

Inhibition of Rho-associated protein kinase improves the survival of human induced pluripotent stem cell-derived cardiomyocytes after dissociation

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Abstract. Heart disease remains the leading cause of morbidity and mortality worldwide. Induced pluripotent stem cells (iPSCs) have the ability to differentiate into cardiomyocytes (CMs), rendering this cell type to be a promising pre-cursor of cardiomyocytes for cell-based cardiac regeneration. Obtaining CMs with a high yield and purity coupled with improved subsequent survival could prove to be invaluable for the future cell replacement therapeutic strategies. Rho-associated protein kinase (ROCK) is involved in a wide range of fundamental cellular functions and serves significant roles in cardiac physiology. In the present study, human (h)iPSC-CMs were generated from iPSCs by including glycogen synthase kinase 3 β and Wnt inhibitors in the basal culture media. The possible effect of Y27632, a ROCK inhibitor, on hiPSC-CMs was then investigated. hiPSC-CMs of high purity were harvested with >96% of cells expressing cardiac troponin T. Additionally, treatment with 10 μ M Y27632 significantly improved the viability of dissociated hiPSC-CMs. The effects of ROCK inhibitors Y27632 and fasudil, on the proliferation and apoptosis of hiPSC-CMs were also examined. Treatment with ROCK inhibitors markedly enhanced hiPSC-CM proliferation, by up to 2.5-fold, whilst Y27632 treatment reduced apoptosis in hiPSC-derived CMs under serum starvation and suspension by suppressing the expression of caspase-3. Taken together, data from the present study indicated that ROCK

kinase inhibitors effectively improved the cultural system of hiPSC-derived CMs.

Introduction

Cardiovascular diseases, including heart failure, chronic ischemic heart disease and acute myocardial infarction, remains a leading cause of morbidity and mortality worldwide (1). The recent advent of the human induced pluripotent stem cell (hiPSC) technology and an increasingly refined capacity to differentiate hiPSCs into cardiomyocytes (CMs), provides an unprecedented opportunity for potential applications in disease modeling, drug discovery, toxicity screening and novel cell-based therapies (2). Over the last two decades, a number of protocols for cardiac differentiation have been established based on embryonic body (EB) and monolayer methodologies (3-5). Obtaining CMs of a high yield and purity by improving cell survival may be invaluable for future applications in cell replacement therapy and novel drug screening.

Rho-associated coiled-coil containing kinases (ROCKs) are members of the serine/threonine protein kinase family that are well documented to be involved in the regulation of a wide range of fundamental cellular functions, including cell proliferation, apoptosis, cell mitosis adhesion, cytoskeletal adjustments and muscle cell contraction (6,7). Evidence from a plethora of animal and clinical studies have suggested ROCKs to be important mediators of a number of pathophysiological characteristics associated with cardiovascular diseases, including cardiac remodeling, fibrosis and hypertrophy (6,8). Of note, Y27632, a pharmacological inhibitor of ROCK and the calcium-sensitization pathway of smooth muscle contraction, has been previously applied as a therapeutic drug for the treatment of cardiovascular diseases (9). Apart from Y27632, fasudil is another potent ROCK inhibitor and vasodilator which has been used for the treatment of cerebral vasospasm (10). Indeed, a number of previous studies have indicated that ROCK1 and ROCK2 contribute to pathological CM apoptosis (11,12). Additionally, long-term fasudil and Y27632 treatment has been demonstrated to attenuate CM apoptosis and limit the progression of pathological cardiac fibrosis, hypertrophy and remodeling, which lead to heart

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failure (13,14). However, the effects of the ROCK signaling pathway on hiPSC-derived CMs have not yet been thoroughly investigated.

Therefore, the aim of the present study was to examine whether ROCK inhibitors can be used to increase the survival of hiPSC-derived CMs, which may facilitate the development of applications in stem cell therapy for the treatment of heart disease. Specifically, the potential effects of the ROCK inhibitors, Y27632 and fasudil, were evaluated on hiPSC-derived CMs. The results indicated that ROCK inhibitors promoted hiPSC-CM proliferation, increased the number of viable cells and inhibited the apoptosis of hiPSC-CMs in suspension. The mechanism of these physiological effects mediated by the ROCK inhibitor on hiPSC-CM apoptosis may be via the suppression of caspase-3 expression.

Materials and methods

The differentiation of hiPSC into cardiomyocytes using small molecules. HiPSCs (cat. no. DYR0100 Type Culture Collection of the Chinese Academy of Sciences) were cultured at 37°C and 5% CO₂ in mTeSR™ 1 maintenance medium (Stemcell Technologies, Inc.). Cells were seeded into 35 mm culture dishes (2x10⁵ cells/dish) coated with Matrigel (Corning, Inc.) and passaged once every 2-3 days using ACCUTASE™ cell detachment solution (Stemcell Technologies, Inc.).

For direct differentiation into cardiomyocytes (5), hiPSC first were split at a 1:6 ratio using mTeSR media and cultured for 2 days to ~85% confluence. On day 0, cells were first treated with 6 μM GSK-β inhibitor CHIR99021 (MedChemExpress) diluted in RPMI/B27 without insulin for 48 h, followed by transfer to RPMI/B27 minus insulin supplemented with 3 μg/ml Wnt signaling inhibitor IWP2 (MedChemExpress) for another 3 days. On day 5 following CHIR99021 treatment, the medium was changed to RPMI/B27 without insulin and finally to RPMI/B27 with insulin medium on day 7 for further maintenance until day 21.

To purify hiPSC-CMs, a biochemical purification protocol was used using glucose-depleted DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 4 mM lactate (Sigma-Aldrich; Merck KGaA) on day 15 (15). On day 15, the medium was changed to the purification medium, consisting of glucose depleted DMEM, 500 μg/ml recombinant human albumin (Oryzogen; Wuhan Healthgen Biotechnology, Corp.) and 213 μg/ml l-ascorbic acid 2-phosphate supplemented (Sigma-Aldrich; Merck KGaA) with 4 mM l-lactic acid (Sigma-Aldrich; Merck KGaA). Medium was refreshed every 2 days during the purification process. On day 19, cells were maintained in RPMI/B27 media and allowed to recover for 2 days.

To investigate the effects of Y27632 on hiPSC-CMs, cells were first dissociated and seeded into gelatin-coated 12-well plates (Corning, Inc.) at a density of 3x10⁵ cells/well under a number of different conditions. For controls, hiPSC-CMs were cultured in DMEM supplemented with 20% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) in the absence of Y27632 (Selleck Chemicals). For other treatment groups, cells were cultured in DMEM containing 20% FBS supplemented with various concentrations of Y27632 (5, 10 or 20 μM). After 24 and 48 h, cells were washed in PBS, trypsinized

and counted (n=3). Specifically, 10 μl cells mixed with equal volumes of 0.4% trypan blue dye (Beijing Solarbio Science & Technology Co., Ltd.) were counted using a hemocytometer.

Immunostaining and fluorescent microscopy. Cells were first fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 15 min at room temperature (RT), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 10 min at RT and blocked with 10% goat serum (Sigma-Aldrich; Merck KGaA) at RT for 1 h. Samples were subsequently stained with primary antibodies against cardiac troponin T (cTnT; 1:200; cat. no. 710635; Invitrogen; Thermo Fisher Scientific, Inc.) and α-actinin (Invitrogen; 1:200; cat. no. 710947; Thermo Fisher Scientific, Inc.), dissolved in PBS at 4°C for 12 h and further incubated with Alexa Fluor™ 555-conjugated antibody (1:500; cat. no. A0460; Beyotime Institute of Biotechnology) and Alexa Fluor™ 488-conjugated antibody (1:500; cat. no. A0423; Beyotime Institute of Biotechnology) at RT for 2 h. Nuclei were stained using 1 μg/ml DAPI (Sigma-Aldrich; Merck KGaA) at RT for 5 min. An Optiphot-2 microscope (Nikon Corporation) equipped with a CCD video camera system (Optronics Engineering, Ltd.) and a computer interface (NIS-Elements; version 4.2.0; Nikon Corporation) was used for imaging analysis (magnification, x100). In total, 40 images were taken for each well.

Crystal violet staining. Cells were first washed with PBS, fixed in 4% paraformaldehyde at RT for 15 min and stained with 0.1% crystal violet solution (Sigma-Aldrich; Merck KGaA) at RT for 30 min. After the cells were washed with ddH₂O and air-dried at RT, images were obtained using a color digital camera (Optronics Engineering, Ltd.). Absorbance was subsequently measured at 590 nm using an automatic microplate reader (BioTek™ ELx800™; Omega Bio-Tek, Inc.).

Cell counting kit-8 (CCK-8) assay. Cells were plated into 96-well plates in triplicate at ~10,000 cells/well and subsequently cultured with or without 10 μM Y27632 at 37°C and 5% CO₂ for 24 h. Cells were analyzed using CCK-8 assay according to the manufacturer's protocol (Beyotime Institute of Biotechnology). In total, 10 μl CCK-8 were added per well and incubated for 2 h at 37°C. The absorbance value at a wavelength of 450 nm was measured for each well using an enzyme-linked immunosorbent assay reader (Thermo Lab Systems; Thermo Fisher Scientific, Inc.).

5-bromo-2-deoxyuridine (BrdU) incorporation assay. hiPSC-CM proliferation was evaluated using BrdU incorporation assay. The same number of hiPSC-CMs (1.5x10⁵ cells) were cultured in DMEM supplemented with 20% FBS and 10 μM Y27632 for 24 h after passage on day 21, following which the cells were treated with or without 10 μM Y27632 for 3 or 6 days, and incubation with or without 10 μM fasudil for 6 days (16). Cells were then treated with 10 μM BrdU (Sigma-Aldrich; Merck KGaA) and incubated at 37°C for 6 h. Samples were then fixed using 4% paraformaldehyde at RT for 15 min, permeabilized by 0.1% Triton X-100 at RT for 15 min and acid-washed by 1 M HCl on ice for 10 min. BrdU-positive cells were subsequently detected by immunocytochemistry using mouse anti-BrdU (1:1,000;

cat. no. B2531; EMD Millipore) at 4°C overnight and goat anti-mouse FITC-conjugated secondary antibodies (1:400; cat. no. BA1101; Molecular Probes; Thermo fisher Scientific, Inc.) at RT for 1 h. Nuclei were visualized via 1 µg/ml DAPI staining at RT for 5 min. In total, each dish was divided into 4 regions, where 10 images were taken per region; ~2,000 cells were quantified and normalized to the total cell number in each field using ImageJ software (version 1.8.0; National Institutes of Health).

Annexin V-FITC/propidium iodide (PI) apoptosis detection by fluorescence-activated cell sorting (FACS) assay. Cell apoptosis was assessed using annexin-V-FITC and PI staining kit (BD Biosciences). In total, 5x10⁵ cells were collected by trypsinization at 37°C for 5 min, centrifuged under 200 x g at 4°C for 5 min and washed twice with PBS, resuspended in 500 µl 1X binding buffer and incubated in 5 µl Annexin V-FITC and 5 µl PI solution in the dark for 15 min at RT. Cells were analyzed using an integrated BD Accuri™ C6 system (BD Biosciences). The gates for flow cytometry was set using following steps: Target cell population was circled by detecting unstained cells. Annexin V-FITC single positive cells were detected, following which fluorescence compensation was adjusted to ensure no particles were in the upper left (UL) and upper right (UR) quadrants. PI single positive cells were detected and fluorescence compensation was adjusted to ensure no particles were present in the upper right (UR) and lower right (LR) quadrants. A total of four populations of cells were observed: i) Cells that were viable and not undergoing apoptosis are both FITC-Annexin V and PI-negative (LL); ii) cells undergoing apoptosis were FITC-Annexin V-positive and PI-negative (LR); iii) the cells that are undergoing late apoptosis or necrotic are both positive for FITC-Annexin V and PI (UL and UR).

Reverse transcription-quantitative PCR (RT-qPCR) or semi-quantitative PCR analysis (RT-PCR). Total RNA was extracted and prepared using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from 1 µg RNA using the AccuPower® RT Premix (Bioneer Corporation). The temperature for the reverse transcription reaction was 25°C for 5 min, 42°C for 60 min and 70°C for 5 min, following which the product was used for RT-qPCR and RT-PCR analyses.

RT-PCR was performed using ACTB mRNA as the normalizing internal control and carried out using Taq DNA Polymerase (Takara Bio, Inc.). The thermocycling conditions (total volume per reaction, 20 µl) were as follows: Initial 5 min denaturation at 95°C, followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec and a final extension for 10 min at 72°C. PCR products were resolved by electrophoresis on a 1.5% agarose gel and stained using GelRed® Nucleic acid gel stain (Biotium, Inc.), then visualized via UV transillumination and photographed using a Gel Doc™ EZ System (Bio-Rad Laboratories, Inc.).

qPCR reactions were set up in duplicate using SYBR® Premix Ex Taq™ (Takara Bio, Inc.) and performed using the 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR reactions of 20 µl for each sample

Table I. Primers used for PCR for the present study.

Genes	Sequence (5'-3')	Size (bp)
ACTB	F: AGCGAGCATCCCCAAAGTT R: GGCACGAAGGCTCATCATT	285
TNNT2	F: TTCACCAAAGATCTGCTCCTC GCT R: TTATTACTGGTGTGGAGTGGGTG TGG	166
TNNI3	F: CTGCAGATTGCAAAGCAAGA R: CCTCCTTCTTCACCTGCTTG	379
MYL7	F: GAGGAGAATGGCCAGCAGGAA R: GCGAACATCTGCTCCACCTCA	450
MYL2	F: ACATCATCACCCACGGAGAAG AGA R: ATTGGAACATGGCCTCTGGAT GGA	247
Bax	F: CCCCCGAGAGGTCCTTTTCC R: TGTCCAGCCCATGATGGTTC	160
Bcl-2	F: GAACTGGGGGAGGATTGTGG R: AAAGCCAGCCTCCGTTATCC	164
Caspase-3	F: GGGGATTGTTGTAGAAGTCTA ACT R: AATAACCAGGTGCTGTGGAGTA	178
Caspase-8	F: ATTAGGGACAGGAATGGAACAC R: GGAGAGGATACAGCAGATGAAG	180

ACTB, β-actin; TNNT2, troponin T type 2; TNNI3, troponin I type 3; MYL7, myosin light chain 7; MYL2, Myosin light chain 2; F, forward; R, reverse.

were made and consisted of cDNA (200 µg cDNA after dilution), 2X SYBR Premix Ex Taq, 50X ROX Reference Dye, dH₂O and 10 µM of each gene-specific primer. The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, annealing at 60°C for 34 sec, 72°C for 30 sec and a final extension at 72°C for 10 min. Relative mRNA abundance was calculated using the 2^{-ΔΔC_q} method (17) and normalized to the expression levels of ACTB. The sequences of the primers used are listed in Table I.

Western blot analysis. Cells were prepared and lysed in ice-cold RIPA buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40 and 10 mM EDTA; EMD Millipore) containing freshly added Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.) and Protease Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA). Protein concentrations were determined using Bradford protein assay (Bio-Rad Laboratories, Inc.). In total, 20 µg protein samples were separated by 10% or 12% SDS-PAGE using an electrophoresis system (Bio-Rad Laboratories, Inc.) and transferred onto nitrocellulose membranes. The membranes were subsequently

blocked with 5% non-fat milk for 1 h at RT and probed with a number of different primary antibodies (GAPDH, cat. no. 2118S; cleaved caspase-3, cat. no. 9661S; or caspase-8 cat. no. 4790S; all 1:1,000; Cell Signaling Technology, Inc.) at 4°C overnight. Samples were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (1:2,500; cat. no. SA00001-2; Proteintech Group, Inc.) at RT for 2 h. Blots were visualized using enhanced chemiluminescence (ECL) reagent (Amersham; GE Healthcare Life Sciences), images were captured using the ECL Tanon 5500 system (Tanon Science and Technology Co., Ltd.) and analyzed using Tanon 5500 Multi automatic chemiluminescence/fluorescent image analysis system (Tanon Science and Technology Co., Ltd.).

FACS analysis. Following purification, 5×10^5 iPSC-CMs were trypsinized under 37°C for 5 min, centrifugated at $200 \times g$ at RT for 5 min, fixed and permeabilized with 70% ethanol at 4°C overnight and stained with phycoerythrin-conjugated mouse anti-cTnT antibodies (1:200; cat. no. 564767; BD Biosciences;) for 2 h at 4°C. Cells were washed three times with PBS and analyzed using flow cytometry (BD Accuri™ C6, BD Biosciences).

Analysis of caspase-3 activity. Caspase-3 activity was detected using a caspase-3 Activity Assay kit (cat. no. C1116, Beyotime Institute of Biotechnology). Cells (5×10^5) were cultured in the presence or absence of 10 μ M Y27632 for 3 days in a 6-well plate, which were then washed with PBS, resuspended in the RIPA lysis buffer (as part of the caspase-3 Activity Assay kit) and incubated on ice for 15 min. For each well of a 96-well microplate, cell lysates (50 μ l/well), assay buffer and caspase-3 substrate (Ac-DEVD-pNA, 10 μ l/well) were combined together. Samples were then incubated at 37°C for 2 h and quantified using microplate reader by measuring absorbance at 405 nm.

Statistical analysis. All experiments of the current study were repeated three times and the data are presented as the mean \pm standard deviation. GraphPad Prism 7 (GraphPad Software, Inc.) software was used for the statistical analysis of all data. Statistical differences between two groups were analyzed using Student's t-test (two-tailed unpaired). Comparisons between multiple groups were performed using one-way ANOVA followed by a Dunnett's post hoc multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference. To detect caspase-3 activity, a linear regression line (Microsoft Corporation) was fitted to the concentration: Optical density data. For comparative purposes, caspase-3 activity values were generated for each group using regression analyses.

Results

Cardiac differentiation and characteristics of hiPSC-CMs. hiPSC-CMs were generated from hiPSC using CHIR9901 and IWP-2, inhibitors of GSK3 β and Wnt respectively, in insulin-free medium (Fig. 1A) (5). Following ~8 days after CHIR99021 treatment, spontaneously contracting cells were observed. As presented in Fig. 1B, the expression of cardiac

markers, including troponin T type 2 (TNNT2), troponin I type 3 (TNNT3), MYL7 and MYL2, were revealed to be positive by RT-PCR analysis. In addition, hiPSC-CMs displayed positive expression for cTnT and sarcomeric- α actinin on day 15 (Fig. 1C). FACS analysis demonstrated that the purity of hiPSC-CMs isolated appeared to be high, with >96% cells expressing cTnT on day 21 (Fig. 1D).

ROCK inhibitor markedly promotes the survival of dissociated hiPSC-CMs. In the present study, the potential effects of Y27632, a ROCK inhibitor, were investigated on hiPSC-CMs. To examine if Y27632 exerts an effect on hiPSC-CM viability, cells were cultured in the presence of 0, 5, 10 or 20 μ M Y27632 for 24 h. The number of viable cells increased in a dose-dependent manner, as the concentration of Y27632 increased to 5 and 10 μ M (Fig. 2A). However, at 20 μ M, the number of viable cells was decreased compared with 10 μ M (Fig. 2A). Subsequently, the effects of Y27632 on the viability of hiPSC-CMs, quantified as the number of cells positively staining for the TNNT2, were examined. Y27632 (10 μ M) markedly increased the plating efficiency of hiPSC-CMs (Fig. 2B). The images and values obtained from crystal violet staining demonstrated a significant increase in relative cell viability following 10 μ M Y27632 treatment compared with untreated cells (Fig. 2C). Quantitative analysis of the data obtained from the CCK-8 assay also reported significant increases in relative cell viability in Y27632 treated cells compared with untreated cells (Fig. 2D). Taken together, these data indicated that Y27632 treatment enhanced the viability of hiPSC-CMs.

ROCK inhibitor enhances hiPSC-CMs proliferation. To determine whether the increased cell viability was also due to increased cell proliferation, a BrdU incorporation assay coupled with fluorescent microscopy was performed to measure hiPSC-CM proliferation. After cells were cultured for 3 or 6 days in the absence or presence of 10 μ M Y27632, fluorescence images were taken on day 3 (Fig. 3A) and day 6 (Fig. 3B). Significant increases in the numbers of BrdU-positive cells were observed following Y27632 treatment for both days ($P < 0.0001$; Fig. 3A and B). On day 3, the mean number of BrdU⁺ cells counted in fluorescent images were 2.12 ± 0.02 and $4.88 \pm 0.07\%$ (Fig. 3A; $n=3$; $P < 0.0001$). After another 3 days, the proliferation rate of the control was $3.34 \pm 0.01\%$ of BrdU incorporation, compared with $5.7 \pm 0.12\%$ for Y27632 treatment (Fig. 3B; $n=3$; $P < 0.0001$). In addition, the results revealed that BrdU-positive cells was also significantly increased following treatment with fasudil, another ROCK inhibitor, for 6 days (Fig. 3C; $P < 0.0001$). These findings indicated that treatment with ROCK inhibitors, particularly Y27632, resulted in a 2.5-fold increase in hiPSC-CM proliferation.

ROCK inhibitor inhibits the apoptosis of dissociated hiPSC-CMs in serum-free medium. To assess the potential effects of Y27632 and fasudil (both 10 μ M) on hiPSC-CM apoptosis under serum starvation and suspension conditions, hiPSC-CMs suspended in serum-free media were seeded into non-adhesive dishes and cultured for 3 days. Apoptosis was then evaluated using Annexin V/PI staining. Following treatment with fasudil, number of apoptotic cells was significantly decreased compared with control ($P < 0.0001$; Fig. 4A).

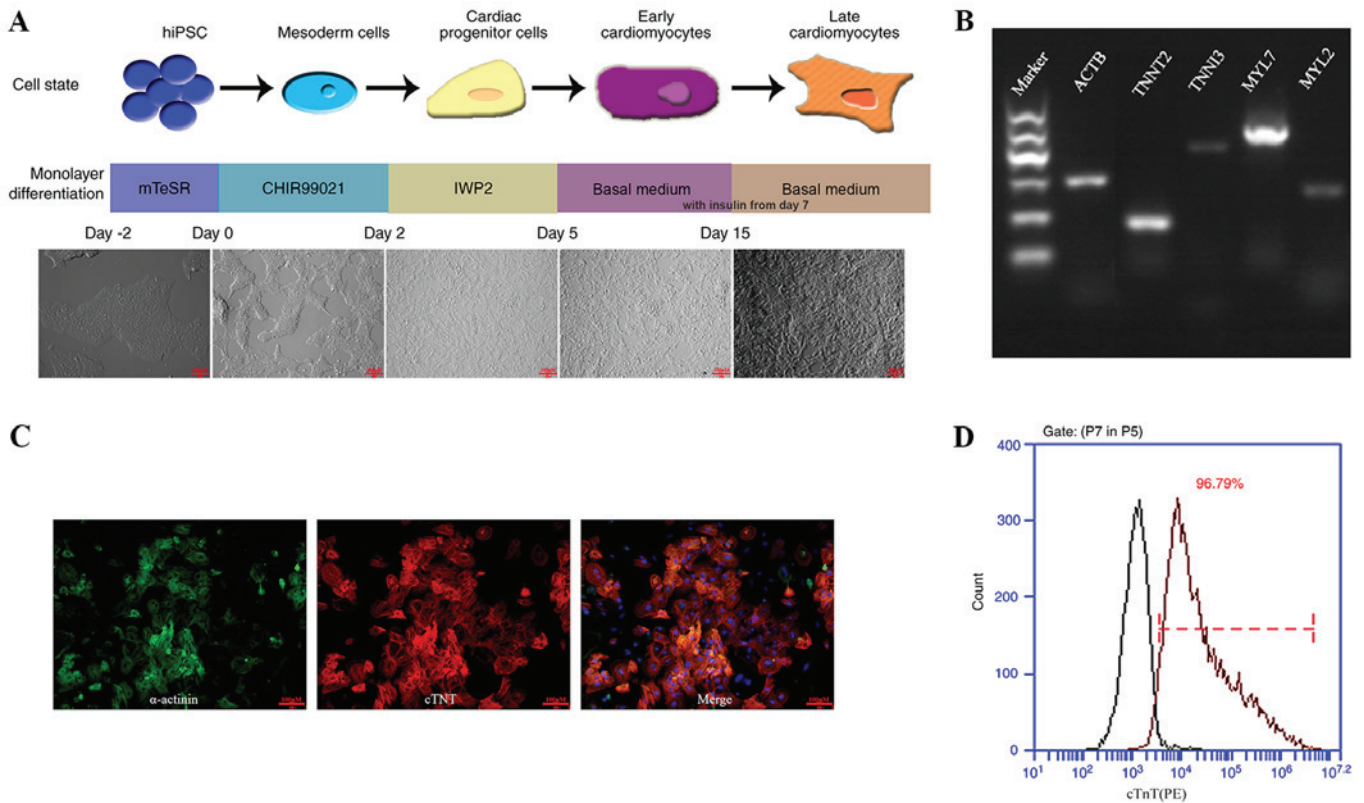


Figure 1. hiPSC differentiation into hiPSC-CM and characterization. (A) Schematic illustration of experimental pipeline applied for the differentiation of human iPSC into cardiomyocytes. The basal medium was supplemented with insulin from day 7 onwards. (B) Reverse transcription-PCR analysis revealed that cardiac gene expression was positive 30 days after the differentiation process commenced. (C) On day 15, cells were purified and re-plated onto 0.1% (wt/vol) gelatin-coated coverslips. Immunostaining for α -actinin (green) and cTnT (red) shows sarcomere organization. (Scale bar, 100 μ m). (D) Representative dot plots for cTnT populations. Left peaks represent the isotype control. Numbers in plots indicate the percentage of cTnT-positive cells after day 21 of differentiation. hiPSC, human induced pluripotent stem cell; CM, cardiomyocytes; ACTB, β -actin; TNNT2, troponin T type 2; TNNI3, troponin I type 3; MYL7, Myosin light chain 7; MYL2, Myosin light chain 2; hiPSC, human induced pluripotent stem cells; CM, cardiomyocyte; cTnT, cardiac troponin T.

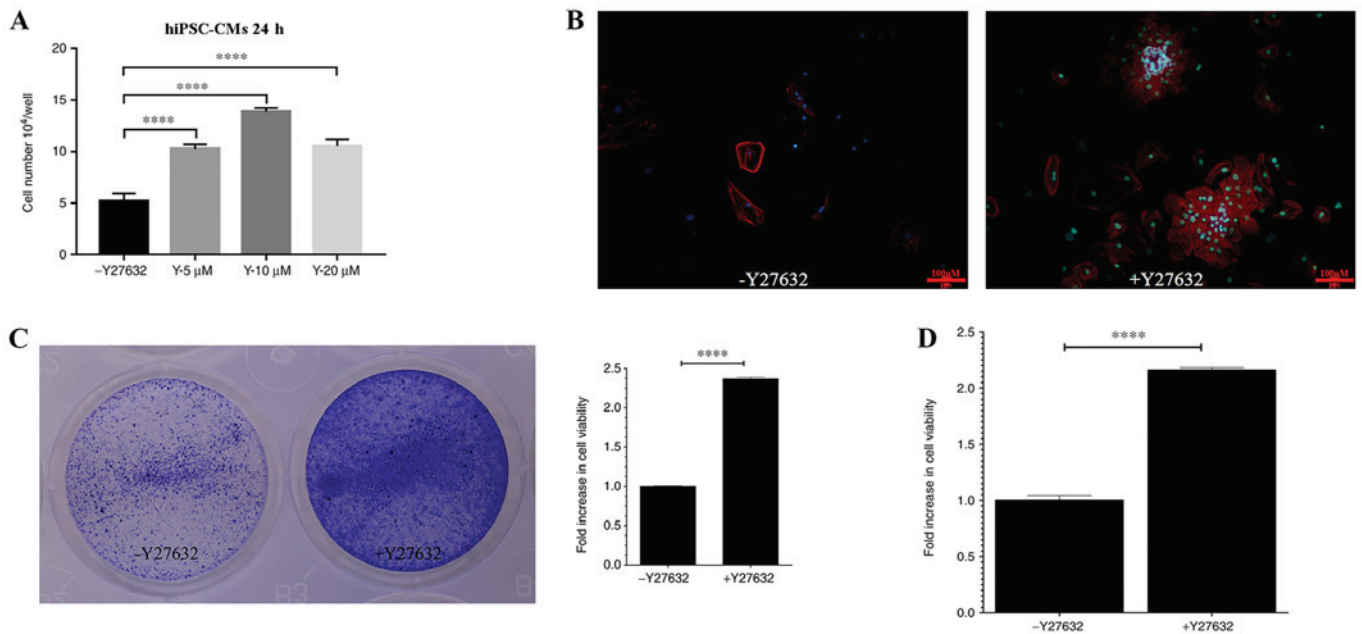


Figure 2. ROCK inhibitor markedly improved hiPSC-CM viability. (A) Y27632 promotes the numbers of surviving cells following passage by single-cell dissociation in a dose-dependent manner. **** P <0.0001 (n =3). (B-D) Dissociated hiPSC-CMs were plated onto 0.1% (wt/vol) gelatin-coated dishes and treated with or without 10 μ M Y27632 for 24 h. (B) Immunostaining of hiPSC-CM markers 24 h after 10 μ M Y27632 treatment. cTnT (red), DAPI (blue). Scale bar, 100 μ m. (C) The total number of cells were quantified using crystal violet staining following 10 μ M Y27632 treatment. **** P <0.0001 (n =3). (D) Effect of 10 μ M Y27632 treatment on the viability of hiPSC-CMs was examined using a Cell Counting kit-8 assay. **** P <0.0001. ROCK, Rho-associated kinase; hiPSC-CMs, human induced pluripotent stem cells-derived cardiomyocytes; cTnT, cardiac troponin T.

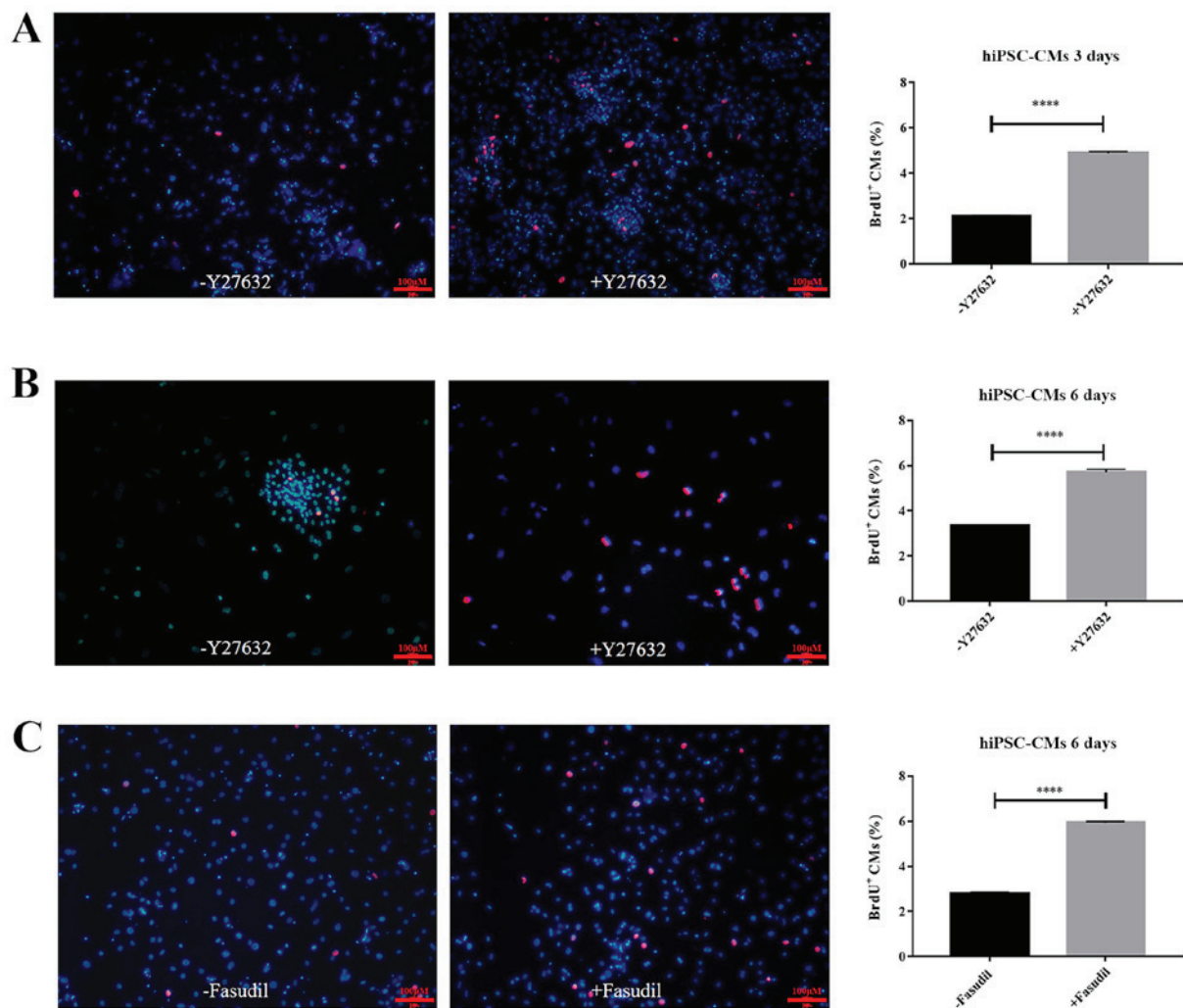


Figure 3. Effects of ROCK inhibitor on the proliferation of hiPSC-CMs, as reported using a BrdU assay. BrdU⁺ cells are presented in red and nuclei were stained in blue. (A) Cells were cultured with or without Y27632 for 3 and (B) 6 days. (C) Cells were treated with or without fasudil for 6 days. **** $P < 0.0001$. ROCK, Rho-associated kinase; hiPSC-CMs, human induced pluripotent stem cells-derived cardiomyocytes; BrdU, 5-Bromo-2-deoxyUridine.

In addition, Y27632 treatment also significantly reduced cell apoptosis compared with control ($P < 0.001$; Fig. 4B). Therefore, treatment with ROCK inhibitors significantly reduced hiPSC-CM apoptosis and necrosis, increasing survival under conditions of stress.

To examine the potential mechanism of Y27632 on the inhibition of apoptosis, mRNA levels of Bcl-2, Bax, caspases-3 and -8 were detected after dissociated hiPSC-CMs were cultured in suspension in the presence or absence of Y27632 for 72 h. The expression of caspase-3 and -8 were significantly reduced by Y27632 ($P < 0.001$), whilst levels of Bcl-2 and Bax did not exhibit significant differences (Fig. 4C and D).

Subsequent western blot analysis revealed that Y27632 significantly reduced the protein expression of cleaved caspase-3 and caspase-8 ($P < 0.0001$; Fig. 4E). The colorimetric assay of caspase-3 activity is based on the spectrophotometric detection of pNA, which is produced by caspase-3-mediated cleavage of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). The level of caspase-3 activity demonstrated that treatment with Y27632 reduced caspase-3 activity ($P < 0.01$; Fig. 4F). These results suggested that the preventive

effects of Y27632 on hiPSC-CMs apoptosis could be due to the suppression of caspase-3 activation.

Discussion

Since being generated from a culture of human fibroblasts using a cocktail of four transcription factors (Oct3/4, Sox2, c-Myc and Klf4) in 2007, human iPSC technology has been widely used for disease modeling, drug discovery and the development of cell therapies (18,19). Progress in hiPSC-derived cardiomyocytes has provided a platform for personalized cardiovascular medicine (20). The development of effective approaches in preventing cell death will facilitate the application of hiPSC-derived CMs in multiple research areas. In the present study, the effects of the ROCK inhibitor, Y27632, were evaluated in hiPSC-CMs.

Significant improvements have been made in the differentiation of pluripotent stem cells into cardiomyocytes over the past two decades, which progressed from an original efficiency of 5-10% to >90% (20,21). In the present study, a directed cardiac differentiation method using small molecules (CHIR99021 and IWP2) that modulate Wnt signaling were

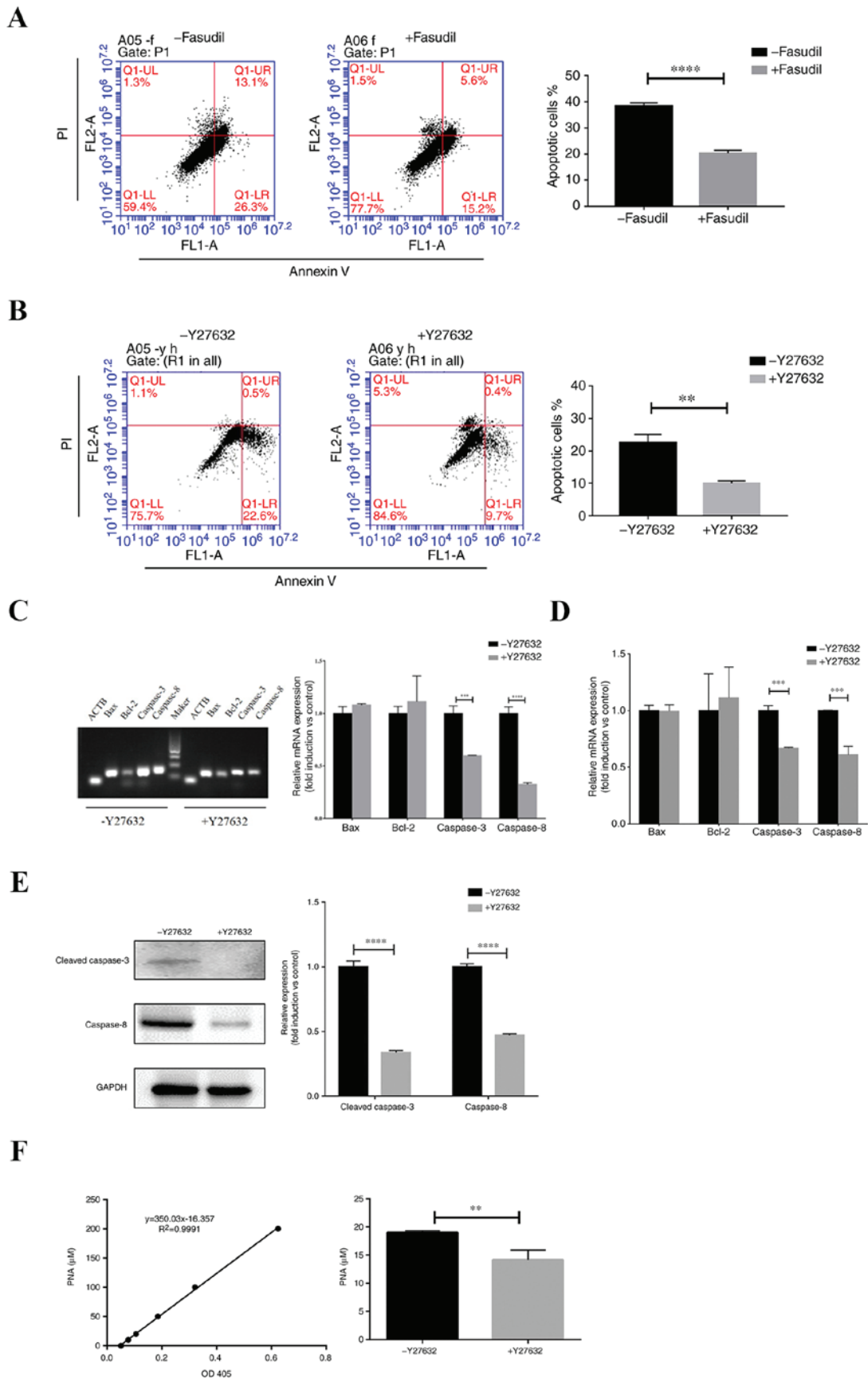


Figure 4. ROCK inhibitors prevent the apoptosis of dissociated hiPSC-CMs in serum-free medium. (A) Following treatment with or without fasudil for 72 h, cells were collected and analyzed by flow cytometry using Annexin V-FITC/PI staining. ****P<0.0001. (B-E) hiPSC-CMs were cultured for 3 days in the presence or absence of Y27632. (B) Flow cytometric analysis were used to detect apoptosis. **P<0.01. (C) The expression of Bcl-2, Bax and caspases 3 and 8 were detected by reverse transcription-PCR and (D) quantitative PCR. ****P<0.0001. (E) The expression of cleaved caspase-3 and caspase-8 were analyzed by western blotting, with GAPDH as the loading control. ****P<0.0001. (F) Y27632 treatment reduced the activity of caspase-3 in detached hiPSC-CMs in suspension culture. **P<0.01. ROCK, Rho-associated kinase; hiPSC-CMs, human induced pluripotent stem cells-derived cardiomyocytes; PI, propidium iodide; PNA, p-nitroaniline; OD, optical density.

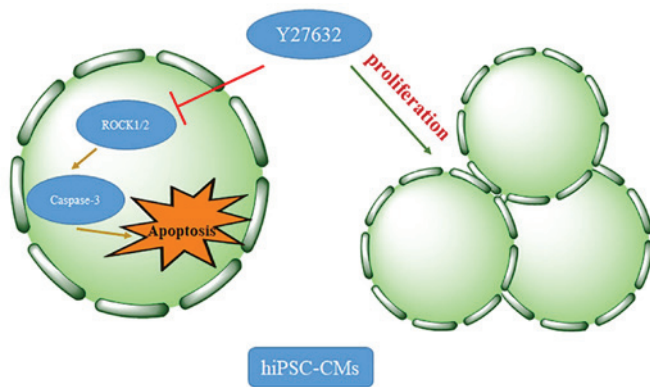


Figure 5. Summary of the effects of the ROCK inhibitor Y27632 on hiPSC-CM physiology as reported in the present study. ROCK, Rho-associated kinase; hiPSC-CMs, human induced pluripotent stem cells-derived cardiomyocytes.

applied, which achieved an efficiency of ~96.79%. Small molecules are advantageous compared with growth factors in that they are more cost effective, easy to store, more stable and more amenable to quality control (22).

Although structurally and functionally immature hiPSC-CM limits its application in drug development and cell therapy, their fetal characteristics may provide a possibility for hiPSC-CMs to proliferate (23). Uosaki *et al* (24) identified four chemical compound groups: Inhibitors of glycogen synthase kinase-3, p38 mitogen-activated protein kinase, Ca^{2+} /calmodulin-dependent protein kinase II and activators of extra cellular signal-regulated kinase, all of which synergistically enhanced the proliferation of cardiomyocytes derived from both mouse and human PSCs. The present study demonstrated that the ROCK inhibitor Y27632 exhibited proliferative effects on hiPSC-CMs. Recently, Mohamed *et al* (25) screened a list of cell-cycle regulators expressed in proliferating fetal cardiomyocytes. It was demonstrated that the overexpression of cyclin-dependent kinase (CDK) 1, CDK4, cyclin B1 and cyclin D1 efficiently induced cell division in the post-mitotic mouse, rat and hiPSC-derived cardiomyocytes (25). However, the potential relationship between ROCK inhibition and cell-cycle regulation requires further investigation.

Y27632 has been demonstrated to suppress cardiac cell apoptosis *in vivo* and *in vitro*. Previous studies have demonstrated that ROCK inhibitors enhanced post-ischemia cardiac function, suppressed cardiac cell apoptosis and decreased the accumulation of neutrophils in the heart following ischemia and reperfusion injury in mice (26-29). Bian *et al* (30) reported that the pretreatment of Y27632 and 3-aminobenzamide reduced myocardial infarction size and cardiomyocyte apoptosis via the poly (ADP-ribose) polymerase/ERK signaling pathway. More recently, Dong *et al* (31) evaluated the cardioprotective effects of Y27632 and demonstrated that Y27632 attenuated myocardial injury by inhibiting the activation of the MAPK and NF- κ B signaling pathway, suppressing apoptosis and the inflammatory response. The relationship between the Y27632-mediated inhibition of apoptosis in the present study with the MAPK and NF- κ B signaling pathways merits further exploration.

ROCK inhibitors have been previously applied in tissue engineering to improve cardiac cell engraftment for a number

of cardiovascular diseases based on cell therapy. Recently, Zhao *et al* (32) revealed that preconditioning with Y27632 increased the engraftment rate of transplanted hiPSC-CMs in a murine myocardial infarction (MI) model by a reduction in the number of apoptotic hiPSC-CMs but reported no changes in proliferation. In the present study, the suppressive effects of Y27632 on hiPSC-CM apoptosis was potentially caused by the suppression of expression and activity of caspase-3 and caspase-8. The results of proliferation were not consistent with those of Zhao *et al* (32), presumably due to different sources of hiPSC and differential experimental conditions, including treatment time and culture environments.

Additionally, previous studies have suggested that ROCK kinase is involved in cell adhesion to the extracellular matrix by modulating integrin avidity, which exerts further effects on downstream physiological functions (7,33,34). In particular, Martewicz *et al* (35) demonstrated the involvement of a mechanotransduction pathway, RhoA/ROCK, in the structural reorganization of hPSC-derived cardiomyocytes after adhesion. Treatment with Y27632 prevented the structural reorganization of the sarcoplasmic reticulum (SR) after attachment, modulating SERCA2 localization and promoting calcium cycling. They also suggested that SR structural reorganization was observed in hPSC-CMs derived from an embryonic bodies-based differentiation protocol that is distinct from monolayer-based differentiation protocols (35). Recently, Yan *et al* (36) demonstrated that the application of Y27632 promoted the gene expression of matrix metalloproteinases 2/3 and Notch-1 signaling to modulate Yes-associated protein-1 localization in the regulation of efficient patterning of cardiovascular spheroids following mesoderm formation from hPSC. Therefore, the possibility that ROCK kinase-associated integrin signaling and receptors may be associated with cardiomyocyte maturation, physiological function, proliferation and apoptosis, which has not been examined in depth in the present study, cannot be ruled out (37,38).

In conclusion, the present study demonstrated that treatment with the ROCK inhibitor Y27632 significantly promoted the survival of dissociated hiPSC-CMs by increasing proliferation and attenuating apoptosis (Fig. 5). The application of Rho-kinase inhibitors will be beneficial for the culturing of CMs, which may facilitate the development of applications of hiPSC-derived CMs for multiple research areas. However, the associated mechanisms require further elucidation.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NQ and YW conceived and designed the experiments of the current study. MK and MJ performed the experiments. MK wrote the manuscript. HW and YY analyzed the data. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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