Co-cultured the MSCs and cardiomyocytes can promote the growth of cardiomyocytes

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Abstract Recently, the incidence of myocardial infarction has been increasing annually. Now cell therapy is a major new strategy in the treatment of this public health challenge. Most recently, evidences showed that MSCs can reduce the area of infarction and improve the heart function. In our study we found that MSCs could promote cardiomyocytes proliferation, inhibit the apoptosis of cardiomyocytes and promote cardiomyocytes autophagy function. These functions could be a therapeutic effect on myocardial infarction. At the same time, we first revealed that MSCs may achieve these functions by the activation of VEGF signaling pathways.

Keywords Cardiomyocytes · MSCs · Myocardial infarction

Abbreviations

MSCs	Marrow mesenchyme stem cells
LV	Left ventricular
VEGF	Vascular endothelial growth factor
SD rat	Sprague-Dawley rats
DMEM	Dulbecco's Modified Eagle's medium
FBS	Fetal bovine serum
PBS	Phosphate buffer saline
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethylsulfoxide
ROS	Reactive oxygen species
FCA	Flow cytometry assay

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Introduction

Cardiovascular diseases account for the highest mortality of the world (Singh et al. 2016). The World Bank estimates that by 2030, 23 million Chinese patients will experience myocardial infarction annually (Zhang et al. 2015). Adjunctive pharmacotherapy, procedural techniques and stent technology in the treatment of patients with MIs have been significant advances. Antiplatelet agents reduce patient morbidity and mortality. Percutaneous coronary intervention

(PCI) is one of the primary treatments of patients with acute ST segment elevation MI. The use of glycoprotein IIb/IIIa antagonist can reduce infarct size. However, the incidence and mortality due to MI continues to be high despite all these recent advances (Reddy et al. 2015). Adult mammals do not possess the capacity for natural heart regeneration unlike lower vertebrates. Stem cell therapy as one of the potential strategies was utilized in the cure of myocardial infarction (Singh et al. 2016).

Mesenchymal stem cells (MSCs) have been used for cardiovascular regenerative therapy for decades (Zeng et al. 2017 #8236). MSCs can differentiate into several cell types such as mesodermal lineage cells. At present, many studies have indicated that autologous MSCs transplantation can play a certain therapeutic effect on myocardial infarction. Transplantation of MSCs into infarcted myocardium attenuated left ventricular (LV) chamber dilation, reduced LV fibrosis, decreased infarct size and improved LV function. In addition, transplantation of MSCs into infarcted myocardium resulted in reduced fibrosis, improved cardiac function and remodeling (Zeng et al. 2017).

VEGF-mediated angiogenesis plays a critical role in the repair of ischemia/infarction, which is characterized by reduced blood supply to the heart (Kajdaniuk et al. 2011; Tang et al. 2011). Also VEGF contributed to the repair of the infarcted heart by inducing angiogenesis and stimulating the regeneration of cardiomyocytes (Zentilin et al. 2010).

In our present study, when MSCs and cardiomyocytes were co-cultured, we found the growth of cardiomyocytes was promoted. At the same time a series of proteins that promote the formation of cardiomyocytes have been promoted. We also found that these phenomena are closely related to the VEGF pathway. The findings indicated that co-cultured of MSCs and cardiomyocytes can simulate the promoting effects of VEGF, which may provide a new solution for the treatment of cardiomyocytes injury.

Materials and methods

Isolation of rat MSCs

Sprague-Dawley (SD) rats, weighing 250–300 g, were obtained from the China Medical University (Shenyang City, China). They were kept in normal

circadian, 26–27 °C conditions and fed with normal feed. The experimental protocol was reviewed and approved by the Ethics Committee of the Shenyang Northern Hospital (Shenyang City, China). Isolation and primary culture of MSC were performed according to previously reported method (Hatzistergos et al. 2010). Simply as follows, 8-week-old SD rats were used as donors. Under general anesthesia with ether, 100 µL bone marrow was aspirated from the tibia and femur with a 20-gauge needle attached to a 10 mL syringe containing 0.5 mL DMEM (Dulbecco's Modified Eagle Medium) and 40 U/mL heparin (Bioss, Beijing, China). 25 mL of DMEM (Invitrogen, Beijing, China) was added to neutralize collagenase activity. The resulting solution was filtered using a 0.22µm nylon membrane. The filtrate was centrifuged at 1300 rpm for 6 min at 25 °C, and the supernatant was removed. The concentration of cells in suspension was adjusted to 5×10^5 mononuclear cells/mL DMEM, supplemented with 20% FBS (fetal bovine serum) (Invitrogen, Beijing China), penicillin (100 U/mL) (Bioss, Beijing, China) and streptomycin (100 µg/mL) (Bioss, Beijing, China) at 37 °C, 5% CO₂ and 95% air. After 3 days, RBCs and neutrophils were removed by Histopaque (Bioss, Beijing, China) density gradient. At passage 3, the MSCs were identified.

Fluorescence-activated cell sorting analysis

P3 MSCs cells were characterized by an Epics XL flow cytometer (ACEA NovoCyte, Hangzhou, China). Cells were washed 3 times in PBS (phosphate buffer saline). Cells were added to a centrifuge tube (1×10^6 /tube), fixed by methanol for 15 min and incubated with 1 µg of the following specific antibodies to rat including mouse anti-CD29 antibody, mouse anti-CD44 antibody, and mouse anti-CD45 antibody, mouse anti-CD90 antibody (all antibodies were brought from BOSTER, Beijing, China) over night at 4 °C. At the next morning, the cells in each sample were washed with PBS for three times and incubated either with 1 µg of FITC-conjugated goat anti-mouse IgG antibody (BOSTER, Beijing, China) to test CD29, CD44, CD45, and CD90. The cells (1×10^6) were incubated with 1 µg of FITC-conjugated mouse anti-rabbit monoclonal antibodies specific to rabbit CD29, CD90, CD44, CD45, or FITC-conjugated isotype-matched immunoglobulin G (Santa Cruz, Beijing,

China) for 1 h at 4 °C. After washing, the samples were analyzed using an Epics XL flow cytometer.

Cell culture

H9C2 cells, rat cardiomyocytes (Procell, Wuhan, China) were starved in DMEM (Invitrogen) containing 10% FBS (Invitrogen, Beijing, China). As the papers by Huang et al. (2014) and by Yuan et al. (2013) described, MSCs and H9C2 cells were cocultured 1:1 in trans-well chambers. In order to study the co-culture effect of MSCs on the proliferation of H9C2 cells, we cultured H9C2 cells using a novel trans-well chamber with four different conditions up to 6 days. Group 1 (H9C2 cells group): H9C2 cells were seeded in the well directly (lower chamber) at the density of 1×10^5 /well in a 24-well plate and were cultured with culture medium (DMEM-10% FBS); Group 2 (Co-culture group): H9C2 cells were seeded in the well directly (lower chamber) at the density of 1×10^5 /well and MSCs were seeded in the upper chamber at the density of 1×10^5 /well in a 24-well plate. The cells were cultured with culture medium (DMEM-10% FBS); Group 3 (VEGF group): H9C2 cells were seeded in the well directly (lower chamber) at the density of 1×10^5 /well in a 24-well plate and cells were overexpressing VEGF by transfection of VEGF plasmid; Group 4 (Co-culture + VEGF inhibitor (Lenvatinib) group): H9C2 cells were seeded in the well directly (lower chamber) at the density of 1×10^5 /well with 15 μ M of Lenvatinib, an inhibitor of VEGF (sc-488530, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and MSCs were seeded in the upper chamber at the density of 1×10^5 /well in a 24-well plate. Group 5 (VEGF + VEGF inhibitor (Lenvatinib) group): H9C2 cells were seeded in the well directly (lower chamber) at the density of 1×10^5 /well in a 24-well plate and H9C2 cells were overexpressing VEGF by transfection of VEGF plasmid then VEGF inhibitor was added. The cells were cultured in culture medium with 15 μ M of Lenvatinib. Group 6 (Co-culture + co+si-FAK group): H9C2 cells were seeded directly into the well (lower-chamber) at the density of 1×10^5 /well transfected with pRS vector/pRSshFAK and MSCs were seeded into the upper-chamber at the density of 1×10^5 /well in a 24-well plate. Group 7 (Co-culture + co+si-cyclin d1 group): H9C2 cells were seeded directly into the well (lower-chamber) at the density of 1×10^5 /

well transfected with pRS vector/pRS-shcyclin d1 and MSCs were seeded in the upper-chamber at the density of 1×10^5 /well in a 24-well plate.

Transfection of sh-RNA

To stably silence FAK/cyclin d1, H9C2 cells were transfected with a set of shRNA constructs against human FAK/cyclin d1, pRS-shFAK/cyclin d1 (Shanghai GeneChem Company, Shanghai, China), and then selected with puromycin (1.5 μ g/mL). The pRS vector (Yeast Expression with multiple cloning sites, Resistance(s): Ampicillin, Growth Temperature: 37 °C, Growth Strain(s): DH5alpha—Fig. 1) was used as control. After silencing FAK/cyclin d1 cells were co-cultured with MSCs as Group 56 and Group 6 and 7. Transfection process: 2 μ g plasmid was diluted with serum-free culture medium. A corresponding amount of transfection reagent in serum-free culture medium was used, the final volume was 100 μ l. After gentle mixing them, the mixture was added into the medium of H9C2 cells. After 4 h, the transfection medium was replaced by culture medium and the culture was continued to develop at 37 °C. 24 h after transfection, 400 μ g/ml ampicillin was used to screen, and the monoclonal culture was further selected.

Hypoxia induction

The H9C2 cells were plated into 6-well plates at a density of 2×10^4 cells per well. At 70% confluence, cells were incubated with CoCl₂ at 150 μ mol/L for 24 hours, followed by a two hour incubation in CoCl₂ free medium.

Transfection of plasmid

Cells were transfected with VEGF (Shenggong, Shanghai, China). The primers sequence is (F:5′–3′: GAAGTGGTGAAGTTCATGGATGTC, R:5′–3′:CG ATCGTTCTGTATCAGTCTTTCC) Vector (Shenggong, Shanghai, China) was used as a control. The VEGF plasmid was transfected into H9C2 cells for the experiment. After transfection, the expression of VEGF was detected in the cells by western blot and real-time PCR, which showed that the VEGF was overexpressed in the cells.

Fig. 1 pRS vector plasmid

Multi-lineage differentiation

Adipogenic differentiation of MSCs was performed at passage three. Third passage MSCs were seeded in 6-well plates at a density of 2×10^4 cells per well. After 24 h, the medium was replaced for fat inducing medium (1 μ M/L dexamethasone, 10 μ g/mL insulin, 200 μ M/L indomethacin and 0.5 mmol/L isobutylmethylxanthine).

Osteogenic differentiation of MSCs was also done with third passage MSCs. Third passage MSCs were seeded in 6-well plates at a density of 2×10^4 cells per well. After 24 h, the medium was replaced for bone inducing medium (1% glutamine, 100 μ M/L ascorbic acid, 10 mM β -glycerophosphate, and 50 nM dexamethasone).

Oil red O stain

The cells were washed once with PBS. The cells were fixed with 10% neutral formalin in PBS for 20 min at room temperature and then rinsed 3 times with PBS. The fixed cells were treated with 0.1% Oil red O for 5 min. Then the cells were washed three times with water and the images of stained cells were analyzed under a microscope.

Alizarin red staining

The cells were washed once with PBS. The cells were fixed with 10% neutral formalin in PBS for 20 min at room temperature and then rinsed 3 times with PBS. The fixed cells were treated either with 0.1% alizarin red for 30 min. Then the cells were washed three times with water and the images of stained cells were analyzed under a microscope.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays

H9C2 cells (in the lower chamber) were co-cultured with MSCs (in the upper chamber) were cultured in a 24-well plate with different conditions as described above for 0, 3, 6, 9 and 12 days. At the end of the incubation, the potency of cellular proliferation was measured by MTT assay. For this, 5 mg/mL of MTT was added to each well. After 4 h DMEM medium was replaced by DMSO (dimethylsulfoxide). Then the optical density at 490 nm was measured using a Microplate Reader (BIO-RAD, Shanghai, China).

Table 1 Primer sequences for detection of mRNA expression

Name	Forward primer (5' → 3')	Reverse primer (5' → 3')
VEGF	GAGGGCAGAATCATCACGAA	GGGAACGCTCCAGGACTTAT
Desmin	TGCAGCCACTCTAGCTCGTA	CATGTCCATCTCCACCTG
cTnT	AAGAATGTCTGACATAGA	TCTTTGGTGAAGGAGG
FAK	TGGCTCCAGAGTCAATCAA	TTCCATCCTCCCTGTCAAT
Cyclin d1	CCGAGGAGCTGCTGCAAATGGAG	GAAATCGTGCGGGGTCATTGCG
Bcl-2	GGTGAAGTGGGGGAGGATTG	GGCAGGCATGTTGACTTCAC
Bax	AGCTGAGCGAGTGTCTCAAG	GTCCAATGTCCAGCCCATGA
Caspase 9	GGCCCTTCCAGGAGCGAG	TCACGCCACAGTTTCCCGGA
Atg5	ACAGATGACAAAGATG	CTCATAACCTTCTGAAA
Gapdh	CTCTGCTCCTCTGTTCGAC	GCGCCCAATACGACCAAAATC

Cell staining and viability analysis

Fluorescein diacetate (FDA) and propidium iodide (PI) stainings were used to determine live cells and dead cells, respectively. Basically, the live cells were stained into green color by FDA solution, the dead cells into red color by PI solution. Fluorescein diacetate and propidium iodide solutions were purchased from Sangon Biotech (Shanghai, China). Cell staining was performed with the method as recommended by the manufacturer's manual. Briefly, after different treatments, the cells were spun down by centrifugation, the pellet was suspended in 500 μ L of $1 \times$ PBS. 100 μ L of the cells (approximately 2×10^4 cells) were stained for 20 min by adding 50 μ L of FDA and PI solution that contained 400 μ g/mL of FDA and 5 mg/mL of PI. At the end of staining, the cells were washed for three times with $1 \times$ PBS, followed by suspension with 100 μ L $1 \times$ PBS and transfer into a 96-well plate. To analyze viability, the stained cells including green and red cells were initially analyzed by flow cytometry (Beckman Coulter, Shanghai, China); the data were then statistically analyzed. The viability was obtained by using the number of live cells divided by that of total cells. The value of the viability represents the mean of three independent experiments with standard deviation (SD).

Reverse transcription and quantitative real-time PCR

Total RNA of cells was isolated by TRIzol (Invitrogen, Beijing, China) according to the protocol (Guo et al. 2015). Complementary DNA was synthesized by

reverse transcription of total RNA using a RT reaction kit (Promega, Shanghai, China). Real-time PCR was performed using an Mx 3000P real-time PCR system (Applied Biosystems, Shanghai, China) according to the manufacturer's instruction and SYBR Premix Ex Taq (TaKaRa, Beijing, China) as a DNA-specific fluorescent dye. Primer sequences were used as indicated in as Table 1.

All the reactions were repeated at least three times. Gene expression levels were calculated relative to gapdh by using StratageneMx 3000P software.

Western blot analyses

To determine the expression of protein, whole cell extracts were prepared from cells or tissues using a lysis buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA), supplemented with proteinase and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). The cell lysates were collected and centrifuged at 4 °C for 20 minutes at 13,000 rpm. Proteins (25 μ g) from each sample were subjected to 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Target proteins were probed with specific antibodies—cyclin d1, VEGF, FAK, FAK^{p-Tyr397}, bax, bcl-2, MHC, cTnT, desmin, atg5 and gapdh (Santa Cruz, Beijing, China).

ROS (reactive oxygen species) production assay

The activity of ROS was measured, respectively, by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The experiment was performed according to the manufacturer's instructions. All standards and samples were run in duplicate. The

Fig. 2 MSCs have the ability to promote the expression of myocardial protein **a** the MSCs cells (1×10^6) were characterized by flow cytometer the results showed that they were positive for CD29, CD44 and CD90 and negative for CD45 was negative. **b** Oil Red O staining was done to identify MSCs after adipogenic differentiation. The left panel was the 100 times under the optical microscope, the right panel was the 200 times under the optical microscope. **c** Alizarin red staining was done after 14 days of osteogenic induction. The left panel was of the 100 times under the optical microscope (scale bars 100 μ m), the right panel was of the 200 times under the optical microscope (scale bars 10 μ m). **d, e** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 3 (VEGF group) were collected and the indicated proteins and mRNA levels were analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $^{**}P < 0.05$ versus H9C2 cells group. **f, g** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 4 (Co-culture + VEGF inhibitor group) were collected and the indicated proteins and mRNA levels were analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $^{**}P < 0.05$ versus H9C2 cells group. **h, i** H9C2 cells in Group 1 (H9C2 cells group), Group 4 (Co-culture + VEGF inhibitor group) and Group 5 (cells + VEGF + - VEGF inhibitor group) were collected and the indicated proteins and mRNA levels were analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM

protein concentration was determined using the BCA Protein Assay Kit (Bioss, Beijing, China).

Statistical analysis

All data were analyzed with GraphPad Prism version 6.0 (GraphPad, USA). Differences were analyzed by analysis of variance (ANOVA) test. Statistical

significance was defined as $P < 0.05$. All experiments were repeated three times.

Results

MSCs have the ability to promote the myocardial protein

MSCs were isolated from rabbit and purified by a flow cytometry. The flow cytometry assay (FCA) showed that they were positive for CD29, CD44 and CD90 and negative for CD45 (Fig. 2a). These findings indicated that MSCs have been isolated from rabbit tissues and can be used for further experiments. After adipogenesis differentiation, we carried out Oil Red O staining to determine the ability of the cells to become fat, Oil Red O staining showed that red large droplets occupied almost the entire cytoplasm, blue nuclei stayed close to the cell membrane (Fig. 2b). After 14 days of osteogenic induction, alizarin red staining showed typical calcified nodules as red deposits (Fig. 2c). By co-culture of MSCs and cardiomyocytes, we found that MSCs can promote the expression of myocardial protein markers to a certain extent, which is similar to VEGF (Fig. 2d, e). To determine whether the function of MSCs is related to VEGF, we added the VEGF inhibitor group. The western blot and real-time PCR showed that after the addition of VEGF inhibitors the promoting role of MSCs had been weakened (Fig. 2f, g). After that, we compared the effects of VEGF

Table 2 The viability of cells in different groups

Group	0 day	3 day	6 day	9 day	12 day
Group 1	100	83.87 $\pm$ 3.98	67.06 $\pm$ 7.29	49.30 $\pm$ 3.47	42.24 $\pm$ 4.63
Group 2	100	98.76 $\pm$ 2.09	88.85 $\pm$ 5.78	66.63 $\pm$ 6.62	59.99 $\pm$ 3.23
Group 3	100	98.21 $\pm$ 4.38	86.50 $\pm$ 3.25	67.70 $\pm$ 5.17	58.85 $\pm$ 5.49
Group 4	100	94.31 $\pm$ 4.12	75.64 $\pm$ 4.38	60.55 $\pm$ 4.45	52.71 $\pm$ 3.73
Group 5	100	94.89 $\pm$ 4.21	76.82 $\pm$ 6.34	61.08 $\pm$ 5.55	50.87 $\pm$ 6.25

Percentage (%) = Live cells/total cells

◀ **Fig. 3** MSCs have the ability to promote the proliferation of cardiomyocytes **a** the proliferation of H9C2 cells in Group 1 (H9C2 cells group); Group 2 (Co-culture group) and Group 3 (VEGF group) were evidenced by MTT assay. Data are shown as mean $\pm$ SEM. **b, c** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 3 (VEGF group) were collected and the indicated proteins and mRNA levels were analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells group. **d** The proliferation of H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 4 (Co-culture + VEGF inhibitor group) were put into evidence by MTT assay. Data are shown as mean $\pm$ SEM. **e, f** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 4 (Co-culture + VEGF inhibitor group) were collected and the indicated proteins and mRNA levels were analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells group. **g** We transfect pRS-vector/shFAK in co-cultured cells **h** We transfect pRS-vector/shcyclin d1 in co-cultured cells

inhibitors on MSCs and VEGF, and found that VEGF inhibitors had similar effects on MSCs (Fig. 2h, i).

MSCs have the ability to promote the proliferation of cardiomyocytes

The viability of cells in different groups was analyzed by flow cytometry. Cells were stained by FDA and PI solution at different time points, the stained cells were counted with flow cytometry and analyzed for the viability. As shown in Table 2 at a concentration of 1×10^5 cells/mL the highest viability was observed in Group 2 and Group 3. The mechanisms of the effect of MSCs on the proliferation of cardiomyocytes were also studied by MTT assay, we found when MSCs were co-cultured with cardiomyocytes, the proliferation of cardiomyocytes was promoted. In our experiment the VEGF group was used as the positive control. MTT assay showed that when H9C2 cells were co-cultured with MSCs the proliferation of H9C2 cells was almost like that of the VEGF group (Fig. 3a). We also found that MSCs could promote the proliferation of cardiomyocytes by up regulating the

expression of FAK^{p-Tyr397} and cyclin d1 as function of VEGF (Fig. 3b, c). In contrast, when we added VEGF inhibitors to the co-culture group, we found that the inhibitors could inhibit the promotion of H9C2 cells proliferation of MSCs (Fig. 3d). This result proved that MSCs may affect the proliferation of cardiomyocytes by regulating the VEGF pathway. The following experiments also confirmed that VEGF inhibitors could inhibit the promotion of MSCs to cardiomyocytes by downregulating VEGF expression (Fig. 3e, f). After that, we constructed stable cell lines with FAK/cyclin d1 silenced. It was found that MSCs could restore the expression of FAK and cyclin d1 (Fig. 3g, h).

MSCs can inhibit the apoptosis of cardiomyocytes

Cell death may include apoptosis and autophagy (Webster 2012). By western blot and real-time PCR, we found that for overexpression of VEGF, MSCs could also inhibit apoptosis of cardiomyocytes (Fig. 4a, b). In order to find out how MSCs inhibit apoptosis of cardiomyocytes, we used western blotting and real-time PCR to detect caspase 9, bax and bcl-2 in cardiomyocytes, in cardiomyocytes co-cultured with MSCs and in cardiomyocytes to which VEGF was added (Fig. 4a, b). The results showed that MSCs could inhibit the expression of bax and caspase 9 and promote the expression of bcl-2 to inhibit apoptosis of cardiomyocytes. At the same time, we found that the expression of FAK^{p-Tyr397} had been inhibited; we conclude that the effect of MSCs on cardiomyocyte apoptosis may be caused by the effect of VEGF pathway. In the same way we did experiments with the addition of VEGF inhibitors, which proved our inference (Fig. 4c, d).

MSCs can inhibit the level of Atg5 and ROS

Autophagy is a kind of programmed cell death, ATG5 protein as a key regulator of autophagy will increase in the process of autophagy (McCormick et al. 2012).

Fig. 4 MSCs can inhibit apoptosis of cardiomyocytes by the VEGF pathway **a, b** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 3 (VEGF group) were collected and the expression of VEGF, FAK^{p-Tyr397}, FAK, bax and bcl-2 was analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells

group. **c, d** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 4 (Co-culture + VEGF inhibitor group) were collected and the expression of VEGF, FAK^{p-Tyr397}, FAK, caspase 9, bax and bcl-2 was analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells group

Western blot and real-time PCR were used to detect the ATG5 protein in the different groups. The results showed that the H9C2 cells produced less ATG5 when they were either overexpressing VEGF or co-cultured

with MSCs (Fig. 5a, b). By the addition of VEGF inhibitor, we found that VEGF inhibitors could up-regulated the reduced expression of ATG5 caused by MSCs (Fig. 5d, e). Excess generation of reactive

oxygen species (ROS) and cytosolic calcium accumulation play major roles in the initiation of programmed cell death during acute myocardial infarction. Interestingly, we observed a substantial decrease in ROS production on MSCs treatment (Fig. 5c, f). In addition, we added the hypoxia factor (hypoxia induced by cobalt oxide) (Tan et al. 2016); we found that the expression of VEGF was raised after hypoxia for 6 h, when the cardiomyocytes were co-cultured with MSCs the expression of VEGF was similar to its expression during hypoxia (Fig. 5g).

Discussion

Over the past 10 years, stem cell transplantation has been used in the treatment of acute and chronic ischemic heart disease (Hertz et al. 2011).

MSCs has been used as a potential treatment of myocardial infarction methods in clinical experimental studies, clinical and animal experiments (Yao et al. 2012; Zhao et al. 2004). VEGF as a major antigenic factor was investigated for the treatment of myocardial infarction. The experiment which was carried out in MI pigs showed that compared with the saline controls, the VEGF group expressed higher phosphorylated Akt and had higher vascular density, more proliferating cardiomyocytes, and less apoptotic cells in the infarct and peri-infarct zones (Shi et al. 2017 #8237). Thus, cardiac-specific and hypoxia-induced expression of VEGF improves the perfusion and function of porcine MI heart through the induction of angiogenesis and cardiomyocyte proliferation, activation of prosurvival pathways, and reduction of cell apoptosis (Tao et al. 2011).

However, there was no exact study on how MSC regulates the proliferation of cardiomyocytes through

VEGF signaling when cardiomyocytes were co-cultured with MSCs.

In our study we co-cultured MSCs and H9C2 cells through the trans-well chambers (cell number ratio of 1:1). VEGF which was recognized as a promoter to the proliferation of cardiomyocytes was used as a positive control (Zhou et al. 2005). The proliferation of H9C2 cells and cell vitality were detected at different time points, and we found that both MSCs and VEGF could promote the proliferation and vitality of H9C2 cells. MSCs start to differentiation after 3–7 days, we detected the proteins after MSC and cardiomyocytes had been co-cultured for 3 days to do the experiments. We knew that VEGF can promote the differentiation of MSCs; however whether MSC can promote the expression of VEGF has not been known. Through the co-culture of MSCs and cardiomyocytes, we found that the level of VEGF showed a certain degree of increase. In order to investigate how MSCs promote the proliferation of H9C2 cells, we examined the changes of FAK^{P-Tyr397} and cyclin d1 which are important proteins in cell cycle (He et al. 2012; Mello and Tuan 2006), since we knew that both of them can be promoted by VEGF. The results showed that the expression of both FAK^{P-Tyr397} and cyclin d1 can be promoted by MSCs and VEGF. This suggested that the role of MSCs towards cardiomyocytes in promoting proliferation is similar to VEGF, and this function may be achieved through the regulation of the VEGF pathway. After that our results showed that MSCs could inhibit the apoptosis of H9C2 cells by adjusting the expression of caspase 9, bax, bcl-2, ATG5 and ROS. We confirmed that all of these effects can be reduced to a certain extent after the addition of VEGF inhibitors.

All results have proven that co-cultured of MSCs and H9C2 cells promote the expression of myocardial

cell function protein and all of these effects can be reduced to a certain extent after the addition of VEGF inhibitors.

All in all, we confirmed that MSCs can promote the proliferation and regeneration of cardiomyocytes through a series of experiments *in vitro*.

◀ **Fig. 5** MSCs can inhibit autophagy of cardiomyocytes by the VEGF pathway **a, b** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 3 (VEGF group) were collected and the expression of VEGF and atg5 was analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells group. **c** ROS production was analyzed using ROS assay. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells group. **d, e** H9C2 cells in Group 1 (H9C2 cells group), Group 2 (Co-culture group) and Group 4 (Co-culture + VEGF inhibitor group) were collected and the expression of VEGF and atg5 was analyzed by western blot and real-time PCR. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells group. **f** ROS production was analyzed using ROS assay. Data are shown as mean $\pm$ SEM. $**P < 0.05$ versus H9C2 cells group. **g** H9C2 cells group, hypoxia for 6 h, Co-culture group, and Co-culture + hypoxia for 6 h group were collected and the expression of VEGF, cyclin d1, Atg5, bax and bcl-2 was analyzed by western blot

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Authors' Contribution HJ: conceived of the study, carried out the molecular studies. HW: carried out the molecular studies, helped to draft the manuscript. TL: participated in the design of the study and performed the statistical analysis. ZY: participated in its design and coordination and helped to draft the manuscript. RZ: conceived of the study. HH: conceived of the study. **Funding** This research was sponsored by the Science and Technology Project of Liaoning Province (2013225089).

Compliance with ethical standards

Conflict of interest No conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical approval Animal study received China medical university animal care and use committee approval.

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