Original Article
Role of miRNA-21/PTEN on the high glucose-induced EMT in human mesothelial peritoneal cells

Lina Yang[1,2], Yi Fan[2], Xiuli Zhang[3], Lili Gao[2], Jianfei Ma[2]

Departments of [1]Geriatrics, [2]Nephrology, The First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning, P. R. China; [3]Department of Nephrology, Benxi Center Hospital, China Medical University, Benxi 117000, Liaoning, P. R. China

Abstract: Objective: To explore the effects of miRNA-21/phosphatase and tensin homolog (PTEN) on the high glucose-stimulated epithelial-to-mesenchymal transition (EMT) in human peritoneal mesothelial cells (HPMCs). Methods: HPMCs were cultured under control conditions, or with high glucose (HG), HG with miRNA-21 mimic or a miRNA-21 inhibitor. Expression of miRNA-21, α-smooth muscle actin, fibronectin, E-cadherin and PTEN were measured by real time PCR, Western blotting and immunofluorescence staining. Results: Compared with the control, HG induced the EMT, as shown by upregulation of α-smooth muscle actin and fibronectin, and downregulation of E-cadherin. We also found that HG upregulated miRNA-21 expression and downregulated PTEN expression; the miRNA-21 inhibitor attenuated the HG-induced EMT in HPMCs by targeting PTEN; the miRNA-21 mimic increased the HG-induced EMT in HPMCs by targeting PTEN. Conclusions: This study demonstrated that miRNA-21 played a vital role in the HG-induced EMT by targeting PTEN in HPMCs.

Keywords: miR-21, PTEN, EMT, HPMCs

Introduction

It is well known that peritoneal dialysis (PD) is a crucial chronic life-sustaining treatment for end stage renal disease (ESRD), and research has shown that the number of patients undergoing PD in both developing and developed countries has increased over the last decade [1]. Due to its simplicity and economic advantages, PD is widely applied to patients who live in remote and rural areas [1]. However, the high glucose-based solutions have disadvantages, such as high lactate, low pH, glucose degradation products and hyperosmolarity [2, 3], which can lead to morphological and functional changes of the peritoneum, and eventually cause peritoneal fibrosis and ultrafiltration failure [4].

The epithelial-to mesenchymal transition (EMT) is a crucial and complex phenomenon involved in organ fibrosis, including renal fibrosis, liver fibrosis and peritoneal fibrosis [5-7]. The EMT in HPMCs is considered as an early stage of peritoneal fibrosis [8]. Preventing the EMT in patients undergoing PD could alleviate perito-

neal fibrosis, thus protecting the peritoneal mesothelium [9]. The EMT process is accompanied by high expression of mesenchymal markers such as fibronectin (FN) and α-smooth muscle actin (α-SMA), and low expression of epithelial markers such as E-cadherin [10]. In our previous study, we found that high glucose could induce the EMT in HPMCs [11]; however, the underlying molecular mechanisms are not yet fully understood.

microRNAs (miRNAs), which play a key role in regulating targeted genes, have been extensively studied as biomarkers in numerous human diseases [12]. Recent studies have demonstrated that miRNAs are related to the development of the EMT and fibrosis in various organs [13, 14]. Among these miRNAs, miRNA-21 has been shown to be upregulated during HG stimulation [15], and it also induced organ fibrosis in liver [16], lungs [17], heart [18] and kidneys [19].

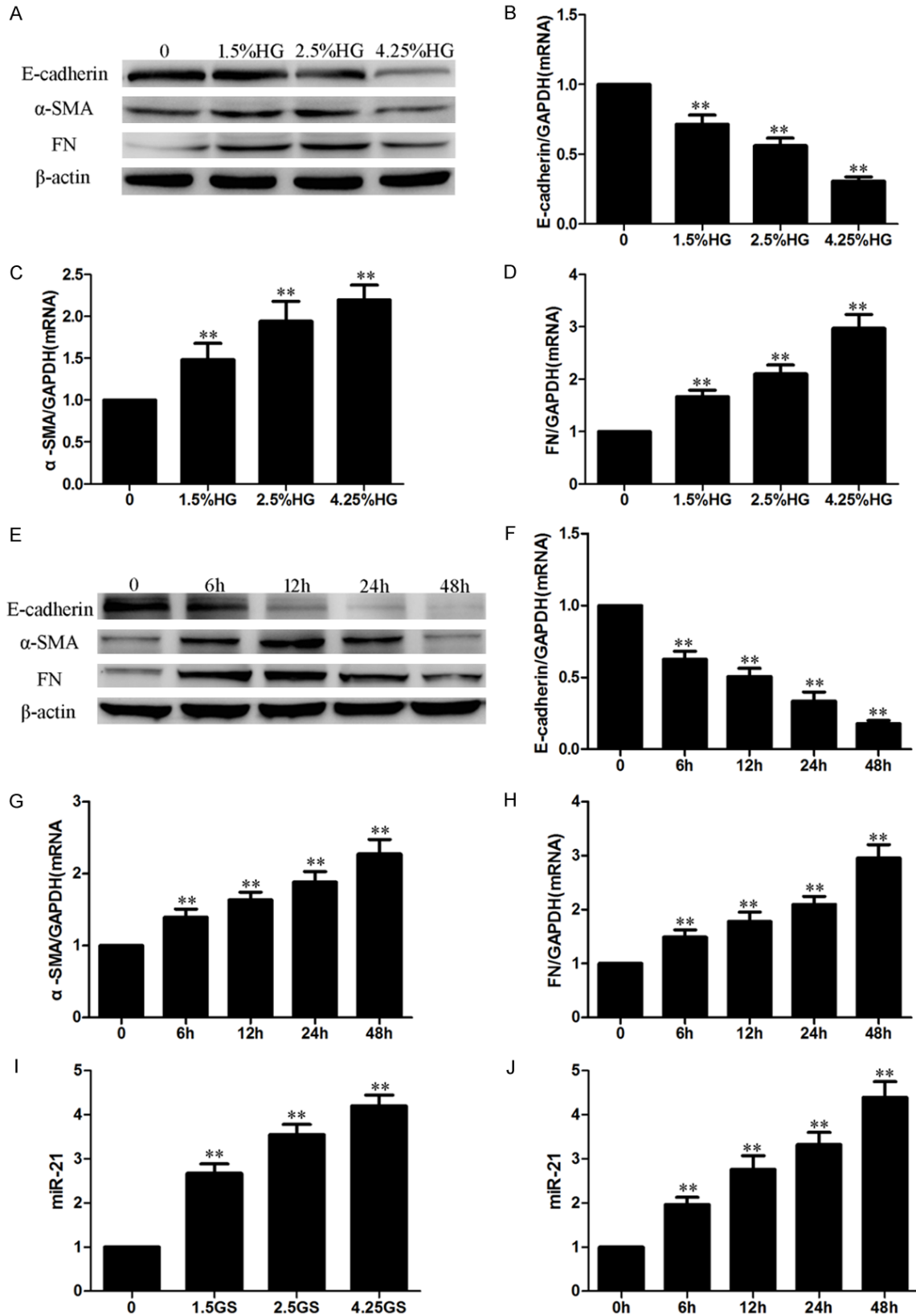However, the effect of miRNA-21 in the EMT process in HPMCs is not completely under-

Figure 1. HG induced the EMT and increased the level of miRNA-21 in HPMCs. HPMCs were cultured with HG of different concentrations (0, 1.5%, 2.5%, 4.25%) for 24 h, or 2.5% HG for different times (0 h, 6 h, 12 h, 24 h, 48 h). (A-D) HG induced the EMT in HPMCs in a dose-dependent manner (A: Western blot; B-D: Real-time PCR). (E-H) HG in-

duced the EMT in HPMCs in a time-dependent manner (E: Western blot; F-H: Real-time PCR). (I, J) HG increased the level of miRNA-21 in a dose-dependent (I) and time-dependent (J) manner (real-time PCR). Each value represents the mean ± SEM (n=6) (**P < 0.01 vs. control).

stood. In the present study, we aimed to investigate the expression of miRNA-21 and phosphatase and tensin homolog (PTEN) in HPMCs, and investigate their potential roles in the EMT in HPMCs.

Materials and methods

Reagents

RPMI 1640, *trypsin*-EDTA and fetal bovine serum (FBS) were obtained from Gibco. Bovine serum albumin (BSA) was purchased from Sigma. Rabbit monoclonal α-SMA, rabbit monoclonal E-cadherin, rabbit monoclonal PTEN, mouse monoclonal FN and mouse monoclonal β-actin antibodies were obtained from Abcam. Goat anti-rabbit and goat anti-mouse HRP-conjugated secondary antibodies and FITC-conjugated secondary antibody were purchased from Beyotime biotechnology (Jiangsu, China). The enhanced chemiluminescence (ECL) kit was purchased from Pierce Biotechnology, Inc. miRNA 21 inhibitor/mimic and miRNA 21 inhibitor/mimic negative controls were purchased from Guangzhou RiboBio Co., Ltd (China). Trizol, Lipofectamine 2000 transfection reagent and DAPI were obtained from Invitrogen.

Culture of HPMCs

As mentioned before [11], HPMCs were cultured in RPMI 1640 medium supplemented with 10% FBS, streptomycin and penicillin. Cells were cultured at 37°C with a 5% CO_2 atmosphere, the RPMI1640 medium was refreshed every 2-3 days. HPMCs were then digested and passaged with a sub-cultivation ratio of 1:3 to 1:4. HPMCs were cultured with HG of different concentrations (control group, 1.5% HG group, 2.5% HG group, 4.25% HG group) for 24 h, or 2.5% HG for different times (0 h, 6 h, 12 h, 24 h, 48 h). HPMCs were then transfected with miR21 mimic/inhibitor with or without HG treatment.

Transfections

HPMCs were transfected with human miRNA 21 inhibitor/mimic and negative controls using

lipofectamine 2000 reagent according to the manufacturer's instructions. Culture medium was removed after 24 h, and HPMCs were unstimulated or stimulated with HG for another 24 h, cells were then collected for real time PCR, western blotting and immunofluorescence staining.

Western blotting

Western blot analysis was carried out as previously mentioned [11]. The 50 ug proteins from HPMCs were separated by 10% SDS-PAGE at 70 V for 2 h and then transferred to a nitrocellulose membrane at 100 V for 1 h. After blocking with 5% non-fat dry milk, the membranes were incubated at 4°C overnight with dilution of 1:1,000 for primary antibodies and 1:5,000 for secondary antibodies. An ECL kit was used to develop the blots, and images were captured using UVP (G-BOX EF). We applied Image J software to measure the intensity of each band and relative expressions were normalized against β-actin. Experiments were repeated at least three times.

Real-time PCR

Trizol reagent was applied to extract total RNA according to the manufacturer's protocol. A Reverse Transcription System kit was used to synthesize first-strand cDNA according to the manufacturer's protocol (Takara). Real-time quantitative PCR was performed using the SYBR Premix Ex Taq II kit (Takara) with ABI 7500 Real-Time PCR System (Applied Biosystems, CA). The relative expression of each gene was measured after normalization with GAPDH. The cDNA sequences were amplified using *specific primers*, and the *sequences were as follows:* E-cadherin (sense) 5'-GGGCTGGA-CCGAGAGAGTTT-3' and (antisense) 5'-CCTTG-TACGTGGTGGGATTGA-3'; α-SMA (sense) 5'-AT-CATCACCAACTGGGACGAC-3' and (antisense) 5'-CTCTTCAGGGGCAACACGAA-3'; FN (sense) 5'-CCAAACCTCAAGCTCCCGTCA-3' and (antisense) 5'-GAGATTCTGCACATCACGGTCA-3'; PTEN (sense) 5'-CGACGGGAAGACAAGTTCAT-3' and (antisense) 5'-AGGTTTCCTCTGGTCCTGGT-3'; and GAPDH (sense) 5'-GCACCGTCAAGGCT-
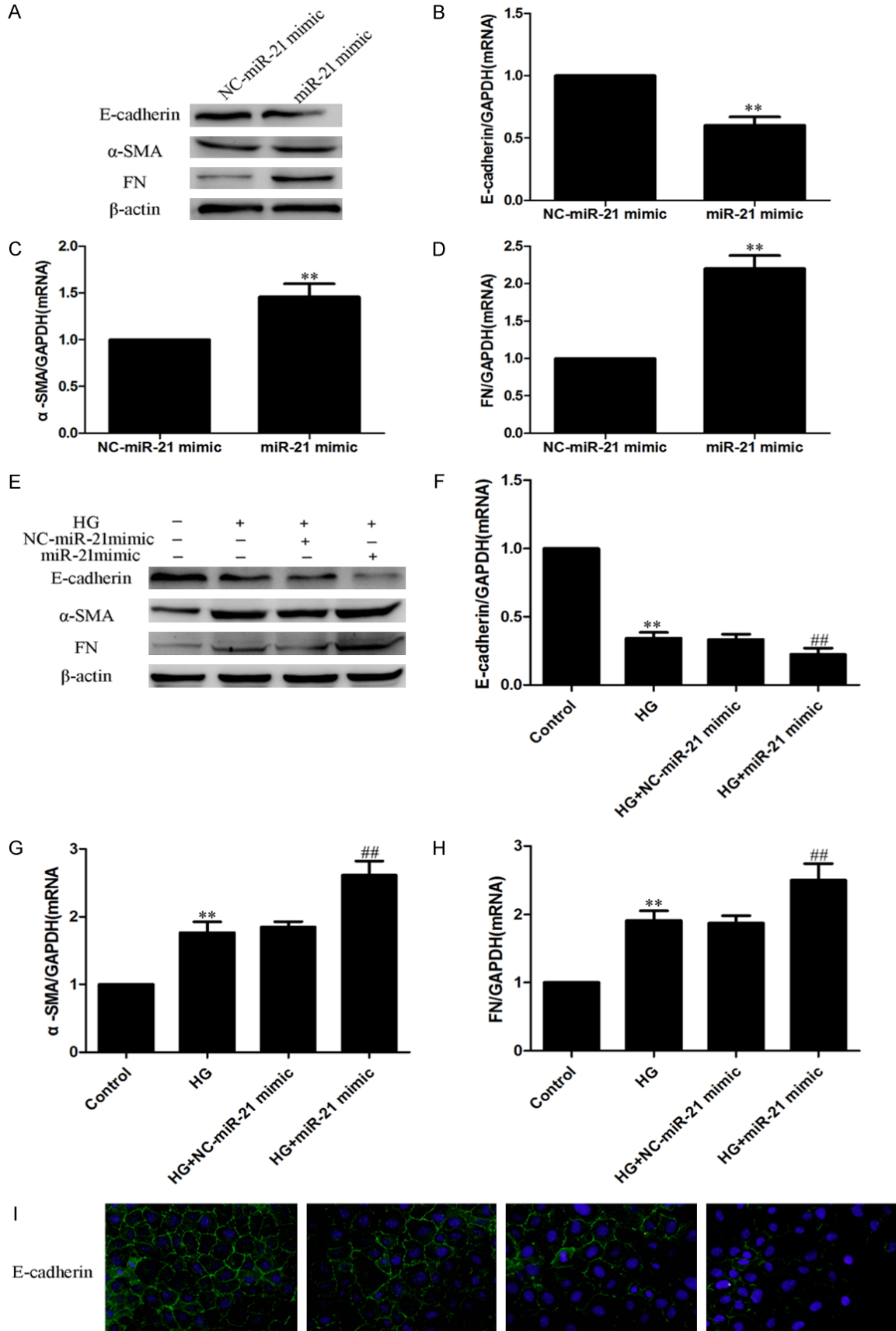
Figure 2. The miR-21 mimic increased the HG-induced EMT in HPMCs. HPMCs were transfected with miR21 mimic with or without 2.5% HG treatment. (A-D) Overexpression of miR-21 exacerbated the EMT in HPMCs. (A: Western blot; B-D: Real-time PCR). (E-I) Overexpression of miR-21 exacerbated the HG-induced EMT in HPMCs. (E: Western blot; F-H: Real-time PCR; I: Immunofluorescence staining of E-cadherin, magnification × 200). Each value represents the mean ± SEM (n=6) (**P < 0.01 vs. control; ##P < 0.01 vs. HG group).
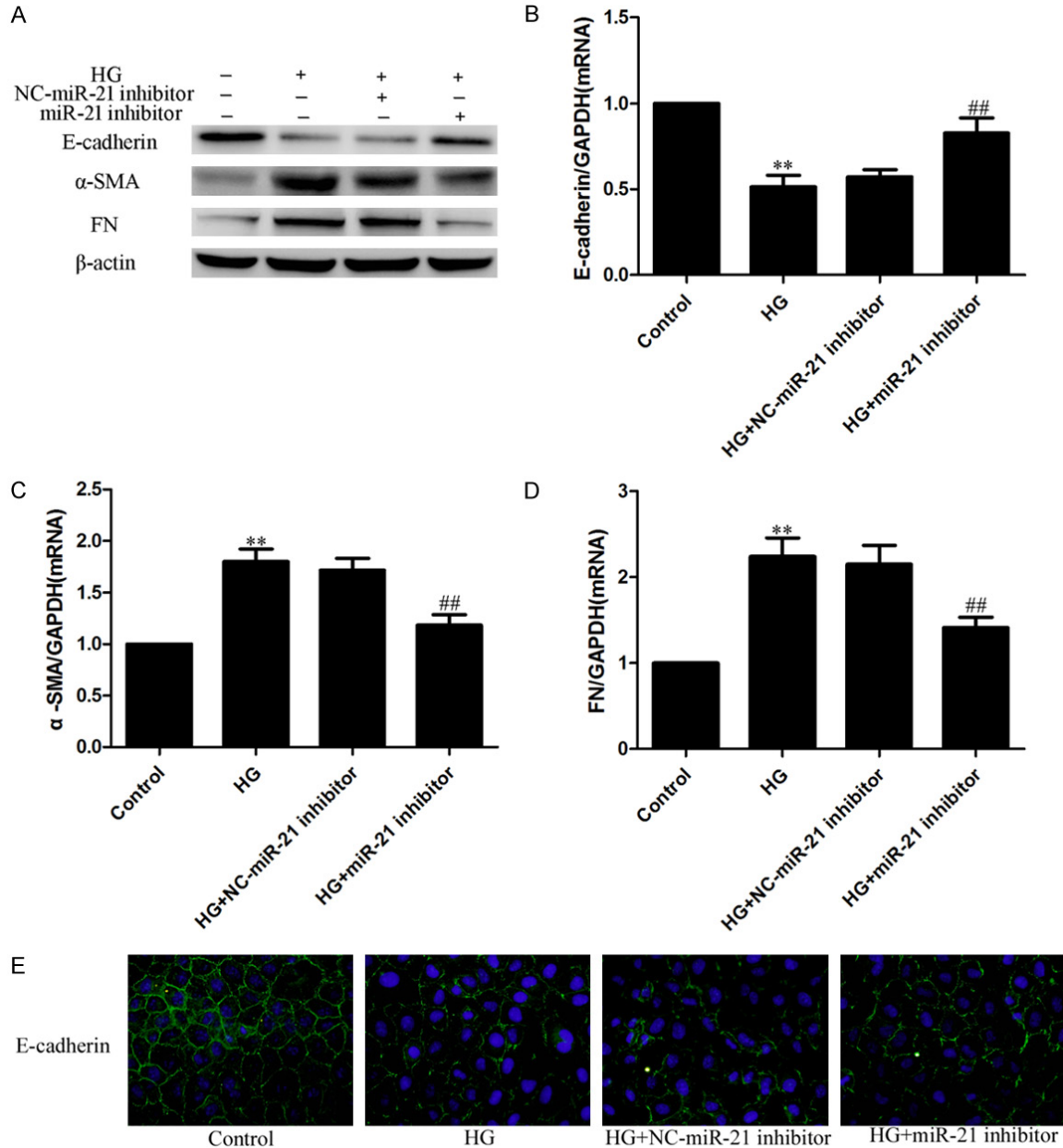

Figure 3. The miR-21 inhibitor alleviated the HG-induced EMT in HPMCs. HPMCs were transfected with miR21 inhibitor followed by 2.5% HG treatment. A: Western blots; B-D: Real-time PCR. Each value represents the mean ± SEM (n=6) (**P < 0.01 vs. control; ##P < 0.01 vs. HG group). E: Immunofluorescence staining of E-cadherin. Magnification × 200.

the primary antibody rabbit anti-E-cadherin (1:200) overnight at 4°C in a humidified chamber. After 3 washes with PBS for 5 min, HPMCs were incubated in FITC-conjugated secondary antibody (1:50) for 1 h at room temperature. The nuclei were stained with DAPI. Images
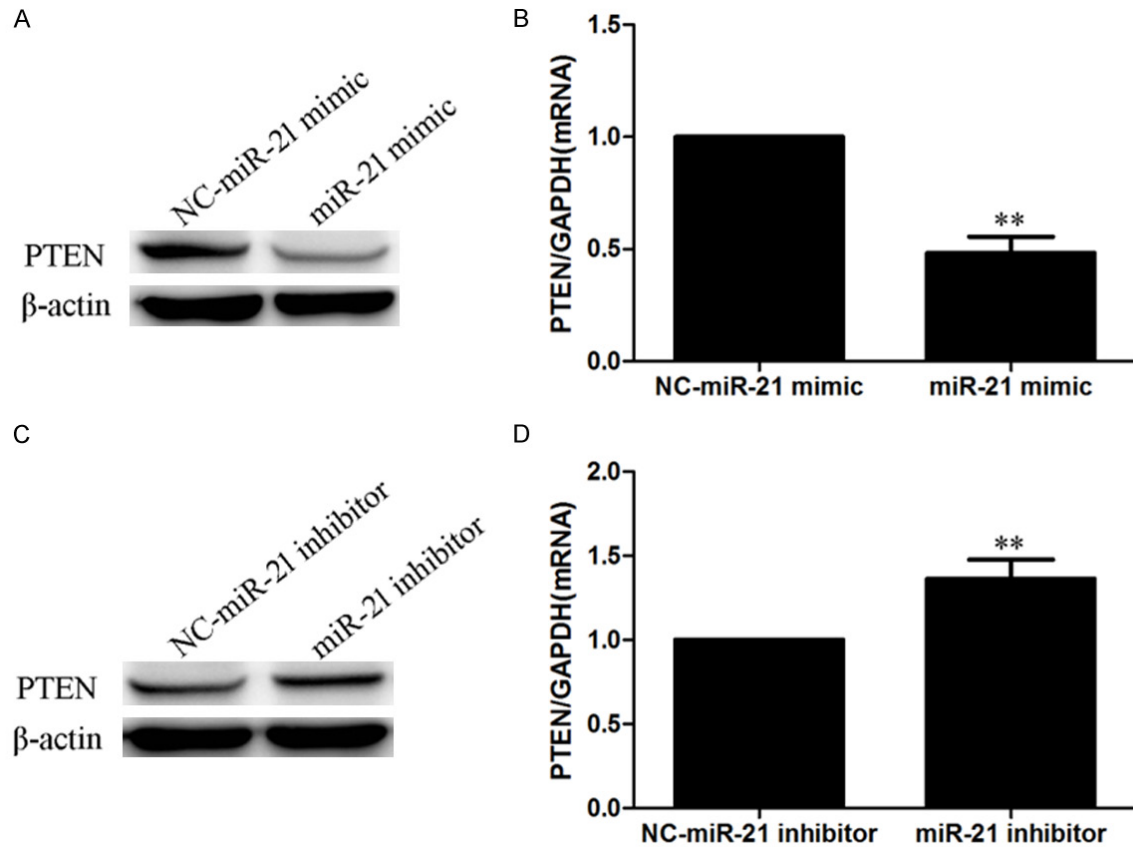
Figure 4. PTEN is a target of miR-21. HPMCs were transfected with miR21 mimic/inhibitor. (A, B) The miR-21 mimic inhibited PTEN expression (A: Western blot; B: Real-time PCR). (C, D) The miR-21 inhibitor upregulated PTEN expression (A: Western blot; B: Real-time PCR). (C: Western blot; D: Real-time PCR). Each value represents the mean ± SEM (n=6) (**$P < 0.01$ vs. miR-21 negative control).

were obtained using a fluorescence microscope (Nikon ECLIPSE Ti).

Statistical analyses

SPSS (version 18) software were used for data analysis. Quantitative data were expressed as means ± SEM. Tukey's multiple comparison test was used for individual comparisons; standard ANOVA methodology was used for multiple group comparisons ($P < 0.05$ was considered to be significant).

Results

HG induced the EMT and increased miRNA-21 expression in HPMCs

In order to explore the role of the HG-induced EMT in HPMCs, we measured the expression of EMT biomarkers in HG-treated HPMCs. Compared with the control group, HG signifi-

cantly increased the expression of α-SMA and FN, and decreased the expression of E-cadherin in a dose-dependent (**Figure 1A-D**) and time-dependent manner (**Figure 1E-H**) in HP-MCs, which means that HG treatment induced the EMT in HPMCs. **Figure 1I**, **1J** showed that HG treatment also remarkably increased miR-21 expression in a dose- and time-dependent manner.

The miR-21 mimic exacerbated the HG-induced EMT in HPMCs

To confirm the role of miR-21 in the EMT in HPMCs, the miR-21 mimic and negative control were transfected into HPMCs; cells were then cultured with or without HG for another 24 h. Western blotting, real-time PCR and immuno-fluorescence indicated that the miR-21 mimic significantly increased the levels of α-SMA and FN, and decreased the level of E-cadherin (**Figure 2A-D**), which suggests that the miR-21
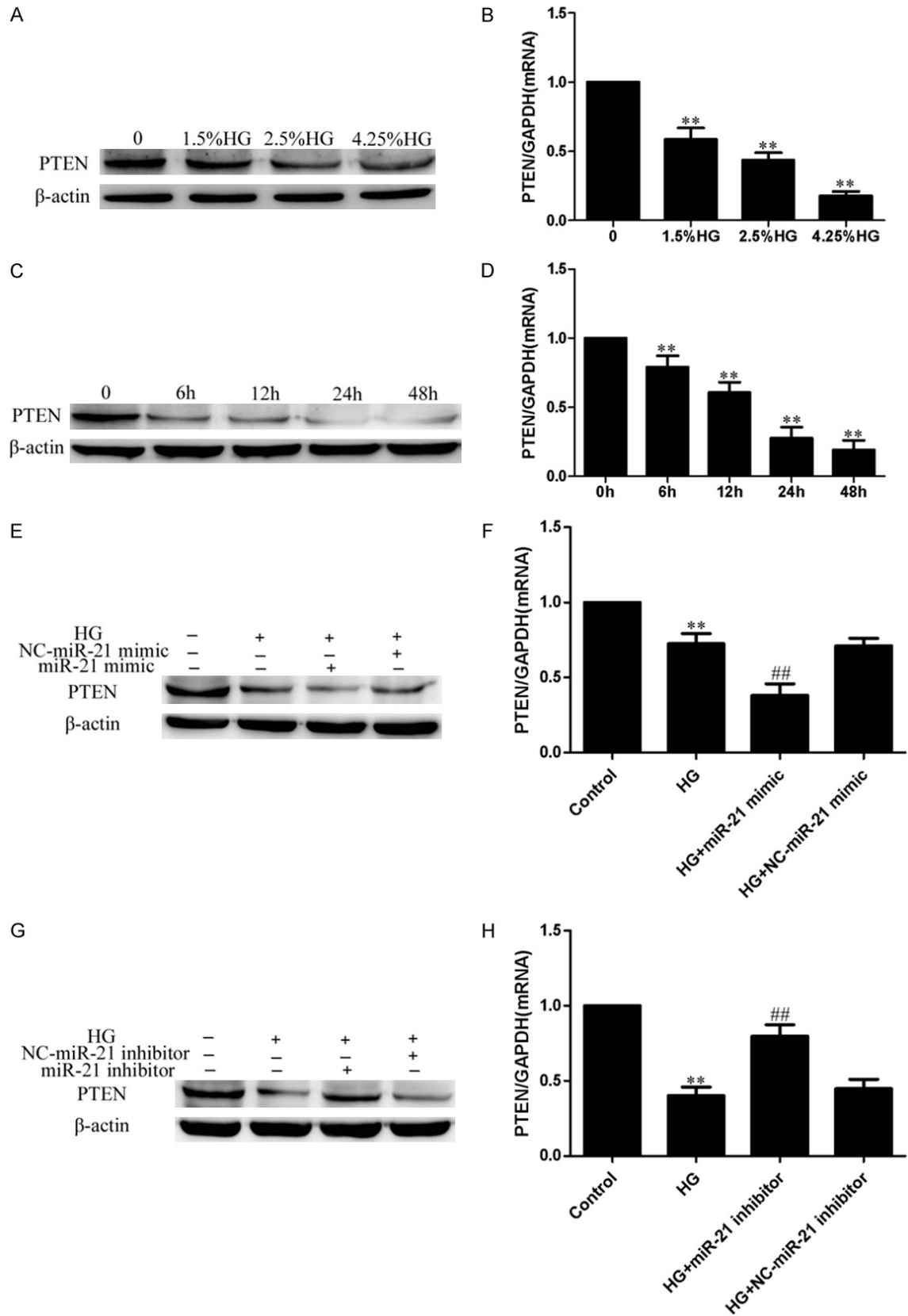
Figure 5. miR-21 directly targeted PTEN in HG-treated HPMCs. HPMCs were cultured with HG of different concentrations (0, 1.5%, 2.5%, 4.25%) for 24 h, or 2.5% HG for different times (0 h, 6 h, 12 h, 24 h, 48 h), then were trans-

fected with miR21 mimic/inhibitor followed by 2.5% HG treatment. (A, B) HG inhibited PTEN expression in a dose-dependent manner (A: Western blot; B: Real-time PCR). (C, D) HG inhibited PTEN expression in a time-dependent manner (C: Western blot; D: Real-time PCR). (E, F) The miR-21 mimic further inhibited PTEN expression in HG-treated HPMCs (E: Western blot; F: Real-time PCR). (G, H) The miR-21 inhibitor partly restored the expression of PTEN in HG-treated HPMCs (G: Western blot; H: Real-time PCR). Each value represents the mean ± SEM (n=6) (**P < 0.01 vs. control; ##P < 0.01 vs. HG group).

mimic can induce the EMT. Moreover, the miR-21 mimic exacerbated the progress of the HG-induced EMT (**Figure 2E-I**). These data demonstrated that miR-21 exacerbated the HG-induced EMT in HPMCs.

The miR-21 inhibitor alleviated the HG-induced EMT in HPMCs

To further explore the role of miR-21 in the EMT, the miR-21 inhibitor and negative control were transfected into HPMCs; cells were then cultured with or without HG for another 24 h. Western blotting, real-time PCR and immuno-fluorescence showed that the miR-21 inhibitor significantly alleviated the upregulation of the mesenchymal markers α-SMA and FN and reduced the downregulation of the epithelial marker E-cadherin in HG-treated HPMCs (**Figure 3A-E**). Therefore, this shows that the miR-21 inhibitor can decrease the HG-induced EMT in HPMCs.

miR-21 directly targeted PTEN in the HG-induced EMT in HPMCs

To verify whether miR-21 targeted PTEN in the HG-induced EMT, PTEN expression was measured after miR-21 mimic and inhibitor transfection in HPMCs with or without HG treatment. Real-time PCR and Western blotting showed that the miR-21 mimic downregulated PTEN expression (**Figure 4A**, **4B**), and that the miR-21 inhibitor upregulated PTEN expression (**Figure 4C**, **4D**).

HG inhibited PTEN expression in a dose- and time-dependent manner (**Figure 5A-D**). PTEN expression was also measured after miR-21 mimic/inhibitor transfection in HG-treated HPMCs. Compared to the HG group, the miR-21 mimic further inhibited PTEN expression in HG-treated HPMCs (**Figure 5E**, **5F**); the miR-21 inhibitor partly restored the expression of PTEN in HG-treated HPMCs (**Figure 5G**, **5H**). These results demonstrated that miR-21 directly targeted PTEN in HG-treated HPMCs.

Discussion

PD is extensively used for the long-term treatment of ESRD; however, HG solutions can lead to morphological and functional injury in the peritoneum, and may eventually cause peritoneal fibrosis and ultrafiltration failure. The HG-induced EMT in HPMCs plays a crucial role in peritoneal dysfunction and fibrosis [8]. Therefore, preventing the EMT could alleviate peritoneal fibrosis, protecting the peritoneal mesothelium in patients undergoing PD [9].

Increasingly more research have shown that miRNAs play a vital role in the process of EMT [20, 21]. miRNA-21 is present in plasma and peritoneal fluid [22-24]. Previous studies have indicated that miRNA21 plays a crucial role in the process of EMT and peritoneal fibrosis in PD [25, 26]. However, the molecular mechanisms of miR-21 in the EMT in HPMCs are still poorly understood.

In the current study, we investigated how miRNA-21 could contribute to the EMT in HG-treated HPMCs. We found that HG could induce the EMT in HPMCs, with a concomitant increase in the level of miRNA-21. We also showed that miR-21 could induce the EMT, and the miR-21 mimic increased this HG-induced EMT in HPMCs, while the miR-21 inhibitor attenuated the HG-induced EMT in HPMCs. Overall, we demonstrated that miRNA-21 was involved in the HG-induced EMT in HPMCs.

microRNAs affect organ EMT and fibrosis by regulating the expression of target genes [27]. Studies have shown that PTEN is related to the miR-21-induced EMT and that PTEN is a direct targeted gene of miR-21 [28-30]. Therefore, we explored whether miR-21 was able to regulate the EMT by targeting PTEN. We measured PTEN expression in this context, and found that miR-21 directly targeted PTEN in the HG-induced EMT in HPMCs.

In summary, this investigation has shown that miR-21 plays a vital role in the HG-induced EMT

in HPMCs, by targeting PTEN. According to the current investigation, we found that miR-21 could be a promising predictor for diagnosing and treating the effects of HG.

Disclosure of conflict of interest

None.

Address correspondence to: Jianfei Ma, Department of Nephrology, The First Affiliated Hospital of China Medical University, 155th Nanjing North Street, Shenyang 110001, Liaoning, P. R. China. Fax: +86 24 83282361; E-mail: majianfei56@sohu.com

References

[1] Jain AK, Blake P, Cordy P and Garg AX. Global trends in rates of peritoneal dialysis. J Am Soc Nephrol 2012; 23: 533-544.

[2] Mortier S, De Vriese AS and Lameire N. Recent concepts in the molecular biology of the peritoneal membrane-implications for more biocompatible dialysis solutions. Blood Purif 2013; 21: 14-23.

[3] Ahmad M, Shah H, Pliakogiannis T and Oreopoulos DG. Prevention of membrane damage in patient on peritoneal dialysis with new peritoneal dialysis solutions. Int Urol Nephrol 2007; 39: 299-312.

[4] Krediet RT and Struijk DG. Peritoneal changes in patients on long-term peritoneal dialysis. Nat Rev Nephrol 2013; 9: 419-429.

[5] Ding X, Ma M, Teng J, Shao F, Teng RK, Zhou S, Zhang Y, Wu E and Wang X. Numb induces e-cadherin adhesion dissolution, cytoskeleton reorganization, and migration in tubular epithelial cells contributing to renal fibrosis. Curr Mol Med 2015; 15: 368-379.

[6] Zhao S, Zhang Y, Zheng X, Tu X, Li H, Chen J, Zang Y, Zhang J. Loss of microRNA-101 promotes epithelial to mesenchymal transition in hepatocytes. J Cell Physiol 2015; 230: 2706-2717.

[7] Strippoli R, Loureiro J, Moreno V, Benedicto I, Lozano ML, Barreiro O, Pellinen T, Minguet S, Foronda M, Osteso MT, Calvo E, Vázquez J, Cabrera ML and Del Pozo MA. Caveolin-1 deficiency induces a MEK-ERK1/2-Snail-1-dependent epithelial-mesenchymal transition and fibrosis during peritoneal dialysis. EMBO Mol Med 2015; 7: 357.

[8] Yáñez-Mó M, Lara-Pezzi E, Selgas R, Ramírez-Huesca M, Domínguez-Jiménez C, Jiménez-Heffernan JA, Aguilera A, Sánchez-Tomero JA, Bajo MA, Alvarez V, Castro MA, del Peso G, Cirujeda A, Gamallo C, Sánchez-Madrid F and López-Cabrera M. Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med 2003; 348: 403-413.

[9] Yu MA, Shin KS, Kim JH, Kim YI, Chung SS, Park SH, Kim YL and Kang DH. HGF and BMP-7 ameliorate high glucose-induced epithelial-to-mesenchymal transition of peritoneal mesothelium. J Am Soc Nephrol 2009; 20: 567-581.

[10] Liu J, Zeng L, Zhao Y, Zhu B, Ren W and Wu C. Selenium suppresses lipopolysaccharide-induced fibrosis in peritoneal mesothelial cells through inhibition of epithelial-to-mesenchymal transition. Biol Trace Elem Res 2014; 161: 202-209.

[11] Yang L, Wu L, Zhang X, Hu Y, Fan Y and Ma J. 25(OH)2D3/VDR attenuates high glucose induced epithelial mesenchymal transition in human peritoneal mesothelial cells via the TGFβ/Smad3 pathway. Mol Med Rep 2017; 15: 2273-2279.

[12] Liang Z and Xi Y. MicroRNAs mediate therapeutic and preventive effects of natural agents in breast cancer. Chin J Nat Med 2016; 14: 881-887.

[13] Zhang Q, Ye H, Xiang F, Song LJ, Zhou LL, Cai PC, Zhang JC, Yu F, Shi HZ, Su Y, Xin JB and Ma WL. miR-18a-5p inhibits sub-pleural pulmonary fibrosis by targeting TGF-β receptor II. Mol Ther 2017; 25: 728-738.

[14] Xu M, Wang G, Zhou H, Cai J, Li P, Zhou M, Lu Y, Jiang X, Huang H, Zhang Y and Gong A. TGF-β1-miR-200a-PTEN induces epithelial-mesenchymal transition and fibrosis of pancreatic stellate cells. Mol Cell Biochem 2017; 431: 161-168.

[15] Lu X, Fan Q, Xu L, Li L, Yue Y, Xu Y, Su Y, Zhang D and Wang L. Ursolic acid attenuates diabetic mesangial cell injury through the up-regulation of autophagy via miRNA-21/PTEN/Akt/mTOR suppression. PLoS One 2015; 10: e0117400.

[16] Noetel A, Kwiecinski M, Elfimova N, Huang J and Odenthal M. MicroRNA are central players in anti- and profibrotic gene regulation during liver fibrosis. Front Physiol 2012; 3: 49.

[17] Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N and Abraham E. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J Exp Med 2010; 207: 1589-1597.

[18] Brønnum H, Andersen DC, Schneider M, Sandberg MB, Eskildsen T, Nielsen SB, Kalluri R and Sheikh SP. MiR-21 promotes fibrogenic epithelial-to-mesenchymal transition of epicardial mesothelial cells involving programmed

cell death 4 and sprouty-1. PLoS One 2013; 8: e56280.

[19] Glowacki F, Savary G, Gnemmi V, Buob D, Van der Hauwaert C, Lo-Guidice JM, Bouyé S, Hazzan M, Pottier N, Perrais M, Aubert S and Cauffiez C. Increased circulating miR-21 levels are associated with kidney fibrosis. PLoS One 2013; 8: e58014.

[20] Li M, Li H, Liu X, Xu D and Wang F. MicroRNA-29b regulates TGF-β1-mediated epithelial-mesenchymal transition of retinal pigment epithelial cells by targeting AKT2. Exp Cell Res 2016; 345: 115-124.

[21] Ma L, Teruya-Feldstein J and Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 2007; 449: 682-688.

[22] Kanaan Z, Rai SN, Eichenberger MR, Roberts H, Keskey B, Pan J and Galandiuk S. Plasma miR-21: a potential diagnostic marker of colorectal cancer. Ann Surg 2012; 256: 544-551.

[23] Cappellesso R, Tinazzi A, Giurici T, Simonato F, Guzzardo V, Ventura L, Crescenzi M, Chiarelli S and Fassina A. Programmed cell death 4 and microRNA 21 inverse expression is maintained in cells and exosomes from ovarian serous carcinoma effusions. Cancer Cytopathol 2014; 122: 685-693.

[24] Komatsu S, Ichikawa D, Takeshita H, Tsujiura M, Morimura R, Nagata H, Kosuga T, Iitaka D, Konishi H, Shiozaki A, Fujiwara H, Okamoto K and Otsuji E. Circulating microRNAs in plasma of patients with oesophageal squamous cell carcinoma. Br J Cancer 2011; 105: 104-111.

[25] Lopez-Anton M, Lambie M, Lopez-Cabrera M, Schmitt CP, Ruiz-Carpio V, Bartosova M, Schaefer B, Davies S, Stone T, Jenkins R, Taylor PR,Topley N, Bowen T and Fraser D. miR-21 promotes fibrogenesis in peritoneal dialysis. Am J Pathol 2017; 187: 1537-1550.

[26] Szeto CC, Chow KM, Kwan BC, Cheng PM, Luk CC, Ng JK, Law MC, Leung CB and Li PK. Peritoneal dialysis effluent miR-21 and miR-589 levels correlate with longitudinal change in peritoneal transport characteristics. Clin Chim Acta 2017; 464: 106-112.

[27] Zou XZ, Liu T, Gong ZC, Hu CP and Zhang Z. MicroRNAs-mediated epithelial-mesenchymal transition in fibrotic diseases. Eur J Pharmacol 2017; 796: 190-206.

[28] Bao L, Yan Y, Xu C, Ji W, Shen S, Xu G, Zeng Y, Sun B, Qian H, Chen L, Wu M, Su C and Chen J. MicroRNA-21 suppresses PTEN and hSulf-1 expression and promotes hepatocellular carcinoma progression through AKT/ERK pathways. Cancer Lett 2013; 337: 226-236.

[29] Zhu W and Xu B. MicroRNA-21 identified as predictor of cancer outcome: a meta-analysis. PLoS One 2014; 9: e103373.

[30] Liu ZL, Wang H, Liu J and Wang ZX. MicroRNA-21 (miR-21) expression promotes growth, metastasis, and chemo- or radio resistance in non-small cell lung cancer cells by targeting PTEN. Mol Cell Biochem 2013; 372: 35-45.