Original Article

Maternally expressed 3 protects the intestinal barrier from cardiac arrest-induced ischemia/reperfusion injury via miR-34a-3p/sirtuin 1/nuclear factor kappa B signaling

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Background: Cardiac arrest (CA), a common disease with a high mortality rate, is a leading cause of ischemia/reperfusion (I/R)-induced dysfunction of the intestinal barrier. Long non-coding RNAs (lncRNAs) play crucial roles in multiple pathological processes. However, the effect of the lncRNA maternally expressed 3 (MEG3) on intestinal I/R injury and the intestinal barrier has not been fully determined. Therefore, this study aimed to investigate the function of MEG3 in CA-induced intestinal barrier dysfunction.

Methods: The oxygen and glucose deprivation (OGD) model in the human colorectal adenocarcinoma Caco-2 cells and *in vivo* cardiac arrest-induced intestinal barrier dysfunction model in Sprague-Dawley (SD) rats were established. The effect and underlying mechanism of MEG3 on the intestinal barrier from cardiac arrest-induced ischemia/reperfusion injury were analyzed by methyl thiazolyl tetrazolium (MTT) assays, Annexin V-FITC/PI apoptosis detection kit, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining, quantitative polymerase chain reaction (qPCR) assays, Western blot analysis, luciferase reporter gene assays, transepithelial electrical resistance (TEER) measurements, immunofluorescence analysis, and enzyme-linked immunosorbent assay (ELISA) assays.

Results: Interestingly, we found that MEG3 could protect Caco-2 cells from oxygen-glucose deprivation (OGD)/reoxygenation-induced I/R injury by modulating cell proliferation and apoptosis. Moreover, MEG3 relieved OGD-induced intestinal barrier dysfunction *in vitro*, as demonstrated by its significant rescue effect on transepithelial electrical resistance and the expression of tight junction proteins such as occludin and claudin-1 (CLDN1), which were impaired in OGD-treated Caco-2 cells. Mechanistically, MEG3 inhibited the expression of inflammatory factors including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon-gamma (IFN)-γ, inflammatory factors including interleukin (IL)-10, and transforming growth factor beta (TGFb)-1, as well as nuclear factor-kappa B (NF-κB) signaling. In response to OGD treatment *in vitro*, MEG3 also activated the expression of sirtuin 1 (SIRT1) by Caco-2 cells via sponging miR-34a-3p. Furthermore, MEG3 relieved CA-induced intestinal barrier dysfunction through NF-κB signaling *in vivo*.

Conclusions: LncRNA MEG3 can protect the intestinal barrier from cardiac arrest-induced I/R injury via miR-34a-3p/SIRT1/NF-κB signaling. This finding provides new insight into the mechanism by which MEG3 restores intestinal barrier function following I/R injury, presenting it as a potential therapeutic candidate or strategy in intestinal injury.

Keywords: Cardiac arrest (CA); intestinal barrier; lncRNA MEG3; miR-34a-3p; nuclear factor kappa B (NF-κB); sirtuin1 (SIRT1)

Introduction

Cardiac arrest (CA) is a common cause of mortality in patients with coronary aorta disorder (1). CA sees the heart rate rise dramatically, and the contractions of cardiac muscle in the ventricular cells become uncoordinated; this causes the heart to pump inefficiently, resulting in unconsciousness and death (2,3). CA-induced microvascular dysfunction, insufficient blood flow, and tissue oxygen transportation lead to systemic ischemia/reperfusion (I/R) injury in various organs, including the brain and intestine (4). Pathophysiological intestinal I/R can also result from acute mesenteric ischemia, surgical procedure, and traumatic shock (5,6). Intestinal I/R can give rise to numerous organ disorders and inflammatory system syndromes and is a major cause of mortality and morbidity in surgical patients (7,8).

After CA, reestablishing the blood flow and oxygen supply are vital in heart and intestine. The reperfusion triggers a cascade of events that may inflict further injury and usually surpasses the initial ischemic damage (9,10). Intestinal I/R destroys the function of the mucosal barrier (11,12). By enhancing the permeability of the intestinal barrier, intestinal I/R induces the translocation of endotoxins and pathogenic bacteria, which eventually progresses to multiple organ failure and sepsis (13,14). Therefore, the intestinal mucosal barrier, which is responsible for defending the body from invasion by pathogenic organisms, should be protected from I/R injury caused by CA (15). However, the mechanism underlying CA-induced intestinal I/R injury and intestinal mucosal barrier dysfunction has not been fully elucidated.

The advances in high-throughput technology have facilitated a profound exploration of the non-coding genome with unparalleled determination. Long non-coding RNAs (lncRNAs) have been found to play crucial roles in multiple physiological and pathological processes (16). LncRNAs have also been observed to impact mRNA paracellular permeability and translation, as well as intestinal cell proliferation and apoptosis susceptibility, to regulate intestinal I/R and the intestinal barrier (17). A recent study reported that lncRNA uc.173 regulates the intestinal barrier by modulating miR-29b/claudin-1 (CLDN1) signaling (18). Maternally expressed gene 3

(MEG3) is an imprinted gene located at chromosome12 in mice and chromosome 14 in humans which expresses in many normal tissues, such as cancer cells, neurons, hepatocytes, cardiac fibroblasts, and ECs. MEG3 is a well-known lncRNA that plays various roles in multiple pathological processes, including cancer progression and inflammatory response (18-20). Many reports reveal that MEG3 could inhibit tumor cell proliferation and induces tumor cell apoptosis as well as autophagy. Those researches suggest that MEG3 exerts an antitumor activity in several cancers. The involvement of lncRNA MEG3 in neurological dysfunction has also been demonstrated in a brain I/R injury mouse model (21). It has been also reported MEG3 can regulate IL-1β abundance to prevent sepsis during lung infection by acting as a decoy of miRNA (22). Piccoli *et al.* identifies the lncRNA Meg3 to be a crucial regulator of cardiac MMP-2, promoting cardiac fibrosis and impairment of diastolic function following pressure overload (23). However, the effect of MEG3 on intestinal I/R injury and the intestinal mucosal barrier remains unclear.

MicroRNAs (miRNAs), a type of small RNAs that modulate mRNA translation, regulate the expression of genes involving in various biological and medical processes, such as including cell cycle regulation, stress response, differentiation, inflammation, and cancer progression (24,25). Several miRNAs, including miR-381-3p, miR-130a, and miR-212, have been reported to participate in the modulation of intestinal I/R injury and the intestinal barrier by targeting various key signaling pathways (26,27). MiR-34a-3p plays a vital role in the development of malignancies including cervical cancer and meningioma (28,29). Moreover, nuclear factor kappa B (NF-κB) signaling, as a critical pathway for multiple cellular processes, contributes to intestinal I/R injury and intestinal barrier dysregulation (30-32). Sirtuin 1 (SIRT1), as a highly conserved NAD+-dependent deacetylases and a critical biological sensor, has been well recognized to inhibit NF-κB signaling in multiple pathological processes, and the miRNA targeting SIRT1 can induce NF-κB activation (33-36). Nevertheless, the relationship between miR-34a-3p, SIRT1/NF-κB signaling, and MEG3, especially in the modulation of intestinal I/R and the intestinal barrier, remains obscure.

In this study, we aimed to explore the role of MEG3

in CA-induced intestinal I/R and intestinal barrier dysregulation. We identified a novel protective role of MEG3 against CA-induced intestinal I/R injury in the intestinal barrier via miR-34a-3p/SIRT1/NF-κB signaling.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-6438).

Methods

Cell culture, treatment and oxygen-glucose deprivation (OGD) model

Human colorectal adenocarcinoma Caco-2 cells were supplied by American Type Tissue Culture Collection (New York, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Solarbio, China) containing 10% fetal bovine serum (Gibco, USA), 0.1 mg/mL streptomycin and 100 units/mL penicillin at 37 ℃ with 5% CO_2. To simulate I/R conditions, an *in vitro* OGD model was established. Caco-2 cells were incubated for 12 h with 5% CO_2 and 1% O_2 with 94% N2. The cells were then cultured for 6 h in normoxic conditions to achieve reoxygenation. The cells were infected with ADV4, ADV4 MEG3, ADV1, and ADV1 MEG3 shRNA. All vectors were synthesized by Genscript (Nanjing, China). The MEG3 shRNA target sequence was: 5'-GCUCAUACUUUGACUCUAU-3'. MiR-34a-3p mimic and inhibitor were obtained from RiboBio (Guangzhou, China). Pyrrolidinedithiocarbamate (PDTC) (Sigma, USA) was used at a dose of 60 mM.

MTT assays

MTT assays were performed to measure the cell viability of Caco-2 cells. Briefly, 2×10^4 Caco-2 cells were put into 96-well plate and cultured for 12 h. Then, 10 μL MTT solution (5 mg/mL) was added, and the cells were cultured for a further 4 h. The medium was discarded, and 150 μL dimethyl sulfoxide (DMSO) was added to the wells. An enzyme-linked immunosorbent assay (ELISA) microplate reader was used to analyze the absorbance at 570 nm (BioTek EL 800, USA).

Analysis of cell apoptosis

Caco-2 cells were cultured at a density of 1×10^5 in 6-well culture plates. Cell apoptosis was measured using an Annexin V-FITC/PI apoptosis detection kit (Keygen Biot Solasodine, China) according to the manufacturer's instructions. Briefly, 1×10^6 cells were collected and washed by binding buffer. The cells were then dyed with Annexin V and propidium iodide (PI) at 25 ℃. Finally, apoptotic cells were measured by flow cytometry.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining

Apoptotic cells were also detected by TUNEL staining kit supplied by Ruisai Biotechnology Co., Ltd. (Shanghai, China) following the manufacturer's manual. Five random fields were selected in each section and the rate of positive cells was analyzed under a fluorescence microscope at 400× magnification.

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

Total RNAs was extracted using TRIzol (Invitrogen, USA). Synthesis of first-strand cDNA was carried out according to the manufacturer's instructions (Invitrogen, USA). Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was conducted using SYBR Premix Ex Taq II kit (Takara, Japan). The standard controls for miRNA and mRNA were U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The quantitative RNA levels were determined in three independent experiments. The primer sequences used were as follows: MEG3 forward: 5'-GTGAAGGTCGGAGTGAACG-3', reverse: 5'-CTCGCTCCTGGAAGATGGTG-3'; miR-34a-3p forward: 5'-ACACTCCAGCTGGG AATCAGCAAGTATACTGCCCTA-3', reverse: 5'-CTCAACTGGTGTCGTGGAGTCGGCAAT TCAGTTGAGTAGGGCAG-3'; SIRT1 forward: 5'-CTTCAGGTCAAGGGATGGTAT-3', reverse: 5'-GCGTGTCTATGTTCTGGGTAT-3'; U6 forward: 5'-GACAGATTCGGTCTGTGGCAC-3', reverse: 5'-GATTACCCGTCGGCCATCGATC-3'; GAPDH forward: 5'-GACAGATTCGGTCTGTGGCAC-3', reverse: 5'-GATTACCCGTCGGCCATCGATC-3'.

Western blot analysis

Caco-2 cells were collected with RIPA (Beyotime, China) with phenylmethylsulfonyl fluoride (PMSF). Identical amounts of protein were divided by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-

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PAGE), and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% milk and incubated with primary antibodies for B-cell lymphoma 2 (Bcl-2, Proteintech, China), Bcl-2-associated X protein (Bax, Proteintech, China), cyclin D1 (Proteintech, China), SIRT1 (Proteintech, China), caspase-3 (Cell Signaling, US), p-p65 (Bioworld Technology, USA), p65 (Bioworld Technology, USA), p-IκB (Bioworld Technology, USA), IκB (Bioworld Technology, USA), and GAPDH (Sigma, USA) at 4 ℃ overnight. Then, the membranes were exposed to the corresponding secondary antibodies for 1 h. Finally, the proteins were visualized using a Bio-Rad imaging system.

Luciferase reporter gene assay

Luciferase reporter gene assays were carried out using the Dual-luciferase Reporter Assay System (Promega, USA). Briefly, samples were transfected with miR-1247-3p mimic, miR-control, and the vector containing MEG3, MEG3 mutant, SIRT1, or SIRT1 mutant fragment using Lipofectamine 2000 (Invitrogen, USA). Luciferase activity was analyzed and Renilla was used for normalization.

Transepithelial electrical resistance (TEER) measurements

To analyze the barrier function of the intestine, TEER was measured. 2×10^5 cells/mL Caco-2 cells were layered on collagen-covered polycarbonate penetrable support supplements (Corning, USA). The medium was replaced every 2 days. The intestinal barrier was measured at 3 weeks by TEER using the Millicell ERS-2 (Electrical Resistance System; Merck-Millipore, USA).

Immunofluorescence analysis

Caco-2 cells were fixed by 4% paraformaldehyde for 30 min, treated with Triton X 100 (0.2%) for 10 min, and then treated with bovine serum albumin (BSA, 2%) for 30 minutes. The slides were incubated with occludin (Proteintech, China) and CLDN1 (Proteintech, China) antibodies at 4 ℃ overnight, and then incubated with secondary antibodies (Proteintech, China) for 1 h at 37 ℃. The slides were stained with Hoechst (Beyotime, China) for 10 min at 25 ℃. A Nikon microscope (Tokyo, Japan) was used to analyze immunofluorescence.

ELISA assays

The levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon-gamma (IFN)-γ, IL-10, and transforming growth factor beta (TGFb)-1 in the culture medium of Caco-2 cells were analyzed with a double-antibody ELISA kit (R&D, USA).

Establishment of a CA rat model

Sprague-Dawley (SD) rats were used to establish a CA-induced intestinal barrier dysfunction model *in vivo*, as previously described (37). Briefly, two acupuncture pins were placed in the fourth rib of the right sternal edge and the left sternal edge. The stimulating wire was attached, and a 60-Hz, 2 mA current was delivered. The current was maintained for 3 minutes to avoid unintentional defibrillation. After 6 minutes, the resuscitation was started; the rats were given adrenaline (2.2 g/100 g) administration, chest compression (200 times/min), and mechanical ventilation (100% FiO_2, 0.65 mL/100 g, 100 breaths/min). The mechanical oxygenation and hemodynamic monitoring were maintained with 21% oxygen for a further 2 h. After recovery, the rats were closely monitored.

Next, the rats were intravenously injected via the tail with ADV4, ADV4 MEG3, ADV1, ADV1 MEG3 shRNA or treated with percutaneous tunneled drainage catheter (PTDC) for further analysis. The serum levels of D-lactic acid and endotoxin in the rats were measured using the corresponding kit (Horseshoe Crab, China). Sections of the intestinal mucosa of the rats were stained with hematoxylin and eosin (HE). Immunohistochemical (IHC) staining with the appropriate antibodies (Proteintech, China) was also performed to analyze the expression levels of occludin and CLDN1 in the intestinal mucosa of the rats. The animal care and experimental procedures were authorized by the Animal Ethics Committee of The First Affiliated Hospital of Xiamen University (20190227), in compliance with the First Affiliated Hospital of Xiamen University guidelines for the care and use of animals.

Statistical analysis

Data were expressed as mean ± SD, and the statistical analysis was performed by GraphPad prism 7. The unpaired Student's

t-test was applied for comparing two groups, and the one-way ANOVA was applied for comparing among multiple groups. P<0.05 were considered as statistically significant.

Results

LncRNA MEG3 protected Caco-2 cells from OGD-induced I/R injury in vitro

Cell proliferation and apoptosis have been reported to be the crucial contributors to intestinal I/R injury (38). To assess the effect of lncRNA MEG3 on intestinal I/R injury *in vitro*, we constructed an OGD model in the human colorectal adenocarcinoma Caco-2 cells by treating them with OGD/reoxygenation (OGD/R) to mimic intestinal I/R injury.

Interestingly, we found that the expression levels of MEG3 were reduced in the OGD cell model, in which the overexpression of MEG3 could rescue this reduction whereas the depletion of MEG3 by shRNA could further decrease the expression in the system (*Figure 1A*), suggesting that OGD-induced intestinal I/R hindered the expression of MEG3 in Caco-2 cells. As expected, cell viability was decreased in OGD-treated Caco-2 cells (*Figure 1B*). Surprisingly, MEG3 overexpression increased the cell viability, but the MEG3 shRNA decreased cell viability in the cells, indicating that MEG3 enhances cell proliferation in response to OGD-induced cell injury (*Figure 1B*). Consistently, cell apoptosis was attenuated by MEG3 overexpression, while the knockdown of MEG3 enhanced cell apoptosis in the OGD-treated cells (*Figure 1C,D*).

Several key proteins, including Bcl-2, Bax, and caspase-3, are involved in the modulation of cell apoptosis and can serve as markers of apoptosis (39,40). Intriguingly, we found that MEG3 overexpression rescued the expression of Bcl-2 in OGD-treated cells and reduced the expression of Bax and caspase-3 induced by OGD treatment, while the depletion of MEG3 exerted the opposite effect (*Figure 1E*). These results suggested that MEG3 can protect Caco-2 cells from OGD-induced I/R injury by modulating apoptosis.

LncRNA MEG3 relieved OGD-induced intestinal barrier dysfunction in vitro

Next, we investigated the role of MEG3 in the regulation of the intestinal barrier *in vitro*. TEER is an indicator of intestinal barrier function (41). Interestingly, the TEER value in Caco-2 cells was significantly reduced after OGD treatment (*Figure 2A*). Strikingly, the OGD-inhibited TEER level was rescued by the overexpression of MEG3 and further decreased by the MEG3 shRNA (*Figure 2A*), suggesting that MEG3 can alleviate the impairment of the intestinal barrier by OGD. Besides, as a control, TEER values in the monolayers were not changed (*Figure 2B*). Tight junction proteins including occludin and CLDN1, play a critical role in maintaining the function of the intestinal barrier (42). Significantly, immunofluorescence revealed that the expression levels of occludin and CLDN1 were reduced in OGD-treated Caco-2 cells; this effect could be rescued by MEG3 overexpression but was reinforced by the depletion of MEG3 (*Figure 2C,D*), indicating that MEG3 relieved OGD-induced intestinal barrier dysfunction *in vitro*.

NF-κB signaling might be involved in the protective mechanism of MEG3 against OGD-induced I/R injury in vitro

Next, we attempted to determine the mechanism underlying MEG3's mediation of the intestinal barrier in OGD-induced I/R injury. Surprisingly, we found that the expression levels of inflammatory factors including IL-1β, TNF-α, IFN-γ, IL-10, and TGFb-1 in the culture medium of OGD-treated Caco-2 cells were significantly attenuated by MEG3 overexpression, whereas cells transfected with MEG3 shRNA enhanced the expression levels of IL-1β, TNF-α, IFN-γ, IL-10, and TGFb-1 (*Figure 3A,B,C,D,E*). These observations suggested that MEG3 protects against inflammation in OGD-related cell injury. The phosphorylation of p65 subunits of NF-κB has been reported to be necessary for p65 activation (43). Moreover, the function of p65 can be suppressed by NF-κB inhibitor (IκB), with the phosphorylation of IκB causing IκB degradation and the activation of p65 (44). We found that the levels of p65 phosphorylation (p-p65) and p65 downstream gene cyclin D1 were up-regulated, whereas phosphorylation of IκB (p-IκB) was down-regulated, in OGD-treated Caco-2 cells (*Figure 3F*). Remarkably, these effects could be rescued by MEG3 overexpression and enhanced by the depletion of MEG3 (*Figure 3F*), suggesting that MEG3 impaired NF-κB signaling in the OGD model. Moreover, the NF-κB inhibitor PDTC could reverse the effect of MEG3 depletion on cell proliferation and apoptosis as demonstrated by the increases in cell viability and Bcl-2 expression, as well as the decreased expression of Bax (*Figure 3G,H*). Taken

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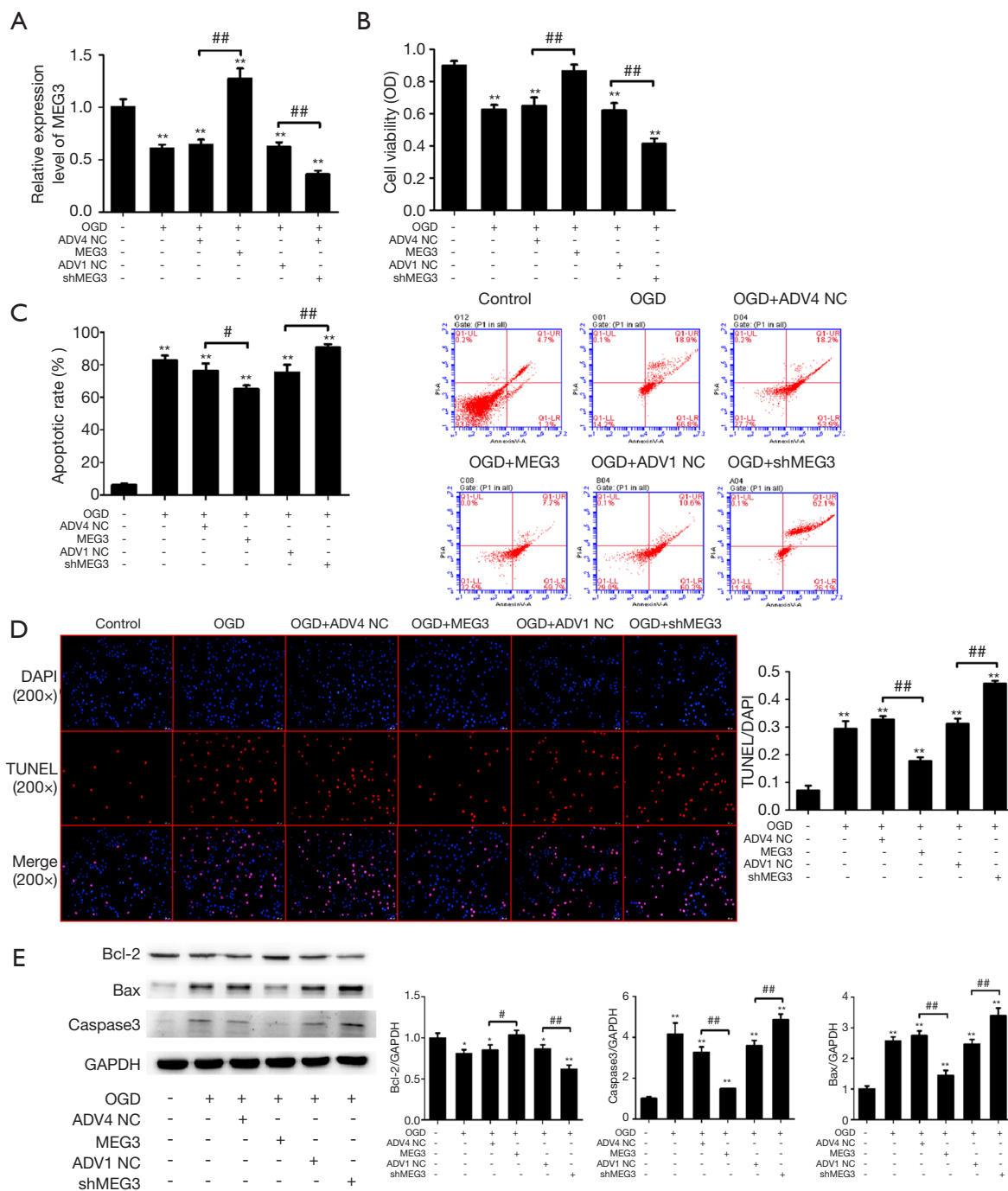Huang et al. Role of LncRNA MEG3 in Cardiac arrest

Figure 1 MEG3 protects Caco-2 cells from OGD induced I/R injury *in vitro*. (A-E) Untreated Caco-2 cells and oxygen and glucose deprivation-treated Caco-2 cells were infected with ADV4, ADV4 MEG3, ADV1, or ADV1 MEG3 shRNA as indicated. (A) The expression levels of lncRNA MEG3 were measured by qPCR. (B) Cell viability was analyzed by MTT assays. (C) Cell apoptosis was assessed by flow cytometry. (D) Cell apoptosis was assessed by TUNEL staining. Mean ± SD of at least three experiments is shown. (E) The expression levels of Bcl-2, Bax, caspase-3, and GAPDH were detected by western blot. Statistically significant differences are indicated: **P<0.01, #P<0.05, ##P<0.01. MEG3, maternally expressed 3; OGD, oxygen and glucose deprivation; qPCR, quantitative polymerase chain reaction; Bcl-2, B-cell lymphoma 2; Bax, B cell lymphoma/leukmia-2 (Bcl-2) associated x protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviation; I/R, ischemia/reperfusion.
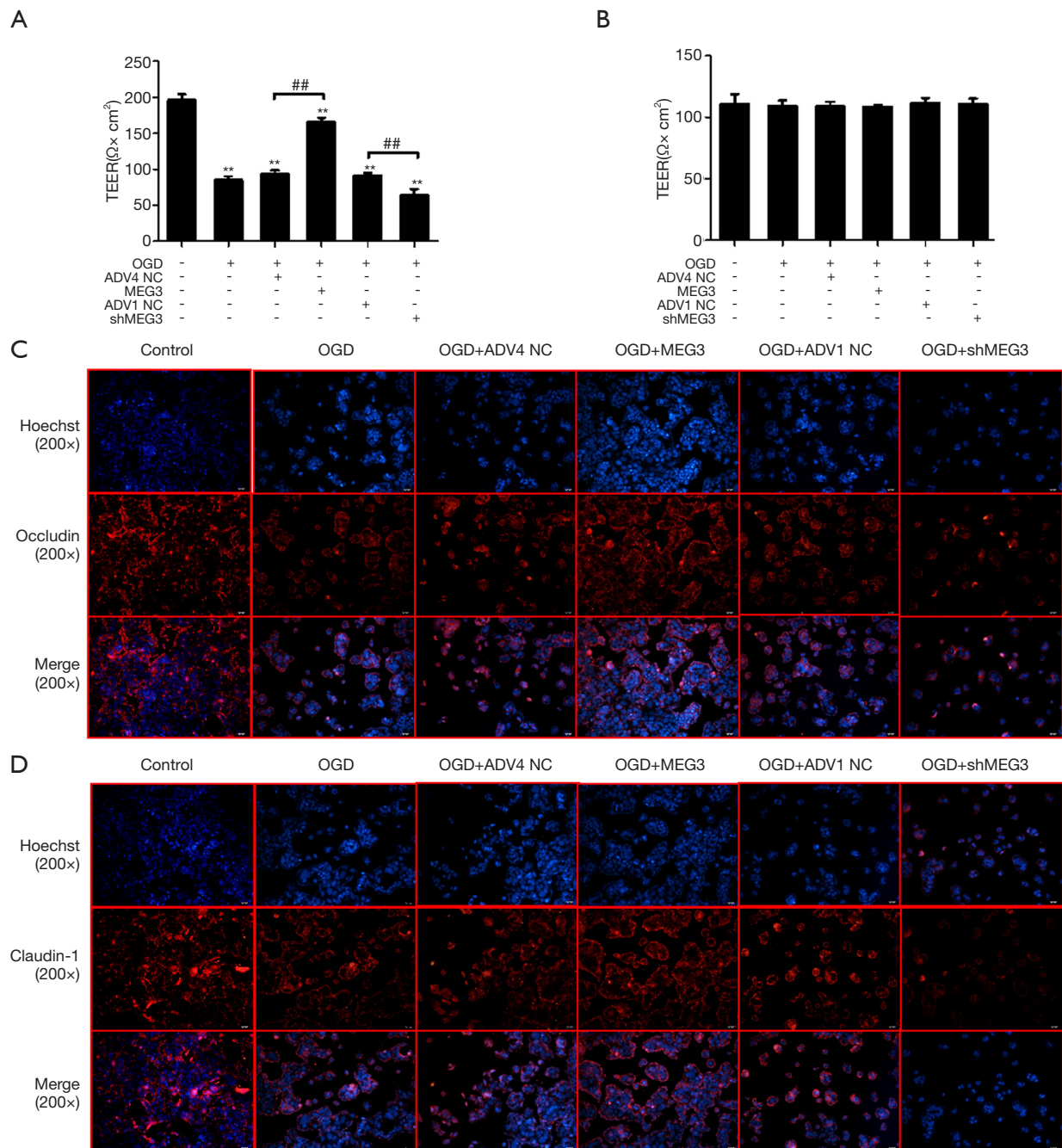
Figure 2 MEG3 relieves OGD-induced intestinal barrier dysfunction *in vitro*. (A,B) Untreated Caco-2 cells and oxygen and glucose deprivation-treated Caco-2 cells were infected with ADV4, ADV4 MEG3, ADV1, or ADV1 MEG3 shRNA as indicated. (A,B) The transepithelial electrical resistance (TEER) levels were measured, with the TEER values in the monolayers serving as a control; (C,D) the expression levels of occludin and claudin-1 were measured by immunofluorescence analysis. Mean ± SD of at least three experiments is shown. Statistically significant differences are indicated: **P<0.01, ##P<0.01. SD, standard deviation; OGD, oxygen and glucose deprivation.

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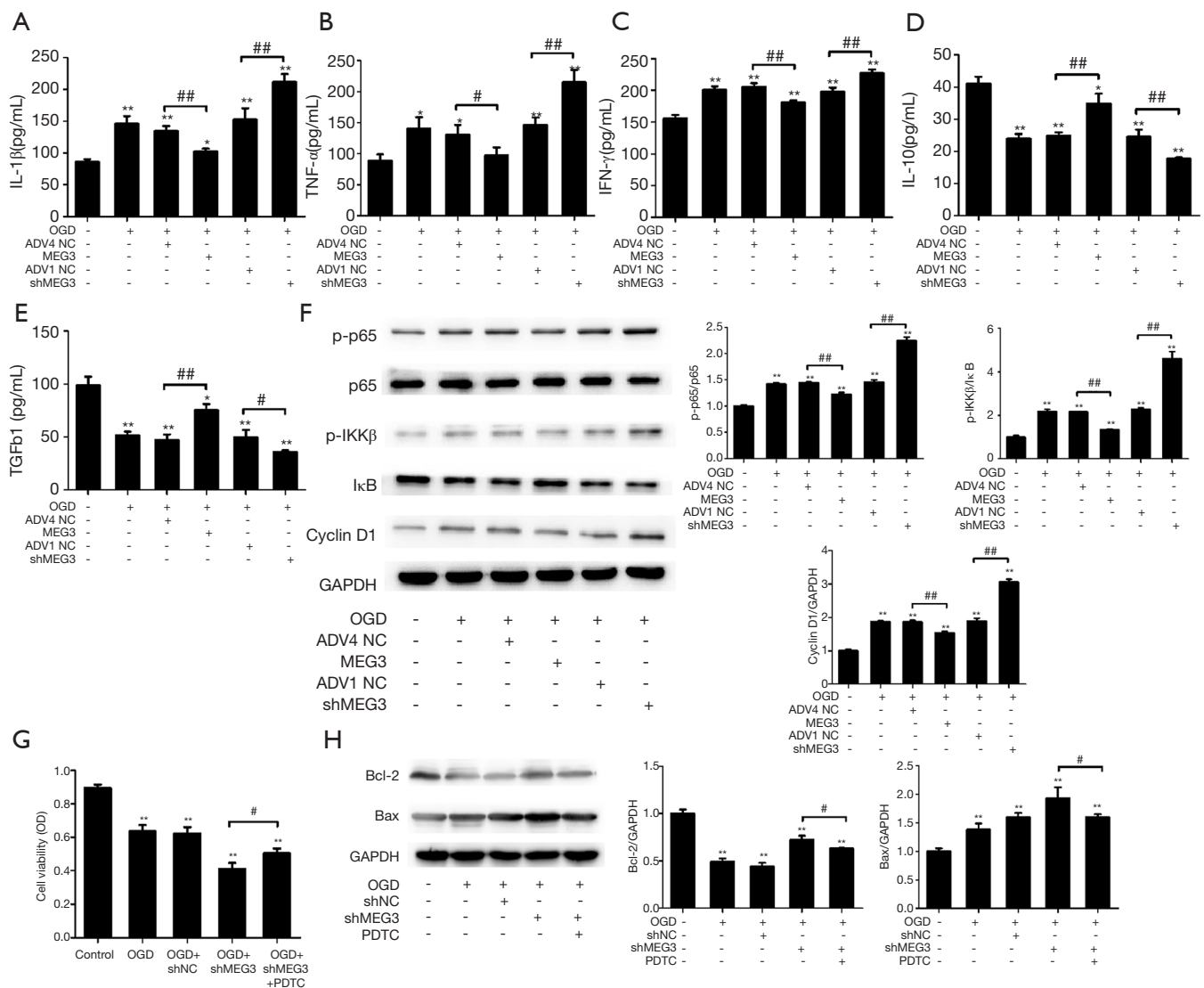
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Figure 3 NF-κB signaling is involved in the protective effect of MEG3 against OGD-induced Caco-2 cell I/R injury. (A-F) Untreated Caco-2 cells and OGD-treated Caco-2 cells were infected with ADV4, ADV4 MEG3, ADV1, or ADV1 MEG3 shRNA as indicated. (A-E) The levels of IL-1β, TNF-α, IFN-γ, IL-10, and TGFb-1 in the culture medium of the cells were determined by ELISA assays. (F) The expression levels of phosphorylation of p65 (p-p65), p65, phosphorylation of IκB, IκB, cyclin D1, and GAPDH were measured by Western blot analysis. (G,H) Untreated Caco-2 cells or OGD-treated Caco-2 cells were infected with ADV1, ADV1 MEG3 shRNA or treated with PDTC as indicated. (G) Cell viability was analyzed by MTT assays. (H) The expression levels of Bcl-2, Bax, and GAPDH were detected by western blot. Mean ± SD of at least three experiments is shown. Statistically significant differences are indicated: *P<0.05, **P<0.01, #P<0.05, ##P<0.01. IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-gamma-γ; IL-10, inflammatory factors including interleukin -10; TGFb-1, transforming growth factor beta-1; ELISA, enzyme-linked immunosorbent assay; NF-κB, nuclear factor-kappa B; MEG3, maternally expressed 3; OGD, oxygen and glucose deprivation; I/R, ischemia/reperfusion.

together, these findings indicated that NF-κB signaling may be involved in MEG3-induced protective function against OGD-induced I/R injury *in vitro*.

MEG3 attenuated OGD-induced I/R injury in vitro by sponging miR-34a-3p

Next, we further investigated the mechanism by which MEG3 modulates the activation of NF-κB signaling. MiRNAs have been reported to be involved in lncRNA-mediated NF-κB signaling (45,46). Therefore, we hypothesized that miRNA was involved in MEG3-regulated p65 inactivation. We performed a bioinformatics analysis using the DIANA tools web server (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php). Interestingly, we identified that MEG3 contained a binding sequence of miR-34a-3p (*Figure 4A*). Furthermore, we observed that miR-34a-3p mimic significantly reduced the luciferase activity of wild-type MEG3 but did not affect that of MEG3 with mutation of the miR-34a-3p binding site (*Figure 4B*), which indicated that MEG3 can was able to target miR-34a-3p. Meanwhile, OGD treatment enhanced the expression of miR-34a-3p in Caco-2 cells in which this effect was impaired by MEG3 overexpression and enhanced by MEG3 depletion (*Figure 4C*). Remarkably, the inhibition of MEG3 by OGD treatment was enhanced by miR-34a-3p mimic, whereas miR-34a-3p inhibitor rescued MEG3 expression (*Figure 4D*), which indicated that MEG3 responds to OGD treatment *in vitro* by sponging miR-34a-3p. Moreover, miR-34a-3p mimic inhibited the cell viability of OGD-treated cells, while miR-34a-3p inhibitor enhanced the cell viability (*Figure 4E*). More importantly, in OGD-treated Caco-2 cells miR-34a-3p mimic impaired MEG3 overexpression-induced cell proliferation and reversed MEG3 overexpression-inhibited cell apoptosis (*Figure 4F,G,H*). These results suggested that MEG3 attenuated OGD-induced I/R injury *in vitro* by sponging miR-34a-3p.

MEG3 activated SIRT1 expression in response to OGD treatment in vitro by sponging miR-34a-3p

Next, we further explored the potential target of miR-34a-3p in the MEG3-mediated NF-κB signaling pathway. SIRT1 has been shown to inhibit NF-κB signaling, and miRNA can target SIRT1, thereby activating p65 function (36,47,48). We performed a bioinformatics analysis using Targetscan (http://www.targetscan.org/vert_72/) to identify the miR-34a-3p-targeted site within SIRT1 3' untranslated

region (UTR) (*Figure 5A*). Luciferase reporter gene assays showed that the luciferase activity of wild-type SIRT1 but not that of SIRT1 with mutation of the miR-34a-3p binding site was reduced by miR-34a-3p mimic (*Figure 5B*), indicating that miR-34a-3p can target SIRT1. The expression levels of SIRT1 were reduced by OGD treatment and further inhibited by miR-34a-3p mimic but enhanced by miR-34a-3p inhibitor (*Figure 5C*); these observations suggest that miR-34a-3p is able to down-regulate SIRT1. Moreover, the overexpression of MEG3 significantly rescued OGD-decreased SIRT1 expression, while miR-34a-3p mimic impaired this rescue (*Figure 5D*). These findings suggested that MEG3 activated the expression of SIRT1 in response to OGD treatment *in vitro* by sponging miR-34a-3p.

MEG3 protected Caco-2 cells from OGD-induced I/R injury in vitro via miR-34a-3p/NF-κB signaling

We further explored the function of MEG3/miR-34a-3p/NF-κB signaling in OGD-induced I/R injury in Caco-2 cells. Interestingly, we found that the NF-κB inhibitor PDTC rescued the decreased expression of MEG3 and blocked the expression of miR-34a-3p induced by OCD in a feedback event (*Figure 6A,B*). Significantly, PDTC attenuated the upregulation of IL-1β and TNF-α levels caused by MEG3 depletion and further consolidated the inhibitive effect of MEG3 overexpression on IL-1β, TNF-α, IFN-γ, IL-10, and TGFb-1 expression (*Figure 6C,D,E,F,G*). Similar results were observed in the western blot analysis of p-p65, p-IκB, and cyclin D1 (*Figure 6H*). Moreover, PDTC attenuated the MEG3 overexpression-inhibited on cell apoptosis and MEG3 depletion-induced cell apoptosis as demonstrated by increased Bcl-2 expression and decreased Bax expression (*Figure 6I*). Together, our findings suggested that MEG3 protected Caco-2 cells from OGD-induced I/R injury via miR-34a-3p/NF-κB signaling *in vitro*.

MEG3 relieved CA-induced intestinal barrier dysfunction via NF-κB signaling in vivo

Next, we examined the role of MEG3 in the CA-induced intestinal I/R injury and intestinal barrier dysfunction *in vivo* by establishing a CA-induced I/R rat model. We found that the levels of lactic acid and endotoxin in the serum of the CA-treated rats were elevated. Moreover, these increases were blocked by MEG3 overexpression and reinforced by MEG3 depletion (*Figure 7A,B*). Meanwhile, PDTC attenuated the increases in the levels of lactic

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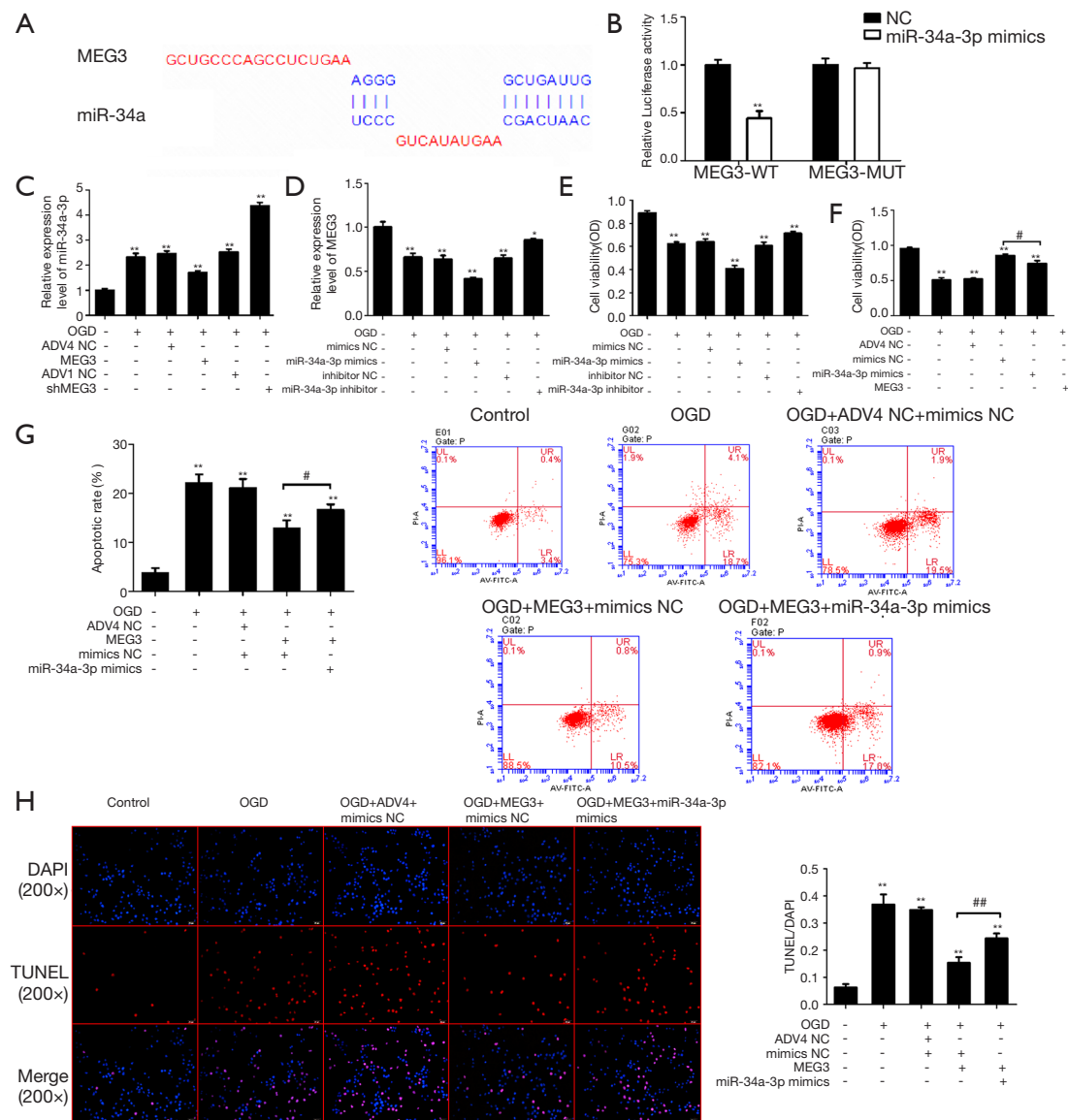Huang et al. Role of LncRNA MEG3 in Cardiac arrest

Figure 4 MEG3 sponges miR-34a-3p to protect against OGD-induced Caco-2 cell I/R injury. (A) The interaction of MEG3 and miR-34a-3p was identified by bioinformatics analysis using the DIANA tools web-server (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php). (B) The luciferase activities of wild-type MEG3 (MEG3-WT) and MEG3 with mutation of the miR-34a-3p binding site (MEG3-MUT) were analyzed by luciferase reporter gene assays in Caco-2 cells treated with the control or miR-34a-3p mimic. (C) Caco-2 cells and OGD-treated Caco-2 cells were infected with ADV4, ADV4 MEG3, ADV1 or ADV1 MEG3 shRNA, as indicated. The expression levels of miR-34a-3p were measured by qPCR. (D,E) Caco-2 cells and OGD-treated Caco-2 cells were treated with control, miR-34a-3p mimic, or miR-34a-3p inhibitor, as indicated. (D) The expression levels of MEG3 were determined by qPCR. (E) Cell viability was analyzed by MTT assays. (F,G) Caco-2 cells and OGD-treated Caco-2 cells were infected with ADV4, ADV4 MEG3, control, or miR-34a-3p mimic, as indicated. (F) Cell viability was analyzed by MTT assays. (G) Cell apoptosis was assessed by flow cytometry. (H) Cell apoptosis was assessed by TUNEL staining. Mean ± SD of at least three experiments is shown. Statistically significant differences are indicated: *P<0.05, **P<0.01, #P<0.05, ##P<0.01. MEG3, maternally expressed 3; OGD, oxygen and glucose deprivation; qPCR, quantitative polymerase chain reaction; SD, standard deviation; I/R, ischemia/reperfusion; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.
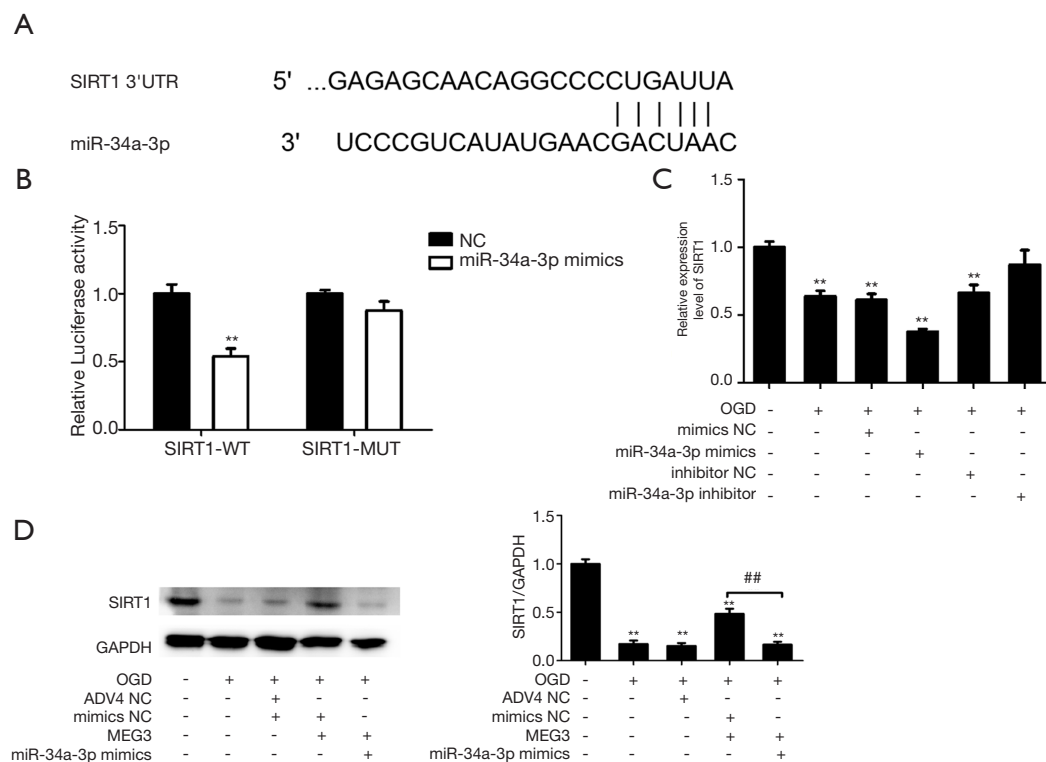
Figure 5 MEG3 activates the expression of SIRT1 by sponging miR-34a-3p in response to OGD treatment *in vitro*. (A) The interaction between SIRT1 and miR-34a-3p was identified by bioinformatics analysis using Targetscan (http://www.targetscan.org/vert_72/) (B) The luciferase activity of wild-type SIRT1 SIRT1-WT) and SIRT1 with mutation of the miR-34a-3p binding site (SIRT1-MUT) were analyzed by luciferase reporter gene assays of Caco-2 cells treated with control or miR-34a-3p mimic. (C) Caco-2 cells or OGD-treated Caco-2 cells were treated with control, miR-34a-3p mimic, or miR-34a-3p inhibitor, as indicated. The expression levels of SIRT1 were analyzed by qPCR. (D) Caco-2 cells or OGD-treated Caco-2 cells were infected with ADV4, ADV4 MEG3, control, or miR-34a-3p mimic as indicated. The expression levels of SIRT1 were measured by western blot. Mean ± SD of at least three experiments is shown. Statistically significant differences are indicated: **$P<0.01$, ##$P<0.01$. MEG3, maternally expressed 3; OGD, oxygen and glucose deprivation; qPCR, quantitative polymerase chain reaction; SD, standard deviation; SIRT1, sirtuin 1.

acid and endotoxin induced by MEG3 depletion but enhanced the decreases in these levels caused by MEG3 overexpression (*Figure 7A,B*). Similar results were observed by HE staining of sections of intestinal mucosa from the rats (*Figure 7C*). Furthermore, the expression levels of occludin and claudin-1 in the intestinal mucosa of the rats were significantly reduced by CA-induced intestinal I/R, and this effect could be rescued by MEG3 overexpression but reinforced by the depletion of MEG3 (*Figure 7D,E*). Additionally, PDTC reversed the occludin and CLDN1 expression caused decreased by MEG3 depletion but further enhanced the MEG3 overexpression-elevated expression of these genes (*Figure 7A,B*). Taken together, our results suggested that MEG3 alleviated CA-induced barrier

dysfunction *in vivo* via NF-κB signaling.

Discussion

Intestinal I/R causes intestinal barrier dysfunction through various mechanisms (49). Nurr1 has been reported to support the restoration of the intestine after I/R injury via repressing p21 (50). Phosphatase and tensin homolog (PTEN) perform an essential role in relieving renal dysfunction after I/R by preserving the capacity of the intestinal barrier (51). MitoQ defends the function of the intestinal barrier by alleviating mitochondrial DNA damage via the Nrf2/ARE pathway (52). Ginsenoside Rb1 was shown to inhibit intestinal I/R-induced oxidative
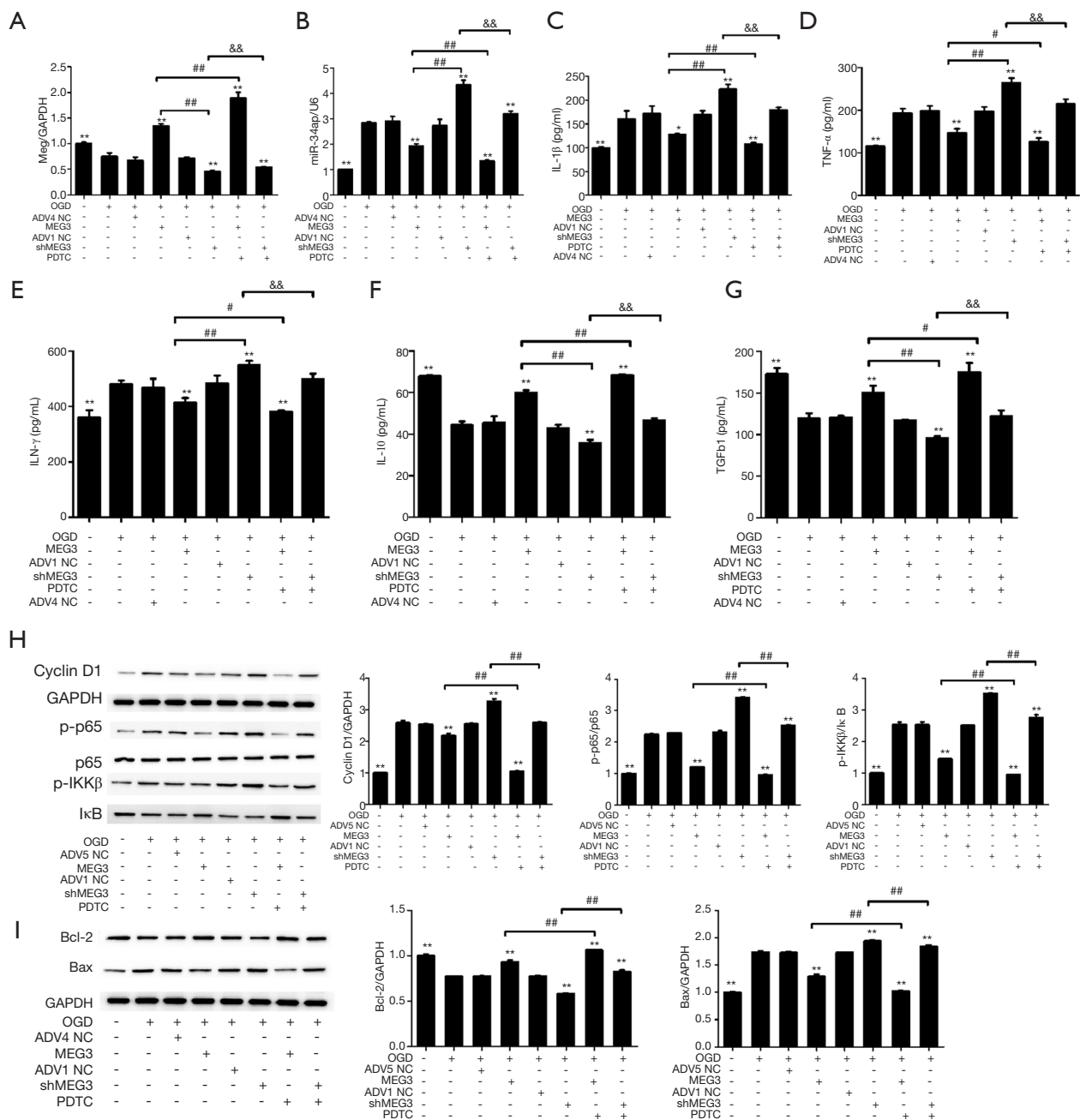
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Figure 6 MEG3 protects Caco-2 cells from OGD-induced I/R injury via miR-34a-3p/NF-κB signaling. (A–I) Untreated or OGD-treated Caco-2 cells were infected with ADV4, ADV4 MEG3, ADV1, or ADV1 MEG3 shRNA or treated with PDTC as indicated. (A) The expression levels of MEG3 were analyzed by qPCR. (B) The expression levels of miR-34a-3p were measured by qPCR. (C-G) The levels of IL-1β, TNF-α, IFN-γ, IL-10, and TGFb-1 in the culture medium of the cells were determined by ELISA assays. (H) The expression levels of phosphorylation of p65 (p-p65), p65, phosphorylation of IκB, IκB, cyclin D1, and GAPDH were assessed by western blot. (I) The expression levels of Bcl-2, Bax, and GAPDH were detected by western blot. Mean ± SD of at least three experiments is shown. Statistically significant differences are indicated: *P<0.05, **P<0.01, #P<0.05, ##P<0.01, &&P<0.01. NF-κB, nuclear factor-kappa B; MEG3, maternally expressed 3; OGD, oxygen and glucose deprivation; I/R, ischemia/reperfusion; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-gamma-γ; IL-10, inflammatory factors including interleukin 10; TGFb-1, transforming growth factor beta-1; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.
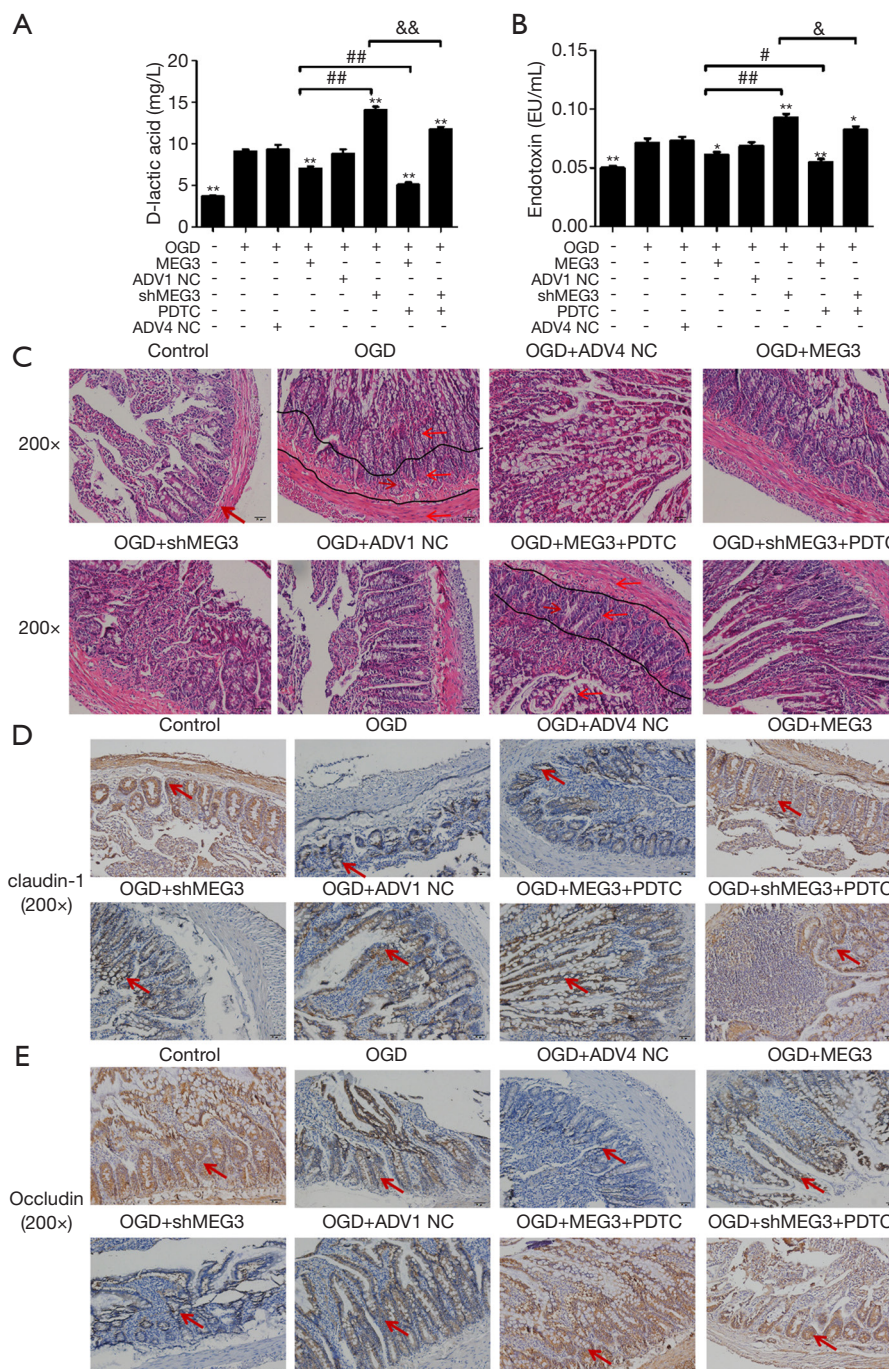
Figure 7 MEG3 relieves CA-induced intestinal barrier dysfunction through NF-κB signaling *in vivo*. (A-E) An *in vivo* CA model was established to assess the effect of CA on intestinal barrier function. Sprague-Dawley rats were intravenously injected with ADV4, ADV4 MEG3, ADV1, or ADV1 MEG3 shRNA via the tail, or treated with PTDC as indicated. (A,B) The levels of D-lactic acid and endotoxin in the serum of the rats were measured by using the corresponding test kits. (C) The intestinal injury was analyzed by hematoxylin and eosin (HE) staining in sections of intestinal mucosa from the rats. (D,E) The expression levels of occludin and claudin-1 in the intestinal mucosa of the rats were determined by immunohistochemical (IHC) staining. Mean ± SD of at least three experiments is shown. Statistically significant differences are indicated: *P<0.05, **P<0.01, #P<0.05, ##P<0.01, &P<0.05, &&P<0.01. The observation positions in (C-E) are marked with red arrows. NF-κB, nuclear factor-kappa B; MEG3, maternally expressed 3; CA, Cardiac arrest.

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stress and inflammation by activating the PI3K/Akt signaling pathway (53). G protein-coupled estrogen receptor was reported to shield the intestine from I/R damage in rats by preserving the cell proliferation of crypt (54). Glial cell line-derived neurotrophic factor (GDNF) plays a vital role in the intestinal barrier in severe I/R injury (13). LncRNAs have also been found to participate in intestinal I/R injury and intestinal barrier disruption. LncRNA H19 plays a restoring role in intestinal I/R-induced intestinal barrier dysfunction (55). Repression of lncRNA NEAT1 can help to overcome the inflammatory response in inflammatory bowel disease (IBD) via modulating the intestinal barrier and through exosome-mediated macrophage polarization (56). Long noncoding RNA SPRY4-IT1 regulates intestinal barrier capacity by tempering the expression of tight junction proteins (57). In the present study, we not only demonstrated that lncRNA MEG3 protected human colorectal adenocarcinoma Caco-2 cells from OGD-induced I/R injury but also identified that MEG3 alleviated OGD-induced intestinal barrier dysfunction *in vitro*. Our results offer new evidence that lncRNA participates in the modulation of intestinal I/R injury and mucosal barrier. Moreover, systemic I/R, as a primary post–cardiac arrest syndrome, is a leading cause of intestinal injury and intestinal barrier dysfunction (58). Here, we also showed that MEG3 relieved CA-induced intestinal injury and barrier dysfunction *in vivo*. Our study provides convincing evidence of the protective role of lncRNA in CA-related intestinal barrier.

MiRNAs have been reported to be involved in the modulation of intestinal I/R and the intestinal barrier. The dysfunction of the intestinal barrier provoked by I/R was shown to up-regulate miRNA-21 (59). Meanwhile, miRNA-378 was observed to protect against intestinal I/R insult by repressing cell apoptosis of the intestinal mucosa (60). MicroRNA-682–modulated decrease of PTEN in intestinal epithelial cells has been shown to enhance intestinal I/R injury (61). Furthermore, the repression of miR-34a-5p relieves intestinal I/R-induced apoptosis as well as the activation of oxygen species via modulating SIRT1 signaling (62). The decrease of IRAK1 by microRNA-146a can also protect the intestine against I/R injury (63).

Our study novelty reported that miR-34a-3p contributed to OGD-induced I/R injury in Caco-2 cells *in vitro* and is sponged by lncRNA MEG3. Our study provides valuable information that miRNA promotes intestine I/R injury. Moreover, a previous study showed that *Escherichia coli* nissle

1917 preserves the function of the intestinal barrier by restraining NF-κB-regulated activation of the myosin light chain kinase (MLCK) signaling pathway (64). Nontoxic doses of Ochratoxin A (OTA) worsen the Deoxynivalenol (DON)-induced dysfunction of the intestinal barrier by activating the NF-κB pathway in IPEC-J2 cells (65). Interference of CREB-modulated zonula occludens-1 (ZO-1) and enhancement of NF-κB-mediated IL-6 by MCT4 impair the function of the intestinal barrier (66). Furthermore, numerous studies have shown that SIRT1 blocks the activation of NF-κB signaling; however, miRNA targeted to SIRT1 is able to rescue this inactivation (33-36). Moreover, SIRT1-mediated p65 inhibition is involved in I/R injury (67). SIRT1/NF-κB signaling plays a pivotal role in the modulation of the intestinal barrier (68). Meanwhile, Xue *et al.* find that down-regulation of MEG3 leads to the inhibition of inflammation and induces M2 macrophage polarization via miR-223/TRAF6/NF-κB axis, thus alleviating VMC (69). Chen *et al.* reveal that MEG3 participates in caerulein-induced inflammatory injuries by targeting the miR-195-5p/FGFR2 regulatory axis via mediating the NF-κB pathway in HPDE cells (70). Bao *et al.* report that LncRNA MEG3 activates the NF-κB signal in GBC cells to affect the proliferation and apoptosis of GBC cells (71). Furthermore, Tong *et al.* find that MEG3 could alleviate HG-inducing apoptosis and inflammation via inhibiting NF-κB signaling pathway by targeting miR-34a/SIRT1 axis (72). Those researches reveal the significant relationship between MEG3 and NF-κB pathway. Our mechanism study showed that MEG3 inhibited the expression of inflammatory factors such as IL-1β and TNF-α, as well as NF-κB signaling. MEG3 activates SIRT1 expression by sponging miR-34a-3p in response to the OGD treatment *in vitro*. These data uncover an innovative mechanism underlying the response to intestinal I/R injury.

In conclusion, we discovered that lncRNA MEG3 exerted a protective effect on intestinal barrier from CA-related I/R injury via miR-34a-3p/SIRT1/NF-κB signaling *in vivo* and *in vitro*. Our finding gives a new insight into the mechanism by which MEG3 restores the function of the intestinal barrier in response to I/R injury, presenting a potential therapeutic candidate for intestinal injury.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-6438

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-6438

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-6438). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (NO.: 20190227) granted by the Animal Ethics Committee of the First Affiliated Hospital of Xiamen University, in compliance with the First Affiliated Hospital of Xiamen University guidelines for the care and use of animals.

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(English Language Editor: B. Draper)