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Regulation and mechanism of miR-146 on renal ischemia reperfusion injury

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Aim: MicroRNAs (miRs) are endogenous substances that act as important diagnostic and treatment targets in renal diseases. miR-146 plays an important role in the development of endotoxin tolerance through NF-κB pathway, but the underlying mechanism is not clearly understood. The aim of this study was to determine the molecular regulation and function of miR-146 and also the expression of miR-146 in an experimental model of renal ischemia reperfusion injury (IRI). *Methods:* IRI was induced in mouse by bilateral IRI for 45 min followed by reperfusion. The male mice were randomized as: sham, I/R, I/R+miR-146, and I/R+antago-miR-146 groups. Renal function, histological damage, and cell apoptosis were evaluated at 24 h after reperfusion. *Results:* Overexpression of miR-146 protected renal function. Renal cells with upregulated miR-146 had lower plasma levels of blood urea nitrogen (BUN) and creatinine, decreased apoptosis and active caspase-3 protein expressions. miR-146 was shown to have a role in renal IR injury. miR-146 has a protective effect on renal function and plays a significant role in apoptosis. IGSF1 acts as a target of miR-146. IGSF1 rescued the effects of miR-146 on renal IRI. miR-146 protected renal function by activation of PI3K/AKT. *Conclusion:* These findings suggest that miR-146 might regulate apoptosis and can cause injury in I/R via targeting IGSF1 and also exert renal protection property.

1. Introduction

Ischemia reperfusion injury (IRI) is characterized by sudden temporary impairment of the blood supply, leading to histological damage and functional failure of various organs. In kidney, IRI leads to the development of acute kidney injury (AKI) which is often developed in approximately 5% of hospitalized patients, and is associated with high mortality rate and long hospitalization (Chang et al. 2010; Mehta and Chertow 2003). Despite a lot of research, the pathogenesis of renal IRI still remains unclear. The disease has become a major challenge for clinicians due to the increasing incidence of renal IRI because there is no specific treatment (Bagshaw et al. 2008; Bonventre and Yang 2011; Saikumar et al. 2012; Sharfuddin and Molitoris 2011; Xu et al. 2012). Generally, IRI occurs due to generalized or localized impairment of oxygen and nutrient delivery, and removal of waste from the renal cells (Le Dorze et al. 2009). This leads to the accumulation of nitrogenous wastes including blood urea nitrogen (BUN) and creatinine. As a result of this, tubular epithelial cells are injured and in severe cases develop apoptosis and necrosis (Bonventre and Yang 2011). There is growing evidence that autophagy deficiency in tubular epithelial cells is a major cause of AKI (Jiang et al. 2012; Livingston and Dong 2014).

Several studies suggested the role of proteins like IL-18 (Melnikov et al. 2002), NGAL (Han et al. 2002) and KIM-1 (Mishra et al. 2005) in renal I/R injury. MicroRNAs (miRs) have gained great attention in the recent years for their role in IRI. miRs are a class of short, non-encoding RNA (19-25 nucleotides), which are involved in many biological, pathological and physiological process through the regulation of the target gene 3'UTR (Krol et al. 2010). These miRs act as post-transcriptional regulators in the immune system including endotoxin tolerance (Baltimore et al. 2008). In a previous study, it has been observed that several miRs (including miR-146) are involved in the regulation of toll-like receptor (TLR4) signaling pathway during endotoxin tolerance (Quinn et al. 2012). It is reported that miR-146a expression occurs in an NF-κB-dependent manner under LPS-mediation (Taganov et al. 2006). The target genes for miR-146 are TRAF6 and IRAK-1, which also promote the inflammation stimulated by pro-inflammatory cytokines (e.g. TNF-α). These findings suggest that miR-146 plays a role in TLR/NF-κB pathway by negative feedback regulation of TLR and cytokine receptor signaling (El Gazzar et al. 2011). In a mouse model of renal IRI, increased TLR4 was observed following ischemia and the development of kidney IRI was dependent on TLR4/MyD88 signaling pathway (Saba et al. 2014). Hence, in this study we decided to investigate the regulation and mechanism of miR-146 on renal IRI by using a mouse model of AKI.

2. Investigations and results

2.1. *Effect of miR-146 on the renal function*

Blood samples were collected to assess the renal function using levels of serum creatinine and BUN. The levels of serum creatinine and BUN were significantly increased in the renal IRI group compared to sham ($P < 0.01$). But, the levels were decreased by overexpression of miR-146 ($P < 0.05$). However, suppression of miR-146 by the addition of an antagonist showed contrary results (Fig. 1A and 1B, $P < 0.05$).

2.2. *miR-146 inhibited tissue apoptosis*

TUNEL assay was performed to assess the apoptosis rate in the renal cells. The results revealed that the percentage of TUNEL positive cells was significantly increased in the renal IRI ($P < 0.01$), but the percentage was decreased by overexpression of miR-146 (P

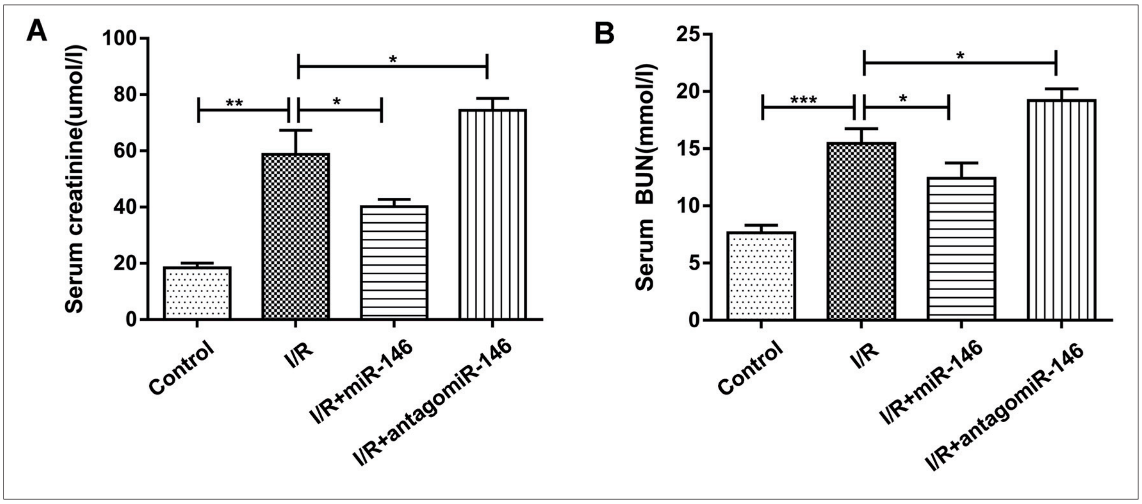

Fig. 1: microRNA-146 protects renal function in IRI mice. Blood was collected at 24 h after reperfusion to determine levels of serum creatinine (A) and BUN (B). Data shown are means + SD. The data were analyzed with one-way ANOVA plus the Tukey post hoc multiple-comparisons test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

< 0.05). However, suppression of miR-146 by the addition of an antagonist showed contrary results (Fig. 2A, $P < 0.05$).

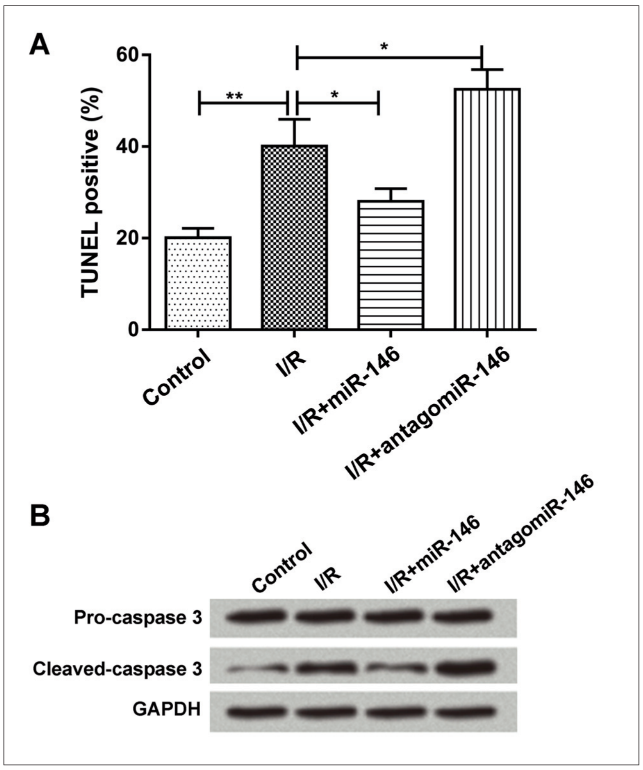

Fig. 2: Effect of miR-146 on renal histology and the expression of caspase 3. (A) The percentage of TUNEL positive cells was calculated. (B) The expression levels of caspase 3 were detected. The groups were Control, I/R, I/R+miR-146 and I/R+antagomiR-146. The data are presented as mean + SD. *$P < 0.05$; **$P < 0.01$.

2.3. Expression of caspase-3 proteins in kidneys

To determine the role of miR-146 in renal IRI, we determined caspase-3 expression by western blot after 24 h of reperfusion. The results showed no significant differences between pro-caspase-3 expressions among all groups. However, cleaved caspase-3 levels were significantly increased in renal IRI, but the levels were decreased by overexpression of miR-146. The data showed that upregulation of miR-146 in IRI could suppress apoptosis (Fig. 2B).

2.4. Immunoglobulin superfamily member 1(IGSF1) as a target of miR-146

Dual luciferase reporter assay was performed to assess the activity of IGSF1. It was significantly reduced by co-transfection with miR-146 mimics and the vector carrying 3'-UTR of IGSF1 compared with negative control. On the contrary, co-transfection with miR-146 inhibitor and the vector carrying 3'-UTR of IGSF1 obviously increased the luciferase activity. These results showed that miR-146 was bound directly to a specific site in the 3'-UTR of IGSF1 mRNA (Fig. 3A and 3B).

To confirm the role of miR-146 in the regulation of IGSF1 expression, qRT-PCR and Western blot analyses were also performed. qRT-PCR analysis showed that transfection with miR-146 inhibitors demonstrated no significant effect on the mRNA levels of IGSF1 compared to the negative control (Fig. 3C). Similarly, Western blotting results showed that transfection of miR-146 mimics significantly suppressed the protein levels of IGSF1, whereas the miR-146 inhibitor transfection dramatically promoted the protein levels of IGSF1 compared to negative control (fig. 3D). These data suggested that miR-146 suppressed the expression of IGSF1 in a post-transcriptional manner.

2.5. IGSF1 rescued the effects of miR-146 on renal IRI

Finally, we determined the effect of IGSF1 inhibition on renal IRI. The results showed that levels of serum creatinine ($P < 0.05$), BUN ($P < 0.01$), TUNEL positive ($P < 0.01$) and caspase-3 levels were significantly decreased by overexpression of miR-146. However, overexpression of IGSF1 decreased the protective effect of serum creatinine levels ($P < 0.05$), serum BUN levels ($P < 0.01$), and TUNEL positive ($P < 0.01$). These results suggested the harmful role of IGSF1 on the protective effects of miR-146 on renal IRI (Fig. 4A-4D).

2.6. miR-146 protected renal function by activation of PI3K/AKT

Western blot was performed to assess the effect of miR-146 on P13K/AKT signal pathway. Results demonstrated that overexpression of miR-146 promoted the levels of p-AKT ($P < 0.005$) and p-ERK ($P < 0.01$). The addition of antagonist showed suppressive results of p-AKT ($P < 0.05$) and p-ERK ($P < 0.05$) compared to I/R-miR-146 group (Fig. 5A and 5B).

3. Discussion

In the present study, we explored the regulation and mechanism of miR-146 in renal IRI using mouse AKI model. We found that

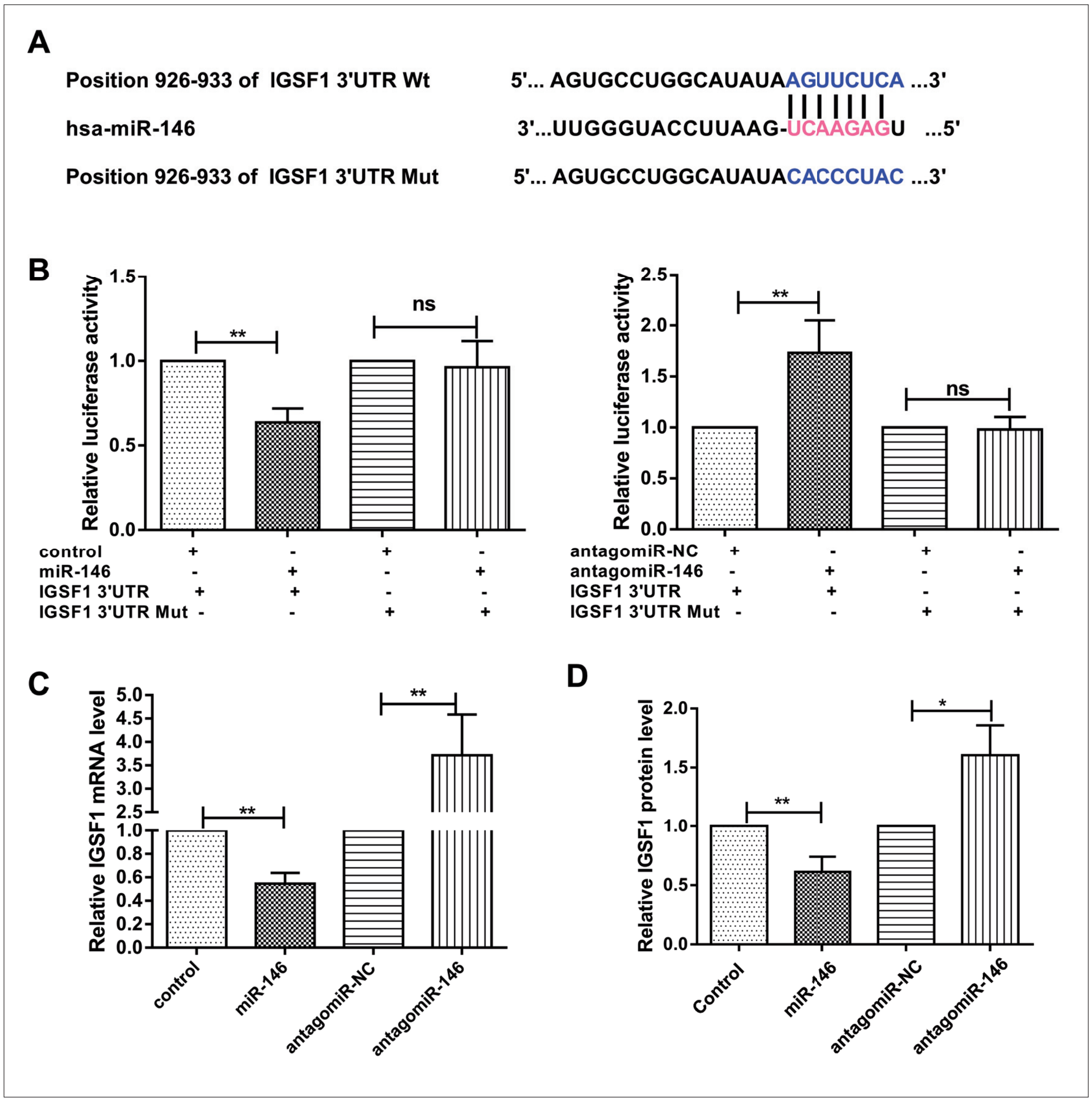

Fig. 3: Effect of miR-146 on regulation of IGSF1. (A) Sequence alignment between miR-146 and the 3'-UTR. (B) Luciferase assay was performed after co-transfection with IGSF1 and miR-146 mimics or inhibitor. (C) The relative mRNA levels of IGSF1 were determined after miR-146 mimics or miR-146 inhibitor transfection by real-time PCR. (D) The protein levels of IGSF1 were determined after miR-146 mimics or miR-146 inhibitor transfection by Western blot. The data presented as mean + SD. **P* < 0.05; ***P* < 0.01.

miR-146 protected the renal function in mice and also inhibited tissue apoptosis. Immunoblot analysis of the caspase 3 pathway showed a significant change in the protein expression levels which indicates apoptosis signaling through miR-146. It is well known fact that dysfunction and death of proximal tubular epithelial cells is mainly responsible for the pathological and clinical effects of AKI (Molitoris 1999).

In ischemic models of humans with acute tubular necrosis, renal tubular cells die due to apoptosis as well as necrosis (Bonegio and Lieberthal 2002). Therefore, it is important to determine the relation between these two forms of cell death to loss of renal tubular cells in acute tubular necrosis. It is believed by many investigators that apoptosis leads to ischemic renal dysfunction. This is determined by multiple approaches such as Hoechst staining, caspase-3 activity, apoptosis-inducing factor or cytochrome c release, TUNEL, conformational Bax activation, and loss of intact B-cell lymphoma 2 show apoptosis after ischemia. But there is still a drawback in each technique and it might be a limitation to our study i.e. TUNEL assays detect necrotic as well as apoptotic cells, and hence it cannot be used as a sole method for quantification of apoptosis. Despite the limitation, our results showed that the percentage of TUNEL positive cells was significantly decreased following overexpression of miR-146. A possible reason for this could be the fact that suppression of autophagy sensitizes tubular cells to apoptosis induced by I/R injury that results in the highest expression of caspase-3 and more TUNEL-positive cells.

Several studies have identified that most of the tissue miRNAs are detected in the body fluid under normal conditions and these circulating miRNAs can act as markers in disease diagnosis and severity (Ng et al. 2009; Wang et al. 2010; Wang et al. 2009), including acute and chronic kidney disease (Aguado-Fraile et al. 2013; Trionfini et al. 2015). However, in the disease condition, the expression profile of miRNA was different which depend on the nature and type of disease (Chen et al. 2008). The role of miRNAs in renal IRI is well known but the underlying mechanism still remains unclear. The results of our study showed that miR-146 protected the renal function in mice which was measured by the decreased levels of serum creatinine and BUN. This is in agreement with other studies which evaluated the role of several miRNAs in AKI or renal IRI models. Bijkerk et al. (2014) have shown that miR-126 has protective effects against renal IRI in mice. They showed that overexpression of miR-126 in the hematopoietic compartment of mice increased the vasoprotective potential which in turn decreased the renal injury. In another study, it was observed that miR-24 antagonism

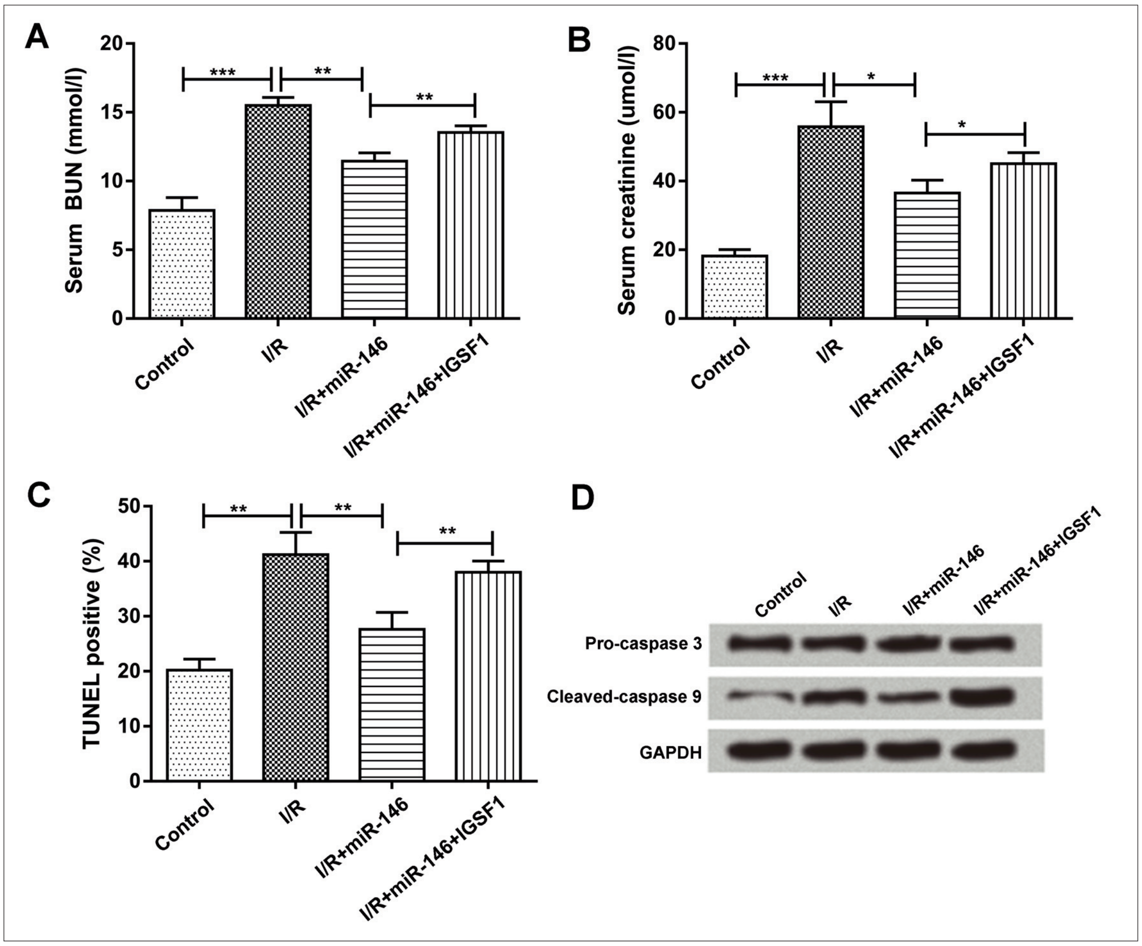

Fig. 4: Effect of miR-146 on PI3K/AKT. The serum creatinine, BUN and TUNEL positive were measured in terms of percentage in Control, I/R, I/R+miR-146 and I/R+miR-146+IGSF1 groups. The data are presented as mean + SD. *$P < 0.05$; **$P < 0.01$.

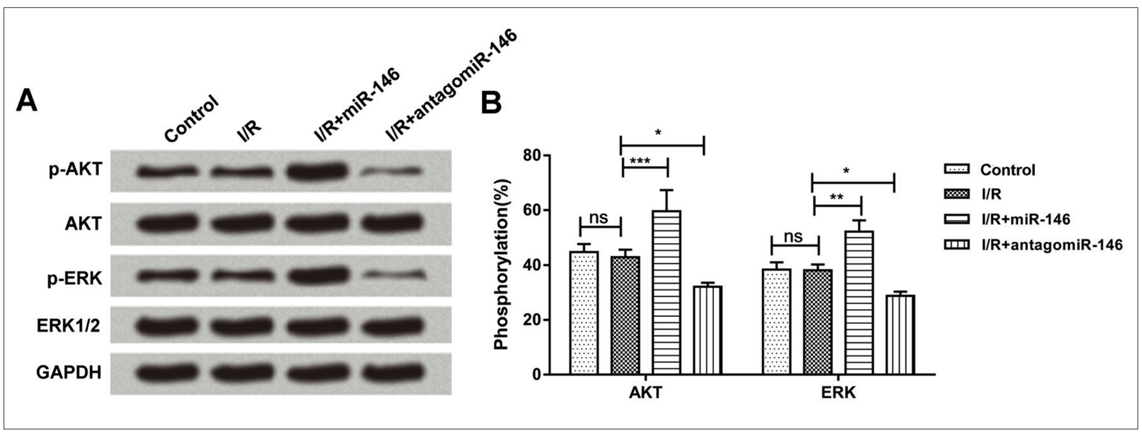

Fig. 5: miR-146 protected renal function by activation of PI3K/AKT. The phosphorylation was measured in terms of percentage in Control, I/R, I/R+miR-146 and I/R+antagomiR-146 groups. The data are presented as mean + SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

prevented renal reperfusion injury in vivo (Lorenzen et al. 2014). This was the first study which showed that inhibition of miRNA might be a potential therapeutic option for the treatment of AKI.

Taganov et al. (2006) have reported that miR-146a/b induced the response to LPS and pro-inflammatory mediators and the induction process was regulated by NF-κB. They also found that TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) genes were the targets of miR-146. Thus, miR-146 plays a significant role in improving the innate immune response by negative feedback mechanism (including downregulation of the TRAF6 and IRAK1 genes). The results from these studies suggest that autophagy is a protective mechanism in renal I/R models and the activation of which might be effective in I/R injury, and possibly AKI.

Moreover, the results from our study revealed that miR-146 protected renal function by activation of PI3K/AKT. It is known that in many cell types the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway can mediate cell survival (Aguado-Fraile et al. 2013), including renal cells (Chen, Ba et al. 2008). Originally, the PI3K/Akt pathway was identified to play a critical role in regulating cell growth and survival (Bijkerk et al. 2014), but recently it has been implicated in the protection of liver and kidney against I/R injury by moderating the inflammatory response (Hausenloy et al. 2005; Joo et al. 2006).

In the clinical setting, AKI is considered as a complex disorder with clinical manifestations such as elevation in serum creatinine to renal failure (Fry 1994). AKI accounts for 40-80% deaths in the intensive care setting and about 13% of AKI patients, in turn, are developing chronic kidney disease (Ravingerova et al. 2012; Sano et al. 2010). It is therefore, a need for determining the pathogenesis for developing new and efficient treatment strategies.

In the present study, miR-146 demonstrated anti-apoptotic properties by suppressing IGSF1 expression in renal IRI and also exhibited significant functional protection in IRI. Thus, this could be a useful target to study the complex physiopathology of renal IRI.

4. Experimental

4.1. Animals

A mouse model of IRI was created by using ischemia/reperfusion (I/R) in the 8-week old male C57BL/6 mice which were purchased from Vital River Laboratory Animal Co., Ltd. (Beijing, China). This study was approved by the Ethics Committee of Guangzhou First People's Hospital of Guangzhou Medical University, and is in accordance with the principles of the Declaration of Helsinki. Mice were randomized to sham (n=6), I/R (n=6), I/R+miR-146 (n=6), and I/R+antagomiR-146 (n=6) groups.

4.2. Kidney I/R injury induction

An IRI model of I/R injury of mouse kidneys was performed according to a previous report, with some modifications. Briefly, anesthesia was induced in the mice using Buddha butyl; the pedicle was then isolated and occluded for 35 min, followed by reperfusion. The mice were then allowed to exsanguinate for three days; their kidneys were removed and frozen in liquid nitrogen for subsequent experiments. Sham-operated animals underwent identical surgical treatment of renal pedicle isolation except pedicle occlusion.

4.3. Assessment of renal function

Blood samples were collected from orbit angular vein at 24 h after reperfusion. The blood was centrifuged at 4500 rpm for 10 min and serum was separated. The renal function was determined using levels of serum creatinine and blood urea nitrogen (BUN).

4.4. Transfection of miRNAs

miRNAs (including miR mimics, miR- inhibitor and negative control) were obtained from Gene Pharma Co., Ltd. (GenePharma, China). miRNA transfection was carried out by seeding the cells into 24-well plates and growing in the antibiotic-free medium overnight until 30–50% confluence was reached. About 0.4 nmol miRNAs was mixed with 15 µl Lipofectamine™ 2000 transfection reagent, and the cells were maintained 5% CO2 incubator (at 37°C, for 6 h). The medium was then replaced with fresh medium for 48 h and quantitative PCR was done to confirm the expression of miR.

4.5. Real-Time RT-PCR

The quantitative RT-PCR was performed using the total RNA which was reverse-transcribed. Using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA), total RNA was isolated from tissues or cultured cells. Extracted RNA was reverse-transcribed using Omniscript RT kit and random primers. Real-time RT-PCR was performed using Rotor-Gene RG-3000 Real-Time Thermal Cycler (Corbett Research, Australia). The miRNA qPCR detection kit (Gene Copoeia, USA) was used to determine the expression of miR and SYBR® Premix Ex TaqTM II kit (Takara, Dalian, China) was used to amplify Atg4B.

The total RNA from kidney cells were initially denatured at 94 °C for 2 min; 94 °C for 20 s, 59 °C for 15 s and 72 °C for 15 s. Plate reading for 40 cycles was done at 2 s, and a melt curve was generated from 65 to 95 °C. The relative expression levels for mRNA were normalized using the $2^{-\Delta\Delta CT}$ method relative to small nuclear RNA U6 and β-actin, respectively.

4.6. Western Blot

The proteins from cells were extracted using RIPA lysis buffer (Beyotime, Nantong, China). The proteins were separated by SDSPAGE and blotted onto a pre-wet nitrocellulose membrane (GE Healthcare, Germany). The membranes were blocked in 10% defatted milk in phosphate-buffered saline (PBS) at 4 °C for 2 h, and then probed with different primary antibodies. The illuminance was scanned using the Typhoon scanner (Amersham Biosciences, Piscataway, N.J., USA). All experiments were performed in triplicate.

4.7. Luciferase reporter assays

Firefly luciferase reporter plasmids containing 3'-UTR of the IGSF1 gene and empty luciferase vectors were obtained from Promega. Cells were co-transfected with the luciferase reporters (50 ng/well) together with 10 nM miR-146 mimic, antagomiR-146 or scrambled miRNA using Lipofectamine 2000 (Invitrogen). Luciferase activity was analyzed 48h post-transfection using Dual-Luciferase Reporter Assay System (Berthold Centro) following the manufacturer's instructions.

4.8. TUNEL

Sections were stained by using a fluorescent terminal deoxynucleotidyl transferase (dUTP) nick-end labeling (TUNEL) assay kit (Roche Diagnostics, Mannheim, Germany). The percentage of TUNEL positive cells was calculated as described earlier.

4.9. Statistical analysis

The results were expressed as mean+SD. Data were analyzed using one-way ANOVA plus the Tukey post hoc multiple comparisons test for comparing the mean values among multiple groups. All analyses were made using GraphPad Prism 5.0 statistical software package and P value of <0.05 was accepted as statistically significant.

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