MicroRNA-146a overexpression alleviates intestinal ischemia/reperfusion-induced acute lung injury in mice

GEHUI LI[1*], MIN XU[2*], HAO WANG[3], XIAOFEI QI[1], XIAOGUANG WANG[1], YONG LI[1], JING SUN[1] and YUANTAO LI[1]

[1]Department of Anesthesiology, Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University, Shenzhen, Guangdong 518028; [2]Department of Anesthesiology, Xiangyang No.1 People's Hospital, Hubei University of Medicine, Xiangyang, Hubei 441000; [3]Department of Food Safety, Market Supervision Administration of Shenzhen Municipality, Shenzhen, Guangdong 518040, P.R. China

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Abstract. Previous studies have shown that microRNAs (miRs), such as miR-146a play an important role in the pathogenesis of intestinal ischemia/reperfusion (I/R)-induced injury; however, the role of miR-146a in intestinal I/R-induced acute lung injury has not been elucidated. An intestinal I/R-induced injury mouse model was established in the present study by clamping the superior mesenteric artery and expression levels of miR-146a in intestinal and lung tissue samples were evaluated using reverse transcription-quantitative PCR (RT-qPCR). Intestinal and lung histopathological characteristics in mice with intestinal I/R-induced injury were assessed by hematoxylin and eosin staining, and mRNA and protein expression levels in intestinal and lung tissue samples were evaluated using RT-qPCR and western blotting, respectively. miR-146a expression was significantly downregulated in the intestinal and lung tissue samples of mice with intestinal I/R-induced injury. Intestinal I/R injury-induced histopathological changes in the lung and intestines, and pulmonary edema in mice transduced with an adenoviral miR-146a-overexpression vector (the miR-146a overexpression group) were alleviated. mRNA expression levels of TNF-α, IL-1β, IFN-γ and TGF-β1, and protein expression levels of TNF receptor-associated factor 6, phosphorylated-p65 NF-κB, cleaved caspase-3 and cleaved caspase-9 in lung and intestinal tissue samples were downregulated in I/R-miR-146a-overexpressing mice, compared with those from the I/R-negative control group. Thus, the present study identified that pre-treatment with the miR-146a overexpression vector alleviated intestinal I/R-induced acute lung injury in mice.

Correspondence to: Dr Yuantao Li, Department of Anesthesiology, Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University, 2004 Hongli Road, Shenzhen, Guangdong 518028, P.R. China
E-mail: yuantaoli6788@126.com

*Contributed equally

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Introduction

The etiologies of intestinal ischemia-reperfusion (I/R) injury include artery occlusion, intestinal obstruction, intestinal transplantation and abdominal trauma (1,2). The intestine is a reservoir of bacteria and endotoxins; thus, intestinal I/R injury may cause the spread of intestinal bacteria and endotoxins, as well as secretion of various inflammatory mediators and cytokines. Such physiological changes may induce local intestinal injury or distant lung injury by activating systemic inflammatory responses or may even result in multiple organ dysfunction (3). Intestinal I/R-induced acute lung injury, a severe life-threatening condition, is associated with high mortality rates (~40% in Washington, USA) (4). Currently, the mechanisms underlying intestinal I/R-induced acute lung injury have yet to be completely elucidated. Thus, there is an urgent need to identify novel biomarkers and devise effective therapeutic strategies for treating this affliction.

MicroRNAs (miRs) are single-stranded non-coding RNAs (length, 19-23 nucleotides), and miRs that suppress expression of target mRNAs at the post-transcriptional level by epigenetic regulation are critical for the regulation of various processes, including cellular proliferation, differentiation, apoptosis, metabolism and immunity (5,6). Previous studies reported that various miRs, including miR-29b-3p (7), miR-381-3p (8), miR-483-5p (9) and miR-145 are involved in the pathogenesis of intestinal I/R injury or acute lung injury (10); however, to the best of our knowledge, only a few studies have examined the role of miRs in intestinal I/R-induced acute lung injury thus far. miR-146a is one of the key miRs involved in inflammatory responses (11,12) and Zeng *et al* (13) observed that miR-146a overexpression contributes to the suppression of inflammatory responses during lipopolysaccharide-induced acute lung injury. Chassin *et al* (14) found that miR-146a-mediated downregulation of interleukin 1 receptor associated kinase 1 expression alleviates small intestinal I/R-induced injury in mice and humans; however, the role of miR-146a in

the pathogenesis of intestinal I/R-induced acute lung injury is not entirely understood.

The NF-κB signaling pathway is closely associated with secretion of inflammatory factors and its activation increases the expression of cytokines and promotes I/R-induced lung injury (15). Furthermore, intestinal I/R-induced lung injury may occur due to activation of the NF-κB signaling pathway (16). TNF receptor-associated factor 6 (TRAF6) activates the NF-κB signaling pathway, promotes the production of pro-inflammatory cytokines and induces I/R-induced injury (17,18). Thus, preventing inflammatory responses through TRAF6/NF-κB signaling has been suggested as a possible treatment of intestinal acute I/R-induced lung injury. A previous study found that miR-146a alleviated I/R-induced injury by targeting TRAF6 and silencing the NF-κB pathway (19); however, the effects of miR-146a/TRAF6/NF-κB on intestinal I/R-induced lung injury remain unclear.

The aim of the present study was to examine the role of miR-146a in the progression of intestinal I/R-induced acute lung injury in a mouse model. Furthermore, the effects of miR-146a overexpression on histopathological and molecular changes associated with intestinal I/R-induced acute lung injury were investigated.

Materials and methods

Animals and experimental groups. Forty-two male C57BL/6 mice (age, 7-10 weeks) were maintained at room temperature (25±2˚C) with 60% humidity under a 12-h light/dark cycle. The mice had free access to water and chow. Before the experiments, the mice were allowed to acclimatize to the laboratory conditions for at least two weeks. All animal experiments adhered to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (revised 1996) and were approved by the Institutional Animal Ethical Committee of the Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University. The 42 mice were randomly assigned to the following groups (with six per group in seven groups): Normal, sham, model, normal-negative control (NC) and I/R-NC (negative groups), normal-miR-146a and I/R-miR-146a group (miR-146a overexpression groups).

Mice in the normal group were not subjected to any procedures and the sham group mice received the same treatment as the model group mice with the exception of superior mesenteric artery occlusion. The model group mice were subjected to superior mesenteric artery occlusion for 60 min, followed by reperfusion for 120 min. In the I/R-NC group mice, an adenoviral miR-NC vector was injected into the tail vein 60 min before intestinal I/R surgery and in the I/R-miR-146a group, an adenoviral miR-146a overexpression vector was injected in the tail vein 60 min before intestinal I/R surgery. In the normal-NC group mice, an adenoviral miR-NC vector was injected into the normal mice and in the normal-miR-146a group, an adenoviral miR-146a overexpression vector was injected into the normal mice.

Surgical protocol. The mice were fasted for 12 h after which they were anesthetized using pentobarbital sodium (50 mg/kg bodyweight; intraperitoneal injection). During surgery, the mice were allowed to breathe spontaneously. A midline laparotomy was performed to expose the intestine, the superior mesenteric artery was exposed and was occluded using a microvascular clamp for 60 min to elicit intestinal ischemia. Ischemia was determined based on the lack of pulse in the mesentery and pale coloration of the small intestine. Subsequently, reperfusion was initiated by removing the clamp. Reperfusion was determined based on the reoccurrence of pink coloration of the small intestine and enhanced intestinal peristalsis. The abdomen was temporarily covered using a sterile plastic wrap to minimize evaporation. Reperfusion was induced for 120 min; the I/R time was set as previously described (20). Body temperature (36-38˚C) was maintained during the procedure using heating pads. The mice were euthanized by intraperitoneal injection with pentobarbital (140 mg/kg bodyweight) 12 h after completion of intestinal I/R surgery. Intestinal and lung tissue samples were collected immediately, after which they were shock-frozen in liquid nitrogen and stored in -80˚C until analysis. Intestinal and lung tissue sections for histopathological analysis were fixed in 10% formalin for 15 min at 25˚C.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse-transcribed to cDNA using the miRNA First Strand cDNA Synthesis kit (Sangon Biotech Co., Ltd.) for miR-146a mRNA or using the Prime Script RT Reagent kit (Takara Biotechnology Co., Ltd.) for TNF-α, IL-1β, IFN-γ and TGF-β1 mRNAs. RT-qPCR analyses were performed using an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Green qPCR Super Mix (Invitrogen; Thermo Fisher Scientific, Inc.). The qPCR thermocycling conditions were as follows: Denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 65˚C for 32 sec. The genes U6 and GAPDH were used for normalization of miRs and mRNA expression, respectively. Relative expression levels of the respective target gene were calculated according to the $2^{-\Delta\Delta Cq}$ method (21).

The following PCR primers were used: GAPDH, forward 5'-TGGCCGTGGGGCTGCCCAG-3' and reverse 5'-GGAAGGCCATGCCAGTGAGC-3'; TNF-α, forward 5'-CTAGTGGTGCCAGCCGATGG-3' and reverse 5'-GGCTCTTGACGGCAGAGAGG-3'; IL-1β, forward 5'-TGTCGGACCCATATGAGCTG-3' and reverse 5'-TCCTTTGAGGCCCAAGGCCA-3'; IFN-γ, forward 5'-TCTTCTTGGATATCTGGAGG-3' and reverse 5'-CCTGATTGTCTTTCAAGACT-3'; TGF-β1, forward 5'-GTGGAGCAACATGTGGAACT-3' and reverse 5'-AAGACAGCCACTCAGGCGT-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'; miR-146a, forward 5'-ACACTCCAGCTGGGTGAGAACTGAATTCCA-3' and reverse 5'-CTCAACTGGTGTCGTGGA-3'.

Adenovirus-mediated overexpression of miRs in vivo. miR-146a overexpression (Agctctgagaact gaattccatgggttatatcaat gtcagacctgtgaaat tcagttcttcagct) and NC (CCGGGAACTGGGGTGCGTGTGATCTCGAGATCACACGCACCCCAGTTTTTTTG) were synthesized by Shanghai GenePharma Co., Ltd. and were combined with the pDC316-mCMV-enhanced green fluorescent protein (EGFP) plasmid. Subsequently, pDC316-mCMV-EGFP, pBHG and pDC316-CMV-GFP

plasmids (cat. no. PD-01-64, AdMax™ Adenovirus System; Microbix Biosystems, Inc.) were added dropwise to the cells whilst being gently mixed, followed by culturing for 24 h. Subsequently, the virus was amplified over three generations, and cells were collected and subjected to three freeze-thaw cycles at -80˚C and 37˚C, after which they were centrifuged at 10,000 x g at 4˚C for 10 min to separate the virus-containing supernatant. The mice were injected in the tail vein with 100 μl solution containing adenoviral miR-146a overexpression vector or miR NC vector (multiplicity of infection=$6x10^7$ pfu/ml; Shanghai GeneChem Co., Ltd.) 60 min before intestinal I/R surgery. The mice were euthanized 12 h after surgery (20). Intestinal and lung tissue samples were excised for histopathological analysis, measurement of mRNA and protein expression levels and to record their wet weight.

Histopathological examination. Sections of the jejunum and of the lower lobes of the right lungs were embedded in paraffin and cut into sections (thickness, 5 μm). The sections were transferred to glass slides and subjected to hematoxylin and eosin (H&E) staining for 4 h at 25˚C. The degree of histological injury was evaluated through blinded analysis of hand counts performed by two experienced investigators using a light microscope (BX51 microscope; Olympus Corporation). Histopathological scores of intestinal tissue samples were obtained according to a previous study (22) and were based on three parameters as follows: Severity of inflammation [based on polymorphonuclear neutrophil infiltration: None (score=0), slight (score=1), moderate (score=2), severe (score=3)]; depth of injury [none (score=0), mucosal (score=1), mucosal and submucosal (score=2), transmural (score=3)]; and crypt damage [none (score=0), basal one-third damaged (score=1), basal two-thirds damaged (score=2), only surface epithelium intact (score=3), entire crypt and epithelium lost (score=4)]. The scores where then summed with a maximum possible score of 10. Five high-magnification fields were randomly selected and were scored to produce an average intestinal injury score for each mouse.

Lung histological scoring was performed based on histological changes, such as alveolar congestion, alveolar wall edema, inflammatory cell infiltration and hemorrhage where each standard was scored between 0 (normal) and 4 (severe) as follows: 0, No or very mild injury; 1, mild injury; 2, medium injury; 3, severe injury; 4, very severe injury (23). The sum of all scores was processed as a total score of lung tissue pathology. Five high-magnification fields were randomly selected and were scored to produce an average lung injury score for each mouse.

Lung tissue wet-to-dry (W/D) weight ratio. The wet weight of lung tissue samples was recorded immediately following tissue excision. The tissue samples were then dried in an incubator at 60˚C for three days, after which the dry weight was recorded. Lung W/D weight ratios were calculated to assess tissue edema.

Western blotting. Intestinal and lung tissue samples were washed using phosphate-buffered saline and were homogenized in lysis buffer (Thermo Fisher Scientific, Inc.) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.). Cell lysates (30 μg/lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the resolved proteins were then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk for 2 h at 25˚C and subsequently incubated overnight at 4˚C with the following primary antibodies: Anti-TRAF6 [cat. no. ab33915; 1:1,000 (v/v); Abcam], anti-p-p65 NF-κB [cat. no. ab239882; 1:500 (v/v); Abcam], anti-p65 NF-κB [cat. no. ab32536; 1:500 (v/v); Abcam], anti-cleaved caspase-3 [cat. no. ab214430; 1:1,000 (v/v); Abcam], anti-cleaved caspase-9 [cat. no. ab77814; 1:1,000 (v/v); Abcam] and anti-GAPDH [cat. no. ab181602; 1:15,000 (v/v); Abcam]. The membrane was then washed with three times for 10 min each in TBST (0.1% Tween-20) and was incubated with the horseradish peroxidase-conjugated Goat Anti-Rabbit IgG H&L antibodies (cat. no. ab6721; 1:20,000 (v/v); Abcam) for 2 h at room temperature. Protein bands were detected using a chemiluminescent peroxidase substrate, ECL (Amersham; Cytiva). The protein band intensity was analyzed using Quantity One software (Bio-Rad Laboratories, Inc.) and target protein band intensities were normalized to GAPDH.

Statistical analysis. All statistical analyses were performed using SPSS 22.0 software (IBM Corp.). Differences between two groups were evaluated using Student's t-test and differences between multiple groups were tested using a one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-146a are downregulated in the lungs and intestines of mice with intestinal I/R injury. Expression levels of miR-146a in intestinal and lung tissue samples were analyzed using RT-qPCR (Fig. 1). miR-146a expression in the intestines and lungs was significantly downregulated in the model group when compared with the sham group (0.28-fold change in intestinal tissues and 0.26-fold change in lung tissues). Differences in miR-146a expression between the normal and the sham group were not significant (1.09-fold change in intestinal tissues and 1.08-fold change in lung tissues). These findings indicate that miR-146a is involved in the development of intestinal I/R-induced acute lung injury in mice. Additionally, no significant differences between the normal and sham group mice were observed regarding W/D weight ratio and IL-1β, TNF-α, IFN-γ and TGF-β1 mRNA expression (Fig. S1A), suggesting that the sham surgery had no significant effect.

Overexpression of miR-146a during intestinal I/R-induced injury. The role of miR-146a during intestinal I/R-induced injury was further evaluated by injecting the mice with an adenoviral miR-146a overexpression vector or with an adenoviral miR-NC vector. EGFP fluorescence images of lung tissue samples demonstrated that the adenoviral vector was successfully infected in the lung tissue (Fig. S2).

RT-qPCR analysis revealed that miR-146a expression in the intestine and lung tissue samples was significantly upregulated in miR-1461-overexpressing mice, compared with I/R-NC mice (4.34-fold change in intestinal tissues and 3.87-fold change in lung tissues; Fig. 2). No significant difference between the normal, normal-NC (normal mice injected with the adenoviral

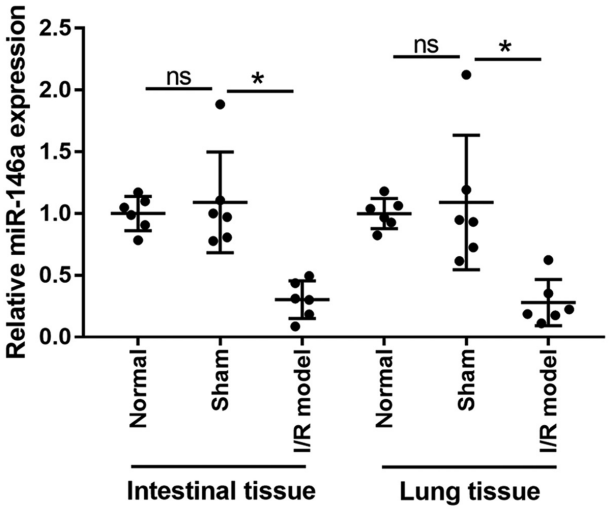

Figure 1. miR-146a expression was significantly inhibited in mice with intestinal I/R-induced injury. The relative expression levels of miR-146a in intestinal and lung tissue samples of mice were analyzed by reverse transcription-quantitative PCR in normal, sham and intestinal I/R-induced mice (n=6). *P<0.05. miR, microRNA; ns, not significant; I/R, ischemia/reperfusion.

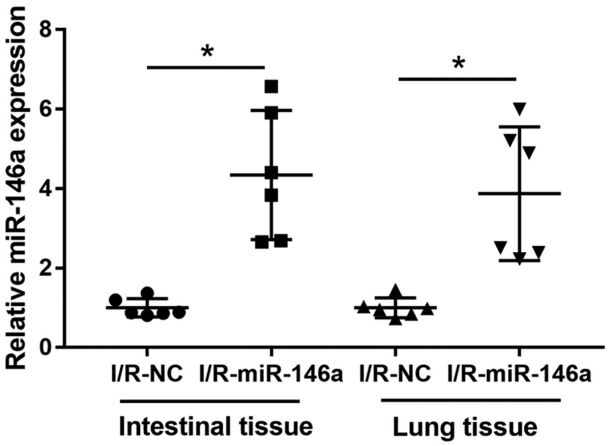

Figure 2. miR-146a expression was significantly increased in mice with intestinal I/R-induced injury after injection with adenovirus-miR-146a. The efficiency of adenovirus-mediated miR-146a overexpression was determined by reverse transcription-quantitative PCR to assess miR-146a expression in intestinal and lung tissue samples of mice with intestinal I/R-induced injury (n=6). *P<0.05. miR, microRNA; ns, not significant; I/R, ischemia/reperfusion; NC, negative control.

NC vector) and normal-miR-146a (normal mice injected with adenoviral miR-146a overexpression vector) groups regarding W/D weight ratios and IL-1β, TNF-α, IFN-γ and TGF-β1 mRNA expression (Fig. S1B), suggesting that the adenoviral vector exerted no significant effect on the normal mice.

Overexpression of miR-146a alleviated morphological changes in intestinal and lung tissue samples of mice with intestinal I/R-induced injury. The lungs of I/R-NC mice exhibited severe histopathological changes, including alveolar congestion, exudate and infiltration of inflammatory cells when compared with the lungs of the sham group mice (6.88-fold change; Fig. 3); however, these histopathological changes associated with intestinal I/R-induced acute lung injury were substantially less severe in the I/R-miR-146a group mice, as evidenced by a significantly decreased lung injury score than that in I/R-NC group mice (0.53-fold change). The intestinal tissue samples from the I/R-NC group mice exhibited severe intestinal crypt injury, widespread mucosal destruction and disintegrated intestinal villi, when compared with the sham group mice (5.67-fold change, Fig. 3); however, miR-146a overexpression markedly alleviated these intestinal histopathological changes associated with intestinal I/R-induced injury, as evidenced by a significantly decreased intestinal injury score (0.5-fold change). Compared with the sham group, the lung injury and intestinal score significantly increased in the I/R-miR-146a group mice (3.63- and 2.83-fold change, respectively).

Overexpression of miR-146a alleviated pulmonary edema in mice with intestinal I/R injury. The effect of miR-146a overexpression on lung injury in mice with intestinal I/R-induced injury was evaluated by examining the W/D weight ratio of lung tissue samples (Fig. 4). The W/D weight ratio of lung tissue samples from the I/R-NC group was significantly higher than that of the sham group lung tissue samples (1.48-fold change) and that of the miR-146a-overexpressing lung tissue samples was significantly lower than that of the lung tissue samples from the I/R-NC group (0.78-fold change). The W/D weight ratio of the lung tissue samples from the miR-146a overexpression group was significantly higher than that of the lung tissue samples from the sham group (1.15-fold change). There was no significant difference in the W/D weight ratio between the normal and the sham group (1.04-fold change).

Overexpression of miR-146a downregulated the expression of inflammatory markers in intestinal and lung tissue samples of mice with intestinal I/R-induced injury. The effect of miR-146a overexpression on mRNA expression levels of proinflammatory cytokines, such as IL-1β, TNF-α, IFN-γ and TGF-β1 in intestinal and lung tissue samples of mice with intestinal I/R-injury was evaluated using RT-qPCR (Fig. 5). mRNA expression levels of IL-1β, TNF-α, IFN-γ and TGF-β1 were significantly upregulated in the intestine and lung tissue samples from mice in the I/R-NC group compared with those from mice in the sham group (4.98-, 4.27-, 6.84- and 6.42-fold change in intestinal tissue, respectively; and 4.71-, 6.42-, 4.79- and 5.46-fold change in lung tissue, respectively). mRNA expression levels of IL-1β, TNF-α, IFN-γ and TGF-β1 in the intestine and lungs were significantly downregulated in miR-146a-overexpressing mice compared with I/R-NC mice (0.32-, 0.36-, 0.32- and 0.23-fold change in intestinal tissue, respectively; and 0.36-, 0.26-, 0.35- and 0.38-fold change in lung tissue, respectively). mRNA expression levels of IL-1β, TNF-α, IFN-γ and TGF-β1 in the lung and intestinal tissues were significantly higher in I/R-miR-146a group than those in sham group (1.61-, 1.53-, 2.16- and 1.49-fold change in intestinal tissue, respectively, and 1.69-, 1.69-, 1.70- and 2.08-fold change in lung tissue, respectively).

Overexpression of miR-146a decreased the expression of apoptosis-related proteins in mice with intestinal I/R injury by inhibiting the TRAF6/NF-κB signaling pathway. Western blotting (Fig. 6) revealed that, compared with the sham group, protein levels of TRAF6, p-p65 NF-κB, p65 NF-κB, cleaved caspase-3 and cleaved caspase-9 in intestinal and lung tissue

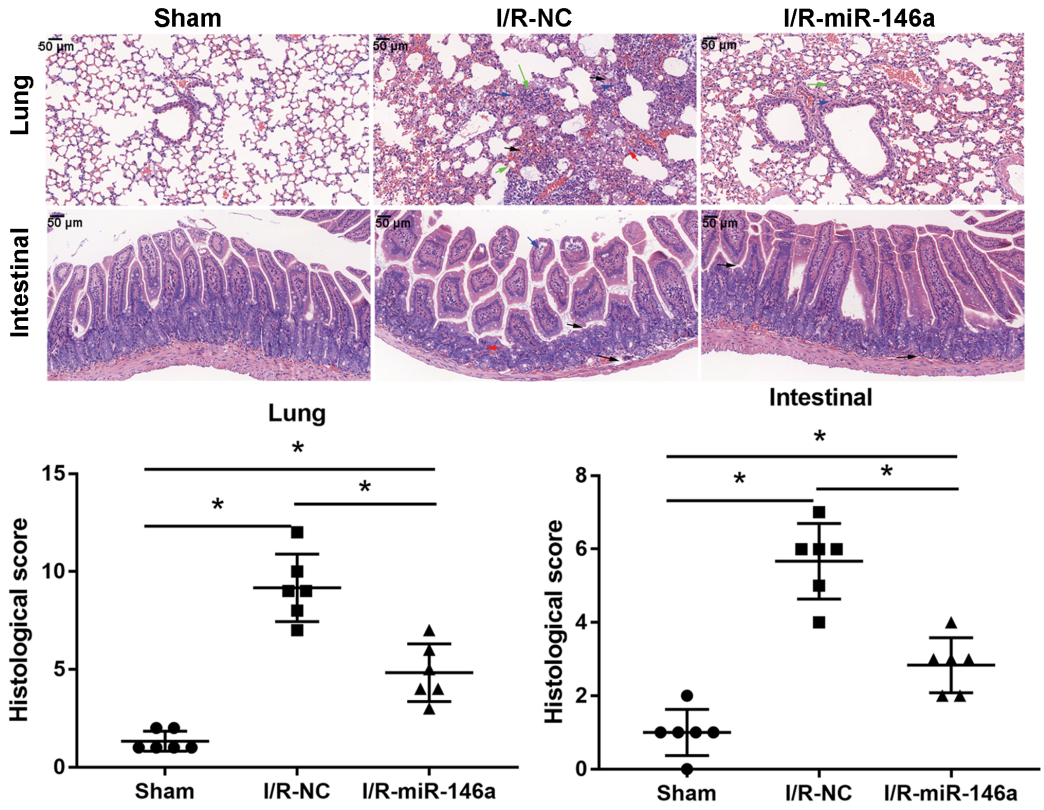

Figure 3. miR-146a overexpression alleviates intestinal I/R-induced acute lung injury. Effects of miR-146a overexpression on the morphology of intestinal and lung tissue samples in an intestinal I/R-induced injury mouse model were assessed by hematoxylin and eosin staining (magnification, x400; n=6). In lung tissue samples: Blue arrow, inflammation (score=2); red arrow, alveolar edema (score=2); blank arrow, hemorrhage (score=4); green arrow, alveolar wall edema (score=4). In intestinal tissue samples: Blue arrow, inflammation (score=2); red arrow, crypt damage (score=2); blank arrow, mucosal destruction and disintegrated intestinal villi (score=3). *P<0.05. miR, microRNA; ns, not significant; I/R, ischemia/reperfusion; NC, negative control.

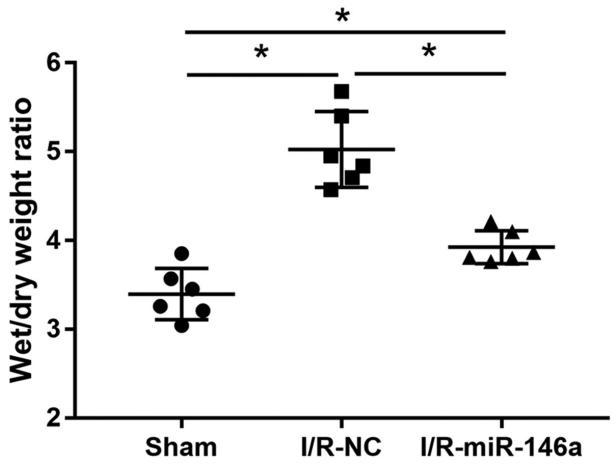

Figure 4. miR-146a overexpression significantly reduced the wet/dry weight ratio of lung tissue samples in intestinal I/R-induced injury mice. Pulmonary edema in mice with intestinal I/R-induced injury was evaluated by measuring the wet/dry weight ratio of lung tissue samples (n=6). *P<0.05. miR, microRNA; ns, not significant; I/R, ischemia/reperfusion; NC, negative control.

samples were significantly upregulated in the IR-NC group (2.60-, 2.31-, 2.61- and 2.19-fold change in intestinal tissues, respectively; 2.61-, 2.46-, 2.40- and 2.24-fold change in lung tissues, respectively). And compared with the IR-NC group, those proteins were significantly downregulated in IR-miR-146a group mice (0.60-, 0.64-, 0.53- and 0.67-fold change in intestinal tissues, respectively; 0.68-, 0.59-, 0.56- and 0.69-fold change in lung tissues, respectively) as shown in Fig. 6. Protein levels of TRAF6, p-p65 NF-κB, p65 NF-κB, cleaved caspase-3 and cleaved caspase-9 in the intestinal and lung tissue samples were significantly higher in the IR-miR-146a group when compared with those in the sham group (1.56-, 1.48-, 1.39- and 1.46-fold change in intestinal tissue, respectively; and 1.76-, 1.44-, 1.35- and 1.54-fold change in lung tissue, respectively) as presented in Fig. 6.

Discussion

The present results demonstrate that miR-146a expression was significantly downregulated in the intestinal and lung tissue samples of an intestinal I/R-induced injury mouse model. Overexpression of miR-146a alleviated histopathological changes in lung and intestinal tissue samples, reduced pulmonary edema and downregulated the expression of inflammatory and apoptotic markers in the intestinal and lung tissue samples of mice with intestinal I/R-induced injury. Moreover, miR-146a overexpression suppressed apoptotic responses in intestinal and lung tissue samples of mice with intestinal I/R-induced lung injury by inhibiting the TRAF6/NF-κB signaling pathway.

Intestinal I/R, a severe clinical condition that leads to local intestinal damage or injury to distant organs including the lungs is associated with high morbidity and mortality rates (24). The pathological mechanism underlying intestinal I/R-induced acute lung injury is complex and is currently not completely

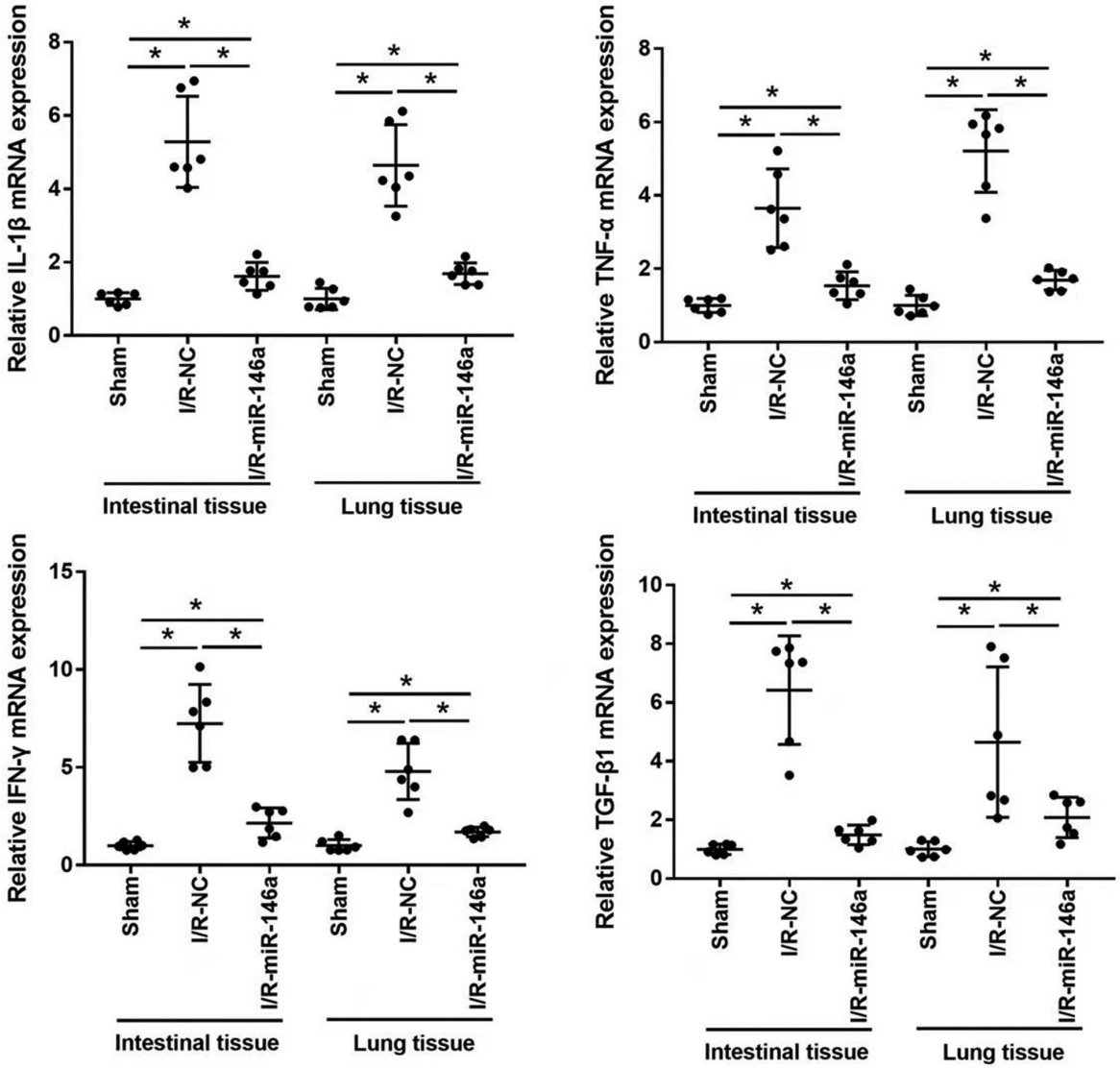

Figure 5. miR-146a overexpression significantly reduced proinflammatory cytokine expression in intestinal I/R-induced injury mice. Effects of miR-146a overexpression on the mRNA expression levels of IL-1β, TNF-α, INF-γ and TGF-β1 in the intestinal and lung tissue samples of mice with intestinal I/R-induced injury were determined by reverse transcription-quantitative PCR (n=6). *P<0.05. miR, microRNA; I/R, ischemia/reperfusion; NC, negative control.

understood. miRs are associated with the pathogenesis of acute lung injury (25) and Kong *et al* (26) found that miR-216a alleviates lipopolysaccharide-induced acute lung injury by regulating the Janus kinase 2/STAT3 and NF-κB signaling pathways. He *et al* (27) observed that miR-146a expression was downregulated in the plasma of patients with mesenteric ischemia, in IEC-6 cells and in small intestinal tissues of ischemia and I/R rat models. In addition to causing local damage, intestinal I/R contributes to distant organ injury and the lungs are the most vulnerable organ in this respect. Consistent with the results of He *et al* (27), the present study observed that miR-146a expression was significantly downregulated in the intestinal and lung tissue samples of mice with intestinal I/R-induced injury, suggesting that miR-146a is associated with the development of intestinal I/R-induced acute lung injury in mice.

Furthermore, the present study investigated the role of miR-146a in mice with intestinal I/R-induced injury. Histopathological changes, such as acute lung injury and intestinal injury were markedly ameliorated in tissue samples of miR-146a-overexpressing mice, compared with those of I/R-NC mice. Additionally, the lung W/D weight ratio was significantly lower in miR-146a-overexpressing mice than that in I/R-NC mice. These results indicate that miR-146a overexpression protects mice against intestinal I/R-induced acute lung injury. Previous studies reported that miR-146a, a key mediator of inflammatory responses, is involved in the pathogenesis of intestinal I/R-induced injury (11); for example, Chassin *et al* (14) observed that miR-146a suppresses inflammatory response and alleviates I/R-induced small intestine injury in mice and humans by inhibiting interleukin 1 receptor associated kinase 1 expression.

Proinflammatory cytokines play a key role in local intestinal I/R-induced lung injury (28) and TNF-α, IL-1β, IFN-γ and TGF-β1 activities are frequently used to assess the degree of inflammation during intestinal I/R-induced injury. A previous study suggested that TNF-α, IL-1β and IL-6 are the key inflammatory mediators during intestinal I/R-induced acute lung injury (29). In the present study, mRNA expression levels of

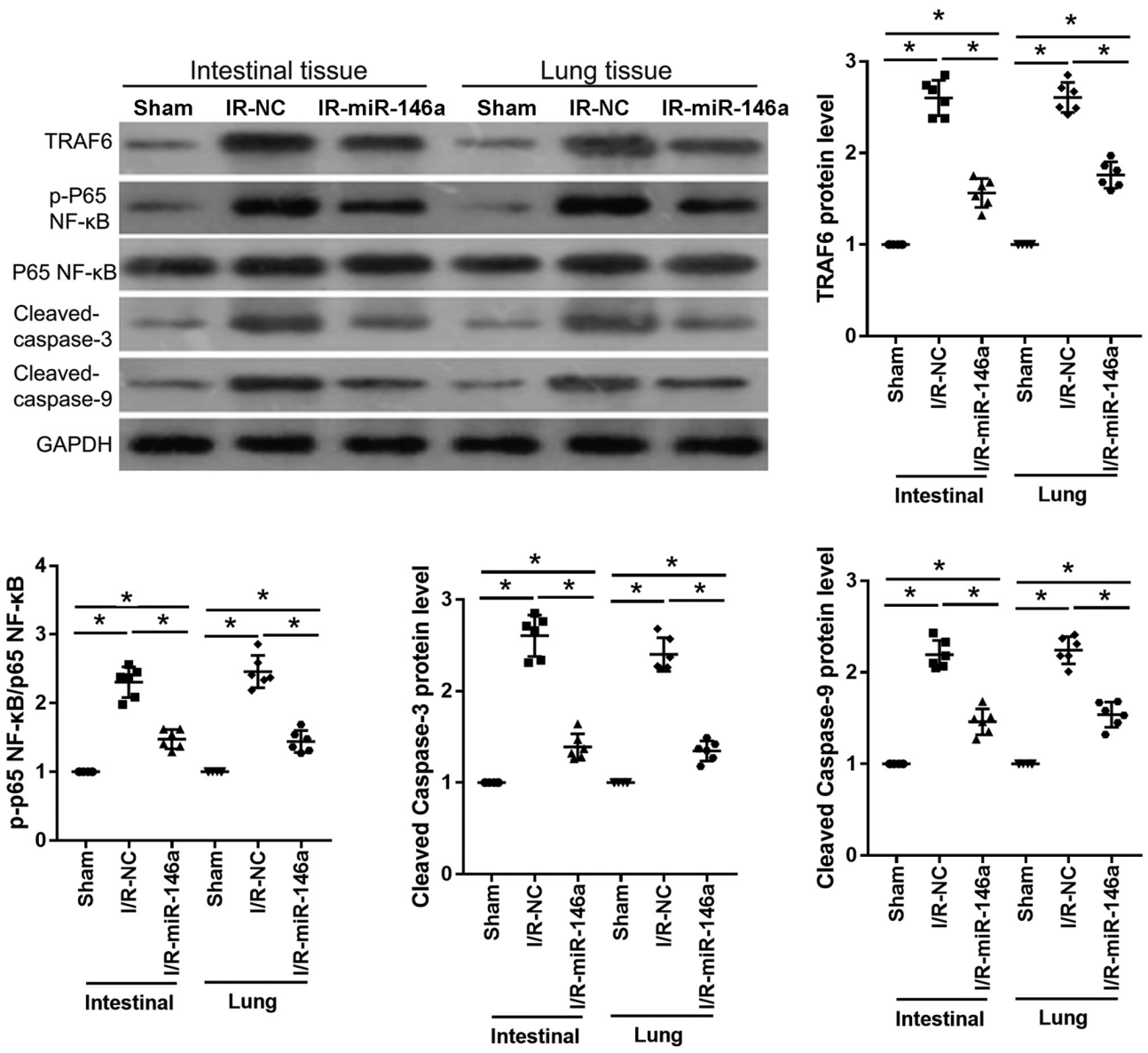

Figure 6. miR-146a overexpression significantly downregulated the NF-κB signaling pathway in intestinal I/R-induced injury mice. Effects of miR-146a overexpression on TRAF6, p-p65 NF-κB, cleaved-caspase 3 and cleaved-caspase 9 expression levels in intestinal and lung tissue samples of mice with I/R-induced injury were evaluated by western blotting (n=6). *P<0.05. miR, microRNA; ns, not significant; I/R, ischemia/reperfusion; NC, negative control; TRAF6, TNF receptor-associated factor 6; p, phosphorylated.

TNF-α, IL-1β, IFN-γ and TGF-β1 in the lung and intestinal tissue samples were significantly downregulated in miR-146a-overexpressing mice, compared with I/R-NC mice. This suggests that miR-146a overexpression alleviates intestinal I/R-induced acute lung injury in mice partly by reducing the secretion of proinflammatory cytokines. Lung epithelium and endothelium injuries also contribute to the development of acute lung injury, which is associated with the loss of cells through apoptosis (30). A previous study demonstrated the role of miR-146 in regulating apoptosis and pathogenesis of intestinal I/R (27). In the current study, the role of miR-146a in regulating apoptotic responses in a mouse model of intestinal I/R-induced injury was assessed by evaluating expression levels of the apoptosis markers, cleaved caspase-3 and cleaved caspase-9 in intestinal and lung tissue samples. Protein expression levels of TRAF6, p-p65 NF-κB, p65 NF-κB, cleaved caspase-3 and cleaved caspase-9 in intestinal and lung tissues were significantly downregulated in miR-146a-overexpressing mice when compared with I/R-NC mice. These results suggest that miR-146a overexpression may attenuate apoptotic responses in intestinal and lung tissues by inhibiting the TRAF6/NF-κB signaling pathway.

Furthermore, the adenoviral miR-146a overexpression vector was injected in the tail vein of mice 60 min before intestinal I/R surgery, suggesting that miR-146a inhibits the acute lung injury process. These findings suggested that pretreatment with the adenoviral miR-146a overexpression vector acted as a preventive measure for treating intestinal I/R-induced acute lung injury. Hence, pretreatment with the adenoviral miR-146a overexpression vector was only suitable for planned intestinal I/R surgery in clinical practice. However, whether miR-146a treatment improves lung injury induced by intestinal ischemia requires further investigation, which is a potential limitation of the present study. Hence, miR-146a treatment may not be applicable to unplanned intestinal I/R surgery in clinical practice.

In conclusion, the present study demonstrated that miR-146a was significantly downregulated in the intestine and lungs of an intestinal I/R-induced mouse model. miR-146a overexpression alleviated intestinal I/R-induced acute lung injury in mice through modulation of inflammatory responses and apoptosis. Thus, miR-146a may serve as a potential target to prevent intestinal I/R-induced acute lung injury.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GL, MX and YTL conceived and designed the study. HW and XQ developed the methodology. GL, MX, HW and XQ conducted the experiments and collected the data. GL, MX, XW, YL and JS analyzed and interpreted the data. GL drafted the manuscript and MX and YTL revised the manuscript. GL and MX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments conducted adhered to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (revised 1996) and were approved by the Institutional Animal Ethical Committee of the Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University (Shenzen, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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