Effects of transplantation of hypoxia-inducible factor-1α gene-modified cardiac stem cells on cardiac function of heart failure rats after myocardial infarction

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Abstract

Objective: To evaluate the effects of transplantation of hypoxia-inducible factor-1α (HIF-1α) gene-modified cardiac stem cells (CSCs) on the cardiac function of heart failure rats after myocardial infarction (MI).

Methods: Twenty-four Sprague–Dawley rats were randomly divided into three groups: HIF-1α-modified CSCs group, single CSCs group, and model group. The model of heart failure after MI was established by thoracotomy-left anterior descending coronary artery ligation, followed by injection of modified CSCs, single CSCs, and PBS, respectively, 2 weeks later. The results were observed 4 weeks later.

Results: CSCs were infected with recombinant adenovirus. HIF-1α mRNA level of HIF-1α-enhanced green fluorescent protein (EGFP)+CSCs group significantly surpassed those of EGFP+CSCs and CSCs groups ($p<0.001$). Left ventricular ejection fractions (LVEFs) of HIF-1α+CSCs+MI and CSCs+MI groups significantly increased compared with the model group ($p<0.001$). The difference between LVEFs before and after transplantation was positively correlated with the survival rate of CSCs in infarction border zone ($r=0.867$, $p<0.001$). The apoptosis rate of HIF-1α+CSCs+MI group was significantly lower than that of CSCs+MI group ($p=0.008$). The expression of vascular endothelial growth factor protein in HIF-1α+CSCs+MI group significantly increased, compared with that of MI group ($p<0.001$). The capillary density of HIF-1α+CSCs+MI group significantly exceeded that of CSCs+MI group ($p<0.001$).

Conclusion: Transplantation of either HIF-1α-modified CSCs or single CSCs reduced cardiomyocyte apoptosis in rats with heart failure after MI, promoted vascular regeneration in infarct area, and improved cardiac function. Particularly, HIF-1α-modified CSCs had more significant effects. *(Anatol J Cardiol 2018; 20: 318-29)*

Keywords: cardiac function, cardiac stem cell, hypoxia-inducible factor 1α, myocardial infarction

Introduction

Cardiac stem cells (CSCs) are undifferentiated cells that can differentiate into cardiomyocytes under certain conditions (1). They are mainly capable of cloning, differentiating into mature cardiomyocytes, vascular endothelial cells, and smooth muscle cells, as well as partially activating and migrating in the injured myocardium (2). After being separated from myocardial tissue *in vitro*, CSCs are cultured, screened, and transplanted into damaged myocardial tissue to stimulate blood vessel regeneration through differentiation into cardiomyocytes, which can improve ischemic myocardial microcirculation and participate in the treatment of heart failure. However, CSCs are often subjected to apoptosis in the hypoxic environment, seriously affecting transplantation efficacy (2). Hypoxia-inducible factor-1α (HIF-1α), as a nuclear protein with transcriptional activity, can be stably expressed under hypoxic conditions and control the expressions of over 100 genes downstream (3). After being expressed, these genes can participate in angiogenesis, glucose metabolism, and apoptosis and can maintain tissue and cell homeostasis under hypoxic conditions (4). Therefore, we herein transduced HIF-1α gene into CSCs to overexpress HIF-1α protein, aiming to evaluate the effects of HIF-1α-modified CSCs on the cardiac functions of rats with myocardial infarction (MI) and the changes of capillary density and cardiomyocyte apoptosis in the infarction border zone.

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Methods

Experimental animals and reagents

SPF 1-day-old male Sprague–Dawley (SD) rats and 3-month-old ones (250–280 g) were purchased from Experimental Animal Center of our hospital. Reporter gene containing enhanced green fluorescent protein (EGFP), HIF-1α adenovirus vector (Ad-EGFP-HIF-1α), and EGFP-containing adenovirus vector (Ad-EGFP) were bought from Shanghai Genomeditech Co., Ltd. (China). This study was approved by the Animal Ethics Committee of our hospital, and great efforts have been made to minimize the suffering of animals.

Isolation and culture of cardiomyocytes

The live heart was taken from a 1-day-old SD rat, quickly put into a plate containing PBS (containing 100 U/mL penicillin and 100 μg/mL streptomycin), and washed twice to remove the pericardium and other tissues. Then the heart was placed into a small beaker, immediately transferred to a clean bench, and 1 ml of PBS was added. The tissue was sheared into paste-like tissue blocks of less than 2 mm^3, pipetted by a straw to disperse cells, and washed twice with PBS. After the supernatant was discarded, the lower layer of tissue block was inserted into an EP tube, mixed with 0.25% trypsin free of calcium and magnesium and 0.02% EDTA, digested by shaking for 10–15 min at 37°C, and then centrifuged at 1000 rpm for 5 min. The resulting solution was discarded, and Iscove's modification of Dulbecco's medium (IMDM) with serum was added. The tissue was inoculated with the cover area of 25 cm^2 and cultured in an incubator at 37°C with 5% CO_2 and saturated humidity.

Identification of CSCs

Well-grown cells were selected and inoculated onto a coverslip for adherent growth. The coverslip full of cells was carefully tweezed 2 days later to label CSCs using the immunofluorescence assay. The cells on the coverslip surface were fixed in 4% paraformaldehyde solution at 37°C for 1 h. The coverslip was rinsed thrice with PBST for 5 min, and the cells were blocked with 5% bovine serum albumin at 37°C for 1 h, mixed with 1:100 dilution of anti-c-kit antibody after the background impurities were removed, incubated in a humidifying box at room temperature for 2 h, rinsed thrice with PBST for 5 min, mixed with 1:500 dilution of streptavidin-FITC conjugate, and incubated in dark at 37°C for 1 h. Afterwards, the cells were stained with 1:800–1000 dilution of DAPI for 3–5 min, mildly rinsed thrice with PBS for 5 min, and observed under a fluorescence microscope.

Purification of CSCs by flow Cytometry

Cells were digested with 0.25% trypsin, rinsed once with PBS, then resuspended with PBS, mixed with PBS containing 2% fetal bovine serum (FBS), and blocked for 1 h. Every 106 cells were mixed with 1 μl of anti-c-kit antibody diluted at 1:100, which reacted at room temperature for 20–60 min, washed once with PBS, centrifuged to remove the supernatant (800–1000 rpm×5 min), resuspended with PBS, then mixed with streptavidin-FITC conjugate diluted at 1:500, reacted in dark at room temperature for 30 min, washed once again with PBS, mixed with PBS into single-cell suspension, and detected by flow cytometry. Purified c-kit-positive CSCs were collected and inoculated into a cell culture flask.

Transfection of CSCs with recombinant adenovirus

CSCs transfected with recombinant adenovirus were divided into an experimental group, i.e., CSCs transfected by HIF-1α-EGFP recombinant adenovirus (HIF-1α-EGFP+CSCs group), a negative control group transfected by EGFP adenovirus (EGFP+CSCs group), and a blank group (single CSCs group). Well-grown CSCs were suspended with 0.25% trypsin, counted, then inoculated into a culture flask at the density of 3×10^5/mL. The cells were cultured in an incubator, and the culture medium was discarded 1 day later. Then the cells were washed thrice with PBS, mixed with 1 mL of serum-free medium, and transfected with virus suspensions containing Ad-EGFP-HIF-1α and Ad-EGFP, respectively, according to the best multiplicity of infection (MOI) (pre-determined MOI=300). After 2 h, the cells were further cultured with IMDM containing 10% FBS. After 72 h, the transfection results were observed using fluorescence microscopy, and the expression of HIF-1α gene was evaluated by RT-qPCR and Western blotting. Finally, the cell suspension was concentrated to 10^6/mL and transplanted into rats of each group, 0.1 mL in each rat.

Detection of HIF-1α mRNA expression by RT-qPCR

Total RNA was extracted from cell sample for reverse transcription into cDNA. The expressions of *HIF-1α* gene and *GAPDH* gene were detected by RT-qPCR. According to the RT-qPCR curve, the Ct values (threshold cycle number) of *HIF-1α* and *GAPDH* genes were obtained, and relative quantification was performed by using the $^{\Delta\Delta}$Ct method. The RT2 Profiler PCR Array Data Analysis System was used for data analysis. The primer sequences for detecting *HIF-1α* gene were (5′-3′) (HIF-1α-F: tatgagccagaagaacttttgggc; HIF-1α-R: CACCTCTTTTTGC AAGCATCCTG). The primer sequences for detecting *GAPDH* gene were (5′-3′) (GAPDH-F: ATG ATTCTACCCACGGCAAG; GAPDH-R: CTGGA AGATGGTGATGG GTT).

The PCR reaction system was 15 μl, comprising 5.7 μl of ultrapure water, 7.5 μl of 2×SYBR Mix premix, 0.15 μl of forward primer (10 μM), 0.15 μl of reverse primer (10 μM), and 1.5 μl of template mixed on ice. The reaction conditions were set as pre-denaturation at 95°C for 10 min; PCR cycle: 95°C, 15 s, and then 60°C, 60 s, a total of 40 cycles; melting chain temperature: 60°C→95°C.

Detection of HIF-1α protein expression by Western blotting

After total protein of cell sample was extracted, the sample was loaded for SDS-PAGE. After electrophoresis, the protein was electronically transferred to a nitrocellulose membrane.

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The membrane was placed in TBST for 10 min and blocked in blocking solution [TBST solution containing 5% bovine serum albumin (BSA)] at room temperature for 2 h. Then the membrane was washed twice with TBST for 10 min and incubated overnight at 4°C with HIF-1α primary antibody (diluted with TBST solution containing 5% BSA). After incubation with primary antibody, the membrane was washed thrice with TBST solution for 10 min each time, then incubated with PV-6001 goat anti-rabbit IgG/HRP secondary antibody (diluted with TBST solution containing 5% BSA) for 2 h at room temperature, and washed thrice with TBST, 10 min each time. Color development was conducted according to the ECL kit's instructions, followed by reaction in dark for 3–5 min and X-ray exposure for 1–2 min.

The membrane was analyzed using the Image J system, with the true value of all blots=system value–background value in the same box.

Animal grouping and establishment of MI model

Rats were anesthetized with 3% pentobarbital sodium. The 4th and 5th intercostal incisions were made under sterile conditions. An ophthalmic swaged needle was inserted along the central lower edge of the left auricle, with the depth through myocardium of about 1 mm. The left anterior descending coronary artery was ligated, and the heart rate and echocardiography (ECG) changes were simultaneously recorded. The whitening of the left ventricular anterior wall and the ST segment elevation shown on synchronous ECG were regarded as the successful signs of ligation. After transplantation, 200,000 U of penicillin was intramuscularly injected into each rat for three consecutive days.

Cell transplantation

Twenty-four SD rats with successful modeling were randomly divided into three groups 24 weeks later, according to the digital table method: model group, empty vector group (CSCs+MI group), and experimental group (HIF-1α+CSCs+MI group). The rats were anesthetized with 3% pentobarbital sodium and subjected to second thoracotomy. CSCs transfected with 0.1 mL of 1×10^6/mL Ad-HIF-1α-EGFP and 1×10^6/mL Ad-EGFP as well as PBS were injected into four sites of the infarction border zones of experimental group, empty vector group and model group (0.025 mL per site) respectively. Then 2×10^5 U of penicillin was routinely injected intramuscularly for three consecutive days.

The criteria for successful modeling were as follows. After ligation, the myocardial tissue in apex cordis of the anterior descending branch and some of the left ventricular anterior wall changed from red to gray, with the local myocardial contractility reducing. ECG showed continuous elevation of the arch in the ST segment, and cardiac ultrasound suggested left ventricular ejection fraction (LVEF) ≤50% two weeks later.

Cardiac ultrasound test of cardiac function

Cardiac function was tested by the same operator before modeling, 2 weeks after modeling and 1, 2, 3, 4 weeks after transplantation. Cardiac ultrasound was performed, and all values were measured thrice to obtain the averages.

Observation of transplanted CSCs by microscopy

Rats were sacrificed 4 weeks after transplantation. The myocardial tissue was taken, and paraffin sections were prepared. Histopathological changes were observed after HE staining. The myocardial tissue in the infarction border zone of each group was taken to prepare frozen sections. Yellowish green CSCs were observed by fluorescence microscopy at an excitation wavelength of 490 nm. Five infarction border zones with the highest survival density of CSCs were selected for each section, and the average number of CSCs in each visual field (10×20) was the survival rate.

Detection of cardiomyocyte apoptosis by TUNEL assay

The operation was conducted according to the instructions of TUNEL assay kit. Five non-overlapping 200× visual fields were randomly selected for the myocardial tissue of the infarction border zone to count apoptotic cardiomyocytes and total ones. Apoptosis rate of cardiomyocytes=number of apoptotic cardiomyocytes/total number of cardiomyocytes×100%.

Detection of serum cardiac troponin-T (cTn-T) and myocardial enzyme levels by ELISA

Blood was collected from rats that were sacrificed 4 weeks after transplantation and centrifuged at 3000 r/min for 20 min to collect the serum. Then, serum levels of cTn-T, creatine kinase (CK), CK-MB, and aspartate aminotransferase (AST) were detected by ELISA. Sample, standard substance, and HRP-labeled detection antibody were added into microwells precoated with cTn-T, CK, CK-MB, and AST antibodies, incubated and thoroughly washed. 3,3′,5,5′-Tetramethylbenzidine was used for color development, which turned into blue under the catalysis of peroxidase, and finally into yellow in the presence of acid. The color intensity was positively related with the level of cTn-T, CK, CK-MB or AST. The sample concentration was calculated by measuring the optical density at 450 nm with a microplate reader.

Immunohistochemical staining and calculation of capillary density

Immunohistochemical staining was performed on the surface of vascular endothelial cells labeled with platelet-endothelial cell adhesion molecule (PECAM-1/CD31) by the ABC method. The cells were incubated with rabbit anti-rat CD31 primary antibody (1:100 dilution) at 37°C for 2 h, then added dropwise with PV-6001 goat anti-rabbit IgG/HRP secondary antibody at room temperature, and incubated at 37°C for 37 min. The number of capillaries in each group was observed under the microscope according to the results of staining. Five infarction border zones with the highest capillary density were selected for each section, and the average number of vessels in each visual field (10×10) was capillary density.

Detection of gene expression changes by Western blot

According to a previous study (5), 10% separation gel, and 5% concentrated gel were prepared according to the molecular weight of vascular endothelial growth factor (VEGF) protein. The VEGF protein level of each group was detected quantitatively. VEGF protein was incubated with HIF-1α primary antibody overnight at 4°C and with PV-6001 goat anti-rabbit IgG/HRP secondary antibody at room temperature for 2 h.

The membrane was analyzed using the Image J system, with the true value of all blots=system value-background value in the same box.

Statistical analysis

All data were analyzed by SPSS 21.0. Normally distributed continuous variables were tested with repeated measures ANOVA, and non-normally distributed ones were analyzed with the nonparametric Friedman test. After ad-hoc test (F test), multiple comparisons (pairwise and simultaneous comparisons tests, post-hoc tests) were conducted with suitable multiple comparison tests. P<0.05 was considered statistically significant. All data were expressed as mean±standard deviation (x±SD).

Results

Growth and morphology of primary CSCs

After the rat's cardiac tissue was primarily cultured for 7–8 days, round CSCs with small volume and strong refractivity began to grow out of tissue edge (Fig. 1a).

Immunocytochemical identification of CSCs

Through anti-c-kit antibody and streptavidin-FITC markers, c-kit+CSCs were observed after visible light irradiation at the wavelength of 490 nm, which were yellowish green (Fig. 1b).

Growth and morphology of passaged CSCs

The passaged cells began to adhere to the wall within 24 h and reached 90% confluence after 6–7 days. The round CSCs

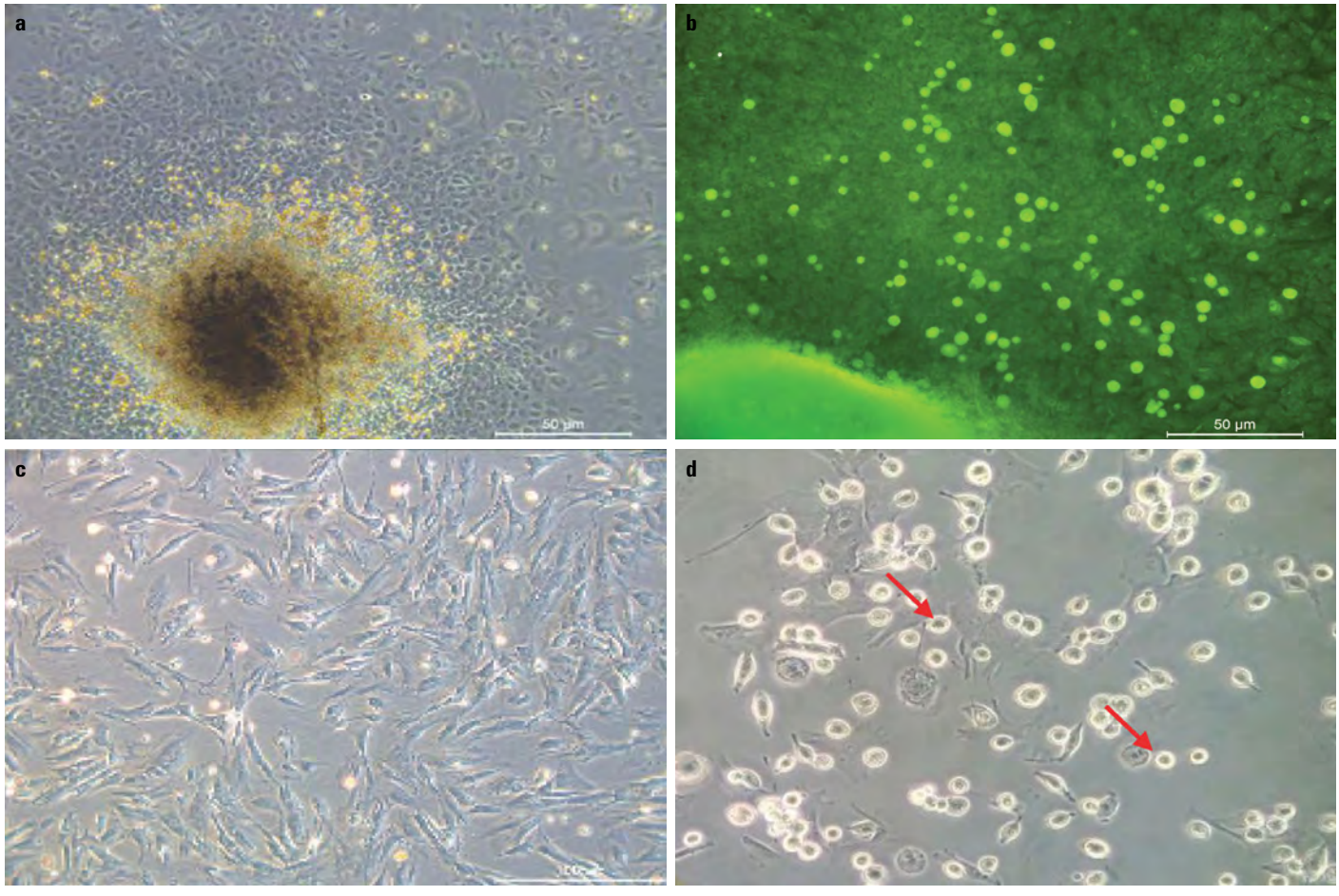

Figure 1. Identification of C-kit+CSCs in primary cell culture and growth of CSCs after flow cytometry. (a) Growth and morphology of primary CSCs. On the 7th day, round, small cells began to grow out from the edge of tissue section (magnification: 10×10); (b) immunocytochemical identification of CSCs. Visible yellow-green CSCs labeled with FITC (magnification: 10×10); (c) growth and morphology of passaged CSCs. Small round bright cells in subculture were still undifferentiated (magnification: 10×40); (d) flow cytometry results. After flow cytometry, CSCs continued to adhere to the wall, a small amount of which were regular and long spindle-shaped, then differentiating into cardiomyocytes (magnification: 10×40)
CSC- cardiac stem cell

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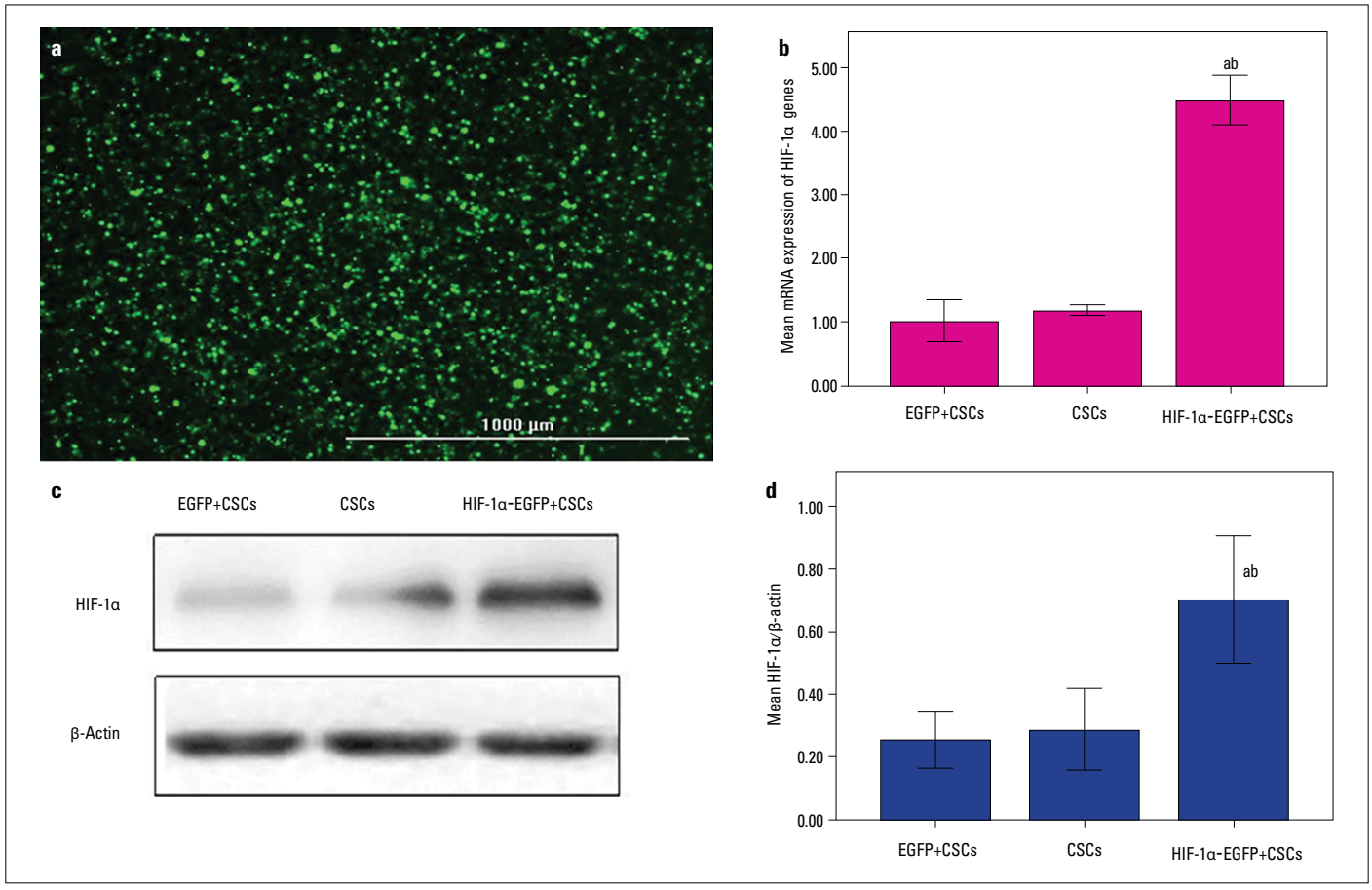

Figure 2. Vector-infected CSCs and HIF-1α mRNA and protein expressions after infection. (a) Fluorescence image for infection results. Third-generation CSCs were infected with fluorescent adenoviral vector, which all grew well. MOI=300, 10×10; (b) PCR results for HIF-1α mRNA expressions; (c) Western blot results for HIF-1α protein expressions; (d) histogram for Western blot results. Compared with MI group, [a]*P*<0.05; compared with MI+CSCs group, [b]*P*<0.05.

CSC - cardiac stem cell; HIF-1α - hypoxia-inducible factor-1α; MOI - multiplicity of infection; MI - myocardial infarction

also had small volume and strong refractivity. Besides, they remained undifferentiated (Fig. 1c).

Flow cytometry results

C-kit+CSCs were identified and recovered by flow cytometry. The purity of CSCs in cultured cells was about 13%. The cells then differentiated into myocardial ones (Fig. 1d).

Transfection results

CSCs were successfully transfected with recombinant adenovirus. They grew well after transfection, with high infection efficiency and no toxic side effects. The transfection efficiency was the best at MOI of 300 (Fig. 2a).

RT-qPCR results

The HIF-1α mRNA level of the HIF-1α-EGFP+CSCs group (4.483±0.156) was significantly higher than those of the EGFP+CSCs group (1.107±0.13) and the CSCs group (1.18±0.03) (p<0.001). EGFP+CSCs and CSCs groups had similar levels (p=0.206) (Fig. 2b).

Western blot results

The HIF-1α/β-actin ratio of the HIF-1α+CSCs group (0.702±0.195) was significantly higher than those of EGFP+CSCs and CSCs groups (p<0.001). The EGFP+CSCs group (0.253±0.087) had a similar HIF-1α/β-actin ratio to that of the CSCs group (0.287±0.124) (p=0.573) (Fig. 2c and 2d).

Cardiac ultrasound results

The rat heart standard ultrasound image is exhibited in Figure 3a. There were significant differences in the indices of cardiac function between the groups 4 weeks after transplantation (p<0.001). Compared with the model group (31.51±4.34), LVEF values of the HIF-1α+CSCs+MI group (43.99±4.95) and the CSCs+MI group (39.41±3.72) significantly increased, with that of the HIF-1α+CSCs+MI group changing more significantly (p<0.001) (Table 1 and Fig. 3b).

Survival of CSCs after transplantation

They were transplanted CSCs labeled by EGFP in the MI+CSCs+HIF-1α group (Fig. 4a) and the MI+CSCs group (Fig. 4b),

Table 1. Doppler echocardiography results

Parameter	MI (n=7)	MI+CSCs (n=6)	MI+CSCs+HIF-1α (n=7)
LVEF (%)			
Before modeling	70.52±1.94	69.52±1.34	70.17±1.86
2 w after modeling	35.10±3.36	35.25±3.64	34.86±2.77
1 w after transplantation	30.84±4.25	35.25±3.80[a]	35.11±3.34[a]
2 w after transplantation	31.07±4.26	38.72±3.42[a]	38.27±3.44[a]
3 w after transplantation	31.59±4.30	39.48±3.74[a]	43.96±4.85[a, b]
4 w after transplantation	31.51±4.34	39.41±3.72[a]	43.99±4.95[a, b]
F_{group}	9.237		
P_{group}	0.002		
F_{time}	1438.222		
P_{time}	<0.001		
$F_{group*time}$	24.006		
$P_{group*time}$	<0.001		
LVFS (%)			
Before modeling	40.38±1.69	39.75±2.50	39.63±2.07
2 w after modeling	17.94±2.79	18.40±4.33	17.82±3.96
1 w after transplantation	17.33±2.74	20.88±3.99[a]	20.54±4.11[a]
2 w after transplantation	17.46±2.87	22.38±4.06[a]	23.94±4.15[a]
3 w after transplantation	17.75±2.94	23.17±4.15[a]	25.31±4.46[a, b]
4 w after transplantation	17.84±2.98	23.10±4.17[a]	25.23±4.42[a, b]
F_{group}	5.398		
P_{group}	0.015		
F_{time}	319.082		
P_{time}	<0.001		
$F_{group*time}$	6.607		
$P_{group*time}$	0.001		
LVDd (mm)			
Before modeling	8.40±0.40	8.46±0.99	8.29±0.26
2 w after modeling	8.71±0.57	8.90±0.38	8.74±0.38
4 w after transplantation	8.83±0.57	8.80±0.38	8.12±0.37[a, b]
F_{group}	3.785		
P_{group}	0.047		
F_{time}	3.766		
P_{time}	0.061		
$F_{group*time}$	0.936		
$P_{group*time}$	0.422		
LVDs (mm)			
Before modeling	5.81±0.37	5.68±0.68	5.76±0.43
2 w after modeling	7.14±0.40	7.25±0.12	7.18±0.34
4 w after transplantation	7.25±0.41	6.76±0.16[a]	6.08±0.48[a, b]
F_{group}	5.484		
P_{group}	0.015		
F_{time}	104.405		
P_{time}	<0.001		
$F_{group*time}$	7.750		
$P_{group*time}$	<0.001		

[a]: Compared with MI group, $P<0.05$; [b]: Compared with MI+CSCs group, $P<0.05$.
MI- Myocardial infarction; CSC- cardiac stem cell; HIF-1α- hypoxia inducible factor-1α; LVEF- left ventricular ejection fraction; LVFS- left ventricular fraction shortening; LVDd- left ventricular end diastolic diameter; LVDs- left ventricular end systolic diameter

but the MI group did not have EGFP-labeled CSCs (Fig. 4c). The survival rate of the HIF-1α+CSCs+MI group (12.391±1.881) was significantly higher than that of the CSCs+MI group (6.756±1.181) four weeks after transplantation (p<0.001). The difference value of LVEF before and after transplantation was positively correlated with the survival rate of CSCs in the infarction border zone (r=0.867, p<0.01).

VEGF expression in infarction border zone

The VEGF protein levels detected by Western blot are shown in Fig. 5a. The expression of VEGF protein in the HIF-1α+CSCs+MI group (4.747±0.969) significantly increased, compared with that of the MI group (1.17±0.088) 4 weeks after transplantation (p<0.001). In addition, VEGF protein expression level was positively correlated with capillary density in the infarction border zone 4 weeks after transplantation (r=0.912, p<0.001) (Fig. 5b).

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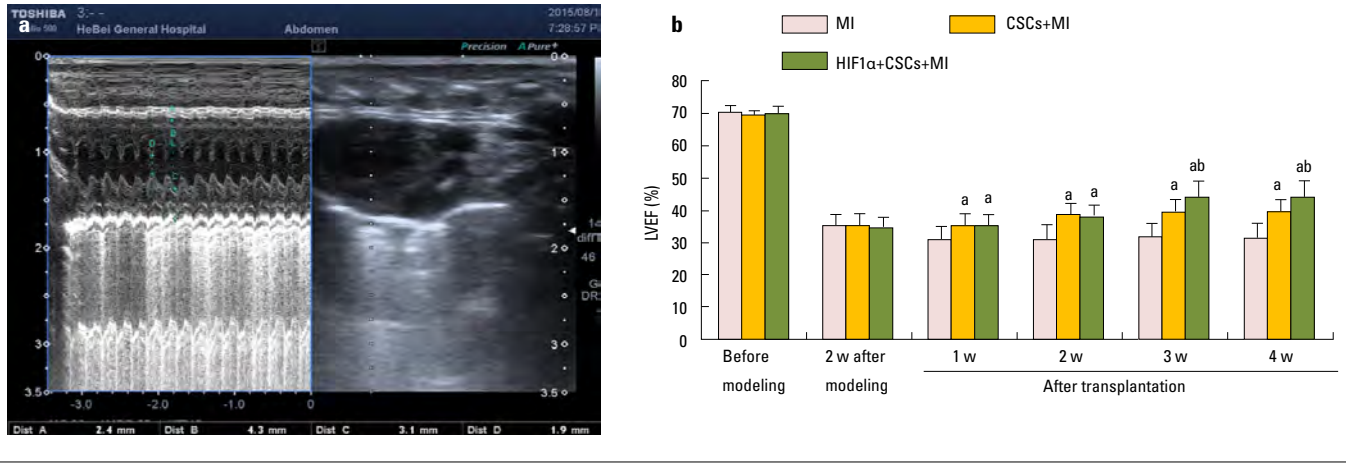

Figure 3. Effects of HIF-1α-modified CSCs transplantation on LVEF of rats with MI. (a) Rat heart standard ultrasound image; (b) LVEF values. There were significant differences in the indices of cardiac function between the groups 4 weeks after transplantation. Compared with the model group, LVEF values of MI+CSCs+HIF-1α and MI+CSCs groups significantly increased, with that of the MI+CSCs+HIF-1α group changing more significantly (P<0.05). Compared with MI group, [a]P<0.05; compared with MI+CSCs group, [b]P<0.05.
CSC - cardiac stem cell; HIF-1α - hypoxia-inducible factor-1α; LVEF - left ventricular ejection fraction; MI - myocardial infarction

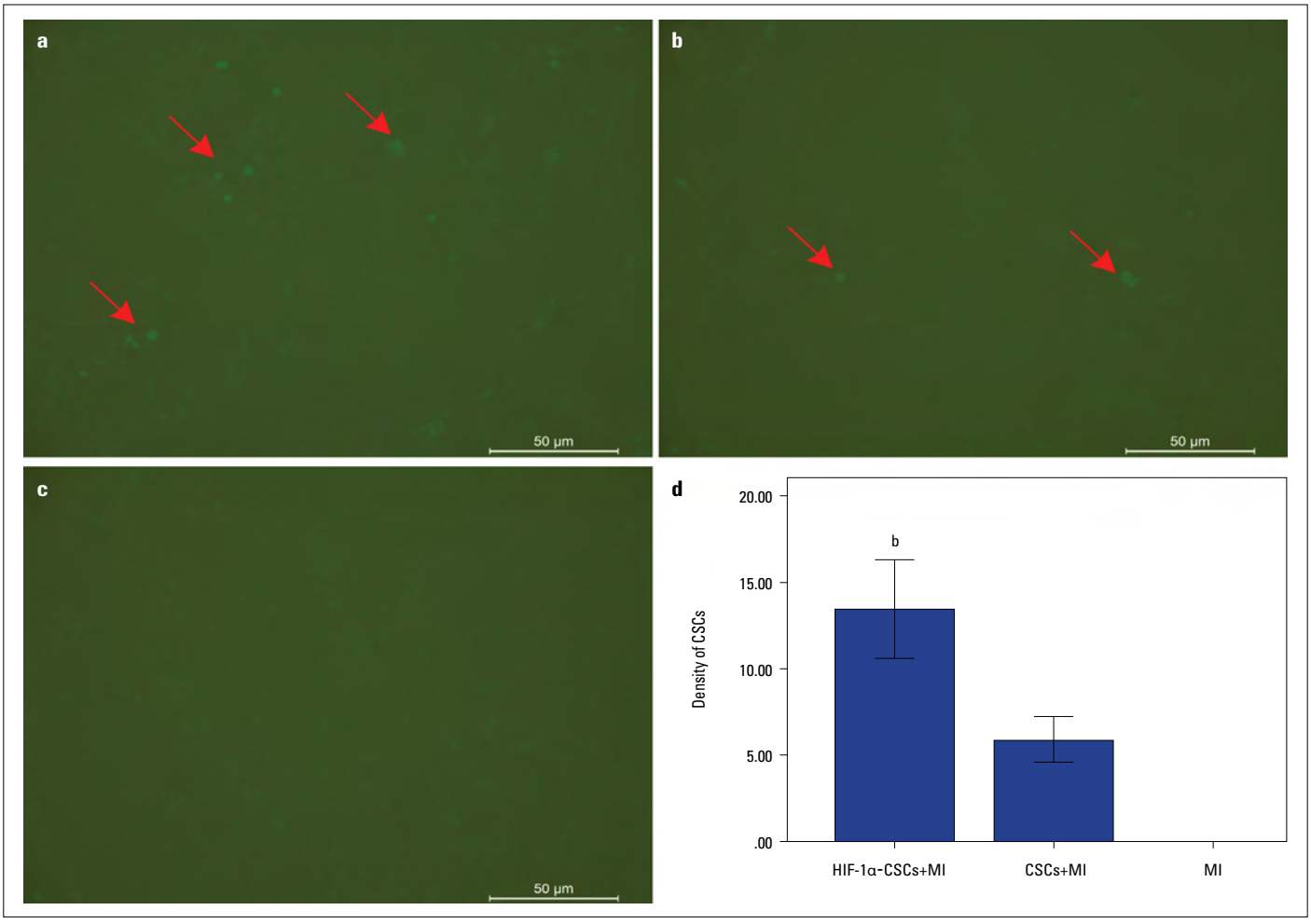

Figure 4. Apoptosis rate and survival rate of CSCs in infarcted peripheral zone. After 4 weeks of transplantation, CSCs were still visible under fluorescence microscope. (a) MI+CSCs+HIF-1α group; (b) MI+CSCs group; (c) MI group; (d) density of CSCs. There were transplanted CSCs labeled by EGFP in the MI+CSCs+HIF-1α group and the MI+CSCs group, but the MI group did not have EGFP-labeled CSCs. Compared with MI+CSCs group, [b]P<0.05.
CSC - cardiac stem cell; HIF-1α - hypoxia-inducible factor-1α; MI - myocardial infarction

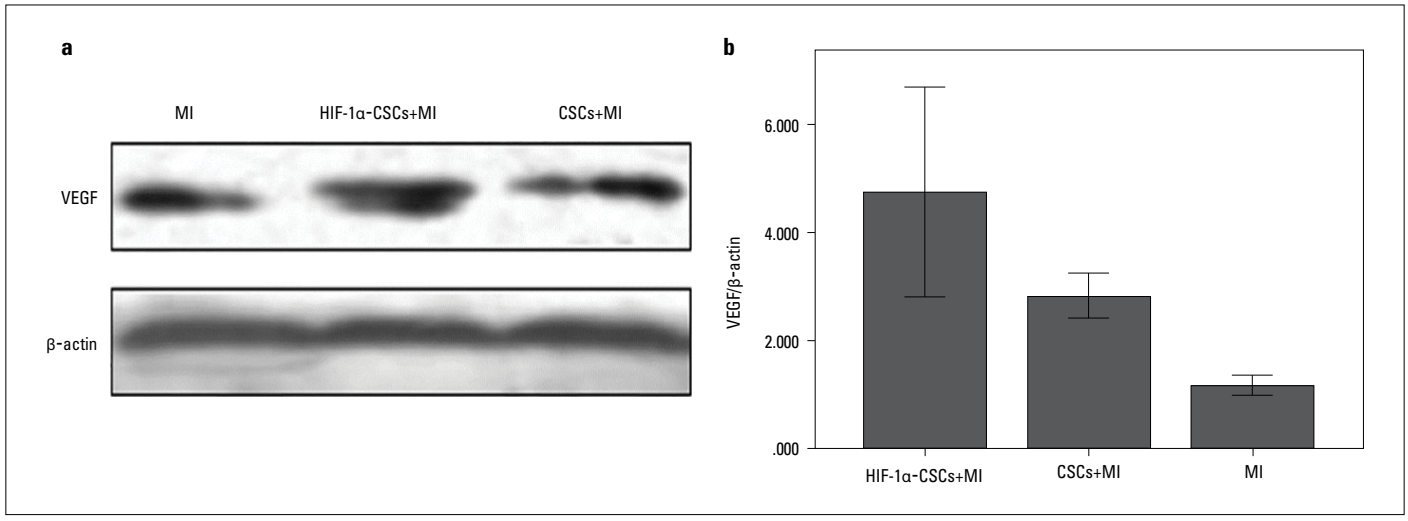

Figure 5. VEGF expression in infarction border zone. (a) VEGF protein levels detected by Western blot; (b) histogram for Western blot results. The expression of VEGF protein in the CSC+HIF-1α+MI group significantly increased compared with that of the MI group four weeks after transplantation. Compared with MI group, [a]*P*<0.05.

MI - myocardial infarction; VEGF - vascular endothelial growth factor

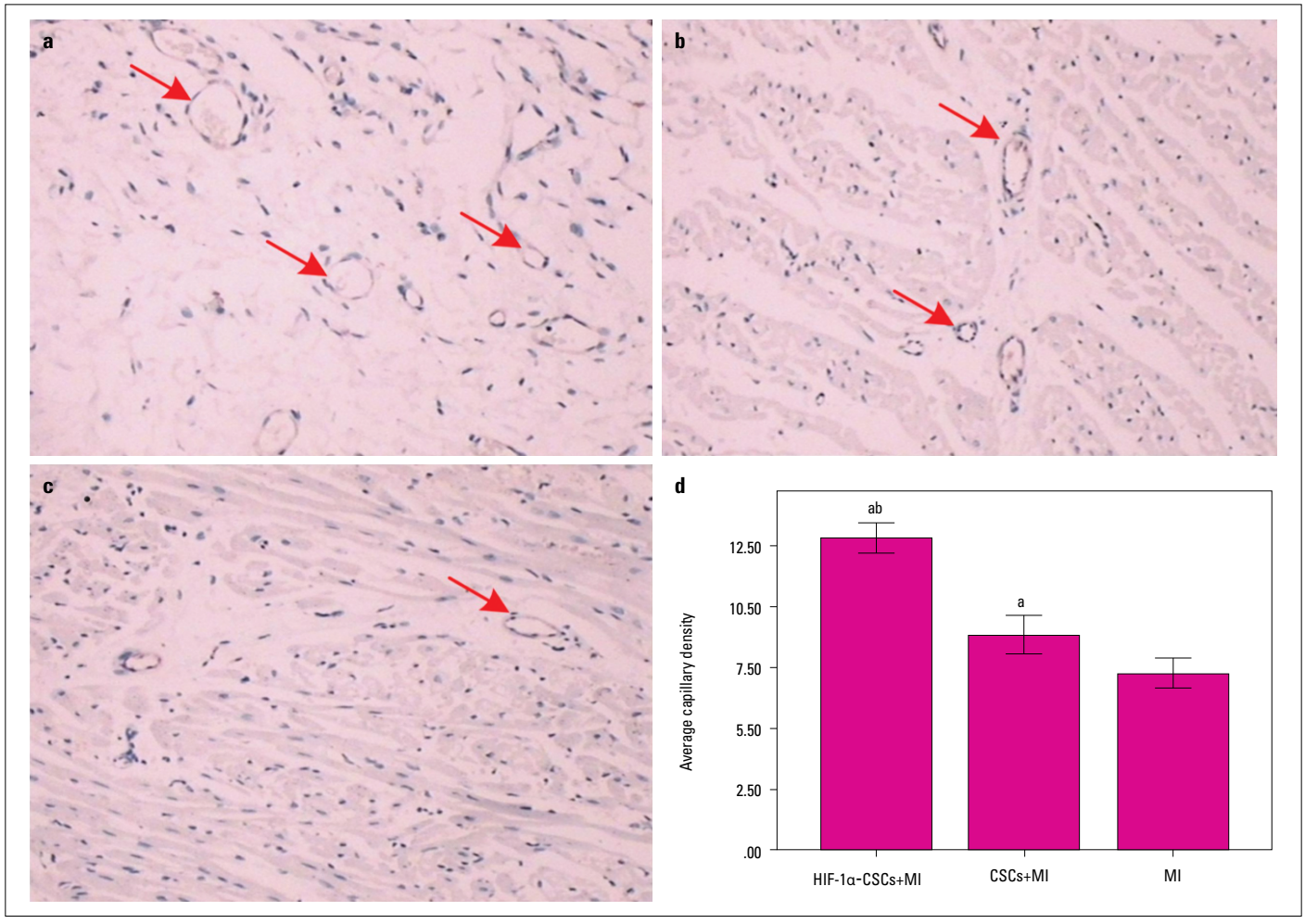

Figure 6. Capillary density in infarcted marginal zone. The infarcted area was stained with brown capillaries. (a) MI+CSCs+HIF-1α group; (b) MI+CSCs group; (c) MI group; (d) average capillary density. The capillary density of the MI+CSCs+HIF-1α group was significantly higher than that of the MI+CSCs group. Compared with MI group, [a]*P*<0.05; compared with MI+CSCs group, [b]*P*<0.05

CSC - cardiac stem cell; HIF-1α - hypoxia-inducible factor-1α; MI - myocardial infarction

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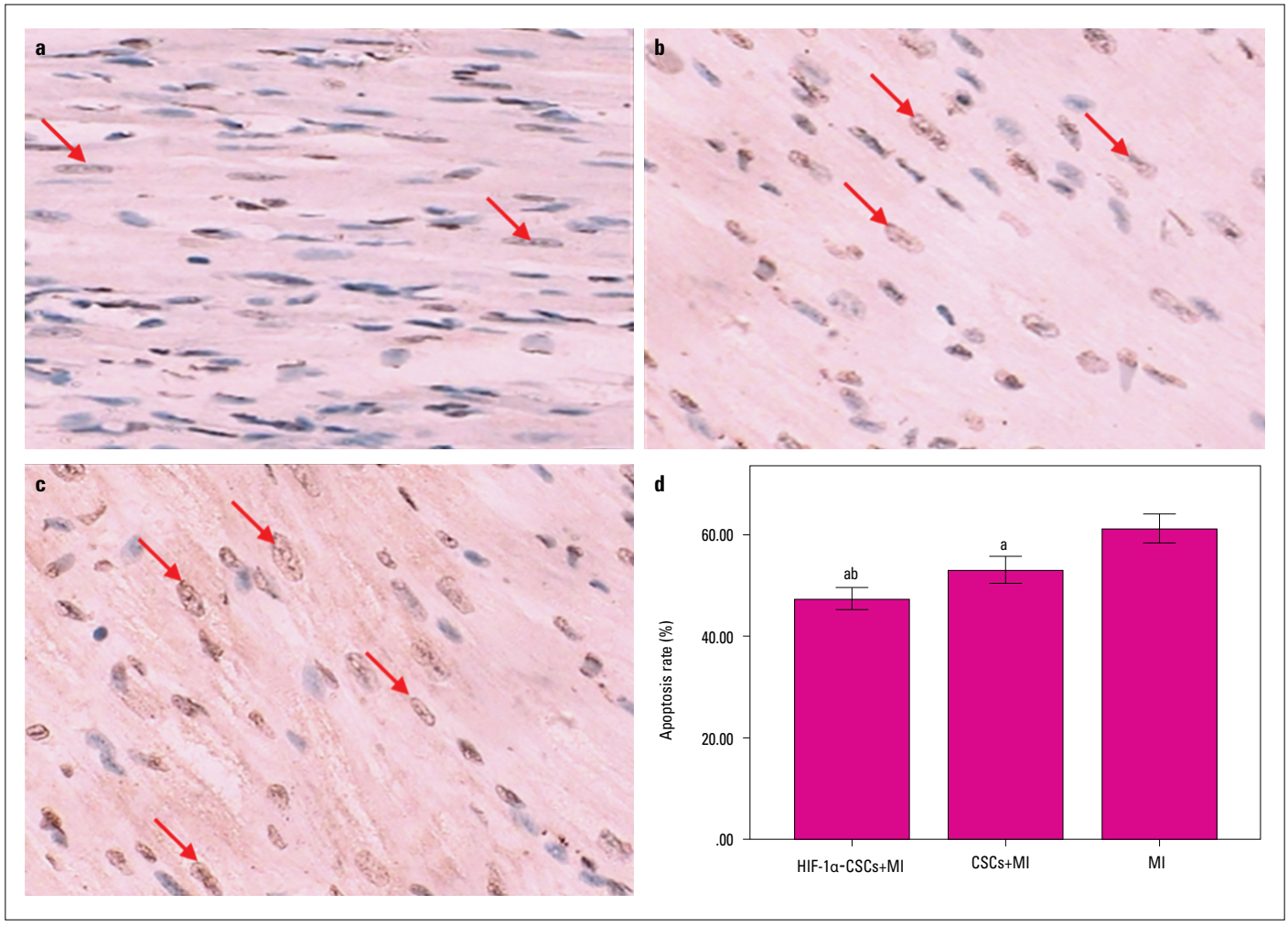

Figure 7. Apoptosis rate of CSCs in infarcted peripheral zone. After 4 weeks of transplantation, the apoptosis of cardiomyocytes in infarcted marginal zone was measured by TUNEL assay, and the apoptotic ones were stained brown; (a) MI+CSCs+HIF-1α group; (b) MI+CSCs group; (c) MI group; (d) apoptosis rate. The apoptosis rate of the MI+CSCs+HIF-1α group was significantly lower than that of the MI+CSCs group, and the apoptosis rate of the MI+CSCs group was significantly lower than that of the MI group. Compared with MI group, [a]*P*<0.05; compared with MI+CSCs group, [b]*P*<0.05.

CSC - cardiac stem cell; HIF-1α - hypoxia-inducible factor-1α; MI - myocardial infarction

Angiogenesis in infarction border zone

The capillary density of the HIF-1α+CSCs+MI group (12.82±0.86, Fig. 6a) was significantly higher than those of the CSCs+MI group (8.86±1.08, Fig. 6b) and MI group (7.26±0.83, Fig. 6c) (p<0.05). After MI, LVEF was positively correlated with the capillary density of infarction border zone (r=0.716, p<0.001) (Fig. 6d).

Cell apoptosis in infarction border zone

Apoptotic cells were found in all the three groups 4 weeks after transplantation, which had brown nuclei (Fig. 7). The apoptosis rate of the HIF-1α+CSCs+MI group [(47.40±3.06) %], (Fig. 7a) was significantly lower than that of the CSCs+MI group (53%±3.65%, Fig. 7b) (p=0.008), and the apoptosis rate of the CSCs+MI group was significantly lower than that of the MI group (61.20%±3.91%, Fig. 7c) (p=0.002). LVEF was negatively correlated with the apoptotic rate of cardiomyocytes 4 weeks after transplantation (r=–0.667, p<0.001) (Fig. 7d).

Serum cTn-T and myocardial enzyme levels 4 weeks after transplantation

Four weeks after transplantation, ELISA showed that the serum cTn-T levels followed a descending order of MI group [(0.08±0.01) ng/ml]>CSCs+MI group [(0.05±0.01) ng/ml]>HIF-1α+CSCs+MI group [(0.03±0.02) ng/ml] (p<0.001, p<0.000, p=0.034) (Fig. 8a). Besides, the CSCs+MI group had an AST level of [(123.17±7.27) U/L], which was significantly higher than that of the HIF-1α+CSCs+MI group [(109.22±5.08) U/L] but significantly lower than that of the MI group [(137.67±6.97) U/L] (p<0.001) (Fig. 8d). In addition, the CK level of the CSCs+MI group [(478.66±32.94) U/L] was similar to that of the MI group [(506.53±39.14) U/L] (p=0.194), both significantly exceeding that of the HIF-1α+CSCs+MI group [(406.93±36.33) U/L] (p=0.003) (Fig. 8b). The CK-MB levels followed the same trend (Fig. 8c).

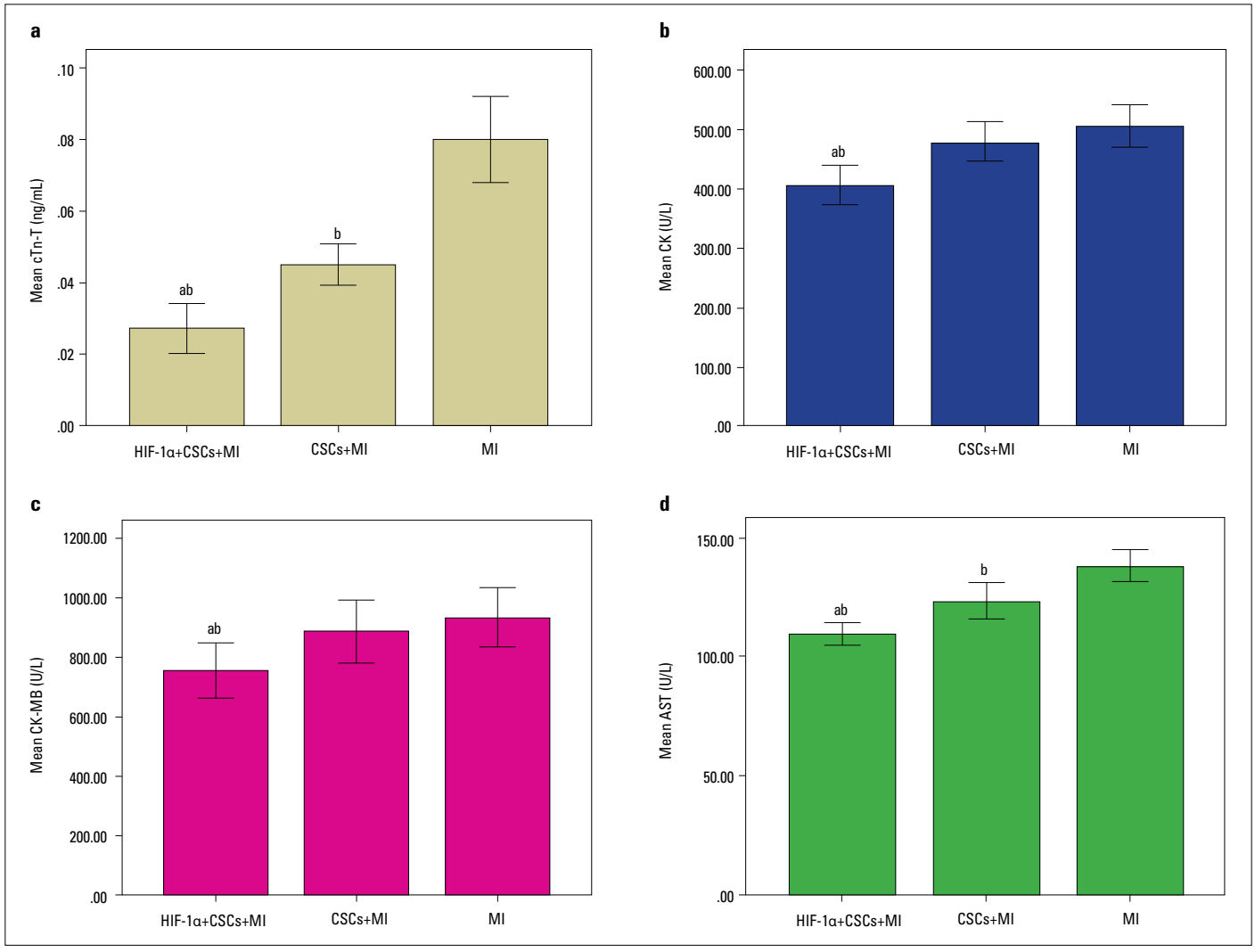

Figure 8. Serum levels of (a) cTn-T, (b) CK, (c) CK-MB, and (d) AST in different groups 4 weeks after transplantation. Four weeks after transplantation, ELISA showed that the serum cTn-T levels followed a descending order of MI group>MI+CSCs group>MI+CSCs+HIF-1α group. Compared with MI group, [a]$P<0.05$; compared with MI+CSCs group, [b]$P<0.05$.

AST - aspartate aminotransferase; cTn-T- cardiac troponin-T; CK - creatine kinase; CK-MB - creatine kinase-MB; CSC - cardiac stem cell; MI- myocardial infarction

Discussion

Up to date, a large number of animal experiments have confirmed the unique advantages of stem cells in the treatment of MI (6). Stem cells may treat human diseases by repairing tissues and organs. However, stem cell transplantation is mainly limited by very low survival rates in harsh environments such as ischemia and hypoxia (7). To solve this problem, changing the genetic structure of antiapoptotic gene through adenoviral transfection before cell transplantation is highly necessary, thereby improving the abilities of stem cells in survival, metabolism, proliferation or differentiation.

HIF-1α protein can accumulate in endothelial cells under hypoxic conditions and bind VEGF gene promoter SENP-1 (8, 9), thus inducing VEGF expression (10-12). In this study, we detected the changes in VEGF and capillary density in the infarction border zones of different groups. Notably, the HIF-1α+CSCs+MI group had significantly increased VEGF protein expression in the infarction border zone compared with that of the MI group 4 weeks after cell transplantation. Also, VEGF can promote the migration of multiple cells such as endothelial cells and vascular smooth muscle cells (13-16). In this study, 4 weeks after cell transplantation, the capillary density of the infarct border zone was found to be significantly increased in both HIF-1α+CSCs+MI and CSCs+MI groups, which was more significant in the former group. Therefore, CSCs modified by HIF-1α gene facilitated VEGF protein expression and capillary regeneration.

In recent years, Chen et al. (17) and Ishida et al. (18) studied the role of stem cells in protecting left ventricular function and limiting left ventricular remodeling (19). In a similar study, CSCs were injected into the heart of patients with ischemic cardio-

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myopathy through coronary arteries in a SCIPIO trial, and LVEF was significantly improved a few months later (20). However, the survival time of CSCs was still suspicious after transplantation in these studies, and the correlation between the improvement of cardiac function and the survival rate of CSCs was not analyzed, so the test results need to be further validated (21).

In this study, we dynamically monitored the changes in cardiac ultrasonography before and after transplantation and identified the survival rate of CSCs 4 weeks after transplantation by fluorescence microscopy. Four weeks after transplantation, LVEF values of HIF-1α+CSCs+MI and CSCs+MI groups increased significantly, of which the value was significantly higher in the former group. The survival rate of CSCs in the HIF-1α+CSCs+MI group was significantly higher than that of the CSCs+MI group, and the difference value of LVEF before and after transplantation was positively correlated with the survival rate of CSCs in the infarction border zone. Hence, CSCs transplantation improved the cardiac function, and transplantation of HIF-1α gene-modified CSCs worked significantly better.

This study used cardiac ultrasound to detect cardiac function, and the ultrasonic measurement data at all stages conformed to the cardiac function changes of animal models at different stages. Genetically modified CSCs transplantation was evidently beneficial to cardiac function and LVEF.

C-kit+CSCs are the most abundant stem cells in the myocardium, including two subgroups, i.e., myogenic and vasculogenic subgroups (22). The former mainly expresses c-kit, which mainly differentiates into cardiomyocytes, and the latter expresses endothelial cell marker KDR in addition to c-kit, which primarily differentiates into endothelial cells and vascular smooth muscle cells. The two kinds of CSCs can specifically differentiate into cardiomyocytes, endothelial cells, and vascular smooth muscle cells *in vitro* (23). In addition, Beltrami and Rota found by implanting c-kit+cells into the MI site of animals that these cells differentiated into mature cardiomyocytes, and expressed myocardial specific myosin heavy chain and myocardial striated protein. In this study, CSCs transplantation increased LVEF, and the difference value of LVEF after infarction was positively correlated with the survival rate of CSCs in the infarction border zone.

Study limitations

Damaging cardiac tissues can specifically attract stem cells, which may be driven by signals in the local microenvironment of ischemic myocardium, allowing further maturation into functional tissues. We herein have not identified these signals and will endeavor to clarify them in future studies.

Conclusion

In summary, we conducted a quantitative study on the survival rate, capillary density, apoptosis rate, and VEGF content of CSCs in the infarction border zone and analyzed the correlation between the difference value of LVEF after infarction and the survival density of CSCs in the infarction border zone. CSCs transplantation improved the cardiac function. Furthermore, transplantation of HIF-1α gene-modified CSCs significantly prolonged the survival time of CSCs and improved the cardiac function compared with those of single CSCs transplantation, and the improvement of LVEF was positively correlated with the survival rate of CSCs in the infarction border zone. CSCs transplantation combined with genetic engineering provides a new idea for the clinical treatment of MI.

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References

1. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114: 763-76. [CrossRef]
2. Doppler SA, Deutsch MA, Lange R, Krane M. Cardiac regeneration: Current therapies-future concepts. J Thorac Dis 2013; 5: 683-97.
3. Tal R. The role of hypoxia-inducible factor-1 alpha in preeclampsia pathogenesis. Biol Reprod 2012; 87: 134. [CrossRef]
4. Hinkel R, Lebherz C, Fydanaki M, Wuchrer A, El-Aouni C, Thormann M, et al. Angiogenetic potential of Ad2Hif-1αVP16 after regional application in a preclinical pig model of chronic ischemia. Curr Vasc Pharmacol 2013; 11: 29-37. [CrossRef]
5. Song J, Wu C, Korpos E, Zhang X, Agrawal SM, Wang Y, et al. Focal MMP-2 and MMP-9 activity at the blood-brain barrier promotes chemokine-induced leukocyte migration. Cell Rep 2015; 10: 1040-54.
6. Shuren L, Man W, Qing G. GW25-e0245 Effects of allogenic bone marrow mesenchymal stem cells modified by bcl-2 gene transplantation on cardiac dysfunction rabbits after acute myocardial infarction[J]. J Am Coll Cardiol 2014; 64(16 Supplement): C34.
7. Hoover-Plow J, Gong Y. Challenges for heart disease stem cell therapy. Vasc Health Risk Manag 2012; 8: 99-113. [CrossRef]
8. Xu Y, Zuo Y, Zhang H, Kang X, Yue F, Yi Z, et al. Induction of SENP1 in endothelial cells contributes to hypoxia-driven VEGF expression and angiogenesis. J Biol Chem 2010; 285: 36682-8. [CrossRef]
9. Ahluwalia A, Tarnawski AS. Critical role of hypoxia sensor--HIF-1α in VEGF gene activation. Implications for angiogenesis and tissue injury healing. Curr Med Chem 2012; 19: 90-7. [CrossRef]
10. Zhu H, Yang X, Ding Y, Liu J, Lu J, Zhan L, et al. Recombinant human endostatin enhances the radioresponse in esophageal squamous cell carcinoma by normalizing tumor vasculature and reducing hypoxia. Sci Rep 2015; 5: 14503. [CrossRef]
11. Zimna A, Kurpisz M. Hypoxia inducible factor-1 in physiological and pathophysiological angiogenesis: applications and therapies. Biomed Res Int 2015; 2015: 549412. [CrossRef]

12. Li M, Ding R, Wen Q. Reducing effects of recombinant human endostatin combined with paclitaxel under different delivery order on expressions of serum VEGF and HIF-1α in lung cancer xenografts in mice. Tumor 2016; 36: 264-71.
13. Ahn GO, Seita J, Hong BJ, Kim YE, Bok S, Lee CJ, et al. Transcriptional activation of hypoxia-inducible factor-1 (HIF-1) in myeloid cells promotes angiogenesis through VEGF and S100A8. Proc Natl Acad Sci U S A 2014; 111: 2698-703. [CrossRef]
14. Zhou Z, Reddy K, Guan H, Kleinerman ES. VEGF(165), but not VEGF(189), stimulates vasculogenesis and bone marrow cell migration into Ewing's sarcoma tumors in vivo. Mol Cancer Res 2007; 5: 1125-32.
15. Banerjee S, Mehta S, Haque I, Sengupta K, Dhar K, Kambhampati S, et al. VEGF-A165 induces human aortic smooth muscle cell migration by activating neuropilin-1-VEGFR1-PI3K axis. Biochemistry 2008; 47: 3345-51. [CrossRef]
16. Ball SG, Shuttleworth CA, Kielty CM. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. J Cell Biol 2007; 177: 489-500. [CrossRef]
17. Chen YL, Sun CK, Tsai TH, Chang LT, Leu S, Zhen YY, et al. Adipose-derived mesenchymal stem cells embedded in platelet-rich fibrin scaffolds promote angiogenesis, preserve heart function, and reduce left ventricular remodeling in rat acute myocardial infarction. Am J Transl Res 2015; 7: 781-803.
18. Ishida O, Hagino I, Nagaya N, Shimizu T, Okano T, Sawa Y, et al. Adipose-derived stem cell sheet transplantation therapy in a porcine model of chronic heart failure. Transl Res 2015; 165: 631-9. [CrossRef]
19. Chimenti I, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A, et al. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circ Res 2010; 106: 971-80. [CrossRef]
20. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, et al. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet 2011; 378: 1847-57. [CrossRef]
21. Chugh AR, Beache GM, Loughran JH, Mewton N, Elmore JB, Kajstura J, et al. Administration of cardiac stem cells in patients with ischemic cardiomyopathy: the SCIPIO trial: surgical aspects and interim analysis of myocardial function and viability by magnetic resonance. Circulation 2012; 126 (11 Suppl 1): S54-64. [CrossRef]
22. Hosoda T. C-kit-positive cardiac stem cells and myocardial regeneration. Am J Cardiovasc Dis 2012; 2: 58-67.
23. Bearzi C, Leri A, Monaco FL, Rota M, Gonzalez A, Hosoda T, et al. Identification of a coronary vascular progenitor cell in the human heart. Proc Natl Acad Sci USA 2009; 106: 15885-90. [CrossRef]