Multiple Mechanisms in Renal Artery Stenosis-Induced Renal Interstitial Fibrosis

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\textbf{Introduction}

Renal artery stenosis (RAS) is a prevalent and important cause of secondary hypertension. Patients with RAS often develop renovascular hypertension and ischemic nephropathy, which significantly increase the risk of cardiovascular morbidity and mortality. RAS has been demonstrated to induce renal fibrosis and atrophy, eventually leading to renal failure [1].

Our previous studies in mice that were subjected to acute renal ischemic injury suggested that peritubular capillary (PTC) loss due to ischemia is directly correlated with the development of renal fibrosis and atrophy. In RAS-induced renal fibrosis. Our findings also suggest that inflammatory macrophages and Wnt/β-catenin signaling participate in these pathological processes. Therefore, multi-target therapeutic strategies may significantly contribute to the prevention of renal interstitial fibrosis and the preservation of renal function in patients with RAS.

\textbf{Key Words}
Renal artery stenosis · Renal interstitial fibrosis · Pericyte · Wnt/β-catenin signaling · Macrophage
roles in microvessel formation, maturation, permeability, and stability [7, 8]. Despite these, we sought that the potential mechanism that underlies the development of RAS remains unclear.

Macrophages are inflammatory cells that are recruited in most kidney diseases. Recruited macrophages play critical roles in experimental and human renal disease, and these cells are implicated in the induction of injury, renal repair and fibrosis [9, 10]. Our previous studies have demonstrated that macrophages promote kidney repair via the Wnt signaling pathway and the upregulation of an autophagy protein that enhances the removal of necrotic debris [3, 11]. However, certain data have shown that macrophages are involved in renal fibrosis in a unilateral ureter obstruction (UOU) model [12]. Therefore, the precise mechanism mediated by macrophages requires further investigation in the RAS model.

Wnt/β-catenin signaling plays a role in kidney development and diseases. Dysregulated Wnt/β-catenin signaling is observed in certain types of kidney diseases, including obstructive nephropathy, and the delivery of a Wnt antagonist, the DKK1 gene, reduces β-catenin accumulation and attenuates renal interstitial fibrosis in a mouse UUO model [13, 14]. Here, we investigated the role of Wnt/β-catenin signaling in RAS-induced pathological processes.

Potential therapies for the treatment of RAS have not shown significant effects [15]. Renal dysfunction may persist despite the obvious amelioration of renal artery occlusion and kidney perfusion via the stenting procedure [16]. The failure of stenting for renal repair demonstrates that regardless of postocclusion interventions, RAS initiates the self-destruction program of kidneys in a nearly irreversible direction. Reexpansion of the renal arteries cannot terminate the self-destruction program, which strongly indicates the existence of a more complex pathological process than previously thought [17]. Our knowledge of the relevant pathological mechanisms is in its infancy, despite the positive therapeutic effects that have recently been achieved [18]. Therefore, an understanding of the pathophysiological processes of RAS will aid the development of novel targeted therapeutics for the treatment of human renovascular hypertension.

In this study, a two-kidney, one-clip Goldblatt mouse model was investigated for its suitability as experimental model of in vivo RAS-induced renal fibrosis. The potential mechanisms underlying RAS-induced renal fibrosis were explored from two perspectives, macrophage involvement and Wnt/β-catenin signaling.

Materials and Methods

Ethics Statement and Animals

Eight- to 9-week-old male C57BL/6 mice [initial body weight (BW) of 20–23 g, 2nd Affiliated Hospital of Harbin Medical University Laboratories] were used in our study. All experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The Animal Experiments Committee of Harbin Medical University approved all animal care and experimental procedures.

Animal Model

In total, 40 male C57BL/6 mice were randomly divided into four groups: a sham-operated control (sham) group and two-kidney, one-clip (2K1C)-operated groups at 7, 14, and 28 days after surgery (n = 10 each). The 2K1C Goldblatt model was induced as described previously [19]. In brief, male mice were anesthetized intraperitoneally with sodium pentobarbital (50–60 mg/kg). The left kidney was exposed through a small flank incision under aseptic conditions, and the renal artery was carefully dissected free of the renal vein. A U-shaped silver clip (0.12 mm internal diameter) was then placed around the left renal artery, resulting in the partial occlusion of renal perfusion. Sham-operated mice that underwent the same surgical procedure without placement of the silver clip served as controls.

Blood Pressure Measurement

Systolic blood pressure (SBP) was measured using a tail cuff according to the manufacturer’s instructions (Softron BP-98A, Tokyo, Japan). Measurements were obtained in each conscious mouse immediately before surgery and every week after surgery for the following 4 weeks in all groups. The mice were pre-warmed to 36°C for 15–20 min in a sack before each measurement. The average of three pressure readings was obtained for each measurement.

Blood and Tissue Samples

Renal function was assessed by measuring serum creatinine levels using the kinetic Jaffe method. The renal weight index was calculated as the dry clipped-kidney weight (CKW)/final BW (mg/g). The ratio of CKW to the nonclipped-kidney weight (NCKW) was also calculated.

Histological Studies Using Light Microscopy

Renal tissues for light microscopy were fixed in 10% neutral buffered formalin for 24 h, dehydrated, embedded in paraffin, and sectioned at 2 or 4 μm for histological staining with hematoxylin-eosin (HE) and picrosirius red stain using standard techniques. The degree of tubulointerstitial injury was determined in the HE-stained tissue sections. In particular, a semiquantitative scoring system was used to assess the tubulointerstitial injury index, as previously described [20]. In brief, injury was graded from 0 to 4: grade 0, normal; grade 1, changes affecting less than 25% of the sample; grade 2, changes affecting 25–50% of the sample; grade 3, changes affecting 50–75% of the sample, and grade 4, changes affecting >75% of the sample. Interstitial fibrosis was quantified as the Sirius red-positive area in picrosirius red-stained paraffin sections.

Morphological Studies Using Transmission Electron Microscopy

The tissues for transmission electron microscopy were cut into blocks (1 mm3) and fixed in cold 2.5% glutaraldehyde at 4°C for

Cui/Chen/Peng/Ma/Zhu/Li/Wei/Li   Nephron Exp Nephrol
DOI: 10.1159/000366481
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DOI: 10.1159/000366481

4 h. The renal tissues were then washed in 1 M phosphate buffer (pH 7.2) three times, post-fixed in 1% osmium tetroxide for 2 h, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections (80–90 nm) were double-stained with uranyl acetate and lead citrate, examined, and photographed using a Hitachi 7650 transmission electron microscope (Tokyo, Japan).

Immuno staining

The kidney tissues for immunostaining were fixed in periodate-lysine-parafomaldehyde buffer for 2 h, followed by 18% sucrose overnight, as described previously [21]. The 4-μm frozen sections were then incubated with primary antibody at 4°C overnight, washed three times for 5 min each in PBS, and incubated with secondary antibody for 1 h at room temperature. Primary antibodies against the following proteins were used for immunolabeling: anti-α-smooth muscle actin (α-SMA)-Cy3 (1:200, Sigma, USA), rat anti-mouse F4/80 (1:200; eBioscience, USA), rabbit anti-mouse platelet-derived growth factor receptor-β (PDGFRβ; 1:200; eBioscience, USA), rabbit anti-mouse α-smooth muscle actin antigen (α-SMA)-Cy3 (1:200, Sigma, USA), rat anti-mouse F4/80 (1:200; eBioscience, USA), rabbit anti-mouse β-catenin (1:200; Abcam, UK), and rabbit anti-mouse β-catenin (1:200; Abcam, UK). The secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit and 594-conjugated goat anti-rabbit antibodies (1:400; Jackson ImmunoResearch Laboratories, USA). Nuclei were stained using 4, 6-diamidino-2-phenylindole (DAPI). Specific cells in tissue sections were quantified as described previously [21]. In brief, the sections were colabeled with DAPI, and the cells were identified as positive for α-SMA, PDGFRβ, F4/80, or CD31 if more than 75% of the cell area surrounding the nucleus exhibited Cy3 fluorescence or Alexa Fluor 488 or 594 fluorescence. Specific cells were counted in 10 random cortical interstitial fields per mouse. PTC loss was evaluated using CD31-labeled kidney sections, and each image was divided into 256 squares. One square without a PTC was considered positive for loss, and the final score was presented as the percentage of positive squares.

Quantitative Real-Time PCR Analysis

Renal tissue was homogenized using TRIzol Reagent (Invitrogen, USA), cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer’s protocol. Quantitative real-time PCR was performed using methods described previously [22], and 2× SYBR Green PCR Master Mix (Applied Biosystems, USA) was used according to the manufacturer’s instructions. The sequences of the primer pairs used for real-time PCR are listed in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000366481). Experimental cycle threshold (CT) values were normalized to β-actin, and fold differences in gene expression were determined using the 2-ΔΔCT method.

Western Blotting Analysis

The preparation of kidney tissue homogenates and Western blotting analysis were carried out as previously described [23]. For detection of β-catenin, 80 μg of protein extracted from the kidney cortex was denatured with loading buffer, separated by SDS-PAGE, and transferred to a PVDF membrane. After blotting, the blocking blots were incubated with rabbit anti-mouse β-catenin (1:10,000, Abcam, UK) overnight, washed 3 times/15 min and subsequently incubated with goat anti-rabbit IgG HRP antibodies (1:10,000, Abcam, UK). All Western blot results were normalized to GAPDH.

Statistical Analysis

All data are expressed as the means ± SD. Statistical analysis was performed using GraphPad Prism 5.0. Significance was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni’s test. Only p values less than 0.05 were considered significant.

Results

Blood Pressure, Renal Function, and Renal Morphological Changes in 2K1C Mice

Unilateral RAS caused a significant elevation of SBP relative to the sham SBP (fig. 1a). The 2K1C surgery did not affect serum creatinine, as the nonclipped kidneys possessed abundant compensatory renal function (fig. 1b). However, renal morphological changes were validated. The ratio of the CKW to the NCKW decreased significantly at day 14, and this ratio dropped progressively through day 28 after 2K1C surgery indicating renal atrophy (fig. 1c). This result was confirmed by the ratio of the CKW to the BW (fig. 1d).

RAS-Induced Tubular Injury and Renal Interstitial Fibrosis Involvement in PTC Loss and Macrophage Infiltration

Significant tubular epithelial cell necrosis, inflammatory cell infiltration, and tubular atrophy were observed on day 7 after 2K1C surgery, and these conditions worsened over time (fig. 2a). These results were validated by semiquantitative histopathological analysis (fig. 2b). However, the contralateral kidneys showed minimal alterations, with no significant glomerular sclerosis, interstitial fibrosis, tubular atrophy, or interstitial inflammation (online suppl. fig. 1A–D). Following 2K1C surgery, the clipped kidneys developed interstitial fibrosis (upper panel of fig. 2c), and picrosirius red staining demonstrated that this fibrosis worsened over time (fig. 2d). Furthermore, we detected myofibroblast expression using immunofluorescence staining of α-SMA because myofibroblasts are the major cells that generate and deposit collagen I and collagen III. Upregulated expression of α-SMA was observed at day 7 after 2K1C surgery, and this expression peaked at day 28 (lower panel of fig. 2c, e). These data demonstrate that RAS induces tubular injury and renal interstitial fibrosis.

Maintenance of the microvasculature is critical for the prevention of progressive renal disease [2, 22]. CD31 was used as a marker of the endothelium to evaluate PTC density, and figure 2f shows that PTCs were lost in the clipped kidneys compared with the sham kidneys. Renal fibrosis...
was enhanced with increased PTC rarefaction. This result is expressed graphically in figure 2g.

The roles of monocytes/macrophages in the development of tissue fibrosis have been increasingly recognized. Many different inflammatory triggers converge into a single pattern of chronic inflammation, with fibrosis targeting the kidney interstitium. Ischemia due to PTC rarefaction is the core problem associated with chronic inflammation in the kidney. We investigated the role of macrophages in this process after 2K1C surgery, and we observed significant increases in macrophages in the clipped kidneys of 2K1C mice compared with sham mice (fig. 2h). Compared with the sham kidney, the contralateral kidneys did not show significant macrophage infiltration (online suppl. fig. 1E and F). In the clipped kidneys, we found that the increase in the number of F4/80+ macrophages peaked at day 14 and then decreased at day 28 after 2K1C surgery; it might be induced by significantly reduced blood supply due to serious PTC loss and lead to F4/80+ macrophage infiltration. The same result is expressed graphically in figure 2i.

Migration and Proliferation of Pericytes in RAS-Induced Renal Fibrosis

Recent evidence has shown that pericytes play vital roles in renal fibrosis in the pathological setting of the UUO model [5, 21]. However, the role of pericytes in RAS-induced renal fibrosis is not clear. Therefore, we investigated the roles of pericytes in this pathological process. We observed that the number of PDGFRβ-positive pericyte-derived myofibroblasts was significantly increased over time in the interstitium of the clipped kidneys compared to sham kidneys (fig. 3a, b). Compared with the sham kidney, the contralateral kidney did not...
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DOI: 10.1159/000366481

Sham

- 7 days
- 14 days
- 28 days

**HE**

- Sham
- 7 days
- 14 days
- 28 days

**Sirius red**

- Sham
- 7 days
- 14 days
- 28 days

**α-SMA**

- Sham
- 7 days
- 14 days
- 28 days

**CD31**

- Sham
- 7 days
- 14 days
- 28 days

**F4/80**

- Sham
- 7 days
- 14 days
- 28 days

Tubulointerstitial injury index

- Sham
- 7 days
- 14 days
- 28 days

Sirius red-positive area/HPF (%)

- Sham
- 7 days
- 14 days
- 28 days

α-SMA-positive area/HPF (%)

- Sham
- 7 days
- 14 days
- 28 days

PTC loss/HPF (%)

- Sham
- 7 days
- 14 days
- 28 days

F4/80-positive cells/HPF

- Sham
- 7 days
- 14 days
- 28 days

(For legend see next page.)
Fig. 2. RAS-induced tubular injury and renal interstitial fibrosis involve PTC loss and macrophage infiltration. 

- **a**: Representative light microscopy images of HE-stained kidney sections. Scale bar, 250 μm.
- **b**: Graph showing the semi-quantitative determination of tubulointerstitial lesions.
- **c**: Representative light microscopy images of picrosirius red-stained kidney sections (scale bar, 250 μm) and immunofluorescence images of α-SMA-stained kidney sections (scale bar, 100 μm). The area of picrosirius red-positive staining (d) or α-SMA-positive staining (e) per high-power field (HPF) was used to evaluate the fibrotic area in the renal interstitium.
- **f**: Immunofluorescence images of CD31-labeled PTCs from the sham and 2K1C groups on days 7, 14, and 28. Scale bar, 50 μm.
- **g**: The PTC loss (%) per HPF was quantitatively assessed to identify PTC rarefaction.
- **h**: Immunofluorescence images of CD31/CD34-labeled macrophages from the sham and 2K1C groups on days 7, 14, and 28. Scale bar, 100 μm. 1 Morphometric quantification of the number of F4/80-positive cells per HPF was used to evaluate the degree of macrophage infiltration. The data are the means ± SD. **p < 0.01, ***p < 0.001, 2K1C-7d, 2K1C-14d, and 2K1C-28d vs. sham.

Fig. 3. Migration and proliferation of pericytes in RAS-induced renal fibrosis. 

- **a**: Representative confocal images of PDGFRβ-labeled pericytes in the sham and 2K1C groups on days 7, 14, and 28. Scale bar, 100 μm.
- **b**: Morphometric quantification of the PDGFRβ-positive area in each HPF was used to evaluate the proliferation of pericytes in the renal interstitium. Confocal images of kidney sections colabeled with PDGFRβ and CD31 in the sham group. Scale bar, 30 μm. The thin and thick arrows represent PTC endothelium and pericytes, respectively.
- **d**: Electron microscopy images revealing the relationship between pericytes and the PTC endothelium under physiological conditions. CBM = Capillary basement membrane; EC = endothelial cell; Tu = tubule.
- **e**: Representative electron microscopy image of the clipped kidney on the 7th day after 2K1C injury. CF = Collagen fibers.
- **f**: Pathological migration of pericytes and detachment from the PTC wall in RAS-induced renal fibrosis. g = Glomeruli. Scale bar, 50 μm. The data are the mean ± SD. ***p < 0.001, 2K1C-7d, 2K1C-14d, and 2K1C-28d vs. sham.
show significant alterations (online suppl. fig. 1g, h). Pericyte-endothelial cell cross-talk is fundamentally important for kidney peritubular microvasculature stability [24]. We found that normal pericytes were adherent to capillaries (fig. 3c, d), but pericytes after RAS were detached and removed from the capillaries (fig. 3e, f). These changes were accompanied by reduced PTC density.

RAS Induced the Activation of Wnt/β-Catenin Signaling

Recent findings have indicated that Wnt protein ligands promote renal interstitial fibrosis by interacting with their receptors in the Wnt/β-catenin pathway [25]. Renal fibrosis is significantly associated with elevated β-catenin activity [22]. The relative mRNA levels of Wnt ligands were determined by quantitative real-time RT-PCR analysis, and our results demonstrated that renal expression of multiple Wnts including wnt2b, wnt4, wnt5b, wnt7b, wnt10a, and wnt10b were upregulated in the clipped kidneys compared with the sham kidneys (fig. 4a–c). As shown in figure 4d, immunostaining revealed that β-catenin expression was weak in normal kidneys. However, β-catenin expression increased after RAS induction and was predominantly localized to and increased in renal tubular epithelial cells (fig. 4d, f). Similar to the immunostaining results, Western blotting indicated that the β-catenin protein level was also upregulated in the clipped kidneys, and a substantial increase in renal β-catenin protein was observed in a time-dependent manner (fig. 4e, g). Our data suggest that increased Wnt/β-catenin signaling participates in the development of RAS-induced renal fibrosis.

Discussion

RAS is a highly prevalent chronic disease in the elderly population, which is also affected by hypertension and diabetes mellitus [26]. This study revealed that following 2K1C surgery, the clipped kidneys developed aggravated interstitial fibrosis and tubular epithelial injury over time. RAS also induced obvious PTC loss and inflammatory macrophage infiltration. Furthermore, more pericytes were found in the clipped kidneys, and these cells detached from the endothelial cells and migrated to the interstitium. Wnt/β-catenin signaling was also upregulated. Our findings suggest that multiple mechanisms are involved in RAS-induced renal interstitial fibrosis.

RAS can cause chronic and sustained injuries in the starved kidney due to the significantly reduced blood supply. Such injuries may represent a comprehensive and thorough disruption of renal functions and a self-destructive property [27]. However, researchers involved in the CORAL randomized controlled trial concluded that stent revascularization should not be a general choice for preventing renal events in patients with RAS [28]. A combination of the angiotensin II receptor blocker telmisartan and the angiotensin-converting enzyme inhibitor ramipril was verified to reduce proteinuria. However, the ONTARGET researchers cautioned that a worsening of major renal outcomes was possible [29]. Taken together, these results indicate the necessity of additional in-depth exploration of the mechanisms underlying RAS.

The kidney peritubular microvasculature has recently received increasing attention because this fragile vasculature may not regenerate normally following injury. Regeneration failure of the peritubular microvasculature may predispose kidneys to chronic ischemia and trigger chronic inflammation, tubular atrophy, and interstitial fibrosis, which are the hallmarks of chronic kidney disease. Several previous studies have demonstrated that the loss of PTCs is a characteristic feature of progressive renal diseases [30]. The clipped kidneys presented obvious PTC loss, especially in areas of high α-SMA expression. Our results suggest that PTC regression is a primary event, and PTC rarefaction may be associated with renal fibrosis in the stenotic kidneys of RAS, indicating that promoting the repair and regeneration of injured microvasculature could be a crucial target in patients with RAS. Additionally, our findings indicate that although RAS significantly increased SBP, most of the insults suffered by ischemic kidneys were not due to hypertension as no apparent morphological alterations could be detected in the contralateral kidneys. Several researchers have shown that the angiogenic factors angiopoietin-1, angiopoietin-2, and vascular endothelial growth factor A are released by pericytes, and serve as important regulators of vascular growth and integrity [21, 31, 32]. Pericytes are specifically involved in the development, maturation, stabilization, and remodeling of the vasculature [33]. Pericyte-endothelial cell cross-talk is fundamentally important for the maintenance of the kidney peritubular microvasculature [34]. Consistent with these previous findings, our study demonstrated a significant increase in the pericyte number in the clipped kidneys. Our finding also suggests that pericyte detachment and migration from capillaries might be essential for the development of microvascular rarefaction and interstitial fibrosis in the stenotic kidneys. However, the question of whether blocking pericyte-endothelial cell cross-talk preserves renal function and pre-
vents capillary rarefaction and renal fibrosis requires further investigation.

Macrophages are present in large numbers in all forms of kidney disease with inflammation. The recruitment of macrophages may contribute to kidney repair at early stages [35], but the continued presence of abnormal quantities of inflammatory macrophages and the phenotypic switch can ultimately cause renal fibrosis. Macrophages produce paracrine signaling molecules, likely signal to neighboring scar-forming cells to promote proliferation and liberate scar-forming extracellular matrix proteins [36]. The present study demonstrated a significant increase in the macrophage number in the interstitium of clipped kidneys, whereas few macrophages were observed in sham kidneys. These results suggest that macrophages are involved in the pathological processes

Fig. 4. Activated Wnt/β-catenin signaling mediates renal fibrosis in the 2K1C mouse model. a–c Quantitative real-time PCR was used to determine mRNA levels of Wnt/β-catenin pathway ligands in the whole kidney after 2K1C injury. Relative mRNA levels were determined after normalization with β-actin and expressed as fold induction over sham controls. d Representative immunofluorescence images showing β-catenin expression and localization in the kidneys in the sham and 2K1C groups at days 7, 14, and 28. Scale bar, 50 μm. f Graph showing quantification of the β-catenin fluorescence intensity. e, g Western blot analysis demonstrates a dramatic increase in renal β-catenin abundance after 2K1C surgery. Relative β-catenin levels (fold induction over sham controls) were reported after normalizing to GAPDH. Numbers (1 and 2) indicate each individual animal. The data are the mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ##p < 0.01, 2K1C-7d, 2K1C-14d, and 2K1C-28d vs. sham.
Our results also demonstrated that macrophages increase the production of Wnt ligands, which contribute to renal repair and fibrosis. Our previous study also confirmed that several Wnt ligands are activated in the RAS setting, and these ligands significantly increase the expression of β-catenin in tubules. These results suggest an involvement of the canonical Wnt/β-catenin signaling pathway in RAS. The sustained activation of Wnts may induce the upregulation of its downstream effector, β-catenin, which further promotes the process of renal interstitial fibrosis. Previous studies of chemically induced renal fibrosis have suggested additional roles for Wnt-4 in the fibrotic disease process, which is consistent with the findings of our study. Moreover, injection of recombinant sFRP4 into the kidney transiently downregulates β-catenin and fibronectin levels and reduces the number of myofibroblasts associated with the development of renal fibrosis [25]. The latter result suggests that secreted Wnt inhibitors suppress the development of an induced fibrosis response. Our previous study also demonstrated that macrophages increase the production of Wnt ligands, which contribute to renal repair and fibrosis [3]. Here, we provide the first demonstration that the Wnt/β-catenin signaling pathway is involved in RAS. Thus, selective suppression of the Wnt/β-catenin signaling pathway might be an effective therapeutic strategy in patients with RAS. However, the significance of these associations requires verification with conditional knock-out mouse models in future studies.

In summary, we successfully demonstrated the pathological evolution of fibrogenesis in an injured kidney in which multiple pathological processes, such as tubular atrophy, inflammatory macrophage infiltration, PTC rarefaction, migration of pericytes, and proliferation of pericytes, were observed. Our study is the first to validate the joint participation of macrophage accumulation and the reactivation of the Wnt/β-catenin signaling pathway in RAS.

In conclusion, our study elucidates multiple mechanisms by which occlusion of the renal artery causes renal interstitial fibrosis, and these insights may provide multiple therapeutic targets for the prevention of renal interstitial fibrosis and the preservation of renal function in patients with RAS.

Acknowledgements

This study was supported by research grants from the National Basic Research Program of China 973 (No. 2012CB517803 and No. 2012CB517602), the National Natural Science Foundation of China (No. 81070569 and 81370812), the Special Grade of China Postdoctoral Science Foundation (No. 201003463), the Research Fund for the Doctoral Program of the Ministry of Education of China, and the Heilongjiang Postdoctoral Science Research Foundation (No. LBH-Q10026).

Disclosure Statement

The authors declare that there are no conflicts of interest.

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