DNA Hypomethylation of miR-30a Mediated the Protection of Hypoxia Postconditioning Against Aged Cardiomyocytes Hypoxia/Reoxygenation Injury Through Inhibiting Autophagy

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Background: Ischemic postconditioning (IPostC) is an endogenous protective mechanism to reduce ischemia-reperfusion (I/R) injury. However, whether IPostC protects aged cardiomyocytes against I/R injury is not fully understood. Considering the protective function of microRNA 30a (miR-30a) against ischemia-induced injury in H9C2 cells, its role in the protective effects of IPostC on I/R injury of aged cardiomyocytes was investigated further.

Methods and Results: To mimic I/R and IPostC in vitro, the aged cardiomyocyte model for hypoxia postconditioning (HPostC) treatment was established by 9 days of incubation with 8 mg/mL D-galactose and then followed by exposure to hypoxic environment. HPostC significantly alleviated hypoxia/reoxygenation (H/R) injury and reduced autophagy of aged cardiomyocytes, as evidenced by decreased LC3B-II expression and increased p62 by Western blot. Quantified by quantitative real-time polymerase chain reaction (qRT-PCR), miR-30a was increased in aged cardiomyocytes treated with HPostC compared with I/R injury group. Overexpression of miR-30a by LV3-rno-miR-30a mimic promoted cardioprotective effect of HPostC in aged cardiomyocytes by suppressing BECN1-mediated autophagy, all of which was abrogated by knockdown of miR-30a expression. Epigenetic analyses demonstrated that HPostC reduced DNA methyltransferase 3b-mediated DNA hypomethylation levels at miR-30a promoter, leading to upregulation of miR-30a.

Conclusions: HPostC protected aged cardiomyocytes survival against H/R injury via DNMT3b-dependent activation of miR-30a. miR-30a could be a potential therapeutic target for ischemic myocardial infarction.

Key Words: Autophagy; DNA methylation; Hypoxia postconditioning; Hypoxia/reoxygenation; miR-30a

The incidence and mortality of cardiovascular diseases keep increasing due to aging population in the intensification, as old patients with aged myocardial are more prone to myocardial infarction (MI) and heart failure.^{1,2} After MI, ischemia/reperfusion (I/R) can cause additional injury such as cellular metabolic dysfunction and structural damage due to rapid reperfusion of the ischemic or hypoxia myocardium.^{3,4} It was reported that ischemic postconditioning (IPostC) can significantly protect cardiomyocytes against I/R injury through brief intermittent

episodes of I/R at the onset of reperfusion after a prolonged period of ischemia or hypoxia.⁵ Evidence has indicated the urgent role of autophagy in the I/R injury of the heart by degrading long-lived proteins and organelles of cells.⁶ However, the exact relation and the related mechanism of autophagy in the cardioprotective effects of IPostC on aged myocardial I/R injury remains unclear.

MicroRNAs (miRNAs) are a class of endogenous, non-coding small ribonucleic acids containing 21–24 nucleotides.⁷ Evidence has indicated that miRNAs involved

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Table. Primer Sequences for Real-Time Polymerase Chain Reaction

Gene	GenBank	Primer sequences
β -actin	NM_031144.3	Forward: 5'-TGTCACCAACTGGGACGATA-3' Reverse: 5'-GGGGTGTGTAAGGTCTCAA-3'
DNMT3b	NM_001003959.1	Forward: 5'-GATGATGGAGATGGCTCTGATA-3' Reverse: 5'-GGCTGGAGATACTGTTGCTGTT-3'

in a wide range of biologic processes, such as autophagy,⁸ proliferation,⁹ differentiation, and apoptosis, negatively modulate gene expression through binding of the target gene to the 3'UTR.¹⁰ For example, the regulation of miRNAs on autophagy was mediated by autophagy-related genes such as BECN1, light chain 3B (LC3B) and p62.¹¹ Recently, it was reported that pathophysiological variation, including the process of cardiac remodeling and heart failure, can affect the abundance of many miRNAs.^{12,13} For example, miR-30a was found to abnormally expressed in the cardiac tissue of murine model with myocardial I/R.¹⁴ Although miR-30 has been demonstrated to have an important role in heart disease, whether it can mediate the protective effect of HPostC on I/R injury in aged myocardium has not been investigated.

Aberrant miRNA expression is often associated with epigenetic regulation such as DNA methylation and histone modifications.¹⁵ DNA methylation is recognized as the most important epigenetic regulation for gene expression,¹⁶ which is catalyzed by DNA methyltransferases (DNMTs). Among them, DNMT1 (maintenance DNMT), DNMT3a and DNMT3b (de novo DNMTs) are mainly responsible for modulating and maintaining DNA methylation levels of gene promoter.¹⁷ Alteration of DNA methylation level in miRNA promoter directly results in the change of its expression level.¹⁸ Vera et al¹⁹ reported that the increase of miR-7 methylation level could act as a potential clinical epigenetic biomarker for the identification of ovarian cancer patients with poor response to platinum-derived therapy. Undoubtedly, there is a crucial role for DNA methylation in many types of diseases, but its role in aged myocardial I/R injury is still poorly understood.

In this study, we explored the underlying mechanism of miR-30a in the cardioprotective effect of hypoxia post-conditioning (HPostC) on aged cardiomyocyte hypoxia/reoxygenation (H/R) injury. The results indicated that HPostC upregulated the miR-30a level through DNMT3b-mediated hypomethylation of the miR-30a promoter in aged cardiomyocytes, thereby inhibiting autophagy.

Methods

Cell Culture, Lentiviral and Adenoviral Infection

Rat cardiomyocytes (H9C2 cells) purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in the Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 100 U/mL, 100 μ g/mL streptomycin in a normoxia incubator (5% CO₂ and 37°C). Lentiviral vectors encoding Rattus miR-30a mimic (LV3-rno-miR-30a mimic: 5'-TGTAACATCCTC GACTGGAAG3-3'), miR-30a inhibitor (LV3-rno-miR-30a inhibitor: 5'-CTTCCAGTCGAGGATGTTTACA-3'), and empty vector (LV3-rno-miR-neg: 5'-GTTCTCCGAACG TGTCACGT-3') were obtained from GenePharma Co.

Ltd (Shanghai, China). The adenovirus expressing Rattus BECN1, the adenovirus expressing Rattus shRNA targeting BECN1 and DNMT3b, and negative control adenovirus (Ad-NC) were provided by GenePharma Co. Ltd (Shanghai, China). Cardiomyocytes were infected with lentivirus or adenovirus following the reported protocols.²⁰

Establishment of H/R and HPostC Models

Aged cardiomyocytes were induced by treatment of H9C2 cells with 8 mg/mL D-galactose (Sigma-Aldrich, G0750) for ~9 days. To induce H/R injury, aged cardiomyocytes were cultured at 37°C in a hypoxic incubator (Thermo Fisher Scientific, USA, 3131) filled with pre-mixed gas (5% CO₂, 1% O₂ and 94% N₂) for 180 min followed by 120 min of reoxygenation. To establish HPostC model, aged cardiomyocytes were cultured under hypoxia for 180 min, followed by 3 cycles of 5 min of hypoxia and 5 min of reoxygenation before 120 min reoxygenation (Supplementary Figure A).

Senescence-Associated β -Galactosidase (SA- β -gal) Assay

A senescence-associated- β -galactosidase staining kit (Solarbio Science & Technology Co., Ltd., Beijing, China; G1580) was used according to the manufacturer's instructions. Briefly, the cells were washed in phosphate buffered saline (PBS), fixed in fixative solution for 15 min at room temperature, and incubated with a staining solution mix overnight at 37°C. The percentage of senescent (SA- β -gal-positive) cells was calculated in five randomly chosen fields of view for each sample.

Immunofluorescence and Phalloidin Staining

Aged cardiomyocytes were fixed with 4% paraformaldehyde for immunofluorescence and phalloidin staining. The Rabbit anti-BECN1 antibody (Abcam; ab207612, IF 1:200) was used in this study. Phalloidin conjugated to phalloidin-iFluor™ 488 Conjugate (AAT Bioquest®, USA) was used to stain actin filaments (F-actin). After washing 3 times with PBS, samples were incubated with secondary antibody for 1 h at 37°C, followed by washing with PBS and nuclei staining with 4',6-diamidino-2-phenylindole (DAPI) in the dark. The immunofluorescence images were captured under a Zeiss LSM800 Meta Laser Scanning confocal microscope (Carl Zeiss, Jena, Germany).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from aged cardiomyocytes and reverse-transcribed with a reverse transcriptase kit (Takara, Otsu, Japan). For qRT-PCR, the mRNA level of DNMT3b was analyzed using SYBR Green assay kit (Takara) on a FTC3000 qRT-PCR System (Funglynn Biotech Inc, Toronto, Canada). Both β -actin and U6 were used as internal controls for template normalization. Relative quantification of mRNA was calculated using the $2^{-\Delta\Delta CT}$ method.²¹ The primers for miR-30a-5p were synthesized by Ruibo Biotechnology (Guangzhou, China). The sequences of

Figure 1. Hypoxia postconditioning (HPostC) inhibits hypoxia/reoxygenation (H/R)-induced autophagy in aged cardiomyocytes. (A,B) Rat cardiomyocytes (H9C2 cells) treated with different concentrations of D-galactose (0, 4, 8 and 16 mg/mL) at different times (3, 6 and 9 days) were identified using β -galactose staining (20 $\times$). Blue color (black arrows) indicates aged cells. (C,D) Representative immunofluorescence images and quantitative analysis of dead (red) and live (green) aged cardiomyocytes in the Normoxia, H/R and HPostC groups (40 $\times$; Bar=50 μ m). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). (E) Aged cardiomyocyte cytoskeletons were examined by fluorescein-conjugated phalloidin staining (40 $\times$; Bar=20 μ m). F-actin fiber was indicated in green and nuclei were indicated as blue. (F) Detection of LC3B protein in Normoxia, H/R, and HPostC groups. Chloroquine (CQ) was used to evaluate the autophagic flux. LC3B-II expression level before and after CQ intervention was quantified. (G) Western blot and quantification analysis of p62 level in the Normoxia, H/R and HPostC groups. (H) Fluorescence image of aged cardiomyocytes after infection with adenovirus expressing mRFP-GFP-LC3. Effect of H/R and HPostC on the formation of autophagosome and autolysosome were observed under confocal microscopy (100 $\times$; scale Bar=10 μ m). Yellow and red puncta represented autophagosome and autolysosome, respectively. (I) The effects of autophagy on the cell viability of H/R and HPostC-exposed aged cardiomyocytes were evaluated using an autophagy inhibitor, 3-MA (1.25 mmol/L) (10 $\times$; Bar=50 μ m). Data represent the mean $\pm$ SD from 3 independent experiments, * P <0.05, ** P <0.01, n =3.

Figure 2. MicroRNA-30a (miR-30a) facilitates hypoxia postconditioning (HPostC)-induced inhibition of autophagy in aged cardiomyocytes. **(A)** Quantitative real-time polymerase chain reaction (qRT-PCR) assay of miR-30a in the aged cardiomyocytes subjected to Normoxia, hypoxia/reoxygenation (H/R) and HPostC. miR-30a level was normalized against U6. **(B)** Relative miR-30a level was detected by qRT-PCR in the aged cardiomyocytes infected with vehicle control, miR-neg control, LV3-rno-miR-30a mimic and LV3-rno-miR-30a inhibitor. **(C,D)** Representative Western blot and quantitative analysis of LC3B-II and p62 protein level after aged cardiomyocytes were infected with vehicle control, miR-neg control, LV3-rno-miR-30a mimic and LV3-rno-miR-30a inhibitor. **(E)** Fluorescence cell viability assay in miR-30a mimic or inhibitor-transfected cells under or not under H/R or HPostC conditions (10 $\times$; Bar=50 μ m). Data represent the mean $\pm$ SD from 3 independent experiments, * P <0.05, ** P <0.01, n =3.

primers used in qRT-PCR were listed in the **Table**.

Other materials and methods information are available in the **Supplementary Materials** section (**Supplementary File**).

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5.0. Data were expressed as means $\pm$ standard deviation (SD). More than 2 groups were compared using a one-way ANOVA, and a Student-Newman-Kul's test; differences between the 2 groups were evaluated using an unpaired Student's t -test. P <0.05 was considered to be statistically different.

Results

HPostC Inhibited Autophagy and Prevented Aged Cardiomyocyte Death Induced by H/R Injury

Aged cardiomyocytes were established by 8 mg/mL D-galactose treatment of H9C2 cells for 9 days, as evidenced by increased SA- β -gal-positive cells (**Figure 1A,B**). Then, aged cardiomyocytes were exposed to hypoxia or a reoxygenation environment for several cycles to mimic I/R and IPostC as described before.²² After cell viability staining, the number of viable cardiomyocytes in the HPostC group is significantly higher than that of the H/R group (**Figure 1C,D**). Moreover, stress fiber disruption and loss of F-actin resulted from H/R were abrogated by HPostC treatment (**Figure 1E**). These results implied that HPostC promoted survival of aged cardiomyocytes.

To testify whether HPostC can protect aged cardiomyocytes from H/R injury through autophagy, we assessed the expression of autophagy-related proteins, LC3B-II (a marker for autophagy activation) and p62 (a marker for autophagy inhibition), by Western blot. LC3B-II expression was significantly reduced, while p62 levels were significantly upregulated in aged cardiomyocytes under HPostC treatment compared to that of the H/R group (**Figure 1F,G**). It was reported that autophagosome-lysosome fusion can result in LC3B-II degradation;²³ we suspect that the upregulation of LC3B-II in H/R-treated cells may be caused by the increase of autophagosomes synthesis or the blockage of autophagosome digestion in lysosomes. The effect of HPostC on autophagic flux was then investigated using chloroquine (CQ) to limit autophagosome-lysosome fusion. CQ administration increased the LC3B-II level under all investigated conditions, while the variability of LC3B-II in the HPostC and Normoxia groups was significantly lower than that of the H/R group (**Figure 1F**). These data demonstrated that HPostC can inhibit autophagosome synthesis and, accordingly, reduce the autophagy flux. To confirm that, mRFP-GFP-LC3 adenovirus infection was used to monitor the autophagy flux in aged cardiomyocytes. In this experiment, only red puncta implied autolysosome, whereas yellow puncta, the overlaying of red puncta with green ones, represented autophagosome.²⁴ Both the number of autolysosomes and autophagosomes remarkably decreased in the HPostC group (**Figure 1H**). In contrary, both the red and yellow puncta increased in the H/R

Figure 3. MicroRNA-30a (miR-30a) inhibited autophagy by binding with *BECN1* under hypoxia postconditioning (HPostC) treatment. **(A)** Binding sites of miR-30a in the *BECN1*-3'-UTR were predicted using TargetScan (http://www.targetscan.org/vert_71/). **(B)** Schematic representation of *BECN1* wild type (WT)-3'-UTR and mutant (Mut)-3'-UTR sequences was used for the construction of luciferase report vectors (Upper panel). The reporter vectors containing 3'-UTR regions of the WT and Mut *BECN1* were co-transfected into the aged cardiomyocytes with miR-30a mimics or miR-neg. Relative luciferase activities were normalized by the ratio of firefly and Renilla luciferase activities (Bottom panel). **(C)** Representative Western blot and quantitative analysis of *BECN1* levels in aged cardiomyocytes infected with vehicle control, miR-neg control, LV3-rno-miR-30a mimic and LV3-rno-miR-30a inhibitor. **(D)** Representative Western blot and quantitative analysis of *BECN1* expression in aged cardiomyocytes subjected to Normoxia, hypoxia/reoxygenation (H/R) and HPostC. **(E)** Immunofluorescence staining of *BECN1* (red) in the aged cardiomyocytes subjected to Normoxia, H/R and HPostC (60 $\times$; Bar=50 μ m). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). **(F,G)** Representative images of fluorescent LC3 puncta under confocal microscope. Aged cardiomyocytes infected with a mRFP-GFP-LC3 adenovirus vector were then infected with Ad-NC, Ad-sh*BECN1* and Ad-BECN1 under HPostC treatments (100 $\times$; scale Bar=5 μ m). **(H)** The effects of *BECN1* restoration on the cell viability of H/R and HPostC-exposed aged cardiomyocytes were evaluated using fluorescence cell viability assay (10 $\times$; Bar=50 μ m). Data represent the mean $\pm$ SD from 3 independent experiments, * P <0.05, ** P <0.01, n =3.

Figure 4. Hypoxia postconditioning (HPostC) inhibits DNA methylation of microRNA-30a (miR-30a) promoter in aged cardiomyocytes. **(A)** Identification of CpG island from miR-30a sequences using UCSC genome browser and MethPrimer. The middle panel presents the CpG island, relative %GC content, and the rat mRNA was obtained from UCSC (<http://genome.ucsc.edu>). **Lower panel:** MethPrimer database (<http://www.urogene.org/cgi-bin/methprimer2/MethPrimer.cgi>) analysis of these CpG sites in the rat miR-30a promoter region from -2,000 to +200 bp. **(B)** miR-30a promoter activity was measured by dual luciferase assay. **(C)** Schematic location of CpG sites in the miR-30a promoter (**Top**). The blue rectangle is shown as the position of the miR-30a promoter ranging from -2,000 to +80. The blue arrow indicates the NCBI-predicted transcription start site (TSS), and the red vertical bars indicate CpG sites. The red bar (denoted 'BSP') specifies the CpG sites quantified by bisulfite sequencing (BSP). In the DNA methylation pattern shown below, black and white circles represent methylated and unmethylated CpG, respectively. **(D)** Following in vitro methylation with SssI (M.SssI), HhaI (M.HhaI) and HpaII (M.HpaII) methylases, the proximal promoter region of miR-30a was digested with McrBC, HhaI and HpaII to examine the methylation status. **(E)** The activity of the miR-30a proximal promoter differentially methylated with M.SssI, M.HhaI and M.HpaII was assessed using a dual luciferase assay in HEK293T cells. Data represent the mean $\pm$ SD from 3 independent experiments, * $P < 0.05$, ** $P < 0.01$, $n = 3$.

group. Moreover, aged cardiomyocytes were treated with the autophagy inhibitor, 3-MA; H/R-induced cell viability was similar with HPostC while HPostC-induced cell viability increased further, indicating that 3-MA can act in the same way with HPostC to enhance the cell viability and the protective effects through inhibiting autophagic activity (**Figure 1I**). Collectively, these results indicated that HPostC could protect aged cardiomyocytes from H/R injury via inhibiting autophagy.

miR-30a Was Involved in HPostC-Mediated Inhibition of Autophagy in Aged Cardiomyocytes

A previous study reported specific miR-30a expression in mouse cardiac tissue.²⁵ The qRT-PCR assay showed a dramatic increase of miR-30a expression in aged cardiomyocytes exposed to HPostC compared to that of H/R-treated cells (**Figure 2A** and **Supplementary Figure B**). In order to further investigate the effect of miR-30a on autophagy, we established cell lines with miR-30a knock-

down or over-expression by transfecting LV3-rno-miR-30a inhibitors or LV3-rno-miR-30a mimic, respectively (**Figure 2B**). As depicted in **Figure 2C**, over-expression of miR-30a could decrease the LC3B-II level, whereas it is the opposite for miR-30a knockdown. In contrast, p62 protein levels were the opposite to the LC3B-II levels (**Figure 2D**). And cell viability staining elucidated that over-expression of miR-30a induced a significant increase of cell viability under H/R conditions compared with miR-neg, while miR-30a knock-down abolished protection afforded by HPostC (**Figure 2E**). In summary, these data suggested that miR-30a mediated the protection of HPostC against aged cardiomyocyte H/R injury through inhibiting autophagy.

miR-30a Directly Targeted *BECN1* to Attenuate Autophagy of Aged Cardiomyocytes Following HPostC

TargetScan (http://www.targetscan.org/vert_71/) predicted a putative miR-30a complementary site in the 3'-UTR of *BECN1*, meaning that *BECN1* is a potential target gene of

Figure 5. DNA methyltransferase (DNMT)3b contributed to the hypoxia postconditioning (HPostC)-mediated upregulation of microRNA-30a (miR-30a) in aged cardiomyocytes. **(A)** Relative expression of miR-30a was determined by quantitative real-time polymerase chain reaction (qRT-PCR) in the aged cardiomyocytes after treatment with DNMT1, DNMT3a and DNMT3b inhibitors (DC-05, Theaflavin-3 and Nanaomycin A), respectively. **(B,C)** Relative DNMT3b mRNA and protein levels in the aged cardiomyocytes were evaluated by qRT-PCR and Western blot. **(D,E)** The miR-30a methylation level and its expression were analyzed by bisulphite sequencing (BSP) and qRT-PCR after aged cardiomyocytes were infected with Ad-shNC and Ad-shDNMT3b under HPostC. **(F-H)** Representative Western blot and quantitative analysis of LC3B-II, p62 and *BECN1* protein expression in aged cardiomyocytes after infection with Ad-shNC and Ad-shDNMT3b under HPostC. **(I)** Live/dead cell imaging of Nanaomycin A-treated aged cardiomyocytes under hypoxia/reoxygenation (H/R) or HPostC conditions (10×; Bar=50 μm). Data represent the mean ± SD from 3 independent experiments, **P*<0.05, ***P*<0.01, *n*=3.

miR-30a (Figure 3A). A luciferase-reporter assay also confirmed the reliability of this prediction as miR-30a mimic significantly inhibited the luciferase activity of wild-type (WT) 3'UTR of *BECN1*, but not for the mutated type (Mut) (Figure 3B), suggesting that miR-30a can specifically target the *BECN1* 3'-UTR region by binding to their putative sequences. Meanwhile, a Western blot assay indicated a negative relation between miR-30a and *BECN1* under HPostC treatments (Figure 3C). In addition, the *BECN1* protein level markedly decreased in aged cardiomyocytes subjected to HPostC as compared with H/R (Figure 3D and Supplementary Figure C). Consistent with the Western blot analysis, immunofluorescence staining also showed that HPostC dramatically reduced the number of *BECN1*-positive cells compared with H/R (Figure 3E). A mRFP-GFP-LC3 adenovirus reporter was further used to investigate the role of *BECN1* in autophagy flux of aged cardiomyocytes under HPostC. As expected, knock-down of *BECN1* reduced the number of red and yellow puncta

in the aged cardiomyocytes. In contrast, ectopic expression of *BECN1* promoted the total autophagic structures under HPostC treatment (Figure 3F,G). Moreover, upregulating autophagy by restoration of *BECN1* expression prevents HPostC to protect aged cardiomyocytes from H/R-induced cell death (Figure 3H). Therefore, these data verified that miR-30a was involved in the autophagy inhibition under HPostC by targeting *BECN1*.

DNA Hypomethylation Contributes to the Elevated miR-30a Promoter Activity Under HPostC

Next, we explored the mechanism responsible for miR-30a upregulation in aged cardiomyocytes under HPostC. Analysis of the genomic DNA sequence by an UCSC genome browser indicated 2 CpG island regions at the miR-30a whole-genome (Figure 4A, Middle). In addition, MethPrimer software (<http://www.uogene.org/methprimer/>) analysis showed that the potential methylation sites in the miR-30a promoter (−2,000 to +200) contained several CpG

Figure 6. Schematic illustration of the mechanism that hypoxia postconditioning (HPostC) protects aged cardiomyocytes against hypoxia/reoxygenation (H/R) injury. Under HPostC conditions, reduced DNMT3b expression mediated DNA hypomethylation levels at the miR-30a promoter, leading to upregulation of miR-30a. The elevation of miR-30a promoted a cardioprotective effect of HPostC on H/R injury by suppressing *BECN1*-mediated autophagy.

sites (red vertical bars) (**Figure 4A, Bottom**). Based on these predictions, 4 synthesized fragments (−2,000/+71, −1,071/+71, −761/+71, −216/+71) were inserted into the firefly luciferase vector, pGL3. Among of them, the (−761/−216) fragment of miR-30a was the basic core regulation region, as it provided the structural basis for methylation (**Figure 4B**). In addition, bisulfite sequencing (BSP) indicated that 2 core regions of the promoter (−761 to −421, −143 to +80) contain 8 CpG sites relative to the miR-30a's transcription start site (TSS). Compared with H/R, HPostC only decreased miR-30a DNA methylation level at the −761 to −421 region, but not at the −143 to +80 region. These data demonstrated that −261 to −761 was the regulating region of miR-30a transcription (**Figure 4C** and **Supplementary Figure D**). Next, in order to examine whether DNA methylation plays a direct role in regulating miR-30a promoter activity, we methylated the miR-30a promoter fragments using M.SssI, M.HhaI and M.HpaII. Among them, SssI methylated all 5'-CpG-3' sites, HhaI only methylated the CpG site of 5'-GCGC-3', and HpaII methylated the sequence of 5'-CCGG-3'. The successful methylation of these sites was confirmed by enzymatic digestion using enzymes of McrBC, HhaI and HpaII (**Figure 4D**). By using the luciferase activity assay, miR-30a promoter activity was found to be markedly blocked after with M.SssI treatment (methylates 15 CpG sites), which was found to reduce miR-30a promoter activity the most (**Figure 4E**). These results implied that DNA methylation contributes to the suppression of miR-30a promoter activity in H/R injury, which can be reversed by HPostC.

HPostC Inhibits Autophagy via DNMT3b-Mediated DNA Hypomethylation of the miR-30a Promoter in Aged Cardiomyocytes

DNA methylation is a major epigenetic modification catalyzed by DNMTs.²⁶ To determine which DNMTs contributed to the HPostC-induced hypomethylation of miR-30a, we treated aged cardiomyocytes with DNMTs inhibitors. **Figure 5A** indicated that Nanaomycin A (NA),

a DNMT3b inhibitor, significantly enhanced the expression of miR-30a, while no significant effect was observed for DC-05 and Theaflavin-3 (DNMT1- and DNMT3a-specific inhibitors) treatment. These data demonstrated that DNMT3b is the key methyltransferase in the regulation of miR-30a expression under HPostC. Meanwhile, HPostC strongly decreased DNMT3b expression in the aged cardiomyocytes compared to H/R injury (**Figure 5B,C**). Accordingly, knockdown of DNMT3b inhibited the methylation of miR-30a, while enhanced miR-30a level in aged cardiomyocytes (**Figure 5D,E**). Additionally, DNMT3b knockdown significantly suppressed the LC3B-II and *BECN1* level, whereas it upregulated p62 expression under HPostC treatment (**Figure 5F–H**). And cell viability staining elucidated that NA treatment can increase aged cardiomyocytes viability under H/R or HPostC conditions, which implied that NA promotes protection afforded by IPostC against H/R injury (**Figure 5I**). Collectively, it concluded that reduction of DNMT3b expression induced by HPostC could result in the miR-30a DNA hypomethylation and lead to miR-30a activation in the aged cardiomyocytes.

Discussion

MI is the leading cause of human death and disability worldwide, and is caused by myocardial ischemia, or hypoxia due to vascular stenosis.²⁷ Currently, the most effective treatment of MI patients is to restore myocardial perfusion and save the viable myocardium. However, the myocardium is susceptible to another form of injury stemming from reperfusion of the previously ischemic tissue once coronary flow is restored.²⁸ Fortunately, IPostC performed at immediate reperfusion has been successfully applied to patients with acute MI.²⁹ Although some clinical studies have indicated the significant benefit of IPostC for the prognosis of aged patients suffering from acute MI,³⁰ the effect of IPostC on the aged myocardium is still paradoxical. In this study, using H/R and HPostC models to mimic the pathological environment of I/R and IPostC, we

explored the protective effect of HPostC on H/R injury in aged cardiomyocytes and the shielding molecular mechanism. Based on the observation, we found that HPostC could effectively relieve H/R injury in aged cardiomyocytes, which is consistent with a previous clinical report.³⁰ We then elucidated the underlying molecular mechanisms of HPostC alleviating H/R injury, which may contribute to the discovery of therapeutic targets for aged people with MI.

Wang et al³¹ proved that IPostC can reduce I/R injury and improve cell survival by inhibiting autophagy activation and maturation in vivo and in vitro. Although autophagy is related to the protective effect of IPostC,³² the exact role of this effect in the ischemic myocardium is still controversial. In this study, we found that inhibition of autophagy contributed to the HPostC-elicited cardioprotection against H/R injury in aged cardiomyocytes, which is in agreement with a previous study.³¹ However, several other studies have shown that autophagy activation, but not inhibition, is associated with protective effects of IPostC. For example, Wei et al³³ reported that IPostC protects cardiomyocytes against I/R injury by increased autophagy via the upregulation of LC3B-II and *BECN1* expressions. These controversial results may be due to the differences between ischemic models, the duration of ischemic injury and animal age. In any case, it is necessary to clarify the molecular mechanisms of autophagy inhibition in the protection of aged cardiomyocytes from HPostC.

Post-transcriptional regulation of miRNAs has been extensively investigated in many diseases, including heart failure, cardiac hypertrophy, and MI.³⁴ However, the mechanism responsible for miRNA regulation and the contributions of HPostC-regulated miRNAs in towards cardioprotection of aged cardiomyocytes is still limited. Tu et al³⁵ demonstrated that miR-21 is involved in the IPostC-mediated cardiac protection against I/R injury through the PTEN/Akt-signaling pathway. Others have reported that miR-1 and miR-133a may attenuate cardiomyocyte apoptosis by targeting CASP9.³⁶ In this study, we demonstrated that the elevated expression of miR-30a in HPostC-treated aged cardiomyocytes enhanced protective effects against H/R injury through inhibiting *BECN1* expression. Moreover, we found that miR-30a can directly promote degradation of *BECN1* by binding with its 3'-UTR region, which is consistent with findings from a previous report.³⁷ As a mammalian autophagic gene, *BECN1* is essential for autophagy induction and regulation.³⁸ Maejima et al³⁹ has shown that *BECN1* inhibition can reduce cell death of cardiomyocytes induced by I/R injury through inhibiting autophagy. The present study also disclosed that HPostC could suppress *BECN1* expression, thereby inhibiting autophagy in the aged cardiomyocytes.

In addition, miRNAs harboring CpG islands can undergo DNA methylation-associated repression with a similar chromatin context to coding genes.⁴⁰ Andolfo et al⁴¹ reported that methylation of the miR-199b-5p promoter can downregulate its expression in the medulloblastomas. Lopez-Bertoni et al⁴² reported that DNMT-dependent suppression of miRNAs regulates the induction of GBM tumor-propagating phenotype by Oct4 and Sox2. DNMT3b functions as a de novo methyltransferase that establishes new DNA methylation patterns,⁴³ over-expression and hyperactivation of DNMT3b, which can result in hypermethylation of promoter CpG islands and gene silence.⁴⁴ In the present study, we disclosed that HPostC can induce DNA hypomethylation of the miR-30a promoter and

upregulate its expression through inhibiting DNMT3b expression in the aged cardiomyocytes. These data supported the point that DNA hypomethylation contributes to genome instability and transcriptional activation, while DNA hypermethylation leads to gene silence.⁴⁵ This finding reasonably explained why the inhibition of DNMT3b activity can result in the loss of DNA methylation during miR-30a replication. Our observation provided an additional regulatory pathway of miR-30a expression in HPostC-treated aged cardiomyocytes.

In conclusion, HPostC showed a protective effect on the aged cardiomyocytes from H/R injury via DNMT3b-mediated DNA hypomethylation at the miR-30a promoter and thereby upregulated miR-30a transcription, which accordingly suppressed *BECN1* expression and attenuated autophagy (Figure 6). As a result, the discovery of aberrant DNA hypomethylation of miR-30a during HPostC treatment not only showed a new protective mechanism of IPostC on the aged cardiomyocytes from I/R injury, but also provided a potential target for precise therapy to ameliorate aged myocardial injury from I/R as well.

Conflict of Interests

None.

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Supplementary Files

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