Therapeutic potential of miR-21 regulation by human peripheral blood derived-small extracellular vesicles in myocardial infarction

Ji-Young Kang1,*, Hyoeun Kim1, Dasom Mun1, Nuri Yun, PhD2 and Boyoung Joung, MD, PhD1

1 Division of Cardiology, Yonsei University College of Medicine, Seoul 03722, Republic of Korea; 2 Institute of Life Science and Biotechnology, Yonsei University, Seoul 03722, Republic of Korea

Correspondence: Boyoung Joung (cby6908@yuhs.ac) or Nuri Yun (yunnuri@hanmail.net)

Ji-Young Kang (k940205@yuhs.ac), Hyoeun Kim (khe7813@yuhs.ac), and Dasom Mun (christsom@yuhs.ac)
Abstract

Small extracellular vesicles (sEVs) as natural membranous vesicles are on the frontiers of nanomedical research, due to their ability to deliver therapeutic molecules such as microRNAs (miRNAs). The miRNA-21 (miR-21) is thought to be involved in the initiation and development of myocardial infarction (MI). Here, we examined whether miR-21 regulation using human peripheral blood-derived sEVs (PB-sEVs) could serve as a potential therapeutic strategy for MI. First, we examined miR-21 levels in hypoxic conditions and validated the ability of PB-sEVs to serve as a potential delivery system for miRNAs. Further, bioinformatics analysis and luciferase assay were performed to identify target genes of miR-21 mechanistically. Among numerous target pathways, we focused on nitrogen metabolism, which remains relatively unexplored compared to other possible miR-21-mediated pathways; hence, we aimed to determine novel target genes of miR-21 related to nitrogen metabolism. In hypoxic conditions, the expression of miR-21 was significantly upregulated and correlated with nitric oxide synthase 3 (NOS3) levels, which in turn influences cardiac function. The downregulation of miR-21 expression by PB-sEVs loaded with anti-miR-21 significantly improved survival rates, consistent with the augmentation of cardiac function. However, the upregulation of miR-21 expression by PB-sEVs loaded with miR-21 reversed these effects. Mechanistically, miR-21 targeted and downregulated the mRNA and protein expression of striatin (STRN), which could regulate NOS3 expression. In conclusion, we identified a novel therapeutic strategy to improve cardiac function by regulating the expression of miR-21 with PB-sEVs as an miR-21 or anti-miR-21 delivery vehicle and confirmed the miR-21-associated nitrogen metabolic disorders in MI.
Abbreviations list

1 sEVs, small extracellular vesicles
2 miRNAs, microRNAs
3 MI, myocardial infarction
4 PB-sEVs, human peripheral blood derived-small extracellular vesicles
5 NOS3, nitric oxide synthase 3
6 STRN, striatin
7 TEM, transmission electron microscopy
8 NTA, nanoparticle tracking analysis
9 siRNA, small interfering RNA
10 NO, nitric oxide
11 cTnI, cardiac troponin I
Introduction

Small extracellular vesicles (sEVs), which are small (30-200 nm) membrane vesicles secreted by most cell types, have been shown to play an important role in mediating cell-to-cell communication and crosstalk between organs by transferring a variety of signaling molecules such as proteins, mRNAs, and microRNAs (miRNAs) [1-4]. sEVs have gained attention for their ability to serve as nanocarriers for the delivery of drugs and nucleic acid-based molecules without undergoing degradation or exhibiting toxicity and adverse immune reactions [5]. However, a key issue is the very low yield of EVs isolated from cell-culture media, which has restricted wider application of EVs for research and clinical therapy [6-8]. In fact, billions of cells are needed to get sufficient EVs from cell culture media, and such numbers of primary cells are usually not available [7]. This limitation may be overcome by the isolation of EVs from human peripheral blood because (i) peripheral blood can be easily obtained from any human subject, (ii) peripheral blood have been used safely and routinely for blood transfusions. Therefore, human peripheral blood derived-sEVs (PB-sEVs) may act as efficient vehicles for miRNA delivery that is safe, and easily applicable.

Myocardial infarction (MI) is a major cause of death worldwide and is characterized with a significant loss in cardiomyocytes and increases in fibrosis, hypertrophy, and contractile dysfunction, often resulting in poor outcomes [9-13]. At present, treatments such as surgical interventions and thrombolysis are being used; however, it is essential to develop effective alternatives for MI treatment [14].

Several studies have shown that alterations in the expression of miRNAs, a class of small non-coding RNAs, that control the expression of targeted mRNAs, contribute to the pathophysiological consequences of MI [15,16]. As abnormal expression of miRNAs is a key phenomenon in MI pathogenesis, there is a growing interest in attempts to regulate miRNAs expression to normal levels [17]. However, poor stability and inefficient delivery in vivo restrict the clinical applications of miRNAs [18]. Thus, efficient delivery approaches are highly desired.

Among miRNAs, miRNA-21 (miR-21) is known to play a crucial role in several physiological and pathological MI processes by targeting various signaling pathways [19-21].
Taking the advantages of the effects of miR-21 on MI, we propose to deliver miR-21 into cardiac tissue through PB-sEVs to promote the recovery of cardiac function. In the present study, we evaluated whether the regulation of miR-21 expression using PB-sEVs may provide an effective strategy for MI treatment and investigated the mechanisms affected by miR-21 in the MI pathogenesis.
Materials and Methods

Clinical samples

All human studies were ethically approved by the local ethics committee (Institutional Review Board of Severance Hospital (Seoul, Korea) of the Yonsei University Health System; approval no. YUMC 4-2011-0872), and signed informed consent was obtained from all subjects. Human peripheral blood was obtained from relatively healthy subjects without serious cardiac disease at Yonsei University Health System (Seoul, Korea). The clinical profiles of patients are listed in Supplementary Table S1. All patients did not had tachycardia at least 1 month before blood sampling.

Cell culture

H9C2, AC16, and HL-1 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). H9C2 and AC16 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; LM001-05, Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS; US-FBS-500, Young In Frontier, Seoul, Korea) and 1% penicillin-streptomycin (10378016, Gibco, Grand Island, NY, USA) at standard culture conditions (37°C, 5% CO₂, and 21% O₂). In addition, HL-1 cells were cultured in Complete Claycomb Medium (51800C, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% FBS, 1% penicillin-streptomycin, 100 µM norepinephrine (A0937, Sigma-Aldrich; Merck KGaA), and 4 mM L-glutamine (TMS-002-C, Sigma-Aldrich; Merck KGaA) in plates coated with 12.5 µg/ml fibronectin (F1141, Sigma-Aldrich; Merck KGaA) and 0.02% gelatin (214340, BD Biosciences Franklin Lakes, NJ, USA). For hypoxia experiments, cells were placed in Forma™ Series II 3131 Water-Jacketed CO₂ Incubators (Thermo Fisher Scientific, Rockford, IL, USA) with 94% N₂, 5% CO₂, and 1% O₂ at 37°C for 24 h.

Cell viability assay

Cell viability was assessed with CellTiter 96® Aqueous One Solution Cell Proliferation Kit (G3580, Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, H9C2 cells were seeded in 96-well microplates and treated with PB-sEVs. After treatment, cells were incubated with 20 µl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution at 37°C for 2 h,
and the absorbance was measured at 490 nm using a microplate reader (VersaMax; Molecular devices, Sunnyvale, CA, USA).

**sEVs purification**

sEVs were isolated from human peripheral blood using the ExoQuick™ Exosome Precipitation Kit (EXOQ20A-1, System Biosciences, Palo Alto, CA, USA) according to the manufacturer’s protocol. Briefly, 1 ml of human peripheral blood was centrifuged at 3,000 x g for 15 min to remove cellular debris and the supernatant was collected. Then, a total of 250 μl of supernatant was mixed with 63 μl of ExoQuick™ Exosome Precipitation solution for 30 min at 4°C and centrifuged at 1,500 x g for 30 min at 4°C. sEVs pellets were resuspended in 200 μl of Dulbecco’s phosphate-buffered saline (DPBS), aliquoted, and stored at −80°C.

**Transmission electron microscopy**

For transmission electron microscopy (TEM), sEVs were adsorbed to a Formvar-carbon-coated electron microscope grid (Leica Microsystems, Inc., Buffalo Grove, IL, USA) for ~1 min. Then, the samples were stained with 2% uranyl acetate and observed using a JEM-1011 electron microscope (JEOL Ltd., Tokyo, Japan).

**Nanoparticle tracking analysis**

Nanoparticle tracking analysis (NTA) was performed using a NanoSight LM10 instrument (Malvern Instruments, Ltd., Malvern, UK), which captures a video file of sEVs moving under Brownian motion. The following settings were used: measurement temperature of 22°C, 30 frames per second, and a measurement time of 60 seconds. The size distribution and concentration of sEVs were analyzed using NTA v.2.3 software (Malvern Panalytical, Ltd., Malvern, UK) by applying the Strokes-Einstein equation.

**Fluorescence labeling and internalization of sEVs**

Isolated sEVs were labeled using the PKH26 Red Fluorescent Cell Linker kit (PKH26GL, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s protocol. Next, unlabeled or PKH26 red fluorescent dye-labeled sEVs were added to H9C2 cells and incubated for 24 h at 37°C. The cells were washed three times with DPBS, fixed with 4% paraformaldehyde for 30 min, and then stained with 4’,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. The internalization of sEVs by H9C2 cells was observed using a confocal microscope (Zeiss LSM 710; Carl Zeiss, Jena, Germany).
To examine and visualize the localization of miRNAs, FAM-labeled scrambled negative control miRNA were loaded into PKH26 red fluorescent dye-labeled sEVs. After incubation of FAM-labeled scrambled negative control miRNA-loaded PKH26-labeled sEVs for 24 h at 37°C, H9C2 cells were washed three times with DPBS, fixed with 4% paraformaldehyde, stained with 4’,6-diamidino-2-phenylindole (DAPI), and observed using a confocal microscope (Zeiss LSM 710; Carl Zeiss, Jena, Germany).

**miRNA transfection into sEVs**

To transfect miRNAs into PB-sEVs, we used the Exo-Fect™ Exosome Transfection Kit (EXFT20A-1, SBI System Biosciences, USA) following the manufacturer’s instructions. The transfected sEVs were incubated with RNase A (12091021, Thermo Fischer Scientific, USA) for 30 min at 37°C to degrade unloaded miRNAs after using the Exo-Fect™ Exosome Transfection kit. Then, the transfected sEVs were washed twice with Amicon® Ultra Centrifugal Filters (UFC8010, Merck Millipore Ltd., Tullagreen, Ireland) with cold DPBS and used for further experiments.

**Cell transfection**

The negative control miRNA (NC), miR-21, anti-miR-21, negative control small interfering RNA (siRNA), and STRN-siRNA (si-STRN) were synthesized by Bioneer (Daejeon, Korea). After the H9C2 cells had reached approximately 60-80% confluence, the cells were transfected with Lipofectamine™ RNAiMAX (13778150, Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. After transfection for 48 h, the cells were harvested for subsequent analysis.

**Luciferase reporter assay**

The 3’-untranslated region (UTR) of STRN fragment containing the predicted wild-type (WT) binding sites of miR-21 and mutated (MUT) binding sites was amplified and inserted into the pmirGLO vector (E1330, Promega, USA). For luciferase activity analysis, H9C2 cells were seeded in 24-well plates, and co-transfected with the constructed pmirGLO vector and miR-21 or negative control miRNA (NC) using Lipofectamine® 3000 (L3000001, Thermo Fisher Scientific, USA). After 48 h, luciferase activity was measured with the Dual-Glo® Luciferase Reporter Assay System (E2920, Promega, USA) according to the manufacturer’s instructions.
Nitric oxide (NO) measurements

The NO levels were evaluated by colorimetric OxiSelect™ In Vitro Nitric Oxide Assay Kit (STA-802, Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s protocol. Briefly, cell-culture media were obtained after transfection for 48 h and centrifuged at 600 x g for 5 min to remove debris. The supernatants were added to 96-well plates and the reagents were added and incubated at room temperature. The absorbance was measured at 540 nm, by a microplate reader (VersaMax; Molecular devices, Sunnyvale, CA, USA).

Quantitative RT-PCR

To analyze the expression of mRNAs and miRNAs, the total RNA from cultured cells and mice was extracted using the miRNeasy® Mini Kit (217004, Qiagen, Hilden, Germany) according to the manufacturer’s protocol. For cDNA synthesis, the miRNA 1st-Strand cDNA Synthesis Kit (600036, Agilent Technologies, Santa Clara, CA, USA) and the High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Darmstadt, Germany) were used. Quantitative RT-PCR was performed using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (600882, Agilent Technologies, USA) on an AriaMx Realtime PCR System (Agilent Technologies, USA). The PCR primers were synthesized by Cosmogenetech (Daejeon, Korea) and shown as follows: miR-21 5’-CGCCGTAGCTTATCAGACTG-3’; U6 5’-CTCGCTTCGGCAGCACA-3’; STRN-forward 5’-TTACTGGAGCGGATCTCAGG-3’ and STRN-reverse 5’-GCCACCAGAACTGGATATCAT-3’; β-actin-forward 5’-TTGCTGATCCACATCTGCTG-3’ and β-actin-reverse 5’-GACAGGATGCAGAAGGAGAT-3’. The relative expression levels of the mRNAs and miRNAs were calculated using the 2 ^ −ΔΔCq method [22] and normalized against U6 and β-actin, respectively. All experiments were repeated at least three times.

To evaluate RNase resistant rate of sEVs, the miRNA-loaded PB-sEVs were untreated or treated with RNase A (100 μg/ml) for 30 min at 37 °C. Then, the expression of retained miR-21 was measured by quantitative RT-PCR and normalized to U6.

Western blot analysis

Total protein was extracted with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (WSE-7420, ATTO, Tokyo, Japan).
The protein concentration was determined with Pierce™ 660 nm Protein Assay Reagent (22660, Thermo Fisher Scientific, USA), and the proteins were separated with 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein bands were transferred onto polyvinylidene fluoride (PVDF) membranes (IPVH00010, EMD Millipore, Bedford, MA, USA) and the membranes were blocked with 5% bovine serum albumin (BSA) in TBS-Tween 20 (TBS-T). The membranes were incubated overnight at 4°C with primary antibodies diluted according to the instructions, followed by probing with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000; sc-516102, Santa Cruz Biotechnology Inc., Dallas, TX, USA) and visualization using an enhanced chemiluminescence kit (1705061, Bio-rad Laboratories Inc., CA, USA). CD9 (EXOAB-CD9A-1) and CD63 (EXOAB-CD63A-1) were obtained from System Biosciences (USA); Alix (sc-53540), CD81 (sc-166029), NOS3 (sc-376751), STRN (sc-136084), and β-actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology Inc. (USA). β-actin was used as the loading control.

Animal experiments

All animal studies were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (approval reference no. 2018–0143). The present study conformed to the rules of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). All procedures were conducted at Yonsei University College of Medicine (Seoul, Korea). Eight weeks old male C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea), maintained at 20±0.5°C with a humidity of 60±5% under 12h-light/12h-dark cycles, and allowed free access to food and water. The mice were anesthetized by intraperitoneal injections of tiletamine-zolazepam (Zoletil 50, 30 mg/kg) and xylazine (Rompun®, 10 mg/kg) and then intubated and ventilated using a ventilator (Harvard Apparatus Co., Millis, MA, USA). After thoracotomy was performed, the heart was exposed and MI was induced by ligation of the left anterior descending (LAD) coronary artery with a 6-0 silk suture (Ethicon, Somerville, NJ, USA). Then, 100 μl of sEVs containing negative control miRNA (NC), miR-21, or anti-miR-21 (approximately 8-10 × 10^8 particles) were administered by intramyocardial injection into the infarct border zone immediately [23]. The chests were sutured after injections, and the animals were meticulously cared for. The mice
were sacrificed 1 week after treatment, and the heart tissue was quickly collected and fixed in 4% formaldehyde at room temperature. The mice were euthanized by the extraction of heart tissue under anesthesia.

To determine the in vivo distribution of sEVs, PKH26-labeled PB-sEVs were injected into the hearts of MI mice, and mice were sacrificed at 24 h post-injection. PKH26 fluorescence signals were detected with an IVIS® Spectrum in vivo imaging system (PerkinElmer, Waltham, MA, USA). The hearts after 24 h post-injection of PKH26-labeled PB-sEVs were sliced into 4-μm-thick sections and immunofluorescent-stained with anti-cardiac troponin I (cTnI) (ab47003, Abcam, Cambridge, UK). The internalization of sEVs was observed using a confocal microscope (Zeiss LSM 710; Carl Zeiss, Jena, Germany).

To assess cardiac function, echocardiography was performed using a Vevo 2100 system (VisualSonics, Toronto, Ontario, Canada), and left ventricle ejection fraction (LVEF) and left ventricle fractional shortening (LVFS) were measured.

Tissues, including heart, liver, spleen, kidney and lung, were fixed in 4% formaldehyde, embedded in paraffin, and chopped into 4-μm-thick sections. Then, the sections were mounted on normal glass slides and stained with hematoxylin & eosin (H&E). Individual sections of different organs were observed using an inverted microscope (Olympus, Tokyo, Japan).

For immunofluorescence, heart tissue sections were deparaffinized and blocked with 5% bovine serum albumin (BSA). Specimens were incubated with anti-cardiac troponin I (cTnI) (ab47003, Abcam, UK) and anti-NOS3 (ab76198, Abcam, UK) overnight at 4°C, and all specimens were subsequently incubated with appropriate fluorescence-labeled secondary antibodies (ab150077, ab150108, Abcam, UK) for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). All slides were mounted and observed under a confocal microscope (Zeiss LSM 710; Carl Zeiss, Jena, Germany).

Statistical analysis

The data are represented as mean ± standard deviation (SD), and statistical significance between the experimental groups was determined using either the unpaired, two-tailed Student's t-test, or one-way analysis of variance (ANOVA) with a Student-Newman-Keuls post hoc test. Analyses were performed with GraphPad Prism software version 8.0, and p < 0.05 was considered statistically significant.
Results

Expression of miR-21 in hypoxic conditions and its effect on NOS3 expression

We investigated the effects of MI on miR-21 expression and found that miR-21 levels were significantly upregulated in MI mice, compared to those in non-MI mice (p < 0.01) (Figure 1A). Consistent with the changes in miR-21 expression in mice, miR-21 levels were also upregulated in cultured H9C2 cells exposed to hypoxia. The expression of miR-21 gradually increased in a time-dependent manner (p < 0.05), and peaked by 1.95-fold at 36 h after hypoxia treatment (Figure 1B). Recently, miR-21 has been shown to play important roles in cardiovascular disorders, and dysregulation of miR-21 expression is known to be associated with MI [16,24]. We examined several potential target pathways involved in miR-21-mediated effects on MI using miRNA databases and target prediction tools such as TarBase, TargetScan, and miRwalk (Figure 1C). Among the possible miR-21-mediated pathways, nitrogen metabolism remains relatively unexplored compared to other pathways; thus, we focused on nitrogen metabolism. Accordingly, we examined the effects of MI on NOS3 expression to investigate the possibility of its correlation with miR-21 levels. As shown in Figure 1D and E, NOS3 expression decreased in both MI mice and hypoxic H9C2 cells. Similar to the results on H9C2 cells, it was observed that miR-21 levels were also gradually increased in both AC16 and HL-1 cells exposed to hypoxia. In addition, NOS3 expression was decreased under the hypoxic conditions of both cell lines (Supplementary Figure S1). Together, these results suggest the possibility that upregulation of miR-21 expression may lead to a significant decrease in NOS3 expression, resulting in cardiac dysfunction.

Characterization and in vitro functional validation of PB-sEVs

To devise an effective strategy of miRNAs delivery for the clinical application, we isolated PB-sEVs from human peripheral blood. TEM, NTA, and western blot analysis were used to characterize the morphology, size distribution/concentration, and protein markers of PB-sEVs, respectively. TEM of the isolated PB-sEVs revealed typical round particles with a diameter of 30-200 nm (Figure 2A). The concentration of sEVs with sizes from 30 nm to 200 nm was $2.30 \pm 0.34 \times 10^8$ particles/ml, and the mean diameter was $103.3 \pm 10.4$ nm, as measured with NTA (Figure 2B). The results of western blotting showed that the isolated
sEVs were enriched in EV markers (Alix, CD9, CD63, and CD81) (Figure 2C). To visualize uptake of PB-sEVs, PB-sEVs were labeled with PKH26 red fluorescent dye and incubated with H9C2 cells. Most H9C2 cells were positive for PKH26 fluorescence, indicating that the sEVs were internalized into the perinuclear compartment (Figure 2D). In addition, PB-sEVs were taken up by H9C2 cells, but showed no observable toxicity in a dose- and time-dependent manner (Supplementary Figure S2A, B) or did not active inflammatory reactions at 24 h after treatment (Supplementary Figure S2C).

Next, we determined whether PB-sEVs may serve as an ideal delivery system for miRNAs. Isolated PB-sEVs were loaded with either miR-21 or anti-miR-21 using Exo-TargetTM Exosome Transfection reagent (Figure 2E). No significant change was observed in the morphology, size distribution, concentration, and marker proteins of the sEVs after miRNAs loading (Supplementary Figure S3A-C). Under both normoxic and hypoxic conditions, miR-21 expression in H9C2 cells was regulated by miR-21- or anti-miR-21-loaded PB-sEVs. The cells treated with miR-21-loaded PB-sEVs showed higher miR-21 expression than normoxic and hypoxic controls, whereas the cells treated with anti-miR-21-loaded PB-sEVs showed a decrease in miR-21 expression (Figure 2F). The uptake of miRNA-loaded PB-sEVs led to the regulation of miR-21 levels in H9C2 cells in a dose-dependent manner. PB-sEVs alone had no effect on miR-21 expression (Figure 2G). FAM-labeled scrambled negative control miRNA also co-localized with PKH26-labeled PB-sEVs and aggregated at the perinuclear region (Supplementary Figure S3D).

To evaluate the RNase resistance rate of miRNA-loaded PB-sEVs, we treated PB-sEVs with RNase A, an enzyme capable of degrading RNA. RNase treatment alone had no effect on the total quantity of miR-21 in the sEVs, indicative of their RNase resistance ability (Figure 2H). Furthermore, western blot analysis showed that anti-miR-21-loaded PB-sEVs completely reversed the inhibitory effect of miR-21-loaded PB-sEVs on NOS3 protein expression levels (Figure 2I). Taken together, PB-sEVs may serve as an ideal in vivo miRNAs delivery system, and anti-miR-21-loaded PB-sEVs may be useful as a therapeutic tool.

**Effect of anti-miR-21-loaded PB-sEVs in mouse infarcted heart**

We examined the effects of anti-miR-21-loaded PB-sEVs on the recovery of
infarcted hearts (Figure 3A). We first evaluated the uptake and distribution of sEVs in vivo. PKH26 fluorescence was concentrated in the hearts 24 h after intramyocardial injection of PKH26-labeled PB-sEVs, observed using an IVIS® Spectrum in vivo imaging system (Figure 3B). In addition, we performed immunofluorescent staining for cardiomyocyte-specific (cTnI⁺) antigens. PKH26-labeled PB-sEVs co-localized with cTnI⁺ cells at 24 h after injection (Figure 3C). Therefore, these results suggested that PB-sEVs effectively distributed into cardiomyocytes in the hearts. We did not observe any inflammation or changes in morphology of the liver and other organs after PB-sEVs injection (Supplementary Figure S4A). The results of quantitative RT-PCR analysis confirmed that miRNA-loaded PB-sEVs effectively regulated miR-21 levels in the hearts after injection, indicating that PB-sEVs successfully delivered of miRNAs to mouse heart (Figure 3D). Taken together, these results support PB-sEVs as a suitable approach for the effective delivery of therapeutic miRNAs in vivo.

After validating the potential utility of the PB-sEVs platform in vivo, we evaluated whether anti-miR-21-loaded PB-sEVs may lead to cardiac function recovery after MI. As shown in Figure 3E, injections of anti-miR-21-loaded PB-sEVs resulted in a significant improvement in post-MI survival rate. The survival rate of mice injected with miR-21-loaded PB-sEVs was 30% at 1 week post-MI, whereas those injected with anti-miR-21-loaded PB-sEVs showed an increase in survival rate to 62.5%. Cardiac function was determined at 1 week after MI by echocardiography. Mice treated with anti-miR-21-loaded PB-sEVs showed significant augmentation in left ventricle ejection fraction (LVEF) and left ventricle fractional shortening (LVFS), compared to those treated with miR-21-loaded PB-sEVs (Figure 3F). These results show that anti-miR-21-loaded PB-sEVs reduced the post-MI deterioration of cardiac function. In addition, the relative levels of collagen I and collagen III, which are known as myocardial fibrosis markers, were dramatically reduced upon injection of anti-miR-21-loaded PB-sEVs, compared with injection of miR-21-loaded PB-sEVs or negative control miRNA (NC)-loaded PB-sEVs (Figure 3G). Taken together, our in vivo data suggest that anti-miR-21-loaded PB-sEVs exert a cardioprotective effect against MI.

Identification of STRN as a direct target of miR-21

To elucidate the molecular mechanisms underlying the MI pathogenesis driven by
miR-21, we performed bioinformatics analysis using TargetScan algorithm to predict the potential target genes of miR-21. Among the candidate genes, STRN contained an miR-21-binding site at the 3'-UTR (Figure 4A). STRN is a dynamic protein that binds to caveolin-1 and calmodulin, which serve as regulators of MI and could activate NOS3 [25,26]. Considering that miR-21 correlates with NOS3 expression in vitro, STRN may be regulated by miR-21. To test whether STRN is a direct target of miR-21, we generated luciferase reporter constructs by cloning either the wild-type (WT) or mutated (MUT) 3'-UTR of STRN into a pmiR-report vector and co-transfected negative control miRNA (NC) or miR-21 in H9C2 cells. The luciferase activity significantly reduced in the wild-type (WT) 3'-UTR constructs, but no effect was observed upon mutation of the miR-21-binding sites (MUT) (Figure 4B). The mRNA and protein levels of STRN were much lower in MI mice and H9C2 cells under hypoxia than in their representative controls (Figure 4C-F). To further investigate whether miR-21 downregulates STRN expression in H9C2 cells, miR-21 or anti-miR-21 were transfected into cells and the mRNA and protein levels of STRN were evaluated. After 48 h of transfection, treatment with miR-21 significantly decreased the mRNA and protein levels of STRN, whereas anti-miR-21 markedly increased both the mRNA and protein levels of STRN (Figure 4G, H). Taken together, these findings suggest that miR-21 inhibited STRN expression by directly binding to its 3'-UTR.

**Effect of STRN on NOS3 expression**

Our finding that STRN is a direct target gene of miR-21 raised the possibility that STRN levels may intermediate between miR-21 and NOS3 in MI. To test this possibility, we confirmed the role of STRN using STRN-specific siRNAs (si-STRN) inhibiting STRN expression. H9C2 cells transfected with STRN-specific siRNAs (si-STRN) showed a ~50% depletion in STRN level. In addition, NOS3 expression significantly decreased upon downregulation of STRN expression (Figure 5A). We then investigated the effect of miR-21 on STRN expression in H9C2 cells exposed to hypoxia. After exposure to hypoxia for 24 h, STRN expression was significantly downregulated and further decreased in the cells transfected with miR-21, whereas the change was reversed upon anti-miR-21 treatment. Western blot analysis also demonstrated the downregulation of NOS3 expression after hypoxia; the treatment with anti-miR-21 reversed this effect on NOS3 expression (Figure 5B). Likewise, in AC16 and HL-1 cells, hypoxia reduced the STRN and NOS3 expression
compared with normoxic controls, and their expressions were downregulated and upregulated by transfection with miR-21 or anti-miR-21, respectively (Supplementary Figure S5). Furthermore, we found that NO production was reduced after exposure to hypoxia for 24 h compared with normoxic controls. Also, miR-21 overexpression could impair NO production, while anti-miR-21 enhanced NO production, respectively. Taken together, miR-21 attenuated the expression of NOS3, which in turn repressing NO production (Figure 5C). Consistently, the in vivo results revealed that STRN expression levels were significantly downregulated in MI mice compared with non-MI mice, and further decreased in the mice treated with miR-21-loaded PB-sEVs. In contrast, the mice treated with anti-miR-21-loaded PB-sEVs significantly increased STRN expression. Moreover, it was observed that the downregulation of NOS3 expression after MI, which was significantly altered following treatment with miR-21-loaded PB-sEVs or anti-miR-21-loaded PB-sEVs (Figure 5D). In addition, immunofluorescence analysis showed that the loose distribution of NOS3 was reversed after treatment with anti-miR-21-loaded PB-sEVs specific for cardiomyocytes (Figure 5E). Overall, miR-21 could lead to a significant decrease in NOS3 levels in response to decreases in STRN expression both in vitro and in vivo.
Discussion

The major highlights of the present study are as follows: miR-21 expression was significantly upregulated in hypoxic conditions and correlated with the expression of NOS3, thereby conferring cardiac dysfunctions in response to MI. Second, the regulation of miR-21 expression using PB-sEVs had an impact on the pathophysiology of MI in mouse model. Finally, miR-21 targeted and suppressed the mRNA and protein expression of STRN, which may regulate NOS3 expression.

miR-21 and NOS3

miR-21 plays a crucial role in response to MI through numerous target pathways such as apoptosis, fibrosis, and inflammation [27-29]. Of these, nitrogen metabolism has not been well studied; hence, we aimed to determine the new target genes of miR-21 that were related to nitrogen metabolism.

NO has been widely investigated as a mediator of numerous physiological and pathological processes [30,31]. In cardiovascular diseases, NO has been shown to exert cardioprotective roles [31,32]. Heart failure is associated with a decrease in the bioavailability of NO, resulting in deleterious effects through the acceleration of heart failure [33]. Thorsten et al. reported that endothelial-derived NOS3/NO in cardiomyocytes protected cardiomyocytes from ischemia/reperfusion injury [34]. Moreover, many studies have consistently demonstrated that NOS3 activation are thought to be involved in cardioprotective effects and significantly reduced myocardial ischemia/reperfusion injury [35-37]. For instance, NOS3 regulates the myofilament Ca\(^{2+}\) sensitivity via PKG-mediated phosphorylation, myocardial oxygen consumption, and intracellular cAMP levels, which impacts cardiac systolic and diastolic function [38,39]. In addition, NOS3 serves a critical function in anti-inflammatory and anti-apoptotic mechanisms via the tumor necrosis factor-alpha (TNF-α) signaling pathway [40]. Furthermore, the NOS3-modulated expression of stromal cell-derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) has been found to regulate cardiac fibrosis [41]. Altogether, these results suggest that NOS3 may impact on the cardiovascular system. In this study, our data showed that NOS3 expression was decreased in hypoxic conditions, however, that is increased after treatment with anti-miR-21-loaded PB-sEVs. Therefore, downregulation of miR-21 levels increased NOS3
expression, promoted NO production, and eventually contributed to sustained cardioprotection.

**PB-sEVs as a therapeutic tool**

Although significant improvements have been made in the field of nucleic acid-based therapies, the studies evaluating suitable carriers for molecules such as miRNAs are still in progress [42]. The main problem with miRNAs delivery is their instability and inability to effectively penetrate the hydrophobic cell membranes [43]. To circumvent this problem, several approaches such as viral vectors and liposomes have been developed to transport miRNAs, these are associated with shortcomings such as vehicle toxicity, low efficiency of delivery, and immunogenic/inflammation responses [44].

In recent years, sEVs have been introduced as alternative to delivery vehicles of therapeutic miRNAs [45]. Accumulated studies have demonstrated that sEVs not only effectively protect miRNAs from degradation but also rapidly transport them into recipient cells without inducing toxicity and adverse immune reactions [46]. Thus, sEVs have gained attention in nanomedicine applications. However, the yield of EVs isolated from cell-culture media is usually very low, thereby restricting their widely use in research and clinical therapy [6,8]. In addition, if immortalized cells are used to isolate EVs instead as to get sufficient EVs, there’s a risk of transferring oncogenic DNA and retrotransposon elements [47]. Given that peripheral blood can be obtained from any human subject readily and has been used safely and routinely for blood transfusions, isolating sEVs from human peripheral blood itself may allow for effective yield system. We have previously reported that PB-sEVs may serve as a miRNAs delivery system to overcome these limitations [23]. Here, we provided further evidence that PB-sEVs may act as carriers and efficient delivery vehicles for miR-21 both *in vitro* and *in vivo*. PB-sEVs significantly regulated miR-21 levels and enhanced the transcriptional level of the corresponding target genes in both *in vitro* and *in vivo* studies. The use of PB-sEVs as vehicles for the transport of miR-21 may pave the way to strategies for MI treatment.

**STRN as a direct target of miR-21**

STRN is a 780 amino acid protein identified and cloned in the late 1990s [48]. At
the molecular level, STRN has four protein–protein interacting domains as follows: a caveolin-1 (cav-1)-binding domain; a coiled-coil structure; a Ca\textsuperscript{2+}-calmodulin-binding domain, and a large WD-repeat domain at the C-terminal region [25]. STRN also binds to regulatory proteins such as cav-1, Ca\textsuperscript{2+}-calmodulin, 3 G\textalpha, and phosphatase 2A, and could regulate transduction molecules such as NOS3 [25,48]. Lu Q et al. reported STRN as the molecular anchor that localizes estrogen receptor-α (ERα) to the plasma membrane and organizes the ERα-NOS3 membrane signaling complex, a critical regulator of many physiologic and pathophysiologic processes [49]. Garza AE et al. revealed the critical role of STRN, possibly through the modulation of endothelial NO-cGMP pathway, in the regulation of vascular function and blood pressure during changes in dietary sodium intake [50]. Thus, STRN may increase NOS3 expression/activity. Our results show that miR-21 directly targeted the 3’-UTR of STRN and acted as a regulator of STRN mRNA and protein expression, which may result in changes of NOS3 expression levels.

**Study limitations**

The present study has a few limitations. First, considering the variety and complexity of miR-21 targets, the miR-21-STRN-NOS3 pathway may only be one of the multiple pathways that regulate the pathophysiological MI processes; Thus, there is concern for other possible adverse effects. A possible way to overcome this might be a combined use of other miRNAs or diverse molecules with functionality. Second, the direct investigation of STRN and NOS3 in vivo using knockout animal model was not performed in the present study. Also, there is a possibility for effects by other cells and a paucity of sufficient knowledge on the physiological role of STRN in the cardiovascular system. Therefore, further studies will be needed to fully understand the implications of STRN and NOS3. Third, although intramyocardial injection can be highly effective route for sEVs delivery precisely to cardiac in consideration of issues with systemic administration such as mainly accumulation in the liver, spleen, and lungs or rapid clearance of sEVs by the reticular-endothelial system (e.g., macrophages) [46,51,52], this method is not preferable in clinical setting. A targeted delivery strategy to cardiac by additional genetic modification of sEVs is a relevant approach to resolve concerns with intramyocardial injection and may facilitate efficient therapy in clinical settings.
Taken together, we present the evidence of the critical role of miR-21 in the pathogenesis of MI by uncovering the novel function of the miR-21/STRN axis. Furthermore, our results highlight the power of miR-21 regulation using PB-sEVs and provide clues for substantially therapeutic approach for MI.

**Clinical perspectives**

- PB-sEVs may serve as an effective alternatives for MI treatment.
- The miR-21/STRN axis would provide a novel clue for improving the pathogenesis of MI.
- Regulation of miR-21 expression using PB-sEVs provide a potential therapeutic approach for the treatment of MI.

**Competing interests**

The authors declare that there are no competing interests associated with the manuscript.

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**Author Contribution**

JK and BJ designed the experiments. JK conducted the experiments. JK, HK and DM analyzed the data. JK, NY and BJ wrote the manuscript. All authors read and approved the manuscript.
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Figure Legends

Figure 1. The expression of miR-21 is induced by MI and miR-21 controls NOS3 expression.

(A, B) Quantitative RT-PCR analysis of miR-21 expression in mouse heart tissue (A) and H9C2 cells exposed to hypoxia in a time-dependent manner (B). Data are normalized to U6. (C) Screening table for putative target pathways of miR-21 that may be related to the pathophysiology of MI. (D, E) Representative blots and quantified data showing NOS3 expression in mouse heart (D) and H9C2 cells exposed to hypoxia in a time-dependent manner (E). *p < 0.05; **p < 0.01.

Figure 2. PB-sEVs effectively deliver miRNAs to recipient cells.

(A) Representative TEM image of PB-sEVs (scale bar: 100 nm). (B) PB-sEVs size distribution and concentration, as determined with NTA. (C) Western blot analysis of EV marker proteins, Alix, CD9, CD63, and CD81 and intercellular protein β-actin in H9C2 cell lysates and PB-sEVs. (D) Representative confocal microscopy images of H9C2 cells incubated with unlabeled or PKH26 (red)-labeled PB-sEVs. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (E) Schematic depiction of the experimental procedure for miRNAs delivery by PB-sEVs. (F) Quantitative RT-PCR analysis of miR-21 expression under normoxic and hypoxic conditions. Data are normalized to U6. *p < 0.05; **p < 0.01. (G) Expression of miR-21 in H9C2 cells incubated with PB-sEVs alone, negative control miRNA (NC)-loaded PB-sEVs, or miRNA-loaded PB-sEVs in a dose-dependent manner. ***p < 0.001. (H) Expression of miR-21 after treatment with RNase A for 30 min. ***p < 0.001. (I) Representative blots and quantified data showing NOS3 expression in the indicated group. *p < 0.05.

Figure 3. PB-sEVs effectively deliver miRNAs to mouse heart and treatment with anti-miR-21-loaded PB-sEVs protects the heart against MI.

(A) Schematic depiction of the in vivo experimental design and analysis. (B) Representative images of mice injected with unlabeled or PKH26-labeled PB-sEVs in the heart, as captured using IVIS® Spectrum in vivo imaging system. (C) Representative confocal microscopy images of PKH26 (red)-labeled PB-sEVs taken by cTnI+ cardiomyocytes (scale bar: 20μm). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (D) Expression of...
miR-21 in the mice injected with negative control miRNA (NC), miR-21, or anti-miR-21-loaded PB-sEVs 24 h post-treatment. (E) Kaplan-Meier curves showing survival rate (n = 8 in each group). (F) Quantified data showing left ventricle ejection fraction (LVEF) and left ventricle fractional shortening (LVFS) at 1 week post-MI. (G) Representative blots and quantified data showing the expression of collagen I and collagen III in the indicated group. *p < 0.05; **p < 0.01; ***p < 0.001.

**Figure 4. miR-21 directly targets STRN.**

(A) Sequence alignment of miR-21 and 3'-UTR of STRN using TargetScan algorithm. (B) Luciferase assays of H9C2 cells co-transfected with negative control miRNA (NC) or miR-21 and reporter plasmids containing 3'-UTR of wild-type (WT) or mutated (MUT) miR-21-binding sites. (C-F) mRNA and protein expression levels of STRN in mouse heart and H9C2 cells exposed to hypoxia in a time-dependent manner. (G) mRNA expression of STRN in H9C2 cells transfected with negative control miRNA (NC), miR-21, or anti-miR-21. (H) Representative blots and quantified data showing STRN expression in H9C2 cells transfected as in G. *p < 0.05; **p < 0.01.

**Figure 5. miR-21 regulates STRN/NOS3 signaling pathway.**

(A) Representative blots and quantified data showing STRN and NOS3 expression in H9C2 cells transfected with siRNA negative control (si-NC) or siRNA targeting STRN (si-STRN). (B) Representative blots and quantified data showing protein expression in H9C2 cells treated with negative control miRNA (NC), miR-21, or anti-miR-21, followed by hypoxia treatment. β-actin was used as a loading control. *p < 0.05; **p < 0.01. (C) NO levels in H9C2 cells treated as in B. *p < 0.05; **p < 0.01. (D) Representative blots and quantified data showing protein expression in the indicated mice groups. *p < 0.05. (E) Representative confocal microscopy images of cTnI+NOS3+ cardiomyocytes (scale bar: 20μm). Green fluorescence indicated NOS3+ cells. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue).
**Figure A**

| STRN 3' UTR Mut | 5' ...AAATTCAAGGTATGGTATTGCAT... |
| STRN 3' UTR WT | 5' ...AAATTCAAGGTATGGATAAGCTA... |
| mir-21-5p | 3' AGUUGUAGUCAGAC — UAUUCGAU |

**Figure B**

| WT STRN 3' UTR | MUT STRN 3' UTR |
|----------------|----------------|

| Relative luciferase ratio | NC | miR-21 |
|---------------------------|----|--------|
| WT STRN 3' UTR            | 1  | 0.5    |
| MUT STRN 3' UTR           | 1  | 0.5    |

**Figure C**

| Relative expression of STRN mRNA | Non-MI | MI |
|----------------------------------|-------|----|
| Non-MI                           | 1.0   | 0.5|
| MI                               | 0.5   | 0.5|

**Figure D**

| Relative expression of STRN/β-actin (%) | Non-MI | MI |
|----------------------------------------|-------|----|
| Non-MI                                 | 1.0   | 0.5|
| MI                                     | 0.5   | 0.5|

**Figure E**

| Relative expression of STRN mRNA | 0 | 12 | 24 | 36 |
|----------------------------------|---|----|----|----|
| 0                                | 1.0| 1.2| 1.1| 1.0|
| 12                               | 1.2| 1.0| 1.1| 0.9|
| 24                               | 1.1| 1.0| 1.0| 0.9|
| 36                               | 1.0| 0.9| 0.9| 0.8|

**Figure F**

| Relative expression of STRN/β-actin (%) | 0 | 12 | 24 | 36 |
|----------------------------------------|---|----|----|----|
| 0                                     | 1.0| 1.2| 1.1| 1.0|
| 12                                    | 1.2| 1.0| 1.1| 0.9|
| 24                                    | 1.1| 1.0| 1.0| 0.9|
| 36                                    | 1.0| 0.9| 0.9| 0.8|

**Figure G**

| Relative expression of STRN mRNA | NC | miR-21 | anti-miR-21 |
|----------------------------------|----|--------|-------------|
| NC                               | 1.0| 1.2    | 1.0         |
| miR-21                           | 1.2| 1.0    | 1.1         |
| anti-miR-21                      | 1.0| 0.9    | 0.9         |

**Figure H**

| Relative expression of STRN/β-actin (%) | NC | miR-21 | anti-miR-21 |
|----------------------------------------|----|--------|-------------|
| NC                                     | 1.0| 1.2    | 1.0         |
| miR-21                                 | 1.2| 1.0    | 1.1         |
| anti-miR-21                            | 1.0| 0.9    | 0.9         |
