

# Lack of miRNA-17 family mediates high glucose-induced PAR-1 up-regulation in glomerular mesangial cells

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## Research Article

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# Abstract

Up-regulation of thrombin receptor protease-activated receptor 1 (PAR-1) is verified to contribute to chronic kidney diseases, including diabetic nephropathy, however, the mechanisms are still unclear. In this study, we investigated the effect of PAR-1 on high glucose-induced proliferation of human glomerular mesangial cells (HMCs), and explored the mechanism of PAR-1 up-regulation from alteration of microRNAs. We found that high glucose stimulated proliferation of the mesangial cells whereas PAR-1 inhibition with vorapaxar attenuated the cell proliferation. Moreover, high glucose up-regulated PAR-1 in mRNA level and protein expression while did not affect the enzymatic activity of thrombin in HMCs after 48 h culture. Then high glucose induced PAR-1 elevation was likely due to the alteration of the transcription or post-transcriptional processing. It was found that miR-17 family members including miR-17-5p, -20a-5p, and -93-5p were markedly decreased among the eight detected microRNAs only in high glucose-cultured HMCs, but miR-129-5p, miR-181a-5p, and miR-181b-5p were markedly decreased in both high glucose-cultured HMCs and osmotic press control compared with normal glucose culture. So miR-20a was selected to confirm the role of miR-17 family on PAR-1 up-regulation, finding that miR-20a-5p overexpression reversed the up-regulation of PAR-1 in mRNA and protein levels induced by high glucose in HMCs. In summary, our finding indicated that PAR-1 up-regulation mediated proliferation of glomerular mesangial cells induced by high glucose, and deficiency of miR-17 family resulted in PAR-1 up-regulation.

## Introduction

Diabetic nephropathy (DN) is one of the most common diabetic microvascular complications, characterized by continuous proteinuria, glomerular mesangial expansion, abnormal accumulation of extracellular matrix, and thickening of glomerular basement membrane, eventually developing glomerular sclerosis (Chen et al., 2019). Moreover, proliferation of the glomerular mesangial cells occurs at the early stage of DN, and is the main cause of increased synthesis and deposition of the extracellular matrix in glomeruli (Xu et al., 2020, Zhao et al., 2021). Among the influencing factors, chronic inflammation plays a very important role in the early pathological process of DN (Zhu et al., 2018, Tang et al., 2020).

Activation of thrombin receptor protease-activated receptor 1 (PAR-1) signaling has been verified to contribute to chronic kidney diseases, including DN. An early report shows that the mRNA level of PAR-1 rather than PAR-4 is up-regulated in the isolated glomeruli of diabetic db/db mice, and PAR-1 plays a role in the progression of glomerulosclerosis in DN (Sakai et al., 2009). Moreover, recent studies further demonstrate that PAR-1 deficiency or inhibition protects against DN in streptozotocin-induced diabetic mice (Waasdorp et al., 2016, Waasdorp et al., 2018). On the other hand, PAR-1 leads to other chronic kidney diseases, as evidenced by glomerular injury induced by doxorubicin in mice (Guan et al., 2017), renal injury and interstitial fibrosis during chronic obstructive nephropathy (Waasdorp et al., 2019), and microcirculation failure and tubular damage in renal ischemia-reperfusion injury in mice (Guan et al., 2021), indicating that PAR-1 antagonism has therapeutic potential. Importantly, our recent study further demonstrated that PAR-1 up-regulation participated in the early pathological process of DN, and the NLRP3 inflammasome and NF- $\kappa$ B signaling mediated the pro-inflammatory effects of PAR-1 (Tang et al., 2020). Furthermore, our study indicated that PAR-1 up-regulation was not associated with thrombin activity in both the renal cortex of diabetic rats and high glucose-cultured

glomerular mesangial cells, and discussed the possible reasons (Tang et al., 2020). Thus, how high glucose induced PAR-1 up-regulation in renal cortex is still unknown.

MicroRNAs (miRs), an evolutionarily well-conserved class of small, non-coding RNAs, negatively regulate gene expression at the post-transcriptional level in a sequence-specific manner. Reports find that PAR-1 is negatively regulated by several microRNAs, such as miR-190a (Chu et al., 2014), and the microRNA precursor miR-17 family including miR-20a/b, -93, -106a/b, etc. (Saleiban et al., 2014). Moreover, microRNAs are involved in pathogenesis of DN (Fiorentino et al., 2013, Kolling et al., 2017), and alterations of microRNAs result in proliferation of glomerular mesangial cells and matrix expansion (Bera et al., 2017, Wang et al., 2018). Then, whether microRNAs take part in the up-regulation of PAR-1 caused by high glucose in glomerular mesangial cells, there are no reports.

In this study, we found that several members of miR-17 family were markedly decreased among the detected microRNAs in high glucose-cultured human mesangial cells without influence by osmotic press. Further studies confirmed that an increase in miR-20a mimic reversed the up-regulation of PAR-1 induced by high glucose.

## Materials And Methods

### Reagents and antibodies

Dulbecco's modified Eagle medium (DMEM) culture medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, Utah); Hsa-miR-20a-5p mimic (GMUL0163595) from Shanghai GeneChem Chemical Technology, Co., Ltd., China; Cell counting kit-8 (CCK-8) from Dongren Chemical Technology Co., Ltd., Shanghai, China; RIPA buffer (#P0013B), phenylmethanesulfonyl fluoride (PMSF, #ST506), and phosphatase inhibitor cocktail (#P1081) from Beyotime Biotechnology, Nantong, China; Bicinchoninic acid (BCA) protein assay kit (#23225) from Thermo Scientific, Rockford, IL, USA; Trizol reagent from Invitrogen, USA; A cDNA synthesis kit (#RR037A) from Dalian Takara, China; Rabbit anti-PAR-1 antibody (#SAB4500823, 1:1000) from Sigma-Aldrich Company, Shanghai, China; Rabbit anti-GAPDH antibody (#AC001, 1:2000) from ABclonal Biotechnology Co., Ltd., HK; Goat anti rabbit IgG (H+L) secondary antibody (#V926-32211, 1:1000) from Li-Cor, Inc., Lincoln, NE.

### Cell culture and treatments

The cell line human mesangial cells (HMCs) was kindly provided by Dr. Wei of our laboratory. The cells were cultured in DMEM containing 10% FBS. After incubation for 24 h under normal conditions (medium containing 5.6 mmol/L glucose, 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 5% CO<sub>2</sub>, 37 °C) and cell cycle synchronization for 12 h, HMCs were divided into the following groups: normal glucose group (NG, 5.6 mmol/L glucose), high glucose group (HG, 30 mmol/L glucose), and PAR-1 antagonist group (HG+Vor, 30 mmol/L glucose plus 0.1 µmol/L vorapaxar). Vorapaxar was dissolved in dimethylsulfoxide and made into stock solution for use. In addition, an osmotic pressure control (NG+MA, 5.6 mmol/L glucose plus 24.4 mannitol) was designed when the selected microRNAs were determined. After treatments for 48 h, the cells were harvested for indices analysis. The culture time was selected according to the changes of PAR-1 protein

in HMCs cultured with 30 mmol/L glucose for 24 h, 48 h, and 72 h, respectively, in our previous report (Tang et al., 2020).

### **MiR-20a-5p overexpression**

Lentivirus carrying hsa-miR-20a-5p mimic was transfected into HMCs, establishing a stable cell line with miR-20a-5p overexpression. After 72 h of screening with puromycin, samples of cell stable strains were collected. Then the cells were divided into two categories, mimic control and miRNA mimic, and the overexpression efficiency of miR-20a-5p was confirmed. After different treatments for 48 h, PAR-1 mRNA and protein were detected.

### **CCK-8 assay for cell proliferation**

The proliferation of HMCs was analyzed using the CCK-8 method. Briefly, cell suspension (100  $\mu$ l/well,  $1.0 \times 10^6$ /ml) was pre-incubated in a 96-well plate for 24-48 h at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. After the cells were incubated in different groups for 24 h, 10  $\mu$ l of the CCK-8 solution was added to each well of the plate and incubated for 2 h in incubator. After 10  $\mu$ l of 1 % (w/v) SDS added to each well in dark at room temperature, the absorbance was determined at 450 nm using a microplate reader. The net absorbance of the normal glucose group was considered as 100% of the cell proliferation viability.

### **Determination of thrombin activity**

Thrombin activity was assessed by using a fluorometric assay based on the cleavage rate of the synthetic thrombin substrate Boc-Asp (OBzl)-Pro-Arg-AMC. Determination of thrombin activity was performed according to our previous report (Tang et al., 2020). The reaction system was triggered after adding about 60  $\mu$ g of proteins at 37 °C for 50 min, and the optical density value was immediately measured with a fluorescence spectroscopy at the excitation wavelength 360 nm and emission wavelength 465 nm. The net absorbance from the plates of cells cultured with normal glucose was considered as 100% thrombin activity.

### **Real-time qPCR assay**

Total RNA isolation was performed by using trizol reagent, and the RNA was reverse-transcribed into single-stranded cDNA by using different primers corresponding to mRNA or microRNA. The Roche 480 LightCycler® system with SYBR Green dye binding to PCR product was used to quantify target mRNA or miRNA accumulation via fluorescence PCR using human  $\beta$ -actin or U6 as a reference. Human primers of the associated genes used for quantitative PCR in this study were listed in Table 1. For the amplification reaction in each well, a Cp value was observed in the exponential phase of amplification, and the quantification of relative expression levels was achieved using standard curves for both target and endogenous control samples. Relative transcript abundance of a gene is expressed as  $2^{-\Delta\Delta C_p}$  values ( $\Delta C_t = C_{p_{target}} - C_{p_{reference}}$ ).

### **Western blot assay**

Cells were lysed in RIPA buffer with 1 mmol/L PMSF and 1 mmol/L phosphatase inhibitor cocktail at 4°C for 30 min followed by 12,000 $\times$ g centrifugation at 4°C for 15 min to obtain the supernatant. The BCA protein assay was performed to determine the protein concentration according to the manufacturer's instructions. The

protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. The membrane was blocked with 2% milk powder solution for 60 min and incubated overnight at 4°C with primary antibodies including rabbit anti-PAR-1 antibody. The proteins were detected using goat anti rabbit IgG (H+L) secondary antibody. Infrared Imaging System was applied to detect immunoreactive blots. Signal densities on the blots were measured with Image J software and normalized using rabbit anti-GAPDH antibody as an internal control.

## Statistical analysis

All statistical analysis was done with GraphPad Prism 7.0 software. Differences between the groups were assessed using unpaired t-test or one-way of analysis of variance followed by Tukey multiple comparisons test. The data in the different experimental groups were expressed as the mean  $\pm$  SD.  $P < 0.05$  was considered to be statistically significant.

## Results

### HG induced mesangial cell proliferation and PAR-1 up-regulation

HG significantly induced cell proliferation in HMCs after 48 h culture compared with the NG culture ( $p < 0.01$ , Fig. 1A), while co-treatment with a selective inhibitor of PAR-1 Vor inhibited cell proliferation induced by HG in HMCs ( $p < 0.05$ , Fig. 1A). Moreover, HG markedly increased the protein expression of PAR-1 in HMCs ( $p < 0.01$ , Fig. 1B). These data indicated that PAR-1 up-regulation mediated the mesangial cell proliferation stimulated by HG condition.

### Effects of HG on thrombin/PAR-1 signaling in HMCs

To seek for the reason of increased protein expression of PAR-1 induced by HG in HMCs, we examined the alteration of thrombin/PAR-1 signaling. It was found that HG did not change the enzymatic activity of thrombin (Fig. 2A), but elevated the mRNA level of PAR-1 ( $p < 0.01$ , Fig. 2B) in HMCs, suggesting that PAR-1 up-regulation induced by HG was likely due to the alteration of transcriptional or post-transcriptional level.

### Prediction and determination of microRNAs combining with 3'-UTR of PAR-1 mRNA

By using the TargetScan 7.1, miRDB, and miRWalk bioinformatics platforms, we predicted potential microRNAs that could combine with the 3'-UTR of F2R (the gene symbol of PAR-1). We chose 14 human microRNAs that were predicted to target the 3'-UTR of F2R with higher score: miR-17-5p, miR-93-5p, miR-106a-5p, miR-106b-5p, miR-20a-5p, miR-20b-5p, miR-190a-5p, miR-190b-5p, miR-448, miR-129-5p, miR-29a-5p, miR-214-3p, miR-181a-5p, and miR-181b-5p.

Next, we selected and examined 8 microRNAs: miR-17-5p, miR-20a-5p, miR-93-5p, miR-190a-5p, miR-129-5p, miR-181a-5p, miR-181b-5p, and miR-214-3p in HMCs cultured with HG. We found that miR-17-5p, miR-20a-5p, and miR-93-5p were significantly decreased in HG-cultured HMCs and unchanged in NG plus MA culture (each  $p < 0.01$ , Fig. 3A) compared with NG culture. Moreover, miR-190a-5p (Fig. 3A) and miR-214-3p (Fig. 3B) showed unchanged in neither HG-cultured or NG plus MA cultured HMCs. However, miR-129-5p, miR-181a-5p, and miR-181b-5p were markedly decreased in both HG-cultured and NG plus MA cultured HMCs compared

with NG culture ( $p < 0.05$  or  $p < 0.01$ , Fig. 3B), suggesting that osmotic pressure rather than HG affects the alteration of these microRNAs in HMCs. Moreover, we afforded the sequence alignment information of these microRNAs combining with the 3'-UTR of F2R, and miR-129-5p and miR-181a-5p have two binding sites (Fig. 4). So the results displayed that miR-17-5p, miR-20a-5p, and miR-93-5p may participate in the up-regulation of PAR-1 caused by HG.

### **Overexpression of miR-20a-5p in HMCs**

To further verify that whether loss of miR-17 family indeed led to the up-regulation of PAR-1 caused by HG, a member miR-20a-5p was selected for further study. So the HMCs with miR-20a-5p overexpression was established. Fig. 5A showed that LV-hsa-miR-20a as well as mimic control totally infected the cells. Q-PCR data indicated that miR-20a-5p was dramatically increased in LV-hsa-miR-20a group compared with mimic control group ( $p < 0.01$ , Fig. 5B), indicating that the HMCs overexpressed with miR-20a-5p was successfully obtained.

### **Overexpression of miR-20a-5p decreased PAR-1 mRNA and protein levels in HG condition**

PAR-1 mRNA level ( $p < 0.01$ , Fig. 6A) and protein expression ( $p < 0.01$ , Fig. 6B) were both significantly decreased in HMCs transfected with miR-20a-5p mimic compared with mimic control, indicating that miR-20a-5p negatively regulated PAR-1 transcription. Further, PAR-1 mRNA level ( $p < 0.01$ , Fig. 6A) and protein expression ( $p < 0.01$ , Fig. 6B) were markedly attenuated in HG-cultured HMCs overexpressed with miR-20a-5p, compared with HG-cultured HMCs transfected with mimic control. Thus, miR-20a-5p deficiency mediated the up-regulation of PAR-1 caused by HG in HMCs.

## **Discussion**

Glomerular mesangial cells are a class of important renal inherent cells, and cell proliferation of mesangial cells is one of the early pathological manifestations of DN. In the present study, firstly we found that chronic high glucose stimulated cell proliferation of HMCs, a human glomerular mesangial cell line, accompanied by unchanged thrombin activity but up-regulation of its receptor PAR-1. Moreover, PAR-1 inhibition decreased PAR-1 protein level and attenuated cell proliferation in high-glucose cultured HMCs. Secondly, we predicted potential microRNAs that could combine with the 3'-UTR of F2R, and determined 8 of 14 microRNAs, finding that miR-17 family members including miR-17-5p, -20a-5p, and -93-5p were markedly decreased due to high glucose rather osmotic press in high glucose-cultured HMCs. Finally, we confirmed that miR-20a-5p overexpression reversed the up-regulation of PAR-1 induced by high glucose in HMCs. Our findings partially clarify the role of miR-17 family in PAR-1 up-regulation in DN.

PAR-1 up-regulation contributes to DN. In 2009, Sakai et al. report that PAR-1 promotes mesangial expansion and abnormal urinary albumin excretion in DN in mice (Sakai et al., 2009). Recently, Waasdorp et al. find that PAR-1 deficient mice develop less kidney damage after induction of diabetes, as evidenced by diminished proteinuria, plasma cystatin C levels, expansion of the mesangial area, and tubular atrophy, and PAR-1 signaling in mesangial cells leads to the increased proliferation and expression of matrix proteins, indicating that PAR-1 may be an attractive therapeutic target to pursue in DN (Waasdorp et al., 2016). Furthermore, Waasdorp et al. verify that streptozotocin-induced diabetic mice treated with vorapaxar, a selective inhibitor of

PAR-1, do not show significant albuminuria, mesangial expansion, and glomerular fibronectin deposition, suggesting that PAR-1 inhibition prevents the development of diabetic nephropathy in this preclinical animal model for type 1 diabetes (Waasdorp et al., 2018). Moreover, our recent study demonstrated that a natural product sarsasapogenin alleviated DN in rats by down-regulating PAR-1 (Tang et al., 2020). Additionally, dual blockade of PAR-1 and PAR-2 additively ameliorates DN in male type 1 diabetic Akita mice (Mitsui et al., 2020). Taken together, PAR-1 plays an important role in the development of DN.

Inflammation mediates the effects of PAR-1 in the pathological process of DN. An early report indicates that activation of PAR-1 amplifies crescentic glomerulonephritis and augments inflammatory renal injury (Cunningham et al., 2000). Recently, PAR-1 antagonism ameliorates kidney injury and tubulointerstitial fibrosis by inhibiting ERK1/2 and transforming growth factor- $\beta$ -mediated Smad signaling, and suppressing oxidative stress, pro-inflammatory cytokine overexpression, and macrophage infiltration into the kidney (Lok et al., 2020). Moreover, edoxaban, an inhibitor of activated factor X, inhibits renal tubulointerstitial injury by attenuating PAR-1 mediated macrophage infiltration and inflammatory molecule up-regulation in unilateral ureteral obstruction mice (Horinouchi et al., 2018). Further, our recent study demonstrated that PAR-1 participated in the pathogenesis of DN through activating the NLRP3 inflammasome and NF- $\kappa$ B signaling (Tang et al., 2020). These studies demonstrate that chronic inflammation mediates the effects of PAR-1 in chronic kidney diseases, including DN.

Deficiency of microRNAs results in PAR-1 up-regulation in renal inherent cells in high glucose. PAR-1 is the prototype receptor of thrombin, and PAR-1 up-regulation can be due to increased thrombin activity, but enzymatic activity of thrombin was unchanged in high glucose-stimulated mesangial cells (Tang et al., 2020). A microRNA pathway is another mechanism to regulate gene expression. Mature microRNAs regulate the expression of their target genes by mRNA degradation or translational inhibition. It is reported that miR-190a inhibits cell migration and invasiveness of breast cancer by targeting PAR-1 expression (Chu et al., 2014). Moreover, our previous results indicated that miR-190a alleviated neuronal damages by decreasing PAR-1 expression in high glucose-cultured SH-SY5Y cells, a commonly used cell line of central neurons (unpublished data). However, in the present study, miR-190a level was not affected by high glucose in HMCs, although PAR-1 was up-regulated in high glucose-cultured HMCs. MiR-20a, miR-20b, miR-17, miR-93, miR-106a, and miR-106b all belong to miR-17 family, and Saleiban et al. report that PAR-1 is post-transcriptionally regulated by miR-20b in human melanoma cells (Saleiban et al., 2014). In our study, three members of miR-17 family miR-17-5p, miR-20a-5p, and miR-93-5p were found to be significantly decreased in HG-cultured HMCs without influence by osmotic press. Unfortunately, the seed region of miR-129-5p and miR-181a/b-5p has two binding sites with the 3'-UTR of F2R, but they were decreased by not only high glucose but also mannitol in HMCs. Nevertheless, such effect needs to be further confirmed. Based on these results, the relationship of miR-20a-5p and PAR-1 was further studied by using HMCs overexpressed with miR-20a-5p. Our results showed that miR-20a-5p overexpression reversed PAR-1 up-regulation induced by HG in HMCs. Thus, deficiency of miR-17 family at least miR-20a-5p participates in the up-regulation of PAR-1 caused by HG in HMCs.

## Conclusions

The present study demonstrated that PAR-1 up-regulation mediated proliferation of glomerular mesangial cells stimulated by chronic high glucose, and expression deficiency of miR-17 family contributed to the up-

regulation of PAR-1 caused by high glucose. Furthermore, miR-20a acted as a representative to confirm the role of miR-17 family in regulation of PAR-1 expression in mesangial cells. Our study primarily clarify the role of miR-17 family in PAR-1 up-regulation in the pathogenesis of chronic kidney diseases.

## Declarations

**Ethical Approval** Not Applicable.

**Consent to Participate** All authors consent for participation.

**Consent to Publish** All authors consent for publication.

**Authors Contributions** Liu YW and Gou LS conceived and designed research. Tang ZZ conducted experiments. An XF contributed new reagents or analytical tools. Tang ZZ and Gu PP analyzed data. Gu PP and Liu YW wrote the manuscript. All authors read and approved the manuscript, and all data were generated in-house and that no paper mill was used.

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**Compliance with ethical standards** Not Applicable.

**Competing Interests** The authors declare no conflict of interest.

**Availability of data and materials** The data and materials used during the current study are available from the corresponding author on reasonable request.

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## Tables

Table 1 Primer sequences of the associated genes used for quantitative PCR.

Gene	Forward 5'to3'	Reverse 5'to3'
PAR-1	CGCAGAGCCCGGGACAATGG	CGGTGCGGGCAGACAACA
$\beta$ -actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG
miR-17-5p	CGCAGAGCCCGGGACAATGG	CGGTGCGGGCAGACAACA
miR-20-5p	TGGGTAAAGTGCTTATAGTGC	AGTGCAGGGTCCGAGGTATT
miR-93-5p	ATGGTTGGGCAAAGTGCTGTTCGT	CGTATCCAGTGCAGGGTCCG
miR-129-5p	CAACGCGACATTTAGATGCGTG	GTGACACTTTTTGCGGTCTGG
miR-214-3p	CGCTAAATTCATAACCGGCCAA	ACTCACACAGCAGGCACA
miR-190a-5p	ACACTCCAGCTGGGGTGATATGTTTGATAT	CTCAACTGGTGTCGTGGAGTCG
miR-181a-5p	GCGGCAACATTCAACGCTGTCGGTGAGT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTG
U6	GCGTCGTGAAGCGTTC	GTGCAGGGTCCGAGGT

## Figures

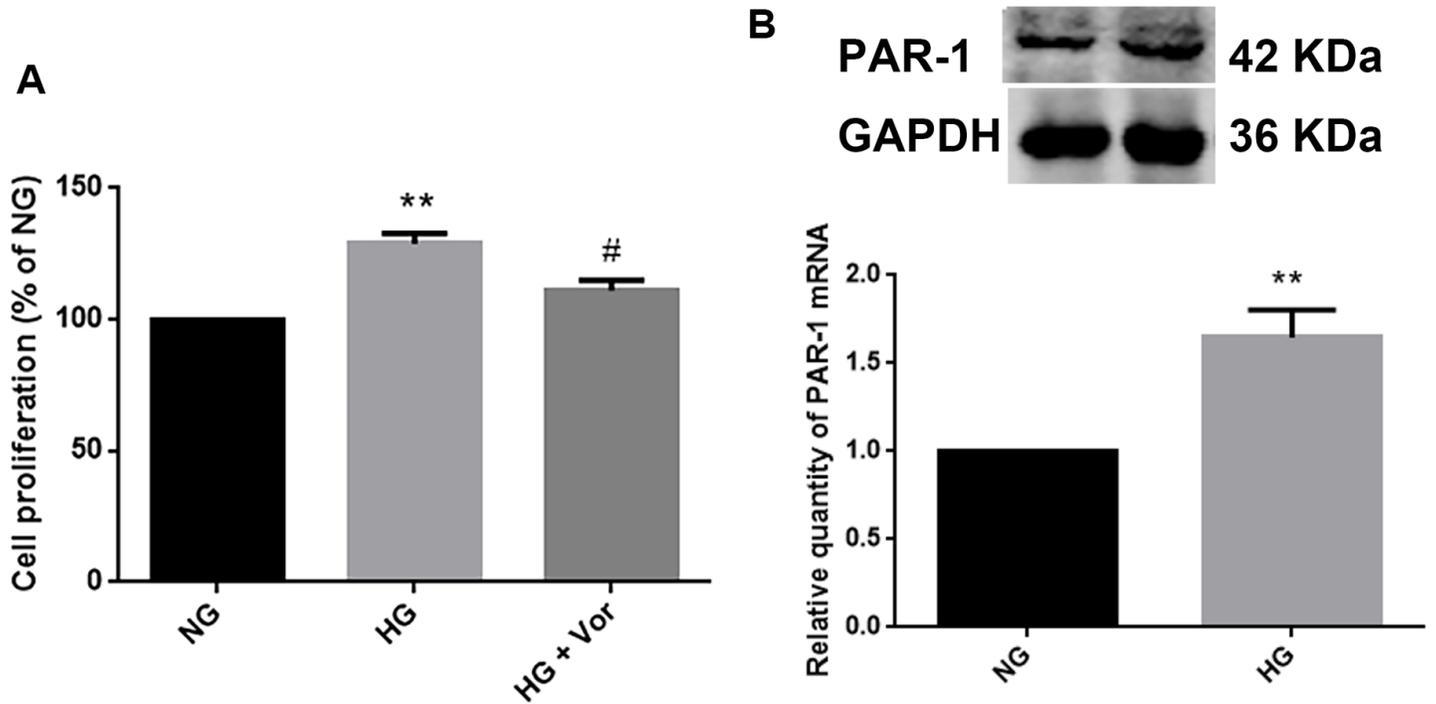
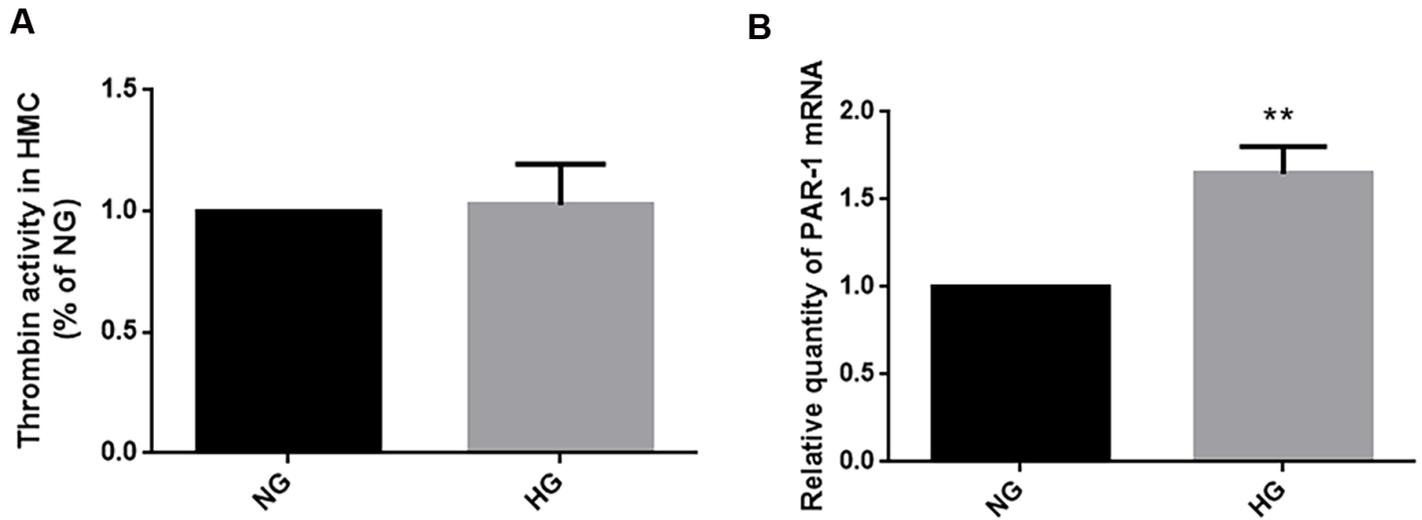


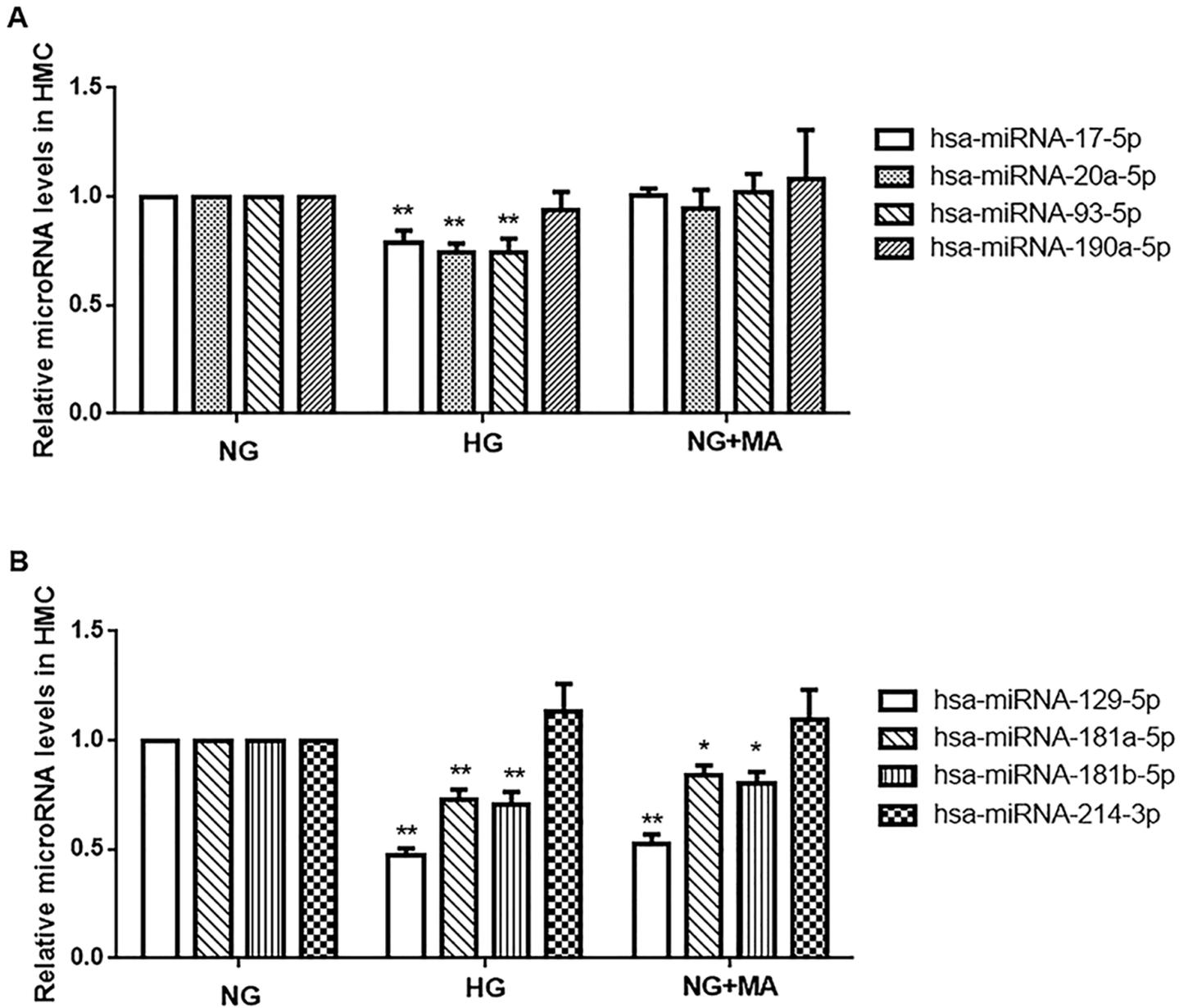
Figure 1

Effects of high glucose on cell viability (A) and PAR-1 protein expression (B) in HMC cells. NG, HG, HG+Vor represent 5.6 mmol/L glucose, 30 mmol/L glucose, and 30 mmol/L glucose plus 0.1  $\mu$ mol/L vorapaxar (Vor), a selective inhibitor of PAR-1, respectively. One-way ANOVA with Tukey post-test was used for the analysis of statistical significance. Mean  $\pm$  SD, n = 3 (cell viability), n = 4 (PAR-1 protein). \*\* p < 0.01, vs. NG; # p < 0.05, vs. HG.



**Figure 2**

Effects of high glucose on thrombin activity (A) and PAR-1 mRNA level (B) in HMC cells. NG and HG represent 5.6 mmol/L and 30 mmol/L glucose, respectively. Mean  $\pm$  SD, n = 4 (thrombin activity), n = 3 (PAR-1 mRNA). \*\*  $p < 0.01$ , vs. NG.



**Figure 3**

Effects of high glucose on the chosen microRNAs in HMC cells. Decreased microRNAs due to high glucose stimulation in HMC cells (A); Decreased microRNAs due to the effect of osmotic pressure in HMC cells (B). MicroRNA levels were assayed by stem-loop RT-qPCR. NG, HG, NG+MA represent 5.6 mmol/L glucose, 30 mmol/L glucose, and 5.6 mmol/L glucose plus 24.4 mmol/L mannitol (MA), respectively. Mean  $\pm$  SD, n = 4. \* p < 0.05, \*\* p < 0.01, vs. NG.

## miR-17 family

Position 342-349 of F2R 3'-UTR 5' GTGCAGTATAGAATAGGCACTTT A 3'  
Hsa-miR-17-5p 3' GAUGGACGUGACAUUCGUGAAAC 5'

Position 342-349 of F2R 3'-UTR 5' GTGCAGTATAGAATAG GCACTTTA 3'  
Hsa-miR-20a-5p 3' GAUGGACGUGAUAUUCGUGAAAU 5'

Position 342-349 of F2R 3'-UTR 5' GTGCAGTATAGAATAGGCACTTT A 3'  
Hsa-miR-93-5p 3' GAUGGACGUGCUUGUCGUGAAAC 5'

Position 1799-1819 of F2R 3'-UTR 5' CTCTACTAAAAATGCAAAAAA 3'  
Hsa-miR-129-5p 3' CGUUCGGGUCUGGCGUUUUUC 5'

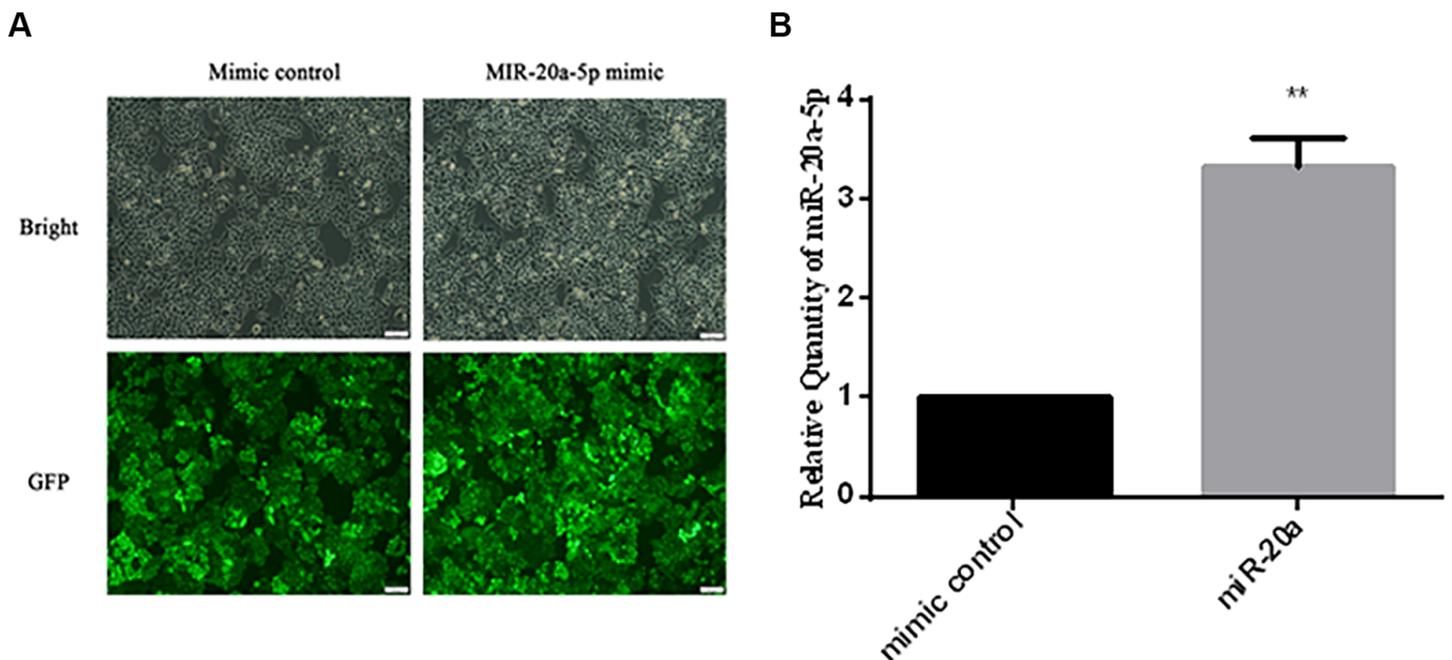
Position 1955-1975 of F2R 3'-UTR 5' GCAAGACTCCATCTCAAAAAA 3'

Position 221-243 of F2R 3'-UTR 5' ATGCTACAGTAATAAATTGAATGT 3'  
Hsa-miR-181a-5p 3' UGAGUGGCUGUCGCAACUUACAA 5'

Position 715-739 of F2R 3'-UTR 5' GAGCATAAGTCCTCTAGTGAATGTA 3'

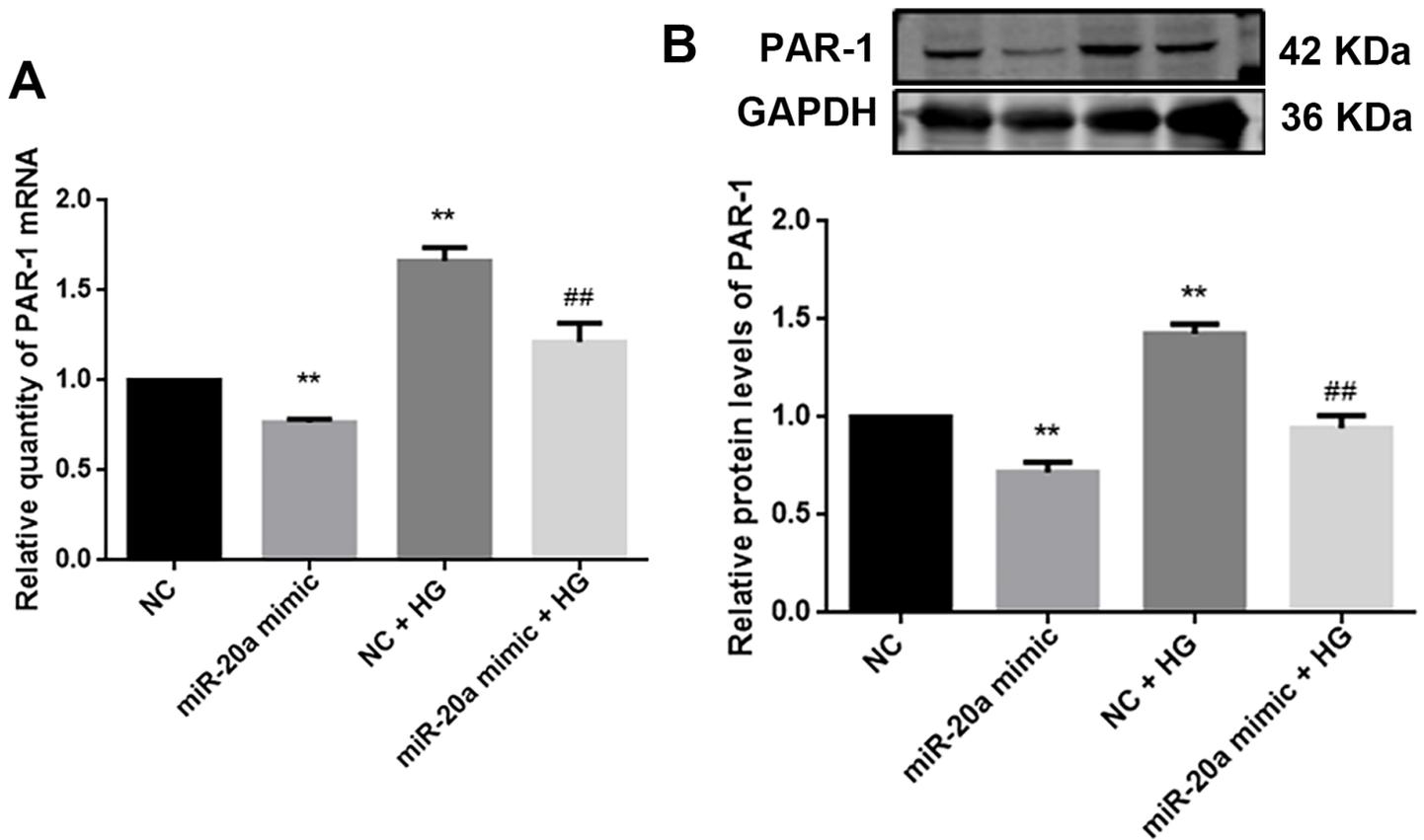
Figure 4

Sequence alignment information for the seed region of three members of miR-17 family as well as miR-129-5p and miR-181a-5p with the 3'-UTR of F2R (the gene symbol of PAR-1) by using miRDB bioinformatics platform.



**Figure 5**

Confirmation of miR-20a-5p overexpression in HMC cells. Typical pictures of lentivirus (LV) infection by using LV-hsa-miR-20a-5p mimic and mimic control (A); miR-20a-5p level in LV-hsa-miR-20a-5p mimic and mimic control (B). Mean  $\pm$  SD, n = 3. \*\* p < 0.01, vs. mimic control. Scale bar: 100  $\mu$ m.



**Figure 6**

Effects of miR-20a-5p overexpression on PAR-1 mRNA level (A) and protein expression (B) in HMC cells. NC and NC+HG represent mimic control and mimic control cultured with 70 mmol/L glucose, respectively. One-way ANOVA with Tukey post-test was used for the analysis of statistical significance. Mean  $\pm$  SD, n = 3 (mRNA), n = 4 (protein). \*\* p < 0.01, vs. NC; ## p < 0.01, vs. NC+HG.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AllWBbandsHMCmiR.pdf](#)