

HIF-1 α overexpression improves the efficacy of human induced pluripotent stem cell-derived cardiomyocytes for cardiac repair following myocardial infarction

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Received June 26, 2024; Accepted October 22, 2024

DOI: 10.3892/mmr.2024.13405

Abstract. Myocardial infarction (MI) is the leading cause of death worldwide and currently there are limited therapies that can regenerate the lost cardiac cells following MI. To enhance the therapeutic effects of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) transplantation for treatment of MI, the present study sought to increase the pro-angiogenic effect of hiPSC-CM by overexpressing a mutated hypoxia-inducible factor 1- α (HIF-1 α ; P402A; P564A) via lentivirus transfection. Morphology and the electrophysiology of the genetically engineered cell were both unchanged. The present study demonstrated that the proangiogenic factors in the conditioned medium of the HIF-1 α -overexpressing hiPSC-CM (HIF-CM) were upregulated and subsequently resulted to the rescue of the tube forming ability and migratory ability of the hypoxia-injured human umbilical vein endothelial cells. Using a MI mouse model, the present study demonstrated that the transplantation of HIF-CM greatly improved cardiac function, decreased scar size, promoted the concentration of the proangiogenic factors in circulation and promoted the neovessel formation in mice with MI. In conclusion, HIF-1 α -overexpressing hiPSC-CM could increase the angiogenesis of endothelial cells and mediate cardioprotection in mouse following MI.

Introduction

Cardiovascular diseases (CVDs) encompass a broad spectrum of disorders affecting the heart and blood vessels, representing a significant global health challenge (1). Among these, myocardial infarction (MI) stands out as a critical and potentially life-threatening event (2). MI happens when disrupted blood flow in the ischemic heart

causes a great deal of cardiomyocyte death, eventually leading to pathological left ventricular (LV) remodeling and heart failure (3). The inherent limited regenerative capacity of the heart impedes self-restoration post-MI, accentuating the lasting effect. Despite medical progress in promptly addressing obstructed blood flow, there are no FDA-approved drugs to regenerate the lost cardiomyocyte during ischemia (4).

Cell transplantation using human induced pluripotent stem cells (hiPSCs) has been proved as a promising therapeutic approach to improve cardiac function following an ischemic event (5). hiPSCs can be induced to fully differentiate into cardiomyocytes (hiPSC-CM) with spontaneous beating, expression of cardiac markers like cardiac troponin T (cTnT) and myocardial heavy chain (MHC) and sarcomeric α actin (SAA) (6). When transplanted into the infarcted area, hiPSC-CMs integrate with existing cardiac cells, contributing to the regeneration of functional myocardium (7). This therapeutic approach aims to enhance heart function, alleviate symptoms and reduce the risk of heart failure post-MI. Although this method offers a novel therapeutic perspective of restoring cardiac function, more work needs to be conducted to optimize engraftment and improve the long-term efficacy of hiPSC-CM transplantation.

Hypoxia-inducible factor 1- α (HIF-1 α) is a ubiquitously expressed, master regulator of genes that allow adaptation to hypoxic conditions (8). Target genes of HIF-1 α include vascular endothelial growth factor (VEGF), erythropoietin, glycolytic enzymes, glucose transporters and other factors critical to vascularization, metabolic regulation, cell proliferation and survival (9). Controlling vascularization by modulating the HIF pathway may be a valuable strategy in patients with ischemic diseases. Previous studies report that overexpressed HIF-1 α in mesenchymal stem cells and exosomes derived from these cells mediates cardioprotection in MI by enhanced angiogenesis, which paved the road for the use of HIF-1 α in stem cell transplantation (10,11). In addition, HIF-1 α is unstable and usually degraded by a prolyl hydroxylase (PHD; prolyl hydroxylase domain) at two specific prolyl residues, Pro402 and Pro564 (12). Thus, a mutated HIF-1 α (P402A, P564A) was used in the present study to enhance its stability (13).

The present study sought to investigate the effects of the MHC promoter driven HIF-1 α overexpression in hiPSC-CM

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Key words: hypoxia-inducible factor 1- α , induced pluripotent stem cell, cardiomyocyte, myocardial infarction, angiogenesis

on hypoxia-injured human umbilical vein endothelial cells (HUVECs) and ischemic heart. It showed that the proangiogenic paracrine effects of the iPSC-CMs were enhanced by HIF-1 α overexpression, which then resulted in the rescue of the migratory ability and angiogenic function of hypoxia-injured HUVECs. Moreover, HIF-1 α overexpressed hiPSC-derived cardiomyocytes exhibited a strong cardioprotective effect on MI heart by promoting neovessel formation in the ischemic border zone. This therapeutic effect was also blocked by an angiogenesis inhibitor, rapamycin.

Materials and methods

Culture and cardiomyocyte differentiation of hiPSCs. Geltrex (cat. no. A1413302; Thermo Fisher Scientific, Inc.) was diluted 1:100 in ice-cold DMEM/F-12 (Thermo Fisher Scientific, Inc.) to coat a 6-well plate (1 ml/well). The coated plates were incubated at 37°C for ≥ 1 h before use. Prior to hiPSC cell plating, colonies were disaggregated into single cells using Accutase (cat. no. A1110501; Thermo Fisher Scientific, Inc.) to achieve a uniform cell suspension. Human iPSCs (passage 10) from the American Type Culture Collection were then seeded onto Geltrex-coated wells at a density of 2×10^5 cells/well, using Essential 8 Medium (E8; cat. no. A1517001; Thermo Fisher Scientific, Inc.) supplemented with 10 μ M Rock inhibitor (Y27632; cat. no. 1254; Tocris Bioscience) for 24 h. Subsequently, medium was changed to freshly prepared E8 without Y27632, with daily medium exchanges. For cardiomyocyte differentiation, GiWi protocol (14) was used as follows: upon reaching 80–90% confluency after two or three days, cells were exposed to 10 μ M CHIR99021 (cat. no. 4423; Tocris Bioscience) in RPMI 1640 (cat. no. 61870; Thermo Fisher Scientific, Inc.) supplemented with B27-without insulin (cat. no. A1895601; Thermo Fisher Scientific, Inc.) for 24 h. The medium was changed to RPMI-B27 without insulin for 48 h. On day 3, cells received 10 μ M IWP2 (cat. no. 3533; Tocris Bioscience) in RPMI-B27 without insulin for 48 h. Subsequent medium changes occurred every 48 h, with hiPSC-CM purification using RPMI-B27 with insulin supplemented with a 4 mM DL-lactate solution over four consecutive days starting on day 7. The Embryo Research Oversight process was not necessary for the present study.

Cloning and generation of HIF-1 α -overexpressing hiPSC-CMs. The backbone vector aMHC-mCherry-Rex-Blasticidin (cat. no. 21228; Addgene, Inc.) was used for the reconstruction of a lentiviral vector containing the mutated cDNA of HIF-1 α (P402A, P564A; cat. no. 52636; Addgene, Inc.) (13). Mutation of Pro402 and Pro564 in HIF-1 α would enhance its stability under normoxic conditions. The HIF-1 α cDNA was amplified using the Q5 High-Fidelity DNA Polymerase (New England BioLabs, Inc.), then cloned into the backbone vector aMHC-mCherry-Rex-Blasticidin using the NEBuilder HiFi DNA Assembly Kit (New England BioLabs, Inc.). Lentivirus (2nd generation) was packaged using 293T cells (cat. no. CRL-3216; American Type Culture Collection) by transfecting psPAX2 (7 μ g; cat. no. 12260; Addgene, Inc.), pMD2.G (3 μ g; cat. no. 12259; Addgene, Inc.), and the backbone vectors (10 μ g) or the HIF-1 α expressing vector (10 μ g) with packaging plasmids by Lipofectamine[®] 3000 (Invitrogen;

Thermo Fisher Scientific, Inc.) according to manufacturer's instruction. Culture medium was collected at 24, 48, and 72 h after transfection. Lentivirus was concentrated from culture medium by PEG 8000 precipitation. HIF-1 α overexpression hiPSC cell line was established by lentiviral transduction (at 5 multiplicity of infection (MOI)) and blasticidin selection (5 μ g/ml; Gibco; Thermo Fisher Scientific, Inc.). The transfected hiPSCs were maintained in mTeSR Plus medium (cat. no. 100-0276; STEMCELL Technologies) supplemented with 5 μ g/ml blasticidin and passaged for at least 2 passages (~ 6 days) before conducting further experiments. Reverse transcription-quantitative (RT-q) PCR was performed to evaluate HIF-1 α expression level in hiPSC-CM.

RT-qPCR. TRIzol[®] (Thermo Fisher Scientific, Inc.) was used to isolate the total RNA from cells (at 90% confluency). RT was executed using the PrimeScript RT reagent kit (Takara Bio, Inc.). HIF-1 α , VEGF, angiopoietin 1 (Ang-1), fibroblast growth factor 1 (FGF-1) and platelet-derived growth factor receptor alpha (PDGFRA) expression levels in hiPSC-CMs were assessed through the SYBR Green (cat. no. 12369010; Invitrogen) assay following the manufacturer's instructions. GAPDH served as the control. RNA extraction, cDNA synthesis, and qPCR were all performed according to the manufacturer's protocols. Primer details are provided in Table I. The $2^{-\Delta\Delta C_q}$ method (15) determined relative mRNA expression and each assay was conducted in triplicate. Thermocycling conditions were as follows: 50°C for 2 min and 95°C for 10 min 1 cycle, 95°C for 15 sec and 60°C for 1 min 40 cycles.

HUVECs culture. HUVECs were purchased from American Type Culture Collection (cat. no. PCS-100-010; passage 3) and maintained according to the manufacturer's protocol. In brief, cells were cultured on a 10 cm dish and maintained in EGM-2 Endothelial Cell Growth Medium-2 BulletKit (cat. no. CC-3162; Lonza Group Ltd.) For hypoxic culture, HUVECs were cultured in a standard incubator composed of 94% N₂, 5% CO₂ and 1% O₂ for 48 h. Hypoxia-induced HUVECs were used in the following Matrigel and migration assays.

Microelectrode array (MEA)-based analysis. The MEA, also referred to as multielectrode arrays, consists of multiple small electrodes embedded in the culture surface of the well. Electrically active cardiomyocytes were cultured on top of these electrodes. CytoView MEA 24-well plates (Axion BioSystems, Inc.) were pre-coated with Geltrex and incubated at 37°C for 1 h. After dissociating cardiomyocytes from six cell plates, 100,000 wild type hiPSCs cardiomyocytes (WT-CM) or HIF-1 α -overexpressing hiPSC-CM (HIF-CM) per well were seeded onto the electrodes of the CytoView MEA 24-well plate and the assay performed at 48 h later. Data were calculated using Axion BioSystems Integrated Studio software (version 2.4; Axion BioSystems, Inc.).

ELISA assay. The concentration of three proangiogenic factors, VEGF, PDGF and Ang-1, were evaluated in the conditioned medium of WT-CM and HIF-CM and blood. Cardiomyocytes were cultured in fresh medium for 48 h and then the conditioned medium was collected for the subsequent ELISA assay. Protein concentrations were determined by VEGF, Ang-1

Table I. Reverse transcription-quantitative PCR Primers.

Gene name	Forward primer (5'-3')	Reverse Primer (5'-3')
GAPDH	GCCTCAAGATCATCAGCAATGC	CCACGATACCAAAGTTGTCATGG
HIF1A	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
PDGFRA	TGGCAGTACCCCATGTCTGAA	CCAAGACCGTCACAAAAAGGC
VEGF-A	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
Ang-1	AGCGCCGAAGTCCAGAAAAC	TACTCTCACGACAGTTGCCAT
FGF1	CTCCCGAAGGATTAAACGACG	GTCAGTGCTGCCTGAATGCT

HIF1A, hypoxia-inducible factor 1 α ; PDGFRA, platelet-derived growth factor receptor A; VEGF-A, vascular endothelial growth factor A; Ang-1, angiopoietin 1; FGF1, fibroblast growth factor 1.

and PP775 ELISA kits purchased from Beyotime Institute of Biotechnology (cat. nos. PV963, PA033 and PP775, respectively) and the experiment was performed according to the manufacturer's instructions.

Matrigel assay. HUVECs were used for *in vitro* tube formation assay as described previously (16). A total of 40,000 HUVECs suspended in HIF-1 α -hiPSC-CMs conditioned medium or hiPSC-CMs conditioned medium were applied to 100 μ l of Matrigel (BD Biosciences) coated wells (at 37°C for 1 h) in an 8-well glass chamber slide (BD Falcon; BD Biosciences) and then incubated at 37°C for 24 h. The number of formed tubes were counted and averaged with the help of a computer assisted fluorescent microscope (Olympus Corporation) at 20x magnification. Five random fields were examined for each biology replication.

Migration assay. HUVECs (5x10⁴ cells/well) were seeded in top chamber of the Transwell plates in FBS-free media with membrane inserts without Matrigel coating. Then 0.6 ml DMEM supplemented with 10% FBS as attractant was added to the well of the plate (lower compartment). The plate was incubated at 37°C for 16 h. Then cells that migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde at room temperature for 10 min, stained with DAPI (room temperature for 5 min) and observed under a fluorescence microscope (Olympus Corporation) at 20x magnification. Five random fields were examined for each biology replication. The migrated cells were quantified and normalized per mm².

Animals. A total of 10 Immunodeficient NOD/SCID mice, female, aged 8~12-weeks-old, weighed 18~25 g were purchased from Liaoning Changsheng Biotechnology co., Ltd., housed at Animal Facility of the Fourth People's Hospital of Shenyang (Liaoning, China) at 18~23 °C with 40-60% humidity on a 12-h light/dark cycle with free access to water and standard rodent food. All animal procedures were approved by the Ethics Review Committee of the Fourth People's Hospital of Shenyang (Liaoning, China) and were performed in accordance with the Guidelines for the Care and Use of Research Animals (version 2017.8) established by the Fourth People's Hospital of Shenyang (Liaoning, China) (17).

Mouse MI model. Mouse MI model was performed according to a previous study (18). Briefly, mice were anesthetized

with inhaled isoflurane (5% for induction, 1.5-2.0% for maintenance), intubated and ventilated with a small animal respirator before left thoracotomy was performed and the fourth intercostal space was entered using scissors and blunt dissection. An 8-0 silk suture was placed through the myocardium into the anterolateral LV wall around the left anterior descending (LAD) coronary artery. The suture was tied resulting in permanent ligation of the LAD and subsequent MI was confirmed via the use of the electrocardiogram (ECG) monitor. After LAD ligation, animals were assigned to four treatment groups: Vehicle group, WT-CM group, HIF-CM group and HIF-CM + Rapamycin group. Specifically, Vehicle group received 30 μ l DMEM medium; WT-CM group received 1x10⁶ wildtype hiPSC-derived cardiomyocyte in 30 μ l DMEM medium; HIF-CM group received 1x10⁶ HIF-1 α -overexpressed hiPSC-derived cardiomyocyte in 30 μ l DMEM medium; HIF-CM + Rapamycin group received 1x10⁶ HIF-1 α -overexpressed hiPSC-derived cardiomyocyte together with rapamycin (10 μ g/kg) intraperitoneally administered in the mice. The injected cells were delivered into the infarct border zone by three intramyocardial injections (equal volume; 10 μ l/site). After chest closure, buprenorphine (0.1 mg/kg; Buprenex; Reckitt Benckiser Pharmaceuticals Inc.) was administered subcutaneously immediately following surgery and every 8-12 h for 72 h. Animal health was monitored every 8-12 h for the first 72 h post-surgery and then at least every 3 days afterwards. All study animals recovered well from the surgery. The study was continued for 28 days before termination. Animals were humanely sacrificed by cervical dislocation under anesthesia with 5% isoflurane. Failure to detect respiration and absence of a heartbeat for >5 min was used to confirm death.

Echocardiography. The mice underwent anesthesia with 1.5% isoflurane and was positioned supine on a heated platform equipped with embedded ECG leads (FUJIFILM Visual Sonics) to maintain body temperature. Ultrasound coupling gel, heated to 37°C, was applied to the chest area. Using a linear array transducer (18-23 MHz) on the mid-ventricular level, two-dimensional B-mode parasternal long and short axis views were acquired using Vevo 2100 (FUJIFILM VisualSonics). One-dimensional M-mode images were then captured in the short-axis view to measure cardiac wall and chamber dimensions. Offline analysis involved measuring LV

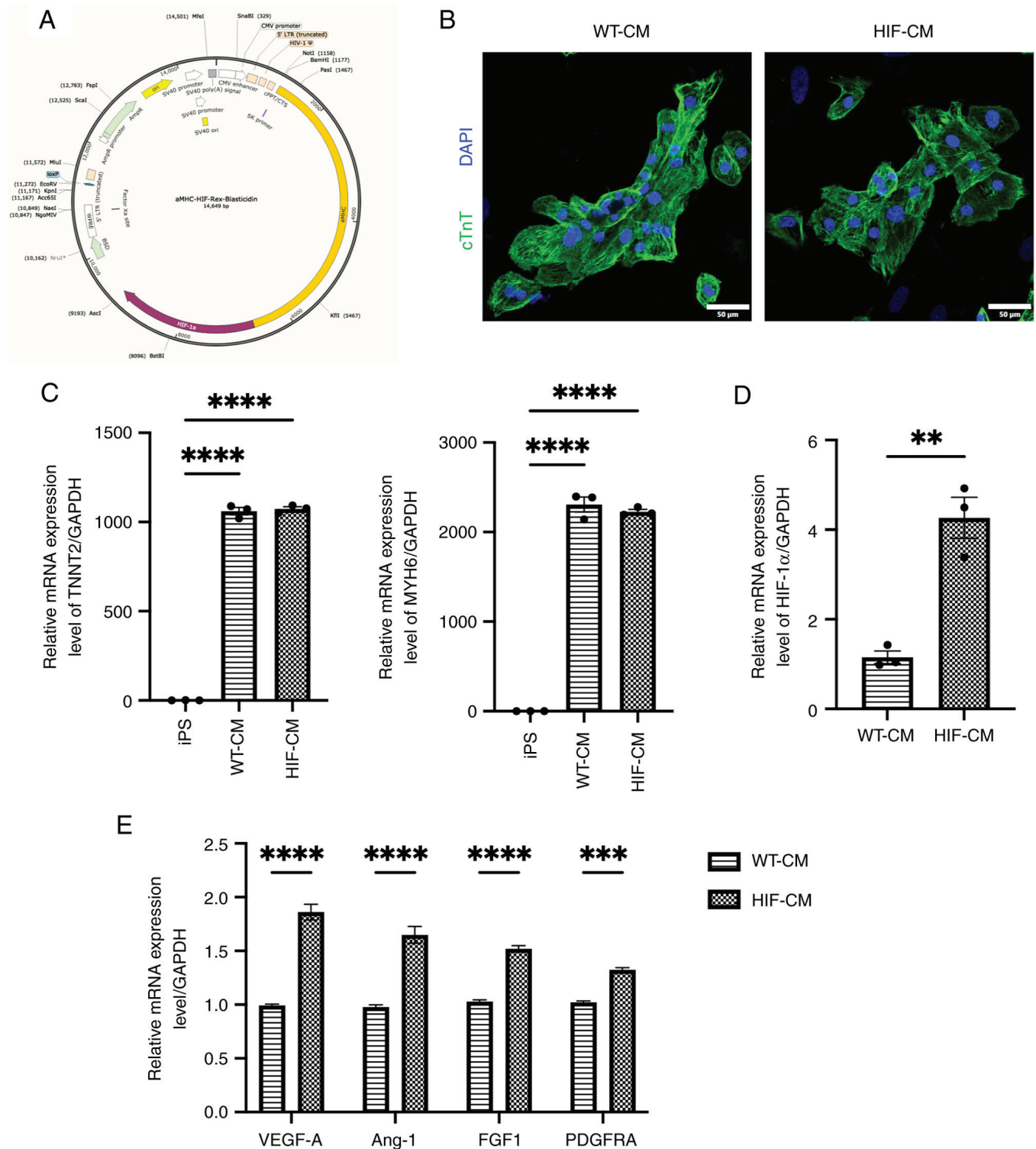


Figure 1. HIF-1 α overexpression in HIF-CM. (A) The plasmid that express HIF-1 α driven by the α -MHC promoter is used for lentiviral transduction in hiPSC. (B) Immunofluorescent staining against cTnT shows the successful differentiation of WT-CM and HIF-CM. 20X magnification. (C) RT-qPCR was used to examine cardiomyocyte specific gene TNNT2 and MYH6. (D) RT-qPCR was used to validate the success overexpression of HIF-1 α in cardiomyocytes. (E) The expression levels of pro-angiogenic factors were evaluated by RT-qPCR. HIF-1 α , hypoxia-inducible factor 1-alpha; HIF-CM, HIF-1 α -overexpressing hiPSC-CM cardiomyocytes; α -MHC, α -myosin heavy chain; cTnT, cardiac troponin T; WT-, wild-type-; CM, cardiomyocytes; RT-qPCR, reverse transcription-quantitative PCR; iPS, induced pluripotent stem cells. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

chamber size and wall thickness from at least three consecutive beats, averaging results. Parameters included LV wall thickness at the intraventricular septum and posterior wall during systole and diastole, as well as LV internal dimensions (LVID) during systole and diastole. LV percent fractional

shortening (FS) and ejection fraction (EF) were subsequently calculated based on the M-mode measurements.

Measurement of infarct size. The hearts were excised at 28 days after MI and the infarct size was evaluated as

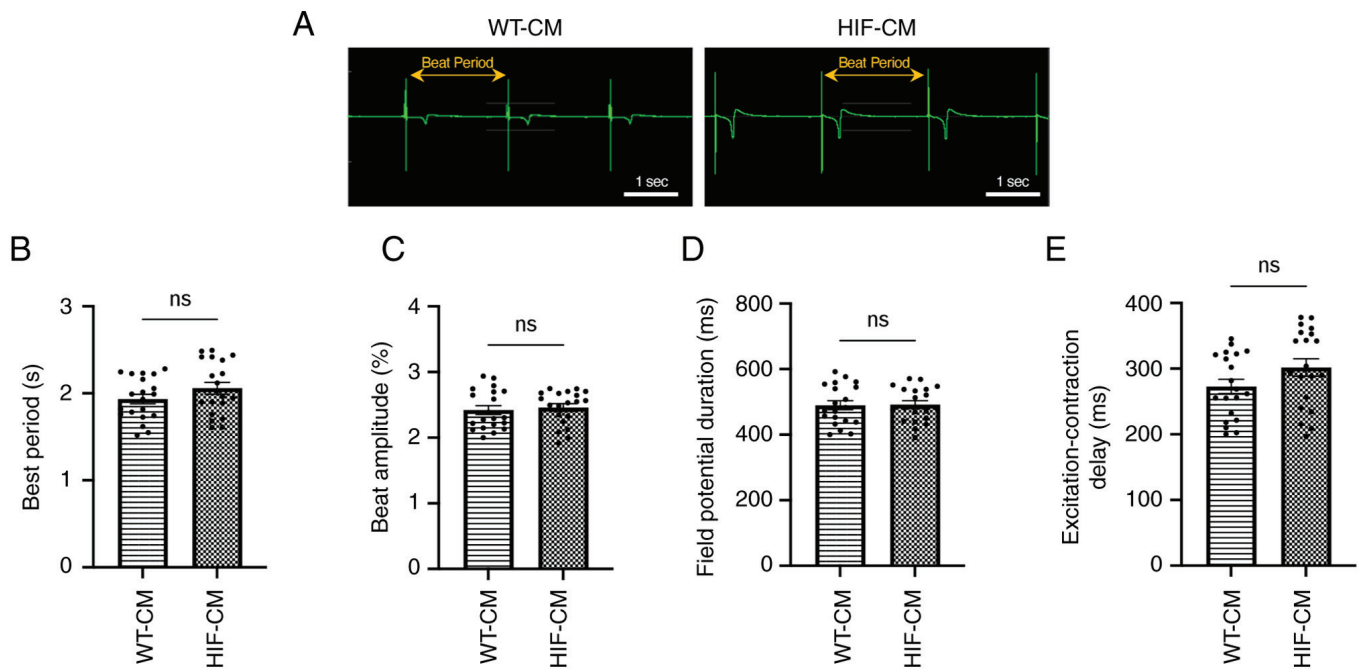


Figure 2. Microelectrode array analysis shows HIF-1 α overexpression in hiPSC-CM does not change the electrophysiology property. (A) Representative field potential recordings are displayed for WT-CM and HIF-CM. Field potential recordings were used for calculation of (B) beat period and (C) amplitude, (D) field potential duration and (E) excitation-contraction delay in WT-CM and HIF-CM groups. $n=20$. ns, not significant; HIF-1 α , hypoxia-inducible factor 1-alpha; HIF-CM, HIF-1 α -overexpressing hiPSC-CM cardiomyocytes; WT-, wild-type-; CM, cardiomyocytes.

previously described (19). In summary, the hearts were fixed overnight in a 4% paraformaldehyde solution at 4°C, followed by dehydration in 30% sucrose for 12 h at 4°C. Subsequently, the hearts were embedded in optical cutting temperature compound. Then 10- μ m-thick short-axis sections spanning from the base to the apex were cut from the entire ventricles and affixed to glass slides. For each mouse, five sections were treated with Bouin's solution and stained using 0.04% Sirius Red/0.1% Fast Green collagen staining (MilliporeSigma). The sections of the left ventricle were imaged by an Olympus SZ61 Stereo light microscope at 4.5x magnification. The microscope was able to capture the whole cross-section of the left ventricle, thus, one image was taken for each slide. The percentage of fibrotic length was calculated using the arc length of the fibrotic area divided by the circumference of the left ventricle. The percentage fibrotic area was calculated using the area of the fibrotic tissue (red) divided by the area of the left ventricle. All these parameters were quantified by ImageJ (National Institutes of Health, version 1.52d).

Immunostaining. Heart sections (8 μ m) were fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.1% Triton X-100 at room temperature for 3 min, blocked with 5% donkey serum (Sigma-Aldrich, cat. no. D9663) at room temperature for 20 min and then incubated with anti-CD31 antibody (cat. no. ab28364, Abcam; 1:200), anti-alpha smooth muscle actin (α -SMA; cat. no. ab5694; Abcam; 1:200) and anti-cTnT (cat. no. MAB1874; Bio-Techne; 1:50) at 4°C overnight. After washing with PBS for 3 times, sections were incubated with Alexa Fluor 488 Anti-Rabbit secondary antibody (Thermo Fisher Scientific, Inc.) and DyLight 549 Anti-Rabbit secondary antibody (Thermo

Fisher Scientific, Inc.) in dark at room temperature for 1 h. The sections were then washed with PBS and mounted with Vectashield (cat. no. H-1800; Vector Laboratories, Inc.). Images were captured under a fluorescent microscope (Olympus Corporation) at 20X magnification. Five random fields were examined for each biology replication.

Statistical analysis. The data were expressed as mean \pm SEM. Significance was assessed using the unpaired Student's t-test for two-group comparisons or one-way ANOVA with Tukey's multiple comparison test for comparisons among three groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HIF-1 α is overexpressed in hiPSC-derived cardiomyocytes. hiPSC cell line was purchased from American Type Culture Collection and maintained in Essential 8 medium for 30 passages. To specifically overexpress HIF-1 α in cardiomyocytes, but not hiPSC, an MHC promoter was applied to drive the HIF-1 α expression and the overexpression plasmid was transduced into hiPSC by lentiviral transduction (Fig. 1A). Cardiomyocytes differentiation was induced in both wild-type hiPSC and HIF-1 α -transduced hiPSC using the GiWi protocol (20). Further characterization by immunostaining with cTnT (Fig. 1B) and RT-qPCR (Fig. 1C) showed that cTnT and MYH6 expression levels were significantly increased in both WT-CM and HIF-CM, indicating the successful CM differentiation. Immunostaining also did not indicate any morphologic changes between WT-CM and HIF-CM. HIF-1 α expression was increased by more than 4-fold in HIF-1 α overexpressed hiPSC-derived cardiomyocytes (HIF-CM) compared with

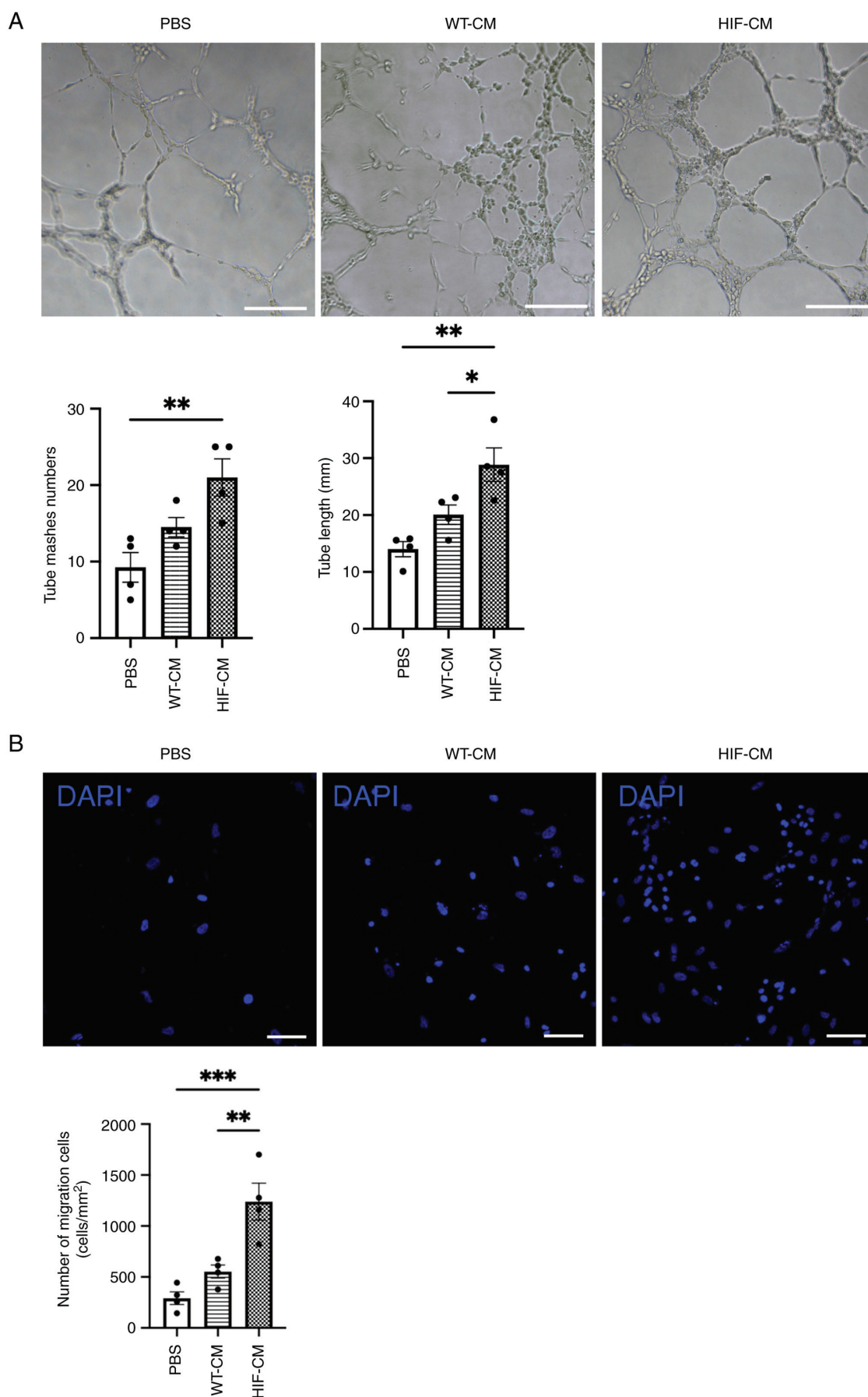


Figure 3. Conditioned medium from HIF-CM increases the migration and tube formation of hypoxia-induced HUVECs. (A) Representative figures of tube constructs formed by HUVECs on Matrigel. Tube meshes numbers and tube length were quantified. Scale bar, 50 μ m. (B) Representative figures of migrated HUVECs using a modified Boyden chamber. Migrated cell numbers were quantified. Scale bar, 50 μ m. n=4; *P<0.05, **P<0.01, ***P<0.001. HIF-CM, HIF-1 α -overexpressing hiPSC-CM cardiomyocytes; HUVECs, human umbilical vein endothelial cells; WT-, wild-type-; CM, cardiomyocytes.

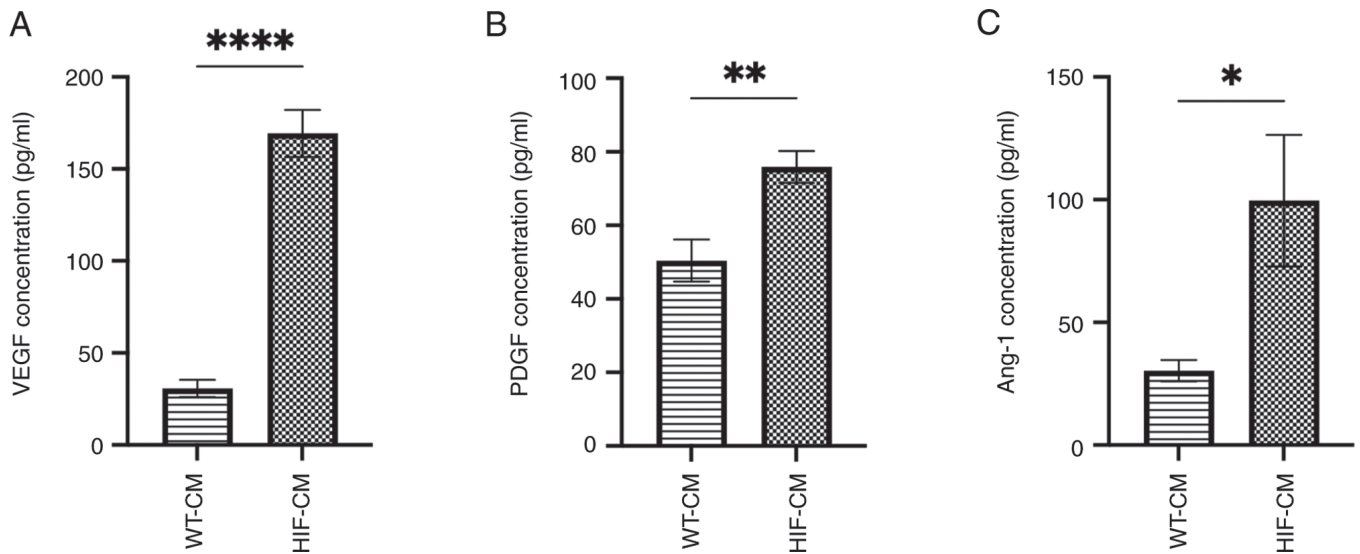


Figure 4. Upregulated downstream proangiogenic factors in the conditioned medium of HIF-CM. Protein expression level of (A) VEGF, (B) PDGF and (C) Ang-1 detected by ELISA in the conditioned medium of hiPSC-CM, respectively (n=5). *P<0.05, **P<0.01, ****P<0.0001. HIF-CM, HIF-1 α -overexpressing hiPSC-CM cardiomyocytes; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor receptor; Ang-1, angiopoietin 1; WT-, wild-type-; CM, cardiomyocytes.

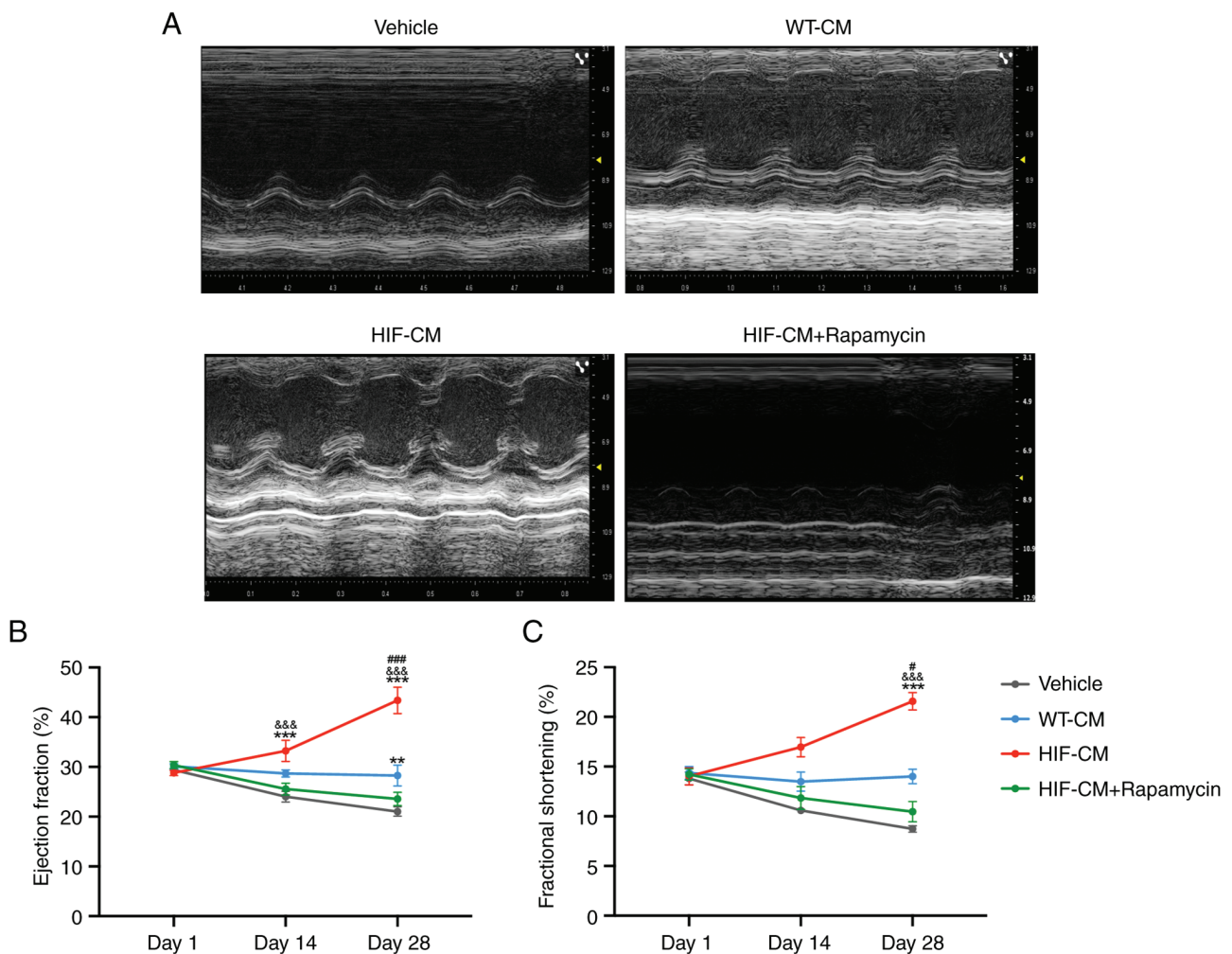


Figure 5. HIF-CM transplantation promotes cardiac function following MI. (A) Echocardiographic images were acquired from mice in Vehicle group, WT-CM group, HIF-CM group and HIF-CM + rapamycin group on days 1, 14 and 28 after MI induction and used to calculate (B) LVEF and (C) LVFS. n=5. **P<0.01 vs. Vehicle, ***P<0.001 vs. Vehicle; &&&P<0.01 vs. HIF-CM + Rapamycin; #P<0.05 vs. WT-CM, ###P<0.01 vs. WT-CM. HIF-CM, HIF-1 α -overexpressing hiPSC-CM cardiomyocytes; MI, myocardial infarction; WT-, wild-type-; CM, cardiomyocytes; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening.

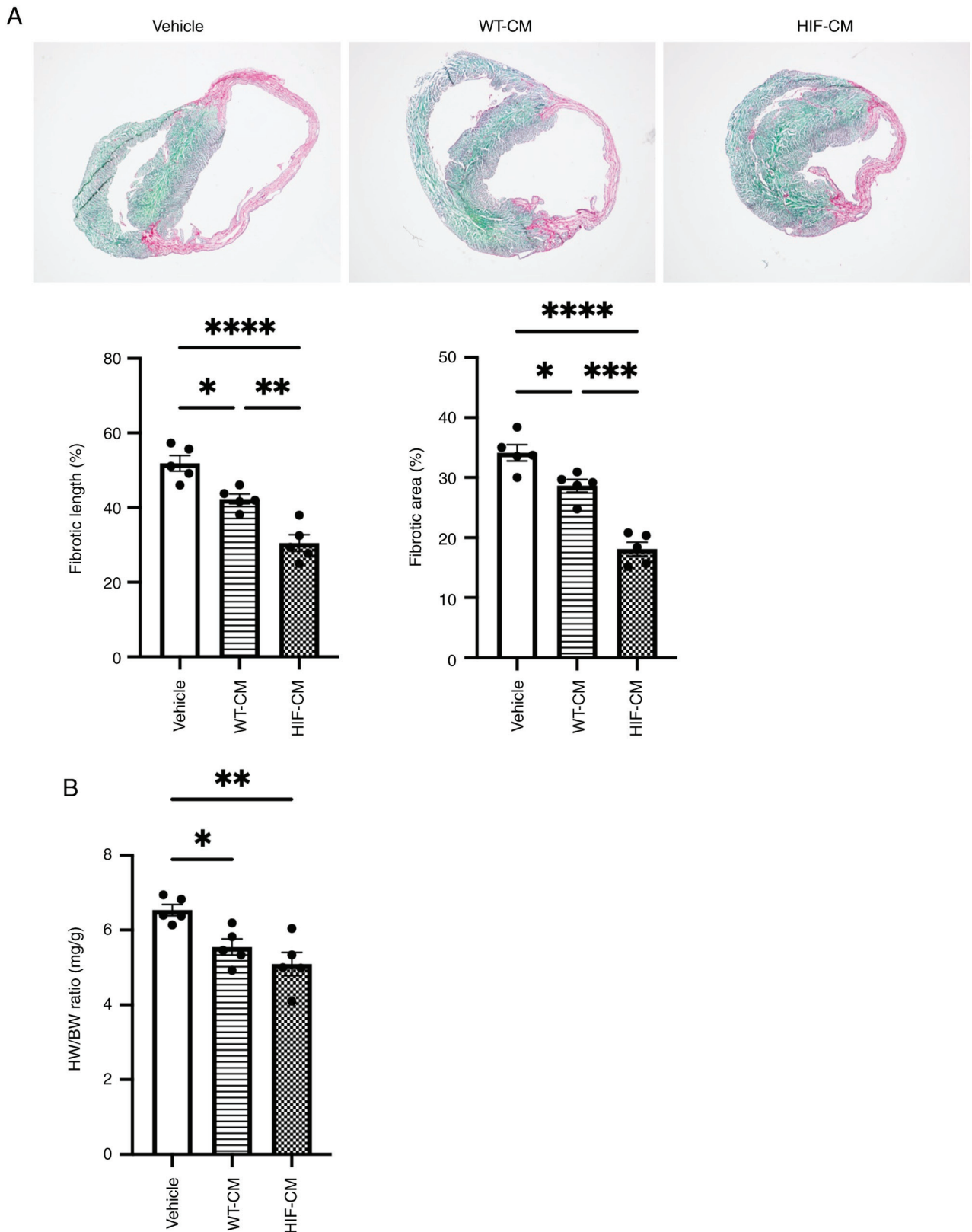


Figure 6. HIF-CM transplantation decreases scar formation and reduces cardiac hypertrophy. (A) LVs collected from mice on day 28 were sectioned and stained with Sirius Red and Fast Green to visualize fibrotic (red) and normal (green) tissue; then, fibrotic length ratio and fibrotic area ratio were quantified. Images were taken at 4.5x magnification. (B) HW and BW of each mouse was recorded to calculate HW/BW ratio. HIF-CM, HIF-1 α -overexpressing hiPSC-CM cardiomyocytes; HW, heart weight; BW, body weight; WT-, wild-type-; CM, cardiomyocytes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

WT-CM (Fig. 1D; $P < 0.01$). Moreover, the expression levels of pro-angiogenic factors, Vascular endothelial growth factor A, ang-1, FGF-1 and PDGFRA, were all elevated in the HIF-CM

group compared with WT-CM (Fig. 1E). In addition, MEA assay was performed to investigate the electrophysiological properties of the hiPSC-CMs. As shown in Fig. 2, the beat

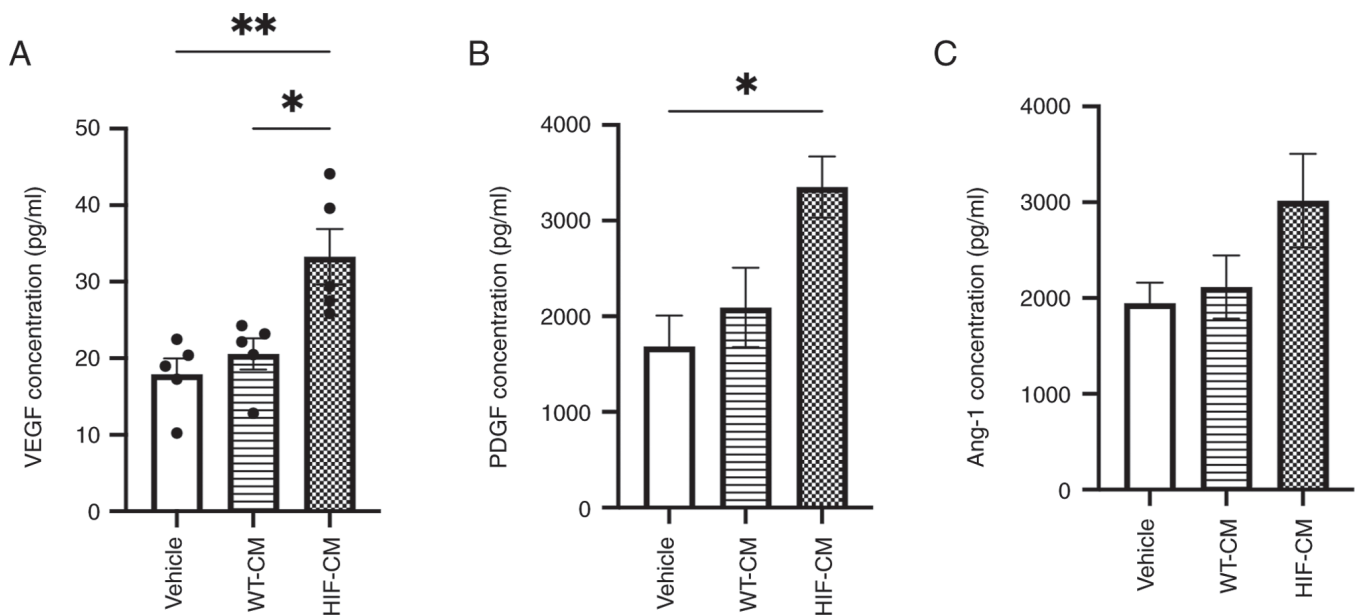


Figure 7. Upregulated proangiogenic factors in the circulation of HIF-CM-treated mice on day 7. Protein expression level of (A) VEGF, (B) PDGF and (C) Ang-1 detected by ELISA in the circulation of the treated animals (n=5). *P<0.05, **P<0.01; ns, not significant. HIF-CM, HIF-1 α -overexpressing human induced pluripotent stem cell-derived cardiomyocytes; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor receptor; Ang-1, angiopoietin 1.

period, beat amplitude, field potential duration and the excitation-contraction delay were not statistically different between WT-CM and HIF-CM, indicating the transgene did not alter the electrophysiology of the CMs.

In vitro angiogenesis and migration are rescued in hypoxia-injure HUVECs following treatment with conditioned medium collected from HIF-1 α overexpressed cardiomyocytes. Conditioned medium were collected from HIF-CM and WT-CM. Hypoxia-injured HUVECs were then cultured in conditioned medium collected from HIF-CM and WT-CM for 24 h. HIF-CM derived conditioned medium promoted tube formation of HUVECs; by contrast, WT-CM derived conditioned medium had less effect in inducing the tube formation (Fig. 3A). To examine the migratory effects, HUVECs were then cultured in the upper chamber of a Transwell plate; conditioned medium from HIF-CM and WT-CM were added in the lower chamber of the plate. More cells migrated towards the lower chamber with conditioned medium from HIF-CM (Fig. 3B). In addition, to determine if these promotional effects were related to the enhanced proangiogenic paracrine effect of the HIF-CM, the concentration of three proangiogenic factors, VEGF, PDGF and Ang-1, which are also regulated by HIF-1 α , were evaluated in the conditioned medium (Fig. 4A-C). Upregulated secretion of these three proangiogenic factors in the conditioned medium of HIF-CM was observed, which probably contributed to the rescue of the angiogenesis and migration in hypoxia-injured HUVECs.

HIF-1 α overexpresses cardiomyocytes enhanced cardiac function recovery and decreased scar size following acute MI. Transplantation of HIF-1 α overexpressed cardiomyocytes significantly improved echocardiographic parameters, EF and FS, in mice with MI. Echocardiography performed at 1, 14 and 28 days (Fig. 5A) following cell transplantation demonstrated that

LV contractility was markedly improved in the HIF-CM-treated group compared with that in the Vehicle and WT-CM groups (P<0.01 vs. Vehicle, WT-CM; Fig. 5B and C). Notably, a fourth group, in which the mice received HIF-CM intramyocardially and rapamycin (an angiogenesis inhibitor) intraperitoneally, was included to serve as an intervention group. The therapeutic effect mediated by HIF-CM was blocked by this angiogenesis inhibitor, indicating HIF-CM promoted cardiac function recovery by promoting angiogenesis.

Scar size was also evaluated 4 weeks after MI induction by Fast Green/Sirius Red staining to indicate fibrotic tissue (Fig. 6A). Although WT-CM transplantation could decrease scar size compared with Vehicle treatment, HIF-CM further decreased scar size when compared with WT-CM. Heart weight was recorded after sacrificing the mice to calculate heart/body weight ratio (Fig. 6B). The results showed that HIF-CM significantly reduced the Heart Weight/Body Weight ratio compared with other two groups, suggesting that HIF-CM could prevent the cardiac hypertrophy following MI. Altogether, transplantation of HIF-1 α overexpressed cardiomyocytes presented significant LV functional recovery compared with WT-CM group following MI.

Angiogenesis is significantly improved following transplantation of HIF-1 α overexpressed cardiomyocytes. Supported by the improved cardiac function and reduced scar size, the present study sought to evaluate the mechanism of this therapeutic effect. It first revealed that concentration of angiogenic factors (VEGF and PDGF) in the circulation was elevated at day 7 post MI with HIF-CM transplantation (Fig. 7), which is in accordance with the *in vitro* study. On day 28, mice were sacrificed and the angiogenesis in the peri-infarction area of the infarcted hearts evaluated. Immunostaining with CD31 reflected neovascularization in the peri-infarct myocardium four weeks after MI (Fig. 8A). The averaged fluorescent

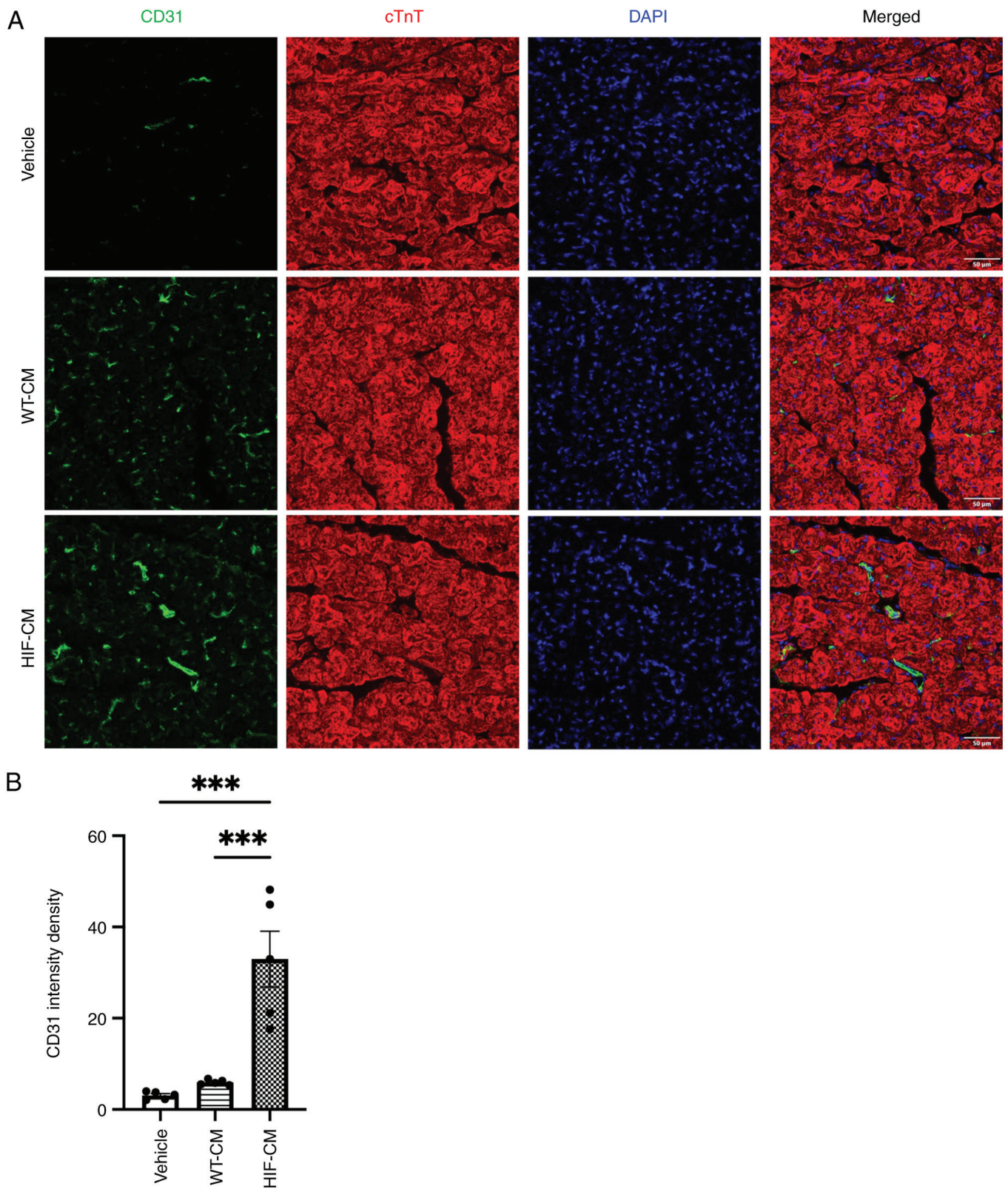


Figure 8. HIF-CM transplantation increases angiogenesis following MI. (A) Cardiac tissue from the injection zone were collected on day 28 and stained for the presence of cTnT and CD31 to evaluate the angiogenesis activity. 20x magnification. (B) CD31 intensity density was quantified using ImageJ. HIF-CM, HIF-1 α -overexpressing hiPSC-CM cardiomyocytes; MI, myocardial infarction; cTnT, cardiac troponin T; WT-, wild-type-; CM, cardiomyocytes. ***P<0.001.

intensity density in the ischemic border zones of left ventricle was significantly greater in the HIF-CM-transplanted group compared with the WT-CM-transplanted group and Vehicle group (Fig. 8B).

Discussion

Previous studies have demonstrated the therapeutic effects of hiPSC-derived cardiomyocytes in a murine

infarction model (5,21). Although these cells were able to exert myocardial protection to a certain degree, the therapeutic efficacy of the transplanted cells still needs to be improved (22). Previous studies using HIF-1 α overexpressed stem cells exhibited sufficient cardioprotection in MI (10,23), which prompted the present study to genetically engineer iPSC-derived cardiomyocytes as well by HIF-1 α overexpression.

The present study genetically engineered an iPSC clone overexpressing mutated HIF-1 α (P402A and P564A), which could enhance its stability under normoxia (13). The iPSC clone carries HIF-1 α gene that is expressed under control of a cardiac-specific promoter, MHC promoter (24). After cardiac differentiation, the present study was able to obtain contracting CMs with a purity of 85%. CMs showed a clear mature phenotype with strong expression of cTnT and MHC. Moreover, iPSC-derived CMs also showed high expression of HIF-1 α as determined by RT-qPCR. Conditioned medium was collected from cardiomyocytes differentiated from HIF-CM and WT-CM. In the tube formation assay the present study was able to effectively enhance the tube formation of hypoxia-injured HUVECs using conditioned medium from HIF-CM compared with WT-CM. The data also showed that conditioned medium from HIF-CM promoted the migration of hypoxia-injured HUVECs in Transwell assay. These effects were probably caused by the enhanced secretion of proangiogenic factors, VEGF, Ang-1 and PDGF. These findings showed the paracrine effects of HIF-CM, which are also in accordance with previous reports (10,11,25).

Following intramyocardial injection into ischemic myocardium, the HIF-CM group exhibited enhanced neovascularization compared with the WT-CM group. It was hypothesized that enhanced neovascularization was induced by the overexpression of HIF-1 α . HIF-1 α has been described as a master regulator of genes including VEGF, erythropoietin and other factors critical to vascularization (26). In the MI model of the present study, HIF-CMs significantly improved cardiac functions following injection into the infarct area. The echocardiographic data suggested that following transplantation of HIF-CM enhanced angiogenesis could be a critical reason for the survival of the surrounding cardiomyocytes in the ischemic myocardium. Future experiments will have to establish whether HIF-1 α overexpression may improve the myocardial connection in our model.

In summary, the present study demonstrated that the direct intramyocardial transplantation of HIF-1 α -engineered iPSC-derived cardiomyocytes led to substantial functional improvement and mitigated adverse remodeling 28 days post-acute MI, as evidenced by both echocardiography and histological morphology assessment. The preservation of LV thickness in the infarct zone prevented progressive LV dilatation. Future investigations delving into the mechanical mechanisms by which transplanted cells confer therapeutic effects would enhance our understanding and potentially advance stem cell therapy for ischemic heart diseases.

Acknowledgements

Not applicable.

Funding

The present study was supported by Natural Science Foundation of Liaoning Province (grant no. 20170540527).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JD and LX conceived and designed the project. JD and TW acquired and analyzed data. JD and LX wrote and revised the manuscript. All authors read and approved the final manuscript. JD and LX confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by The Fourth People's Hospital of Shenyang Ethics Review Committee (Liaoning, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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