

MicroRNA-29b suppresses TGF- β -induced epithelial-mesenchymal transition in renal interstitium of spontaneously hypertensive rats

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To the Editor: Hypertension is a well-known cardiovascular disease in which the kidney is an important target due to its role in the regulation of fluid, electrolyte balance, and blood pressure.^[1] Glomerulosclerosis, renal tubule interstitial fibrosis, and proteinuria are the main pathological characteristics of hypertensive kidney injury.^[2]

Multiple studies have indicated that microRNAs (miRNAs, miRs) participate in the progression of chronic kidney disease; such disease is caused by heterogeneous triggers and is characterized by progressive loss of kidney function.^[3] miR-29 has been identified as a potent renoprotective regulator. Evidence from our laboratory revealed that miR-29b protected renal tubular epithelial cells against angiotensin II-induced epithelial-mesenchymal transition (EMT).^[4] However, the effects of miR-29b in animal models of hypertension-induced nephropathy remain unknown.

We therefore used spontaneously hypertensive rats (SHRs), a well-known cardiovascular disease model characterized by severe hypertension and renal dysfunction, to investigate whether renal injury is determined by miR-29b function. We hypothesize that chronic hypertension will interfere with pathologic remodeling of the kidney and cause renal damage and that miR-29b will favor the alleviation of renal dysfunction and interstitial fibrosis. To investigate the role of miR-29b during the progression of hypertensive renal insufficiency, rats were randomly assigned to five groups of five animals each: (1) normotensive Wistar-Kyoto (WKY) rats treated with physiological saline (WKY Group); (2) SHR rats treated with physiological saline (SHR Group); (3) SHR rats treated with nonspecific negative control lentiviral vectors (SHR+C Group); (4) SHR rats treated with high-expression-miR-29b lentiviral vectors (SHR+M Group); and (5)

SHR rats treated with miR-29b-inhibiting lentiviral vectors (SHR+I Group).

To overexpress or downregulate miR-29b, we obtained lentiviruses expressing a mature miR-29b mimic (lv-miR-29b) or a miR-29b inhibitor (lv-miR-29b-In). We also acquired a nonspecific lentivirus (lv-miRC) for use as a nonspecific negative control. The upregulation and inhibition of miR-29b was caused by administering these reagents via intrarenal parenchymal injection. Four weeks postinjection, rats were euthanized by cervical dislocation. The left kidney was dissected into four parts. One part was used for polymerase chain reaction (PCR) analysis, and two parts were immersed for immunohistochemical assay and Sirius red staining. The final part was snap-frozen in liquid nitrogen for Western blot analysis. To explore miR-29b-induced changes in renal function, blood samples were collected for detection of blood urea nitrogen (BUN) and serum creatinine (SCr) before and after viral transfection. Kidney samples were homogenized in ice-cold lysis buffer containing protease inhibitor cocktail.

Fifteen-week-old male normotensive WKY rats and SHRs were obtained for this study. Body weight and tail-cuff systolic blood pressure were measured at 1-week intervals. SCr and BUN were higher in SHRs than in WKYs, and both showed a statistically significant increase in SHRs between baseline and the 4-week time point. Consistent with the increased concentrations of serum SCr and BUN, SHRs showed markedly elevated urinary protein content compared to WKY rats. Once again, SHRs further exhibited a statistically significant increase within a 4-week time frame. These findings suggest the development of hypertensive renal injury. Four weeks after transgene delivery, SHRs treated with lv-miR-29b showed attenuated levels of SCr and BUN compared to the rats treated

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with lv-miRC [Supplementary Figure 1A and B, <http://links.lww.com/CM9/A870>]. Moreover, miR-29b overexpression in SHR (SHR+M) lowered the urinary protein content by 6.75% compared with the urinary protein content in the SHR which treated with nonspecific negative control lentiviral vectors (SHR+C). Urinary protein levels were remarkably elevated in compliance with increased SCr and BUN in the SHR+I group compared with the SHR+C group [Supplementary Figure 1C, <http://links.lww.com/CM9/A870>]. These results suggest that overexpression of miR-29b inhibits hypertension-induced renal injury and that knockdown of miR-29b enhances renal injury. For all three renal function measurements at 4 weeks, the measured values in the WKY group were significantly lower than the measured values in the SHR+C and SHR+I groups but were not significantly different from the values in the SHR+M group. Furthermore, the measured values in the SHR group were not significantly different from the measured values in the SHR+C group but were significantly higher than the measured values in the SHR+M group and significantly lower than the measured values in the SHR+I group.

Next, we investigated the effect of miR-29b levels on extracellular matrix (ECM) accumulation by measuring the level of collagen I in the kidneys. As shown in Supplementary Figure 2, <http://links.lww.com/CM9/B50>, the mRNA and protein expression levels of the ECM component collagen I were dramatically decreased in rats following lv-miR-29b administration compared to controls, while its expression was significantly increased when treated with the inhibitor lv-miR-29b-In [Supplementary Figure 2A-C; <http://links.lww.com/CM9/B50>]. To further confirm the expression of collagen I in the kidneys, we used rat renal tissue for immunohistochemical staining. As expected, SHRs exhibited more collagen I deposition in the renal interstitial structure [Supplementary Figure 2D and E; <http://links.lww.com/CM9/B50>], consistent with PCR and Western blot data. In addition, the positive areas of collagen I on the kidney sections were significantly decreased with lv-miR-29b administration compared to control virus administration [Supplementary Figure 2F and G; <http://links.lww.com/CM9/B50>]. In contrast, lv-miR-29b-In treatment caused a statistically significant increase in collagen I-stained areas relative to controls [Supplementary Figure 2H; <http://links.lww.com/CM9/B50>].

Moreover, Sirius red staining was performed for morphometric evaluation of ECM deposition [Supplementary Figure 2J-N; <http://links.lww.com/CM9/B50>]. As shown, kidneys with lv-miR-29b administration displayed only 3.29% renal fibrosis (interstitial staining), whereas kidneys with lv-miR-29b-In treatment showed more severe interstitial staining than the controls [Supplementary Figure 2O; <http://links.lww.com/CM9/B50>]. These results demonstrate that miR-29b upregulation efficiently protects against the development of chronic hypertensive interstitial fibrosis 4 weeks following lentivector transfection.

To investigate the effects of miR-29b on EMT, a critical injury mechanism in chronic fibrosis, we measured the expression of α -smooth muscle actin (α -SMA, a

myofibroblast marker) in kidneys 4 weeks after transfection. Of note, lv-miR-29b treatment resulted in a reduction in α -SMA synthesis to less than baseline levels, both at the gene and protein levels [Supplementary Figure 3A-C, <http://links.lww.com/CM9/A870>]. In contrast, the mRNA and protein levels of α -SMA were markedly increased following lv-miR-29b-In administration [Supplementary Figure 3A-C, <http://links.lww.com/CM9/A870>].

Similar results were obtained through immunohistochemical staining. More α -SMA-positive tissue staining was detected in SHRs than in WKYs [Supplementary Figure 3D and E, <http://links.lww.com/CM9/A870>], suggesting that the development of hypertension induced α -SMA expression. In the SHR+M group, positive staining for α -SMA was significantly reduced compared to positive staining for α -SMA in the SHR+C group [Supplementary Figure 3F and G, <http://links.lww.com/CM9/A870>]. Meanwhile, there was significant α -SMA-positive tissue staining in the SHR+I group 4 weeks posttransfection; positive areas were localized around the renal tubulointerstitium [Supplementary Figure 3H, <http://links.lww.com/CM9/A870>]. Taken together, these observations revealed that overexpression of miR-29b strongly suppresses the α -SMA myofibroblast marker, which suggests inhibited development of EMT.

Transforming growth factor- β (TGF- β) is a critical molecule in the process of renal fibrosis. Therefore, in the present study, the mechanisms underlying hypertensive renal fibrosis were assessed by measuring alterations in TGF- β expression. First, we investigated the mRNA levels of TGF- β with lv-miR-29b gene delivery. Our data showed suppressed mRNA levels of TGF- β compared to negative controls [Supplementary Figure 4A, <http://links.lww.com/CM9/A870>]. To further confirm our results, Western blot analysis was performed. As shown in Supplementary Figure 4B, <http://links.lww.com/CM9/A870>, we observed decreased protein levels of TGF- β in lv-miR-29b-treated rats, with levels reduced to 1/3 of the controls [Supplementary Figure 4C, <http://links.lww.com/CM9/A870>]. Consistent with earlier analysis, immunostaining revealed low expression of TGF- β in rats treated with lv-miR-29b [Supplementary Figure 4G, <http://links.lww.com/CM9/A870>]. In contrast, miR-29b knockdown resulted in remarkably increased mRNA and protein levels of TGF- β in SHRs compared to the controls. Meanwhile, immunohistochemical analysis identified strong staining in kidney sections from lv-miR-29b-In-treated animals [Supplementary Figure 4H, <http://links.lww.com/CM9/A870>]. Taken together, this negative association between miR-29b and mRNA/protein expression of TGF- β suggests a strong link between miR-29b and TGF- β .

The present study yields several important findings about the effects of miR-29b on renal protection against hypertension. First, miR-29b ameliorates hypertensive renal dysfunction by reducing serum levels of creatinine and urea nitrogen in parallel to decreased proteinuria. Second, miR-29b attenuates ECM accumulation and suppresses the development of EMT, as indicated by decreased expression of collagen I and α -SMA, respectively. Finally, we proved the inhibitory effects of miR-29b

overexpression on TGF- β activation by demonstrating significantly reduced gene and protein expression of TGF- β . Considering the pivotal role that TGF- β plays in fibrotic processes,^[5] we hypothesized that miR-29b may exert its protective effects by modulating TGF- β . To further verify our supposition, TGF- β -related ECM accumulation and EMT were measured during the subsequent procedures. Peritubular capillaries become atrophic when compressed by collagen, and the distance between tubular cells and capillaries increases, thereby worsening the damage and favoring the development of renal failure.^[2] In the present study, SHR rats exhibited more collagen deposition than normotensive WKY rats, indicating more severe ECM accumulation under hypertensive conditions. Moreover, overexpression of miR-29b reduced the expression of collagen I, whereas downregulation of miR-29b with inhibitors induced the expression of collagens *in vivo*. Complementary to the present study, our previous research demonstrated that miR-29b modulated collagen expression in renal tubular epithelial cells *in vitro*.^[4] Moreover, Qin *et al*^[6] reported that increased expression of miR-29b could block progressive renal fibrosis, while downregulation of miR-29b was associated with a significant upregulation of collagen I in wild-type mice. In addition to the major effect on ECM proteins, miR-29b additionally inhibits EMT. Moreover, we demonstrated an inverse correlation between the miR-29b level and the myofibroblast marker α -SMA. Collectively, these results suggest that miR-29b plays a protective role by suppressing hypertension-induced EMT in SHR rats.

TGF- β is crucial in fibrotic diseases. In our study, downregulation of TGF- β was observed via overexpression of miR-29b in SHR rats. In contrast, knockdown of miR-29b was associated with increased TGF- β content in the kidney. Interestingly, studies in human trabecular meshwork cells revealed that miR-29b suppresses the expression of TGF- β at the promoter, transcript, and protein levels.^[7] Our results add evidence to the negative regulatory effects of miR-29b on the expression of TGF- β , suggesting some level of crosstalk between TGF- β and miR-29b.

Taken together, we found that miR-29b may serve as a novel antifibrotic factor by reducing ECM accumulation

and inhibiting EMT via downregulation of TGF- β . Elevated expression of miR-29b could have beneficial effects against hypertensive kidney injury and prevent pathogenic renal fibrosis. Overexpressing miR-29b could be a promising therapeutic strategy for the treatment of hypertension-induced nephropathy.

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Conflicts of interest

None.

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