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Original Article

Cardioprotective role of mesenchymal stem cell-secreted follistatin-like protein 1 in a rat model of ischemia/reperfusion injury

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Abstract: Objective: Follistatin-like protein 1 (FSTL1) is a secreted glycoprotein that has been implicated in multiple pathological processes. The aim of this study is to investigate the protective role of FSTL1 secreted by mesenchymal stem cells (MSCs) during myocardial ischemia/reperfusion injury. Methods: siRNA against FSTL1 (siFSTL1) and FSTL1 expression plasmid were employed to modulate expression of FSTL1. Effects of FSTL1 expression on H9C2 cell viability were evaluated by CCK-8 assay and EdU proliferation detection kit. Effects of FSTL1 expression on myocardium injury under ischemia/reperfusion were also assessed in a rat model of ischemia/reperfusion injury. Under this model, myocardium infarction area was calculated. Histological changes were analyzed by Hematoxylin and eosin staining. Associated biomarkers were all determined by ELISA assay. Apoptosis was also detected by TUNEL assays. Results: knockdown of FSTL1 in MSCs promoted, while overexpression of FSTL1 inhibited H9C2 cell viability without significantly affecting cell apoptosis. Under the ischemia/reperfusion injury (IRI) model, mice treated with natural MSC supernatants (containing FSTL1) exhibited significantly smaller infarction areas and minimal histological changes. Myocardium injury biomarkers, including serum lactate dehydrogenase (LDH), creatine phosphorkinase (CK) and malondialdehyde (MDA) were accordingly decreased under MSC supernatants treatments. However, MSC supernatants with FSTL1 knockdown blunted the above effects. In addition, myocardium apoptosis was inhibited by MSC supernatant treatments, whereas it was promoted after knockdown of FSTL1. Conclusions: Mesenchymal stem cell protects myocardium from ischemia/reperfusion injury through secreting FSTL1.

Keywords: Mesenchymal stem cell, FSTL1, ischemia/reperfusion injury

Introduction

Acute myocardial infarction is the result of abrupt occlusion of a nutritional blood vessel in the heart. As a result, the myocardium distal to the occlusion site becomes ischemic. If the occlusion of an artery is unrelieved, ischemia will cause permanent damage to the myocardium previously supplied by the occluded artery [1]. Timely restoration of blood flow to the ischemic myocardium (termed "reperfusion") is currently the standard treatment for patients suffering from acute myocardial infarction [2]. Unfortunately, reperfusion itself may cause adverse effects; such as directly accelerate the ischemic condition and additionally injure the myocardium, which is referred to as "myocardial ischemia/reperfusion injury (IRI)". The IRI is reported to account for up to 50% of final infarct size in the infarct myocardium [3]. Reperfusion injured myocardium mainly by the release of oxygen free radicals, the overload of intracellular calcium, and alterations in cell metabolism [4].

Follistatin-like 1 (FSTL1, Gene ID: 11167), also referred to as TSC36, is a secreted extracellular glycoprotein that belongs to the follistatin family of proteins, though the homology is limited [5]. FSTL1 was originally cloned from a rat osteoblast cell line as a transforming growth factor β (TGF β)-inducible gene [5]. Substantial studies have reported that FSTL1 was involved in human cancer progression and autoimmune diseases. Overexpression of FSTL1 in cancer cells have been shown to suppress tumor growth and invasion [6-8]. FSTL1 was also shown to be elevated in systemic autoimmune

diseases and correlated with disease activity in patients with rheumatoid arthritis [9]. It was also recently demonstrated that FSTL1 was a serum biochemical marker reflecting the severity of joint damage in patients with osteoarthritis [10]. However, only scanty data have been presented with regard to the functional roles of FSTL1 in vascular diseases. Based on currently available data, FSTL1 was detectable in the medium of cardiac myocytes [11] and endothelial cells (ECs), and its expression was even upregulated in response to ischemic stress [12].

To date, the secretion of FSTL1 by mesenchymal stem cells (MSCs) has not been investigated, and no functional analysis of FSTL1 has been performed in the setting of ischemia/reperfusion injury. In the present study, we tested whether FSTL1 secreted by MSCs has any effects on the cellular behaviors in H9C2 cells under ischemic hypoxemia conditions. Besides, we also examined the effects of FSTL1 secretion by MSCs on the myocardium injuries in a rat model of IRI. Our observations indicate that mesenchymal stem cell-secreted FSTL1 exert potent cardioprotective effects on rats under ischemia/reperfusion stress. This protective role is time-dependent.

Materials and methods

Rat model of ischemia/reperfusion injury (IRI)

Ligation of left anterior descending (LAD) artery was used to induce regional myocardial IRI. Protocols for myocardial IRI operations were in accordance with previous studies [13, 14]. Briefly, thirty six 200 g Wistar rats were purchased from SLRC Laboratory Anima (Shanghai, China) and divided evenly into four groups, namely 1) control group; 2) IRI group; 3) IRI+MSC group: 1×10^6 MSCs were injected into the abdomen of each rat under IRI; 4) IRI+MSC FSTL1 siRNA group: MSCs were transfected with specific siRNA against FSTL1 (siFSTL1) prior to injection. Then 1×10^6 of FSTL1-depleted MSCs were injected into each rat abdomen under IRI. For each group (n=9), rats were further divided into three subgroups (n=3 for each subgroup) according to the observation time: 1 day, 3 days, and 7 days. For each observed time point, rats were scarified and cardiac muscle tissues at the infarct section was dissected and fixed in 10% formalin for 24 h. The tissues were embedded in paraffin and sagittally sliced at 5 μm. The sections were stained with hematoxylin and eosin (HE) staining. Ten randomly selected fields (400×) of each slide were observed seriously by two independent investigators who were blinded to our study. All the procedures were approved by an ethical committee from Hospital. All efforts were made to minimize sufferings.

Myocardial infarct size determination

Before dissecting the heart, 0.5 ml of 2% methylene blue were injected into the left ventricle once LAD was ligated externally at the previous occlusion site, to stain the non-ischemic area. After excision, the whole heart was washed with pre-cooled PBS. Left ventricular was weighted and stored into -80°C freezer for 5 min. Thereafter, the left ventricle was cut into slices of 5 mm thickness and incubated in 1% TTC-phosphate buffer (PH=7.4) for 10 min at 37°C. The infarct size was determined by pressure-controlled injection of 15 ml tetrazolium red into the LAD. The non-infarct areas were at intensely red, whereas infarct tissues remained pale. The infarct part was sliced and weighted. Myocardial infarct size (MIS) was presented as the weight of infarct area/the whole left ventricle. Moreover, the pale part and entire left ventricle were subjected to digital photography.

ELISA assay of LDH and CK

Myocardial serum lactate dehydrogenase (LDH) and creatine phosphorkinase (CK) were measured using commercially available ELISA kit under the manufacturer's instructions (Catalog No. SEB864Ra for LDH, and SEA479Ra for CK from Life Science Inc., Wuhan, China).

Determination of SOD activity and MDA concentration

Before measuring, 9-fold volume of NaCl medium was mixed together with the sample to get the 10% (v/v) solution. The mixture was centrifuged at 4000 rpm/min for 10 min and measured the concentration of the tissue protein with Comassie blue protein assay kit. For SOD activity, xanthine oxidase was used and the absorbance was collected at 550 nm. For MDA concentration, the thiobarbituric acid was included and the absorbance was acquired at 532 nm. The standard curves of SOD activity and MDA concentration were explored according to the manufacture's recommendation.

Determination of apoptosis with TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed with an Apoptosis Detection kit (Catalog No. 11767291910, Roche, Basel, Switzerland) following the manufacturer's instructions. Brown-yellow granules could be observed in the cytoplasm in apoptotic cells, which was positive data. Otherwise, the normal cells contained blue particles in the cytoplasm. The number of apoptotic cells randomly in five high-power fields (×400 magnifications) was calculated. The apoptosis index (AI) was expressed as the number of apoptotic cells/100 cells (%).

Cell culture and treatments

H9C2 cell and mesenchymal stem cell were commercially from American Type Cultural Collection (ATCC, Chicago, USA). H9C2 cells and MSC were cultured in DMEM solution (Catalog No. 11995-065, Gibco, New York, USA) supplemented with 10% FBS (Catalog. No. 10437-010, Gibco) and 1% antibiotics. For the sake of ischemic hypoxemia model, serum-free DMEM with 100 nM deferoxamine (DFO) solution (Catalog No. D9533, Sigma, St. Louis, USA) was used to incubate with H9C2 cells. All cell culture was maintained in an incubator under 5% CO_2 at 37°C.

Plasmid/siRNA transfection and collection of supernatants containing FSTL1

MSC was seeded into a six-well plate and incubated till the density reached 80% at 37°C. For knockdown of FSTL1, specific siRNA against FSTL1 (siFSTL1) was designed and chemically synthesized by Sigma (St. Louis, USA). For transfection, siFSTL1 and the expression plasmid of FSTL1 were transfected into MSCs with lipofectamine 2000 (Catalog No. 11668-019, Invitrogen, Shanghai, China) according to the manufacturer's instructions. 6 h post-transfection, the OPTIM-MEM medium was replaced with fresh DMEM supplied with 10% FBS for another 24 hours. The supernatants from the cultured MSCs with distinct treatments were collected respectively for subsequent analysis.

Flow cytometry analysis

Flow cytometry was explored on a FACSCanto II instrument (BD Biosciences). The MSC was digested with 0.25% trypsin without ethylene diamine tetraacetic acid (EDTA) and pipetted several times to obtain the single cell suspension ($2\sim3\times10^5$). The cells were washed with phosphate-buffered saline (PBS) twice and the supernatant was discarded. After adding 100 μl binding buffer and 5 μl TITC-labeled Annexin-V (20 μg/ml) to each well, the cells were incubated for 30 min in room temperature. After that, 5 μl protease inhibitor (PI, 50 μg/ml) was added into each well and the plate was kept in the dark for 5 min. Then 400 μl binding buffer was added into each sample and flow cytometry was performed immediately with FACScan. The reaction was executed in less than one hour.

Cell viability assay

CCK-8 (Catalog No. CK04, Dojindo, Kumamoto, Japan) assay was performed to determine the cell viability. When cell confluence reached 90%, the H9C2 cells were seeded into 96-well plate (300 cells/well). MSCs were also cultured in thermostatic incubator for 24 h and the supernatant was obtained to for treatment of the H9C2 cells at a 96-well plate. Forty-eight hours after the co-culture of MSCs supernatant and H9C2 cells, 100 μl CCK-8 was added into each well and mixed gently with 100 μl DMEM medium. The plate was incubated for another 3 h and the cell viability was acquired by collecting the absorbance at 450 nm with MRX II microplate reader (Dynex, Chantilly, VA, USA).

Western blot analysis

Cells were washed with ice-cooled PBS three times and mixed with lysis buffer (Cell Signaling Technology, Danvers, MA, USA) for 2 h at 4°C. The total protein concentration and quality were identified by BCA protein quantification kit (Catalog No. 23227, Thermo Fisher Scientific, Rockford, IL, USA). An equal amount of 40 μg protein was loaded into each lane in a 12% SDS-PAGE gel. After electrotransferring to a PVDF membrane (Catalog No. IPVH304F0, Millipore, Billerica, MA, USA), the non-specific sites were blocked by shaking the membrane with blocking buffer containing 5% bull serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h on ice. Then the membrane was incubated with the primary rabbit anti-FSTL1 antibody (Catalog No. ab71548, Abcam, Cambridge, MA, USA) diluted in the blocking buffer overnight. After washing the membrane for 5 min with TBST four times, the horseradish peroxidase-labeled secondary antibody (Catalog No. ab150077, Abcam,

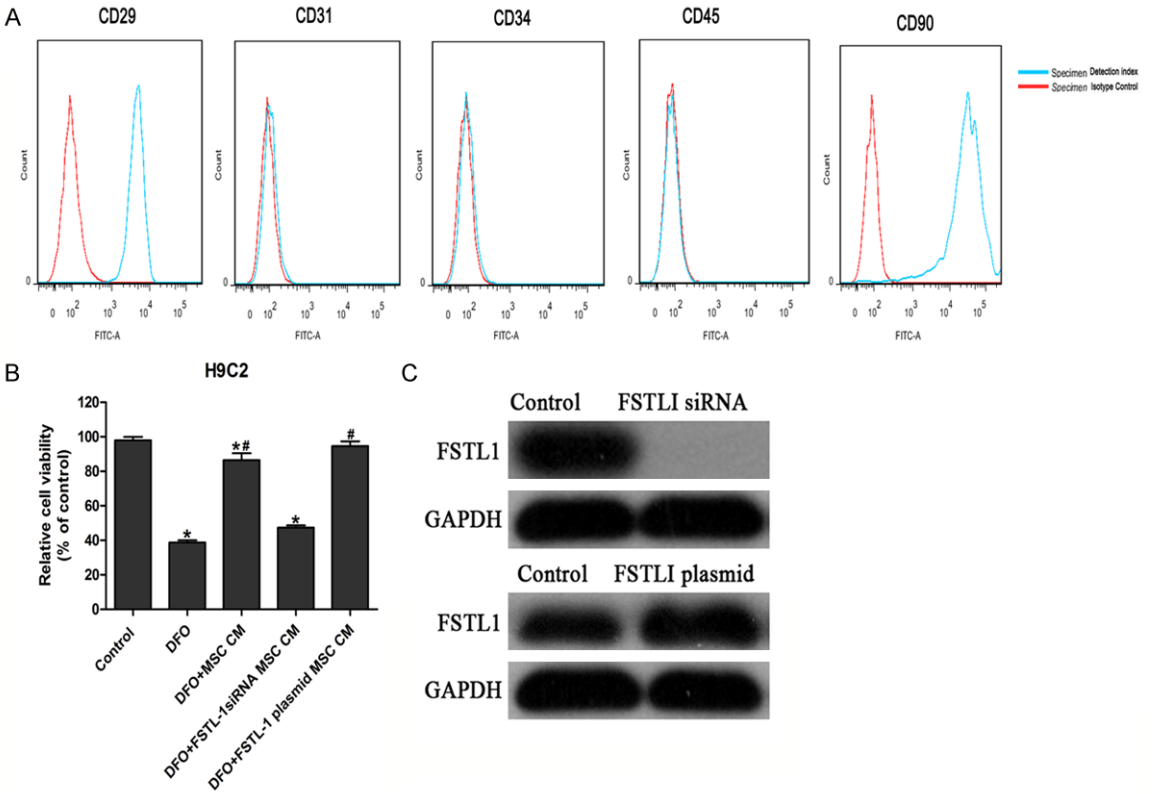

Figure 1. FSTL1 promotes H9C2 cell viability and protects H9C2 cells against ischemic hypoxemia *in vitro*. A. Flow cytometry assay was performed to detect the molecular biomarkers on the surface of MSC, including CD29, CD31, CD34, CD45 and CD90. The red curve represented specimen detection index, and the blue one was specimen isotype control. B. Relative cell viability in different experimental groups. Control: H9C2 cells without any stimulation; DFO: cells treated with deferoxamine; DFO+MSC CM: cells co-incubated with deferoxamine and the supernatant of the MSC culture medium; DFO+FSTL1 siRNA MSC CM: cells co-incubated with deferoxamine and the supernatant of the FSTL1 siRNA-treated MSC culture medium; DFO+FSTL1 plasmid MSC CM: cells co-incubated with deferoxamine and the supernatant of the FSTL1 plasmid-treated MSC culture medium. *P<0.05 *vs.* control, #P<0.05 *vs.* DFO. C. Western blot assay showed the FSTL1 and GAPDH protein levels when MSC was transfected with FSTL1 siRNA or plasmid.

Cambridge, MA, USA) was added and incubated for 2 h at 4°C. After the final wash with TBST, the detection reagents (GE Healthcare, Piscataway, NJ, USA) were mixed to cover the surface of the PVDF membrane. The dilution for primary antibody is 1:1000 and the secondary antibody is 1:2000. For autoradiography, film was exposed as described under the manufacturer's instruction. GAPDH was also included as an internal control.

EdU click-iT reaction

EdU incorporation was detected using the Click-iTEdU Imaging Kit (Catalog No. C10446, Invitrogen; Carlsbad, CA, USA). Each step was performed at room temperature. H9C2 cells were permeabilized in 1.0% TritonX 100 for 25 min and rinsed three times in PBS for 10 min each time. At the meantime, the Click-iT kit azide and buffer additive were taken out from a -20°C freezer and allowed to thaw in a dark box. During the third PBS rinse, the reaction cocktail was added with azide. Cells were then incubated in the EdU cocktail for 30 min and washed three times in PBS for 15 min per time. The azides were coupled to either an Alexa Fluor 488 (green) or Alexa Fluor 350 (hyacinthine) fluorophore. Upon completion of the reaction, cells were mounted in Vectashield mounting medium onto glass slides and sealed with clear glycerol.

Statistical analysis

The results were exhibited as means ± standard derivate (SD). Statistical analysis was carried out with the Student's *t*-test. P<0.05 was considered significant. All experiments were repeated in triplicate.

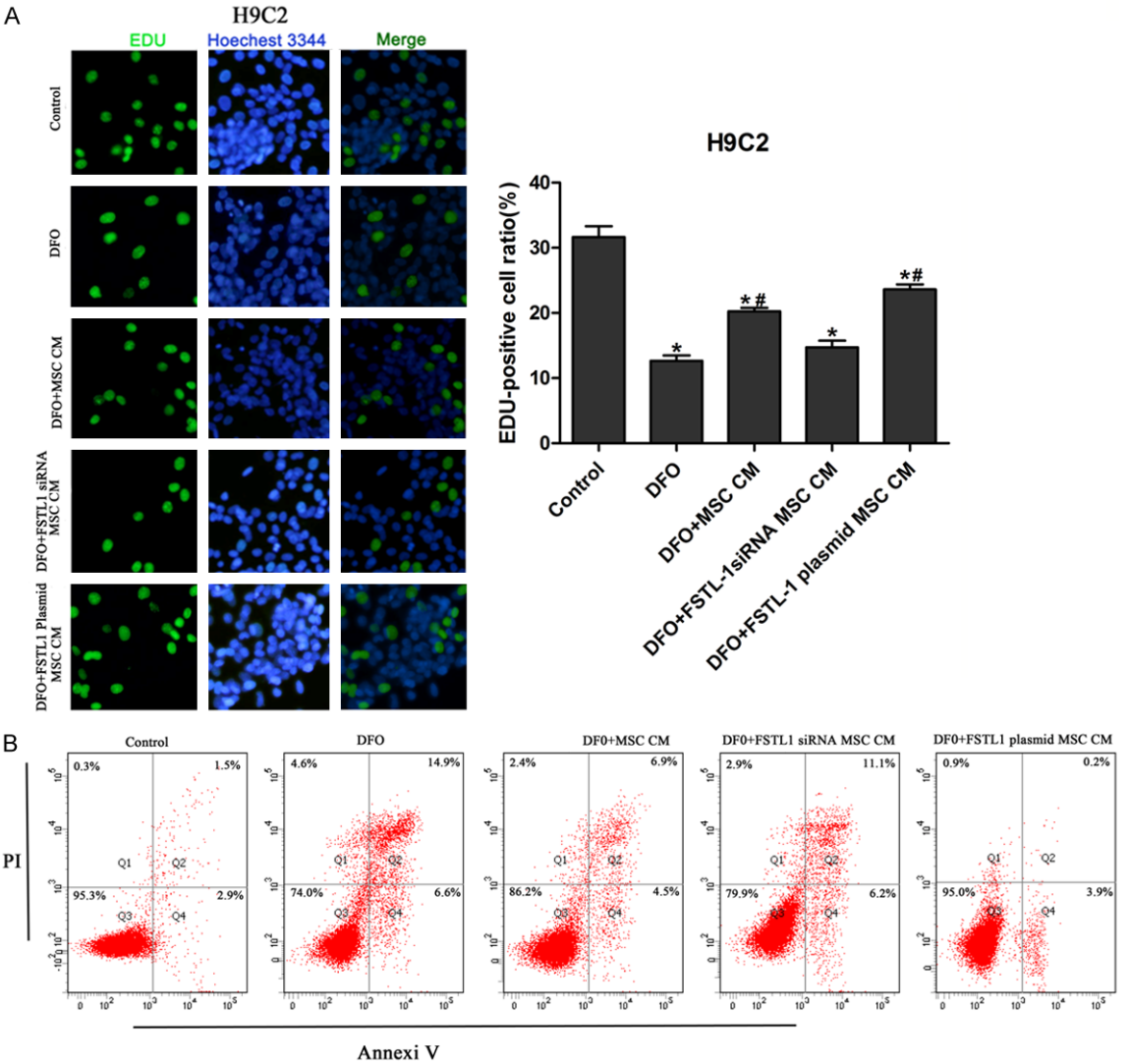

Figure 2. FSTL1 increases H9C2 cell proliferation rate and causes little change on apoptosis *in vitro*. A. EdU click-iT reaction was performed to measure the proliferation rate. Control: H9C2 cells without any stimulation; DFO: cells treated with deferoxamine; DFO+MSC CM: cells co-incubated with deferoxamine and the supernatant of the MSC culture medium; DFO+FSTL1 siRNA MSC CM: cells co-incubated with deferoxamine and the supernatant of the FSTL1 siRNA-treated MSC culture medium; DFO+FSTL1 plasmid MSC CM: cells co-incubated with deferoxamine and the supernatant of the FSTL1 plasmid-treated MSC culture medium. *P<0.05 *vs.* control, #P<0.05 *vs.* DFO. B. TUNEL assay showed that late stage apoptosis rate was increased to 14.9% when H9C2 cells were treated with DFO and to 6.9% when cells were co-incubated with the supernatant of MSC culture medium. Early stage apoptosis was promoted by DFO, but was partially inhibited by MSCs CM. Treatments of H9C2 cells with MSCs natural supernatant or supernatants from FSTL1-overexpressed MSCs reduced the early and late stage apoptosis. No significant differences between groups were observed though.

Results

FSTL1 promotes H9C2 cell viability and protects H9C2 cells against ischemic hypoxemia in vitro

The cultured mesenchymal stem cell (MSC) expresses CD73, CD90 and CD105 on their surface, while lacking the expression of CD14, CD19, CD34, CD45 and CD79a [15]. To investigate the accuracy of our MSC, flow cytometry was performed to detect the molecular biomarkers on the surface of MSC. It was shown in **Figure 1A** that CD29 and CD90 were highly positive on the surface, whereas the CD31, CD34 and CD45 were negative, which suggested the MSC in our study maintained the biological activities of bone marrow mesenchymal

stem cells. Then we checked the H9C2 cell viability after different treatment with the supernatant of MSC culture medium (CM). To this end, we employed specific siRNA against FSTL1 and FSTL1 expression plasmid. MSCs were initially transfected with siFSTL1 or FSTL1 expression plasmid. The corresponding supernatants were collected and treated with H9C2 cells, respectively. In addition, the deferoxamine (DFO) solution was used to treat H9C2 cells to induce ischemic hypoxemia. CCK-8 assay showed that H9C2 cell viability was decreased by approximately 60% when treated with DFO solution. After the supernatant of MSC culture medium (CM) was added into DFO-treated H9C2 cells, the cell viability was increased to 90% compared with the control group. However, compared with the control group, siRNA-mediated knockdown of FSTL1 caused decreases of H9C2 cell viability to 48%, whereas overexpression of FSTL1 in MSCs resulted in approximate 97% cell viability which was fairly close to the control cells (**Figure 1B**). These observations suggested that the supernatant of MSCs CM which contained FSTL1 was protective against ischemic hypoxemia in myocardial H9C2 cells. In addition, supernatants from FSTL1-depleted or overexpressed-MSCs CM were collected, respectively, and subjected to western blot analysis. Our results showed that FSTL1 protein level in MSC was significantly knocked down when FSTL1 siRNA was transfected, whereas it was highly increased in the FSTL1 plasmid-transfected group (**Figure 1C**). The highly efficient transfection further confirmed that data observed in **Figure 1B** were convincible.

FSTL1 increases H9C2 cell proliferation rate but causes minimal change on apoptosis in vitro

To further explore the role of FSTL1 on cell proliferation and apoptosis, EdU click-iT reaction and TUNEL assay were performed here. EDU-positive cell rate was extremely decreased when H9C2 cells were incubated with DFO. When the supernatant of the culture medium of MSCs was added into H9C2 cells, the proliferation rate was increased and further enhanced when MSCs were transfected with FSTL1 plasmid. On the contrary, the EDU-positive cells were significantly decreased when FSTL1 was depleted from the supernatant of MSCs CM (**Figure 2A**). Then we examined the role of FSTL1 on cell apoptosis with flow cytometry assay. **Figure 2B** showed that the late stage apoptosis rate was increased to 14.9% when H9C2 cells were treated with DFO, and to 6.9% when co-incubated with the supernatant of MSC culture medium. Early stage apoptosis was promoted by DFO, but was partially inhibited by MSCs CM. Generally; treatments of H9C2 cells with MSCs natural supernatant or supernatants from FSTL1-overexpressed MSCs reduced the early and late stage apoptosis. However, there was no significant difference between groups. These results show that H9C2 cells exhibit lower proliferation rate when cells are under hypoxemia. MSCs-secreted FSTL1 shows its ability to increase cell proliferation rate but has little effects on H9C2 cell apoptosis.

FSTL1 rescues the myocardial infarction caused by IRI in vivo

We next examined whether FSTL1 exerted the similar role in an IRI rat model. Myocardial infarction area was extremely higher in IRI group compared with the control one. The infarction was relieved when IRI mice were injected with the supernatant of MSC CM. However, the infarction area became even remarkable when MSC was transfected with siFSTL1 and the FSTL-depleted supernatant were injected into IRI mice (**Figure 3A**). The MSCs supernatant-mediated relief of IRI was time-dependent (**Figure 3A**). Subsequent H&E staining showed that compared with the normal myocardium, the IRI mice showed abnormal fibers which were unrhythmically arranged and some of which were fractured and necrotic. Interstitial at the margin of the infarct area was infiltrated with abundant inflammatory cells (**Figure 3B**). This abnormality was even significant when FSTL1 was knocked down. However, histological abnormality was significantly milder when IRI rats were treated with MSC supernatant, though a small amount of neutrophils were still observed. These data suggest that while IRI induces myocardial injuries in the rat model, MSC protects myocardium against IRI by secreting FSTL1.

FSTL1 affected myocardial injury-related biomarkers

The cardioprotective role of FSTL1 raised our interest to investigate whether FSTL1 affected

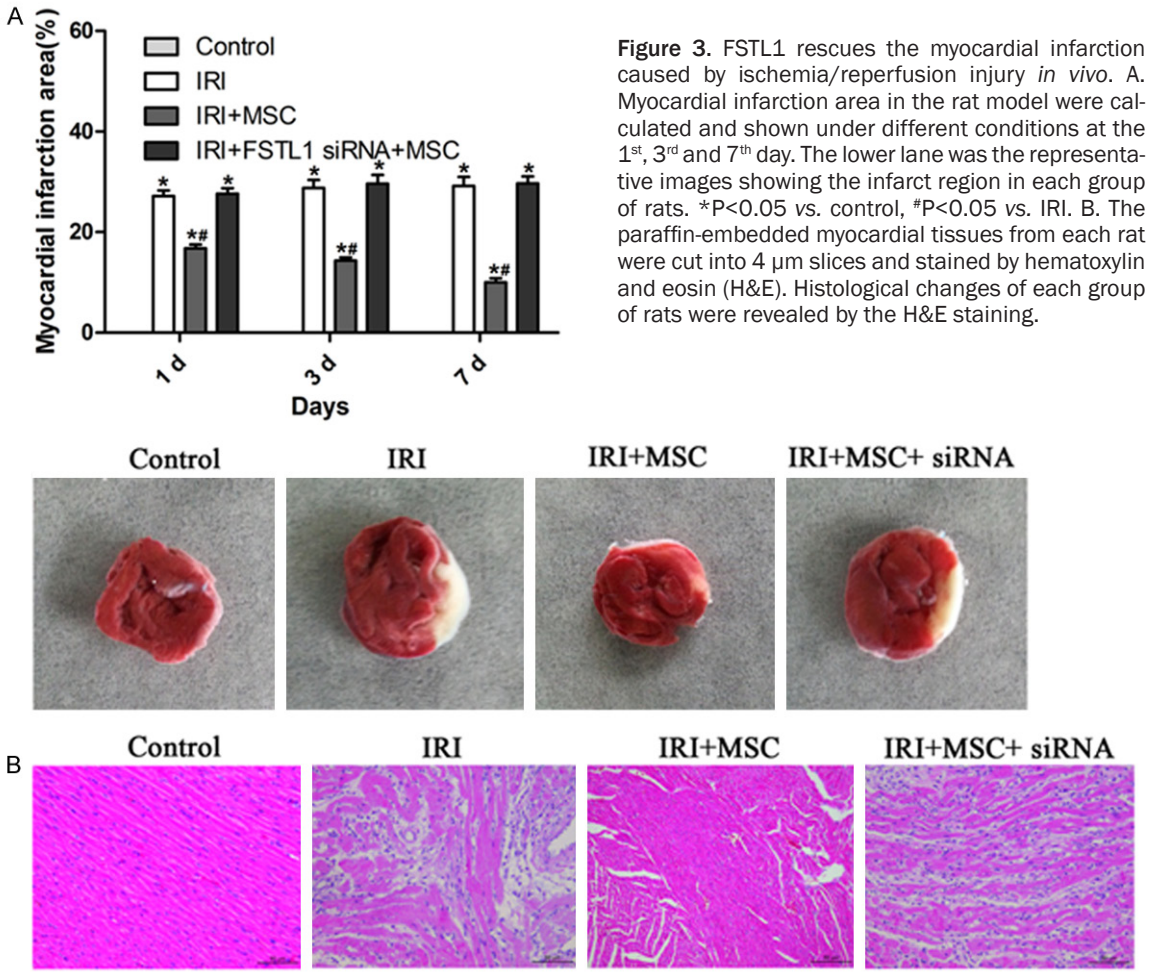

Figure 3. FSTL1 rescues the myocardial infarction caused by ischemia/reperfusion injury *in vivo*. A. Myocardial infarction area in the rat model were calculated and shown under different conditions at the 1st, 3rd and 7th day. The lower lane was the representative images showing the infarct region in each group of rats. *P<0.05 *vs.* control, #P<0.05 *vs.* IRI. B. The paraffin-embedded myocardial tissues from each rat were cut into 4 μm slices and stained by hematoxylin and eosin (H&E). Histological changes of each group of rats were revealed by the H&E staining.

the expression levels of common myocardial injury biomarkers. Hence, we selected four common biomarkers, including creatine phosphate kinase (CK), lactic dehydrogenase (LDH), malondialdehyde (MDA) and superoxide dismutase (SOD). The former three are indicators of myocardial injury, while SOD represents a cardioprotective factor. Our results showed that compared with the control rats, IRI rats exhibited significantly higher CK and LDH levels. However, the levels of CK and LDH in IRI rats were significantly decreased by MSCs supernatant. Interestingly, MSCs supernatant-mediated decreases of CK and LDH in IRI rats were blunted by siRNA-mediated knockdown of FSTL1 in MSCs (**Figure 4A** and **4B**). These observations suggest that while CK and LDH were induced after IRI, MSCs-secreted FSTL1 inhibited their releases which were consistent with the protective effects of FSTL1 on myocardium. Similar result was also observed with respect to MDA (**Figure 4C**). On the contrary, SOD level which indicated myocyte self-protection was decreased in IRI rats. SOD level was increased by MSCs natural supernatants, but was decreased again after FSTL1 was depleted from MSCs (**Figure 4D**). The FSTL1-mediated above effects were even remarkable with time extended. These data further confirm that MSCs-secreted FSTL1 relieved myocardial injury under ischemia/reperfusion conditions.

MSCs-secreted FSTL1 relieved IRI-induced myocyte apoptosis in the rat model of IRI

The role of MSCs-secreted FSTL1 in cell apoptosis was also assessed in the rat model of IRI with the aid of TUNEL assay. On the first day after IRI surgery, the myocardial cell apoptosis rate was 24-fold higher in IRI rats than the control ones. The myocyte apoptosis rate in IRI rats were decreased by up to 30% after treatments of rats with MSCs natural supernatants. However, the MSCs supernatant-mediated

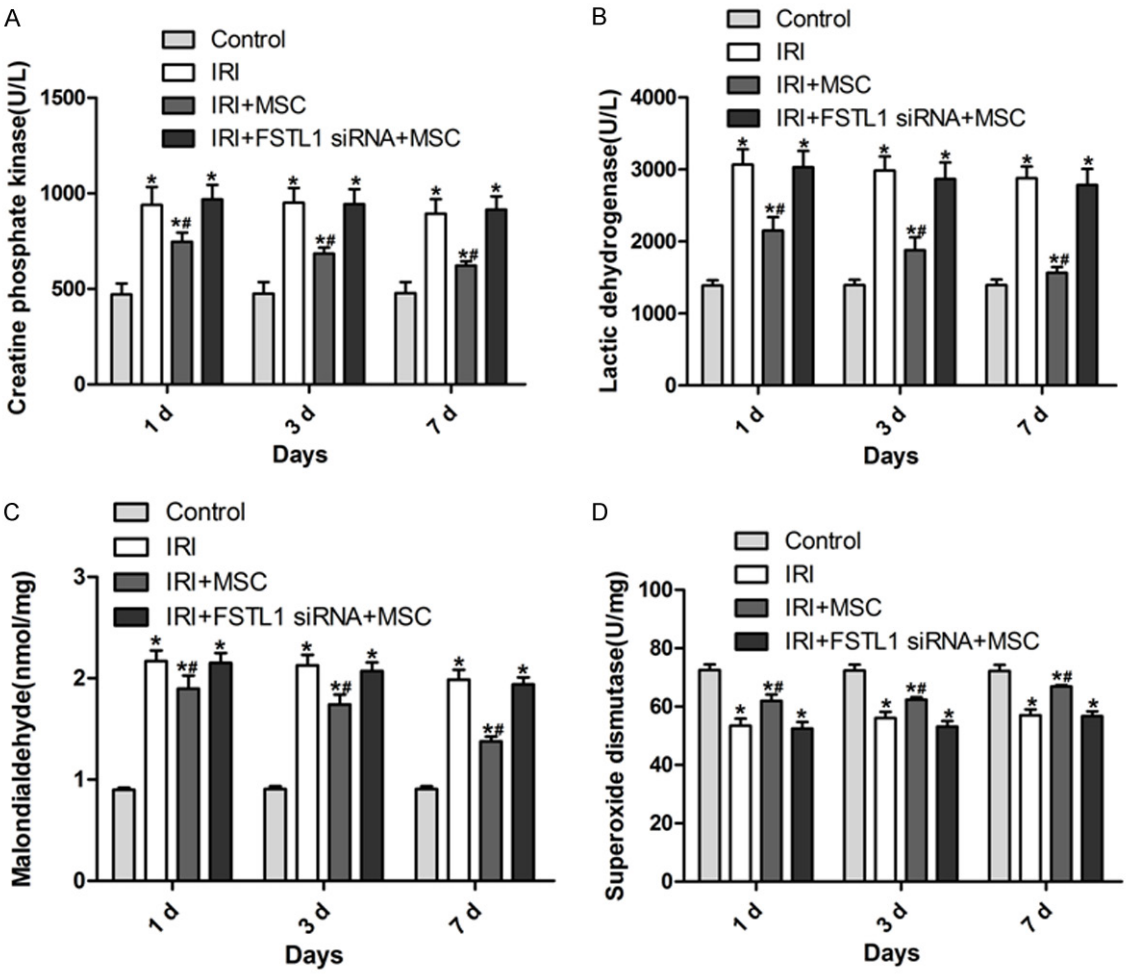

Figure 4. FSTL1 affected myocardial injury-related biomarkers. Four biomarkers indicating myocardial injury were selected and detected by ELISA assays. A-C. For creatine phosphate kinase (CK, U/L), lactic dehydrogenase (LDH, U/L) and malondialdehyde (MDA, nmol/mg), they indicates the myocardial injuries by ischemia/reperfusion. The results showed that compared with the control rats, IRI rats exhibited significantly higher levels of CK, LDH and MDA. However, their levels in IRI rats were significantly decreased by MSCs supernatant. MSCs supernatant-mediated decreases of CK, LDH and MDA in IRI rats were blunted by siRNA-mediated knockdown of FSTL1 in MSCs. D. Superoxide dismutase (SOD, U/mg) level represents myocyte self-protection under IRI. It was decreased in IRI rats and increased by MSCs natural supernatants in IRI rats. But it was decreased again after FSTL1 was depleted from MSCs. Their levels were detected on the 1st, 3rd, and 7th day, respectively. *P<0.5 *vs.* control, #P<0.05 *vs.* IRI.

relief of myocyte apoptosis was greatly reversed after MSCs were depleted of FSTL1 (**Figure 5**, left panel). These findings could also be reflected by the representative images showing the apoptosis in each group (**Figure 5**, right panel). Moreover, with the progressive apoptosis induced by IRI, MSCs-secreted FSTL1 increasingly relieved the myocyte apoptosis in a time-dependent manner (**Figure 5**, left panel). These data suggest that while IRI induces myocyte apoptosis, MSCs-secreted FSTL1 functions as an anti-apoptotic factor.

Discussion

Cardiovascular disease is a global health problem [16]. In particular, ischemic heart disease is featured with high morbidity and mortality rates. Hence, ischemic heart disease has aroused medicine and public concern worldwide [1, 17]. It is estimated that myocardial infarction will be the leading cause of mortality by 2020 according to the World Health Organization [18]. IRI is defined as the damage to myocardial tissue when blood supply is

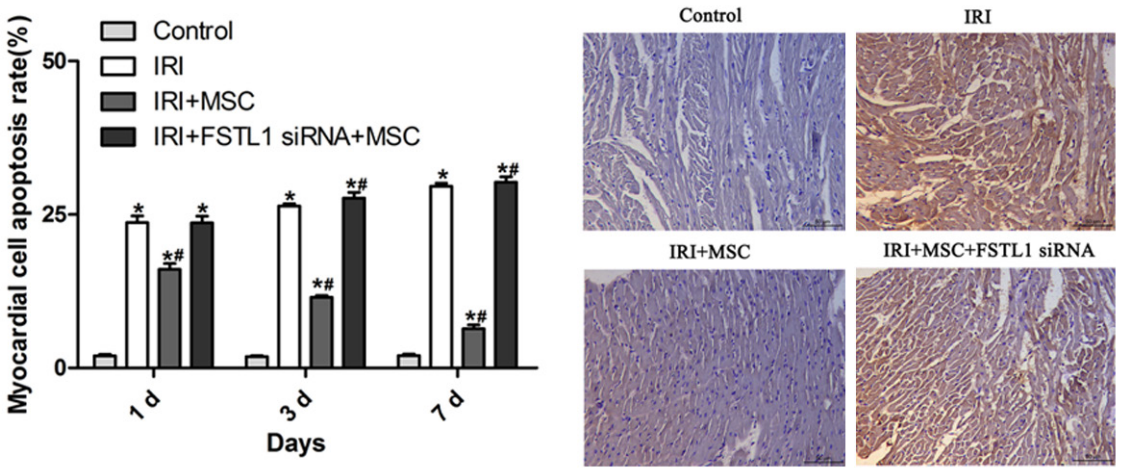

Figure 5. MSCs-secreted FSTL1 relieved IRI-induced myocyte apoptosis in the rat model of IRI. Myocyte apoptosis was evaluated on the 1st, 3rd, and 7th day, respectively. Left panel: on the first day after IRI surgery, the myocardial cell apoptosis rate was 24-fold higher in IRI rats than the control ones. The myocyte apoptosis rate in IRI rats were decreased by up to 30% after treatments of rats with MSCs natural supernatants. MSCs supernatant-mediated relief of myocyte apoptosis was greatly reversed after MSCs were depleted of FSTL1. These effects were time-dependent. Right panel: the representative images showing the apoptosis in each group. Magnification: 400×. *P<0.5 *vs.* control, #P<0.05 *vs.* IRI.

restored after a period of ischemia. Serious results from IRI include inflammation infiltrates, oxidative damage, overload of intracellular calcium, apoptotic and necrotic myocytes death and cardiac dysfunction [19-21]. Currently, evolving therapies have been proposed as to the treatment of IRI. These include therapies targeting to reduce ischemic injury, reperfusion injury, or targeting to reduce both damages. However, these strategies have only been progressed to clinical phase II trials. Novel therapeutic strategies are still in great need.

In the present study, we investigated the potential protective roles of FSTL1 in myocardial IRI. In the ischemic hypoxemia conditions, H9C2 cells were significantly inhibited from proliferation. However, treatment of H9C2 cells with the supernatants of MSCs culture medium which contained FSTL1 significantly rescued the myocardial IRI. Overexpression of FSTL1 further increased H9C2 cell viability. On the contrary, siRNA-mediated knockdown of FSTL1 in MSCs, which caused depletion of FSTL1 in the supernatant, blunted H9C2 cell viability. These findings suggest that FSTL1 which is secreted by MSCs could protect H9C2 cells against ischemic hypoxia. Moreover, in the rat model of IRI, rats from the IRI group underwent significant myocardial infarction, whereas supernatant of MSCs significantly relieved damage to the rats in a time-dependent manner, as evidenced by the decreased infarction size, less histological lesion, the decrease of myocyte injury factors and the increase of cardioprotective factor superoxide. The MSCs supernatant-mediated relief of IRI was blocked by siRNA-mediated knockdown of FSTL1 in MSCs. In addition, FSTL1-mediated protection of myocardial IRI was associated with inhibition of myocyte apoptosis. These in vivo data strongly suggest that FSTL1 is a cardioprotective factor which is secreted by MSCs.

Previously, FSTL1 was reported to be secreted by the heart and was cardioprotective under the regulation of Akt signaling [11]. Unlike previous report, our evidence showed that FSTL1 could be secreted by MSCs and exerted potent cardioprotective roles in rats under IRI. The serum and heart detectable FSTL1 levels suggested its diagnostic values [22]. In our study, the immunodetectable FSTL1 protein in the supernatant of MSCs would suggest that MSCs-secreted FSTL1 could also have therapeutic utilities. The hearts secret biomarkers that serve as therapeutic or diagnostic agents, of which atrial natriuretic peptide and brain natriuretic peptide are two widely used hormones that serve as therapeutic and diagnostic bio-

markers [23, 24]. Therefore, the identified factors secreted by MSCs or heart could also be considered candidates for clinical applications.

Proteins from the follistatin family are generally believed to function by binding to and modifying members of the TGFβ superfamily [25]. For instance, follistatin can bind to growth and differentiation factor 8 (GDF8) as well as activin A and B, and a subset of bone morphogenic proteins (BMP) including BMP-2, -4, and -7 [26]. However, it is unclear whether FSTL1 functions to protect myocardial IRI in a similar mechanism as follistatin. Currently, the mechanisms underlying the biological activities of FSTL1 in various pathological processes remain largely unknown, especially in the IRI process. The only known fact is that FSTL1 is regulated by Akt signaling when maintaining its cardioprotective roles. Downstream targets of *FSTL1* remain to be elucidated.

In all, the present study investigated the protective roles of FSTL1 from myocardial IRI when secreted by MSCs. MSCs-secreted FSTL1 protected H9C3 cells against ischemic hypoxemia injury in vitro. Moreover, the natural supernatants of MSCs protected myocardium from IRI in vivo, whereas knockdown of FSTL1 in MSCs lost its ability to exert the cardioprotective roles in myocardial injury of ischemia/reperfusion. The identification of FSTL1 as a key secretion by MSCs is of great significance as regard with the therapeutic and diagnostic strategies of ischemic heart disease.

Acknowledgements

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Disclosure of conflict of interest

None.

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