Original Research

Prevention of renal ischemia-reperfusion injury by short hairpin RNA of endothelin A receptor in a rat model

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Abstract

Endothelin A receptor (ETaR) is a key molecule involved in a variety of biological events such as vessel contraction and inflammatory response in ischemia-reperfusion (I/R) injury. RNA interference using short hairpin RNA (shRNA) is a powerful tool to silence gene expression. Here, the effect of ETaR shRNA on I/R injury in rats was studied. A more effective shRNA sequence out of two constructed into plasmid vectors was selected using the A-10 cell line, and was then applied to a rat model. Twenty-eight male Sprague-Dawley rats were randomized into four groups: Sham, shRNA, vector and phosphate-buffered saline (PBS). Renal I/R injury was induced by clamping the left renal pedicle for one hour followed by reperfusion for 24 h. ETaR shRNA (100 μg) plasmid was administered by renal vein injection 48 h before clamping. The expression of both ETaR mRNA and protein was lowered by ETaR shRNA treatment compared with that in the vector and PBS groups; serum creatinine and blood urea nitrogen were significantly decreased; the semi-quantitative score of renal structural damage was improved; the mRNA level of endothelin 1 (ET-1), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), macrophage inflammatory protein 2 (MIP-2) and monocyte chemoattractant protein 1 (MCP-1) was reduced, but nitric oxide (NO) production in kidney tissues was increased ($P < 0.05$). In conclusion, ETaR shRNA partially silenced ETaR expression in I/R injury kidneys, reduced the mRNA level of ET-1, inflammatory mediators including TNF-α, IL-6, MIP-2 and MCP-1, increased NO production, and ultimately improved renal function and structure.

Keywords: kidney, ischemia-reperfusion injury, endothelin A receptor, RNA interference

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Introduction

Ischemia-reperfusion (I/R) injury is inevitable in kidney transplantation, which causes a series of acute and chronic renal adverse events, including delayed graft function,[1] acute rejection and chronic allograft dysfunction. I/R injury affects a variety of renal cells, including tubular epithelial cells as well as endothelial cells. Prevention of I/R injury can effectively reduce acute and chronic renal injury, and improve the quality of 'marginal kidneys' to expand the donor pool.[2]

Endothelin (ET) is not only a strong endogenous vasoconstrictor, but is also a regulator for many other functions. There are three isoforms of ET, including ET-1, ET-2 and ET-3. The ET system regulates renal blood flow, glomerular filtration rate, water metabolism and acid–base balance. ET-1, secreted by vascular endothelial cells, exerts biological effects through the endothelin A receptor (ETaR), which is primarily expressed on the membrane of vascular smooth muscle cells. The interaction between ET-1 and ETaR is involved in blood vessel contraction, cell proliferation and inflammatory response. The expression level of ET-l and ETaR in the kidney is significantly elevated after renal I/R, which induces vascular smooth muscle contraction and vasospasm.[3] The effects and mechanisms of endothelin on renal I/R injury are complicated, and so far have not been well defined. RNA interference using small interfering RNA (siRNA) or short hairpin RNA (shRNA) is a powerful tool to silence gene expression in mammalian cells at the post-transcriptional level.[4] siRNA or shRNA has been successfully delivered into renal tissues in rodent[5–7] and swine[8,9] models for prevention of kidney I/R injury.

Tumor necrosis factor-α (TNF-α) is an important cytokine associated with I/R injury, which is secreted at an early stage after harmful stimulation, and mediates the induction of other chemokines and attracts neutrophil infiltration, resulting in inflammation.[10] Interleukin 6 (IL-6) is produced by T-cells, fibroblasts, monocytes, macrophages and endothelial cells, and has a wide range of biological functions in immune response and inflammation.[11] In addition, chemokines attract leukocytes migrating to the inflammatory site. Monocyte chemoattractant protein 1 (MCP-1) is an important chemokine, which mainly activates and attracts mononuclear macrophages.[12] Macrophage inflammatory protein 2 (MIP-2) is another CXC class chemokine, which is involved in chemotaxis and activation of a variety of immune cells, especially neutrophils.[13]

In this study, ETaR shRNA was used to suppress the expression of ETaR in a rat I/R injury model. The silencing effect of ETaR shRNA was assessed at mRNA and protein levels and its interaction with ET-1. The mRNA expression of cytokines and chemokines, as well as the production of nitric oxide (NO), was also evaluated. It was demonstrated for the first time that efficient silencing of ETaR by shRNA protected the kidneys against I/R injury in rats.

Materials and methods

Animals

Male Sprague-Dawley rats were purchased from The SLAC Laboratory Animal Center (Shanghai, China). The rats were maintained under specific pathogen-free conditions. Six- to eight-week-old rats were used for the experiments. The experimental protocol was approved by the Committee of Animal Care of Fudan University.

ETaR shRNA Design

Two target sequences of ETaR gene were selected. The oligonucleotides containing sequences specific for ETaR (#1: 5′ GATCCCGCCACAACACAGAACGGAGCTTCAAGAGAGCTCCGTTCTGTGTTGTGGTTTTTTCCAAA 3′; #2: 5′ GATCCCGTGCGCTCATAGCCAGCCTGTTCAAGAGACAGGCTGGCTATGAGCGCATTTTTTCCAAA 3′) were synthesized and annealed. An ETaR shRNA expression vector, which expresses shRNA under the control of the rat U6 promoter and cGFP genes, was constructed by inserting pairs of annealed DNA oligonucleotides into a pRNAT-U6.1/Neo shRNA expression vector that had been digested with BamHI and HindIII (Genescript, Nanjing, China). This co-expression system allows tracking the expression and distribution of shRNA by detecting green fluorescence under a fluorescence microscope.

In vitro silencing of the ETaR Gene

A-10 cells (ATCC, Manassas, VA, USA) were cultured and transfected with two different sequences of ETaR shRNA using lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. Briefly, cells were plated into six-well plates (10^5 cells per well) and allowed to grow overnight to reach 90% confluence. Cells were transfected with 4.8 μg ETaR shRNA or negative control plasmids in Opti-MEM I medium (Invitrogen) for five hours, and then incubated in complete medium for 24 h before being collected to extract the total RNA. The vehicle alone and transfection reagent were used as negative controls.

Renal I/R injury model and shRNA administration

Rats were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg), and placed on a heating pad to maintain their body temperature during the surgery. Following abdominal incisions, renal pedicles were bluntly dissected and a microvascular clamp was placed on the left renal pedicle for 60 min. During the procedure, animals were kept at a constant temperature (37°C). Following ischemia, the clamps were removed, along with the right kidney nephrectomy. For the group of sham control, only abdominal incision and renal pedicle dissection were performed, and after waiting for 60 min, right kidney nephrectomy was performed. Then, the incisions were sutured, and the animals were allowed to recover with free access to food and water. Blood was collected and the left kidney was harvested for analysis 24 h after reperfusion.

To investigate the *in vivo* silencing effect and protective efficacy of ETaR shRNA on the renal tissue of I/R injury, the rats were administered with ETaR shRNA 48 h before the operation. ETaR shRNA plasmid DNA (100 μg) were diluted in 1 mL of phosphate-buffered saline (PBS) and delivered into the kidney through the renal vein by a retrograde 'hydrodynamic' injection.[14,15] Rats were treated with 1 mL of ETaR shRNA, PBS or the control vector.

Assessment of renal function

Blood samples were obtained from the inferior vena cava 24 h post-ischemia. Serum creatinine and blood urea nitrogen (BUN) were examined by the core laboratory at the SLAC Laboratory Animal Center to assess the renal function.

Histology detection

At 24 h post-ischemia, kidneys were dissected from rats, and tissue slices were fixed in 10% formalin and processed for histology examination using standard techniques. Formalin tissue was embedded in paraffin and 5-μm sections were stained with hematoxylin and eosin. These sections were blindly examined by a pathologist. The percentage of histological changes in the cortex and medulla was scored using a semi-quantitative scale designed to evaluate the degree of the necrosis in tubular and glomerular areas, tubular vacuolization and cast formation on a five-point scale based on injury area of involvement. The scale is as follows: 0 = <10%; 1 = 10–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100%.

Western blot analysis of ETaR

Kidney tissues were homogenized in RIPA buffer containing 1 mmol/L phenylmethanesulfonyl fluoride. Homogenates were centrifuged for 10 min at 10,000 *g* under 4°C, and the protein concentration was determined in supernatants using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Twenty micrograms of proteins were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were incubated with rabbit anti-ETaR antibody (Millipore) diluted 1:200 overnight at 4°C, or rabbit anti-GAPDH antibody (Abcam, Cambridge, UK) diluted 1:10,000 as the loading control. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:20,000 for one hour and developed by enhanced chemiluminescence detection reagent (Thermo Scientific) for one minute, and finally exposed to Kodak BioMax MS (Rochester, NY, USA) film for two to five minutes. Densitometry analysis of blots was performed using the VersaDoc imaging system (Bio-Rad, Hercules, CA, USA) with the Quantity One program.

Expression of ETaR, ET-1, TNF-α, IL-6, MCP-1 and MIP-2 mRNA in renal tissues

Total RNA was extracted from silenced and non-silenced cells or renal tissues using the Trizol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed using a SYBR PrimeScript RT-PCR Kit (Takara Bio, Otsu, Shiga, Japan). Reaction mixtures consisted of 2 μL cDNA, SYBR Premix Ex Taq (Takara Bio), and forward and reverse primers in a volume of 25 μL. Realtime polymerase chain reaction (PCR) was performed on a Rotor-Gene 3000 realtime rotary analyzer (Corbett Research, Sydney, Australia) with initial denaturation for 30 s at 95°C and a two-step amplification program (5 s at 95°C followed by 30 s at 60°C) repeated 45 times. Melt curve analysis was used to check the amplification of a single specific product. Relative expression was determined using the $2-\Delta\Delta CT$ method of normalized samples according to the manufacturer's protocol in relation to a calibrator sample and β-actin was used as a reference. Each PCR run included a calibrator sample, a non-template control and a sample without reverse transcriptase. All measurements were performed in triplicate. Primers used for the amplification of rat ETaR, ET-1, TNF-α, IL-6, MCP-1, MIP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. The expression of each targeting gene was normalized to GAPDH, a housekeeping gene.

Measurement of NO production

Kidney tissues were homogenized in RIPA buffer and centrifuged at 10,000 *g* under 4°C for 10 min. The protein concentration in the supernatant was determined using a Pierce BCA protein assay kit (Thermo Scientific). Total NO production in kidney tissues was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, using a modified Griess reaction method following the protocol provided with a Total Nitric Oxide Assay Kit (Beyotime Institute of Biotechnology, Nantong, China).

Table 1 Sequences of used primers

	Primers	5′–3′
ETaR	Forward	ACATGCTGGCTGGTTCTGGAG
	Reverse	ATGGGCAGGAGTGTAGCCAGTC
ET-1	Forward	ACCTGGACATCATCTGGGTCAAC
	Reverse	TTTGGTGAGCACACTGGCATC
TNF-α	Forward	AACTCGAGTGACAAGCCCGTAG
	Reverse	GTACCACCAGTTGGTTGTCTTTGA
IL-6	Forward	CCACTTCACAAGTCGGAGGCTTA
	Reverse	GTGCATCATCGCTGTTCATACAATC
MCP-1	Forward	CTATGCAGGTCTCTGTCACGCTTC
	Reverse	CAGCCGACTCATTGGGATCA
MIP-2	Forward	TCATGAAGTTTGTCTCAACCCTGAA
	Reverse	GCGAGGCACATCAGGTACGA
GAPDH	Forward	GGCACAGTCAAGGCTGAGAATG
	Reverse	ATGGTGGTGAAGACGCCAGTA

ETaR, endothelin A receptor; ET-1, endothelin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; MIP-2, macrophage inflammatory protein 2; MCP-1, monocyte chemoattractant protein 1

Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons between groups were performed using one way analysis of variance test. Histopathology data were analyzed using χ^2 analysis. Statistical significance was determined as $P < 0.05$.

Results

In vitro gene silencing of ETaR

To knock down ETaR expression, two sequences specifically targeting the ETaR gene (ETaR shRNA#1 and #2) were designed. The targeting sequences were cloned into the shRNA expression vector that encodes a cGFP reporter gene as described previously. The silencing efficiency of two different sequences at the mRNA level was assessed using realtime PCR (Figure 1). The ETaR shRNA#1 was more potent in silencing the ETaR gene than shRNA#2 ($P < 0.05$), with empty vector and liposome as the controls. Hence, we applied the ETaR shRNA#1 in all subsequent *in vivo* experiments of this study.

In vivo gene silencing of ETaR

The plasmid vector containing ETaR shRNA and cGFP was constructed and administered into the kidney. In the plasmid group, much more green fluorescence was visualized in the frozen section of renal biopsies in comparison with the PBS group (Figure 2).

The expression of ETaR in the kidney was significantly increased by I/R injury at both mRNA and protein (Figures 3a and b) levels. The increased ETaR mRNA expression induced by I/R injury in the kidney was markedly suppressed after treatment with ETaR shRNA ($P < 0.05$ versus PBS or vector-treated group, Figure 3a). The

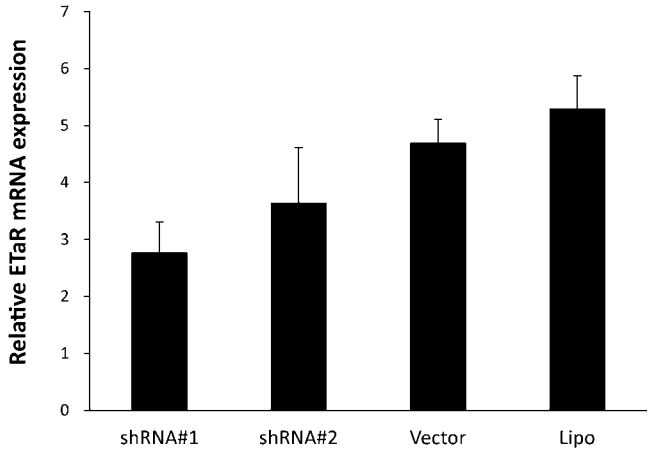

Figure 1 ETaR shRNA inhibits ETaR mRNA expression *in vitro* detected by realtime PCR. Relative quantity of ETaR mRNA is expressed as mean ± SEM from at least three independent experiments. The empty vector and liposome were used as the controls. The ETaR shRNA#1 was more potent than shRNA#2 ($P < 0.05$) in silencing the ETaR gene. ETaR, endothelin A receptor; shRNA, short hairpin RNA; PCR, polymerase chain reaction

reduction of ETaR protein was also observed in the kidneys of shRNA-treated rats (Figure 3b). In addition, the level of ET-1 mRNA in the kidney was also decreased in the ETaR shRNA-treated rats in comparison with that in the control rats (Figure 3c).

ETaR shRNA protects renal function against I/R injury

Given that shRNA appeared to block not only the up-regulation of ETaR, but also the ET system in the rats exposed to I/R injury, it was hypothesized that shRNA could improve renal function. Both serum creatinine and BUN (Figures 4a and b) were increased significantly in the I/R injury group compared with the unclamped controls 24 h after reperfusion. The treatment with ETaR shRNA before induction of I/R injury significantly prevented the increase of serum creatinine and BUN induced by I/R injury (Figure 4a and b).

ETaR shRNA prevents renal tissue damage against I/R injury

To demonstrate further the beneficial effect of ETaR shRNA on renal I/R injury, the histopathology changes of I/R injury were examined in the kidney. Compared with the unclamped kidneys, a much larger area of tubular necrosis, vacuolization and cast formation was observed in the kidneys with I/R injury (Figure 5A). However, rats pretreated with ETaR shRNA demonstrated significant attenuation of pathological damage in the kidney, which was not significantly different from normal (Figure 5B).

ETaR shRNA inhibits I/R-induced cytokine and chemokine production

To determine whether ETaR shRNA affected renal expression of TNF-α, the expression of TNF-α in the kidney was measured by realtime PCR. Renal expression of TNF-α was reduced by pretreating the kidney with ETaR shRNA compared with the control group (Figure 6a). The expression of IL-6 in the kidney was also measured and a same trend as TNF-α was shown (Figure 6b). Along these lines, the expression of the chemokine MCP-1 and MIP-2 was investigated in the kidneys with or without ETaR shRNA treatment. As shown in Figures 6c and d, the expression of MCP-1 and MIP-2 in the renal tissues with I/R injury was higher than that of unclamped tissues. Administration of ETaR shRNA before induction of I/R injury prevented the up-regulation of MCP-1 and MIP-2.

ETaR shRNA increased NO production

To determine whether ETaR shRNA affected renal synthesis of NO, we measured the production of NO in renal tissues. The level of NO was significantly increased in the kidneys pretreated with ETaR shRNA compared with the PBS or vector control group (Figure 7).

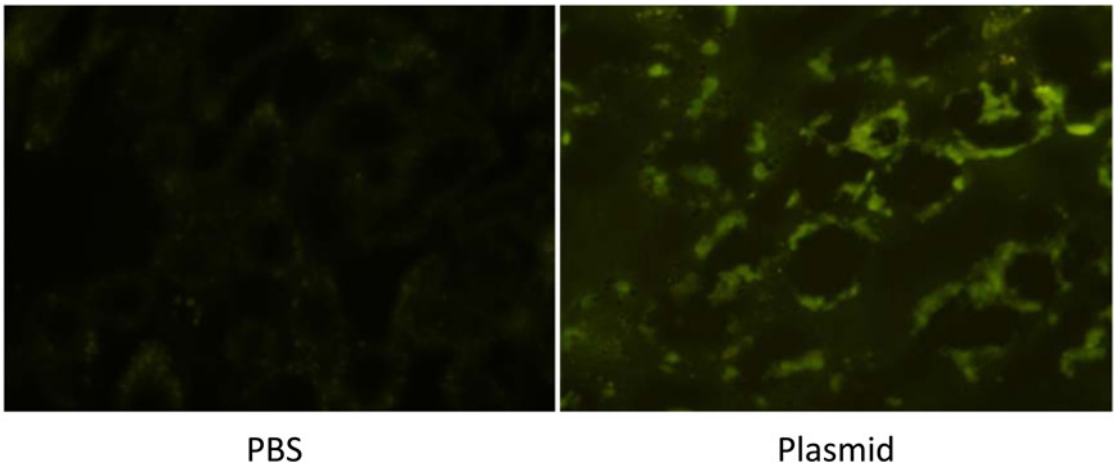

Figure 2 The frozen sections of renal biopsies were examined under a fluorescence microscope. The plasmid vector containing ETaR and cGFP was constructed and administered into the kidney. In the plasmid group, more green fluorescence was visualized in the frozen section of renal biopsies in contrast to the PBS group. ETaR, endothelin A receptor

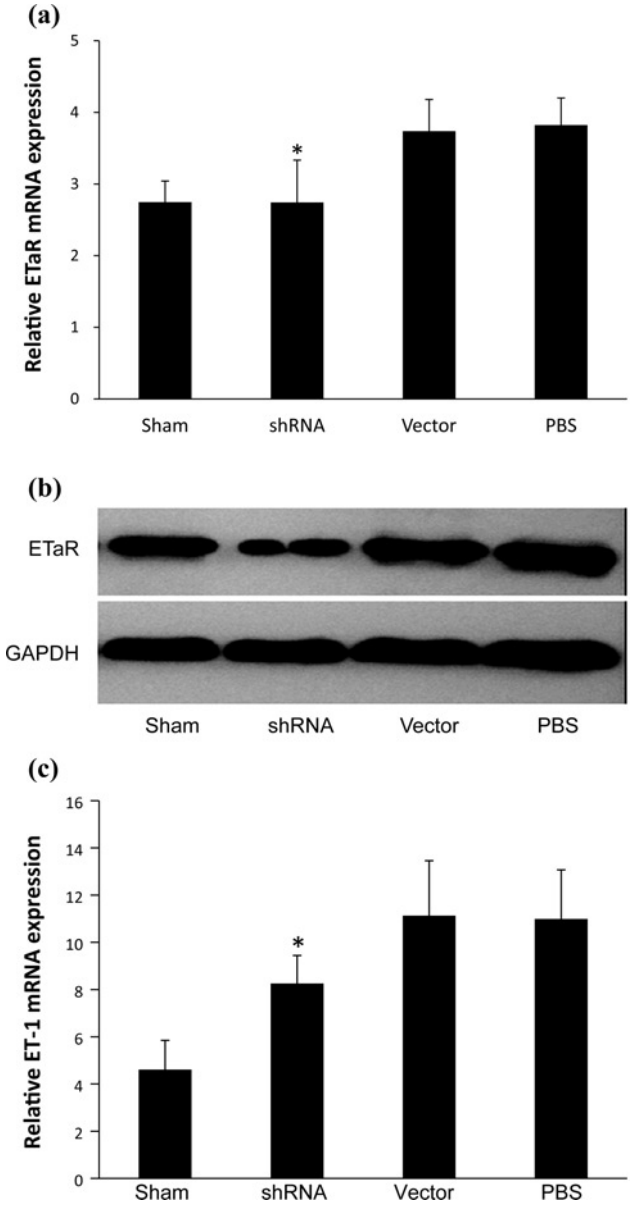

Figure 3 Silencing ETaR with ETaR shRNA *in vivo*. (a) Transcripts of ETaR and GAPDH were determined by quantitative realtime PCR. Relative quantity of ETaR mRNA is expressed as mean ± SEM, $n = 7$. Statistical significance as compared with controls was denoted at $P < 0.05$. (b) The ETaR protein expression in the kidney after gene silencing detected by Western blot. (c) Expression of ET-1 mRNA was determined by realtime PCR. Statistical significance as compared with controls was denoted at $P < 0.05$. ETaR, endothelin A receptor; PCR, polymerase chain reaction; shRNA, short hairpin RNA; PBS, phosphate-buffered saline GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Discussion

Short- and long-term post-transplant renal function is greatly affected by the degree and duration of I/R injury.[16] I/R injury is not only directly linked to the viability of the donor kidney, but it also subsequently increases the rejection rate, because destroyed cells in the kidney release a large number of pathogens.[17,18] Therefore, how to effectively prevent I/R injury in renal transplantation is very important for renal function recovery and long-term graft survival.

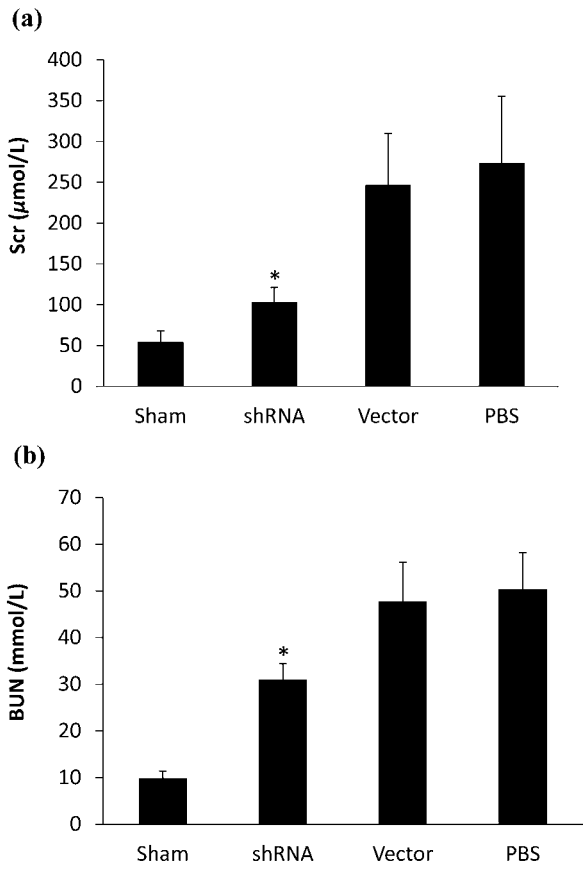

Figure 4 shRNA reduces the level of serum creatinine (a) and BUN (b) in rats with renal I/R injury. Data are shown as mean ± SEM, $n = 7$. Statistical significance as compared with controls was denoted at $P < 0.05$. Scr, serum creatinine; shRNA, short hairpin RNA; BUN, blood urea nitrogen; I/R, ischemia-reperfusion

RNA interference is widely applied in a variety of organisms in order to induce specific post-transcriptional gene silencing.[19,20] siRNA or shRNA are a class of double-stranded RNA molecules and play crucial roles in the downstream biological functions of targeting genes.[21–23] In this study, in order to optimize the silencing effects, two shRNA sequences targeting ETaR were designed. The result from this *in vitro* study using A-10 cells showed that both sequences of ETaR shRNA are able to effectively silence the expression of ETaR, but ETaR shRNA#1 was more efficient than ETaR shRNA#2. So ETaR shRNA#1 was selected for further *in vivo* study.

The plasmid vector containing ETaR shRNA and cGFP was constructed, transfected into cells or administered into the kidney in this study. In order to improve the delivery efficiency of shRNA into the target organ *in vivo*, hydrodynamic retrograde renal vein injection was used.[24] In the plasmid group, green fluorescence was observed in the frozen sections from renal biopsies, indicating the efficient delivery of ETaR shRNA plasmid into the kidney. However, it was difficult to identify the specific location of ETaR shRNA plasmid in the kidney, even through it was supposed to be an endothelial cell-oriented delivery. The evidence here at least suggested that liposome-assisted transfection could be avoided *in vivo*, although this method can increase transfection efficiency in a variety of cells.

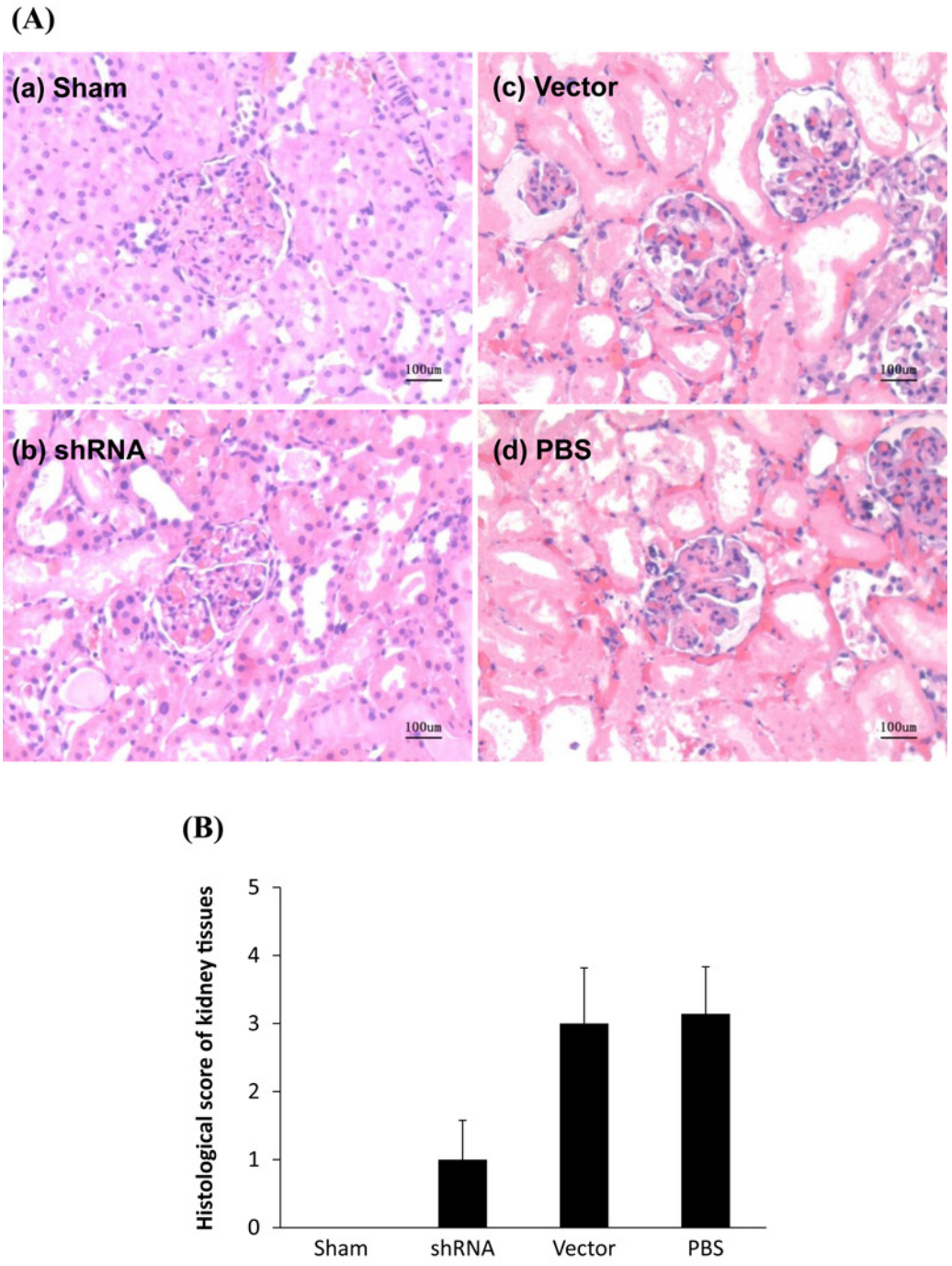

Figure 5 (A) ETaR shRNA protects renal structure against I/R injury evaluated in H&E sections. Normal unclamped kidney (a), ETaR shRNA-treated I/R kidney (b), control vector-treated I/R kidney (c) and PBS-treated I/R kidney (d) are illustrated. (B) The percentage of histological changes in the cortex and medulla was semi-quantitatively scored. ETaR, endothelin A receptor; shRNA, short hairpin RNA; I/R, ischemia-reperfusion; H&E, hematoxylin and eosin

We also detected the expression of ETaR mRNA and protein in kidney tissues to further verify the silencing efficiency of ETaR shRNA. It was shown that ETaR shRNA can effectively knock down the expression of ETaR at both mRNA and protein levels. In this study, we used vector-mediated shRNA that had the following advantages: stable silencing efficiency *in vivo* in terms of less susceptible to physical and chemical degradation factors; low cost; low cytotoxicity; and low immunogenicity. However, some disadvantages also exist: relatively low transfection efficiency and longer priming time *in vivo*. In contrast, the chemical synthesized siRNA of 20–25 nucleotides in length can significantly reduce the onset time and can be more suitable for acute models. However, the high cost of large-scale synthesized siRNA and the high rate of degradation before onset *in vivo* remain to be settled.

The ET system plays an important role in renal I/R injury. Effective ET antagonists reduce renal I/R injury.[25] ETaR antagonist has achieved exciting effects in different aspects of diseases, such as pulmonary arterial hypertension.

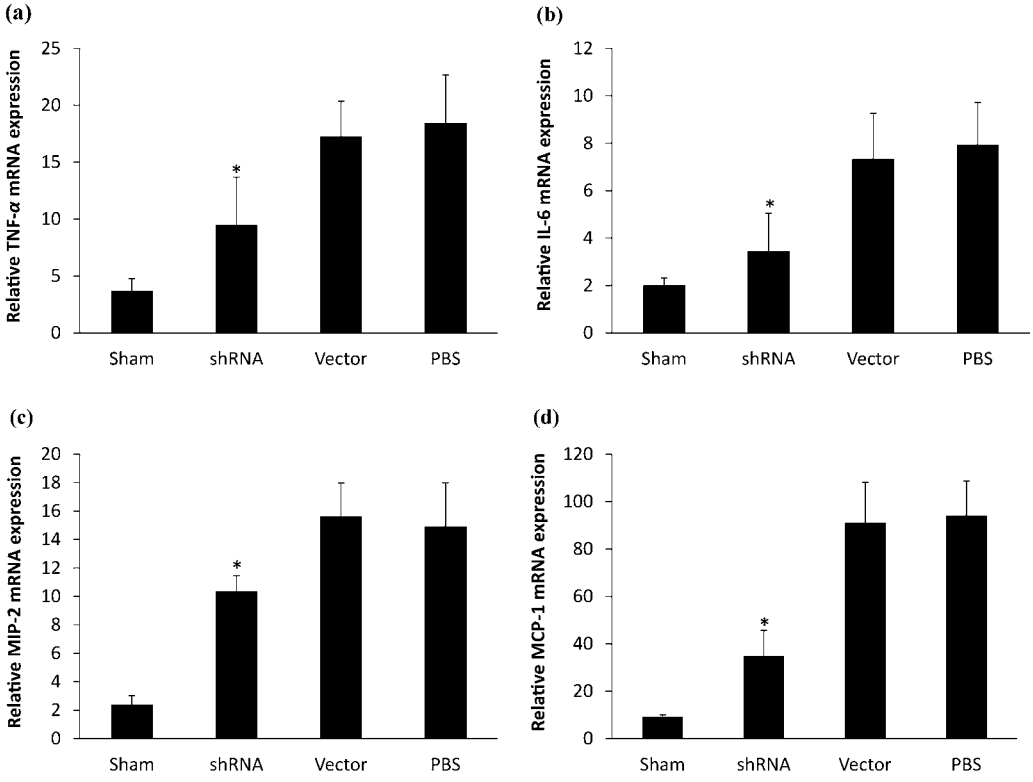

Figure 6 Expression of (a) TNF-α, (b) IL-6, (d) MCP-1 and (c) MIP-2 in renal tissues 24 h after reperfusion ($n = 7$). ETaR shRNA down-regulates the expression of TNF-α, IL-6, MCP-1 and MIP-2 in renal tissues. Statistical significance as compared with controls was denoted at $P < 0.05$. ETaR, endothelin A receptor; shRNA, short hairpin RNA; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; MIP-2, macrophage inflammatory protein 2; MCP-1, monocyte chemoattractant protein 1; PBS, phosphate-buffered saline

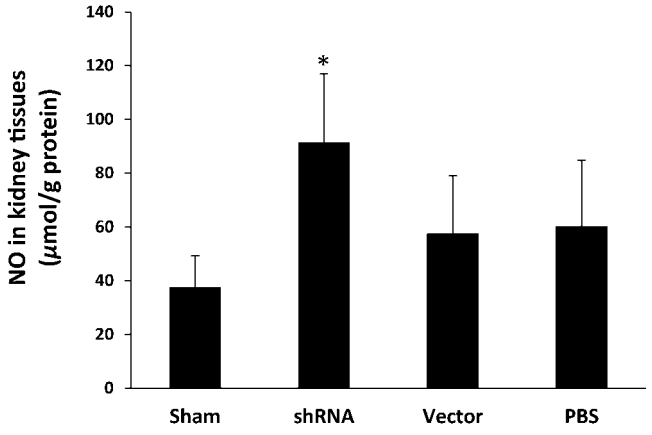

Figure 7 Production of NO in renal tissues 24 h after reperfusion ($n = 7$). ETaR shRNA increased the level of NO (μmol/g protein) in renal tissues. Statistical significance as compared with controls was denoted at $P < 0.05$. NO, nitric oxide; ETaR, endothelin A receptor; shRNA, short hairpin RNA; PBS, phosphate-buffered saline

However, the specificity of available drugs targeting ETaR is not as good as shRNA, which can precisely inhibit gene expression in response to the complementary sequence of existing mRNA with high efficiency. Besides, ET-receptor antagonists have a few side-effects, including increasing liver enzymes,[26] cardiological side-effects,[27,28] headache,[27,29] peripheral edema and fluid retention,[30] which limit their clinical application and efficacy. In this study, we tried to find a new approach to inhibit ETaR with high specificity and low side-effects. Therefore, the gene therapy utilizing shRNA, a relatively new approach, would be a better alternative to ETaR antagonists. The results from this study showed that ETaR shRNA not only knocked down the expression of ETaR, but also reduced ET-1 mRNA level in the kidney. At the same time, renal function and kidney tissue structure were also significantly improved. It suggests that ETaR shRNA can effectively silence ETaR mRNA, which subsequently coordinated the expression of ET-1 mRNA. The synergistic interaction between ETaR and ET-1 eventually reduced I/R injury.

ETaR shRNA downregulated the expression of ET-1 mRNA; however, this does not mean that the shRNA of ETaR is unspecific, but more likely due to a feedback response, as shRNA silencing is based on the complementary sequence binding of targeted existing mRNA. For instance, as ETaR shRNA specifically suppressed the expression of ETaR, the binding of ET-1 to ETaR cannot be achieved. Alternatively, more ET-1 has to bind to ETbR, which is mainly located on the surface of endothelial cells and consequently produces more NO.[31] As we know, NO inhibits the expression of ET-1 mRNA.[32] The downregulation of ETaR, therefore, could cause the downregulation of ET-1 mRNA. Furthermore, we detected the production of NO in renal tissues, and observed that the level of NO was significantly increased in the kidneys pretreated with ETaR shRNA

compared with the PBS or the vector control group. This, at least, could be indirect evidence for the existence of this feedback regulation, but remains to be further investigated.

The mRNA levels of TNF-α, IL-6, MCP-1 and MIP-2 in renal tissues were significantly decreased in the ETaR shRNA group compared with the control group in this study. Similarly, the downregulation of mRNA expression of these inflammatory mediators might be due to the responses of feedback loops, rather than unspecific characteristics of ETaR shRNA. The down-regulation of TNF-α could reduce the inflammatory cascade. The reduction of IL-6 could avoid further expansion of the inflammatory response. In addition, the meliorated MCP-1, MIP-2 expression might inhibit aggregation of inflammatory cells in I/R kidney. The down-regulation of these cytokines and chemokines may be due to the silencing of the ETaR gene, as well as the reduction of ET-1 mRNA expression. All of these orchestrated changes could further control the development of inflammation and vascular endothelial cell injury associated with renal I/R injury. However, ET-1-ETaR signaling may affect a variety of biological events, such as cell contraction, migration, proliferation and survival.[32] ETaR is a key molecule involved in blood vessel contraction and inflammatory response of I/R injury. In this study, we mainly focused on inflammatory responses associated with the protective effects of ETaR shRNA on renal I/R injury.

In conclusion, this study successfully constructed vector-mediated ETaR shRNA, which is an efficient tool to knock down the expression of ETaR at mRNA and protein levels in kidney tissues. Down-regulation of ETaR by shRNA correspondingly reduced the mRNA expression of ET-1, as well as other cytokines and chemokines, and subsequently improved renal function and structure, although the action mechanism of the ET system, as well as ETaR shRNA, in renal I/R injury needs to be further investigated.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. YJ, TZ and YO performed the animal experiments; ZZ, YQ and MX conducted the molecular biological analyses; YJ, ZZ and BY wrote the manuscript; and RR and TyZ contributed to the revision of the manuscript. YJ and ZZ contributed equally to this work.

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