Periostin renders cardiomyocytes vulnerable to acute myocardial infarction via pro-apoptosis

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Abstract

Aims As a severe cardiovascular disease, acute myocardial infarction (AMI) could trigger congestive heart failure. Periostin (Postn) has been elucidated to be dramatically up-regulated in myocardial infarction. Abundant expression of Postn was also observed in the infarct border of human and mouse hearts with AMI. This work is dedicated to explore the mechanism through which Postn exerts its functions on AMI.

Methods and results The expression of Postn in AMI mice and hypoxia-treated neonatal mouse cardiomyocytes (NMCMs) was quantified by qRT-PCR. The biological functions of Postn in AMI were explored by trypan blue, TUNEL, flow cytometry analysis, and JC-1 assays. Luciferase activity or MS2-RIP or RNA pull-down assay was performed to study the interaction between genes. Postn exhibited up-regulated expression in AMI mice and hypoxia-treated NMCMs. Functional assays indicated that cell apoptosis in NMCMs was promoted via the treatment of hypoxia. And Postn shortage could alleviate cell apoptosis in hypoxia-induced NMCMs. Postn was verified to bind to mmu-miR-203-3p and be down-regulated by miR-203-3p overexpression. Postn and miR-203-3p were spotted to coexist with small nucleolar RNA host gene 8 (Snhg8) in RNA-induced silencing complex. The affinity between Snhg8 and miR-203-3p was confirmed. Afterwards, Snhg8 was validated to promote cell apoptosis in hypoxia-induced NMCMs partially dependent on Postn. Furthermore, vascular endothelial growth factor A (Vegfa) was revealed to bind to miR-203-3p and be implicated in the Snhg8-mediated AML cell apoptosis and angiogenesis.

Conclusions miR-203-3p availability is antagonized by Snhg8 for Postn and Vegfa-induced AMI progression.

Keywords Snhg8; miR-203-3p; Postn; Vegfa; Acute myocardial infarction

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Introduction

Acute myocardial infarction (AMI) is featured with increasing incidence and high mortality.1 AMI could trigger left ventricular dilatation, heart failure, and sudden cardiac death.2 It has been discovered that the myocardium border zone exhibited much more cardiomyocyte apoptosis. Cardiomyocyte apoptosis could be caused by many reasons, including hypoxic injury.3–5 Besides, AMI initiation was revealed to be related with hypoxia of coronary microvasculature.6 Moreover, the mortality of AMI could be conspicuously decreased by early diagnosis and efficacious treatment.7,8 Therefore, in order to provide more valuable biomarkers for AMI treatment, it is imperative to study the molecular mechanisms underlying hypoxia-induced AMI.

The matricellular protein—periostin (Postn)—has been disclosed to be highly expressed in the infarct border of human and mouse hearts with AMI.9 Postn has been reported to participate in many diseases. For instance, Postn promotes colorectal tumourigenesis through integrin–FAK–Src pathway-mediated YAP/TAZ activation.10 Besides, Postn was analysed to be significantly up-regulated in myocardial infarction (MI) mice than in control group.11 However, the specific molecular mechanism through which Postn regulates AMI occurrence was rarely mentioned. We decided to quest for the role of Postn in AMI. MicroRNAs (miRNAs) are small
non-coding RNAs, characterized by the length of 18–25 nucleotides and the regulation on messenger RNAs (mRNAs). The consequential roles of miRNAs in cardiomyocyte apoptosis have also been uncovered. Long non-coding RNA (lncRNA) lacks protein-coding ability but possesses a length of over 200 nucleotides. Previous investigations have verified active participation of lncRNAs in various biological and pathological processes as well as cardiovascular diseases. More importantly, the regulation of miRNA on its target mRNA could be abrogated by the lncRNA, which could bind to the miRNA, thereby enabling mRNA to exert its functions in diseases.

The primary purpose of this work was to figure out the functions of Postn in AMI occurrence. The potential regulatory mechanism of Postn in AMI would also be explored.

Methods

Animal model

Male C57BL/6 mice (20–30 g) were procured from Shanghai Slack Laboratory Animal Research Center (Shanghai, China) and utilized with the ethical approval from the Animal Care and Use Committee of Fuwai Central China Cardiovascular Hospital. For AMI model, mice were anaesthetized with 40 mg/kg sodium pentobarbitone and 12.5 mg/kg xylazine, and then the 1.1–1.5 cm incision was created on the left of sternum. Muscle layers of chest wall were dissected bluntly, and hearts were pushed out in the thoracic cavity. The chest was closed after occluding the left anterior descending coronary artery. Each surgical procedure was conducted under sterile conditions. Twenty-four hours later, mice were treated with xylazine and sodium pentobarbitone and sacrificed for isolating hearts. Mice in sham group (control) were only treated with opening chest procedures.

Cell culture and treatment

After anaesthetizing mice with 5% isoflurane inhalation anaesthesia, mice hearts were minced and put in 0.25% trypsin and 0.1 mM BrdU (Solarbio, Beijing, China). Dispersed neonatal mouse cardiomyocytes (NMCMs) were maintained in the DMEM (Invitrogen, Carlsbad, CA) adding 10% FBS (Gibco, Grand Island, NY) under normoxia of 5% CO2 and 37°C. To induce hypoxia, cell samples were cultured at 37°C under hypoxia conditions of 1% O₂, 5% CO₂, and 94% N₂.

Quantitative real-time PCR

Total RNAs were extracted from mice hearts and cultured NMCMs by the application of TRIzol (Invitrogen); 500 ng of total RNA was used for cDNA synthesis, and qPCR was implemented with SYBR Green I (Roche, Basel, Switzerland). Relative RNA levels were calculated on the basis of threshold cycle (Ct) values, standardized to U6 and GAPDH for each sample.

Transfection

The shRNAs specific to Postn and Snhg8, as well as the control shRNAs (GenePharma, Shanghai, China), were transfected into cultured cell samples using Lipofectamine 2000 (Invitrogen) as required by supplier. The miR-203-3p mimics/inhibitor and NC mimics/inhibitor, as well as pcDNA3.1/Postn, pcDNA3.1/vascular endothelial growth factor A (Vegfa), and NC pcDNA3.1 vectors, were all procured from GenePharma; 48 h later, all samples were reaped.

Trypan blue staining assay

After cell transfection, samples from different groups were seeded at 5 × 10⁴ cells per well in the 24-well plates at 37°C for 48 h. Samples were then washed, trypsinized, and treated with trypan blue dye (Beyotime, Shanghai, China). Samples were counted in cell counting chamber.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay was processed for cell apoptosis in line with the instruction of One-Step TUNEL Apoptosis Assay Kit (Beyotime). After DAPI staining for detection of cell nucleus, TUNEL-positive cell samples were counted.

Flow cytometry apoptosis analysis

Flow cytometry apoptosis analysis was processed using Annexin V assay kit (BD Biosciences, San Jose, CA) as instructed by supplier. Cell apoptosis was monitored by adding Annexin V-fluorescein isothiocyanate and propidium iodide. Results were analysed via flow cytometry with FACSCalibur (BD Biosciences).

JC-1 assay

JC-1 assay was developed with 10 nM JC-1 (Beyotime) using EnSpire Reader analysis (PerkinElmer, Waltham, MA) as per the user manual. Mitochondrial membrane potential was measured through the ratio of fluorescence at 590 to 530 nm emission.
**RNA immunoprecipitation assay**

The cultured NMCM cell samples from various groups were harvested for co-transfection with pMS2-GFP and pSL-MS2, pSL-Postn for 48 h, and then exposed to RNA immunoprecipitation (RIP) RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA). Ago2-RIP was conducted by use of anti-Ago2 antibody and control IgG antibody. Precipitated RNA fractions were subjected to quantitative real-time PCR.

**Dual-luciferase reporter assay**

Postn promoter was amplified by PCR for inserting to luciferase reporter pGL3 (Promega, Madison, WI) and then co-transfected with miR-203-3p mimics or NC mimics in hypoxia-induced NMCM cell samples. The wild-type (Wt) and mutated (Mut) miR-203-3p binding sites to Postn, Snhg8, or Vegfa were obtained and cloned into pmirGLO reporter vector (Promega) for co-transfecting with miR-203-3p mimics or NC mimics; 48 h later, samples were collected for Dual-Luciferase Reporter Assay System (Promega).

**RNA pull down**

RNA pull down was processed by application of Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, Waltham, MA) using miR-203-3p biotin probe. The pull downs were purified for quantitative real-time PCR.

**Fluorescent in situ hybridization**

Snhg8 fluorescent in situ hybridization probe was specifically designed by RiboBio (Guangzhou, China). Samples were treated with DAPI for nuclear staining and then observed under confocal microscope (Zeiss, Jena, Germany).

**Tube formation**

Neonatal mouse cardiomyocytes in different groups were seeded at $4 \times 10^5$ cells per well in the 49-well plates containing the 150 μL pre-cooled Matrigel in 5% CO$_2$ at 37°C; 24 h later, samples in each well were imaged under microscope.

**Statistical analysis**

Results from the independent bio-triplications were shown as the mean ± standard deviation. Data differences between groups were monitored via Student’s t-test and one-way analysis of variance applying the SPSS 13.0 (SPSS Inc., Chicago, IL). P-value <0.05 was seen as the threshold of statistical significance.

**Results**

**Periostin shortage could weaken dispersed neonatal mouse cardiomyocytes injury triggered by hypoxia**

To begin with, an AMI model and a sham model were developed to detect Postn expression. As shown in Figure S1A, the expression of Postn was remarkably up-regulated in AMI mice heart. Postn also displayed high expression in hypoxia-induced NMCMs (Figure 1B). Sh-Postn#1/2/3 was transfected to interfere Postn expression in hypoxia-induced NMCMs (Figure 1C). Then, EdU and trypan staining assays revealed that cell viability was impaired under hypoxic condition. This impairment could be largely compensated by Postn silencing (Figure 1D–1G). Afterwards, TUNEL assay suggested that the accelerated cell apoptosis in hypoxia-induced NMCMs could be reversed by Postn knockdown (Figure 1H and 1I). It was also examined by flow cytometry analysis that the absence of Postn could restore the enhanced cell apoptotic ability under hypoxic condition (Supporting Information, Figure S1A and S1B). JC-1 assay demonstrated that the facilitated cell apoptosis aroused by hypoxia was suppressed in sh-Postn#1/2-transfected cells (Supporting Information, Figure S1C and S1D). In conclusion, high expression of Postn could enhance cell injury in hypoxia-induced NMCMs.

**Periostin is regulated by miR-203-3p in acute myocardial infarction**

Then, it was figured out from ENCORI website (http://starbase.sysu.edu.cn/index.php) that nine miRNAs could potentially bind to Postn. Next, MS2-RIP assay revealed that mmu-miR-203-3p, mmu-miR-741-3p, and mmu-miR-325-3p could bind to Postn (Figure 2A). The functions of these three miRNAs were investigated in AMI. EdU assay manifested that only overexpression of miR-203-3p could enhance cell viability in hypoxia-treated NMCMs (Figure 2B). Flow cytometry assay disclosed that cell apoptosis could be restrained by miR-203-3p overexpression rather than enforced expression of the other two miRNAs (Figure 2C). Luciferase reporter assay was carried out to further assess the affinity between miR-203-3p and Postn. As depicted in Supporting Information, Figure S2A, there existed no interaction between miR-203-3p and Postn promoter. Therefore, bioinformatics instruments were employed to probe the binding sites between miR-203-3p and Postn 3’UTR (Supporting Information, Figure S2B). The luciferase activity of Postn-Wt promoter was evaluated to be reduced by miR-203-3p overexpression.
small nucleolar RNA hostgene 8 binds to miR-203-3p and promotes cell apoptosis

To look for which lncRNA could sponge miR-203-3p, ENCORI was applied and a total of 14 lncRNAs were spotted. RNA pull-down assay indicated that three candidates were
enriched in miR-203-3p biotin probe group (Figure 3A). RIP assay demonstrated that only small nucleolar RNA host gene 8 (Snhg8), miR-203-3p, and Postn were detected in anti-Ago2 group, suggesting the coexistence of these three genes in RNA-induced silencing complex (RISC) (Figure 3B). Fluorescent in situ hybridization assay illustrated that Snhg8 mainly localized in the cytoplasm of hypoxia-induced NMCMs (Figure 3C). The binding sites between Snhg8 and miR-203-3p were then obtained (Figure 3D). The luciferase activity of Snhg8-Wt reporter was weakened in miR-203-3p mimic-transfected cells, proving the combination between miR-203-3p and Snhg8 (Figure 3E). Snhg8 was effectively knocked down in
Vascular endothelial growth factor A competes with Snhg8 to bind to miR-203-3p

Despite that Postn was involved in Snhg8-mediated AMI cell apoptosis, Postn could only account for roughly half of the effects caused by Snhg8. Therefore, we wondered whether Snhg8 could target other mRNAs; 104 mRNAs were screened out in ENCORI to bind to miR-203-3p (Figure 4A), among which five mRNAs were analysed to be evidently down-regulated (with log2 < −2) in miR-203-3p inhibitor-transfected cells (Figure 4B). RNA pull-down assay suggested that of five candidates, only Vegfa could bind to miR-203-3p (Figure 4C). And the coexistence of Snhg8, Vegfa, Postn, and miR-203-3p...
in RISC was also verified in RIP assay (Figure 4D). The binding sites between Vegfa and miR-203-3p were acquired in Figure 4E. And the affinity between Vegfa and miR-203-3p was validated by luciferase reporter assay (Figure 4F). In addition, miR-203-3p overexpression was proved to inhibit Vegfa expression in hypoxia-induced NMCMs (Figure 4G). Besides, enforced expression of miR-203-3p could elevate the suppressed Vegfa expression in sh-Snhg8#1-transfected cells (Figure 4H). Considering the function of Vegfa in angiogenesis, angiogenesis was investigated in hypoxic condition. Results manifested that angiogenesis was inhibited in hypoxia-treated human umbilical vein endothelial cells (HUVECs) (Figure 4I). Vegfa expression was increased by the transfection of pcDNA3.1/Vegfa (Supporting Information, Figure S4A). As shown in Supporting Information, Figure S4B, overexpression of Vegfa could enhance the angiogenesis suppressed by the treatment of hypoxia. In sum, Vegfa is also regulated by Snhg8 in hypoxia-induced NMCMs.
Periostin and Vascular endothelial growth factor A are implicated in small nucleolar RNA host gene 8-mediated acute myocardial infarction occurrence

In order to determine the involvement of Postn and Vegfa in Snhg8-mediated AMI occurrence, Vegfa was successfully overexpressed (Figure 5A). As illustrated in Figure 5B and 5C, the protected cell viability induced by Snhg8 depletion could be impaired by overexpression of Vegfa and Postn. It was also uncovered that enforced expression of Vegfa and Postn could compensate for the Snhg8 deficiency-triggered inhibitive effects on cell apoptosis in hypoxia-induced NMCMs (Figure 5D and 5E and Supporting Information).

**Figure 5** Postn and Vegfa are implicated in Snhg8-mediated AMI occurrence. (A) qRT-PCR of overexpression efficiency of Vegfa. (B, C) EdU and trypan blue staining assays of cell viability in different transfected hypoxia-induced NMCMs. (D, E) TUNEL and flow cytometry assays of cell apoptosis in different groups. *P < 0.05 and **P < 0.01.
Figure S5A). Vegfa and Postn were successfully silenced in hypoxia-induced HUVECs as well as Snhg8 was effectively overexpressed in hypoxia-induced HUVECs (Supporting Information, Figure S5B). In the end, it was unveiled that the enhanced angiogenesis in sh-Snhg8#1-transfected cells could be restrained by Vegfa and Postn overexpression (Supporting Information, Figure S5C). In a word, Snhg8 regulates cell injury in AMI dependent on Vegfa and Postn.

Discussion

Previous literature elucidated that Postn could serve as a target in therapeutic treatment of cardiac fibrosis. According to one previous study, it has been confirmed that Postn induces proliferation of differentiated cardiomyocytes and promotes cardiac repair, which is opposite to our study. In our work, conspicuously high expression of Postn was detected in AMI mice heart and hypoxia-induced NMCMs. Cell apoptosis was evaluated to be promoted under hypoxia treatment. Moreover, Postn could pose promising effects on cell apoptotic ability of hypoxia-induced NMCMs, implying that Postn could accelerate cell injury in AMI.

MiRNAs could regulate the expression of their downstream target mRNAs. Therefore, ENCORI website was utilized to figure out the potential miRNA, which could regulate Postn. Then, it was verified that Postn could bind to miR-203-3p, miR-741-3p, and miR-325-3p. Only miR-203-3p overexpression could affect cell viability and impair cell apoptosis. Hence, miR-203-3p was chosen to be studied. miR-203-3p has been investigated in several diseases. As an illustration, miR-203-3p was sponged by lncRNA Gm6135 in diabetic nephropathy. Down-regulated miR-203-3p could initiate type 2 pathology during schistosome infection. Nevertheless, rare research mentioned miR-203-3p in AMI. Data from our study revealed that miR-203-3p could bind to Postn and was down-regulated in NMCMs under hypoxic condition. Moreover, miR-203-3p overexpression could suppress Postn level.

miRNA has also been reported as critical components in the competing endogenous RNA regulatory network constructed by IncRNA. Through the miRNA binding elements possessed by IncRNA, IncRNA could bind to miRNA and block the functions of miRNA. LncRNAs are important engagers in AMI. For instance, IncRNA Mirt1 silencing restrains AMI via inhibiting NF-kB activation. LncRNA NEAT1 is suppressed in early-onset MI sufferers. LncRNA ZFAS1 could impair cardiac contractile function in MI by binding with SERCA2a. In current work, Snhg8 was identified as the molecular sponge of miR-203-3p. The existence of Snhg8 in RISC and the localization of Snhg8 in cytoplasm verified the hypothesized competing endogenous RNA role of Snhg8. Besides, the affinity between Snhg8 and miR-203-3p was also validated. Snhg8 could up-regulate Postn expression via sequestering miR-203-3p. It was disclosed that Postn could only partially account for the Snhg8-mediated AMI cell injury. We went on to search for the other mRNAs that could as well be regulated by Snhg8. Then, Vegfa was found to combine with miR-203-3p and exist in RISC. Vegfa expression could also be promoted by Snhg8. As an angiogenic factor, Vegfa has been supported to promote angiogenesis. In our investigation, Vegfa could also promote angiogenesis. In the end, it was disclosed that Snhg8 enhanced cell apoptosis in AMI via targeting Vegfa and Postn.

In conclusion, the availability of miR-203-3p was antagonized by Snhg8 for the Vegfa and Postn-triggered AMI cell injury. We will further verify the results of this experiment in subsequent studies due to the opposite functions of Postn in different researches.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) Flow cytometry assay of NMCMs apoptosis under hypoxic condition. (B) Flow cytometry assay of cell apoptosis when Postn was knocked down. (C) JC-I assay of cell apoptosis under nomoxia or hypoxia treatment. (D) JC-I assay of cell apoptotic ability with downregulated Postn. **P < 0.01.

Figure S2. (A) Luciferase reporter assay of the affinity between miR-203-3p and Postn promoter. (B) The binding sites between miR-203-3p and Postn. (C) Luciferase reporter assay of the combination between miR-203-3p and Postn. (D) qRT-PCR of miR-203-3p expression in NMCMs under nomoxia or hypoxia treatment. (E) qRT-PCR of miR-203-3p expression in NC mimics or miR-203-3p mimics-transfected cells. **P < 0.01.

Figure S3. (A) qRT-PCR of overexpression of Postn. (B-C) EdU and trypan blue staining assays of cell viability in cell transfected with sh-NC or sh-Snhg8#1 or sh-
Figure S4. (A) qRT-PCR of overexpression efficiency of Vegfa in hypoxia-induced HUVEC cells. (B) Tube formation assay of tube length in cells transfected with pcDNA3.1 or pcDNA3.1/Vegfa. **P < 0.01.

Figure S5. (A) JC-1 assays of cell apoptosis in different groups. (B) qRT-PCR of interference efficiency of Vegfa, Postn and the overexpression efficiency of Snhg8. (C) Tube formation assay of tube length in cells transfected with pcDNA3.1 or pcDNA3.1/Snhg8 or pcDNA3.1/Snhg8 + sh-Vegfa#1 or pcDNA3.1/Snhg8 + sh-Vegfa#1 + sh-Snhg8#1. *P < 0.05, **P < 0.01.

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