FDNC5/Irisin improves the therapeutic efficacy of bone marrow-derived mesenchymal stem cells for myocardial infarction

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Research

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Abstract

Background: The beneficial functions of bone marrow mesenchymal stem cells (BM-MSCs) decline with decreased cells survival, limiting their therapeutic efficacy for myocardial infarction (MI). Irisin, a novel myokine which is cleaved from its precursor fibronectin type III domain-containing protein 5 (FNDC5), is believed involved in a cardioprotective effect but little was known on injured BM-MSCs and MI repair yet. Here, we investigated whether FNDC5 or irisin could improve the low viability of transplanted BM-MSCs and increase their therapeutic efficacy after MI.

Methods: BM-MSCs, isolated from dual-reporter firefly luciferase and enhanced green fluorescent protein positive (Fluc⁺– eGFP⁺) transgenic mice, were exposed to normoxic condition and hypoxic stress for 12 h, 24 h, and 48 h, respectively. In addition, BM-MSCs were treated with irisin (20 nmol/L) and FNDC5-OV in serum deprivation (H/SD) injury. Furthermore, BM-MSCs were engrafted into infarcted hearts with or without FNDC5-OV.

Results: Hypoxic stress contributed to increased apoptosis, decreased cells viability and paracrine effects of BM-MSCs while irisin or FNDC5-OV alleviated these injuries. Longitudinal *in vivo* bioluminescence imaging illustrated that FNDC5-MSCs treatment improved the survival of transplanted BM-MSCs, which ameliorated the increased apoptosis and decreased angiogenesis of BM-MSCs *in vivo*. Furthermore, FNDC5-MSCs therapy significantly reduced fibrosis and alleviated injured heart function.

Conclusions: The present study indicated that irisin or FNDC5 improved BM-MSCs engraftment and paracrine effects in infarcted hearts, which might provide a potential therapeutic target for MI.

Background

The treatments focus on retaining the function of the remaining cardiomyocytes, but myocardial infarction (MI) still remains a major cause of morbidity and mortality worldwide^(1,2). Recently, mesenchymal stem cells (MSCs) transplantation has held great promise for multiple diseases, such as MI⁽³⁻⁶⁾. The illustrated underlying therapeutic mechanisms include engraftment⁽⁷⁾, paracrine⁽⁸⁾ and other effects, like exosomes⁽⁶⁾. These mechanisms play a role on the premise that the engrafted MSCs survives for a certain period. However, many studies have shown that only a small number of transplanted MSCs eventually survive to play protective effects⁽⁹⁻¹¹⁾. Also, evidences indicated that under myocardial ischemia conditions, hypoxic stress caused increased apoptosis of MSCs and reduce its therapeutic effects^(1,12). Therefore, it is reasonable to assume that promoting the low engrafted MSCs survival in ischemia myocardial tissue may be the key to improving stem cells therapeutic efficacy after MI.

FNDC5 is a transmembrane protein that possesses two domains (fibronectin III and carboxy-terminal respectively) located in the cytoplasm⁽¹³⁾. Previous study has indicated that FNDC5 can be cleaved by an unknown enzyme and then the extracellular domain of FNDC5 was named as irisin⁽¹⁴⁾. In addition, Irisin

was recognized as a myokine secreted from skeletal muscle and heart^(15,16). Furthermore, irisin has been considered as a multifunctional peptide which is involved in regulating cardiovascular function^(13,17,18). Meanwhile, accumulating studies demonstrated that irisin was involved in cardioprotective effect, such as anti-apoptosis, increased cells viability and anti-oxidative stress by various pathways⁽¹⁹⁻²⁰⁾.

However, the protective effect of FNDC5/Irisin in hypoxia-induced the low viability of transplanted BM-MSCs is still unclear. Therefore, we hypothesized that Irisin may protect against increased apoptosis and paracrine dysfunction of BM-MSCs induced by hypoxia. Moreover, genetically modified BM-MSCs overexpressing FNDC5 may promote the functional survival of transplanted BM-MSCs and increase the cardiac protective effect.

Methods

Animal

Adult male C57BL/6 mice (8-12 weeks of age, 20-25g) were provided by the PLA Rocket Force Characteristic Medical Center (Beijing, China). Meanwhile, Fluc⁺–eGFP⁺ transgenic mice [Tg(*fluc-egfp*)] were purchased from Contag Laboratory (Stanford, CA, USA). Mice were created on the C57BL/6 background to stably express both firefly luciferase (Fluc) and enhanced green fluorescence protein (eGFP) in all tissues and organs. As alleged up front⁽¹⁾, mice were placed into a temperature-controlled animal facility with a 12-h light/dark cycle (light cycle, 8:00 a.m. to 8:00 p.m.), with tap water and rodent chow provided ad libitum. All animal experiments were performed by a protocol approved by the Animal Care and Use Committee of the PLA Rocket Force Characteristic Medical Center (ID: 5034) and were in compliance with the Guidelines for the Care and Use of Laboratory Animals, as published by the National Academy Press.

Isolation, culture and treatments of BM-MSCs

BM-MSCs were isolated and expanded as described⁽¹²⁾. Briefly, bone marrow was flushed from the femoral and tibia of adult Tg (*fluc-egfp*) mice with fetal bovine serum (FBS)-free Dulbecco's modified Eagle's medium (DMEM). After passing through a 70-μm strainer and centrifugation at 1200 rpm for 5 min at room temperature, the cell pellet was re-suspended in DMEM supplemented with 20% FBS and incubated at 37 °C in an atmosphere containing 5% CO₂. After 24 h, the medium was replaced to remove the nonadherent cells, and then was completely replaced every 3 days. Third-passage BM-MSCs with optimal growth at the third generation were applied for different treatments to avoid contamination with other types cell. Then, cells were treated with irisin (20 nmol/L) in the presence or absence of hypoxia for 48 h⁽¹⁹⁾.

FNDC5 over-expression

As described previously ⁽²¹⁾, FNDC5 cDNA was cloned from cells. The primer sequences were as follows: 5'-ATG CAC CCC GGG CCG CCC CG- 3' (forward) and 5'-GTC CCC TCT CTC CCT GAG C-3' (reverse). The PCR product and the pIRES2-EGFP vector (from Clontech) were digested by EcoRI and BamHI restriction sites. And the FNDC5 fragment was ligated into the pIRES2-EGFP vector that was used as control. Then, Lipofectamine and plasmids were diluted in Opti-MEM (Gibco), separately. The two solutions were then mixed in a ratio of 1:1 and incubated at 20–25 ° C for 5 minutes. Once BM-MSCs reached 60-70% fusion, the mixture was added into the cells, and then incubated and processed for the indicated time.

Hypoxia/serum deprivation injury

Hypoxia/ serum deprivation (H/SD) injury was performed for the hypoxic stress of BM-MSCs as described previously ⁽²²⁾. Briefly, after being replaced in glucose-free DMEM without FBS, BM-MSCs were exposed to hypoxia (94% N₂/5% CO₂/1% O₂) with an anaerobic system (Thermo Forma) at 37 °C for 12, 24, and 48 h, respectively. Also, BM-MSCs in the control group incubated under normoxic conditions (37 °C in 95% air, 5% CO₂) with full medium for equivalent periods.

Measurement of BM-MSCs apoptosis

BM-MSCs apoptosis was determined by flow cytometry with an Annexin V-FITC/PI Kit (Merck) according to the manufacturer's instructions ⁽²³⁾. In brief, cells were re-suspended in 200 µL of binding buffer. Cells were incubated with 10 µl of Annexin V solution and 5µl propidine iodide (PI) at room temperature for 30 minutes respectively. The cells were immediately analyzed on a FACSC-LSR (Becton, Dickinson and Company, San Jose, CA). Meanwhile, the Caspase-3 activity was measured using a Caspase-3 Assay kit (Clontech, MountainView, Calif) according to the manufacturer's instructions for 3 times. And these assays were performed in a blinded manner.

Cell viability assay

The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously ⁽²⁴⁾. Briefly, BM-MSCs were plated in 96-well plates at 1x10⁵ cells/well. After an overnight incubation,

BM-MSCs with different treatments were incubated for 72 h. Then, MTT solution (Sigma) was added into a final concentration of 0.5 g/L to each well. These cells in 96-well plates were cultured in a 5% CO₂ incubator at 37 °C for 4 h, and further the medium was aspirated. Next, 200 µL dimethyl sulphoxide (DMSO) was added into each well. The absorbance was determined at a wavelength of 490 nm. In addition, optical density (OD) values of each group were detected in six duplicate wells and their averages were calculated. We did all assays blindly.

Furthermore, the viability of cells was also assessed by bioluminescence imaging (BLI) with the IVIS Kinetic system (Caliper, Hopkinton, MA, USA) ⁽²³⁾. In brief, BM-MSCs were plated in 24-well plates (5 x10⁴ per well). Cells were given different treatments, and then the medium was removed. BM-MSCs were

incubated with D-Luciferin reporter probe (4.5 µg/mL) and further measured by the IVIS Xenogen Kinetic system (Caliper Life Sciences, USA).

Determination of VEGF, bFGF, IGF-1, and HGF

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the concentrations of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1, and hepatocyte growth factor (HGF) secreted by BM-MSCs following the manufacturer's instructions. All samples and standards were measured blindly for three times.

Myocardial infarction model

MI was accomplished by ligation of the left anterior descending (LAD) artery as described previously^(3,25). In brief, mice (8-12 weeks of age, 20-25g) were randomized into four groups: Sham group(Sham); MI group; MI+MSCs group; MI + FNDC5-MSCs group. C57BL/6 mice were anesthetized with isoflurane and mechanically ventilated. The heart was exposed by left thoracotomy. Then, LAD artery was permanently ligated with a 6-0 silk suture. When the anterior wall of the left ventricle (LV) turned pale and characteristic electrocardiographic (ECG) changes were recorded, success of the ligation was confirmed. At last, the chest and skin were sealed, and mice were placed on the ventilator until they woke up. Mice in Sham-operated control group underwent the same operation except that the suture below the left coronary artery was not ligated. Completely randomized design and blinding were adopted in animal experiments.

BM-MSCs transplantation

As described previously^(3,23), BM-MSCs transplantation was performed immediately after MI. In brief, BM-MSCs were collected and randomly divided into the different groups separately. The suspended cells (1×10^6) were injected directly into the peri-infarcted areas [at 2 sites near the peri-infarct zone (medial and lateral zones)] by a Hamilton syringe with a 29-gauge needle (in 20 mice in every group).

***In vivo* evaluation of BM-MSCs engraftment**

BLI was performed to track transplanted BM-MSCs using an IVIS[®] Kinetic system (Caliper, Hopkinton, MA, USA)⁽¹⁾. After intraperitoneal injection with D-luciferin (375 mg/kg body weight), recipient mice were anesthetized with isoflurane and imaged for 10 mins on days 1, 7, 14, 21, 28 until sacrificed. Peak signals (photons/s/cm²/sr) from a fixed region of interest (ROI) were analyzed with Living Image[®] 4.0 software (Caliper, MA, USA). All these assays were performed in a blinded manner.

Histological analysis of fibrosis and angiogenesis

As described previously⁽²⁶⁾, fast green/Sirius red stain was performed to detect fibrosis in cardiac muscle in 4-week post-procedure. Fibrosis was evaluated by measuring the collagen area as a proportion of the total left ventricular area with Imaging Pro Plus software. Meanwhile, the capillary density was

determined by CD31 immunohistochemistry. Vessels in the peri-infarct zone were counted in randomly chosen 5 high-power fields (HPFs, magnification x400). The results are expressed as vessels per HPF.

Echocardiographic measurements

Cardiac function was measured under anesthesia with 2 % isoflurane by transthoracic echocardiography at baseline, 7 days, and weekly until sacrifice at 4-week post-operation with a 30-MHz transducer on a Vevo® 2100 ultrasound system (VisualSonics, CA, USA) ^(23, 25). Briefly, mice were anesthetized (2% isoflurane and oxygen) and put in a supine position. M-mode images and grayscale two-dimensional parasternal short-axis images at the mid-papillary level of each mouse were recorded. Measurements were carried out offline by a single observer in a group-blinded manner. The left ventricular end-systolic diameter (LVESD) and end-diastolic diameter (LVEDD) were measured from M-mode images. Meanwhile, left ventricular end-systolic volume (LVESV) and left ventricular end-diastolic volume (LVEDV) were also measured to calculate left ventricular ejection fraction (LVEF) and fractional shortening (FS) with the following equations: $LVEF = (LVEDV - LVESV)/LVEDV \times 100\%$, $LVFS = (LVEDD - LVESD)/LVEDD \times 100\%$. All the echocardiographic measurements were performed for three times in a blinded manner.

Determination of myocardial apoptosis

Myocardial apoptosis at 48 h after BM-MSCs transplantation was determined by Terminal-deoxynucleotidyl transferase mediated-dUTP nick-end labeling (TUNEL) assay as previously described ⁽²⁷⁾. In brief, apoptotic cell nuclei and 49,6-diamidino-2-phenylindole (DAPI) (Sigma) stained all cell nuclei. Additional staining was performed with a monoclonal antibody against Troponin I (cTnI, Santa Cruz) for the identification of myocardium. Sections were imaged using confocal microscope (Fluo-View-FV1000, Olympus, Japan). The total number of nuclei and the number of TUNEL-positive nuclei were determined in five random fields from the border zone of the infarct in each sample. Meanwhile, the percentage of apoptotic cells was calculated. All these assays were performed in a blinded manner.

Western blot assay

Cells were collected and dissolved in proteinlysis buffer (Sigma). Then, equivalent protein (50 µg/lane) was separated by electrophoresis on 12% SDS-PAGE gels at 120 V for 1.5 h. Furthermore, they were transferred to PVDF membrane by 300 mV electrophoresis for 1.5h. Cellular membranes were subjected to immune-blotting with primary antibodies overnight at the temperature 4 °C after blocked in 5% nonfat dry milk (BD Biosciences) with 1xTBST at room temperature for 1 h. After incubation with appropriate secondary antibodies binding to horseradish peroxidase, an enhanced chemiluminescence system (Amersham Bioscience) was used to visualize blots bands. Also, we determined densitometric analysis of Western blot with VisionWorks LS, version 6.7.1. ⁽²⁸⁾ The following antibodies were used: rabbit anti-mouse FNDC5(1:1000, Cell Signaling Technology), and rabbit anti-mouse β-actin (1:1000, Abcam).

Statistics analysis

Data analysis was performed with Graph Pad Prism 5.0 (San Diego, CA, USA). All quantitative data are expressed as the Mean $\pm$ SEM. Shapiro-Wilk normality test was performed for checking normality of data distribution. Comparisons of parameters among three or more groups were performed with a One-way analysis of variance (ANOVA). Differences between 2 groups were compared by Student's t test. Bonferroni testing was performed to determine the post-hoc testing. P value < 0.05 was considered as statistical significance.

Results

Hypoxia increased apoptosis of BM-MSCs

To analyze BM-MSCs apoptosis induced by hypoxic stress, flow cytometry and caspase-3 activity assays were performed. Annexin V is considered a marker for early stage apoptosis. Early stage apoptotic cells will only take up Annexin V stain but will remain PI negative. The late-stage apoptotic and necrotic cells will be positive for both Annexin V and PI. The representative flow cytometry results shown in **Figure 1a-c** indicated that the percentage of early stage apoptotic cells under hypoxic condition significantly increased than that in normal condition ($p < 0.05$). In addition, compared with normal condition, cleaved caspase-3 was increased in hypoxia for 12 h. Meanwhile, with the increased hypoxia-time exposure, the expression of cleaved caspase-3 was elevated gradually (**Fig. 1d**, $p < 0.05$), which suggested that hypoxia promoted the cleaved caspase-3 expression level. These data suggested that hypoxic stress increased the apoptosis of BM-MSCs.

Hypoxia exposure decreased BM-MSCs viability

Representative *in vitro* BLI indicated a robust linear correlation between the cells number and average Fluc radiance ($r^2 = 0.98$; **Supplemental figure** in the Data Supplement), suggesting that BLI of Fluc could be reliably used to monitor the viability of engrafted MSCs^{Fluc+GFP+} quantitatively *in vivo*. Furthermore, *in vitro* BLI displayed a remarkable decline of BLI signal intensity in MSCs^{Fluc+GFP+} after H/SD injury compared with normoxia (**Figure 1e-f**, $p < 0.05$). Concurrently, MTT results also showed that hypoxia exposure impaired viability of MSCs^{Fluc+GFP+} after H/SD injury (**Fig. 1g**, $p < 0.05$). Collectively, these data suggested that hypoxic stress decreased the viability of BM-MSCs.

Hypoxic stress inhibited growth factors secretions in BM-MSCs

BM-MSCs involved in cardiac repair and regeneration at least in part by paracrine effects. Therefore, we evaluated the effect of hypoxia on cytokine secretion in BM-MSCs. Results demonstrated that H/SD injury suppressed VEGF secretion compared with that in normoxic group (**Fig. 1h**, $p < 0.05$). Also, the ELISA assay results illustrated that the bFGF, IGF-1 and HGF were restrained in BM-MSCs with hypoxic treatment (**Figure 1i-k**, $p < 0.05$). Taken together, these results showed that H/SD injury contributed to paracrine dysfunction of BM-MSCs.

H/SD injury reduced expression of FNDC5 in BM-MSCs

To explore the effect of hypoxic stress on the protein expression of FNDC5, Western blot was performed. The representative results shown in **Figure 2a** indicated that compared with normoxic group, the protein expression level of FNDC5 was decreased in hypoxia exposure group. Meanwhile, semi-quantitative analysis demonstrated that the expression of FNDC5 was decreased in hypoxia for 12 h compared with normoxic group ($p < 0.05$, **Fig. 2b**). At the same time, the expression level of FNDC5 was restrained gradually after hypoxic exposure for 24 h and 48 h, respectively. In a word, these results showed that hypoxic injury suppressed the expression of FNDC5 in BM-MSCs.

To further increase the expression of FNDC5 in hypoxic BM-MSCs, FNDC5 was over-expressed by transfection. Results suggested that expression level of FNDC5 was promoted in FNDC5-MSCs ($p < 0.05$, **Fig. 2c and 2d**).

FNDC5/Irisin ameliorated BM-MSCs apoptosis induced by H/SD injury

Flow cytometry and caspase-3 activity assays were performed for confirming whether FNDC5/Irisin plays a protective role in hypoxia-induced apoptosis in BM-MSCs. The flow cytometry results illustrated that the percentage of early stage apoptotic BM-MSCs under hypoxia for 48 h significantly increased than that in normal condition (**Figure 3a-c**, $p < 0.05$). Also, the expression of cleaved caspase-3 was promoted under hypoxia for 48 h (**Fig. 3d**, $p < 0.05$). These data suggested that hypoxic stress enhanced the apoptosis of BM-MSCs. Interestingly, FNDC5-OV or irisin administration alleviated the increased apoptosis in hypoxia exposure for 48 h (**Fig. 3a-d**, $p < 0.05$).

FNDC5/Irisin alleviated the reduced viability of BM-MSCs induced by hypoxia

The *in vitro* BLI was performed to confirm the effect of FNDC5/Irisin on cells viability. Representative results in **Figure 4a** displayed that the BLI signal intensity in MSCs^{Fluc+GFP+} in FNDC5-OV group and MSCs+irisin group was significantly increased compared with that in H/SD group (**Figure 4b**, $p < 0.05$). Meanwhile, MTT results demonstrated that FNDC5-OV or Irisin treatment promoted the impaired viability of MSCs^{Fluc+GFP+} under hypoxia condition for 48 h (**Fig. 4c**, $p < 0.05$). Collectively, these results suggested that FNDC5 or Irisin played a protective role in viability of BM-MSCs.

FNDC5/Irisin improved paracrine functions of hypoxic BM-MSCs

To investigate the effects of FNDC5/Irisin on growth factors secretions in BM-MSCs, we performed ELISA assays. The representative results in **Figure 4d-e** demonstrated that the secretions of VEGF bFGF, IGF-1 and HGF were increased after FNDC5-OV or Irisin administration compared with that in hypoxia for 48 h ($p < 0.05$). Taken together, these results illustrated that FNDC5/Irisin ameliorated the paracrine dysfunction of BM-MSCs induced by hypoxia.

FNDC5 enhanced the retention and pro-angiogenic effect of engrafted BM-MSCs

Longitudinal BLI was performed for determining the retention of BM-MSCs transplanted into infarcted hearts. Representative BLI results in **Fig. 5a** showed a progressive decay of BLI signal within 4 weeks

after engraftment in BM-MSCs group. By contrast, FNDC5-OV promoted the retention of engrafted MSCs Fluc+GFP⁺ (**Fig. 5b**, $p < 0.05$). Consistently, the number of capillaries in myocardial tissue was evaluated by CD31 staining. The representative immunohistochemistry results demonstrated that the number of capillaries was reduced in MI group and in MI+BM-MSCs group. And there was no statistical difference between MI group and MI+BM-MSCs group. Interestingly, FNDC5-MSCs increased the number of capillaries compared with that of MI+BM-MSCs group (**Fig. 5c and 5d**, $p < 0.05$). Collectively, FNDC5 over-expression improved the retention and pro-angiogenic effect after MSCs engraftment.

FNDC5-MSCs reduced fibrosis and improved cardiac function after MI

To study the effects of FNDC5 on the therapeutic efficiency of transplanted BM-MSCs, sirius red/ fast green staining was performed to evaluate the fibrosis. Furthermore, echocardiography analysis was performed to evaluate the heart function. Representative sirius red/ fast green staining results in **Figure 6a** showed that BM-MSCs decreased cardiac fibrosis after MI. However, there was no significant difference between MI group and MI+BM-MSCs group statistically. Interestingly, FNDC5-MSCs significantly reduced myocardial fibrosis compared with MI and MI+BM-MSCs group (**Fig. 6a and b**, $p < 0.05$). In addition, echocardiographic analysis revealed that the baseline parameters were similar in all groups. However, the left ventricle (LV) dimensions (LVEDD and LVESD) were increased after MI. Meanwhile, the LV dimensions were decreased in the FNDC5-MSCs group compared with the MI and MI+BM-MSC groups (**Fig. 96c-d**, $p < 0.05$). Moreover, transplantation of BM-MSCs also manifested a trend towards improvement of cardiac performance over the 4 weeks after MI. Interestingly, the apparent benefit of BM-MSCs transplantation was significantly promoted by FNDC5-MSCs (**Figure 6e and 9f**, $p < 0.05$). Taken together, these data suggested that the therapeutic benefits of BM-MSC transplantation after MI is enhanced by FNDC5.

FNDC5-MSCs decreased cardiomyocytes apoptosis after MI

TUNEL assay was performed to confirm the protective effect of FNDC5-MSCs on the apoptosis of cardiomyocytes after MI. As shown by representative photomicrograph (**Figure 7a**), apoptotic cardiomyocytes which were manifested by TUNEL positivity (in green), were more frequently observed in the MI group than that in the sham group ($41.70 \pm 0.69\%$ versus $10.97 \pm 0.58\%$; $p < 0.05$, **Figure 7b**). Furthermore, the apoptosis positive cells were remarkably reduced in the MI+ FNDC5-MSCs group, compared with that in MI and MI+BM-MSCs group, ($41.70 \pm 0.69\%$ vs. $21.47 \pm 0.78\%$; $36.67 \pm 0.66\%$ vs. $21.47 \pm 0.78\%$, $p < 0.05$, **Figure 7b**). Taken together, our data indicated that FNDC5-MSCs decreased apoptosis cardiomyocytes after MI.

Discussion

In the present study, our data indicated that H/SD injury inhibited the protein expression levels of FNDC5 in BM-MSCs. Meanwhile, hypoxic stress involved in BM-MSCs paracrine dysfunction, increased cells apoptosis and viability. Furthermore, FNDC5 over-expression and irisin attenuated the BM-MSCs injury

induced by hypoxia exposure. Interestingly, FNDC5 over-expression elevated the survival of BM-MSCs after transplantation. Furthermore, FNDC5-MSCs transplantation decreased the apoptosis and fibrosis of infarcted myocardium, increased capillary density and finally improved cardiac function. Collectively, our results suggested that FNDC5/irisin may be a proposed optimized strategy of BM-MSCs therapy for MI (Figure 8).

MSCs therapy for MI have achieved a promising stage, but studies also demonstrated that only marginal improvements in cardiac function were observed after the engraftment of MSCs into infarcted heart tissue^(26, 29). The stem cells therapy for clinical application is limited, mainly as a result of the reduced viability of implanted MSCs. Our previous study has revealed a high level of MSCs apoptosis between day 3 and 7 after implantation⁽²³⁾. In the present study, we tracked the transplanted BM-MSCs by BLI and also found similar acute cell death within 1 week after transplantation. Moreover, engraftment with BM-MSCs alone failed to prevent cardiomyocytes death and improve cardiac dysfunction significantly, which was consistent with the results of *Zhang et al.*^(26, 30) Lacks of nutrients and oxygen in ischemic myocardium has been considered as the major factors contributed to the decreased the viability of MSCs after transplantation⁽³¹⁾. Therefore, H/SD *in vitro* cellular model was performed to mimic the *in vivo* ischemic environment⁽³²⁾. Concurrently, our results revealed that H/SD injury reduced cells survival and caused paracrine dysfunction of BM-MSCs⁽¹²⁾. Thus, promoting the low viability of BM-MSCs under hypoxic conditions after transplantation is crucial for elevating the efficiency of cellular therapy.

FNDC5 was recognized as a transmembrane protein with a fibronectin III domain and a carboxy-terminal domain located in the cytoplasm⁽³³⁾. Irisin is the extracellular domain of FNDC5, which is cleaved by enzyme^(14, 34). Meanwhile, irisin has been proved as a multifunctional peptide that could bind to an unidentified receptor to play various effects^(17, 18). *Fatouros et al.*⁽¹⁵⁾ found that irisin was secreted by skeletal muscles. However, *Yu Q. et al.*⁽¹⁶⁾ indicated that the expression of irisin also increased in the heart of hypertrophic mice. They found that angiotensin II-induced myocardial cells hypertrophy was attenuated after administration of irisin *in vitro*, suggesting irisin also could be derived from heart and had a cardioprotective effect on cardiomyocytes dysfunction. In the current study, we found for the first time that hypoxia exposure decreased the protein expression level of FNDC5 in BM-MSCs. Moreover, we also found that irisin/FNDC5 improved survivals of BM-MSCs under hypoxic condition. Recently, genetic modification has attracted great attention as a potential approach to improve the low viability of cells^(8, 35-37). For this reason, we up-regulated the FNDC5 expression in BM-MSCs (FNDC5-MSCs), and transplanted FNDC5-MSCs into infarcted heart. Interestingly, our BLI results suggested the FNDC5 overexpression enhanced the functional survival of BM-MSCs. Furthermore, FNDC5-MSCs significantly reduced fibrosis and apoptosis of cardiomyocytes, with improved cardiac function after MI. Taken together, these date suggested that FNDC5/irisin may be a potential optimizing target for BM-MSCs based cellular therapy for MI.

Although present study bears some clinical relevance, there are several limitations. Firstly, the *in vitro* H/SD cellular model cannot fully mimic the *in vivo* ischemic environment. Secondly, the number of

animal experimental samples was small. Finally, the detailed molecular mechanism by which the protective effect of FNDC5/irisin was not clarified completely. Therefore, research on specific mechanisms warrants further dedicated investigation.

Conclusions

Collectively, our *in vitro* and *in vivo* data embodied the FNDC5/irisin ameliorated hypoxia injury of BM-MSCs. Furthermore, gene modified with FNDC5 over-expression increased the survival of BM-MSCs after engraftment and improved the cardiac functions after MI, suggesting that FNDC5/irisin may be a potential optimizing target for BM-MSCs based cellular therapy for MI.

Abbreviations

FNDC5: Fibronectin type III domain-containing protein 5; BM-MSCs: Bone marrow mesenchymal stem cells; MI: Myocardial infarction; H/SD: Hypoxia and serum deprivation; Fluc: Firefly luciferase; eGFP: enhanced green fluorescence protein; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethyl sulphoxide; OD: Optical density; BLI: Bioluminescence imaging; ELISA: Enzyme-linked immunosorbent assay; VEGF: Vascular endothelial growth factor; bFGF: basic fibroblast growth factor; IGF: Insulin-like growth factor; HGF: Hepatocyte growth factor; LAD: Left anterior descending; LV: Left ventricle; ECG: Electrocardiography; ROI: Region of interest; HPFs: High-power fields; LVESD: Left ventricular end-systolic diameter; LVEDD: Left ventricular end-diastolic diameter; LVESV: Left ventricular end-systolic volume; LVEDV: Left ventricular end-diastolic volume; LVEF: Left ventricular ejection fraction; FS: Fractional shortening; TUNEL: Terminal-deoxynucleotidyl transferase mediated-dUTP nick-end labeling; DAPI: 4,6-diamidino-2-phenylindole

Declarations

Funding

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Availability of data and materials

The data sets supporting the results of this article are included within the article and its additional files.

Authors' contributions

JYD, NZ, ZZ and FC designed the study, drafted the manuscript, and approved its final version. ZZ, JYD and FC acquired data and approved the final version. YC, CX, JMZ and ZTJ revised the article's intellectual content. ZZ and FC are responsible for the integrity of this work. All authors read and approved the final manuscript.

Ethics approval

All procedures were performed in accordance with the institutional guidelines for animal research and were approved by the Animal Care and Use Committee of PLA Rocket Force Characteristic Medical Center.

Consent for publication

Not applicable.

Availability of data and materials

The data sets supporting the results of this article are included within the article and its additional files.

Competing interests

All the authors declare that they have no competing interests.

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Supplemental Figure Legend

Supplemental figure Representative *in vivo* BLI showed a linear relationship between cells number and Fluc reporter gene activity.

Figures

Figure 1

Hypoxia exposure increased apoptosis, reduced the viability and restrained paracrine mechanism of BM-MSCs. a Representative results of the flow cytometry analyses in BM-MSCs under hypoxia for 12h, 24h and 48h. Viable cells: Annexin V-/PI-; Early apoptosis: Annexin V+/PI-; late apoptosis: V+/PI+; Necrotic: V-/PI+. (Scale bars: 20 μ m). b-c Quantification of the apoptotic BM-MSCs. d Histogram illustrated the caspase-3 enzymatic activity in MSCs Fluc+GFP+ after H/SD injury. e In vitro BLI results of BM-MSCs

Fluc+GFP+ under normal conditions and after HS/D injury for 12 h, 24 h and 48 h. f Representative the quantification of BLI assays. g MTT assay indicated the effects of hypoxia (0 h, 12 h, 24 h and 48 h) on viability of MSCs Fluc+GFP+. Representative ELISA assay illustrated the levels of vascular endothelial growth factor (VEGF) (h), basic fibroblast growth factor (bFGF) (i), insulin-like growth factor-1 (IGF-1) (j), and hepatocyte growth factor (HGF) (k) within MSCs under normal conditions and hypoxia for 12 h, 24 h and 48 h. Data are expressed as the means $\pm$ SEM; n = 5; *p < 0.05

Figure 2

Hypoxia restrained the expression of FND5 of BM-MSCs. a and c Representative Western blot results in BM-MSCs. b and d Semi-quantification of FND5 expression. Data are expressed as the mean $\pm$ SEM; n = 5; *p < 0.05

Figure 3

FNDC5/Irisin reduced increased apoptosis of BM-MSCs with hypoxia for 48 h. a Results of the flow cytometry analyses in BM-MSCs under normoxia, hypoxia for 48h, H/SD+FNDC5-MSCs and HS/D+Irisin (Scale bars: 20µm). b-c Representative quantification of the apoptotic BM-MSCs under different treatments. d Histogram illustrated the caspase-3 enzymatic activity in BM-MSCs after various treatments. Data are expressed as the means $\pm$ SEM; n = 5; *p < 0.05

Figure 4

FDNC5 OV/Irisin improved the viability and ameliorated paracrine dysfunction of BM-MSCs under hypoxia for 48 h. a Representative in vitro BLI results of MSCs Fluc+GFP+ with normoxia, hypoxia for 48h, H/SD+FnDC5-MSCs and HS/D+Irisin. b Representative the quantification of BLI assays in different groups. c MTT assay demonstrated the effects of FDNC5 OV/Irisin on viability of MSCs Fluc+GFP+.

Representative ELISA assay illustrated the levels of VEGF (d), bFGF (e), IGF-1 (f), and HGF (g) in BM-MSCs under various groups. Data are expressed as the mean $\pm$ SEM; n = 5; *p < 0.05

Figure 5

FND5 promoted the retention and pro-angiogenic effect of engrafted BM-MSCs. a Longitudinal BLI spatiotemporally tracked MSCs Fluc+GFP+ survival in MI with preconditioned mesenchymal stem cells (MPCMSCs) (top row, n = 10) and FND5-MSCs preconditioned MSCs (MFPCMSCs) (second row, n = 10). Color scale bar values are in photons/s/cm²/sr. b Representative quantitative analysis of Firefly luciferase (Fluc) optical signals on fixed regions of interest (ROI). c Capillaries in the infarct border zone were determined by immunohistochemical staining for CD31-positive cells in different groups (Scale bars= 50 μ m). d Representative capillaries in the infarct border zone. Data are expressed as means $\pm$ SEM; n = 5; *p < 0.05

Figure 6

Evaluation of fibrosis and heart function after different groups. a Masson's trichrome staining indicated left ventricular fibrosis 4 weeks after various treatments (magnification 4x). b Representative quantitative analysis of the fibrotic area. Histograms illustrating heart function parameters: left ventricular end-diastolic diameter (LVEDd, c), left ventricular end-systolic diameter (LVESd, d), left ventricular fractional

shortening (e) and left ventricular ejection fraction (f). Data are expressed as means $\pm$ SEM; n = 5; *p < 0.05

Figure 7

Effects of FNDC5-MSCs on cardiomyocytes apoptosis. a Confocal microscopy showed that TUNEL-positive cardiomyocytes were more frequently observed in the MI group compared with the MI+FNDC5-MSCs group. Apoptotic nuclei were identified as TUNEL positive (green fluorescent) and total nuclei by

DAPI counterstaining (blue fluorescent). Myocardium was stained using a monoclonal antibody against cTnI (red fluorescent). Scale bar represents 20 μm . b Representative TUNEL-positive apoptotic cells. Data are expressed as means $\pm$ SEM; n = 5; *p < 0.05

Figure 8

Proposed hypothesis of FDNC5/Irisin improving the therapeutic efficacy of BM-MSCs for MI. FDNC5/Irisin may protect against promoted apoptosis and paracrine dysfunction of BM-MSCs induced by hypoxia. Furthermore, FDNC5/Irisin may contribute to enhance the low survival of engrafted BM-MSCs and ameliorate cardiac dysfunction after MI.

Supplementary Files

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