EBioMedicine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

By Targeting *Atg7* MicroRNA-143 Mediates Oxidative Stress-Induced Autophagy of c-Kit$^+$ Mouse Cardiac Progenitor Cells

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ARTICLE INFO

Article history:
Received 19 March 2018
Received in revised form 1 May 2018
Accepted 16 May 2018
Available online xxxx

Keywords:
Autophagy
Atg7
Cardiac progenitor cells
MicroRNAs
MI

ABSTRACT

Therapeutic efficiency of cardiac progenitor cells (CPCs) transplantation is limited by its low survival and retention in infarcted myocardium. Autophagy plays a critical role in regulating cell death and apoptosis, but the role of microRNAs (miRNAs) in oxidative stress-induced autophagy of CPCs remains unclear. This study aimed to explore if miRNAs mediate autophagy of c-kit$^+$ CPCs. We found that the silencing of miR-143 promoted the autophagy of c-kit$^+$ CPCs in response to H_2O_2, and the protective effect of miR-143 inhibitor was abrogated by autophagy inhibitor 3-methyladenine (3-MA). Furthermore, autophagy-related gene 7 (*Atg7*) was identified as the target gene of miR-143 by dual luciferase reporter assays. In vivo, after transfection with miR-143 inhibitor, c-kit$^+$ CPCs from green fluorescent protein transgenic mice were more observed in infarcted mouse hearts. Moreover, transplantation of c-kit$^+$ CPCs with miR-143 inhibitor improved cardiac function after myocardial infarction. Take together, our study demonstrated that miR-143 mediates oxidative stress-induced autophagy to enhance the survival of c-kit$^+$ CPCs by targeting *Atg7*, which will provide a complementary approach for improving CPC-based heart repair.

1. Introduction

Ischemic heart disease (IHD) is a leading health problem with high morbidity and mortality. The limited regenerative capacity of adult hearts can't compensate for the lost cardiomyocytes after injury such as myocardial infarction (MI). The enormous loss of cardiomyocytes leads to compromised cardiac function or even heart failure [24]. Recently, cardiac progenitor cells (CPCs) are emerging as a particularly promising approach for heart disease therapy. Lots of reports have demonstrated that CPCs could improve cardiac functions by differentiating into cardiomyocytes and vascular cells, as well as paracrine action [5,18,21]. c-kit$^+$ CPCs transplantation has been demonstrated to alleviate left ventricular (LV) dysfunction in acute and chronic MI to improve the quality of life in animal model [4]. However, the main challenge that CPCs transplantation faced after transplantation is that >90% of transplanted cells will undergo apoptosis, and only <10% CPCs survive in infracted hearts [19].

Oxidative stress is the main reason for the death of c-kit$^+$ CPCs after transplantation. The transplanted c-kit$^+$ CPCs suffer from cell cycle arrest, apoptosis and senescence in response to oxidative stress [1]. Among these, the most predominant form of stem cell death is apoptosis which has been suggested as the key regulatory target for improving the survival of stem cells. Thus, the suppression of CPCs apoptosis will promote the repairment of damaged myocardium after MI. For example, apurinic/apyrimidinic endonuclease/redox factor 1 overexpression inhibited CPCs apoptosis by activating TAK1 and NF-κB signal to promote stem cells survival and improve cardiac functions [2].

Autophagy is a stress adaptation that eliminates aged or damaged cellular components through mechanisms including lysosome-associated digestion. Recent studies showed that autophagy plays an important role in determining the viability and apoptosis of stem cells under oxidative stress. It was found that autophagy induced by H_2O_2 confers stem cells to overcome the apoptosis and enhances its resistance under oxidant stress. Suppression of autophagy exacerbated the apoptosis and reduced the viability of stem cells, and on the contrary,

Abbreviations: *Atg7*, autophagy-related gene 7; CPCs, cardiac progenitor cells; GFP, green fluorescent protein; LV, left ventricular; MI, myocardial infarction; 3-MA, 3-methyladenine; mRFP, monomeric red fluorescent protein; SQSTM1, sequestosome 1; IHD, ischemic heart disease; LVIDd, left ventricular internal diastolic diameter; LVIDs, left ventricular internal dimension at end-systole; LV vol d, left ventricular end diastolic volume; LV vol s, left ventricular end-systolic volume; EF, ejection fraction; FS, fractional shortening; HE, hematoxylin and eosin.

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https://doi.org/10.1016/j.ebiom.2018.05.021

promoting autophagy may enhance the resistance of stem cells to oxidative stress [27].

MiRNAs play important roles in regulating many cellular events, including cell proliferation, differentiation and apoptosis. For example, miR-17–92 cluster has been shown to induce proliferation of cardiomyocytes [9]. It also has been reported that loss of miR-128 promoted cardiomyocytes proliferation and heart regeneration [14]. MiR-16 and miR-26 overexpression inhibited endothelial cell function [15,20]. MiR-210 inhibited antiangiogenic factors to induce angiogenesis [13]. In addition, miR-199a-3p regulated p53 by targeting CABLES1 in mouse cardiac c-kit$^+$ cells to promote proliferation and inhibit apoptosis through a negative feedback loop [17]. It has been reported that miRNAs are involved in autophagy in some cells including miR-106b, miR-124, miR-143, miR-155 and miR-375, etc. [8,10,12,16,23]. However, the role of miRNAs in the autophagy and apoptosis of CPCs under oxidative stress remains unclear. In this study, we aim to investigate whether miR-143 mediates the autophagy to enhance cellular survival and inhibit apoptosis of transplanted CPCs, and thus improve transplantation efficiency and restore cardiac function after MI.

2. Materials and Methods

2.1. Experimental Animals

The neonatal (1–3 days) mice and 6–8 weeks old male C57BL/6 mice (20–25 g) were purchased from the Experimental Animal Center of The Affiliated Second Hospital of Harbin Medical University (Harbin, China). β-actin driven enhanced green fluorescent protein transgenic mice were purchased from Cyagen (Suzhou, China). The mice were housed in SPF condition. Environmental conditions were a temperature of 21 °C ± 2 °C, humidity of 55% ± 10%. Food and water were freely available throughout the experiments. During housing, animals were monitored twice daily for health status. All mice were randomly assigned to receive different treatments, and the experimenters were blind to treatment condition in this study. All groups were each conducted using 6 animals. All experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Harbin Medical University. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Isolation and Culture of c-Kit$^+$ CPCs

The CPCs were isolated and cultured as described in previous literatures [6,18]. In brief, the hearts from neonatal (1–3 days) mice were minced into 1–2 mm^3 pieces and then incubated for 5 min at 37 °C in 0.25% trypsin (Beyotime) and 0.1% collagenase II (GIBCO, Milan, Italy) alternately for three times. The remaining tissues were collected and cultured in high glucose medium containing 10% fetal bovine serum, 100 U/mL penicillin G and 100 μg/mL streptomycin at 37 °C. Cell generation was performed by 0.25% trypsin, and the third passage of CPCs was used in this study.

2.3. Immunofluorescence

CPCs were washed with PBS, and then fixed with 4% paraformaldehyde for 15 min at 37 °C. Cells were blocked in Normal Goat Serum for 30 min at 37 °C after permeabilized with 0.5% Triton X-100 in PBS for 90 min at room temperature, and then CPCs were incubated with primary antibody LC3 (Sigma-Aldrich Cat# L7543, RRID:AB_796155) overnight. The CPCs were incubated with secondary antibody (EarthOx, San Francisco, CA, USA) and DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to counterstain the nucleus. The experimenters were blind to group assignment.

2.4. TdT Mediated dUTP Nick End Labelling (TUNEL)

The assay was performed according to the manufacturer's instructions. In brief, the cells were fixed with 4% paraformaldehyde for 15 min at 37 °C, then washed with PBS for three times. Blocking buffer (3% H_2O_2 in CH_3OH) was added to the wells. The cells were incubated in permeabilizing solution (0.1% Triton in 0.1% sodium citrate) after washed with PBS, then added 50uL viaL 1 and 450uL viaL 2 (Roche) and incubated for 1 h at 30 °C. Nucleus counterstained with DAPI for 15 min at room temperature. All participants were blind to treatment assignment.

2.5. Quantitative Real-Time PCR

Total RNA was extracted from CPCs using TRIzol reagent. PCR was performed using GreenER Two-Step qRT-PCR Kit Universal (Invitrogen). The gene-specific primers sequence for miR-143, forward: GCGGCGGG GTGCAGTGCTGCATC, reverse: ATCCAGTGCAGGGTCCGAGG. RT-Primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAGAG. The GAPDH was used as control. The experimenters were blind to group assignment.

2.6. Transfection

Diluted inhibitor-NC, miR-143 inhibitor, mimics-NC or miR-143 mimics (Genep-harma, Shanghai, China) were mixed with diluted Opti-MEM® I Reduced Serum Medium (Gibco, New York, NY, USA) and then stand for 20 min. The mixture was used to transfection. MiR-143 mimics: 5′ UGAGAUGAAGCACUGUAGCUC 3′, 5′ GCUACAGUGCUU CAUCUCAUU 3′. MiR-143 inhibitor: GAGCUACAGUGCUUCAUCUCA. ATG7 siRNA: GCUAGAGACGUGACACAUATT, UAUGUGUCACGUCUCU AGCTT.

2.7. Live/Dead Cell Staining

Live/Dead Cell Staining Kit for CPCs survival rate was performed. LIVE/DEAD® fixable dead cell stain was diluted by 50 μL DMSO, and then PBS was used to wash the cells after incubated with diluted stain for 30 min. The experimenters were blind to treatment condition.

2.8. EdU Incorporation Assay

Cell-Light EdU Apollo567 in Vitro Kit (RIBOBIO) was used to detect the proliferation rate according to the manufacturers' instructions. In brief, CPCs were incubated with 5-Ethynyl-2′-deoxyuridine (EdU) for 2 h at 37 °C. Cells were fixed with 4% paraformaldehyde for 15 min at 37 °C. Then Apollo Staining reaction liquid was added into the wells to detect the positive cell. Nucleus counter stained with DAPI for 15 min at room temperature. The experimenters were blind to treatment condition.

2.9. Western Blot

CPCs were split to extract total protein with RIPA lysis Buffer (Thermo Scientific Pierce). BCA assay (Thermo Fisher Scientific) was used to determine the concentration. Proteins were separated by SDS-PAGE, and then transferred from the gel to the Pure Nitrocellulose Blotting membrane (Millipore, Bedford, MA, USA). The membrane was incubated with appropriate primary antibodies at 4 °C overnight and then incubated with secondary antibodies for 1 h at room temperature. The primary antibodies used in this study are as following: LC3 (Sigma-Aldrich Cat# L7543, RRID:AB_796155), C-CASPASE-3 (Cell Signaling Technology Cat# 9654S, RRID:AB_10694088), β-actin (ZSGB-Bio Cat# TA-09, RRID:AB_2636897), SQSTM1 (Cell Signaling Technology Cat# 5114, RRID:AB_10624872), ATG7 (Cell Signaling Technology Cat# 8558, RRID:AB_10831194).

Please cite this article as: Ma W, et al, By Targeting *Atg7* MicroRNA-143 Mediates Oxidative Stress-Induced Autophagy of c-Kit+ Mouse Cardiac Progenitor Cells, EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.05.021