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NR4A1 silencing protects against renal ischemia-reperfusion injury through activation of the β-catenin signaling pathway in old mice

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ABSTRACT

Renal ischemia-reperfusion injury (IRI), a major cause of acute kidney injury as well as a contributor to a rapid kidney dysfunction and high mortality rates, is a complex yet not fully understood process. Investigation on the underlying molecular mechanism including the inflammation initiation and progression can help to have a better understanding of the disease, and thereby lead to a potential therapeutic approach. We established renal IRI mouse model groups differing in their ages. These renal IRI mice were treated either only with si-nuclear receptor subfamily 4, group A, member 1 (NR4A1) or together with si-β-catenin by tail vein injection to analyze the role of NR4A1 and β-catenin in the development of renal IRI. Serum creatinine (SCr) and blood urea nitrogen (BUN) levels were examined for renal function analysis. Levels of the apoptosis markers B-cell lymphoma-2 (Bcl-2), Bcl-2 associated protein X (Bax), and cleaved caspase-3 were determined. *NR4A1* gene was up-regulated in the renal tissues of all mice with IRI, which showed a much higher level in the old mice with IRI. si-NR4A1 treatment resulted in reduced SCr and BUN levels and a decrease of cell apoptosis, indicated by lower expression of Bax and cleaved Caspase-3, while in contrast increased levels of Bcl-2 were detected. Interestingly, also the β-catenin level was increased by knockdown of NR4A1. Furthermore, si-β-catenin reversed the effect of knockdown of NR4A1, leading to aggravated renal function damage, severe pathological injury and increased apoptosis. Thus, silencing NR4A1 ameliorates renal IRI *via* β-catenin signaling pathway activation. Down-regulated NR4A1 confirms renoprotective properties against renal IRI *via* the activation of β-catenin signaling pathway in old mice.

1. Introduction

Ischemia-reperfusion injury (IRI) in tissues and organs can lead to ischemic diseases (Wei et al., 2010). IRI-induced renal injuries are the leading cause of acute renal injury related with high chances of mortality (Wan et al., 2014). Renal IRI is a complex pathophysiological process characterized by renal tubular necrosis, apoptosis and inflammatory response. It is commonly observed in hospitalized patients with acute or chronic kidney diseases (Shigeoka et al., 2010; Lee et al., 2012). In general, men show a higher predisposition to severe renal IRI than women due to the polypeptide hormone relaxin. This pregnancy hormone displays renoprotective properties against IRI (Yoshida et al., 2013). Emerging evidences indicate that renal IRI is a common outcome of operations, *e.g.* renal transplantation, partial nephrectomy, surgical revascularization of the renal artery, and suprarenal aortic aneurysms treatment (Shingu et al., 2010). Currently, it has been found that some genes, including caspase-8 and dipeptidyl peptidase 4, fulfill a protective function against renal IRI by increasing the resistance of tubular epithelial cells against apoptosis through short hairpin RNA therapy (Du et al., 2006; Glorie et al., 2012).

The nuclear receptor subfamily 4, group A, member 1 (NR4A1), also known as NGFIB and NUR77, belongs to the NR4A subgroup of nuclear hormone receptors that play an important role in a variety of different cancer types and diseases, including renal injury (Westbrook et al., 2014). NR4A1 is recognized as an immediate-early response gene during IRI and mediates cardiomyocyte apoptosis (Huang et al., 2014). In special cases, NR4A1 is up-regulated in cardiac microvascular IRI, and regulates in a positive manner in the microvascular collapse, endothelial cellular apoptosis and mitochondrial damage (Zhou et al., 2018). In triple-negative breast cancer cells, NR4A1 interacts with the

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transforming growth factor β-induced β-catenin, which then impedes cell migration (Hedrick and Safe, 2017). As an important regulator of cell development, regeneration, and carcinogenesis, the β-catenin signaling pathway is found to reduce hepatocellular damage and inflammatory response *in vivo* in mouse models with liver IRI (Ke et al., 2013). Moreover, Thiagarajan et al. have observed that activation of the β-catenin signaling pathway is cardioprotective against myocardial IRI (Thiagarajan et al., 2017). β-catenin has also been reported to influence the renoprotective effects of Vitamin D and/or pioglitazone on renal IRI (Ali et al., 2018). The functions of NR4A1 and β-catenin in the progression of IRI have been studied independently, and their roles in development and progression of renal IRI remain to be clarified. In this study, we generated a renal IRI mouse model to identify the underlying molecular mechanism of NR4A1 and β-catenin.

2. Materials and methods

2.1. Ethics statement

The current study was conducted under the approval by the Ethics Committee of the Second Xiangya Hospital. All animals involving procedures were performed in strict accordance with the recommendation of the Guide for Care and Use of Laboratory Animals.

2.1.1. Bioinformatics prediction

The Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) of National Center for Biotechnology Information Search database (https://www.ncbi.nlm.nih.gov/) was used to search renal IRI-related gene expression microarray data. The microarray data of GSE39548 (containing 4 control renal tissue specimens and 4 renal IRI tissue specimens) and GSE52982 (containing 5 renal IRI tissue specimens of young mice and 5 renal IRI tissue specimens from old mice) were obtained. Limma installation package of R software was used for differential analysis with $|\log2FoldChange| > 2.0$ and p value $< .05$ as the threshold. Heat maps of differentially expressed genes were drawn by using the pheatmap package.

2.1.2. Animal treatment

A total of 90 C57BL/6 mice [20 of 3 months old (young group) and 70 of 23–24 months old (old group)] purchased from Experimental Animal Center, Medical College of Wuhan University (Hubei, China) were reared under specific conditions. Then, the mice in the young group were randomly assigned into the sham group and the IRI group, containing 10 individuals in each group. Mice in the old group were randomly allocated into 7 groups, (i) sham, (ii) IRI, (iii) IRI + si-negative control (NC) (IRI mice injected with si-NR4A1 NC *via* caudal vein), (iv) IRI + si-NR4A1 (IRI mice injected with si-NR4A1 *via* caudal vein), (v) si-NC + si-Ctrl (IRI mice injected with si-NR4A1 and si-β-catenin NC *via* caudal vein), (vi) si-NR4A1 + si-Ctrl (IRI mice injected with the NC of si-NR4A1 and si-β-catenin *via* caudal vein), and (vii) si-NR4A1 + si-β-catenin (IRI mice injected with si-NR4A1 and si-β-catenin *via* caudal vein) groups. *In vivo* gene silencing was conducted by fluid dynamics and the injection procedures were performed as previously reported (Hamar et al., 2004).

2.1.3. IRI model in mice

Prior to operation, mice underwent an 8–12 h period of food fasting and granted free access to water. IRI models were constructed as described by Zhang, Ying, et al. (Zhang et al., 2016). In brief, mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg for old mice and 60 mg/kg for young mice), followed by an incision along the ventral midline to expose the kidneys. Bilateral renal pedicles were isolated and clamped by microaneurysm clip (728,816, Harvard Apparatus, Holliston, MA, USA) for a specific amount of time (35 min for young mice and 28 min for old mice), during which, the color of the kidney was attentively observed. Renal blood flow block changed the color from dark red into dark purple. Then the clip was loosened and kidney reperfusion was confirmed by the color change from dark purple back to dark red. Next, the mice were injected subcutaneously with 1.5 mL normal saline, and the incision was sutured, followed by conventional feeding. Mice in the sham group were only treated with bilateral kidney separation.

2.1.4. Renal function analysis

After 48 h of renal IRI model induction, the serum samples of the mice in each group were collected and stored at −80 °C. Serum creatinine (SCr) level was determined using QuantiChrom Uric Acid Assay Kit (Bioassay systems, CA, USA) and the level of blood urea nitrogen (BUN) was detected by Infinity Ammonia Liquid Stable Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.1.5. Hematoxylin-eosin (HE) staining

After 48 h of renal IRI model induction, paraffin-embedded renal tissue specimens were sliced into 3 μm sections and stained with HE. Severity of tubulointerstitial injury was determined by semi-quantitative assessment based on the area of counted renal tubular injuries. The assessment parameters/scoring criteria were as follows: 0 point (no injury); 1 point (injury area of ≤10%); 2 points (injury area of 11% - 25%); 3 points (injury area of 26% - 45%); 4 points (injury area of 46% - 75%) and 5 points (injury area of ≥76%). Ten high-power visual fields of junction of skin and spinal cord in each section were selected (× 400).

2.1.6. Immunohistochemistry (IHC)

After 48 h of renal IRI model induction, the renal tissue sections were dewaxed using xylene, dehydrated by gradient alcohol, followed by antigen retrieval in citrate buffer. After the addition of 3% H_2O_2 to block endogenous peroxidase activity, the sections were blocked with (normal) goat serum working solution for 1 h at 37 °C to reduce non-specific staining. Subsequently, (primary) polyclonal rabbit-α-NR4A1 antibody (ab153914, 1: 500, Abcam Inc., Cambridge, MA, USA), and polyclonal rabbit-α-p-β-catenin antibody (ab53050, 1: 500, Abcam Inc., Cambridge, MA, USA) were incubated with the sections overnight at 4 °C, followed by thawing at room temperature for 30 min. Then, horseradish peroxidase (HRP)-labeled (secondary) goat-α-rabbit immunoglobulin G (IgG) antibody (ab6721, 1: 5000, Abcam Inc., Cambridge, MA, USA) was added for 1 h incubation. Finally, the sections were developed by using diaminobenzidine, counterstained by hematoxylin, differentiated in 1% hydrochloric acid, dehydrated, cleared, mounted and observed under the microscope. The percentage of positive cells was calculated and analyzed by Image J software.

2.1.7. Western blot analysis

The total protein concentration of renal tissues was tested by a bicinchoninic acid protein assay kit (Sigma-Aldrich Chemical Company, St Louis, MO, USA). Next, the total protein extract (20 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted on polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA), and blocked with 5% skim milk powder for 2 h at room temperature. Primary antibodies against NR4A1 (ab109180, 1: 2000), p-β-catenin (ab27798, 1: 500), Bcl-2-associated X protein (Bax, ab32503, 1: 2000), B-cell lymphoma/leukemia-2 (Bcl-2, ab182858, 1: 2000), cleaved caspase-3 (ab2302, 1: 5000) and β-actin (ab9484, 1: 2000) were incubated with the membranes at 4 °C overnight, followed by thawing for 30 min. Next, the membranes were rinsed with Tris-buffered saline Tween-20 (TBST) (4 times, 15 min each). Corresponding HRP-labeled secondary antibody (ab205718, 1: 5000) was added to the membranes for 1 h at 37 °C, followed by a rinse with TBST (4 times). All above antibodies were purchased from Abcam, Inc. (Cambridge, MA, UK). The Image J software was used to measure the relative protein levels of the target genes.

2.1.8. Terminal deoxyribonucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL) assay

TUNEL kit (Promega Corp., Madison, Wisconsin, USA) was used to measure the apoptosis level of renal cells. Briefly, the paraffin embedded renal tissue sections were dewaxed by xylene, dehydrated in gradient alcohol, and immersed in 3% H_2O_2 for 10 min. Then the sections were hydrolyzed with 50 μL proteinase K solution (20 μg/mL, P6556, Sigma-Aldrich Chemical Co., St Louis, MO, USA) for 20 min for protein removal. In the next step, antigen retrieval was conducted by the addition of citrate for 30 min. After that, the sections were immersed in 50 μL TdT enzyme reaction solution in a 37 °C wet box for 1 h in the dark with TdT enzyme free reaction solution as NC. Subsequently, the samples were incubated with 50 μL peroxisome labeled anti-digoxigenin antibody in a 37 °C wet box for 30 min without light exposure, and developed by 4′,6-Diamidino-2-Phenylindole (C1002, Beyotime Institute of Biotechnology, Shanghai, China) for 10 min. After neutral resin mounting, the samples were observed and photographed under a light microscope. Cells with yellow-brown nucleus were positive apoptotic cells. Ten fields of each sample were randomly selected and the number of positive cells was counted. The apoptotic rate equals the number of positive cells divided by the number of total cells × 100%.

2.2. Statistical analysis

Statistical analyses were performed using the SPSS 22.0 software (IBM Corp, Armonk, NY, USA). Measurement data were represented as mean ± standard deviation. Gaussian distribution was tested by D'Agostino & Pearson omnibus normality test and data with normal distribution between two groups were compared using the Student's *t*-test. p value < .05 was statistically significant.

3. Results

3.1. NR4A1 is expressed at a high level in old mice with renal IRI

GSE39548 microarray data was used in differential gene expression analysis and the results showed that NR4A1 was highly expressed in renal IRI group compared to the control group ($p < .05$) (Fig. 1A) and was much higher in old mice with renal IRI ($p < .05$) (Fig. 1B). IHC was used to confirm the expression of NR4A1 protein between groups and ages. As shown in Fig. 1C, NR4A1 was localized in the nucleus and cytoplasm with expression rate remarkably increased in the IRI group and was more pronounced in old mice with renal IRI ($p < .05$). This demonstrated that NR4A1 was up-regulated in old mice with renal IRI.

3.2. NR4A1 knockdown mitigates renal pathological injury in old mice with renal IRI

To elucidate the effects of NR4A1 in old mice with renal IRI, serum levels of SCr and BUN in kidney tissues were measured. As shown in Fig. 2A & B, SCr and BUN were significantly higher in the IRI and IRI + si-NC groups than in the sham group ($p < .01$). On the other hand, it only slightly increased in the IRI + si-NR4A1 group compared to the IRI + si-NC group ($p < .01$). HE staining was also performed to observe pathological IRI in renal tissues. Staining results showed that mice in the sham group had morphologically clear/distinct renal tubular epithelial cells, and complete basement membrane structure. In contrast to that, mice treated with IRI and IRI + si-NC exhibited renal tubular atrophy, disordered tubular arrangement, and a large amount of inflammatory cell infiltration in the interstitium. Hence, the pathological injury score in mice treated with IRI and IRI + si-NC was remarkably higher than in the sham group. Additionally, mice treated with IRI + si-NR4A1 exhibited complete renal structure and a small amount of inflammatory cell infiltration, and the pathological injury score was significantly lower than mice treated with IRI + si-NC ($p < .01$) (Fig. 2C). Altogether, NR4A1 silencing could alleviate renal pathological injury in old mice with renal IRI.

3.3. Silenced NR4A1 impedes the apoptosis of renal cells in old mice with renal IRI

To investigate on the mechanistic action of NR4A1 in IRI, TUNEL and Western blot analysis were conducted to detect apoptotic renal cells in old mice with IRI. The results showed evident cell apoptosis as well as increased protein levels of Bax and cleaved caspase-3 in IRI, IRI + si-NC and IRI + si-NR4A1 groups than in the sham group but decreased Bcl-2 protein level ($p < .01$).

In comparison to the IRI + si-NC group, cell apoptosis as well as the protein levels of Bax and cleaved caspase-3 decreased in the IRI + si-NR4A1 group while Bcl-2 protein level was elevated ($p < .01$) (Fig. 3A & B). These results suggested that the apoptotic rate of renal cells in old mice with IRI increased and inhibiting the expression of NR4A1 could alleviate its effects.

3.4. NR4A1 silencing promotes β-catenin activation in old mice with renal IRI

Previous studies have showed that the loss of β-catenin can exacerbate renal injury (Zhou et al., 2012). Besides, NR4A1 inhibition can impede β-catenin transcriptional activity (Cui et al., 2016). Thus, it was hypothesized that NR4A1 repressed the β-catenin signaling pathway and lower the immunity of renal IRI in old mice. Therefore, we conducted IHC and Western blot analysis to assess the levels of NR4A1 and p-β-catenin in old mice. The results displayed up-regulated NR4A1 and down-regulated p-β-catenin level in the IRI and IRI + si-NC groups than in the sham group. In comparison to the IRI + si-NC group, NR4A1 expression was significantly reduced while p-β-catenin level was elevated in the IRI + si-NR4A1 group (Fig. 4A & B). The results suggested that p-β-catenin was absent in renal tissues of old mice with renal IRI, and silencing of NR4A1 seemed to promote the activation of β-catenin in renal tissues of old mice with renal IRI.

3.5. Silencing NR4A1 ameliorates renal IRI in old mice via β-catenin signaling pathway activation

To further verify whether NR4A1 affected renal IRI through its interaction with β-catenin, old mice treated with si-NR4A1 were injected with si-β-catenin. In mice treated with si-Nr4a1 + si-Ctrl, NR4A1 expression was diminished, p-β-catenin expression was elevated (Fig. 5A), renal function damage was alleviated (Fig. 5B & C), pathological injury was ameliorated (Fig. 5D) and apoptosis rate decreased (Fig. 5E) in comparison to the si-NC + si-Ctrl treatment. NR4A1 expression in mice treated with si-NR4A1 + si-β-catenin was statistically similar to si-NR4A1 + si-Ctrl, while p-β-catenin expression was reduced. The results also showed striking renal function damage, severe pathological injury and increased apoptosis rate. This demonstrated that NR4A1 silencing activated the β-catenin signaling pathway and thus protected mice against renal IRI.

4. Discussion

Renal IRI is a clinically-relevant problem with high morbidity and mortality rate amongst hospitalized patients with kidney diseases (Qiao et al., 2013). Inflammation has been identified to be the primary cause of renal IRI, and consequently, it is classified as an acute inflammatory process (Kim et al., 2013). It follows a characteristic pathophysiology initiated by a hypoxic kidney and impaired renal blood flow, thus triggering inflammation with increased cell infiltration, protease activity and cytokine levels (Wan et al., 2011). This study explored the *in vivo* effects of NR4A1 on the occurrence and progression of renal IRI in

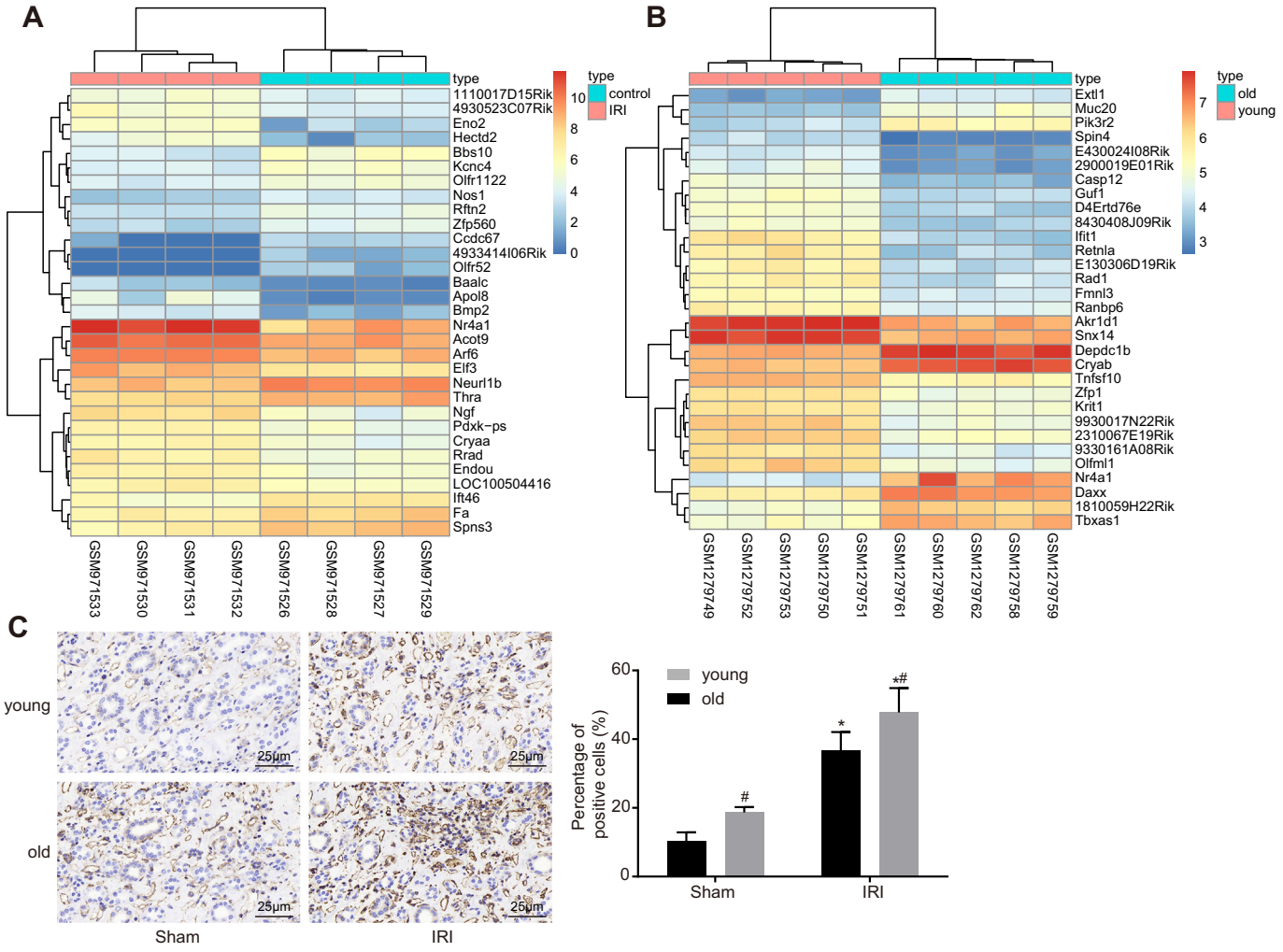

Fig. 1. Old mice with renal IRI exhibit higher expression of NR4A1. (**A**) and (**B**) Heat map of GSE39548 microarray data (4 sham operated mice kidney tissue samples *vs.* 4 renal IRI mice kidney tissue samples) and GSE52982 microarray data (Renal tissue samples from 5 young IRI mice *vs.* 5 old IRI mice) respectively. Abscissa: Sample item. Ordinate: Cluster analysis of differentially expressed genes. Each rectangular field corresponds to a gene expression value, the histogram at the upper right represents color gradation; (**C**) Positive/Higher expression of NR4A1 protein in the sham and IRI groups with old mice (× 400, scale bar = 25 μm), $n = 10$, measurement data were compared by Student's *t*-test; * $p < .05$ *vs.* the sham group; # $p < .05$ *vs.* the young group; IRI, ischemia-reperfusion; NR4A1, nuclear receptor subfamily 4, group A, member 1.

old mice *via* the regulation of β-catenin signaling pathway. The findings indicated an augmented β-catenin expression through down-regulation of NR4A1 leading to the repression of apoptotic capacity of renal cells and reduced injury.

Primarily, this study revealed that NR4A1 was highly expressed in old mice after renal IRI. NR4A1 has been previously found to be highly expressed in cardiac microvascular IRI, in which it induces serine/threonine kinase casein kinase2 α, hence leading to a fatal

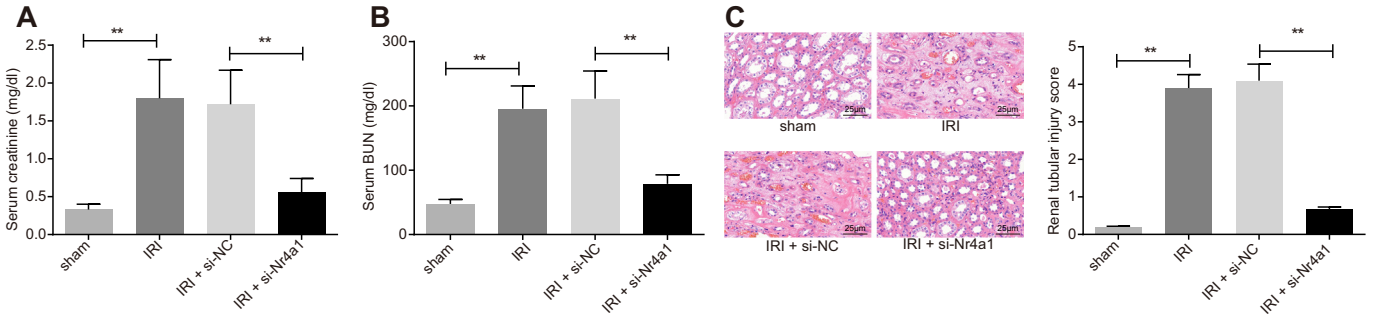

Fig. 2. Inhibition of NR4A1 improves renal function and lessens tissue damage in old mice with renal IRI. (A) Serum level of SCr of mice in the sham, IRI, IRI + si-NC and IRI + si-NR4A1 groups; (B) Serum level of BUN of mice in the sham, IRI, IRI + si-NC and IRI + si-NR4A1 groups; (C) Pathological injury score of mice in the sham, IRI, IRI + si-NC and IRI + si-NR4A1 groups (× 400, scale bar = 25 μm); n = 10, measurement data were compared by Student's *t*-test; ** $p < .01$ *vs.* the sham or IRI + si-NC group; SCr, serum creatinine; BUN, blood urea nitrogen; IRI, ischemia-reperfusion; NR4A1, nuclear receptor subfamily 4, group A, member 1.

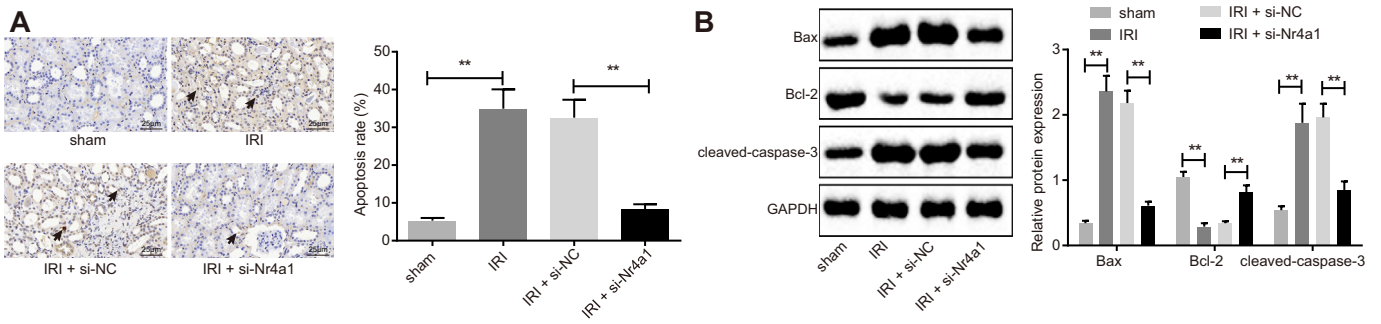

Fig. 3. Inhibition of NR4A1 suppresses the apoptosis of renal cells in old mice with renal IRI. (A) Apoptosis rate of renal cells of mice in the sham, IRI, IRI + si-NC and IRI + si-NR4A1 groups (× 400, scale bar = 25 μm, the black arrow points to apoptotic cells); (B) Protein levels of Bax, cleaved caspase-3 and Bcl-2 in the sham, IRI, IRI + si-NC and IRI + si-NR4A1 groups; n = 10, measurement data were compared by Student's *t*-test; ** $p < .01$ *vs.* the sham or IRI + si-NC group; IRI, ischemia-reperfusion; NR4A1, nuclear receptor subfamily 4, group A, member 1; TUNEL, terminal deoxyribonucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP)-digoxigenin nick end labeling; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma/leukemia-2.

mitochondrial fission (Zhou et al., 2018). Moreover, NR4A1 has been observed to significantly increase in an *in vitro* neural ischemia model (Xiao et al., 2013). It also functions as a pro-oncogene in cerebral IRI by promoting mitochondrial damage and when silenced, capable of decreasing the cerebral infarction area (Zhang and Yu, 2018). More importantly, silencing NR4A1 was found to attenuate the pathological injury in old mice with renal IRI as shown in decreased serum levels of SCr and BUN. Previously, it has also been reported that small interfering RNA targeting Fas or pro-caspase-8 decreases renal tubular injury (necrosis and apoptosis) and serum levels of SCr and BUN, thus protecting the kidneys from IRI (Du et al., 2006). Elevated BUN/SCr has also been shown to function as a contributor to the depraved renal function in patients suffering from decompensated heart failure (Brisco et al., 2013).

Furthermore, silencing NR4A1 expression suppressed cell apoptosis, indicated by the decline of Bax and cleaved caspase-3 and increased Bcl-2. The anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax are well known members of the Bcl-2 family, acting in opposite to regulate apoptosis (Khodapasand et al., 2015). Bcl-2 and Bax are found to exert paramount effects on cell apoptosis, adhesion and migration during the morphological development of kidney (Song et al., 2012). Previous data have shown that the loss of Bcl-2 results in kidney maldevelopment (Sorenson, 2004). The activation of Bax impedes survival of renal epithelial cells, and induces apoptosis as well as organelle membrane injury after metabolic stress occurring during renal ischemia (Wang et al., 2009). Besides, Park et al. have observed a remote ischemic preconditioning method for the treatment of hepatic IRI by downregulating the expression levels of Bax and cleaved caspase-3 (Park et al., 2016). In line with these findings, another study has showed that retinoic acid-mediated inhibition of NR4A1 abates apoptosis of tubular epithelial cells, and improves renal function and renal histologic injury after renal IRI as well as decreases the expression of epithelial-derived proinflammatory cytokines (Balasubramanian et al., 2012).

The Wnt/β-catenin signaling pathway exerts a pivotal action in nephrogenesis (Bridgewater et al., 2008). Renal IRI induces a clear decline of β-catenin, however Wnt/β-catenin activation by Wnt agonist can mitigate renal regeneration and function and lessens inflammation

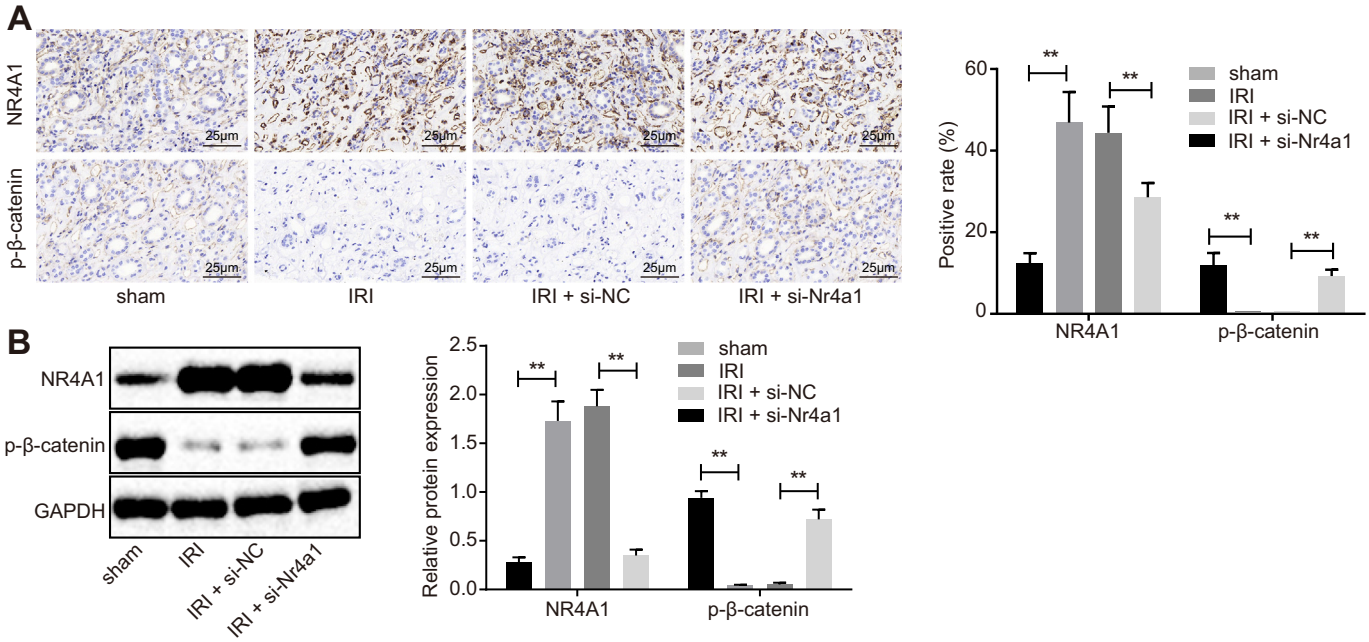

Fig. 4. NR4A1 negatively regulates β-catenin in old mice with renal IRI. (A) Positive levels of NR4A1 and p-β-catenin proteins in the sham, IRI, IRI + si-NC and IRI + si-NR4A1 groups (× 400, scale bar = 25 μm); (B) Protein levels of NR4A1 and p-β-catenin in the sham, IRI, IRI + si-NC and IRI + si-NR4A1 groups; n = 10, measurement data were compared by Student's *t*-test; ** $p < .01$ *vs.* the sham or IRI + si-NC group; IRI, ischemia-reperfusion; NR4A1, nuclear receptor subfamily 4, group A, member 1.

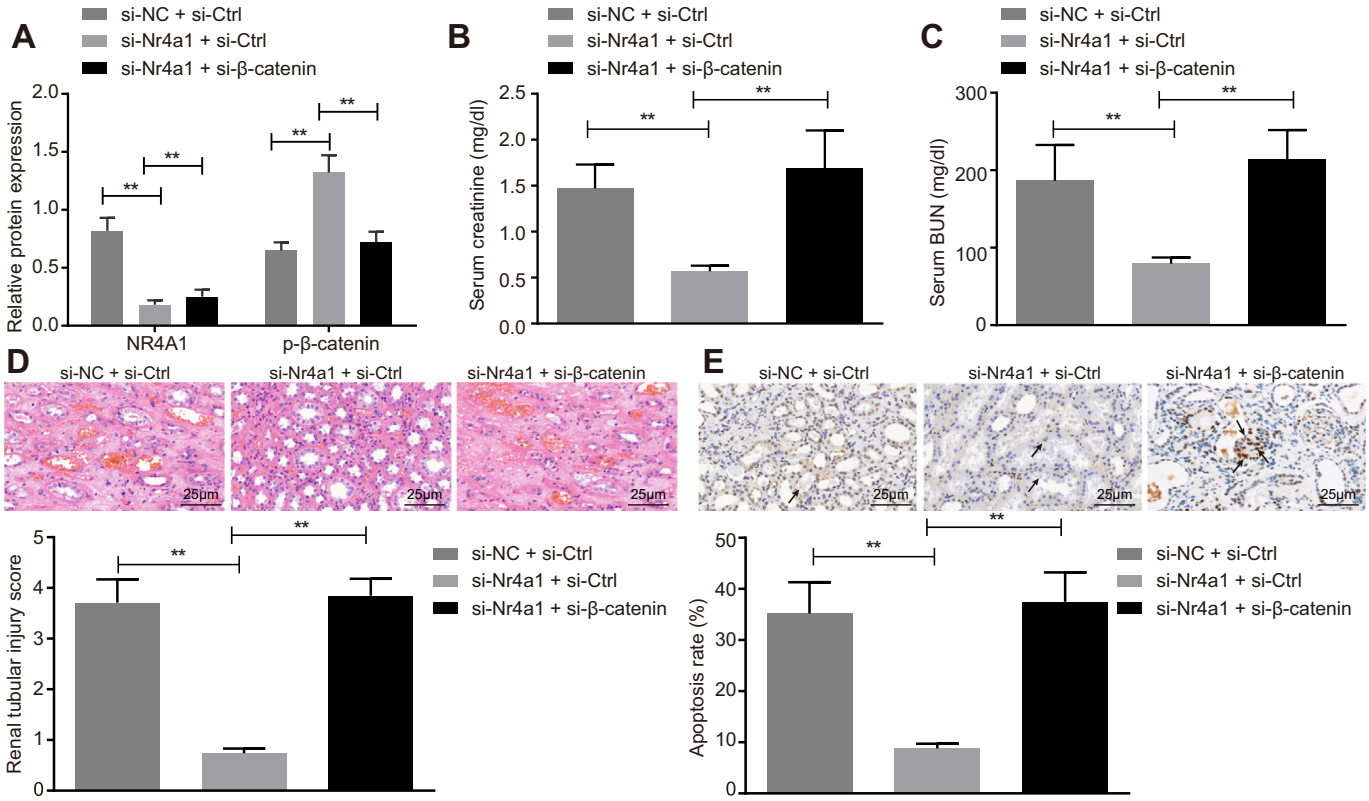

Fig. 5. Inhibition of NR4A1 promotes protection against renal IRI *via* β-catenin activation. (A) Protein levels of NR4A1 and p-β-catenin in mice treated with si-NR4A1 + si-Ctrl and si-NR4A1 + si-β-catenin; (B) Serum level of SCr in mice treated with si-NR4A1 + si-Ctrl and si-NR4A1 + si-β-catenin; (C) Serum level of BUN in mice treated with si-NR4A1 + si-Ctrl and si-NR4A1 + si-β-catenin; (D) Pathological injury score of mice treated with si-NR4A1 + si-Ctrl and si-NR4A1 + si-β-catenin (× 400, scale bar = 25 μm); (E) Apoptosis rate of renal cells of mice treated with si-NR4A1 + si-Ctrl and si-NR4A1 + si-β-catenin (× 400, scale bar = 25 μm, the black arrow points to apoptotic cells); n = 10, measurement data were compared by Student's *t*-test; ** $p < .01$ *vs.* the si-NC + si-Ctrl or si-Nr4a1 + si-Ctrl group; IRI, ischemia-reperfusion; NR4A1, nuclear receptor subfamily 4, group A, member 1; SCr, serum creatinine; BUN, blood urea nitrogen.

in the kidneys of adult male mice after IRI (Kuncewitch et al., 2015). After treatment with IRI, mice with β-catenin-knockout hepatocytes are significantly more vulnerable to liver injury, and mice with up-regulated Wnt-β-catenin signaling pathway in hepatocytes become more resistant to hepatic I/R injury (Lehwald et al., 2011). Consistent with our findings, NR4A1 may be involved in the progression of IRI in old mice *via* its interaction with the β-catenin signaling pathway. Additionally, it has been indicated that restored β-catenin expression by murine Mucin 1 protects kidney function and morphology by suppressing the levels of pro-apoptosis-related genes (p53, Bax and cleaved caspase-3) in a mouse model of IRI (Al-Bataineh et al., 2016). Furthermore, NR4A receptors, consisting of Nurr1, NR4A1 and Nor1, harbor repressive effects on β-catenin, which is crucial for the development and function of osteoblasts (Rajalin and Aarnisalo, 2011). Others have shown that NR4A1 also suppresses angiotensin II-induced vascular remodeling by inhibiting its transcriptional activity and promoting β-catenin degradation (Cui et al., 2016). Altogether, NR4A1 mediated β-catenin inhibition can accelerate the occurrence and progression of IRI. However, whether NR4A1 mediated other signaling pathways in IRI is still to be investigated.

5. Conclusion

These findings underscore the role of inhibited NR4A1 expression in renal IRI *via* its regulation of β-catenin, whereby RNA interference of NR4A1 can provoke the activation of β-catenin signaling pathway and attenuate renal function injury and suppress renal cell apoptosis. However, both young and old mice IRI model were employed in the current study so that the association with aging in IRI should be taken into account for further investigation. Besides, due to the limited funding and experiment condition, the knowledge of the mechanisms by which they acted was still lacking, highlighting more *in vitro* study to dissect the definitive molecular base. Collectively, these findings may help to develop novel therapeutic strategies to treat renal IRI by repression of NR4A1 as target.

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Declararion of Competing interest

The authors declare no conflicts of interest.

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