rAAV9-mediated supplementation of miR-29b improve Angiotensin-II induced Renal Fibrosis in mice

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Short report

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Abstract

Background Renin-angiotensin-aldosterone system activation is the critical factor in renal remodeling and dysfunction. Our previous study suggested that miR-29b might attenuate angiotensin II-induced renal interstitial fibrosis in vitro. In the present study, we aim to determine that recombinant adeno-associated virus mediated miR-29b delivery can protect AngII-induced renal fibrosis and dysfunction.

Method Mice were treated with AngII via osmotic mini-pumps or PBS. Recombinant adeno-associated virus serotype 9 vectors were produced with the rBac-based system in SF9 cells. rAAV9-miR-29b or rAAV9-control-miR were injected into the kidney of a mouse model of AngII infusion. Role of miR-29b in renal fibrosis were assessed by Q-PCR, Western blot and histological examination.

Results In AngII-induced fibrotic kidney, miR-29b expression was down-regulated. rAAV9-miR-29b delivery significantly reversed renal dysfunction as indicated by decreased blood serum creatinine in AngII-infused mice. As far to organ remodeling, tubulointerstitial fibrosis and deposition of extracellular matrix deposition such as Collagen type I and type III were significantly decreased in kidney tissue of rAAV9-miR-29b delivered mouse.

Conclusion Our results show the great potential of rAAV9 as an applicable vector for miR-29b delivery for antifibrogenic factors in the treatment of renal dysfunction caused by tubulointerstitial fibrosis.

Background

Renal interstitial fibrosis is one of main characters of many chronic kidney disorders, contributes to end-stage function failure. Multiple extracellular matrix (ECM) components, particularly collagen type I and III, have crucial role in interstitial fibrosis (Zeisberg and Neilson, 2010). Collagen expression often correlates with interstitial fibrosis severity. Therefore, inappropriate collagen accumulation is an important therapeutic target for kidney fibrosis. Experiment evidences showed that tubular epithelial cells gradually lose their epithelial characteristics before fibrosis, which leads to epithelial mesenchymal transition (EMT) [1]. Activation of renin-angiotensin II-aldosterone system (RAAS) signaling, the classic risk factor of information and remodeling, is a key mechanism involving kidney remodeling (Almeida et al. 2020). Therefore, in addition to the essential etiology control, anti-fibrosis have been considered to be a therapeutic approach to protect kidney function. Because of limitations of RAAS blockers in the treatment of chronic kidney disorders such as side effects of renal impairment and aldosterone escape, the prevention of renal fibrosis is far to satisfactory.

MicroRNAs are described to regulate gene expression by mRNA degradation or by translation inhibition. Emerging evidences demonstrate microRNAs (miRs) play a pathological role in kidney diseases. Lots of studies demonstrated that microRNAs either affected by RAAS inhibitors or RAAS effectors (DuPont et al. 2016). So miRs might be novel therapeutic target for RAAS overactivation induced kidney remodeling.

In our previous studies, we observed that miR-29b was downregulated in the kidney of spontaneously hypertensive rats and AngII-instigated NRK52E cells, that suggested it might protect fibrosis by suppressing the deposition of ECM and preventing EMT (Pan et al. 2016). There's no miR target as much number of collagens and ECM related genes as miR-29b does (Liu et al., 2016). Bretschneider et al. (Bretschneider et al. 2016) reported that activated mineralocorticoid receptor (MR) reduced miR-29b abundance in vascular smooth muscle cells. MR is a risk factor for tissue fibrosis. And MR antagonist was limited for preventing renal fibrosis because of clinical side effects. Based on these findings, we hypothesized that *miR-29b* may be a novel therapy target of renal interstitial fibrosis by inhibiting ECM deposition. To test this hypothesis, we used baculovirus-insect cell expression system to deliver synthetic pre-miR-29b to the kidneys. In a mouse model of renal interstitial fibrosis induced by AngII infusion, we observed the effects of miR-29b overexpression on ameliorating renal interstitial fibrosis and dysfunction.

Methods

Generation of recombinant adeno-associated virus serotype 9 vector

Recombinant adeno-associated virus serotype 9 (rAAV9) vectors were produced with the rBac-based system in SF9 cells as described previously (Chen, 2012). rAAV9 vectors were packaged with single-stranded DNA containing enhanced green fluorescent protein (GFP) gene, which was driven by the mice cytomegalovirus (CMV) and U6 promoter (AAV9-CMV-U6-GFP). Vector titers were determined by quantitative polymerase chain reaction (qPCR) on vector genomes.

In vivo gene transduction by rAAV9 vectors

All animal experiments were performed according to the guide of Institutional Animal Care and Use Committee of Zhejiang University. C57/BL6J wild type mice were purchased from the Shanghai institute of biological science and used for study. 8-week-old male mice were divided into four groups (n = 6 per group). After mouse being fully anesthetized by injection of 50mg/g of 0.8% pentobarbital, the left kidney was exposed and injected in five sites with rAAV9, 1×10^{10} IVP/site. Kidneys were injected with vehicle, rAAV9-random miR, or rAAV9-miR-29b. Additional animals underwent the same procedure with injection of PBS serve as age-matched controls.

Identification of in vivo transduction efficiency

GFP expression in mouse kidney determined by both western blot and fluorescent analysis. Once the kidney tissues were harvested, there were immediately prepared for fresh frozen sections at 5µm thickness. GFP expression was tested using fluorescent microscopy (Germany, Leica). At least three images of 200× magnification were captured from the each section. The transduction efficiency of rAAV9 vectors was assessed by GFP expression, which was assessed by both fluorescence signal intensity and western blot. Image J 32 software package was used for analysis.

Western blot

Equal content of proteins were loaded on and separated by 10% and 8% SDS-PAGE, The protein extracted after rAAV9 transduction kidney tissue was loaded on and separated by 12% SDS-PAGE gel electrophoresis. The protein bands were transferred to PVDF membranes (Bio-Rad, 162–0255). After blocking, membranes were incubated at 4°C for at least 16h with primary antibodies against anti-GFP antibody and HRP-labeled anti-GAPDH antibody (1:5000, Cell Signalling Technology). Membranes were washed in TBST for three times every 5 minutes. Membranes were then incubated at room temperature for 2h with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2500, Cell Signaling Technology). The relative protein content was analyzed by Image J software and normalized to GAPDH protein levels in the same sample.

A mouse model of AngII-induced renal fibrosis

The mouse of three groups were treated with continuous AngII (1.44mg/kg/day) infusion for 28 days by using an Alzet micro-osmotic pump (Model 2004; Durect Co., Cupertino, CA). One group received continuous PBS infusion. After 28 days infusion, blood was collected from orbital cavity. Kidney samples were harvested and immersed in RNA preservation solution or liquid nitrogen. The Level of serum creatinine was measured with an automated biochemical analyzer (Beckman coulter Au5800, Tokyo, Japan) and Accuras Auto CRE kit (Shino-Test Corporation, Tokyo, Japan). Levels of urea nitrogen were measured by using Urease ultraviolet rate kit(Beckman,USA).

Histology and Immunohistochemistry

To evaluate renal remodeling such as the degree of tubular injury and fibrosis. Samples were mounted on normal glass slides and stained with both Masson trichrome stain and Sirius red stain for collagen deposition examination. For the collagen volume fraction (CVF) analysis in the tubular basement membrane and interstitial space, the percentage of cortex fibrosis was quantified by using the Image J software. Five fields of vision w for each slice (magnification, 200×) were selected and analysis of CVF (CVF = collagen stain/ total area of each vision). Samples for sirius red stain visualized under polarized light. We quantified the pixels of type I and type III collagens images using the histogram of Photoshop CC 2017 software.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Slides were also deparaffinized and followed by rehydrate in gradient ethanol of 95%, 90%,80% and 70%, respectively. Then slides were washed in distilled water. The objective tissue was marked with liquid blocker pen. Slides were incubated 20 minutes at room temperature after adding permeabilizing working solution and further digested with 1µg/ml proteinase K(Servicebio, G1205) for 25 minutes. Next TUNEL reagents(Roche, 11684817910),TdT and dUTP, were mixed at ratio of 1:9 and added to objective tissue. Then slides were placed in a flat wet and incubated for 2h. Then they were mounted with DAPI (Servicebio, G1012). Negative controls for this procedure included a slide without TdT enzyme or pretreated with DNase I before the normal TUNEL procedure. Photographs of slides were captured by inversed fluorescent microscope (Leica, Germany).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

For miR analysis, to determine the enrichment of mature miR-29b, homogenized kidney specimens were isolated using a miRNeasy Mini Kit (Qiagen, 217004) and 1 ug of each purified RNA was reverse transcribed by using the miScript® II RT Kit (Qiagen, 21861). Real-time qRT-PCR was performed using a miScript SYBR Green PCR Kit (Qiagen, 218073) and the miScript Primer Assay (Qiagen, CAS00006566). The levels of miR-29b were normalized by U6. Data are expressed relative to the control group. For mRNA analysis, reverse-transcription of total RNA was performed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, RR047A) and RNAs were quantified using a SYBR Green RT-PCR Kit (Takara, RR420A). Gene expression levels were quantified by the $2^{-\Delta\Delta C_t}$ method. Real time qPCR conditions were set up according to reagent instructions. *miR-29b* level was quantified using the miScript Primer Assay (Qiagen, CAS00006566). GAPDH and U6 small nuclear RNA was used for normalization. All of the mice primers for the qRT-PCR are listed below.

Collagen I forward: 5'-TGCCGTGACCTCAAGATGTG-3'

Collagen I reverse: 5'-CACAAGCGTGCTGTAGGTGA-3'

Collagen III forward: 5'-GCGGAATTCCTGGACCAAAAGGTGATGCTG-3'

Collagen III reverse: 5'-GCGGGATCCGAGGACCACGTTCCCCATTATG-3'

α -SMA forward: 5'-ACTCTCTTCCAGCCATCTTTCA-3'

α -SMA reverse: 5'-ATAGGTGGTTTCGTGGATGC-3'

GAPDH forward: 5'-GCAGTGGCAAAGTGGAGATTG-3'

GAPDH reverse: 5'-AGAGATGATGACCCTTTTGGCTCC-3'

U6 forward: 5-GCGCGTCGTGAAGCGTTC-3

U6 reverse: 5-GTGCAGGGTCCGAGGT-3

Statistical analysis

Data are presented as the mean $\pm$ standard error (SEM). Differences among groups were assessed using Student's *t* test or one-way ANOVA. All analyses were performed using GraphPad Prism 6, $P < 0.05$ was considered statistically significant and we corrected significance of multiple comparisons using Holm-Sidak method. Images were processed using ImageJ, Adobe Photoshop CS5, and Adobe Illustrator CC 2017 software.

Results

A genomic sequence with the pre-miR-29b was PCR amplified from the sequence of mice miR-29b precursor and cloned into the sequence-complementary AAV genome vector with U6 promoter (Fig. 1A). In our preliminary experiment, the rAAV9-GFP particles were injected in kidney, and GFP expression was

evaluated for efficiency of rAAV9 gene transduction to the mouse kidney. Animals were killed 3 weeks or 5 weeks after injection for quantifying GFP expression. GFP is robustly expressed at 5 weeks, which is higher than at 3 weeks. The transduction effect was confirmed by Western blot analysis (Fig. 1B) and microscopy of fresh frozen sections (Fig. 1C). rAAV9-mediated miR-29b gene transfer in kidney tissue in the setting of AngII-induced renal fibrosis (Fig. 1D) restored the expression of the mature miR-29b in kidneys.

Increased concentration of Ang II modulate fibrosis by the direct effects on the ECM and by regulating the expression of other factors (Valls-Lacalle et al., 2018). To clarify the role of miR-29b in renal interstitial fibrosis, we used the AngII infusion as renal interstitial fibrosis model. In preliminary experiment model, we found significant fibrosis after continuous AngII infusion at 28 days (Fig. 2) As shown in Fig. 2A and B, anti- α -SMA staining and Masson trichrome staining indicated that, compared with control group, the AngII-infused mice all showed the features of renal interstitial fibrosis. As shown in Fig. 2C, real-time PCR further confirmed the decrease of miR-29b levels in the renal tubular epithelium in fibrotic kidney (Fig. 2C).

On the basis of the results, we postulated that miR-29b might protect AngII-induced renal interstitial fibrosis. To test this hypothesis, we improved the AngII-induced downregulation of miR-29b in mouse kidney by delivering rAAV9-miR-29b into the kidneys via in situ injection. Delivery of rAAV9-miR-29b effectively increased the levels of miR-29b. As shown by Masson trichrome staining, Sirius red stain and anti- α -SMA staining of mouse renal tissue sections in Fig. 3A, B and C, delivery of miR-29b-expressing significantly ameliorated the tubular interstitial fibrosis and EMT induced by AngII. The results suggest that delivery of miR-29b can significantly prevent renal interstitial fibrosis.

As shown in Fig. 4, delivery of miR-29b-expressing vectors significantly mitigated the reduction of miR-29b in mouse kidneys after AngII infusion. However, delivery with miR mimics control vector didn't have the same effect. qRT-PCR analysis indicated that the expression of α -SMA, collagen type I and collagen type III was increased in AngII infused kidney. These could be significantly inhibited by rAAV9-miR-29b transfer, but not by negative control -rAAV9-miR (Fig. 4).

rAAV9-miR-29b delivery reduced renal tubular injury in mice. Apoptosis of renal tubular epithelial cells, serum creatinine and urea nitrogen levels were used to assess tubule injury. Increased miR-29b expression reduced renal tubule cell apoptosis (Fig. 5, A and B) and attenuated the tubular injury parameters in blood (Fig. 5, C). Finally, closely associated with the fibrotic process, rAAV9-miR-29 delivery protected tubular epithelial cells.

Discussion

The results in our study suggest the delivery of rAAV9-miR-29b inhibited renal interstitial fibrosis and injury by suppressing ECM deposition. These results suggest that miR-29b has a pivotal role during the process of renal interstitial fibrosis and might be a potential therapeutic target of tissue fibrosis. This is the first report to show that delivery of exogenous miR-29b with rAAV9 vector ameliorated renal interstitial

fibrosis in vivo. The ability of a single miR to regulate the level of hundreds of genes expression (Arash et al., 2012) suggests that modulation of individual miRs could influence multiple signal pathways. Previous study (Liu et al., 2010) have shown that miR-29b might be a master regulator of more than 20 collagens and other genes related to ECM, which might be relevant to the development of renal interstitial fibrosis in rats. Thus, therapeutically supplementing exogenous miR-29b may therefore alleviate the deleterious effect of ECM deposition associated with renal interstitial fibrosis.

In the present study, we assessed the persistent effect of miR-29b gene expression in AngII-induced renal interstitial fibrosis in vivo mediated by rAAV9 vector. Our results showed that continuous AngII infusion induced pathological changes characterized by excessive ECM deposition in the renal interstitium. Many ECM related genes like collagens and fibrogenic factors like TGF-beta are the targets of miR-29b. So the pathologic changes could be significantly reversed by overexpressing miR-29b in the kidney after AngII infusion. We presented a novel therapeutic strategy for the treatment of preexisting fibrosis based on kidney delivery of miR-29b. Our results demonstrated that miR-29b supplementation reversed interstitial fibrosis and kidney dysfunction by decreasing ECM deposition. Our results are consistent with other studies showing the effects of miR-29b in cardiac fibrosis and diabetic (van Rooij ET AL., 2010). The present study showed an important aspect of the pivotal role that miR-29b plays in the attenuation of RAAS activation-induced fibrosis, a key pathological feature of kidney injury. It has been verified that some miRs like miR-200a, miR-200c and miR-141, play a key role in the pathological remodeling of many organs by targeting a variety of ECM related proteins (Xiong et al., 2012). In line with previous findings, our results suggest that miR-29b could be a potential therapy target of fibrosis by targeting multiple collagen proteins.

We hypothesized that rescuing miR-29b expression in mice kidney reduces renal fibrosis and protects renal function. We used rAAV vector-mediated gene transfer technology to demonstrate that AngII-induced renal miR-29b decrease could be restored by the reexpression of miR-29b in mice kidney. rAAV vectors provide sustained long-term gene expression with low immunological consequences. In the present results, we do not see any adverse effects of rAAV9 vector on the renal morphology and function in mouse. So rAAV9 vector may keep a stable and sustained expression of miRs in vivo without producing harmful immune response. rAAV vectors have been applied in some clinical trials (Schievenbusch et al. 2010). Some studies had verified that compared with rAAV2 and rAAV8, rAAV9 was the most efficacious serotype for kidney gene transfer (Schievenbusch et al., 2010; Shen et al., 2018). Compared with other organs, such as liver and heart, the efficiency of gene transduction in kidney is lower. Systemic administration of rAAV vector by intravenous injection provides efficient transduction of liver and heart but provides poor transduction in the kidney. We used in situ injection of rAAV-9 vector carrying GFP to determine the magnitude and distribution of gene expression in the kidney. Our results suggested that this method of kidney gene delivery is safe and effective. Kidney injection did not impair renal function in PBS-infused mice.

Study Limitation

The present study was focused on evaluating the early effects of increasing miR-29b expression in the kidney in vivo using an rAAV9 under the control of a CMV and U6 promoters. In our pretesting, we infused rAAV9-GFP vectors through tail vein injection in mice and found GFP was strongly expressed in liver and heart tissues. In order to improve the gene transfer to the kidney by rAAV-9, we transduced the rAAV9 vector via in situ kidney injection, which is an invasive operation. It might produce stress in mice.

Conclusion

The administration of rAAV9 vectors via in situ kidney injection achieved a safe and effective way of exogenous gene expression in the kidney. rAAV-mediated supplementation of miR-29 ameliorates interstitial fibrosis and attenuates progressive injury of renal function through reversing organ maladaptive remodeling. Restoration of miR-29b levels might be a novel therapeutic strategy to reverse renal fibrosis.

Abbreviations

ECM: Multiple extracellular matrix

AngII: angiotensin II

EMT: epithelial mesenchymal transition

RAAS: renin-angiotensin II-aldosterone system

MiR: microRNA

rAAV: recombinant adeno-associated virus

GFP: green fluorescent protein

CMV: cytomegalovirus

qPCR: quantitative polymerase chain reaction

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

qRT-PCR Quantitative reverse-transcription polymerase chain reaction

SEM mean $\pm$ standard error

Declarations

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Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Design of the rAAV9 mediated miR-29b transduction in vivo. (A) Schematic representation of miR-29b expression cassette. The expression cassette was comprised of miR-29b stem-loop sequence flanked by its native intron sequence, which preserves the putative hairpin structure and proper endogenous processing. A genomic fragment 800 bp containing the miR-29b precursor was PCR amplified from the mouse miR-29 precursor sequence and cloned into the self-complementary rAAV9. miR-29b under the control of cytomegalovirus and U6 promoter. rAAV9-GFP transduction in renal tissue was tested by GFP (B)western blot and (C) fluorescence in fresh frozen sections of kidney. In AAV-injected kidneys, there is significant EGFP fluorescence in tubule epithelial cells at 5 weeks. (D) Overall design of the study in vivo. Mouse were subjected to continuous AngII or PBS infusion. Two weeks later, when fibrosis was evident, the animals were randomly chosen to receive either recombinant adeno-associated vector type 9 carrying miR-29b (rAAV9-miR-29b) or control vector (rAAV9-random miR) at 5×10^{10} IVP per kidney via in situ injection.

Figure 2

Decrease of miR-29b levels in mouse kidney during angiotensin II-induced renal fibrosis. Representative images of Masson trichrome staining (A) and a-SMA immune stain (B) of kidney with saline or angiotensin II infusion at different time points. Magnification, $\times 200$. Right histogram represents quantitative analysis of tubular interstitial fibrosis. Results are presented as mean $\pm$ SEM from three independent experiments. Each group consisted of six mice. ** $P < 0.01$ compared with control group (CTL). # $P < 0.01$ compared with angiotensin II infusion group (Day 14). Scale bar 50 μ m.

Figure 3

Effects of rAAV9-miR-29b transduction on fibrotic changes in AngII-induced kidneys. **A** Representative Masson staining of kidney sections in each group quantitative analysis of fibrotic areas. Areas with positive blue staining show collagen fibers. Magnification, $\times 200$. **B** Representative sirius red staining of kidney sections and quantitative analysis of fibrotic areas. Area with yellow stain show collagen type I (COL I) and green stain show collagen type III (COL III). Magnification, $\times 400$. **C** Representative images of alpha-smooth muscle actin (a-SMA) immune staining of kidney among the groups (six high-power fields counted per group). Magnification, $\times 200$. Values are mean $\pm$ SEM ($n=6$). * $P < 0.05$ compared with control group. # $P < 0.05$ compared with rAAV9-negative control. Scale bar 100 μm .

Figure 4

Effect of rAAV9-miR-29b delivery on fibrotic markers in kidney. Mature miR-29b levels was significantly increased in rAAV9-miR-29b transduction group. The expression levels of alpha-smooth muscle actin (a-SMA), collagen type I (COL I) and collagen type III (COL III) were evaluated as fold change relative to the control animals at 5 weeks post gene transfer. Values represent mean $\pm$ SE(n=6). Significance of differences: *P<0.05, rAAV9-miR-29b vs. control group, #P<0.05, rAAV9-miR-29b vs. rAAV9-negative control. CTL, control; rAAV9 indicates recombinant adeno-associated vector type 9; NC, negative control.

Figure 5

Effects of miR-29b gene transfer on renal damage and dysfunction in vivo. (A) Assessment of apoptosis. Representative epifluorescence images of TUNEL labeling of kidney histological sections at 5 weeks post gene transfer. Scale bar: 50 μ m. (B) Quantification of TUNEL-positive nuclei. Values are mean $\pm$ SEM measured from 10 000 nuclei. *P<0.05 vs. Control (CTL); #P<0.05 vs. rAAV9-Negative control (NC) (n=6). (C) Assessment of renal function. Renal function was assessed by the level of serum creatinine and urea nitrogen in each group (n=6).