Knockdown of miR-129-5p alleviates intestinal barrier dysfunction induced by ischemia/reperfusion injury via targeting surfactant protein D

Miao Wu
Renmin Hospital of Wuhan University
Jiawei Bai
Renmin Hospital of Wuhan University
Chengtai Ma
Renmin Hospital of Wuhan University
Jie Wei
Renmin Hospital of Wuhan University
Xianjin Du (duxianjin@whu.edu.cn)
Renmin Hospital of Wuhan University

Research Article

Keywords: ischemia/reperfusion injury, intestinal barrier dysfunction, intestinal mucosa permeability, microRNA-129-5p, surfactant protein D

Posted Date: April 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1567299/v1

Abstract

An increasing number of microRNAs (miRs) have been shown to serve crucial roles in intestinal ischemia/reperfusion (I/R) injury. The aim of the present study was to examine the effects of miR-129-5p on I/R injury *in vitro* and *in vivo*. It was revealed that the expression level of miR-129-5p was upregulated, whereas SP-D expression was downregulated in the intestinal tissues of I/R mice. Serum levels of diamine oxidase, FD-4 and D-lactic acid, which are commonly used indicators of intestinal mucosal permeability, were significantly decreased following miR-129-5p knockdown. By contrast, ZO-1 and E-cadherin expression levels were increased following miR-129-5p knockdown, as determined using IHC, which improved the function of the epithelial barrier. In addition, Caco-2 cells were exposed to hypoxia/reoxygenation, which can trigger I/R injury. miR-129-5p expression was increased, whereas SP-D expression was decreased by H/R exposure *in vitro*. Knockdown of miR-129-5p increased ZO-1 and E-cadherin expression in Caco-2 cells. Moreover, overexpression of SP-D improved the function of the epithelial barrier, as indicated by increased transepithelial electrical resistance values and decreased paracellular flux of FD-4. It was also found that liver and lung injury triggered by I/R was attenuated by miR-129-5p knockdown. Collectively, the present study suggested that miR-129-5p knockdown mitigated I/R injury by targeting SP-D.

Introduction

Intestinal ischemia/reperfusion (I/R) injury is an outcome of the activation of a cascade of reactions following the restoration of blood flow to ischemic tissues (1). Ischemia/reperfusion is a severe complication of several clinical conditions, and I/R injury may develop following several different disorders, including infection, mesenteric ischemia and shock (2). Innate immune responses and inflammation are activated following reperfusion of ischemic tissues, which results in hypoxic cell damage (3). In addition, I/R injury can lead to damage of the intestinal mucosa, impairment of local microvasculature, elevated mucosal permeability and multiple organ failure (4, 5).

During reperfusion, intestinal ischemia can result in significant cellular damage, which leads to dysfunction of the epithelial barrier. It is well established that impairment of the intestinal epithelial barrier can result in increased permeability and bacterial translocation (6). Moreover, dysfunction of the intestinal barrier contributes to I/R complications (7). The aim of the present study was to investigate the potential mechanism via which intestinal barrier dysfunction develops.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs of 20–24 nucleotides in length (8). miRNAs can modulate the expression of genes via mRNA degradation or inhibition of transcript translation (9, 10). A growing number of studies have shown that certain miRNAs are involved in I/R injury (11–13). Studies have shown that miR-378 can ameliorate I/R injury by inhibiting intestinal mucosal cell apoptosis (14). It has been reported that miR-125b acts as a biomarker in renal I/R injury (15), whereas miR-200c contributes to cardiac I/R injury by regulating glutaminase-mediated glutamine metabolism (16). In addition, miR-146a can protect the small intestine against I/R injury by repressing NF-κB (17).

miR-129-5p exerts various effects in different types of cancer (18–20). For instance, miR-129-5p can sensitize breast cancer to trastuzumab by inhibiting ribosomal protein S6 (21), whereas miR-129-5p represses the progression of chondrosarcoma via modulation of the Wnt signaling pathway (22). In addition, miR-129-5p can attenuate gastric cancer cell proliferation and epithelial-mesenchymal transition via modulation of high mobility group box 1 (HMGB1) (23).

The present study aimed to examine the effects of miR-129-5p on II/R injury and to determine its potential mechanism of action.

Materials And Methods

Cell culture. Human colon epithelial cancer cell line Caco-2 were purchased from American Type Culture Collection and maintained in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA), 1% non-essential amino acids and 1% glutamine. Cells were incubated at 37˚C in a humidified incubator with 5% CO_2. Hypoxia was induced by culturing the cells in a modular incubator with an O_2 sensor, which allowed the mixing of N_2 and air to achieve hypoxic conditions (2% O_2 and 5% CO_2 balanced with N_2) for 12 h. Cells were transferred to normoxia conditions (21% O_2 and 5% CO_2 balanced with N_2) for reoxygenation for 12 h.

II/R mice model. A total of 24 male C57BL/6 mice (age, 6 weeks; weight, 20–22 g; Shanghai Animal Laboratory Center) were maintained at 22 ± 2˚C and 55–60% humidity with 12-h light/dark cycles. Animal experiments were performed in the specific-pathogen-free Animal Laboratory at Renmin Hospital of Wuhan University. All mice were fed adaptively for 1 week with food and water *ad libitum*. Mice were anesthetized via an intraperitoneal injection of 30 mg/kg pentobarbital. Then, a midline laparotomy was performed. The superior mesenteric artery was identified, separated and clamped. After 45 min of ischemia, the vascular clamp was removed to allow reperfusion. After 0, 4, 8 or 16 h of reperfusion, small intestinal tissues were collected for further evaluation. The mice were divided into the following four groups (n = 6 per group): i) Sham + lentiviral vector (LV)-negative control (NC); ii) sham + LV-anti-miR-129-5p; iii) I/R + LV-NC group; and iv) I/R + LV-anti-miR-129-5p. At the end of reperfusion (4–16 h), a 10-cm segment of the intestine was cut 5 cm away from the ileocecal valve. After the indicated period of reperfusion, mice were sacrificed via cervical dislocation, and the tissue was harvested for further analysis. Filter paper was used to dry the intestinal mucosa, followed by preservation at -80˚C for further analysis.

All animal experiments were performed in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (24). Animal experiments were approved by Ethics Committee of Renmin Hospital of Wuhan University).

LV infection and plasmid transfection. Lentiviral constructs-empty vector (LV-NC), LV-anti-miR-129-5p and LV-miR-129-5p were obtained from Shanghai GenePharma Co., Ltd. The sequences were as follows: LV-miR-129-5p, 5’-CUUUUUGCGGUCUGGGCUUGC-3’; and LV-anti-miR-129-5p, 5’-

GCAAGCCCAGACCGCAAAAAG-3'. Caco-2 cells ($2x10^6$/well) were infected with $1x10^7$ lentivirus transducing units with 5 µg/ml polybrene for 24 h at room temperature. After 48 h, cells were harvested for the following study. To stably infect the cells, 2 µg/ml puromycin was added for 3 days. Mice were injected with LV-NC (10^9 PFU) or LV-anti-miR-129-5p (10^9 PFU) via the tail vein at 12 h prior to ischemia. For transfection, the SP-D overexpression plasmid pcDNA3.1-SP-D was purchased from Shanghai GenePharma Co., Ltd. Briefly, Caco-2 cells ($2x10^6$/well) were seeded into 6-well plates and cultured until they reached 70–80% confluence. Prior to transfection, Lipofectamine® 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), serum-free DMEM and empty pcDNA3.1-NC (1 µg; Shanghai GenePharma Co., Ltd.) or pcDNA3.1-SP-D (1 µg; Shanghai GenePharma Co., Ltd) were mixed and incubated for 30 min at room temperature, and then added to the cells with complete medium containing 10% FBS. After 24 h of transfection at room temperature, cells were harvested for subsequent experiments.

Bioinformatics analysis. starBase (http://starbase.sysu.edu.cn) was used to predict the association between SP-D and miR-129-5p.

Immunofluorescence. Cells were fixed using ice-cold 4% paraformaldehyde for 15 min and then washed with PBS at room temperature. Following fixing, cells were permeabilized using 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) at room temperature for 10 min, and blocked using 3% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The cells were incubated with antibodies against zona occludens 1 (ZO-1; cat. no. ab221547) and E-cadherin (cat. no. ab76055) (both 1:200; Abcam) at 4˚C overnight. Subsequently, cells were incubated with the Alexa Fluor 594 secondary antibody (cat. no. ab150080; Abcam) for 1 h at room temperature. DAPI was used to stain the nuclei at room temperature for 15 min. Cells were imaged using a fluorescence microscope (Olympus Corporation; magnification, x200).

Reverse transcription-quantitative PCR. Total RNA from the intestinal tissues and Caco-2 cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed using a Transcript All-in-one SuperMix qPCR kit (Beijing TransGen Biotech Co., Ltd.) according to the manufacturer's protocol, using a reaction volume of 20 µl. SP-D mRNA expression and miR-129-5p expression were quantified using a SYBR Real-time PCR kit (Beijing TransGen Biotech Co., Ltd.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 5 min; followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec, using a total reaction volume of 25 µl. PCR amplification was performed on an ABI Prism 7900 detection system (Applied Biosystems; Thermo Fisher Scientific, Inc). PCR primers were synthesized by Shanghai GenePharma Co., Ltd. and the sequences of the primers used are listed in Table I. GAPDH was used as the reference gene for SP-D mRNA, and U6 was used as the reference gene for miR-129-5p. Relative gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (25).

Western blotting. Total protein was extracted from cells and small intestinal tissues using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with proteinase inhibitors (Beyotime Institute of Biotechnology). The lysate was centrifuged for 20 min at 12,000 x g at -4˚C, the supernatant was

collected. The protein concentration was determined using a BCA assay (Beyotime Institute of Biotechnology). A total of 50 μg protein was loaded per well on a 10% SDS-gel, resolved using SDS-PAGE and transferred to PVDF membranes (Cytiva). The membranes were blocked using skimmed milk at room temperature for 1 h, and subsequently incubated with primary antibodies against SP-D (cat. no. ab220422), ZO-1 (cat. no. ab276131), E-cadherin (cat. no. ab40772) and GAPDH (cat. no. ab9484) (all 1:1,000; Abcam) overnight at 4˚C. The membrane was incubated with horseradish-peroxidase-bound secondary antibodies (all 1:2,000; cat. nos. ab20571 and ab205718; Abcam) for 2 h at room temperature. ECL western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) was used to visualize the signals. Quantity One software (version 4.6.2, Bio-Rad Laboratories, Inc.) was employed to semi-quantify relative protein expression.

Histopathology and immunohistochemistry (IHC). Lung and liver tissue samples obtained from the II/R mouse model were fixed with 4% paraformaldehyde for 24 h at room temperature, paraffin-embedded and cut into 4-μm sections. After rehydration in a decreasing ethanol series, sections were deparaffinized and stained with Harris hematoxylin solution (Beyotime Institute of Biotechnology) for 5 min at 37˚C and eosin for 1 min at 37˚C. The Mikawa's score (26) was calculated based on the following: i) Alveolar congestion; ii) hemorrhage; iii) infiltration or aggregation of neutrophils in the airspace or vessel wall; and iv) thickness of alveolar wall/hyaline membrane formation. Each feature was scored on a 5-point scale as follows: 0, minimal damage; 1+, mild damage; 2+, moderate damage; 3+, severe damage; and 4+, max. Eckhoff's scores (27) were evaluated by using an ordinal scale as follows: Grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders, and mild to moderate neutrophil infiltration; and grade 3, severe injury with disintegration of hepatic cords, hemorrhage, and severe PMN infiltration.

For IHC of SP-D, mouse small intestinal tissue sections were prepared as previously described. Sections were fixed using 4% methanol at 4˚C for 24 h, washed in PBS and treated with antigen retrieval solution (Dako; Agilent Technologies, Inc.) for 20 min at 90˚C. After incubation in 5% BSA (cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.) at 4˚C for 30 min, the sections were incubated with anti-SP-D antibody (cat. no. ab234260; Abcam; 1: 100) overnight at 4˚C. An ABC kit (Biolead) and 3,3'diaminobenzidine substrates were used to develop the SP-D signal. Then, sections were incubated with an HRP-conjugated polyclonal goat anti-rabbit secondary antibody (1:200; cat. no. GB23303; Wuhan Servicebio Technology Co., Ltd.) for 1 h at room temperature. Histopathological and IHC analysis were performed using a light microscope (Olympus Corporation; magnification, x200).

Transepithelial electrical resistance (TEER) assay. An epithelial voltohmmeter (World Precision Instruments) was used to measure the transepithelial electrical resistance (TER) of the filter-grown Caco-2 intestinal monolayers as previously described (28). To form the monolayers, Caco-2 cells were sub-cultured after partial digestion with 0.25% trypsin and 0.9 mM EDTA in PBS free of Ca^{2+}- and Mg^{2+}. Caco-2 monolayers were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids and 1% glutamine for 3–4 weeks after seeding at a density of $2x10^5$ cells/well.

A Transwell system was used to perform the TEER experiments. Caco-2 cell monolayers with a TEER > 500 Ω cm^2 were used for the assay. The relative TER in the various groups was calculated as a percentage of the control Caco-2 monolayers.

Determination of intestinal permeability in vitro. The permeability of the intestinal barrier in Caco-2 cells was determined based on FITC-dextran 4 (FD-4) fluorescence intensity. Briefly, 1 mg/ml FD-4 was applied to the apical side of the Caco-2 cell monolayer at room temperature for 10 min as described previously in the TEER section. The Caco-2 cell monolayer was subjected to fluorescence analysis, and the fluorescence intensity was measured using an Enspire2300 microplate reader. Serum D-lactic acid was measured using coupled liquid chromatography (Waters Corporation). Liquid chromatography was equipped with a binary solvent delivery pump, an autosampler, a column compartments, a PDA detector and controlled using MassLynx v4.1 software (Waters Corporation). A reverse phase Acquity UPLC BEH C18 column (Waters Corporation; 2.1x100 mm; 1.7 μm) and an Acquity UPLC BEH C18 VanGuard™ pre-column (1.7 μm) from Waters Corporation were used. A total of 1 ml whole blood was collected from the LV-NC mice and LV-anti-miR-129-5p mice to obtain the serum at 6 h after reperfusion via tail vein. D-lactic acid was oxidized using D-lactate dehydrogenase, and the absorbance was measured at a wavelength of 450 nm. Diamine oxidase (DAO) levels were assessed using an ELISA kit (cat. no. CB3349172; Shanghai Lianmai Biological Engineering Co.) according to the manufacturer's protocol. All the mice were administered 750 mg/kg FD-4 via oral gavage for treatment before testing.

Luciferase reporter assay. The dual-luciferase miRNA Target Expression vector pmirGLO (Promega Corporation) was used to generate luciferase reporter constructs. Luciferase activity was assessed using the dual-Glo Luciferase assay system (Promega Corporation). Briefly, cells ($2x10^6$/well) were seeded into 48-well plates for 24 h. Lipofectamine 3000 was used to co-transfect cells with cloned SP-D wild-type (WT) 3'untranslated region (UTR) or SP-D mutant (MUT) 3'UTR (both obtained from Shanghai GeneChem Co., Ltd) and LV-miR-29-5p or LV-anti-miR-129-5p. Then, the mixtures were added to cells and incubated for 24 h at room temperature. Following transfection, the activities of firefly and *Renilla* luciferase were measured by using a Dual-Luciferase Reporter assay system (Promega Corporation). Relative luciferase activity was determined as the ration of firefly to *Renilla* luciferase.

Statistical analysis. SPSS version 22.0 (IBM Corp.) was used for statistical analysis. GraphPad Prism version 7 (GraphPad Software, Inc.) was used to generate the graphs. Three independent experiments were performed. Unpaired Student's t-tests or one-way ANOVA followed by a Tukey's post hoc test were used to compare differences among groups. A Kruskall-Wallis test followed by a Dunn's post hoc test was used to evaluate the Mikawa's and Eckhoff's scores. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-129-5p and SP-D following II/R injury in vivo. First, C57BL/6 mice were subjected to 45 min of intestinal ischemia, followed by 0–16 h reperfusion or sham surgery. As shown in Fig. 1A,

intestinal miR-129-5p expression levels were increased during the first 4 h following the onset of reperfusion and progressively decreased from 4–16 h after reperfusion. Moreover, as shown in Fig. 1B and C, SP-D expression was decreased during the first 8 h. IHC staining identified that SP-D expression was significantly reduced following II/R injury compared with that in the sham group (Fig. 1D). These results suggested that miR-129-5p and SP-D may participate in II/R injury.

Epithelial barrier function and intestinal mucosal permeability are improved by miR-129-5p knockdown in vivo. To investigate the function of miR-129-5p, mice that had undergone I/R injury for 8 h (29) were infected with LV-anti-miR-129-5p or LV-NC. Compared with that in the LV-NC group, miR-129-5p expression was significantly decreased by LV-anti-miR-129-5p *in vivo* (Fig. 2A). The protein expression levels of ZO-1 and E-cadherin were significantly increased following miR-129-5p knockdown compared with those in the LV-NC group (Fig. 2B). Additionally, the serum levels of DAO, D-lactic acid and FD-4 were significantly decreased following miR-129-5p knockdown compared with those in the LV-NC group (Fig. 2C-E). These results indicated that miR-129-5p improved intestinal mucosal permeability and epithelial barrier function *in vivo*.

Analysis of miR-129-5p and SP-D expression during hypoxia/reoxygenation (H/R) injury in vitro. To determine the role of miR-129-5p *in vitro*, Caco-2 cells were exposed to H/R conditions to simulate II/R injury, as previously described (30). miR-129-5p expression was significantly increased in Caco-2 cells that underwent H/R compared with that in the control group (Fig. 3A). Additionally, the mRNA and protein expression levels of SP-D were significantly decreased in the H/R group compared with those in the control group (Fig. 3B-D). These results demonstrated that miR-129-5p and SP-D were involved in H/R injury *in vitro*, which was consistent with the *in vivo* data.

miR-129-5p knockdown improves epithelial barrier function in vitro. Caco-2 cells were infected with LV-anti-miR-129-5p or LV-NC for 48 h. As shown in Fig. 4A, miR-129-5p expression was successfully knocked down by LV-anti-miR-129-5p. SP-D mRNA expression was also increased following miR-129-5p knockdown (Fig. 4B). Moreover, the protein expression levels of ZO-1 and E-cadherin were significantly increased following miR-129-5p knockdown compared with those in the LV-NC group (Fig. 4C). Importantly, compared with that of the LV-NC group, miR-129-5p knockdown significantly improved epithelial barrier function based on the increased TEER values (Fig. 4D) and significantly decreased the paracellular flux of FD-4 (Fig. 4E). These results suggested that miR-129-5p knockdown improved epithelial barrier function *in vitro*.

SP-D is a direct target of miR-129-5p. Bioinformatics analysis was used to predict the association between SP-D and miR-129-5p (Fig. 5A). It was found that miR-129-5p expression was significantly increased by LV-miR-129-5p compared with that in the LV-NC group (Fig. 5B). Luciferase reporter plasmids containing WT-SP-D and MUT-SP-D binding sites were transfected into cells. Compared with that in the NC group, co-transfection of WT-SP-D and LV-miR-129-5p significantly decreased reporter activity in Caco-2 cells, whereas co-transfection of MUT-SP-D and LV-miR-129-5p did not result in a

decrease in luciferase activity (Fig. 5C). Compared with that in the NC group, co-transfection of WT-SP-D and LV-anti-miR-129-5p significantly increased reporter activity in Caco-2 cells (Fig. 5C)

Subsequently, the effect of SP-D on Caco-2 cells was evaluated. Cells were infected with pcDNA3.1-SP-D or LV-miR-129-5p. As shown in Fig. 5D and E, compared with that in the NC group, SP-D expression was significantly increased following transfection with pcDNA3.1-SP-D, whereas co-transfection with LV-miR-129-5p significantly decreased pcDNA3.1-SP-D-induced expression levels. Overexpression of SP-D improved epithelial barrier function, based on the increased TEER values (Fig. 5F) and decreases in paracellular flux of FD-4 observed in the SP-D group compared with those in the NC group (Fig. 5G). miR-129-5p overexpression reversed the effects of SP-D overexpression on Caco-2 cells. These results suggested that SP-D may be a direct target of miR-129-5p.

miR-129-5p knockdown suppresses I/R-induced organ injury. It has been reported that II/R injury can lead to the damage of multiple organs (31). In the present study, lung and liver tissue injury was evaluated using the Mikawa's (26) and Eckhoff's scores (27), respectively. As shown in Fig. 6A and B, miR-129-5p knockdown alleviated II/R-induced lung and liver histological injury (Fig. 6C and D). These results suggested that miR-129-5p knockdown suppressed I/R-induced liver and lung injury.

Discussion

In the present study, it was found that miR-129-5p expression was significantly increased in the mouse models of II/R injury and in the H/R cell models, whereas SP-D expression was significantly decreased. miR-129-5p knockdown reduced intestinal barrier dysfunction, and serum levels of D-lactic acid, DAO and FD-4 were decreased. Moreover, ZO-1 and E-cadherin expression was increased following treatment with LV-anti-miR-129-5p *in vivo*. Consistently, miR-129-5p knockdown improved epithelial barrier function in Caco-2 cells. The results demonstrated that SP-D overexpression increased TEER values and decreased paracellular flux of FD-4. Additionally, miR-129-5p knockdown attenuated mesenteric I/R-induced liver and lung injury.

Several studies have shown that miRNAs affect II/R injury (32–34). However, few reports have examined the role of miR-129-5p in I/R injury. In the present study, it was identified that miR-129-5p expression was increased in II/R injury mouse models and in the H/R cell models. Loss of intestinal barrier function is a hazardous complication of II/R injury, which can result in life-threatening bacterial translocation (35). For example, during the development of intestinal epithelial cells, enterocytes can migrate from the crypt base to the villi (36). The role of miR-129-5p in I/R injury has been determined in other organs. For instance, miR-129-5p can ameliorate I/R injury by targeting HMGB1 in the myocardium (37). In addition, miR-129-5p alleviates myocardial injury by targeting the suppressor of cytokine signaling 2 following I/R injury (38). In the present study, it was demonstrated that miR-129-5p knockdown reduced the dysfunction of the intestinal barrier. Furthermore, levels of D-lactic acid, DAO and FD-4 were decreased by miR-129-5p knockdown, whereas the expression levels of ZO-1 and E-cadherin were increased by LV-anti-miR-129-5p.

SP-D is a member of the collectin family (39), and has been found to have important roles in lung infection (40). SP-D is expressed and secreted by lung tissue, and its expression has also been observed in extrapulmonary tissues, such as the intestinal mucosal surface (41, 42). Alterations in plasma levels of SP-D have been observed in patients with acute respiratory distress syndrome (43). In the present study, it was demonstrated that SP-D expression was significantly decreased in II/R injury, whereas its overexpression ameliorated intestinal barrier dysfunction. Restoration of SP-D expression increased TEER values and decreased paracellular flux of FD-4. In addition, miR-129-5p knockdown improved lung and liver damage. However, whether these mechanisms are observed in humans following I/R injury remains to be determined.

In conclusion, the present study identified that miR-129-5p knockdown exerted beneficial effects on intestinal barrier dysfunction and that SP-D was the target gene of miR-129-5p. However, a novel target gene for management of intestinal barrier dysfunction remains to be identified.

Abbreviations

miR, microRNA; II/R, intestinal ischemia–reperfusion; H/R, hypoxia/reoxygenation ; SP-D, surfactant protein D; ZO-1, zona occludens 1; FD-4, FITC-dextran 4; LV, lentiviral vector; NC, negative control; TEER, Transepithelial electrical resistance; DAO, Diamine oxidase; WT, wild-type; MUT, mutant; UTR, untranslated region

Declarations

Acknowledgements

This study was supported by the Fundamental Research Funds for the Central Universities (2042020kf0109) and Open Foundation of Hubei Key Laboratory (2021KFY025).

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Renmin Hospital of Wuhan University. All animal experiments were based on the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Competing interests

The authors declare that they have no competing interests.

References

1. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Ischemia/Reperfusion. Comprehensive Physiology. 2016;7(1):113–70.

2. Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischemia-reperfusion injury of the intestine and protective strategies against injury. Digestive diseases and sciences. 2004;49(9):1359–77.
3. Chassin C, Hempel C, Stockinger S, Dupont A, Kubler JF, Wedemeyer J, et al. MicroRNA-146a-mediated downregulation of IRAK1 protects mouse and human small intestine against ischemia/reperfusion injury. EMBO molecular medicine. 2012;4(12):1308–19.
4. Clark JA, Coopersmith CM. Intestinal crosstalk: a new paradigm for understanding the gut as the "motor" of critical illness. Shock. 2007;28(4):384–93.
5. Martin B. Prevention of gastrointestinal complications in the critically ill patient. AACN advanced critical care. 2007;18(2):158–66.
6. Jin X, Zimmers TA, Zhang Z, Pierce RH, Koniaris LG. Interleukin-6 is an important in vivo inhibitor of intestinal epithelial cell death in mice. Gut. 2010;59(2):186–96.
7. Kannan L, Kis-Toth K, Yoshiya K, Thai TH, Sehrawat S, Mayadas TN, et al. R-spondin3 prevents mesenteric ischemia/reperfusion-induced tissue damage by tightening endothelium and preventing vascular leakage. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(35):14348–53.
8. Mohr AM, Mott JL. Overview of microRNA biology. Seminars in liver disease. 2015;35(1):3–11.
9. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–33.
10. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425(6956):415–9.
11. Makhdoumi P, Roohbakhsh A, Karimi G. MicroRNAs regulate mitochondrial apoptotic pathway in myocardial ischemia-reperfusion-injury. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2016;84:1635–44.
12. Eltzschig HK, Eckle T. Ischemia and reperfusion–from mechanism to translation. Nature medicine. 2011;17(11):1391–401.
13. Hao J, Wei Q, Mei S, Li L, Su Y, Mei C, et al. Induction of microRNA-17-5p by p53 protects against renal ischemia-reperfusion injury by targeting death receptor 6. Kidney international. 2017;91(1):106–18.
14. Li Y, Wen S, Yao X, Liu W, Shen J, Deng W, et al. MicroRNA-378 protects against intestinal ischemia/reperfusion injury via a mechanism involving the inhibition of intestinal mucosal cell apoptosis. Cell death & disease. 2017;8(10):e3127.
15. Guclu A, Kocak C, Kocak FE, Akcilar R, Dodurga Y, Akcilar A, et al. MicroRNA-125b as a new potential biomarker on diagnosis of renal ischemia-reperfusion injury. The Journal of surgical research. 2017;207:241–8.
16. Liu F, Li Y, Liu G. MicroRNA-200c exacerbates the ischemia/reperfusion injury of heart through targeting the glutaminase (GLS)-mediated glutamine metabolism. European review for medical and pharmacological sciences. 2017;21(14):3282–9.

17. He X, Zheng Y, Liu S, Shi S, Liu Y, He Y, et al. MiR-146a protects small intestine against ischemia/reperfusion injury by down-regulating TLR4/TRAF6/NF-kappaB pathway. Journal of cellular physiology. 2018;233(3):2476–88.
18. Shen R, Pan S, Qi S, Lin X, Cheng S. Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 in gastric cancer. Biochemical and biophysical research communications. 2010;394(4):1047–52.
19. Liu Y, Hei Y, Shu Q, Dong J, Gao Y, Fu H, et al. VCP/p97, down-regulated by microRNA-129-5p, could regulate the progression of hepatocellular carcinoma. PloS one. 2012;7(4):e35800.
20. Dyrskjot L, Ostenfeld MS, Bramsen JB, Silahtaroglu AN, Lamy P, Ramanathan R, et al. Genomic profiling of microRNAs in bladder cancer: miR-129 is associated with poor outcome and promotes cell death in vitro. Cancer research. 2009;69(11):4851–60.
21. Lu X, Ma J, Chu J, Shao Q, Zhang Y, Lu G, et al. MiR-129-5p Sensitizes the Response of Her-2 Positive Breast Cancer to Trastuzumab by Reducing Rps6. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology. 2017;44(6):2346–56.
22. Zhang P, Li J, Song Y, Wang X. MiR-129-5p Inhibits Proliferation and Invasion of Chondrosarcoma Cells by Regulating SOX4/Wnt/beta-Catenin Signaling Pathway. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology. 2017;42(1):242–53.
23. Wang S, Chen Y, Yu X, Lu Y, Wang H, Wu F, et al. miR-129-5p attenuates cell proliferation and epithelial mesenchymal transition via HMGB1 in gastric cancer. Pathology, research and practice. 2019;215(4):676–82.
24. In: th, editor. Guide for the Care and Use of Laboratory Animals. The National Academies Collection: Reports funded by National Institutes of Health. Washington (DC)2011.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–8.
26. Mikawa K, Nishina K, Takao Y, Obara H. ONO-1714, a nitric oxide synthase inhibitor, attenuates endotoxin-induced acute lung injury in rabbits. Anesthesia and analgesia. 2003;97(6):1751–5.
27. Eckhoff DE, Bilbao G, Frenette L, Thompson JA, Contreras JL. 17-Beta-estradiol protects the liver against warm ischemia/reperfusion injury and is associated with increased serum nitric oxide and decreased tumor necrosis factor-alpha. Surgery. 2002;132(2):302–9.
28. Al-Sadi R, Guo S, Ye D, Dokladny K, Alhmoud T, Ereifej L, et al. Mechanism of IL-1beta modulation of intestinal epithelial barrier involves p38 kinase and activating transcription factor-2 activation. Journal of immunology. 2013;190(12):6596–606.
29. Ning JZ, He KX, Cheng F, Li W, Yu WM, Li HY, et al. Long Non-coding RNA MEG3 Promotes Pyroptosis in Testicular Ischemia-Reperfusion Injury by Targeting MiR-29a to Modulate PTEN Expression. Frontiers in cell and developmental biology. 2021;9:671613.
30. Liu L, Yao J, Li Z, Zu G, Feng D, Li Y, et al. miR-381-3p knockdown improves intestinal epithelial proliferation and barrier function after intestinal ischemia/reperfusion injury by targeting nurr1. Cell

death & disease. 2018;9(3):411.
31. Hu Q, Zhou Q, Wu J, Wu X, Ren J. The Role of Mitochondrial DNA in the Development of Ischemia Reperfusion Injury. Shock. 2019;51(1):52–9.
32. Liu Z, Jiang J, Yang Q, Xiong Y, Zou D, Yang C, et al. MicroRNA-682-mediated downregulation of PTEN in intestinal epithelial cells ameliorates intestinal ischemia-reperfusion injury. Cell death & disease. 2016;7:e2210.
33. Zhang B, Tian Y, Jiang P, Jiang Y, Li C, Liu T, et al. MicroRNA-122a Regulates Zonulin by Targeting EGFR in Intestinal Epithelial Dysfunction. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology. 2017;42(2):848–58.
34. Kannan KB, Colorado I, Reino D, Palange D, Lu Q, Qin X, et al. Hypoxia-inducible factor plays a gut-injurious role in intestinal ischemia reperfusion injury. American journal of physiology Gastrointestinal and liver physiology. 2011;300(5):G853-61.
35. Macfarlane GT, Macfarlane S. Factors affecting fermentation reactions in the large bowel. The Proceedings of the Nutrition Society. 1993;52(2):367 – 73.
36. Clevers H, Batlle E. SnapShot: the intestinal crypt. Cell. 2013;152(5):1198- e2.
37. Xing J, Liu J, Liu J, Xu Z. miR-129-5p ameliorates ischemia-reperfusion injury by targeting HMGB1 in myocardium. General physiology and biophysics. 2020;39(5):461–70.
38. Ma R, Chen X, Ma Y, Bai G, Li DS. MiR-129-5p alleviates myocardial injury by targeting suppressor of cytokine signaling 2 after ischemia/reperfusion. The Kaohsiung journal of medical sciences. 2020;36(8):599–606.
39. Seaton BA, Crouch EC, McCormack FX, Head JF, Hartshorn KL, Mendelsohn R. Review: Structural determinants of pattern recognition by lung collectins. Innate immunity. 2010;16(3):143–50.
40. Haagsman HP, Hogenkamp A, van Eijk M, Veldhuizen EJ. Surfactant collectins and innate immunity. Neonatology. 2008;93(4):288–94.
41. Madsen J, Tornoe I, Nielsen O, Koch C, Steinhilber W, Holmskov U. Expression and localization of lung surfactant protein A in human tissues. American journal of respiratory cell and molecular biology. 2003;29(5):591–7.
42. Rubio S, Lacaze-Masmonteil T, Chailley-Heu B, Kahn A, Bourbon JR, Ducroc R. Pulmonary surfactant protein A (SP-A) is expressed by epithelial cells of small and large intestine. The Journal of biological chemistry. 1995;270(20):12162–9.
43. Giannoni E, Sawa T, Allen L, Wiener-Kronish J, Hawgood S. Surfactant proteins A and D enhance pulmonary clearance of Pseudomonas aeruginosa. American journal of respiratory cell and molecular biology. 2006;34(6):704–10.

Tables

Table I. Primers for reverse transcription-quantitative PCR.

Gene	Sequence (5'à3')
GAPDH	F: AAGAAGGTGGTGAAGCAGGC
	R: GTCAAAGGTGGAGGAGTGGG
U6	F: CTCGCTTCGGCAGCACATA
	R: CAGTGCAGGGTCCGAGGTA
miR-129-5p	F: GGGGGCTTTTTGCGGTCTGG
	R: AGTGCGTGTCGTGGAGTC
SP-D	F: TAGATCACATGCCCACCACAT
	R: AGCCCTTAAGCCCTGGAAGTC

miR, microRNA; SP-D, surfactant protein D; F, forward; R, reverse.

Figures

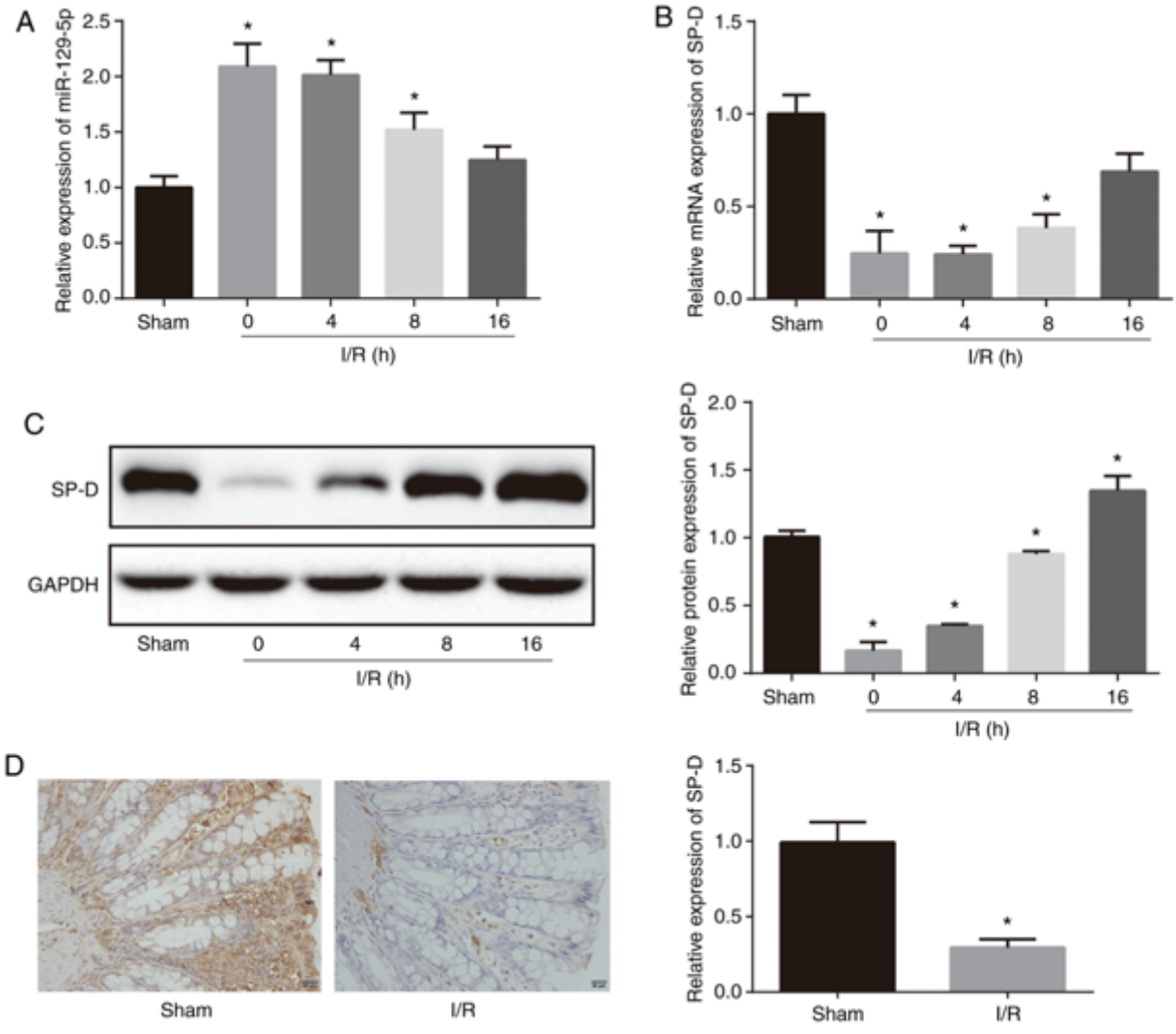

Figure 1

Analysis of miR-129-5p and SP-D expression following II/R injury *in vivo*. (A) miR-129-5p expression was assessed via reverse transcription-quantitative PCR in the intestinal tissues of mice. Mice were subjected to 45 min of intestinal ischemia, followed by 0-16 h of reperfusion or sham surgery. (B) mRNA and (C) protein expression levels of SP-D. (D) Immunohistochemistry analysis of SP-D expression (magnification, x200). Data are presented as the mean ± SD of at least three experiments. *P<0.05 vs. sham. miR, microRNA; SP-D, surfactant protein D; I/R, ischemia/reperfusion.

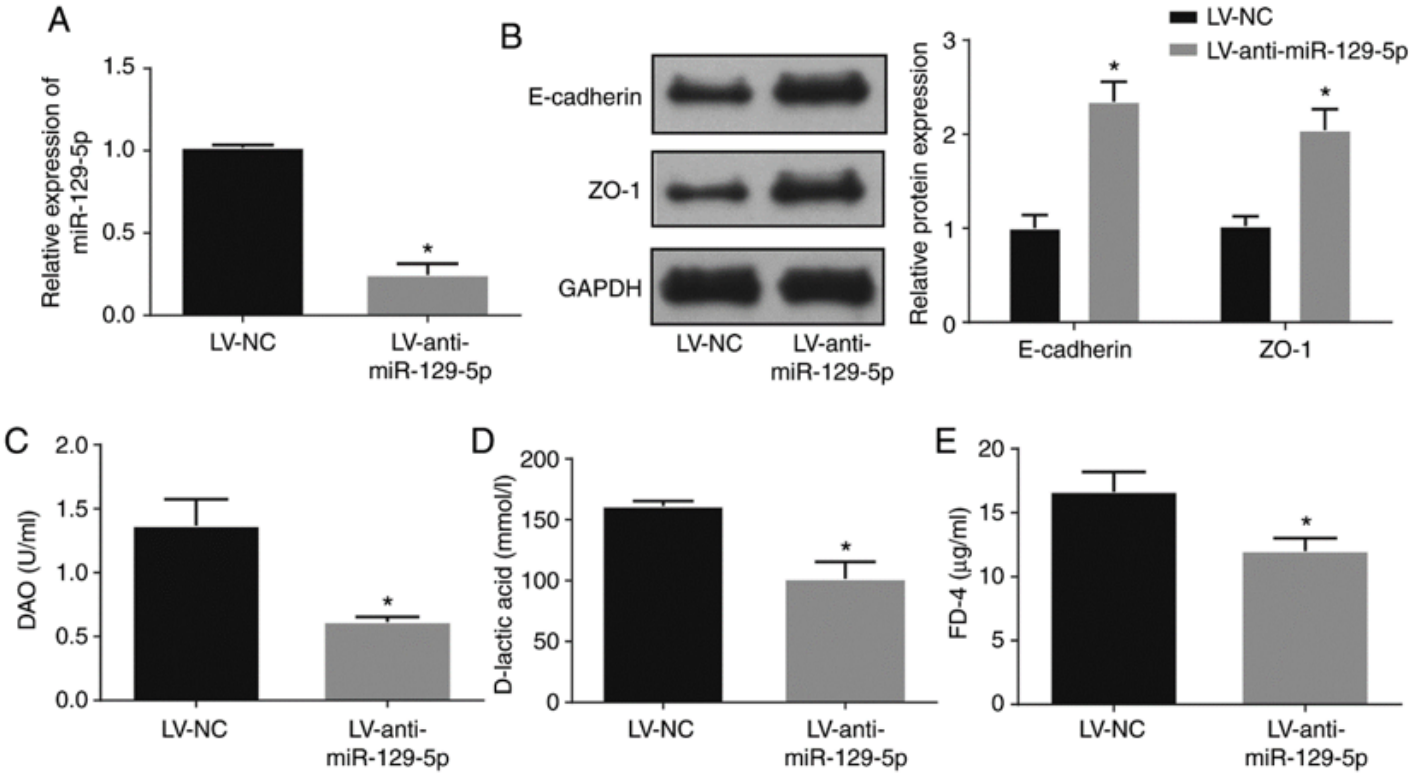

Figure 2

Intestinal mucosal permeability is reduced following miR-129-5p knockdown *in vivo*. (A) miR-129-5p expression in the intestinal tissues of mice. (B) Protein expression levels of E-cadherin and ZO-1. Mice subjected to I/R injury were infected with LV-anti-miR-129-5p or LV-NC. Serum levels of (C) DAO, (D) D-lactic acid and (E) FD-4 in I/R injury mice infected with LV-anti-miR-129-5p or LV-NC. Data are presented as the mean ± SD of at least three experiments. *P<0.05 vs. LV-NC. miR, microRNA; NC, negative control; I/R, ischemia/reperfusion; FD-4, FITC-Dextran 4; DAO, diamine oxidase; ZO-1, zona occludens 1; LV, lentiviral vector.

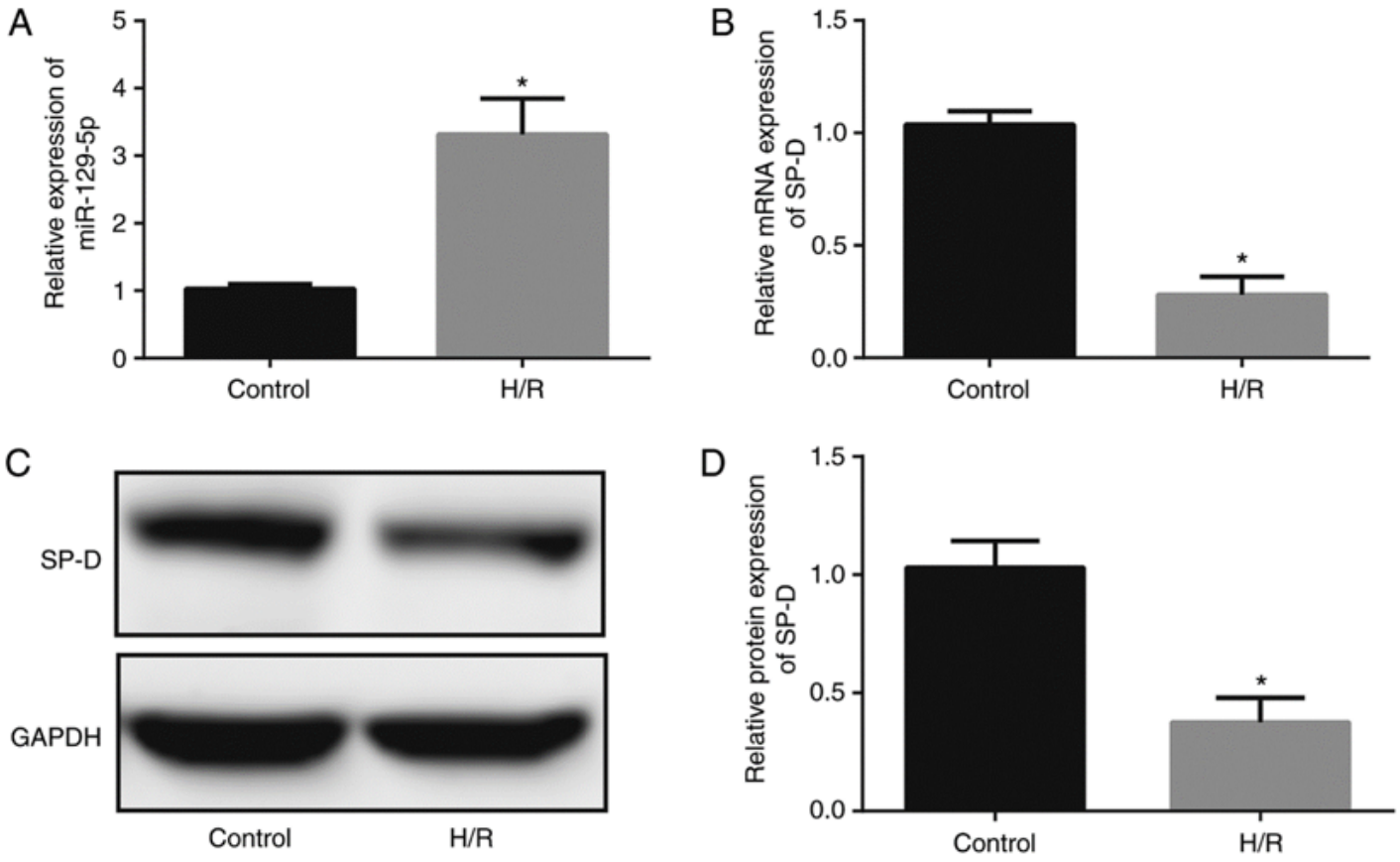

Figure 3

Analysis of miR-129-5p and SP-D expression during H/R injury in Caco-2 cells. (A) Expression level of miR-129-5p. Caco-2 cells were exposed to H/R conditions to simulate intestinal ischemia/reperfusion injury. (B) mRNA expression level of SP-D. Protein expression level of SP-D was (C) determined by western blotting and (D) semi-quantified. *P<0.05 vs. control. miR, microRNA; SP-D, surfactant protein D; H/R, hypoxia/reoxygenation.

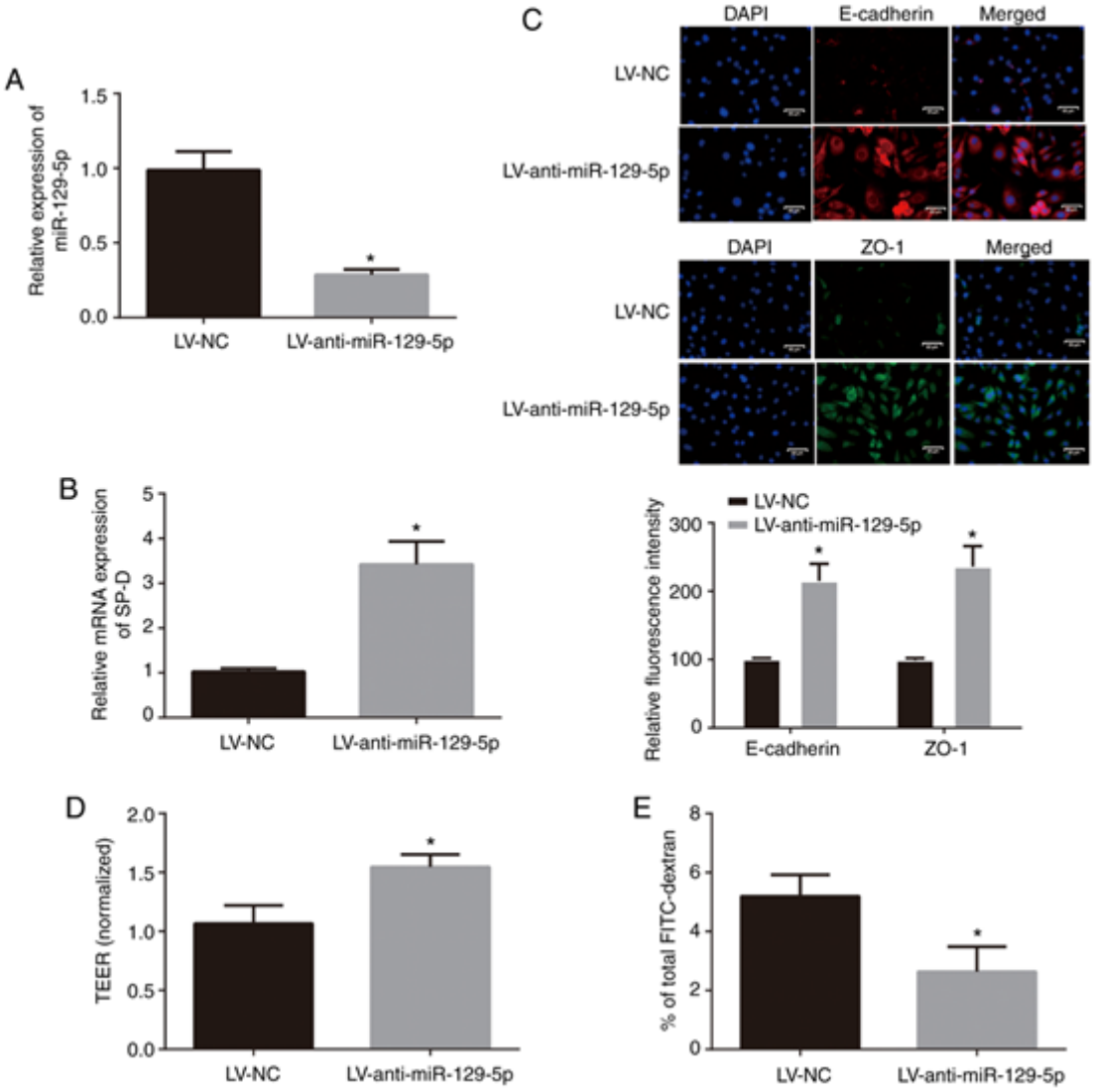

Figure 4

miR-129-5p knockdown increases ZO-1 and E-cadherin expression, and improves epithelial barrier function in Caco-2 cells. (A) Expression level of miR-129 -5p following infection of Caco-2 cells with LV-anti-miR-129-5p or LV-NC. (B) mRNA level expression of SP-D. (C) Cellular distribution of ZO-1 and E-cadherin (scale bar, 50 μm). (D) Changes in epithelial barrier function as indicated by changes in the TEER values. (E) Changes in epithelial barrier function as indicated by changes in FITC-dextran 4 paracellular permeability. Data are presented as the mean ± SD of at least three experiments. *P<0.05 vs. LV-NC. miR, microRNA; NC, negative control; TEER, transepithelial electrical resistance; LV, lentiviral vector; ZO-1, zona occludens 1; SP-D, surfactant protein D.

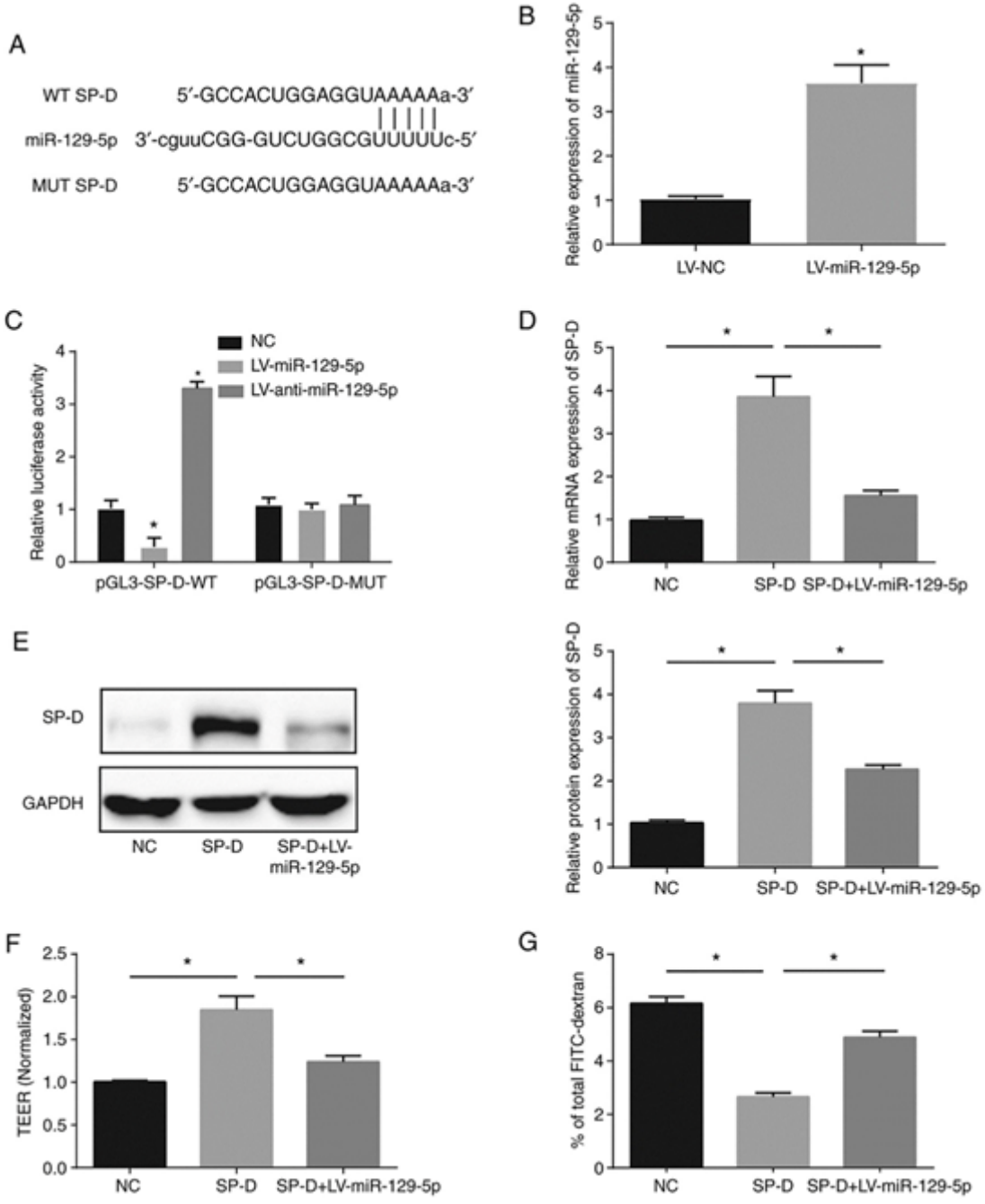

Figure 5

Overexpression of SP-D improves epithelial barrier function in Caco-2 cells. (A) Putative binding site between miR-129-5p and SP-D, and the mutant sites in SP-D-MUT reporter. (B) Expression level of miR-129-5p. Caco-2 cells were transfected with LV-miR-129-5p. (C) Luciferase activity was evaluated in Caco-2 cells co-transfected with SP-D-WT or SP-D-MUT reporter and LV-anti-miR-129-5p or LV-miR-129-5p. (D) mRNA expression level of SP-D. Caco-2 cells were transfected with pcDNA3.1-SP-D or LV-miR-129-5p. (E) Protein expression level of SP-D. Changes in epithelial barrier function as indicated by changes in the (F) TEER values and (G) FITC-dextran 4 paracellular permeability. Data are presented as the mean ± SD of at least three experiments. *P<0.05 vs. LV-NC. miR, microRNA; SP-D, surfactant protein D; NC, negative control; TEER, transepithelial electrical resistance; LV, lentiviral vector; WT, wild-type; MUT, mutant.

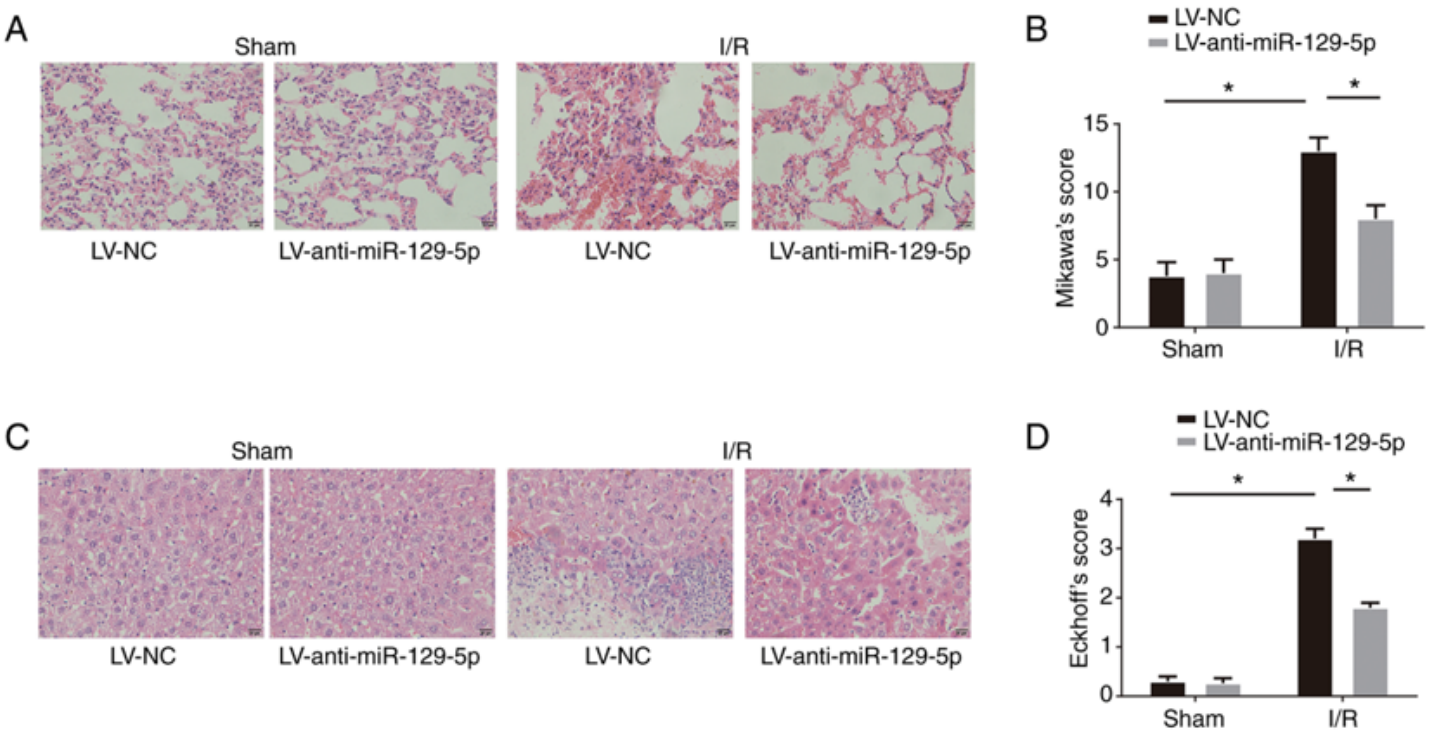

Figure 6

miR-129-5p knockdown reduces II/R induced liver and lung injury. Mice were divided into the following four groups: i) Sham + LV-NC; ii) sham + LV-anti-miR-129-5p; iii) I/R + LV-NC; and iv) I/R + LV-anti-miR-129-5p. (A) Representative images of H&E-stained lung sections from mice (magnification, x200). Lung injury was scored histopathologically using the (B) Mikawa scoring system. (C) Representative images of H&E-stained hepatic sections from mice (magnification, x200). (D) Hepatic injury was scored histopathologically using the Eckhoff scoring system. Data are presented as the mean ± SD of at least three experiments. *P<0.05. miR, microRNA; NC, negative control; I/R, ischemia/reperfusion; LV, lentiviral vector.