MiR-15a-5p suppresses inflammation and fibrosis of peritoneal mesothelial cells induced by peritoneal dialysis via targeting VEGFA

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ABSTRACT

Long-term peritoneal dialysis often ends up with ultrafiltration failure (UFF) which is partially caused by persistent inflammation and fibrosis of peritoneal tissues. However, the mechanism is still unclear. In the current study, the peritoneum from UFF patients demonstrated inflammation and fibrosis which were positively related to expression of VEGFA. The *in vitro* model using human peritoneal mesothelial cells (HPMCs) stimulated by high glucose or advanced glycation end (AGE) product showed consistent changes of inflammation, fibrosis and VEGFA. What's more, we showed that VEGFA was an instigator of inflammation and fibrosis. Several miRNAs have been reported to regulate expression of VEGFA elsewhere. Five of them were selected to test the expression in peritoneum of PD patients. Results suggested that miR-15a-5p was the most significantly down-regulated one. Also, in high glucose or AGE product -stimulated HPMCs, miR-15a-5p decreased. When miRNA mimic was used to restore the expression of miR-15a-5p, high glucose-induced VEGFA was repressed. The predicted binding site between these two molecules was confirmed by dual-luciferase assay. Restoration of miR-15a-5p restrained inflammation and fibrosis of HMPCs. TGF-β1/Smad2 was shown to be the down-stream signaling pathway and their activity was regulated by miR-15a-5p/VEGFA. In conclusion, our present study demonstrates that miR-15a-5p acts as a regulator of VEGFA mRNA and the following inflammation and fibrosis in peritoneal mesothelial cells. The miR-15a-5p/VEGFA pathway may be a potential target for preventing ultrafiltration failure in peritoneal dialysis patients.

INTRODUCTION

Ultrafiltration failure (UFF) caused by peritoneal fibrosis (PF) is currently a challenge for peritoneal dialysis (PD). UFF has been the leading cause of PD dropping out and has been closely associated with patients' survival (Chemek et al., 2018). Several factors, including inflammatory cell infiltration, neoangiogenesis, mesothelial-mesenchymal transition and interstitial fibrosis (mainly caused by extracellular matrix deposition), play important roles in UFF (Jiang et al., 2018). Of all these, inflammation and fibrosis are believed to be directly associated with UFF and therefore obtained most attentions (Davies, 2016). The normal function of peritoneal membrane is essential for the acceptance of PD (Margetts and Bonniaud, 2003). Mesothelial cell, covering the surface of peritoneal membrane, is the first barrier to PD solution. Various ingredients, especially high concentration of glucose, could induce injury to mesothelial cells. Such damage, accompanied with inflammation, may lead to fibrosis and, consequently, decreased efficiency of PD (Krediet et al., 2000). Also, it has been reported that long-term exposure to high concentration of glucose could lead to deposition of advanced glycation end (AGE) product (Kawanishi et al., 2013). The accumulation of AGE product could also be a risk factor for UFF. It has been shown that a reduction in AGE product relieved PD (Honda et al., 1999).

Vascular endothelial growth factor (VEGF) is a family of proteins that was initially reported to promote the formation of vascular endothelial cells. Seven members have been identified in the family, and of which, VEGFA is the most ubiquitously and abundantly expressed member. Previous studies have indicated that over-expressed VEGF is closely related to PF and UFF (Liu et al., 2018; Tang, 2017). However, its role and

related mechanism in peritoneal mesothelial cells need to be further verified.

MicroRNAs, a class of endogenous non-coding small RNAs in eukaryotes, are about 20-22 nucleotides in length and regulate the expression of target genes at post-transcriptional level. By completely or incompletely pairing with bases in the 3'UTR of target mRNAs, miRNAs facilitate degradation of target mRNAs, inhibits translation, therefore influence cell growth, differentiation, apoptosis and tumorigenesis (Guan et al., 2016). Several miRNAs have been reported to regulate VEGFA mRNA in different conditions(Aqeilan et al., 2010). However, the functional one in mesothelial cells during UFF has yet to be known.

Evidence from literature showed that TGF-β1/Smad2 play central roles in regulating extracellular matrix accumulation of HPMCs and fibrosis of peritoneum (Yao et al., 2007). Here we tested their involvement in VEGFA function of high glucose stimulated HPMCs.

In this study, both human peritoneal tissues and cultured mesothelial cells were used to investigate the role of VEGFA in process of peritoneal inflammation and fibrosis. Then the putative regulatory miRNA for VEGFA was discussed. Potential signaling pathway was evaluated. Our study will provide novel evidence to the mechanism of peritoneum fibrosis and inflammation caused by PD which may inspirit potential therapeutic target for preventing UFF.

MATERIALS AND METHODS

Tissue collection

All human peritoneal tissues were collected from the First Affiliated Hospital of Zhengzhou University. Normal peritoneal tissues were excision redundant of abdominal surgical. PD samples were collected from extubating procedure of patients with peritoneal ultrafiltration failure. PD duration of all these patients was at least 3.5 years. These investigations were conducted in accordance with the principles of the Declaration of Helsinki and were approved by the Research Ethics Committee of Zhengzhou University. Informed consent has been obtained from each patient.

Tissue staining

The standard tissues embedded in paraffin were sectioned at 4 μm. Hematoxylin-eosin (HE), Periodic Acid-Schiff (PAS) and Masson staining were carried out as previously described (Li et al., 2017; Otali et al., 2016). The fibrosis degree was assessed by two independent investigators (Q. H and L. S) according to the thickness and compactness of fiber. The final score was the average value.

Cell culture

Human peritoneal mesothelial cells (HPMCs) were a generous gift of Professor Zongpei Jiang from Sixth Affiliated Hospital of Sun Yat-sen University. DMEM/F12 medium was used for cell culture, and the cultural procedure was carried out as previously described (Shang et al., 2017). High glucose (concentration at 75 or 125 mmol/L in cultural medium) was used as a stimulator and mannitol of the same concentration was used as an osmolality control. AGE product at concentration of 50 or 100μg/mL

was also used in our experiment.

Western blotting

All the procedures were performed as described previously (Zhu et al., 2018). Briefly, cell lysate was extracted using RIPA buffer (CWBiotech Co., Beijing, China), and the concentration was measured using BCA protein assay kit (CWBiotech Co., Beijing, China). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed before protein was transferred to polyvinylidene fluoride (PVDF) membranes (Roche, Mannheim, Germany). The membranes were blocked using 3% de-fat milk shaking for 2 hours at room temperature. After that, they were incubated with primary antibodies at 4°C overnight and secondary antibody at room temperature for 2 hours. Enhanced chemiluminescence reagent kit (CWBiotech Co., Beijing, China) was used to detect the bands. Primary antibodies of anti-VEGFA (1:1000, ProteinTech Group, Chicago, IL), anti-Collagen IV, anti-Fibronectin (1:1000, Abcam, Cambridge, MA), anti-Smad2 (1:1000, Cell Signaling Technology, Danvers, MA), anti-p-Smad2 (1:1000, Cell Signaling Technology, Danvers, MA) and anti-GAPDH (1:1000, Cell Signaling Technology, Danvers, MA) were used in this study. Secondary antibodies (Dingguo Changsheng Biotech Co., Ltd.) were used at 1:1000 dilution.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described previously (Xiao et al., 2015). Expression of miRNAs was measured by miR qRT-PCR Quantitation Kit (Novland BioPharma, Shanghai, China) according to the

manufacturer's instruction. Reverse transcription of mRNA was taken out using HiScript Q RT SuperMix for qPCR (Vazyme, Nanjing, China) and expression of VEGFA mRNA was measured by AceQTM qPCR SYBR Green Master Mix (Novland Bio-Pharma, Shanghai, China). Primers for mRNA detection: VEGFA F: 5'-CTAATGTTATTGGTGTCTTCA-3'; R: 5'-TCTCATCTCCTCCTCTTC-3'; GAPDH F: 5'-CTCTGGTAAAGTGGATATTGT-3'; R: 5'-GGTGGAATCATATTGGAACA-3'.

Enzyme-linked immunosorbent assay

Tissue homogenate or the supernatant of treated cells were collected and stored at −80°C. Concentrations of VEGFA (Abcam, Cambridge, MA), interleukin (IL) -6 (Thermo Fisher Scientific, USA), monocyte chemotactic protein (MCP) -1 (Abcam, Cambridge, MA), transforming growth factor (TGF) -β1 (Abcam, Cambridge, MA), IL-17a (Abcam, Cambridge, MA), TNF-α (Abcam, Cambridge, MA) and IL-8 (Thermo Fisher Scientific, USA) were measured by Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction.

Cell transfection

MiRNA mimic targeting miR-15a-5p and siRNA targeting VEGFA were synthesized by RiboBio Co. (Guangzhou, China). The oligodeoxynucleotide sequences used in this study were as follows: miR-15a-5p mimic: Sense: 5'-UAGCAGCACAUAAUGGUUUGUG-3'; Anti-sense: 5'-CACAAACCAUUAUGUGCUGCUA-3'; miR-15a-5p inhibitor: 5'-CACAAACCAUUAUGUGCUGCUA-3'. SiRNA for

VEGFA: 5'-GGAGTACCCTGATGAGATC-3'. Lipofectamine 2000 (GenePharma, Shanghai, China) was used for transfection as previously described (Schneider et al., 2017). Transfection efficiency was measured by RT-PCR or western blotting.

Dual-luciferase reporter assay

To test the direct binding ability between miR-15a-5p and VEGFA mRNA, we performed dual-luciferase reporter assay. The predicted binding sequence was identified and downloaded from TargetScan (http://www.targetscan.org). Recombinant pmirGLO plasmid containing binding sequence was constructed by GenePharma (Shanghai, China). Enzyme activity was measured by Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). The difference in mean values between different groups was examined using one-way ANOVA followed by Duncan's multiple range test. Pearson correlation coefficient was calculated to analyze correlation between two data sets. A P value < 0.05 at two-tailed was considered as statistically significant.

RESULTS

Fibrosis and inflammation in peritoneum of long-term PD patients were associated with increased VEGFA

HE, PAS and Masson staining showed that compact and thick fibrotic tissues were only

found in patients with long-term PD, but not in controls (Figure 1 A). Summarized data indicated that, peritoneum of PD group showed different degrees of fibrosis (Figure 1 B). Expression of VEGFA was semi-quantitatively measured by ELISA and results showed that it was increased in PD group (Figure 1 C). To further evaluate the role of VEGFA in peritoneal fibrosis, we performed the correlation analysis. It was indicated that higher level of VEGFA was closely related with aggravated fibrosis of peritoneal tissues (Figure 1 D). Then we measured the expression of inflammatory factors including IL-6, MCP-1, TGF-β1 and IL-8 in the supernatant of tissue homogenate by ELISA and results showed that all these proteins were significantly increased in PD group (Figure 2 A). We also estimated their correlation with VEGFA. Results indicated that higher level of VEGFA was closely related with increased inflammatory factors of peritoneal tissues (Figure 2 B). These results together suggested that peritoneal inflammation and fibrosis in long-term PD patients were closely related with over-expressed VEGFA.

Over-expressed VEGFA was related with fibrosis and inflammation in cultured HPMCs

HPMCs were cultured *in vitro* and stimulated by either high glucose or AGE product at different concentrations. Cells were collected and total protein was extracted to measure the expression of VEGFA, fibronectin and collagen IV. It was shown that both high glucose and AGE product were associated with promoted expressions of these proteins (Figure 3 A and B). Then the supernatant was collected to measure the concentrations of inflammatory factors by ELISA. Results showed that high glucose or AGE product stimulation resulted in increased release of inflammatory factors (Figure 3 C and D).

Fibrosis and inflammation was regulated by VEGFA in HPMCs

To investigate the role of VEGFA on fibrosis and inflammation, we constructed the specific siRNA targeting VEGFA. Real-time PCR and western blotting showed high gene interference efficiency at protein level (Figure 4 A). SiRNA (or negativecontrol) was transfected into HPMCs before high glucose stimulation. Western blotting showed that the high glucose-induced expression of fibronectin and collagen IV was significantly inhibited by siRNA transfection (Figure 4 B). Also, the over-expressed inflammatory factors were attenuated by the siRNA targeting VEGFA (Figure 4 C). As a whole, it was suggestive that VEGFA may play an important role in regulation of fibrosis and inflammation in HPMCs.

MiR-15a-5p was the significantly down-regulated miRNA in HPMCs

Several miRNAs have been reported elsewhere to regulate expression of VEGFA. Here we detected five of those. RT-PCR using supernatant of tissue showed that miR-15a-5p was the most significantly decreased one in PD patients compared with control (Figure 5 A). Then cultured HPMCs were used as the *in vitro* model of PD to verify the expression of miR-15a-5p. Results showed that it also gradually decreased as the concentration of high glucose or AGE increased (Figure 5 B).

MiR-15a-5p regulates VEGFA expression by directly binding to its mRNA

Next we investigated whether over-expression of miR-15a-5p affect high glucose/AGE-induced fibrosis and inflammation. MiRNA mimic specific for miR-15a-5p was constructed while vector was used as control. RT-PCR showed that miRNA mimic

efficiently increased the expression of miR-15a-5p in HPMCs cultured under a standard condition (Figure 6 A). Under high glucose or AGE product condition, miRNA mimic also restored the expression of miR-15a-5p. In both conditions, negative control showed no effect (Figure 6 B). Then RT-PCR showed that miR-15a-5p mimic significantly restrained high glucose-induced VEGFA mRNA, suggesting the regulatory role of miR-15a-5p in VEGFA mRNA manipulation (Figure 6 C). We further explored the putative mechanism of miRNA-15a-5p-regulated VEGFA. Using Target Scan to predict the binding possibility, it was shown a strong putative binding site between 276 and 283 in VEGFA 3'UTR region (Figure 6 D). To verify this binding effect, a dual-fluorescence reporter system was constructed. HPMCs were co-transfected with miR-15a-5p mimic and luciferase system containing wild type or mutant sequence of the predicted target binding sequence in VEGFA mRNA. Enzyme activity was measured, showing that over-expression of miR-15a-5p significantly decreased enzyme activity in the wild type group. However, such effect was eliminated in the mutant group (Figure 6 E), indicating the identification of the binding sites. These results showed that miR-15a-5p regulated VEGFA expression by directly binding to its mRNA.

Over-expression of miR-15a-5p could inhibit VEGFA, fibrosis and inflammation in cultured HPMCs

HPMCs were transfected with mimic or negative control before high glucose or high AGE product stimulation. Western blotting showed that both high glucose and AGE-induced expression of VEGFA, fibronectin and collagen IV were significantly suppressed by miRNA mimic (Figure 7). ELISA showed that the elevated concentration of

inflammatory factors in supernatant of cultured HPMCs was attenuated by transfection of mimic (Figure 8). All these results indicated that miR-15a-5p may play an important role in regulation of VEGFA, fibrosis and inflammation induced by either high glucose or AGE product.

TGF-β1/Smad2 was the down-stream signaling pathway of miR-15a-5p/VEGFA

The phosphorylation state of Smad2 was tested by western blot. Results showed that high glucose could activate Smad2 by increasing p-Smad2/Smad2 ratio. However, high glucose-induced activation of Smad2 was significantly attenuated by siRNA-VEGFA or miRNA-15a-5p mimic ($P<0.05$). What's more, TGF-β1 could also promote phosphorylation of Smad2 (Figure 9). These results indicated that TGF-β1/Smad2 was an important down-stream signaling pathway of miR-15a-5p/VEGFA.**DISCUSSION**

Long-term PD is a risk factor of peritoneal inflammation and fibrosis which is mainly caused by functional and morphological changes in peritoneal membrane. Continuous stimulation by high glucose and AGE product to mesothelial cells on the surface of peritoneal membrane would lead to membrane injury and dysfunction (Krediet et al., 2000). In the current study, we observed that over-expressed VEGFA was positively correlated with increased fibrosis and inflammation in peritoneum of PD patients. The *in vitro* model, using high glucose or AGE -stimulated HPMCs, showed that VEGFA was the up-stream regulator of fibrosis and inflammatory factors. Among those putative miRNAs, which were reported to regulate VEGFA mRNA elsewhere, miR-15a-5p was the most significantly down-regulated one in peritoneum of PD patients. In cultured HPMCs, we found that miR-15a-5p could regulate VEGFA expression via directly

binding to its mRNA 3'UTR. Restoration of miR-15a-5p could inhibit inflammation and fibrosis of HPMCs. Further experiments showed that TGF-β1/Smad2 was activated by high glucose and regulated by miR-15a-5p and VEGFA. These results imply that miR-15a-5p plays its role via regulating VEGFA mRNA in HPMCs inflammation and fibrosis induced by long-term PD (Figure 10).

VEGFA is the most ubiquitously and abundantly expressed member of VEGF family. Our previous studies have already confirmed that high glucose could promote VEGF expression in peritoneal tissue of PD Sprague-Dawley rats (Guo et al., 2014a; Xiao et al., 2014; Zhao et al., 2011). In the present study, we further tested the expression and function of VEGFA in patient samples and *in vitro* cultured HPMCs. In accordance with the animal studies, elevated VEGFA was showed to be closely related with inflammation and fibrosis in peritoneum of PD patients. In high glucose or AGE product -stimulated HPMCs, VEGFA was found to be an instigator of inflammation and fibrosis. Previous studies indicated that several miRNAs could bind and regulate VEGFA mRNA. However, their roles in PD have not been tested. Here, we detected five of those and results showed that miR-15a-5p was the most significantly down-regulated one. As a result, we focused on its role in VEGFA-mediated mesothelium dysfunction during PD. In cultured HPMCs, high glucose or AGE product inhibited expression of miR-15a-5p. When miRNA mimic was transfected into HPMCs to restore miR-15a-5p, it led to attenuation of over-expressed VEGFA.

MiRNA-15a-5p has been reported to function via binding with different mRNAs in 3'UTR. Previous studies have already confirmed CDKN2B, BCL2L2, AP4, and Bim-

1 (Guo et al., 2014b) to be the target of miR-15a. Here, we also wondered whether miR-15a-5p could target VEGFA mRNA. First, we searched Target Scan to predict the binding efficiency of these two molecules. Results showed an 8-mer paring sequence in 3'UTR of VEGFA mRNA for miR-15a-5p. To confirm this prediction, we performed dual-luciferase reporter assay system and results showed that the enzyme activity was significantly reduced in existence of miR-15a-5p mimic, suggesting a binding effect via the predicted sequences.

MiR-15a-5p has shown important roles in regulating cancer behavior by targeting multiple mRNAs (Alderman and Yang, 2016). Recently, some studies also documented its role in regulating inflammation and fibrosis. In bacterial sepsis model of mice, inflammation at the initial phase of infections was associated with deletion of miR-15a (Moon et al., 2014). During this process, miR-15a was reported to control inflammation via limiting expression of Toll-like receptor 4 (TLR4) which is the receptor of innate immunity. MiR-15a is also an important participator in cellular and humoral immunity. It was reported that peripheral blood mononuclear cells isolated from patients of acute coronary syndrome expressed decreased level of miR-15a (Liu et al., 2014). And by binding to 3'-UTR of coactivator-associated arginine methyltransferase 1 (CARM1), miR-15a modulated the expression of CARM1 and its down-stream chemokines. These results suggested that miR-15a participated in different process of inflammation in different diseases. In our experiments, expression of miR-15a-5p was closely related with inflammatory factors, including IL-6, MCP-1, TGF-β1, IL-8, IL-17a and TNF-α. Up-regulation of miR-15a-5p in HPMCs by miRNA mimic attenuated the concentration of

these inflammatory factors, indicating its role in regulating inflammation of mesothelial cells in PD. As for fibrosis, some studies have documented the anti-fibrotic role of miR-15a. In diabetic heart, which is characterized by gradual myocardial fibrosis, biopsy tissues showed significant down-regulation of miR-15a with marked up-regulation of pro-fibrotic transforming growth factor-beta receptor-1 (TGFbetaR1) and connective tissue growth factor (CTGF). What' more, therapeutic restoration of miR-15a in cardiomyocytes reduced the activation of TGFbetaR1 and CTGF and diminished the differentiation of diabetic human cardiac fibroblasts (Rawal et al., 2017). Similarly, in Chlamydia infection of the reproductive epithelium, miR-15a was down-regulated and was responsible for the epithelial-mesenchyme transition which would lead to fibrosis (Igietseme et al., 2015). In accordance with these studies, our results also showed that restoration of miR-15a-5p in HPMCs by transfection of miRNA mimic could limit the over-expressed fibronectin and collagen IV. All these results suggested that miR-15a-5p play an important role in regulating inflammation and fibrosis of mesothelial cells in PD.

TGF-β1 was reported to be involved in many biological processes, including inflammation and fibrosis. By binding to its specific transmembrane receptors, it could recruit and phosphorylate Smads which then translocate into the nucleus to regulate target gene expression. In proximal tubular epithelial cells, TGF-β1 activated Smad2 is responsible for the expression of fibronectin (Rhyu et al., 2005). In HPMCs, high glucose solution activated TGF-β1-dependent Smad2 and Smad4, which were involved in the fibrosis and functional injury of the peritoneum (Yang et al., 2007; Yao et al., 2004). In our

study, the results indicated that high glucose modulated phosphorylation state of Smad2 partially by up-regulation of TGF-β1. This effect could be inhibited by siRNA targeting VEGFA or miR-15a-5p mimic suggesting miR-15a-5p/VEGFA was the up-stream regulator of TGF-β1/Smad2. The enhanced signaling pathway finally led to expression of fibrosis factors and failure of peritoneal ultrafiltration (Zhang et al., 2005). However, further studies are needed to test the involvement of other members of Smads family.In summary, our results demonstrate that miR-15a-5p is an important regulator of inflammation and fibrosis via directly binding with 3'UTR of VEGFA mRNA in peritoneal mesothelial cells. TGF-β1/Smad2 is the down-stream signaling pathway of miR-15a-5p/VEGFA. Targeting miR-15a-5p/VEGFA may provide us new strategies in prevention and treatment of peritoneal fibrosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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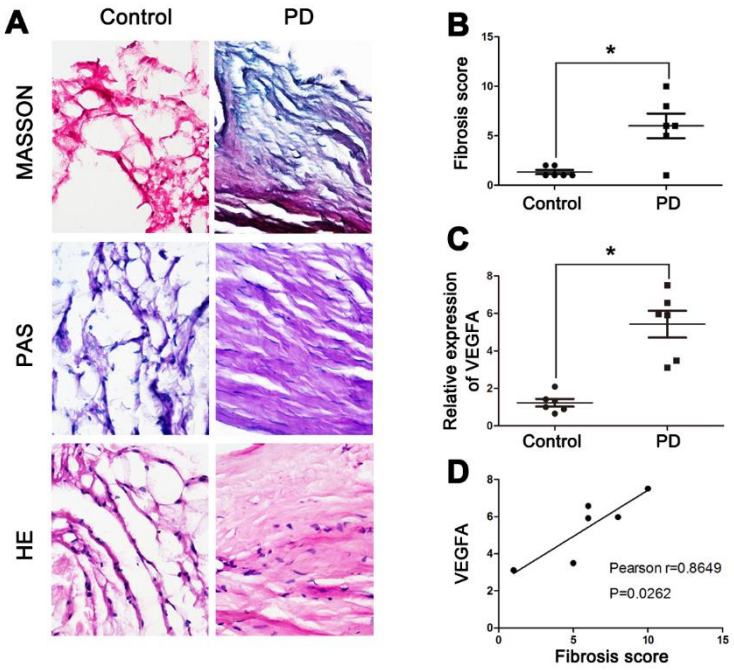
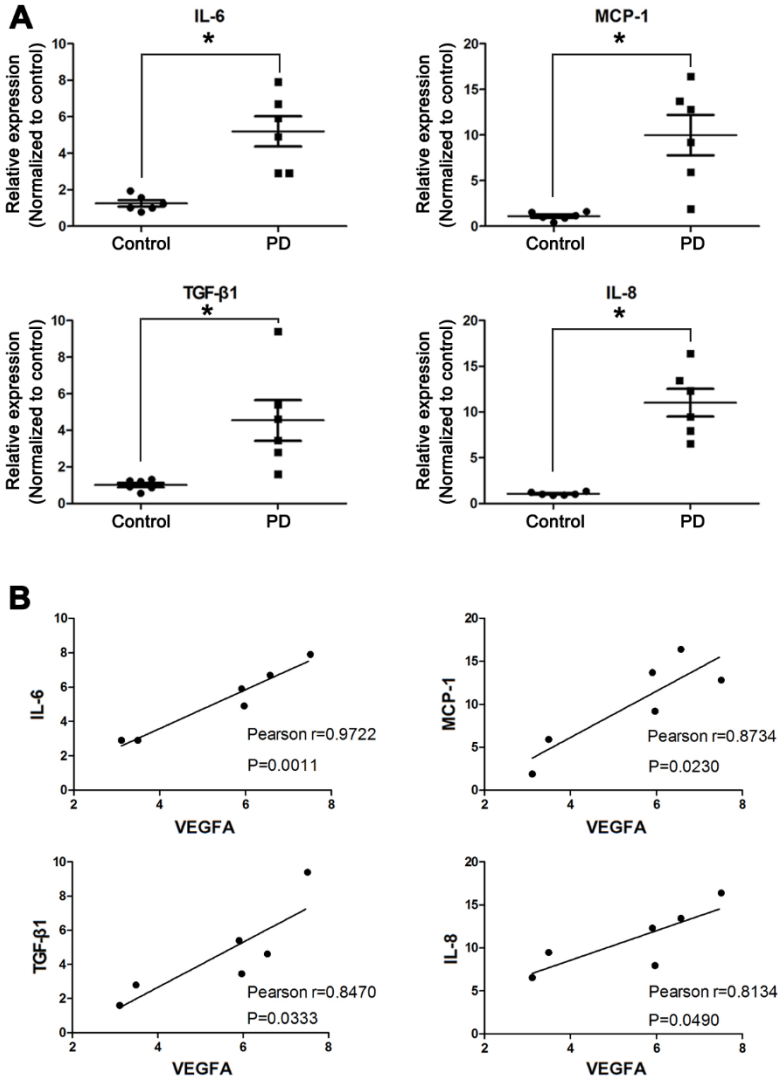
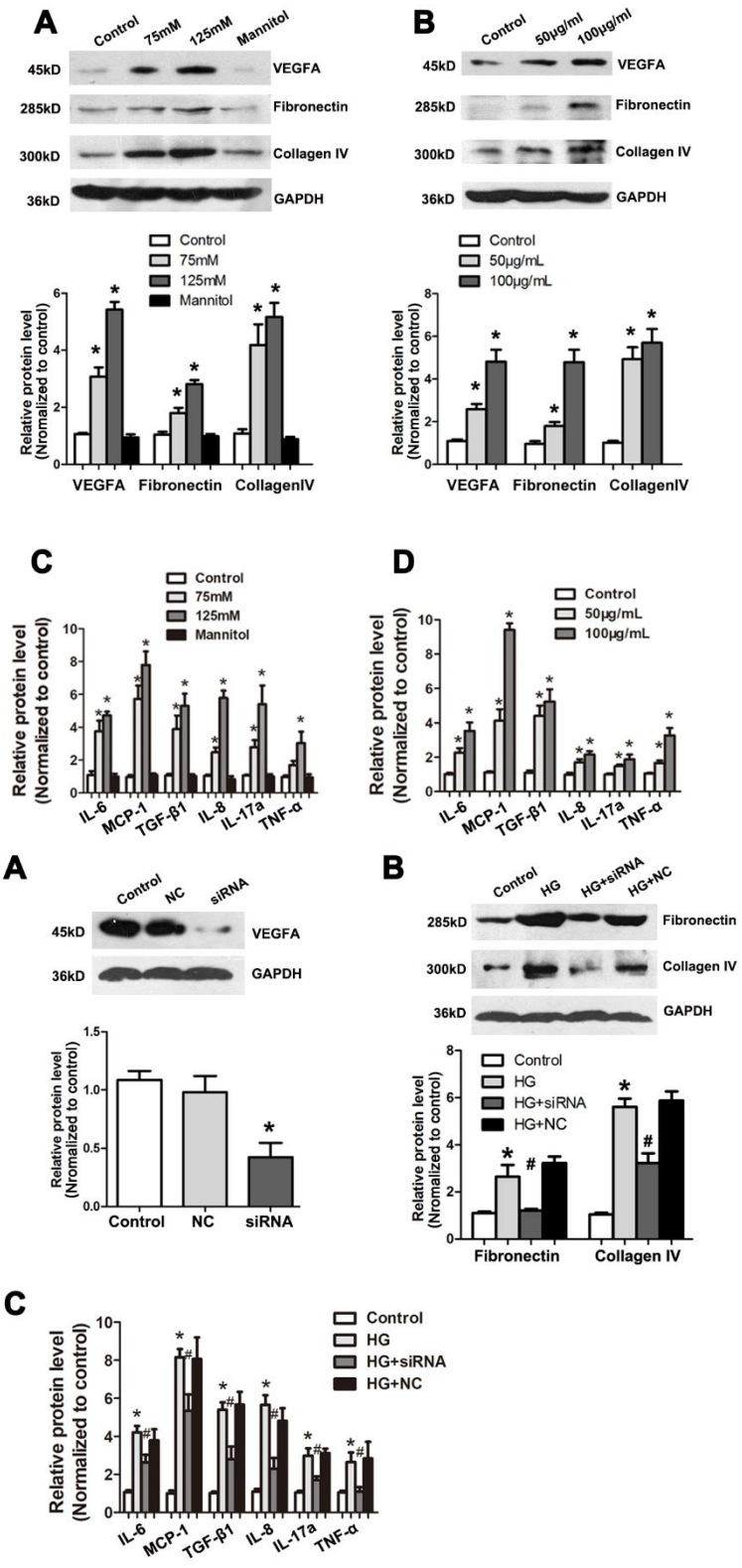


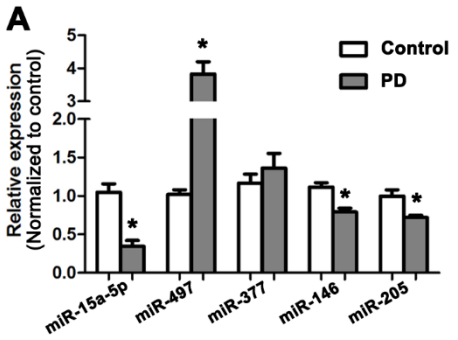

A
IL-6
*
Relative expression (Normalized to control)
Control
PD
MCP-1
*
TGF-β1
*
IL-8
*
B
IL-6
Pearson r=0.9722
P=0.0011
VEGFA
MCP-1
Pearson r=0.8734
P=0.0230
TGF-β1
Pearson r=0.8470
P=0.0333
IL-8
Pearson r=0.8134
P=0.0490

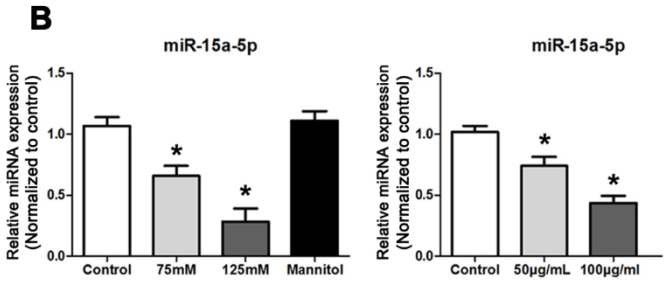
441


A
Control
75mM
125mM
Mannitol
45kD
VEGFA
285kD
Fibronectin
300kD
Collagen IV
36kD
GAPDH
Control
75mM
125mM
Mannitol
Relative protein level
(Nromalized to control)
VEGFA
Fibronectin
CollagenIV
B
Control
50μg/ml
100μg/ml
45kD
VEGFA
285kD
Fibronectin
300kD
Collagen IV
36kD
GAPDH
Control
50μg/mL
100μg/mL
Relative protein level
(Nromalized to control)
VEGFA
Fibronectin
CollagenIV
C
Control
75mM
125mM
Mannitol
Relative protein level
(Normalized to control)
IL-6
MCP-1
TGF-β1
IL-8
IL-17a
TNF-α
D
Control
50μg/mL
100μg/mL
Relative protein level
(Normalized to control)
IL-6
MCP-1
TGF-β1
IL-8
IL-17a
TNF-α
A
Control
NC
siRNA
45kD
VEGFA
36kD
GAPDH
Relative protein level
(Nromalized to control)
Control
NC
siRNA
B
Control
HG
HG+siRNA
HG+NC
285kD
Fibronectin
300kD
Collagen IV
36kD
GAPDH
Control
HG
HG+siRNA
HG+NC
Relative protein level
(Nromalized to control)
Fibronectin
Collagen IV
C
Control
HG
HG+siRNA
HG+NC
Relative protein level
(Normalized to control)
IL-6
MCP-1
TGF-β1
IL-8
IL-17a
TNF-α

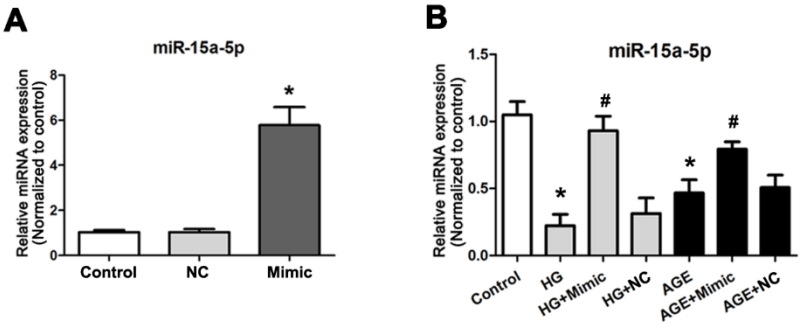
442

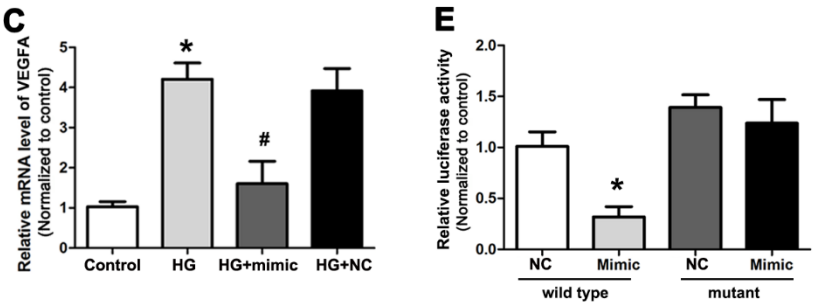
443

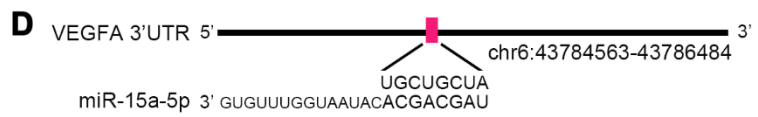

A
Relative expression (Normalized to control)
Control
PD
miR-15a-5p
miR-497
miR-377
miR-146
miR-205


B
miR-15a-5p
Relative miRNA expression (Normalized to control)
Control
75mM
125mM
Mannitol
miR-15a-5p
Relative miRNA expression (Normalized to control)
Control
50μg/mL
100μg/ml

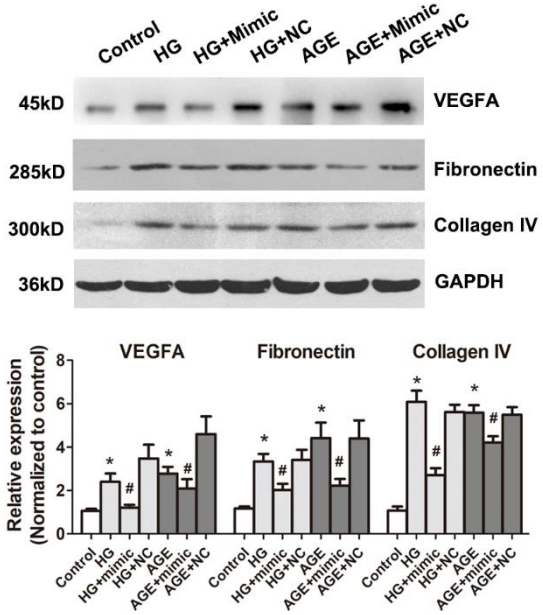
444


A
miR-15a-5p
Relative miRNA expression (Normalized to control)
Control
NC
Mimic
B
miR-15a-5p
Relative miRNA expression (Normalized to control)
Control
HG
HG+Mimic
HG+NC
AGE
AGE+Mimic
AGE+NC

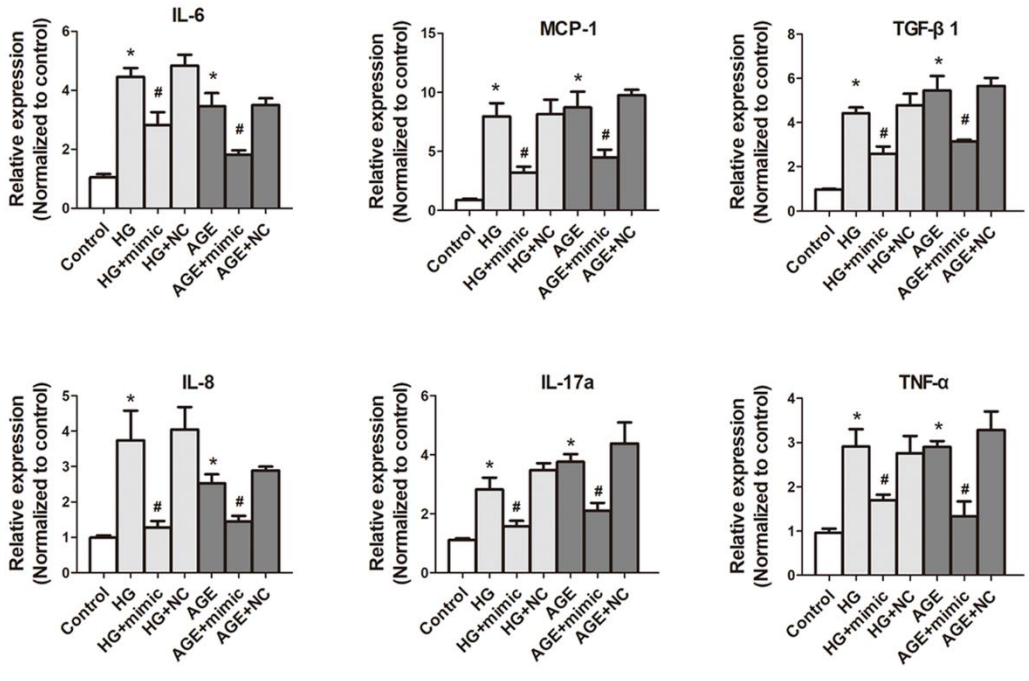

C
Relative mRNA level of VEGFA (Normalized to control)
Control
HG
HG+mimic
HG+NC
E
Relative luciferase activity (Normalized to control)
NC
Mimic
NC
Mimic
wild type
mutant


D
VEGFA 3'UTR 5'
3'
chr6:43784563-43786484
UGCUGCUA
miR-15a-5p 3' GUGUUUGGUAAUACACGACGAU

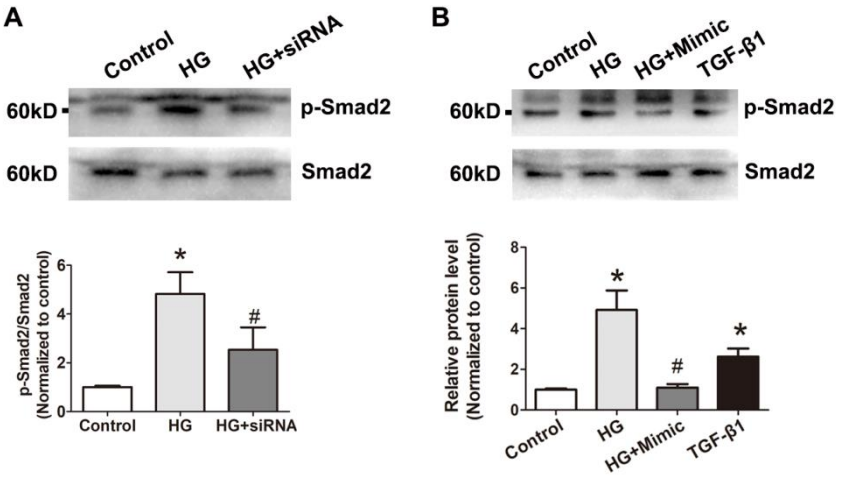
445


Control
HG
HG+Mimic
HG+NC
AGE
AGE+Mimic
AGE+NC
45kD
VEGFA
285kD
Fibronectin
300kD
Collagen IV
36kD
GAPDH
VEGFA
Fibronectin
Collagen IV
Relative expression
(Normalized to control)

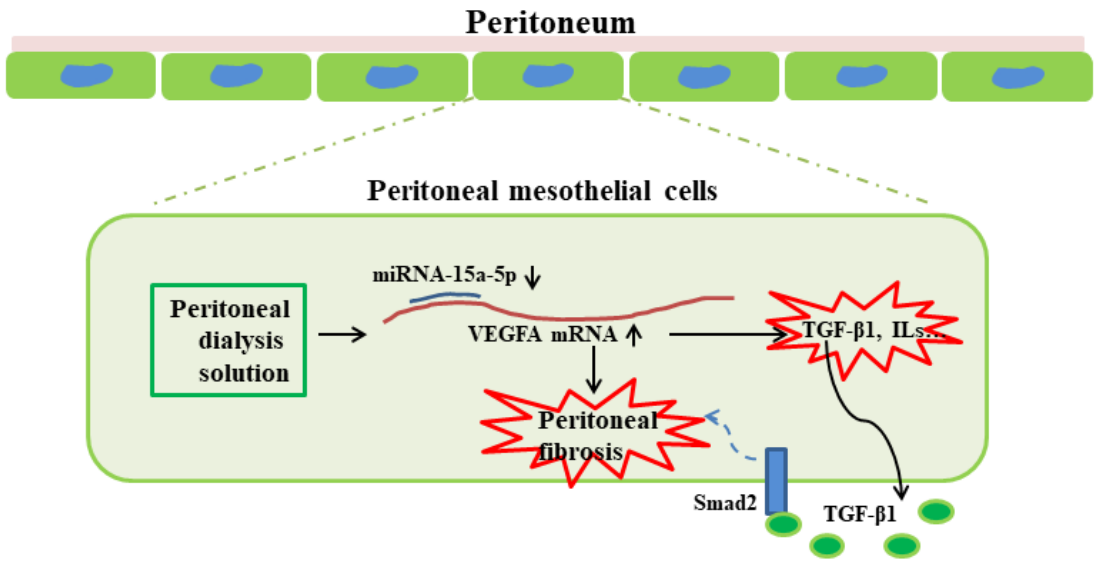
446


IL-6
MCP-1
TGF-β 1
IL-8
IL-17a
TNF-α
Relative expression
(Normalized to control)
Control
HG
HG+mimic
HG+NC
AGE
AGE+mimic
AGE+NC

447


A
Control
HG
HG+siRNA
60kD
p-Smad2
60kD
Smad2
p-Smad2/Smad2 (Normalized to control)
Control
HG
HG+siRNA
B
Control
HG
HG+Mimic
TGF-β1
60kD
p-Smad2
60kD
Smad2
Relative protein level (Normalized to control)
Control
HG
HG+Mimic
TGF-β1

448


Peritoneum
Peritoneal mesothelial cells
Peritoneal dialysis solution
miRNA-15a-5p
VEGFA mRNA
TGF-β1, ILs
Peritoneal fibrosis
Smad2
TGF-β1

449