

Original Article

Long noncoding RNA MANTIS relieved the protein-bound uremic toxin-induced injury on human umbilical vein endothelial cells in chronic kidney disease and end-stage renal disease

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Abstract: This study aimed to explore the role of long, noncoding RNA MANTIS in regulating the protein-bound, uremic toxin-induced injury on human umbilical vein endothelial cells (HUVECs) in chronic kidney disease (CKD) and end-stage renal disease (ESRD). The MANTIS expression in patients with normal kidney function, stage 3 CKD, stage 4 CKD and ESRD was detected. In addition, HUVECs were stimulated with various concentrations of HSA-bound P-cresol (20, 40 and 80 µg/ml) and then transfected with pcDNA-MANTIS, sh-MANTIS and their controls to further investigate the effects of MANTIS overexpression and knockdown on HSA-bound P-cresol-induced HUVECs injury. Furthermore, the regulatory relationships between MANTIS and Sox18, as well as between MANTIS and p38 MAPK or p65 NF-κB pathways were elucidated. MANTIS expression was down-regulated in patients with CKD and ESRD and might be associated with disease severity. In addition, HSA-bound P-cresol induced HUVECs injury and decreased MANTIS expression. Overexpression of MANTIS relieved HSA-bound P-cresol induced HUVECs injury by increasing HUVECs viability, migration and invasion, and inhibiting cell autophagy. Moreover, the effects of MANTIS on HSA-bound P-cresol induced HUVECs injury were through positive regulation of Sox18. Besides, MANTIS overexpression markedly inhibited the activation of p38 MAPK and p65 NF-κB pathways in HSA-bound P-cresol-stimulated HUVECs, which were reversed after overexpression of MANTIS and knockdown of Sox18 synchronously. Our findings reveal that lncRNA MANTIS may relieve the protein-bound uremic toxins-induced HUVECs injury in CKD and ESRD via positive regulation of Sox18 and inhibition of p38 MAPK and p65 NF-κB pathways.

Keywords: Long noncoding RNA, chronic kidney disease, end-stage renal disease, MANTIS, Sox18, p38 MAPK signaling, p65 NF-κB signaling

Introduction

Chronic kidney disease (CKD), a debilitating chronic disease, is defined as an increased urinary albumin excretion, reduced glomerular filtration rate, or both [1]. It is estimated that the prevalence of CKD is 8-16% worldwide [1]. CKD is a common cause of global mortality and morbidity due to its significant effect on cardiovascular risk and outcomes as well as end-stage renal disease (ESRD) [2-4]. ESRD is also confirmed to be associated with high rates of morbidity and mortality, and increased health care use [5]. However, the key molecular mechanism underlying CKD is largely unknown.

Long non-coding RNAs (lncRNAs) are a class of RNAs with a length longer than 200 nucleotides and limited protein-coding capacity, which can engage in numerous biological processes [6-8]. Moreover, lncRNAs are emerging as new players in the initiation and progression of multiple pathological processes [9-11]. In kidney and cardiovascular diseases, lncRNAs have also been found to play a key role [12-14]. Several potential lncRNAs have been identified as relevant to acute and chronic renal epithelial injury through analysis of whole transcriptome profiling of human proximal tubular epithelial cells in hypoxic and inflammatory conditions [15]. However, the key lncRNAs involved in ure-

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Table 1. Summary of the patient details

	Control	Stage 3 CKD	Stage 4 CKD	ESRD
Number of patients	20	18	20	10
Sex (Male/female)	15/5	13/5	16/4	7/3
Ages (Ranges, years old)	35-75	38-77	56-76	32-80
Serum creatinine ($\mu\text{mol/L}$)	83.2 ± 13.34	123.5 ± 23.89	303.6 ± 103.46	588.5 ± 173.75
eGFR (ml/min)	75.5 ± 10.64	43.6 ± 8.35	18.2 ± 6.23	8.2 ± 3.12
Haemoglobin (g/L)	148.4 ± 9.04	133.6 ± 15.32	113.5 ± 10.3	108.8 ± 13.05
Calcium (mM/L)	2.34 ± 0.04	2.32 ± 0.06	2.25 ± 0.08	2.27 ± 0.15
Phosphate (mM/L)	1.04 ± 0.14	1.05 ± 0.16	1.32 ± 0.19	1.56 ± 0.25
Albumin (g/L)	39.4 ± 3.66	37.6 ± 4.38	36.5 ± 3.35	35.2 ± 5.05
PTH (pM/L)	N/A	5.6 ± 2.33	16.5 ± 5.53	29.2 ± 20.35

mic toxin-induced injury on human umbilical vein endothelial cells (HUVECs) in CKD and ESRD are largely unknown. Recently, MANTIS was identified as a differentially-regulated lncRNA that plays a key role in facilitating endothelial angiogenic function in tumors [16]. However, the role of MANTIS in regulating the protein-bound uremic toxins-induced HUVECs injury has not been fully investigated.

In the current study, the MANTIS expression in patients with mild to severe CKD and ESRD was detected. In addition, HUVECs were stimulated with various concentrations of HSA-bound P-cresol to induce the damage to blood vessels caused by toxins. The effects of MANTIS overexpression and knockdown on HSA-bound P-cresol induced HUVECs injury were investigated. Furthermore, the regulatory relationships between MANTIS and Sox18, as well as between MANTIS and p38 MAPK or p65 NF- κ B pathways were elucidated. The objective of this study was to explore the role of MANTIS in regulating protein-bound uremic toxins-induced HUVECs injury, to provide a new insight for the treatment of CKD and ESRD.

Materials and methods

Patient samples

Between Jan 2016 and Feb 2017, 68 patients with normal kidney function ($n = 20$), Stage 3 CKD ($n = 18$), Stage 4 CKD ($n = 20$) and end-stage renal disease (ESRD) ($n = 10$) who received hemodialysis treatment at our hospital were enrolled in this study. The clinical details of the patients are summarized in **Table 1**. Blood samples were obtained by venipuncture before hemodialysis treatment into ethylenedi-

aminetetraacetic acid-containing tubes. Serum was then obtained after centrifugation at 3000 g for 15 min at room temperature and then stored at -80°C . Using the Modification of Diet in Renal Disease formula, estimated glomerular filtration rate (eGFR) was calculated from the serum creatinine. Other routine laboratory measurements including hemoglobin, calcium, phosphate, albumin and parathyroid hormone (PTH) were determined. Ethical approval for this study was obtained from the ethics committee of our hospital and all patients gave informed written consent.

Cell culture

HUVECs were obtained from American Type Culture Collection (ATCC, Manassas, VA) and then cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco) at 37°C . At 80% confluence, HUVECs were treated with the RPMI 1640 medium supplement with 2% FBS for 24 h, and then treated with 20, 40 and 80 $\mu\text{g/ml}$ HSA-bound P-cresol [17-19]. An equivalent volume of methanol solvent was used as a control. After treating for 24, 48 and 72 h, the cells were harvested for subsequent experiments.

Transfection and generation of stably transfected cell lines

The sequence of MANTIS was ligated into the pcDNA3.1 to form the overexpression vector, which was referred to as pcDNA-MANTIS, and pcDNA3.1 was used as the negative control. Short-hairpin RNA targeting MANTIS (sh-MANTIS) was ligated into the U6/GFP/Neo plasmid (GenePharma, Shanghai, China) to construct the knockdown vector. For the analysis of the

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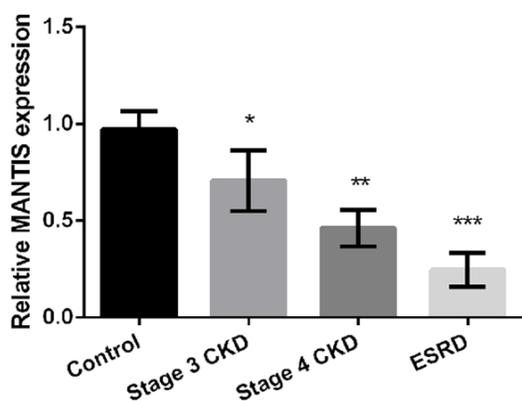


Figure 1. Down-regulation of MANTIS was observed in patients with normal kidney function, stage 3 chronic kidney disease (CKD), stage 4 CKD and end-stage renal disease (ESRD). All experiments were repeated three times and the data were presented as the means \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.01$.

ATG3 functions, small interfering RNA directed against Sox18 (si-Sox18) was also constructed in U6/GFP/Neo plasmid (GenePharma). The plasmid carrying a non-targeting sequence was used as a negative control of sh-MANTIS and si-Sox18 that was referred as to si-NC. Cells were then transfected with these vectors using the lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The harvested cells at 48 h post-transfection were used in the subsequent experiments.

Cell viability assay

Different treated HUVECs (2×10^4 cells/well) were seeded into 96-well culture plates and incubated overnight at 37°C . Subsequently, 10 μl of the CCK-8 reagent in cell-counting kit-8 (CCK-8, Dojindo, Japan) was added to each well. After 4 h of incubation at 37°C , the absorbance at 450 nm was measured using a microplate reader (DTX880, Beckman, Germany).

Migration and invasion assay

Cell migration was detected using Transwell chambers with a pore size of 8 μm (Costar, Switzerland). For the invasion assay, the chambers were pre-coated with BD Matrigel Matrix (BD Biosciences, USA). In brief, differently treated HUVECs were suspended in 200 μl of serum-free medium and then added into the upper compartment of the Transwell chamber,

and the lower compartment was added with 600 μl of complete medium as a chemoattractant. After incubation at 37°C , cells on the upper chamber were removed using cotton swabs, and traversed cells on the lower compartment were fixed with methanol, stained with crystal violet, and counted under a light microscope.

Apoptosis assay

Differently treated HUVECs (2×10^4 cells/well) were seeded in 6-well plates and cultured overnight. The cells were incubated with various concentrations of protein-bound P-cresol for 72 h and then transfected with different plasmids. At the end of the treatment, cells were collected, washed with cold PBS, and then re-suspended in a binding buffer (100 mmol HEPES, Ph 7.4, 100 mmol NaCl, and 25 mmol CaCl_2). Cells were then stained with Annexin V-FITC and PI at room temperature in darkness for 15 min using the Annexin V-FITC/PI Apoptosis Detection kit (BioVision, CA, USA). Apoptotic cells (PI and Annexin V-positive) were then analyzed using fluorescence activated cell-sorting (FACS) flow cytometry (BD Biosciences, San Jose, CA).

RNA extraction and qRT-PCR

Total RNA was extracted from the patients' serum and differently treated HUVECs using Trizol Reagent (Life Technologies) and then quantified with a SmartSpec Plus spectrophotometer (Bio-Rad). For detecting the expression of MANTIS, Sox18 and GAPDH, qRT-PCRs were conducted using a GoTaq 2-Step RT-qPCR System (Promega, Madison, WI, USA) following the protocols recommended by the manufacturer. GAPDH was used as an internal control and the relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Western blot

Differently treated cells were collected and lysed with a cell lysis buffer (Beyotime, Haimen, China) for extracting total protein. About 30 μg of protein extracts per lane were separated on 12% SDS-polyacrylamide gels, which were then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The primary antibodies to LC3BI, LC3BII, Beclin-1, Bcl-2, Bax, cleaved-Caspase-3, cle-

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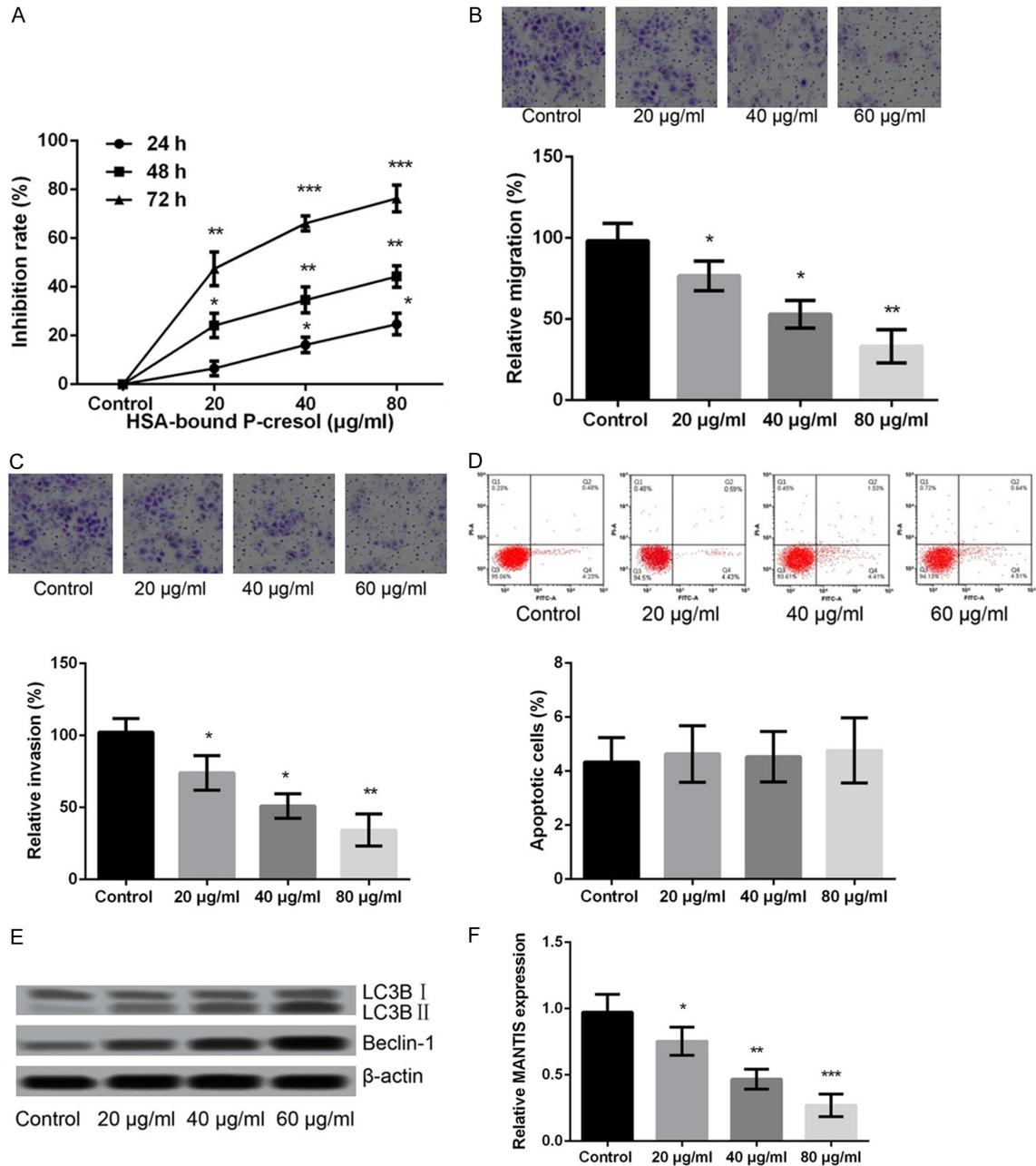


Figure 2. HSA-bound P-cresol induced HUVECs injury and decreased MANTIS expression. HUVECs were treated with 20, 40, and 80 µg/ml HSA-bound P-cresol for 24, 48 and 72 h. A: HUVECs viabilities of different treated groups. B: HUVECs migration of differently treated groups. C: HUVECs invasion of differently treated groups. D: Apoptotic cells of differently treated groups. E: The expression of autophagy-related proteins in differently treated groups. F: The expression of MANTIS in differently treated groups. All experiments were repeated three times and the data were presented as the means ± standard deviation. *P < 0.05, **P < 0.01, and ***P < 0.01.

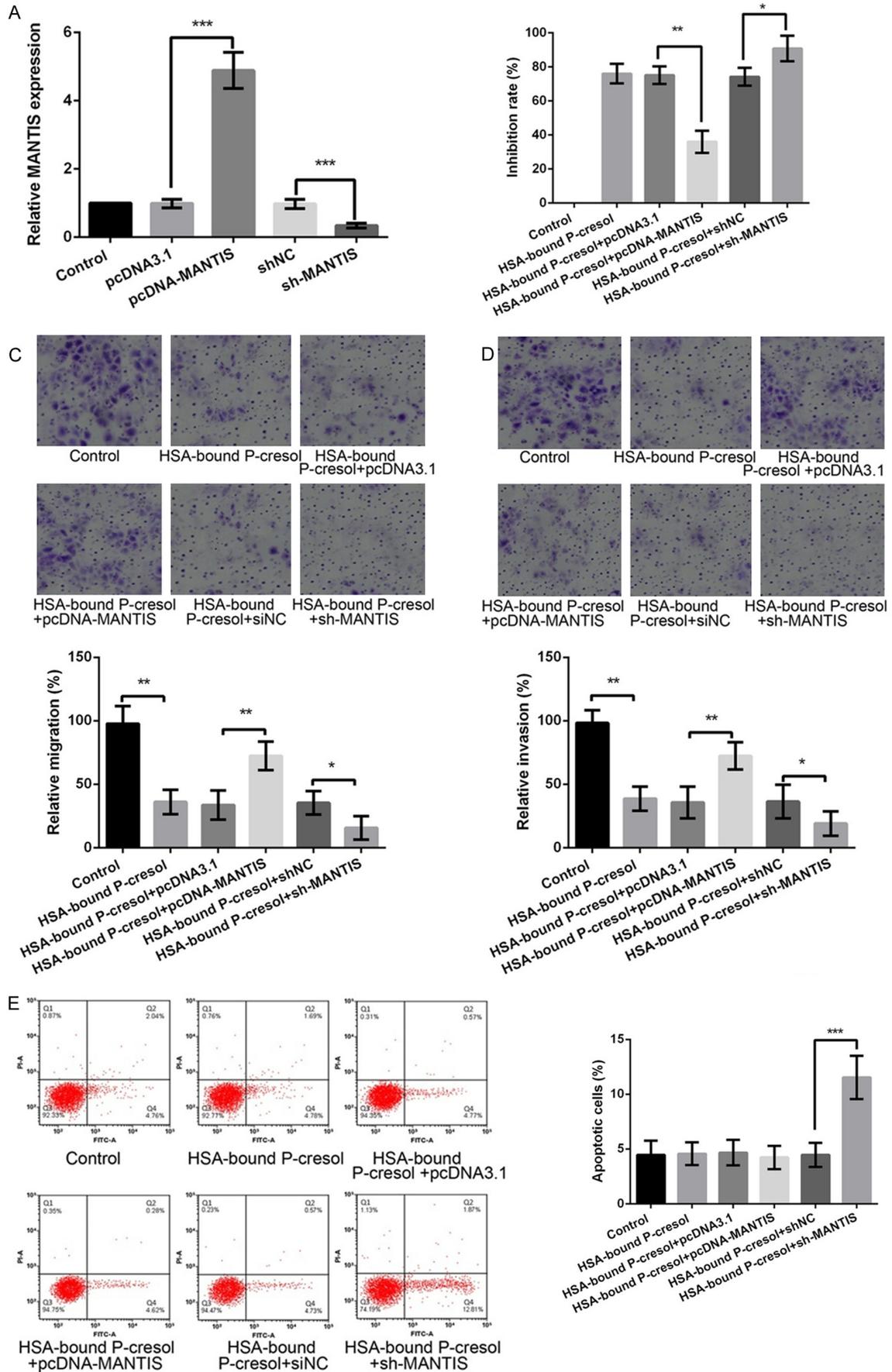
aved-Caspase-9, Sox18, p-p38 MAPK, p38 MAPK, p65 NF-κB, p-p65 NF-κB and β-actin (1:1000, Abcam, Cambridge, MA) were used for immunoblotting of proteins. β-actin was used as the control. The protein signals were revealed after incubation with the recommended secondary antibodies labeled by horseradish peroxidase (1:5000, Abcam) using an

Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).

Statistical analysis

All experiments were carried out in triplicate and the data were presented as the means ± standard deviation. SPSS Statistics 20.0 soft-

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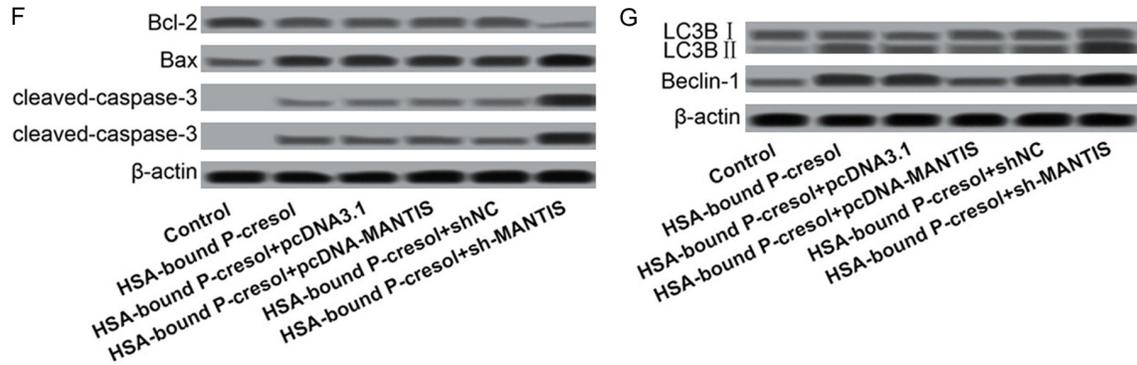


Figure 3. Overexpression of MANTIS relieved HSA-bound P-cresol induced HUVECs injury, while knockdown of MANTIS aggravated this injury. HUVECs were treated with 80 $\mu\text{g/ml}$ HSA-bound P-cresol for 72 h and then transfected with pcDNA-MANTIS, sh-MANTIS and their controls. A: The MANTIS expression in HUVECs transfected with pcDNA-MANTIS, sh-MANTIS and their controls. B: HUVECs viabilities of differently treated groups. C: HUVECs migration of differently treated groups. D: HUVECs invasion of differently treated groups. E: Apoptotic cells of differently treated groups. F: The expression of apoptosis-related proteins in differently treated groups. G: The expression of autophagy-related proteins in differently treated groups. All experiments were repeated three times and the data were presented as the means \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.01$.

ware (IBM, Armonk, NY, USA) was applied for statistical analysis. The statistical differences between the groups were analyzed using two-tailed Student's t-tests or one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Down-regulation of MANTIS was observed in patients with CKD and ESRD

To investigate whether MANTIS played a key role in CKD, the expression of MANTIS in patients with normal kidney function, stage 3 CKD, stage 4 CKD and ESRD was detected. The results showed that compared to patients with normal kidney function (control), MANTIS was significantly down-regulated in patients with stage 3 CKD, stage 4 CKD or ESRD (all $P < 0.05$, **Figure 1**). Moreover, the MANTIS expression in ESRD patients was lowest, implying that the MANTIS expression was associated with the severity of CKD (**Figure 1**).

HSA-bound P-cresol induced HUVECs injury and decreased MANTIS expression

To investigate the role of MANTIS in CKD, HUVECs were stimulated with HSA-bound P-cresol to induce the damage to blood vessels caused by toxins. The results showed that, in comparison with the controls, HSA-bound P-cresol treatment significantly decreased HUVECs viabilities after 48 and 72 h of treatment

(**Figure 2A**), and inhibited HUVECs migration (**Figure 2B**) and invasion (**Figure 2C**) after 72 h of treatment in a dose-dependent manner (all $P < 0.05$). However, HSA-bound P-cresol treatment did not exhibit any effects on HUVECs apoptosis ($P > 0.05$, **Figure 2D**). Moreover, HSA-bound P-cresol treatment resulted in the increased expression of LC3BII/LC3BI and Beclin-1 in HUVECs (**Figure 2E**), indicating that HSA-bound P-cresol promoted cell autophagy. Furthermore, relative to control, HSA-bound P-cresol markedly decreased the expression MANTIS in a dose-dependent manner (**Figure 2F**). According to these data, 80 $\mu\text{g/ml}$ of HSA-bound P-cresol for treating cells for 72 h was used for subsequent experiments.

Overexpression of MANTIS relieved HSA-bound P-cresol induced HUVECs injury

To elucidate the role of MANTIS, the effects of overexpression and suppression of MANTIS on HSA-bound P-cresol induced HUVECs injury were evaluated by assessing HUVECs viability, migration, invasion, apoptosis and autophagy. HUVECs were transfected with pcDNA-MANTIS, sh-MANTIS and their controls. The results showed that the MANTIS expression in pcDNA-MANTIS group was markedly higher than that in the pcDNA3.1 group, while the MANTIS expression in the sh-MANTIS group was markedly lower than that in sh-NC group (**Figure 3A**), suggesting that the transfection was successful. In addition, the results showed overexpression of MANTIS significantly inhibited HSA-bound

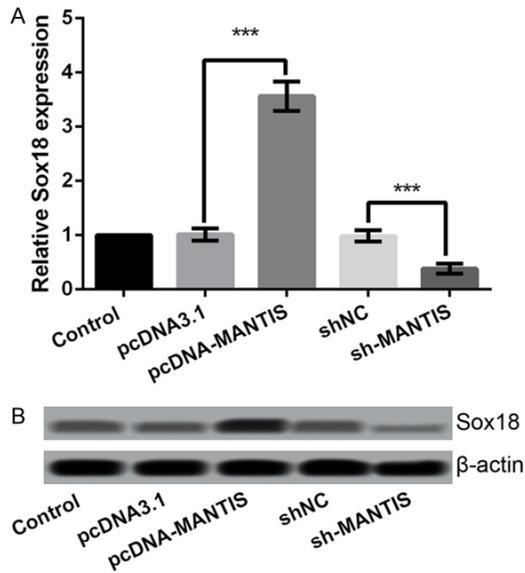


Figure 4. MANTIS positively regulated the expression Sox18. A: The Sox18 mRNA expression in HUVECs transfected with pcDNA-MANTIS, sh-MANTIS and their controls. B: The Sox18 protein expression in HUVECs transfected with pcDNA-MANTIS, sh-MANTIS and their controls. All experiments were repeated three times and the data were presented as the means \pm standard deviation. ***P < 0.01.

P-cresol-induced HUVECs viability (**Figure 3B**), migration (**Figure 3C**) and invasion (**Figure 3D**) (all P < 0.05), while the knockdown of MANTIS had opposite effects (**Figure 3B-D**). Moreover, the results of flow cytometry showed that knockdown of MANTIS markedly promoted HSA-bound P-cresol-induced HUVECs apoptosis (P < 0.05, **Figure 3E**), but overexpression of MANTIS had no obvious effects on HSA-bound P-cresol-induced HUVECs. Also, we found that the expression levels of Bax, cleaved-caspase-3 and cleaved-caspase-3 were markedly increased in HSA-bound P-cresol-induced HUVECs after knockdown of MANTIS, while the Bcl-2 expression was obviously decreased (**Figure 3F**), confirming that knockdown of MANTIS promoted HSA-bound P-cresol-induced HUVECs apoptosis. In addition, the expression of autophagy-related proteins was also investigated. The results showed that the overexpression of MANTIS significantly inhibited HUVECs autophagy via decreasing HSA-bound P-cresol-induced expression of LC3BII/LC3BI and Beclin-1 (**Figure 3G**), while the knockdown of MANTIS markedly promoted HSA-bound P-cresol-induced HUVECs autophagy via the regulation of the expression of these

autophagy-related proteins (**Figure 3G**). These data indicated that overexpression of MANTIS relieved HSA-bound P-cresol induced HUVECs injury by promoting HUVECs viability, migration and invasion and inhibiting autophagy.

MANTIS positively regulated the expression Sox18

A previous study showed that Sox18 plays a key role in promoting the vascularization of HUVECs *in vitro* [20], so we also investigated the relationship between MANTIS and Sox18. The results showed that the expression levels of the Sox18 mRNA (**Figure 4A**) protein (**Figure 4B**) in pcDNA-MANTIS group was markedly higher than those in the pcDNA3.1 group, while the MANTIS expression in the sh-MANTIS group was markedly lower than that of the sh-NC group (all P < 0.001). These data indicated that MANTIS positively regulated the expression of Sox18.

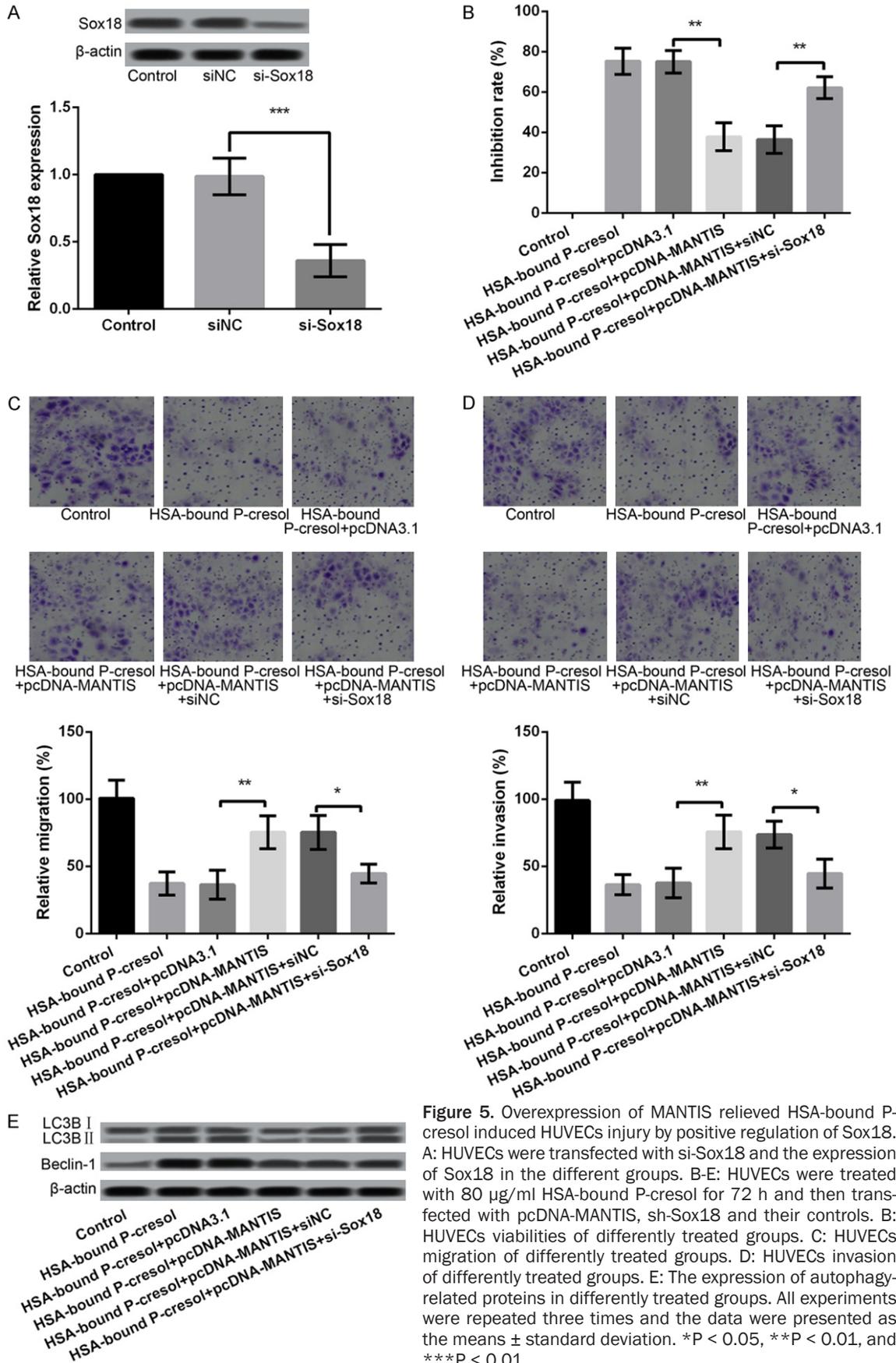
Overexpression of MANTIS relieved HSA-bound P-cresol induced HUVECs injury by positive regulation of Sox18

To further investigate whether MANTIS regulated HSA-bound, P-cresol-induced HUVECs injury by regulation of Sox18, we knocked down the expression of Sox18 by transfection with si-Sox18. The results showed that the Sox18 expression was markedly decreased in the si-Sox18 group compared with that of the siNC group (P < 0.001, **Figure 5A**). Moreover, HSA-bound P-cresol-stimulated HUVECs were co-transfected with pcDNA-MANTIS and si-Sox18. The results showed that knockdown of Sox18 significantly reversed the effects of the overexpression of MANTIS on HSA-bound P-cresol-induced HUVECs viability (P < 0.01, **Figure 5B**), migration (P < 0.05, **Figure 5C**), invasion (P < 0.05, **Figure 5D**) and autophagy (**Figure 5E**) after overexpression of MANTIS and knockdown of Sox18 synchronously. These data indicated that the overexpression of MANTIS relieved HSA-bound P-cresol induced HUVECs injury by positive regulation of Sox18.

Effects of MANTIS on HUVECs were by inhibition of p38 MAPK and p65 NF-κB pathways

The possible signaling pathway involved in MANTIS-mediated HSA-bound P-cresol induced HUVECs injury was explored. As shown in **Figure**

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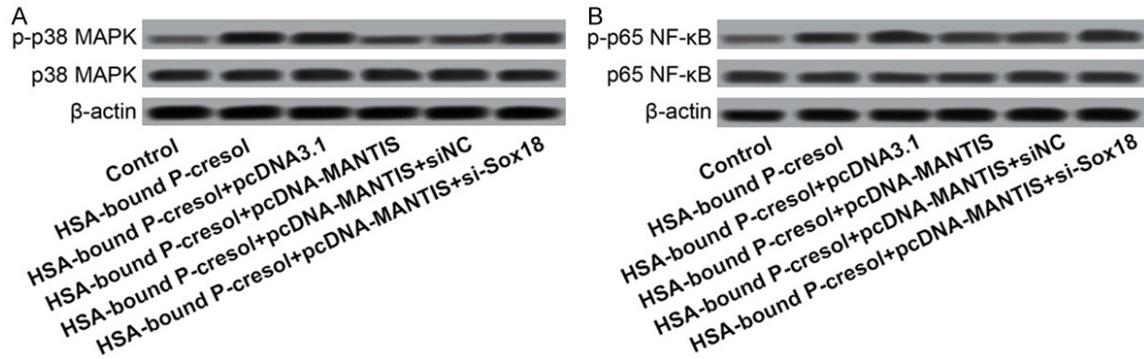


Figure 6. Effects of MANTIS on HUVECs were by inhibition of p38 MAPK and p65 NF-κB pathways. HUVECs were treated with 80 μg/ml HSA-bound P-cresol for 72 h and then transfected with pcDNA-MANTIS, sh-Sox18 and their controls. A: The expression of p38 MAPK and p-p38 MAPK. B: The expression of p65 NF-κB and p-p65 NF-κB.

6, HSA-bound P-cresol markedly increased the expression of p-p38 MAPK and p-p65 NF-κB, which were obviously reversed by overexpression of MANTIS. Moreover, the effects of the MANTIS overexpression on the expression of p-p38 MAPK and p-p65 NF-κB in HSA-bound P-cresol-stimulated HUVECs were further reversed after the overexpression of MANTIS and the knockdown of Sox18 synchronously. These data indicated that effects of MANTIS on HUVECs occurred through the inhibition of the p38 MAPK and p65 NF-κB pathways.

Discussion

In this study, the potential effect of MANTIS on protein-bound uremic toxins-induced HUVECs injury was studied. The results showed that HSA-bound P-cresol induced HUVECs injury and decreased the MANTIS expression. Overexpression of MANTIS relieved the HSA-bound P-cresol induced HUVECs injury by increasing cell viability, migration and invasion, and by inhibiting cell autophagy. Moreover, the effects of MANTIS on HSA-bound P-cresol-induced HUVECs injury were through positive regulation of Sox18. Besides, MANTIS overexpression markedly inhibited the activation of p38 MAPK and p65 NF-κB pathways in HSA-bound P-cresol-stimulated HUVECs, which were reversed after overexpression of MANTIS and knockdown of Sox18 synchronously. Importantly, MANTIS expression was down-regulated in patients with CKD and ESRD and might be associated with disease severity. These data suggest that MANTIS may play a key role in uremic toxin-induced HUVECs injury in the development of CKD and ESRD.

A previous study found that MANTIS facilitates endothelial angiogenic function in tumors possibly by regulating the transcription of key endothelial genes, including Sox18 [16]. Consistent with the findings, we also found that MANTIS positively regulated the expression of Sox18. Sox18 is a key endothelial gene important for angiogenesis [21]. Hoeth et al. demonstrated that the overexpression of Sox18 in endothelial cells played a crucial role in the morphogenesis of the vasculature [22]. Sox18 has also been shown to induce adipose-derived stromal cells to exhibit several endothelial-like features involved in vascular patterning [23]. Young et al. reported that it plays a key role in promoting the vascularization of HUVECs [20]. In this study, the overexpression of MANTIS relieved HSA-bound P-cresol induced HUVECs injury, and knockdown of Sox18 significantly reversed the effects of the overexpression of MANTIS on HSA-bound P-cresol-induced HUVECs injury after the overexpression of MANTIS and the knockdown of Sox18 synchronously. Given the key role of Sox18 in endothelial cells, we speculate that MANTIS may mediate the uremic toxin-induced HUVECs injury through positive regulation of Sox18.

Furthermore, the regulatory relationships between MANTIS and possible signaling pathways, such as p38 MAPK or p65 NF-κB pathways were explored to further elucidate the possible mechanism of MANTIS. In CKD, the P38 MAPK pathway is activated after unilateral ureteral obstruction, and blockade of this pathway reduces the accumulation of the interstitial extracellular matrix and subsequent inflammation [24]. Moreover, NF-κB is also found acti-

vated in renal fibrosis and contributes to kidney inflammation by up-regulating various cytokines and chemokines [25, 26]. In addition, it is reported that hydrogen sulfide could attenuate chronic renal failure in rats by involving the ROS/MAPK and NF- κ B signaling pathways [27]; baicalein protects against the inflammatory response to ameliorate renal fibrosis via down-regulating MAPK and NF- κ B pathways [28]; Astragaloside IV, a component of the traditional Chinese medicinal plant *Astragalus membranaceus*, alleviates renal interstitial fibrosis via the inhibition of the MAPK and NF- κ B signaling pathways [29]; and ulinastatin can exert cardioprotective effects against isoproterenol-induced chronic heart failure via regulating p38 MAPK and NF- κ B pathways [30]. In this study, MANTIS overexpression markedly inhibited the activation of p38 MAPK and p65 NF- κ B pathways in HSA-bound P-cresol-stimulated HUVECs. These data reveal the possible role of p38 MAPK and NF- κ B pathways in mediating protein-bound uremic toxins-induced HUVECs injury and support the therapeutic potential of targeting MANTIS for the treatment of CKD.

Taken together, our findings reveal that lncRNA MANTIS may relieve the protein-bound uremic toxin-induced HUVECs injury in CKD and ESRD via the positive regulation of Sox18 and the inhibition of p38 MAPK and p65 NF- κ B pathways. Targeting MANTIS may provide a new perspective for the treatment of CKD and ESRD.

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Disclosure of conflict of interest

None.

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