IFN- γ Enhances the Efficacy of Exosomes Derived from Mesenchymal Stem Cells in Myocardial Infarction Rats via miR-21 Stimulated by STAT1

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Abstract

Background: Mesenchymal stem cells (MSCs) activated with IFN- γ elicit more powerful physical effects. Exosomes (Exos) secreted from MSCs have protective against myocardial injury. The aim of this study was to investigate whether Exos derived from IFN- γ -pretreated MSCs exhibit more potent cardioprotective function and the underlying mechanisms.

Methods: Exos were isolated from MSCs (Ctrl-Exo) and IFN- γ -primed MSCs (IFN- γ -Exo) and were then delivered to H9c2 cells or human umbilical vein endothelial cells (HUVECs) in vitro under oxygen and glucose deprivation (OGD) condition or in vivo in an infarcted rat heart. RNA sequencing was to identify the different expressed functional transcription factor (TF). Quantitative reverse transcription-PCR (qPCR) was to confirm the upregulated TF and miRNA in IFN- γ -primed MSCs. Dual-luciferase reporter gene assay were to analyze the transcriptional regulation of miRNAs by STAT1. The target of miR-21-5p (miR-21) was disclosed by luciferase reporter assays and qPCR. The function of BTG2 was verified in vitro under OGD condition.

Result: IFN- γ -Exo accelerated migration, tube-like structure formation, and prevented H9c2 from OGD-induced apoptosis. Similarly, IFN- γ -Exo led to further reduction in fibrosis size, reduced cardiomyocyte apoptosis and improved cardiac function compared to Ctrl-Exo. miR-21 was significantly upregulated in both IFN- γ -primed MSCs and IFN- γ -Exo. STAT1 transcriptionally induced miR-21 expression. Up-regulated miR-21 can inhibit the expression of BTG2. BTG2 promoted H9c2 cells apoptosis and reversed the protective effect of miR-21 under OGD environment.

Conclusion: IFN- γ -Exo have enhanced therapeutic efficacy against acute MI possibly through promoting angiogenesis and anti-apoptotic effect through increasing the level of miR-21, which directly targeted on BTG2.

Introduction

IFN- γ produced by activated T cells and natural killer (NK) cells plays physiologically key role in maintaining innate and adaptive immunity responses.¹ As a cytokine that can promote immunomodulation, IFN- γ has been widely studied for its excellent anticancer activity.² After interacting with the recipient cells through IFN- γ receptor (IFNGR), IFN- γ subsequently activates downstream signal transduction pathways and transcriptionally stimulates the expression of various genes involved in immune regulation and other biological activities.³ Studies have illustrated that MSCs activated with IFN- γ elicit more powerful immunomodulatory effects by the upregulation of immunoactive factors.^{4, 5}

MSCs are stromal cells with self-renewal ability and multi-differentiation ability,⁶ and have been used for cardiovascular diseases as regenerative therapy.⁷ Recent studies have shown that the therapeutic effects of MSCs are mainly derived from the paracrine mediated by Exos.⁸ The Exos are 50-200 nm vesicles secreted into the extracellular space, and shuttle a variety of microRNA (miRNA), long noncoding RNA

(lncRNA) and proteins to perform the function of cell-cell communication.⁹ Previous studies have discovered that MSC-derived Exos had the function of treating ischemic heart disease by inhibiting cell apoptosis, promoting angiogenesis and regulating macrophage polarization.^{10, 11}

Subsequently, relevant studies suggest that Exos derived from modified MSCs exhibit more powerful protective efficacy. Wang et al. reveal that compared with normoxic condition, Exos derived from hypoxic condition exert better ability to inhibit cell death through miR-125b.¹² Adiponectin stimulates the release of Exos to enhance the treatment of heart failure in mice driven by MSCs.¹³ Recently, our studies prove that hypoxia induction and macrophage migration inhibitory (MIF) modification can strengthen the therapeutic capacity of MSC-derived exosomes on myocardial infarction (MI) through inhibiting apoptosis and promoting angiogenesis.¹⁴ However, whether Exos derived from MSCs stimulated by IFN- γ perform more efficient protective effects against MI than Ctrl-Exo remains unknown.

Therefore, Ctrl-Exo and IFN- γ -Exo were extracted to treat both H9c2 and HUVECs cells under OGD condition and rat MI models to explore their therapeutic effects and potential mechanisms. On this basis, we found that IFN- γ treatment can heighten the anti-apoptotic ability and angiogenesis of Exos derived from MSCs to better preserve heart function. This progress was partly achieved by the increased expression of cardioprotective miRNA-21 through IFN- γ treatment. This Exo-mediated delivery of miRNAs from MSCs under various treatment conditions might be an effective alternative for MI treatment.

Materials And Methods

Ethics statements

This study protocol conforms to the Guide for the Care and Use of Laboratory Animals [National Institutes of Health, (NIH) Bethesda, MD, USA] and was approved by the Institutional Animal Care and Use Committee of the Nanjing Medical University for Laboratory Animal Medicine (IACUC-2005043).

Cells Culture

Human umbilical MSCs (purchased from the Clinical Center of Reproductive Medicine of Nanjing Medical University) were cultured in α -minimal essential medium (α -MEM) containing 10% fetal bovine serum (FBS). MSCs were exposed to control or 50 ng/ml IFN- γ (300-02, Peprotech, USA) conditions for 48 hours. H9c2 and human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS. All media and reagents used for cell culture were purchased from Gibco (Carlsbad, USA). For normal culture, cells were incubated at 37°C, 21% O₂, and 5% CO₂. Under oxygen and glucose deprivation conditions, cells were cultured under 1% O₂, 5% CO₂, 94% N₂ and serum deprivation conditions.

Exosomes Extraction and Characterization

MSCs (1×10^6) were cultured to 70% confluence and then treated with 6ml of Exosome-free FBS (Gibco) for 2d. The cell culture medium (6ml) was collected in a 15ml centrifuge tube and centrifuged at $1,500 \times g$ for 30 min to remove the cells and debris. Transferred the processed supernatant to another 15ml centrifuge tube containing 2ml RiboTM exosome extraction reagent (for cell culture medium, C10130-2, Ribobio, China). These mixtures were incubated overnight at 4°C , and then centrifuged at $2,000 \times g$ for 30 min. The supernatant was discarded, and the exosomal pellet was resuspended in 100 μl PBS.

The exosomal surface markers were detected using western blotting with anti-TSG101, CD63, and CD81 antibodies (Abcam, UK). The external electron microscope (TEM) was utilized to observe the appearance of Exos. The Exos were fixed with 1% glutaraldehyde, and then coated with 1% phosphotungstic acid on a copper mesh. JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) was used to observe the sample. Nanoparticle tracking analysis (NTA) was applied to analyze the size and distribution of Exos. We recorded and tracked the Brownian motion of Exos in PBS (Carlsbad, California). The particle size distribution data was obtained with Stoke-U.S. A ZetaView PMX 110 system (Particle Metrix, Germany) was used for NTA.

Exosomes uptake assay

In order to demonstrate the uptake of Exos by H9c2 cells and HUVEC cells, Exos were labeled with Dil (red fluorescent dye, C1036, Beyotime, China) and co-cultured with recipient cells at 37°C for 6h or 24h and then washed with PBS and fix with 4% paraformaldehyde for 20 min. The nuclei were stained with 6-diamino-2-phenylindole (DAPI) (0.5g/ml, Beyotime) for 10 min, and observed with a confocal microscope.

Apoptosis assays

Flow cytometry (KeyGEN Biotech, China) detected cell apoptosis. H9c2 cells were seeded in 1×10^5 /6-well tissue culture plates overnight, and treated with different Exos or PBS before OGD. The cells were washed with PBS and stained with Annexin V fluorescein isothiocyanate and propidium iodide apoptosis kit (KeyGen Biotech, China). Flowjo Software version 10.0 (Tree Star, USA) was used to analyze apoptotic cells. TdT-mediated dUTP Gap End Labeling (TUNEL) Apoptosis Detection Kit (Roche, USA) was also used to detect cell or tissue apoptosis according to the manufacturer's instructions. The formula for calculating the percentage of apoptotic nuclei was the total number of TUNEL stained nuclei divided by the total number of TUNEL positive nuclei.

Migration Assay

Cultured HUVECs in a 6-well plate, scraped the confluent layer with the tip of a P200 pipette. Then washed and incubated the cells after adding 100 µg/well of different Exos. Images were taken before and 6h, 12h after incubation, and Image J software (NIH) was used to determine the reduction ratio of the scratch area.

Tube Formation Assay

HUVECs were treated with PBS or different Exos. Then cells were washed with PBS and seeded (30,000 cells/well) in 96 well plates coated with growth factor reduced Matrigel (Corning, United States). After 6h, capillary-like tube formation was observed and photographed. Tube length and number of branches were analyzed with Image J software (NIH).

Quantitative real-time PCR (qRT-PCR)

The total cellular and exosomal RNA was extracted using Trizol reagent (Life Technologies, USA) according to the manufacturer's instructions.¹⁴ A stem-loop-specific primer method was used to measure miR-21-5p expression, as described previously.²⁰ The sequences of primers used in the study were shown in Table S1. The relative expression was calculated using the following equation: relative gene expression = $2^{-(\Delta C_{t\text{sample}} - \Delta C_{t\text{control}})}$. All samples were measured in triplicate.

Transfection experiment

Transfection of miR-21-5p mimics (50 nmol/L) and negative control miRNA (50 -100 nmol/L) synthesized by Guangzhou Ribobio were carried out into H9c2 cells using riboFECTTM CP Reagent (Ribobio, China) according to the instructions of manufacturer. The full-length BTG2 sequence and empty vector as negative control were inserted into a pcDNA 3.1 plasmid (GenePharma, China) to transfect H9c2 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer instructions. STAT1 siRNA and Negative control FAM manufactured by Suzhou GenePharma were delivered into MSCs by Lipofectamine 2000. qRT-PCR were performed to determine transfection efficacy. At 48h after transfection, different groups of cells were harvested for further study.

Western blot

Protein extraction and Western blot (WB) analysis were performed as previously stated.¹⁴ Briefly, cells were washed with PBS and lysed with lysis buffer on ice for 20 minutes. The total cell protein concentration was detected by the BCA Protein Assay Kit. The total protein (20µg) was separated using SDS-PAGE (Invitrogen) and transferred to a PVDF membrane (Roche). The membrane was blocked with 5% bovine serum albumin (0.1%) in TBS-Tween and incubated against the desired antibody. The primary

antibodies used for Western blot analysis are listed in Table S2. Bands were visualized using enhanced chemiluminescence reagents and analyzed using a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst version 1.1).

MI Model, Histological Analysis, and Immunofluorescence Staining

Eight-week-old male Sprague-Dawley (SD) rats obtained from the experimental animal center of Nanjing Medical University were randomly divided into 4 groups: sham operation group (Sham group, n=6), PBS injection group (PBS group, n=6), Ctrl-Exo injection group (Ctrl-Exo group, n=6), and IFN- γ -Exo injection group (IFN- γ -Exo group, n=6). Briefly, as previously described¹⁴, left anterior descending artery (LAD) was ligated, and Exos (50 μ L, 1 μ g/ μ L) or PBS was injected around the infarct area in rats. All surgeries and subsequent analyses were blinded for intervention. Echocardiography (Vevo 3100) was performed to determine the left ventricular ejection fraction (LVEF) and left ventricular short axis shortening rate (LVFS) after 2w and 4w.

The rats were sacrificed 4w after surgery. Inflammatory cell infiltration and cell arrangement was evaluated by Hematoxylin-Eosin (HE) staining. Masson's trichrome staining was used to evaluate fibrosis and collagen area after MI, CD31 immunofluorescence staining was to observe the distribution of microvessels. Apoptosis was detected by TUNEL staining (Roche, USA). The primary antibody was anti-CD31 (ab7388; British abcam). DAPI was used for nuclear counterstaining. The images were further analyzed using a fluorescence microscope (Zeiss, Germany) and Image J software (NIH).

Statistical Analysis

Continuous variables and categorical variables were described as mean $\pm$ SD and percentages, respectively. Independent-Sample T-test was used to compare continuous variables between the two groups. One way Analysis of variance (ANOVA) followed by Tukey's correction was used for comparison of three or more groups. All statistical tests were performed using GraphPad Prism software version 8.0, and $p < 0.05$ was considered statistically significant.

Results

Characterization of control and IFN- γ -primed Exos

Exosomes were isolated from the control MSCs and 50 ng/ml IFN- γ -primed MSCs supernatant and identified the appearance using TEM. The pictures showed that the Ctrl-Exo and IFN- γ -Exo were typical lipid bilayer membrane encapsulated nanoparticles with a diameter between 30 and 150 nm (Fig. 1A). The marker proteins TSG101, CD81, and CD63 were positively expressed in two groups (Fig. 1B). Fig. 1C showed that the peak diameter of Exo were 123.4 nm and 116.2 nm in Ctrl-Exo and IFN- γ -Exo group,

respectively. NTA analysis showed that IFN- γ treatment can increase the number of Exos secreted by MSCs. Similarly, IFN- γ increased the protein concentration in the Exo suspension observed by western blot.

The labeled Exos with Dil dye were co-cultured with HUVEC cells and H9c2 cells for 6h and 24h respectively. Confocal microscopy showed that Dil-labeled Exos can be observed around the nucleus within 6h, and most of the Exos were absorbed within 24h (Fig. 1D, E). In summary, both control MSCs and IFN- γ -primed MSCs can secrete Exos with common vesicle characteristics, and these Exos can be absorbed by H9c2 cells and HUVEC cells in a time-dependent manner *in vitro*.

Pro-angiogenesis and anti-apoptotic effects of IFN- γ -Exo *in vitro*

Both TUNEL staining and flow cytometry showed that the percentage of apoptotic cells were significantly reduced in IFN- γ -Exo group compared with OGD and Ctrl-Exo group under OGD condition (Fig. 2A, B). Fig. 2C showed that the apoptosis-related proteins Bax and Cleaved-caspase-3 were significantly reduced in the IFN- γ -Exo group, while the anti-apoptotic protein Bcl2 increased compared with the OGD and Ctrl-Exo group. Angiogenesis and migration rate of HUVECs significantly increased in the IFN- γ -Exo group compared with control and Ctrl-Exo groups (Fig. 2D, E). These results suggested that IFN- γ -Exo confer superior protective effects on H9c2 and HUVECs compared to Ctrl-Exo *in vitro*.

IFN- γ -Exo exerted stronger cardioprotection against myocardial damage than Ctrl-Exo *in vivo*

AMI rat model was used to determine the cardioprotective effects of MSC-derived Exos. Ctrl-Exo, IFN- γ -Exo (100 μ l, 1 μ g/ μ l), or 100 μ l PBS were intramyocardially injected at the time of surgery (Fig. 3A). Dil-labeled Exo was intramyocardially injected and then colocalized with cardiomyocytes 6h after injection, suggesting an efficient *in vivo* uptake of the Exos by heart tissue (Fig. 3B). LVEF and LVFS from 4 weeks' echocardiography were significantly improved in IFN- γ -Exo group compared with PBS and Ctrl-Exo groups (Fig. 3C), while 2 weeks' result showed no difference between Ctrl-Exo and Ctrl-Exo groups (Fig. S1). Quantification of the infarct area suggested that IFN- γ -Exo can maximize the reduction of the fibrotic scar area after MI (Fig. 3D). The above results indicated that IFN- γ -Exo has a better therapeutic effect against myocardial ischemia and hypoxia injury compared with Ctrl-Exo.

IFN- γ -Exo promote angiogenesis and cardiomyocyte survival *in vivo*

HE staining showed that the degree of inflammatory cell infiltration in the IFN- γ -Exo group was lower than that of the Ctrl-Exo and PBS group (Fig. 4A). TUNEL analysis showed that the proportion of apoptotic cells significantly decreased in the IFN- γ -Exo group compared with PBS and Ctrl-Exo groups (Fig. 4B). CD31 staining found that the number of regenerative blood vessels in the IFN- γ -Exo group was significantly higher than that of the other two groups (Fig. 4C). Combined with *in vivo* and *in vitro* experiments, we found that IFN- γ -Exo can exert a better protective effect on myocardium after ischemia and hypoxia injury.

IFN- γ -Exo attenuates OGD-induced injury in H9c2 cells by upregulating miR-21 expression.

We compared the expression of putative five miRNA in control and IFN- γ -primed MSCs and the results (Fig. S2) showed that miR-21 was the most significantly elevated miRNA in IFN- γ -primed MSCs compared with the control ones (Fig. 5A). Further, we found that miR-21 was significantly enriched in IFN- γ -Exo compared with Ctrl-Exo (Fig. 5B).

In order to investigate the role of miR-21 in regulating apoptosis, miR-21 mimics and negative control (NC) were transfected into H9c2 cells prior to exposure to OGD conditions. Apoptosis caused by ischemia and hypoxia were significantly abrogated by markedly increased miR-21. Moreover, western blot analysis confirmed that the protective effect of miR-21 against OGD-induced injury relied on the upregulation of Bcl2 and decreasing Bax and Cleaved-caspase-3 (Fig. 5C).

STAT1 activator promoted expression of miR-21 in IFN- γ -primed MSCs

In order to explore whether transcription factors (TF) were involved in the transcriptional regulation of miR-21, we employed Illumina HiSeq 2,500 high-throughput sequencing for mRNA expression profiling of control and IFN- γ -primed MSCs to identify the functional transcription factor in IFN- γ -primed MSCs. With a 2-fold change and $P < 0.05$ as the threshold cutoff, we identified 55 significantly differentially expressed TFs, of which 37 were upregulated in IFN- γ -primed MSCs (Fig. 5D). Next, we used the TransmiR v2.0 database¹⁵ (<http://www.cuilab.cn/transmir>) to predict the transcription factors that might regulate miR-21 (Fig. 5E) and then crossed with the upregulated expressed TFs to finally get 3 promising TFs (STAT1, STAT2, and FOXC1) (Fig. 5F). Among them, consistent with sequencing results, STAT1 was the most significantly increased in IFN- γ -primed MSCs compared with control MSCs verified by qPCR (Fig. 5G).

Consistent with previous studies,^{16, 17} we found that STAT1 were induced by IFN- γ in MSCs and cellular miR-21 were significantly upregulated. Further, the IFN- γ induction of miR-21 was abolished following STAT1 downregulation (Fig. 5H). In addition, we cloned the wild-type fragment miR-21 promoter into the upstream region of the luciferase reporter gene (pGL3-basic) to obtain pGL3- miR-21 WT and found that

pGL3-miR-21 WT was activated by STAT1 overexpression. These results suggested potential transcriptional regulation of miR-21 by STAT1 (Fig. 5I).

BTG2 was identified as a target gene of miR-21 and promoted H9c2 cell apoptosis under OGD conditions

Using TargetScan and miRDB, BTG2 was identified as a potential target for miR-21 (Fig. 6A). Luciferase reporter assays confirmed the association between miR-21 and BTG2: the luciferase activity of BTG2-wt in miR-21 transfected cells was significantly inhibited, while that of the BTG2-mut remained unchanged (Fig. 6B). Furthermore, the mRNA and protein levels of BTG2 were significantly decreased in the miR-21 mimic groups, respectively (Fig. 6C, D). The above results indicated that there is a negative regulatory relationship between miR-21 and BTG2.

Subsequently, BTG2 was overexpressed to explore its effect on H9c2 cells apoptosis under OGD conditions. Fig 7A showed that western blot analysis confirmed that BTG2 was successfully overexpressed in H9c2 cells via transfection with BTG2 plasmid. Further experiments revealed that apoptosis induced by ischemia and hypoxia is further aggravated by BTG2 overexpression. The expression of Bax and Cleaved-caspase-3 protein were increased significantly in the BTG2 group, while the expression of Bcl2 protein was decreased. The same trend was verified in TUNEL staining and flow cytometry, that is, the incidence of apoptosis in the BTG2 group was significantly increased compared with ctrl (OGD) and vector group (Fig. 7B, C).

In order to deeply explore the functional relationship between miR-21 and BTG2, we used reply experiment to verify the effect of miR-21 mimics and BTG2 plasmid on H9C2 under OGD conditions. Consistent with the previous findings¹⁸, ischemia and hypoxia not only increase the expression of Bax and Cleaved-caspase-3 but also increase the expression of BTG2, and this elevation were inhibited by miR-21 mimics. However, the anti-apoptotic effect of miR-21 on H9C2 cells under OGD conditions were obviously abrogated by BTG2 overexpression (Fig. 7D). These results indicated that the overexpression of BTG2 reverses the protective effect of miR-21 on OGD-treated H9c2 cells.

Discussion

This study found that compared with Ctrl-Exo, IFN- γ -Exo accelerated migration, tube-like structure formation, and prevented H9c2 from OGD-induced apoptosis; IFN- γ -Exo also reduced fibrosis size, cardiomyocytes apoptosis and improved recovery in cardiac function; Further research has revealed that IFN- γ -Exo attenuates OGD-induced injury in H9c2 cells through upregulating miR-21 expression stimulated by STAT1, which directly targeted on BTG2 (Fig. 8).

Growing evidence shows that Exos released from MSCs can protect ischemic cardiomyocytes from death, improve ventricular remodeling, and preserve heart function.¹⁹ On this basis, how to improve the

therapeutic effect of Exo is a point worthy of our consideration. Researchers have devoted a great deal of efforts to finding a variety of modification methods that can reinforce the therapeutic effect of MSC-derived Exos. Hypoxia, as the most common treatment, elicited MSC-derived Exos exhibit more effective cardioprotection mediated by UCA1 and miR-125b.^{12, 20} Genetic modification can also enhance the treatment of heart disease by adjusting the expression of various miRNA. Exos secreted by MSCs overexpressing GATA-4 retain a large amount of anti-apoptotic miRNA for cardioprotection.²¹ The Exos derived from MSCs modified by MIF can treat acute MI by limiting apoptosis and promoting angiogenesis.¹⁴ Some drugs and cytokines have also been selected to improve the characteristics of stem cells. Atorvastatin, as a common clinical lipid-lowering drug, has also been proven to increase the therapeutic effect by up-regulating the expression of H19.²² IFN- γ , as an immune-related cytokine, enable MSC-derived Exos to show stronger immunomodulation efficacy and therefore perform better in the treatment of colitis. Our research disclosed that Exo derived from IFN- γ -primed MSCs have more sufficient anti-apoptosis and angiogenesis ability compared with the control group both in cardiomyocytes under OGD conditions and MI rat models. These results indicated that IFN- γ treatment may be an alternative to strengthen the therapeutic effect of exosome derived from MSCs, so we have conducted a more in-depth study on the changes in the contents of Exos.

Minimizing the loss of myocardial cells and restoring the microcirculation in the marginal zone of the infarction are common strategies for the treatment of MI.²³ miR-21 is a typical cardioprotective miRNA, which has various therapeutic effects such as anti-apoptosis, anti-fibrosis, and inhibiting inflammation.²⁴ Subsequently, in order to explore the mechanism of miR-21 being upregulated, we used RNA sequencing to explore the transcription factors that were upregulated after IFN- γ treatment and finally selected STAT1 that may regulate the expression of miR-21. Some studies have reported that STAT3 can stimulate the expression of miR-21 by binding to the promoter region.^{25, 26} As an important member of the signal transduction and transcription activator protein family, STAT1 has a similar structure and function with STAT3.²⁷ Our research revealed that STAT1 induced by IFN- γ can upregulate miR-21 expression by binding to the promoter region.

The angiogenesis effect of miR-21 has been extensively studied, so we further explored the anti-apoptotic effect of miR-21 under ischemia and hypoxia.^{28, 29} BTG2 (B cell translocation gene 2) is the first gene found in the BTG/TOB gene family, and it exerts a tumor suppressor effect in various cancer types.³⁰ Previous studies have found that down-regulation of BTG2 by miR-21 can protect cardiomyocytes from doxorubicin treatment.³¹ Functional studies have shown that overexpression of BTG2 can exacerbate the apoptosis of H9c2 cell apoptosis under ischemic and hypoxic conditions. Moreover, BTG2 reversed the protective effect of miR-21 on hypoxia-induced injury in H9c2 cells. At the same time, BTG2 was found to be up-regulated under OGD conditions, which suggests that BTG2 plays an important role in myocardial infarction, so it is worthy of in-depth study.

Conclusions

In summary, in this study, we have documented that Exos derived from control and IFN- γ -primed MSCs attenuate myocardial injury. IFN- γ -Exo exerted superior therapeutic efficacy, which were mainly mediated by increasing the expression of miR-21-5p. Briefly, our findings provide insight into promoting the ability of anti-apoptosis and angiogenesis from MSC-derived Exos and suggest a promising approach to treat ischemic heart disease.

Abbreviations

MSC: mesenchymal stem cells

IFN: interferon

Exos: exosomes

OGD: oxygen and glucose deprivation

MI: myocardial infarction

TF: transcription factors

STAT1: signal transducer and activator of transcription 1

BTG2: BTG anti-proliferation factor 2

BCL2: BCL2 apoptosis regulator

BAX: BCL2 associated X, apoptosis regulator

HUVEC: Human Umbilical Vein Endothelial Cells

Declarations

Ethics approval and consent to participate

Animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals and were approved by Ethics Committee of Nanjing medical university (IACUC-2005043).

Consent for publication

Not applicable.

Availability of data and materials

The data that support the finding of this study are available from the corresponding upon reasonable request.

Competing interests

The authors have declared that no competing interest exists.

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Authors' contributions

JZ, YL, YMM, WZ and FXZ contributed to the design of the study. JZ, YL, WZ, YY, TYW and YQZ performed the experiments. JZ, YL, PCZ and FXZ contributed to the writing the manuscript. JZ, YL and YMM contributed to the material support of the study. All authors read and approved the final manuscript.

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Figures

Figure 1

Figure 1

Characterization of Exos derived from MSCs (A) Cup-shaped morphology of purified Ctrl-Exo and IFN-γ-Exo assessed by TEM. (B) Representative images of western blot showing the exosomal protein markers in Ctrl-Exo and IFN-γ-Exo groups. (C) The particle size distribution and particle concentration were analyzed by nanoparticle tracking analysis (n=3). Confocal images showed that red fluorescence of dye

Dil labeled Exos were endocytosed by HUVECs (D) and H9c2 (E) 6 and 24 h after incubation. Scale bar = 20 μ m

Figure 2

Figure 2

IFN- γ -Exo exhibited better protective effects on HUVECs and H9c2 cardiomyocytes than Ctrl-Exo in vitro. (A) TUNEL analysis (n=3). Green, TUNEL-positive nuclei; blue, DAPI-stained nuclei. Scale bars=100 μ m. (B) Flow cytometric analysis (n=3). (C) Western blot analyzed BAX, BCL-2, and cleaved-caspase-3 protein levels in hypoxic and ischemic H9c2 cells incubated with PBS, Ctrl-Exo and IFN- γ -Exo. Relative protein

levels are presented as the average expression normalized to β -Tublin (n=3). (D) Migration was monitored for 6 and 12 h after scratching in HUVECs cultured with PBS, Ctrl-Exo and IFN- γ -Exo (n=3). (E) Tube formation of HUVECs incubated with PBS, Ctrl-Exo and IFN- γ -Exo (n=3). Data are presented as mean $\pm$ SEM. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's correction. *P< 0.05, **P < 0.01, ***P< 0.001, NS not significant.

Figure 3

Figure 3

IFN- γ -Exo effectively preserved cardiac function in rats with MI in vivo. (A) The flowchart of experimental design in vivo. (B) Dil-labeled Exos were injected into the infarcted heart of rats for 6 h (50 μ g Exos per rat). Representative images of post-MI heart sections stained with Dil-labeled Exos (red), α -actin (green), and DAPI (blue). (C) Representative echocardiographic images showed heart function among the different groups on the 28th day following MI and quantitative analysis of left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) among the different groups (n =6 for sham group, n=5 for PBS group, n=6 for Ctrl-Exo group, n=6 for IFN- γ -Exo group). (D) Masson's trichrome stained myocardial sections on the 28th day following MI in rats treated with PBS, Ctrl-Exo and IFN- γ -Exo. Scar tissue and viable myocardium were identified in blue and red, respectively (n=4). Data were presented as mean $\pm$ SEM. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's correction. *P< 0.05, **P< 0.01, ***P < 0.001, ****P< 0.0001.

Figure 4

Figure 4

IFN- γ -Exo was better at inhibiting apoptosis or promoting angiogenesis than IFN- γ -Exo in vivo. (A) HE staining images at the border zone 4 weeks after MI. (B) Representative fluorescence images of blood vessels in the border zone of ischemic hearts stained with CD31 (red) (3 random fields per animal; n=5). (C) Representative photographs showing the TUNEL-positive cells in the heart tissue among the different groups. Quantitative analysis of the apoptotic rate at the border zone among the different groups (3

random fields per animal; n=5). Data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's correction. *P< 0.05, **P< 0.01, ***P < 0.001, ****P< 0.0001.

Figure 5

MiR-21 was a key component in IFN-γ-Exo -induced cardioprotection in vitro. Quantitative real-time PCR (qRT-PCR) analysis of miR-21 level in control and IFN-γ-primed MSCs (A) and Exos (B) (n =3). (C) Western blot analyzed BAX, BCL-2, and cleaved-caspase-3 protein levels in hypoxic and ischemic H9c2 cells. Relative protein levels were presented as the average expression normalized to β-Tubulin (n=3). (D) Heat

map based on mRNAs sequence values (red represents high expression and green represents low expression) between control and IFN- γ -primed MSCs. (E) Bioinformatics analysis network of the transcription factors interacting with the promoter of miR-21. The yellow circle represents hsa-miR-21, and the blue and red circles represent transcription factors. (F) STAT1, STAT2 and FOXC1 were present in both up-regulated transcription factors and bioinformatics prediction analysis results. (G) qRT-PCR analysis of STAT1 level in MSCs treated with control and IFN- γ (n=3). (H) qRT-PCR analysis of miR-21-5p level in MSCs treated with control, IFN- γ , IFN- γ +si-STAT1 (n=3). (I) Luciferase reporter assay was used to detect the miR-21 promoter reporter activity in 293T cells transfected with vector or STAT1 (n=3). Data was presented as mean $\pm$ SEM. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's correction. *P< 0.05, **P< 0.01, ***P < 0.001, ****P< 0.0001.

Figure 6

Figure 6

MiR-21 directly targeted BTG2 in H9c2 cells. (A) Predicted miR-21 target sequence in BTG2-3' UTRs. Target sequences of BTG2-3' UTRs were mutated. (B) Luciferase assay of 293T cells transfected with BTG2-3' UTR-WT or BTG2-3' UTR-Mut reporter together with mimic NC or miR-21-5p mimic (n=3). (C) qRT-PCR analysis of BTG2 level in H9C2 cells treated with mimic NC and miR-21-5p mimic (n=3). (D) Western blot analyzed BTG2 protein levels in H9c2 cells treated with control, mimic NC and miR-21-5p mimics. Relative protein levels were presented as the average expression normalized to β-Tubulin (n=3). Data were

presented as mean $\pm$ SEM. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's correction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 7

Overexpression of BTG2 aggravated OGD-induced injury damage and reversed the protective effect of miR-21. (A) Western blot analyzed BAX, BCL-2, and cleaved-caspase-3 protein levels in hypoxic and ischemic H9c2 cells treated with PBS, vector and BTG2. Relative protein levels were presented as the average expression normalized to GAPDH (n=3) and TUNEL analysis (n=3) (B) Green, TUNEL-positive

nuclei; blue, DAPI-stained nuclei. Scale bars=200μm. And flow cytometric analysis (n=3) (C). (D) H9c2 cells treated with control, OGD condition, OGD condition+miR-21 and OGD condition+miR-21+BTG2. Relative BAX, BCL-2, and cleaved-caspase-3 protein levels among different groups were presented as the average expression normalized to β-Tubulin (n=3). Data were presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's correction. *P< 0.05, **P< 0.01, ***P < 0.001, ****P< 0.0001.

Figure 8

Figure 8

This study found that compared with Ctrl-Exo, IFN- γ -Exo accelerated migration, tube-like structure formation, and prevented H9c2 from OGD-induced apoptosis; IFN- γ -Exo also reduced fibrosis size, cardiomyocytes apoptosis and improved recovery in cardiac function; Further research has revealed that IFN- γ -Exo attenuates OGD-induced injury in H9c2 cells through upregulating miR-21 expression stimulated by STAT1, which directly targeted on BTG2 (Fig. 8).

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