified by immunostaining markers TRA-1-60 (Red), OCT4 (Red) and SSEA4 (green) with DAPI (blue for nuclei, **B**). hiPSCs viability were assessed by CCK-8 after treatment with Isoxazole (ISX-9, 10–50 μM, **C**), Danazol (DAN, 10–50 μM, **D**) and Givinostat (GIV, 10–200 nM, **E**). Higher cell viability (CCK8 kit) was observed with using ISX9 (25 μM), DAN (25 μM) and GIV (150 nM). Values are mean ± SE; $n = 6$; † *P*-value < 0.05, * *P*-value = 0.01, ** *P*-value = 0.001, $^{\#}$ *P*-value = 0.0001 are significant

The hiPSCs-differentiated cardiac progenitor cells were followed up with PCR and immunostaining to detect the mature cells.

Real-time PCR analysis for gene expression

RNA was isolated from hiPSCs. Total RNA was isolated using RNeasy kit (Qiagen) according to manufacturers' protocols. Quantitative reverse transcription PCR (qRT-PCR) was performed using the Applied Biosystems set of products. The cDNA was prepared using the miScript PCR Starter Kit (Reverse Transcription kit, HiFlex Buffer, Qiagen). The quantitative real-time RT-PCR was performed using the QuantiTect SYBR Green PCR Master mix which is provided in miScript SYBR Green PCR kit (Qiagen) according to the protocol for the Applied Biosystems 7500 Real-Time PCR System. All primers were used as in Table 1. For quantification analysis, the comparative threshold cycle (Ct) method using TaqMan Gene Expression Assays was used. The Ct values of each gene were normalized to the Ct value of GAPDH in the same RNA sample. The gene expression levels were evaluated by the fold change using the equation 2^{-ddCt}.

Western blot analysis

hiPSCs treated with small molecules were collected by using cell culture scraper in Pierce® RIPA buffer (Thermo Fisher Scientific, USA). Protein was measured using Pierce™ BCA protein assay kit (Thermo Fisher Scientific, USA). Protein samples (40 μg) were fractionated by 12% SDS–polyacrylamide gel electrophoresis (ISC BioExpress) and electro-transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h with 1xTBS blocking buffer (Cell Signaling Technology) and 5% nonfat dry milk, followed by incubation with gentle shaking at 4 °C with the primary antibodies diluted in blocking buffer. The primary antibodies used in western blot included: MyoD (1:200, Santa Cruz Biotechnology, sc-377460), Pax7 (1:200, Abcam, ab199010); Pax3 (1:200, Abcam, ab180754), myosin (1:100, Abcam, ab124205), β-catenin (1:100, Abcam, ab6302), desmin (1:500, Abcam, ab8592), myogenin (1:100, Santa Cruz Biotechnology, sc-52903), dystrophin (1:100, Abcam, ab85302), Myf5 (1:100, Invitrogen Thermo Fisher Scientific, PA1-24,450), Nkx2.5 (1:200, PA5-49,431), α-sarcomeric actin (1:500, MA1-21,597), troponin I or T (1:200, Invitrogen, Thermo Fisher Scientific, 701,585 and MA1-16,687, respectively), VE-cadherin (1:500, Abcam, ab232880) and GATA4 (1:200, Santa Cruz Biotechnology, sc-25310). GAPDH (1:100, Santa Cruz Biotechnology, sc-47724) served as the loading control. The membrane was washed 3 times for 5 min each with 1xTBS blocking

Table 1 The PCR primers for real-time PCR (Integrated DNA Technologies, IDT)

Gene product	Primer forward	Primer reverse	Product size (bp)
Myf5	GAGAGAGCAGGTGGAGAACT	TGGACCAGACAGGACTGTTA	116
Desmin	TACACCTGCGAGATTGACGC	CTGGCAAATCGGTCCTCCAA	83
Dystrophin	TCAGGAGAAAGATGCTGTTTTGC	GGTTCTCAATATGCTGCTTCCC	182
B-Actin	CACAGAGCCTCGCCTTTGC	GAGCGCGGCGATATCATCA	80
PAX7	TGGCCGGCTATCAGTACG	CAGAGTGCTCCCCGAGCTT	105
MYOD	AAGGCGCCTACTACAACGAG	AGGCAGTCTAGGCTCGACAC	76
MYOGENIN	TCAACCAGGAGGAGCGTGA	TCTGTAGGGTCAGCCGTGAG	166
MYH-2	CTTTCAAGAGGGACACTGGTCA	TAGCCTGGGAGTGAGACAGTT	77
GAPDH	TTAGGAAAGCCTGCCGGTGA	GGCGCCCAATACGACCAAA	157
Meox1	CAGGACGAACTCCTCGTCAG	GGCTGAGAAGTCAGGGTACG	74
Meox2	TGGTTCCAAAACAGGCGGAT	ACCAGTTCCTTTTCCCGAGC	80
PAX3	CGGCTAGCTGACATTGGTGA	TCCATGTCGTTACTCAGGCG	96
Hand1	ATATAAGCCAGATCCGCAGGG	TTCTTACCGGTTTCTGCCGC	90
Hand2	ATTCCGGGTTAGCTTCGGTG	GGAGGCAGAATCCTCTCGTG	117
Mesp1	CGAGTCCTGGATGCTCTCTG	ATGAGTCTGGGGACGAGACG	98
DKK1	CGGGCGGGAATAAGTACCAG	GGGACTAGCGCAGTACTCATC	95
Cdh4	CAAGGATTCGATGAACGGCA	ACACAGCTGCTGAACTTGACT	87
Lhx2	AACTTCTGTGCCTGGCAACC	AAGAAATTGTGGGGTGAGGGG	107
Myl7 (Mlic-2a)	GGAGTTCAAAGAAGCCTTCAGC	TAGGTCTCCCTCAGGTCTGC	78
Nppa (ANP)	AGCAGTGGATTGCTCCTTGA	GACACGGCATTGTACATGGG	128
Pitx2	CTCCTGTTCGACCGCCC	AGCCCTGACAGAGAAGTGTC	151
Podxl	AACGAAATCTTGGCCTCGGT	TTGCTGAGCCAGGGTTGTAG	81
Tbx1	CGAGGAGACACGATTCACCG	TTGCTGGCAATCTTGAGCTG	72
Tcf15	TGGTCAGAGACAAGGCAGAAC	TCTCCTCAGCTCAGAACGAC	157
Tnnt2	CATCCAGAAGACAGAGCGGAAAAG	CCTCCTCTCAGCCAGAATCTT	79
Isl1	TTTCCCTGAATCTCCCCACTC	ACTACACTGAGGCAAACTTCACA	76
NKX2.5	GTATCCGAGCCTGGTAGGGA	GGACTCAGGGTCATGTTGGG	112
GATA4	TGGAAGCACCAGGAAGCATT	CAGCCGGTTACAGACGAGAG	135
Mef2c	GCAAAGCCTCGGTCTTCATAGA	ACCAGCTGGCTTTGAAGAGAA	92
Tbx5	TCAGTCCCCCGGAACAACTC	GGAACTCTTCACGAAGGGAGG	77
Mlc2v (Mylk3)	AGCTTGCACAGATCTTGTCT	CAAACTTAAACCTGGAGGCAGT	70
Myh6	AGATAGAGAGACTCCTGCGGC	TCTGGGCATCGGTCATCTTG	87
Connexin 43	TCTGAGTGCCTGAACTTGCC	GGGCACCACTCTTTTGCTTA	97

buffer and 0.1% Tween® (TBS/T). The primary antibody reaction was detected by incubating for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000, Cell Signaling Technology) diluted in TBS/T with 5% nonfat dry milk. The membrane was washed and developed using SuperSignal™ West Pico Plus chemiluminescent substrate (Thermo Fisher Scientific, USA). The chemiluminescent signal from blots has been detected by FluorChem™ E System (Thermo Fisher Scientific, USA), and was quantitatively assessed by densitometer.

Immunostaining for characterization of skeletal and cardiac markers

Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 20 min and blocked in 10% goat serum and 0.01% Triton X-100 in PBS for 1 h. The primary antibodies used in immunostaining included: TRA-1-60 (1:50, Thermo Fisher Scientific), Oct4 (1:400, Invitrogen Thermo Fisher), SSEA4 (1:50, Invitrogen Thermo Fisher), MyoD (1:50, Thermo Fisher Scientific), Pax3 (1:50, Abcam), myosin (Skeletal, slow or fast) (1:100, Sigma), β-catanin (1:100, Abcam), desmin (1:50, Abcam), myogenin (1:100, Thermo Fisher

Scientific), dystrophin (diluted solution, Abcam), Myf5 (1:50, Invitrogen, Thermo Fisher Scientific), Nkx2.5 (1:25, Invitrogen), α-sarcomeric actin (1:200, Sigma), troponin I or T (1:300, Invitrogen, Thermo Fisher Scientific), VE-cadherin (1:300, Invitrogen) GATA4 (1:100, Thermo Fischer scientific) and connexin 43 (1:75, Cell Signaling Technology). Primary antibodies were added and incubated overnight at 4 °C, washed and secondary antibodies were added for 1 h. Secondary antibodies used were anti- Rabbit or mouse IgG Fb2 Alexa Fluor 488 or 594 (1:1000, Molecular Probes, Life Technology™). Cell nuclei were stained by NucBlue™ live cell (Molecular Probe, Life Technology™) and covered with Vecta Mount™ medium (Vector Laboratories, CA, USA).

Flow cytometry for troponin-T cardiac markers

Cells were detached as described previously and washed in phosphate-buffered saline (PBS)-1% bovine serum albumin (BSA). Cells were then centrifuged at 5000 g for 2 min before 1×10^5 cells were resuspended in the appropriate primary antibody Alexa Fluor® 647-conjugated cardiac troponin T (1:10, Fisher Scientific, BDB565744) in flow buffer and incubated for 1 h at 48C. For unconjugated antibodies, cells were then washed and resuspended in FITC conjugated anti-mouse (1:10, Invitrogen, Thermo Fisher Scientific 12-7691-82) for 30 min at 48C. Cells were then analyzed using a Becton Dickinson FACS caliber flow cytometer (BD biosciences) using Cell Quest Pro and Flow Jo software.

Statistical analysis

All experiments were performed at least 3 times, and all variable data were expressed as mean ± SEM using IBM SPSS® Statistic version 20. Student's *t*-test was used for comparison of unpaired data between small molecules and control groups. One-way analysis of variance (ANOVA) was used to compare unpaired data between 3 or more groups; followed by Bonferroni post hoc test for comparisons between corresponding time points in the two groups. Discrete variables were expressed as percentage and the Chi-square or Fisher's exact test was used accordingly. A *P*-value of < 0.05 was considered statistically significant.

Results

Firstly, the cytotoxicity of 3 molecules was assessed with different concentration for 48 h. Higher cell viability (CCK8 kit) was observed by using ISX9 (25 μM, $P = 0.001$), DAN (25 μM, $P = 0.001$) and GIV (150 nM, $P < 0.05$) versus controls (Fig. 1C, D).

Givinostat has dual expression of cardiac/skeletal myogenic genes and proteins versus ISX9 and danazole

GIV treatment increased upregulation of cardiac/skeletal myogenic genes (Real Time PCR Assay) versus ISX9, DAN and control after 7 days ($P < 0.05$) (Fig. 2). GIV increased muscle-specific genes such as Myf5 (134.3-fold, $P = 0.0001$), Pax7 (53.2-fold, $P = 0.0001$), myogenin (15.6-fold, $P = 0.0001$), desmin (8.3-fold, $P = 0.001$), MyoD1 (3.5-fold, $P = 0.001$), β-catenin (3.2-fold, $P = 0.001$) and dystrophin (2.8-fold, $P = 0.01$) versus controls (Fig. 2A). Also, GIV increased cardiac-specific genes such as Tbx5 (37-fold, $P = 0.0001$), Nkx2.5 (29.8-fold, $P = 0.0001$), Isl1 (16.7-fold, $P = 0.0001$), GATA4 (0.8-fold), Mef2c (0.1-fold), Myh6 (0.1-fold) and Mlc2v (0.1-fold) versus controls (Fig. 2B).

On the other hand, ISX9 treatment increased skeletal muscle genes such as Pax7 (17.2-fold, $P = 0.0001$), Myf5 (8.7-fold, $P = 0.001$), myogenin (4.5-fold, $P = 0.001$), desmin (2.3-fold, $P = 0.01$), dystrophin (1.2-fold, $P < 0.05$), and β-catenin (0.9-fold), MyoD1 (0.5-fold) versus

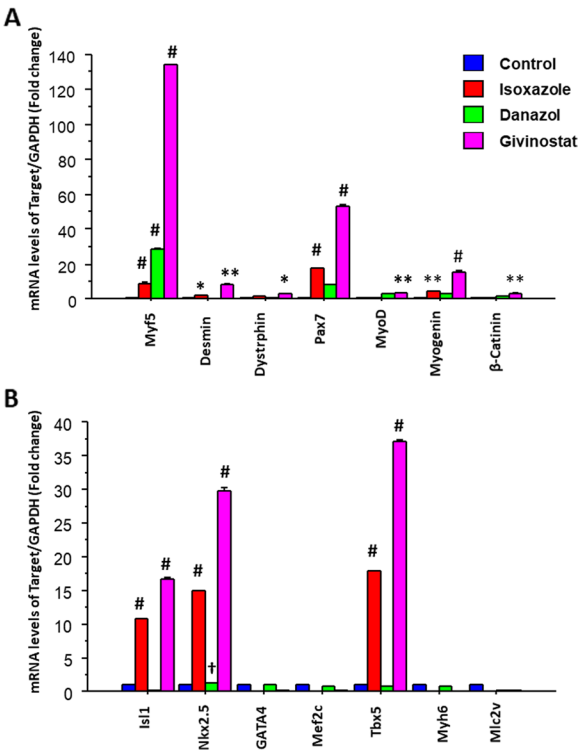

Fig. 2 Givinostat has dual expression of cardiac/skeletal myogenic genes: GIV increased upregulation of cardiac/skeletal myogenic genes (Real Time PCR Assay) versus ISX9, DAN and control after 7 days. **A** Muscle genes included Myf5, Pax7, MyoD1, myogenin, dystrophin, desmin and β-catenin. **B** Cardiac gene included Isl1, Nkx2.5, Tbx5, GATA4, Mef2c, Myh6 and Mlc2v. Values are mean ± SE; $n = 3$; † *P*-value < 0.05, * *P*-value $= 0.01$, ** *P*-value $= 0.001$, # *P*-value $= 0.0001$ are significant

controls (Fig. 2A). Also, ISX9 increased cardiac genes such as Tbx5 (17.9-fold, $P=0.0001$), Nkx2.5 (14.9-fold, $P=0.0001$), Isl1 (10.8-fold, $P=0.0001$), GATA4 (0.1-fold), Mef2c (0.1-fold), Myh6 (0.04-fold) and Mlc2v (0.03-fold) versus controls (Fig. 2B).

Similarly, DAN increased muscle genes such as Myf5 (28.6-fold, $P=0.0001$), Pax7 (7.9-fold, $P=0.001$), MyoD1 (2.9-fold, $P=0.01$), myogenin (2.8-fold, $P=0.01$), β-catenin (1.2-fold, $P<0.05$), dystrophin (1.03-fold, $P>0.05$) and desmin (0.3-fold) versus controls (Fig. 2A). Also, DAN increased cardiac genes such as Nkx2.5 (1.2-fold, $P<0.05$), GATA4 (0.9-fold), Mef2c (0.9-fold), Tbx5 (0.7-fold), Myh6 (0.7-fold), Isl1 (0.2-fold) and Mlc2v (0.2-fold) versus controls (Fig. 2B).

GIV treatment demonstrated dual effect of skeletal and cardiac myogenic protein expression (Western Blot Assay) from hiPSCs versus ISX9, DAN and control after 7 days ($P<0.05$) (Fig. 3A, B).

MyoD was significantly higher expressed with GIV versus ISX9 ($P=0.001$), DAN ($P=0.001$), and control ($P=0.001$). Pax3 was significantly higher expressed with GIV versus ISX9 ($P=0.001$), DAN ($P=0.001$), and control ($P<0.001$). Pax7 was significantly higher expressed with GIV versus DAN ($P=0.001$), and control ($P=0.01$), but non-significant higher versus ISX9 ($P>0.05$). Myosin was significantly higher expressed with GIV versus DAN ($P=0.01$), and control ($P=0.01$), but non-significant higher versus ISX9 ($P>0.05$). β-catenin was significantly higher expressed with GIV versus DAN ($P=0.001$), and control ($P=0.01$), but non-significant higher versus ISX9 ($P>0.05$). Desmin was significantly higher expressed with GIV versus DAN ($P=0.0001$), and control ($P=0.001$), but significantly lower than ISX9 ($P=0.01$). But cardiac genes such as NKX2.5 was non-significantly expressed in GIV versus ISX9, DAN and control ($P>0.05$) (Fig. 3A, B).

Givinostat-induced skeletal myogenic progenitor genes and proteins

GIV increased upregulation of skeletal myogenic genes in hiPSCs-derived skeletal myoblast in first 4 days and further increased after 8 days versus control ($P<0.05$) (Fig. 4A). GIV increased skeletal myogenic genes on Day 4 such as Myf5 (1.8-fold, $P=0.01$), myogenin (1.7-fold, $P=0.01$), Tcf15 (1.4-fold, $P>0.05$), desmin (0.9-fold), Myh6 (0.9-fold), Mesp1 (0.8-fold), MyoD1 (0.7-fold), dystrophin (0.7-fold), β-catenin (0.6-fold), Meox1 (0.6-fold), Meox2 (0.6-fold), Pax3 (0.5-fold), Pax7 (0.4-fold), Tbx1 (0.4-fold) and Myh2 (0.3-fold) versus control. Whereas, GIV increased much higher expression of skeletal myogenic genes on Day 8 such as Pax3 (27.7-fold, $P=0.0001$), Meox1 (21.9-fold, $P=0.0001$), Myf5 (6.3-fold, $P=0.001$), myogenin (6.3-fold, $P=0.001$), Myh2 (5.8-fold, $P=0.001$), Meox2 (4.1-fold, $P=0.001$), dystrophin (3.9-fold, $P=0.001$), Myh6 (3.7-fold, $P=0.001$), Tcf15

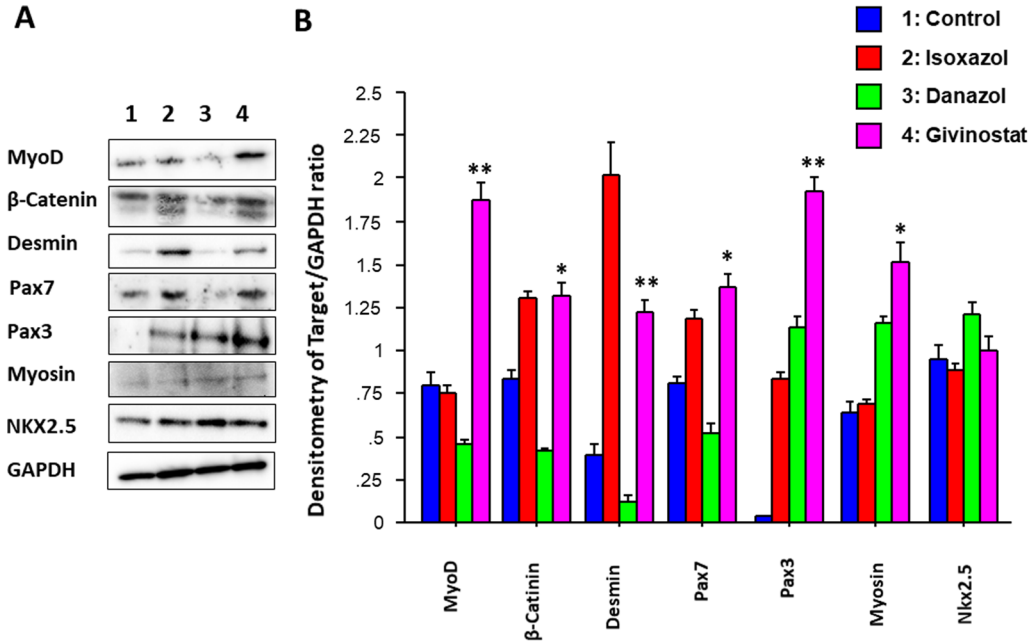

Fig. 3 Givinostat has dual expression of cardiac/skeletal myogenic proteins: GIV had dual cardiac myogenic progenitors and skeletal myogenic progenitor's developments (Western Blot Assay) from hiPSCs versus ISX9, DAN and control after 7 days. Muscle protein expression included Pax3, Pax7, MyoD, myosin, desmin and β-catenin. Cardiac protein expression as Nkx2.5. Values are mean ± SE; $n=3$; † *P*-value < 0.05, * *P*-value = 0.01, ** *P*-value = 0.001, # *P*-value = 0.0001 are significant

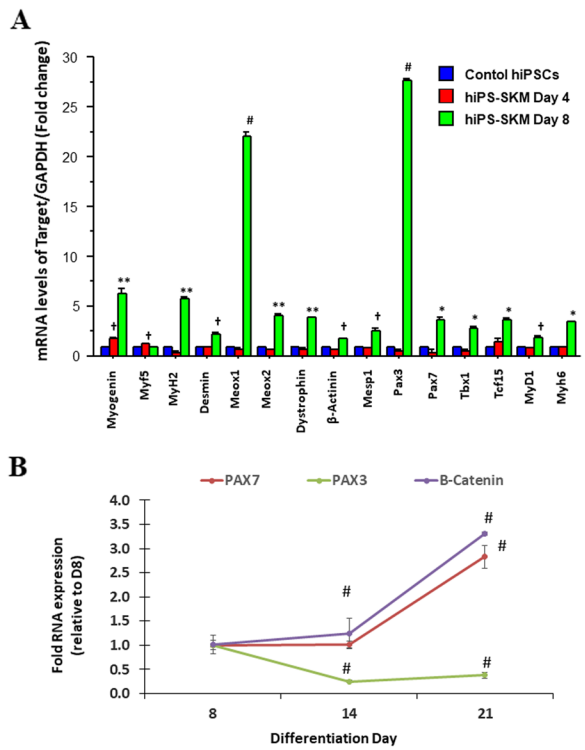

Fig. 4 Givinostat induce higher expression of skeletal myogenic genes: Skeletal muscle genes included Meox1, Meox2, Tcf15, Pax3, Pax7, MyoD1, dystrophin, myogenin, Myh2, Myh6, Tbx1, Mesp1, desmin and β-catenin. GIV increased upregulation of skeletal myogenic genes (Real Time PCR-analysis) in skeletal iPSCs (hiPSC- SKM) at first 4 days and further increased after 8 days versus control (**A**). Skeletal maturity genes were followed up at day 14 and Day 21 with normalized to day 8 (**B**). Values are mean ± SE; $n = 3$; † P-value < 0.05, * P-value = 0.01, ** P-value = 0.001, # P-value = 0.0001 are significant

(3.6-fold, $P = 0.001$), Pax7 (3.5-fold, $P = 0.01$), Tbx1 (2.8-fold, $P = 0.01$), Mesp1 (2.6-fold, $P = 0.01$), desmin (2.1-fold, $P = 0.01$), MyoD1 (1.8-fold, $P < 0.05$) and β-catenin (1.7-fold, $P < 0.05$) versus control (Fig. 4A).

Skeletal maturity genes after 14 and 21 days were assessed for Pax3, Pax7 and β-catenin with normalized to day 8. Pax 3 gradually decreased on day 14 and day 21 versus day 8 ($P < 0.001$), but β-catenin and Pax 7 remained higher on day 14 and day 21 versus day 8 ($P < 0.001$) (Fig. 4B).

GIV increased expression of skeletal myogenic proteins that were assessed by western blots (A) and western blots densitometry (B) analysis after 4–8 days treatment of hiPSCs with GIV using antibodies of Pax3, Pax7, Myf5, MyoD1, dystrophin and desmin versus control ($P < 0.05$) (Fig. 5A, B).

MyoD was significantly higher expressed with GIV on Day 4 ($P = 0.001$) and Day 8 ($P = 0.001$) versus control, but non- significant difference between Day 4 and Day 8 ($P > 0.05$). Pax3 was significantly higher expressed with GIV on Day 4 ($P < 0.0001$) and Day 8 ($P < 0.0001$) versus control, and significantly higher on Day 8 than Day 4 ($P = 0.001$). Pax7 was significantly higher expressed with GIV on Day 4 ($P = 0.001$) and Day 8 ($P = 0.01$) versus control, but non- significant lower on Day 8 than Day 4 ($P > 0.05$). Myosin was significantly higher expressed with GIV on Day 4 ($P = 0.001$) and Day 8 ($P = 0.001$) versus control, and significantly higher in Day 8 than Day 4 ($P < 0.05$). Myf5 was significantly higher expressed with GIV on Day 4 ($P < 0.0001$) and Day 8 ($P = 0.001$) versus control, but non- significant higher on Day 8 than Day 4 ($P > 0.05$). Dystrophin was significantly higher expressed with GIV on Day 4 ($P < 0.0001$) and Day 8 ($P = 0.01$) versus control, but significant lower on Day 8 than Day 4 ($P = 0.01$). Desmin was significantly higher expressed with GIV on Day 4 ($P < 0.0001$) and Day 8 ($P < 0.001$) versus control and significantly lower in Day 8 than Day 4 ($P < 0.0001$). β-catenin was significantly higher expressed with GIV on Day 8 ($P = 0.001$) versus control, and significantly higher in Day 8 than Day 4 ($P = 0.001$), but non-significantly lower expression on Day 4 than control ($P > 0.05$) (Fig. 5A, B).

Morphologically, effect of GIV on differentiation of hiPSCs to myogenic cells was assessed under microscope. The myotube-shaped cells appear after 21 days versus control. Immunostaining analysis revealed that GIV can differentiate the hiPSCs into skeletal myogenic cells. Cells were positive for skeletal myoblast markers as Myf5, Pax3, MyoD, dystrophin, myosin, myogenin, β-catenin and desmin at 1 month (Fig. 6).

Givinostat-induced cardiac myogenic progenitor genes and proteins

GIV increased upregulation of cardiac muscle genes for cardiac iPSCs after 4 days versus control ($P < 0.05$) (Fig. 7A). GIV increased cardiac muscle genes on Day 4 such as Pitx2 (70.5-fold, $P < 0.0001$), Nkx2.5 (48.1-fold, $P = 0.0001$), Tbx5 (37.1-fold, $P = 0.0001$), Hand1 (14.9-fold, $P = 0.001$), MLC2v (9.3-fold, $P = 0.001$), Myl7 (7.5-fold, $P = 0.001$), GATA4 (5.5-fold, $P = 0.001$), ISl1 (4.9-fold, $P = 0.001$), Nppa (ANP, $P = 0.001$) (4.5-fold), Cdh4 (3.2-fold, $P = 0.001$), and Lhx2 (2.7-fold, $P = 0.001$), Mef2c (1.7-fold, $P > 0.05$), Podx1 (0.9-fold), TnnT2 (0.6-fold) versus control (Fig. 7A). Whereas, GIV increased much higher expression of cardiac muscle genes on Day 8 such as Pitx2 (57-fold, $P = 0.0001$), Nkx2.5 (29.6-fold, $P = 0.0001$), Myl7 (16.5-fold, $P = 0.001$), ISl1 (6.9-fold, $P = 0.001$), Hand1 (5.9-fold, $P = 0.001$), TnnT2 (5.7-fold, $P = 0.001$), MLC2v (5.4-fold, $P = 0.001$), Tbx5 (3.9-fold, $P = 0.001$), Cdh4

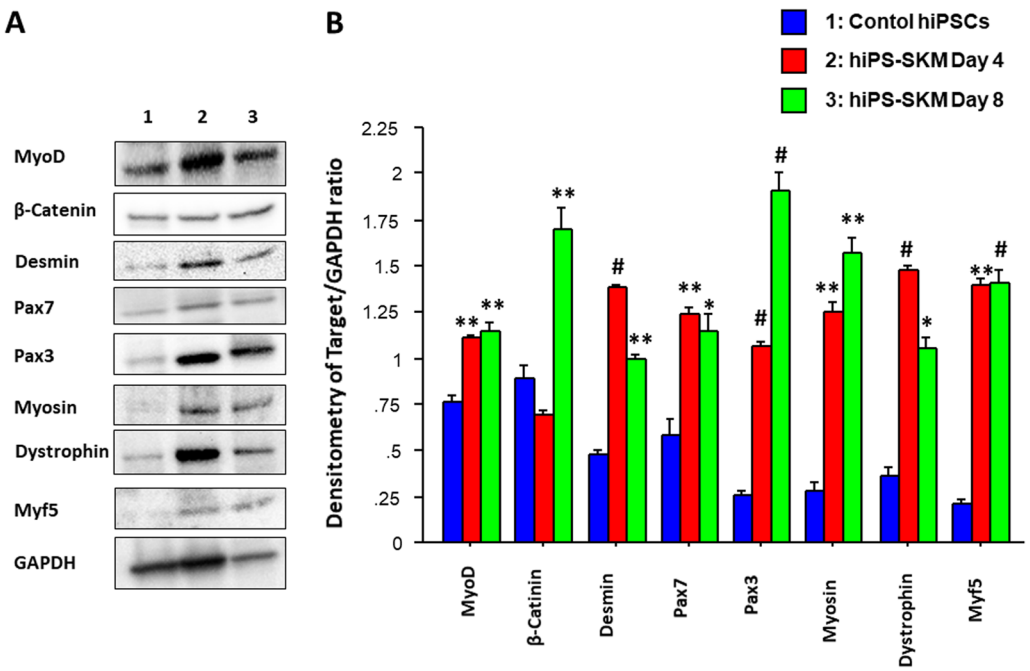

Fig. 5 Givinostat induce higher expression of skeletal myogenic proteins: Western blots (**A**) with densitometry (**B**) analysis after 4–8 days of (hiPSC-SKM) using antibodies of Pax3, Pax7, Myf5, MyoD, dystrophin, β-catenin and desmin versus control. Values are mean ± SE; $n = 3$; † *P*-value < 0.05, * *P*-value = 0.01, ** *P*-value = 0.001, # *P*-value = 0.0001 are significant

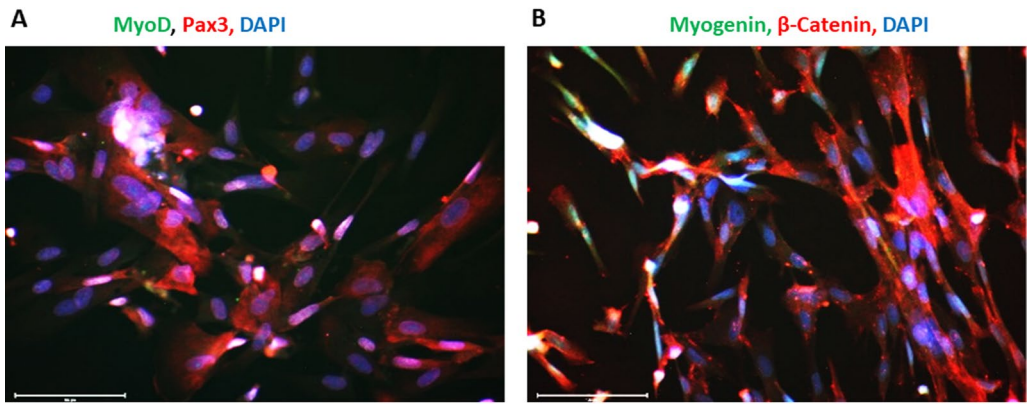

Fig. 6 Effect of GIV on differentiation of hiPSCs to skeletal myogenic cells: Effect of GIV on differentiation of hiPSCs to skeletal myogenic cells. Cells were positive for skeletal myoblast markers Pax3, MyoD, myogenin and β-catenin (40X) at 30 days after immunostaining

(2.9-fold, $P = 0.001$), Lhx2 (2.2-fold, $P = 0.001$), GATA4 (1.9-fold, $P = 0.01$), Nppa (ANP) (0.9-fold), Mef2c (0.6-fold) and Podx1 (0.07-fold) versus control (Fig. 7A).

Cardiac maturity genes after 14 and 21 days were assessed for Nkx2.5, GATA4, TnnT2 and connexin-43 with normalized to day 8. Nkx2.5 was gradually decreased on day 14 and day 21 versus day 8 ($P < 0.01$) and GATA4 was decreased on day 21 versus day 14 ($P < 0.05$), but TnnT2 was little decreased on day 21 versus day 8 and day 14 ($P < 0.05$) (Fig. 7B).

Morphologically, cardiomyocyte-like cells appear amongst differentiating cells after 21 days (Fig. 8). Immunostaining analysis revealed that GIV can differentiate the hiPSCs into cardiac myogenic cells. Cells were positive for different cardiac progenitor's markers such as Nkx2.5, GATA4, TnT, TnI, VE-cadherin,

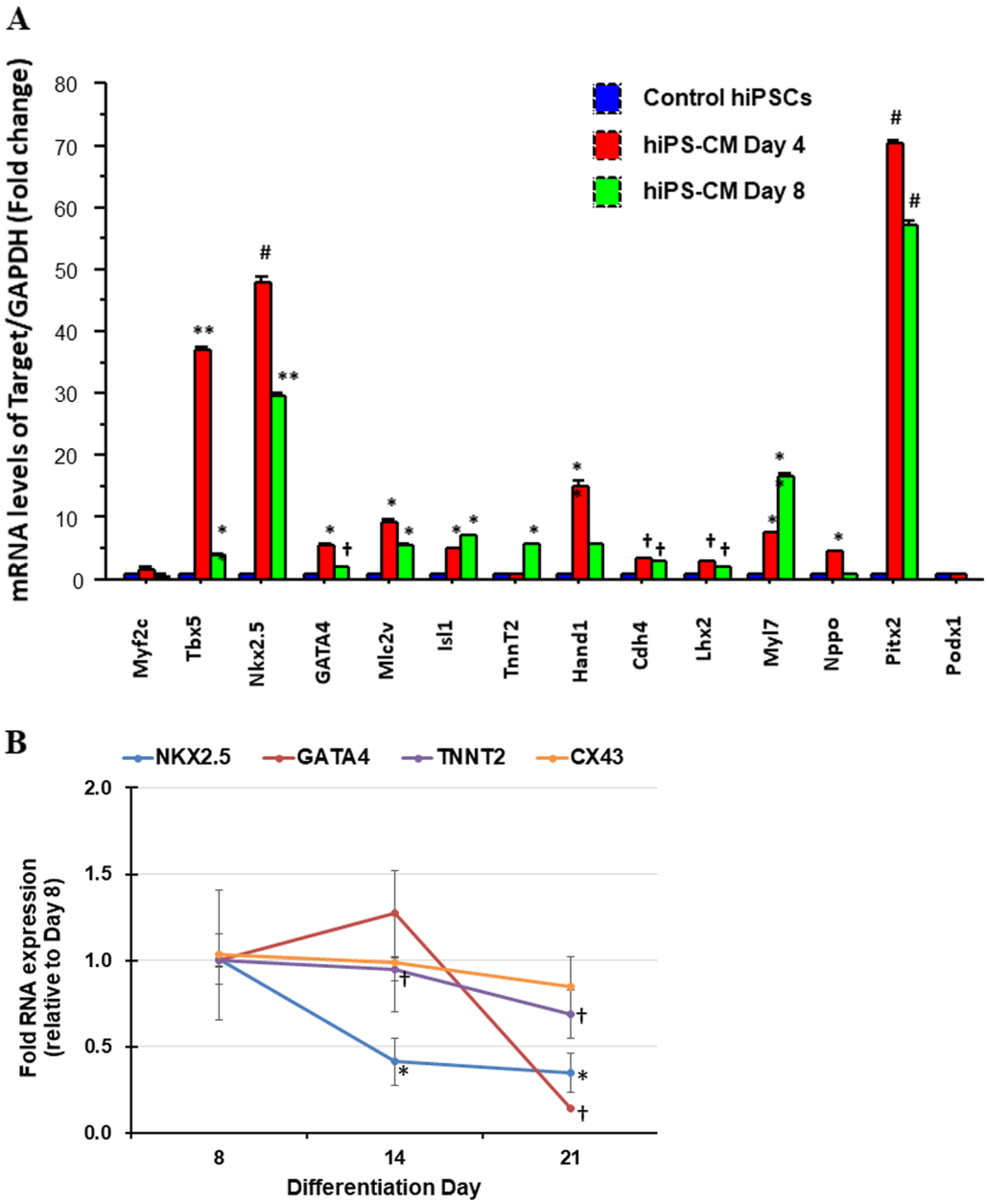

Fig. 7 Givinostat induce higher expression of cardiac myogenic genes: Cardiac genes included Pitx2, ISl1, Nkx2.5, Hand1, GATA4, Tbx5, TnnT2, Myl7, MLC2v, Mef2c, Cdh4 and Lhx2 versus control. GIV increased upregulation of cardiac muscle genes (Real Time PCR-analysis) for cardiac iPSCs (hiPSC-CM) after 4 days. Cardiac maturity genes were followed up at day 14 and Day 21 with normalized to day 8 (**B**). Values are mean ± SE; $n = 3$; † *P*-value < 0.05, * *P*-value = 0.01, ** *P*-value = 0.001, # *P*-value = 0.0001 are significant

α-sarcomeric actin and connexin 43 at 1 month (Fig. 8A). The FACS analysis showed that 90.6% of the population of hiPSC- derived cardiac progenitor was cardiac troponin T-positive (Fig. 8B).

Discussion

However, the in vitro differentiation of hESCs into cardiomyocytes or skeletal myoblast involves a poorly defined, inefficient, and relatively nonselective process

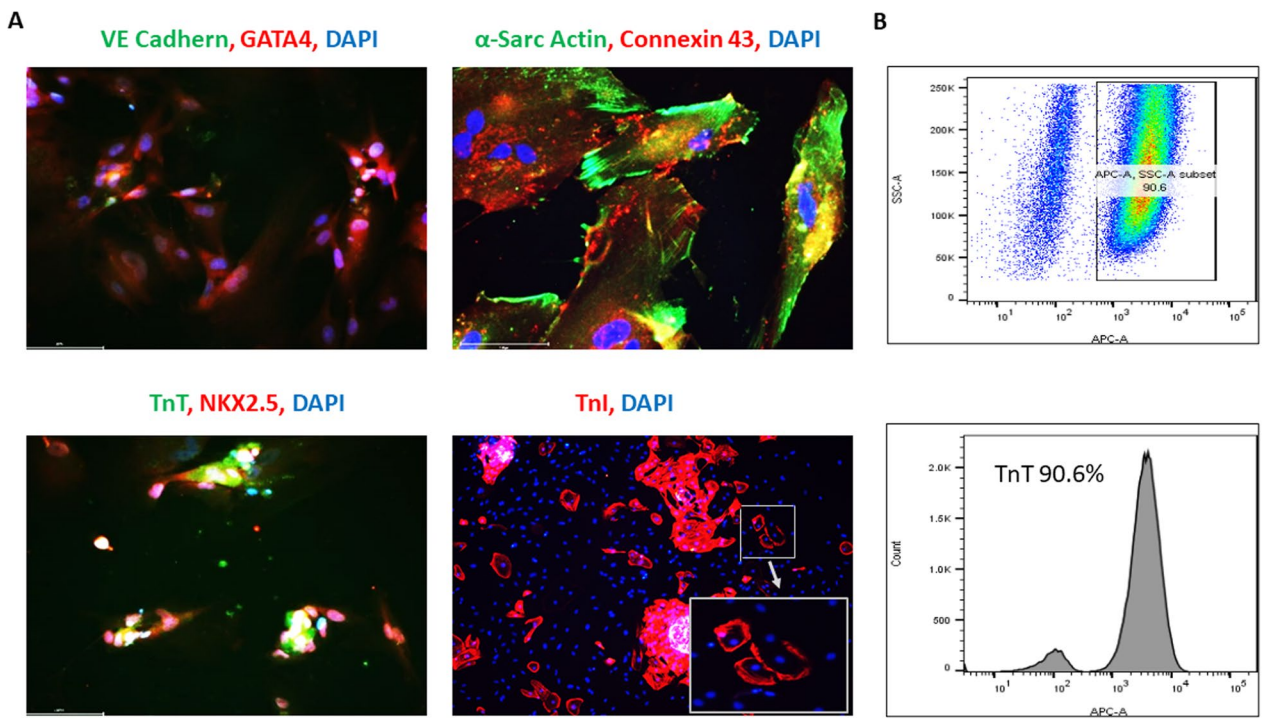

Fig. 8 Effect of GIV on differentiation of hiPSCs to cardiac myogenic cells. **A** Cells were immunostained positive for different cardiac progenitors' markers as Nkx2.5, GATA4, TnT, VE cadherin, α-sarcomeric actin and connexin 43 (40X), but TnI (10X) at 30 days. **B** Flow cytometry analysis of hiPSC-cardiac progenitor stained for Alexa Fluor® 647-conjugated cardiac troponin T. The analysis showed that 90.6% of the population of hiPSC- cardiac progenitor was cardiac troponin T-positive

[9]. Cardiac and skeletal myogenesis involves generation of striated muscles and are traditionally thought of as two separate processes with distinct developmental processes [23, 24]. However, heart and craniofacial skeletal muscles originate from a common progenitor population (cardiopharyngeal mesoderm, CPM) during embryonic development [23]. This may explain the co-existing cardiac and craniofacial defects commonly observed in patients with DiGeorge syndrome [24].

In this study, our concentration of small molecules ISX9 (25 μM) [14, 18], DAN (25 μM) [19] and GIV (150 nM) [4] were similar to other studies using different cell types. Our observation showed pretreatment of hiPSCs with GIV had dual cardiac/skeletal myogenic progenitors more than ISX9 and DAN; and will likely facilitate therapeutic application of hiPSCs with GIV in heart disease or DMD.

Development of skeletal myogenic progenitors

There is still no available cure for DMD, but gene- and cell-based therapy is considerable interest in creating myogenic precursors such as stem cell therapy (Skeletal myoblast, bone marrow-derived stem cells, pericyte, ESCs and iPSCs) or *ex-vivo* stem cell with myogenic gene modification delivery in DMD models [25, 26]. However, ex vivo overexpression of Pax3, Pax7, Myf5, MyoD or dystrophin in ESCs and iPSCs can drive differentiation to myogenic precursors that can be transplanted into animal [7], but its ability to induce direct myogenesis in human pluripotent stem cells has not been demonstrated.

In this study, GIV increased upregulation of skeletal myogenic genes in hiPSCs in first few days versus control ($P<0.05$). GIV increased skeletal myogenic genes and their proteins expression within 1 week such as Pax3 (27.7-fold), Meox1 (21.9-fold), myogenin (6.3-fold), Myf5 (6.3-fold), Myh2 (5.8-fold), Meox2 (4.1-fold), dystrophin (3.9-fold), Myh6 (3.7-fold), Tcf15 (3.6-fold), Pax7 (3.5-fold), Tbx1 (2.8-fold), Mesp1 (2.6-fold), desmin (2.1-fold), MyoD1 (1.8-fold) and β-catenin (1.7-fold) versus control ($P<0.05$). Our results were in agreement with other studies reported the potential myogenic effect after oral treatment with HDACi GIV in mdx mice, the morphological recovery is accompanied by increased muscle strength and exercise performance, and also increased myotube size of human skeletal myoblast in vitro by addition of HDACi GIV (150 nM) or Tricostatin A (50 nM) [4]. Also, oral administration of HDACi GIV in the treatment of DMD (Phase I/II clinical trial) [21].

Our results were in agreement with other studies using different small molecules to enhance the efficiency of hiPSC differentiation into multiple lineages [22]. In another small molecule, Myoseverin [27], retinoic acid [28] or Dorsomorphin [29] was able to generate mononucleate progeny from multinucleate myotubes. Combining the small-molecule glycogen synthase kinase-3 (GSK3β) inhibitor BIO, Forskolin, and FGF2 induced efficient skeletal muscle specification of hiPSCs through the sequential treatment with a WNT activator [15]. The hiPSC-derived cells expressed muscle markers like Pax7, Myf5, MyoD, Myogenin, and MHC and formed mature myotubes in vitro [6, 15]. Upon injection into cardiotoxin-injured tibialis anterior muscles of immunocompromised mice, the hiPSC-derived myogenic progenitors contributed to muscle fibers and the satellite cell pool in the host muscle [15].

The fundamental role of histone acetyltransferases (HATs) and histone deacetylases (HDACs) in regulating muscle development and differentiation [30]. HATs catalyze the transfer of acetyl groups to lysine residues of histones, resulting in the relaxation of chromosomal DNA permissive for transcription [31]. The HATs p300/CBP and p300/CBP-associated factor (PCAF) activate muscle gene expression by acetylation of MyoD and modulation of its recruitment at target loci [32]. GIV can inhibit the activities of HDACs and restore or increase the level of histone acetylation [30]. Therefore, treatment with HDACi promotes the expression of two core components of the myogenic transcriptional machinery, MyoD and BAF60c [30] and upregulates three myogenic microRNA (myomiRs; miR-1.2, miR-133 and miR-206) involved into muscle differentiation in fibro-adipogenic progenitors (FAPs) [31]. MyomiRs in turn target two alternative BAF60 variants- BAF60 A and B, that when incorporated into the Switch/Sucrose Non-Fermentable (SWI/SNF) chromatin-remodeling complex would otherwise promote the activation of the FAPs [31].

Development of cardiac myogenic progenitors

The overall efficiency of the embryoid body (EB) protocol to generate hiPSCs or hESCs-derived cardiomyocytes is low with a minority of EBs developing contracting cardiomyocytes [33]. Also, the ability of hESC or hiPSC- derived cardiomyocytes to mature following transplantation has not been demonstrated [10], but this seems a reasonable expectation.

In this study, GIV with addition of ALK4/5/7 (TGFβ type-I receptor) inhibitors SB431542 for cardiac specification resulted in a cardiac progenitor ~ 80–90% around day 30. This was in agreement with another study reported that using ALK4/5/7 (TGFβ type-I receptor) inhibitors, which was competent for differentiation of human iPSC-derived CPCs to cardiomyocytes [34]. Our results were similar to many previous studied using small molecules for cardiac differentiation from ESC/hiPSCs such as Trichostatin A [35], Cardiogenol C [15], Ascorbic acid [16, 36], Famotidin [37], 1,5-disubstituted Benzimidazoles [38], B-turn peptide mimetic compounds (CW209E) [39] and Resveratrol [40].

The enhanced differentiation efficiency of our protocol in hiPSCs was also similar to other studies that used small molecules such as Wnt/β-catenin pathway inhibitor; IWR-1 [36], XAV0939 [18], Wnt-CD59 [41] enhanced the differentiation of the cardiac progenitors to the ventricular cardiomyocyte lineage [3]. This protocol reliably yields preparations of 30–60% cardiomyocytes, which can then be further enriched to > 90% cardiomyocytes using straightforward physical methods.

Cardiomyocyte differentiation was achieved by treatment of hiPSCs with combination of recombinant growth factors (activin A, BMP-4 and bFGF) in first 2 days before small molecules. This step is required for developing a polarized axis of mesoderm that gives rise to the heterogeneous cell types of the cardiovascular system, including cardiomyocytes and those of the endocardium, vascular endothelium, and the hematopoietic system [42].

In this study, GIV increased cardiac muscle genes expression such as Pitx2 (70.5-fold), ISl1 (6.9-fold), Nkx2.5 (48.1-fold), Hand1 (14.9-fold), GATA4 (5.5-fold), Tbx5 (37.1-fold), TnnT2 (5.7-fold), Myl7 (16.5-fold), MLC2v (9.3-fold), Mef2c (1.7-fold), Cdh4 (3.2-fold), Podx1 (0.9-fold), Nppa (ANP) (4.5-fold) and Lhx2 (2.7-fold) versus control. Our hiPSC-derived cardiac progenitor cells expressed the cardiomyocyte structural markers cTnT, cTnI, GATA4, NKX2.5, α-sarcomeric actin, connexin 43, and MLC2v; but no beats were observed in culture. Interestingly, the expression of connexin 43 correlates with the presence of functional gap junctions. Our results were similar to other studies [22]. Also, ESCs with another small molecule as Cardiogenol C that induced the expression of the cardiac muscle cell-specific transcription factors Mef2c, Nkx2.5, GATA4 and sarcomeric myosin heavy chain (MHC) after 7 days [15]. Sarcomeric MHC is one of the essential motor proteins responsible for cardiac muscle contractibility [43] and was used as a secondary assay for differentiation. Members of the Mef2 family are essential for muscle development. Nkx2.5, one of the earliest lineage-restricted genes to be expressed in cardiac progenitor cells. But α-MHC or atrial natriuretic factor (ANF) that targeted late cardiac differentiation markers [14, 22]. In another study, MESP1-induced CPM cardiac derivatives predominantly expressed atrial-specific genes, including transcription factor (Nr2f2), myosin (MLC2a), ion channel (Kir3.1) and secretory peptide

ANF (Nppa) [22], and ventricular specific genes such as Hand1 and MLC2v were enriched in cardiac progenitors.

Our results were similar to another HDACi (Trichostatin A) enhanced cell survival of hESC/hiPSC with 90% of the treated beating colonies, triggered the degradation of HDAC4, associated with a faster spontaneous rhythmic contraction, a cardiac lineage commitment, increased up-regulation of cardiac actin, and cardiac-specific transcriptional factors (Mef2c) [35, 44, 45]. However, Trichostatin A-mediated acetylation control might enhance cardiac function but not cardiac differentiation during hESC/hiPSC-CM culturing [35, 44]. Also, a member of class II HDAC, HDAC4, binds to the transcription factors SRF and Mef2c and inhibits the expression of cardiac-specific genes in different lines of iPSCs [35, 44, 46, 47], and maybe explain the role of HDACi GIV for induction of cardiomyocytes.

Therefore, preconditioning of donor stem cell with HDACi (Givinostat) before transplantation in myocardial infarction can be useful tool in cardioprotective and myocardial repair [20]. There are two potential evidence that HDACi targets for stroke therapy via upregulate cardio/neuro-protective factors [20, 48–50]: i) knockdown of HDAC4 or inhibition of HDAC4 showed reduced infarct size following myocardial ischemia-induced reperfusion injury. ii) anti-inflammatory effects of HDACis via their down-regulation of pro-inflammatory factors such as Fas-L, IL-6, NF-κB, iNOS, TNF-α, COX-2, MMP-9, MIP-1 and MCP-1; iii) up-regulation of neuroprotective factors or proteins as HSP70, BDNF and gelsolin.

Conclusion

Pretreatment of hiPSCs with GIV represents a viable strategy for producing both cardiac/skeletal myogenic progenitors in vitro for cell therapies against myocardial infarction and Duchenne muscular dystrophy. Thus, approaches to produce homogenous beating cardiomyocytes for practical use in cardiac regenerative medicine, drug research and tissue engineering remain a great need [8]. Moreover, the use of differentiated cells derived from iPSCs is an ideal therapeutic approach based on the advantages to generate patient-specific pluripotent cells and ethical deliberation [33]. However, technical issues and anticipated safety for homogeneity and procancerogenic potential of iPS-derived cells are crucially concerned before the employment in translational and clinical implication.

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Author contributions
IE designed the protocol, made experiments and statistically and wrote the paper and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), University of Illinois at Chicago and The Ohio State University, USA. The committee's reference number is not available.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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