

Blocking Posttranslational Core Fucosylation Ameliorates Rat Peritoneal Mesothelial Cell Epithelial-Mesenchymal Transition

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Abstract

Background: Core fucosylation (CF), catalyzed by α -1,6 fucosyltransferase (Fut8) in mammals, plays an important role in pathological processes through posttranslational modification of key signaling receptor proteins, including transforming growth factor (TGF)- β receptors and platelet-derived growth factor (PDGF) receptors. However, its effect on peritoneal fibrosis is unknown. Here, we investigated its influence on epithelial-mesenchymal transition (EMT) of rat peritoneal mesothelial cells (PMCs) *in vitro* induced by a high-glucose (HG) culture solution.

Methods: Rat PMCs were first cultured in a HG (2.5%) culture solution to observe the CF expression level (fluorescein isothiocyanate-lens culinaris agglutinin), we next established a knockdown model of rat PMCs *in vitro* with Fut8 small interfering RNA (siRNA) to observe whether inhibiting CF decreases the messenger RNA (mRNA) expression and protein expression of Fut8 and reverses EMT status. Rat PMCs were randomly divided into control group, mock group (transfected with scrambled siRNA), Fut8 siRNA group, HG group, HG + mock group, and HG + Fut8 siRNA group. Finally, we examined the activation of TGF- β /Smad2/3 signaling and PDGF/extracellular signal-regulated kinase (ERK) signaling to observe the influence of CF on them.

Results: CF, Fut8 mRNA, and protein expression were all significantly upregulated in HG-induced EMT model than those in the control rat PMCs ($P < 0.05$). Fut8 siRNA successfully blocked CF of TGF- β receptors and PDGF receptors and attenuated the EMT status (E-cadherin and α -SMA and phenotypic changes) in HG-induced rat PMCs. In TGF- β /Smad2/3 signaling, Fut8 siRNA did not suppress the protein expression of TGF- β receptors and Smad2/3; however, it significantly suppressed the phosphorylation of Smad2/3 (relative expression folds of HG + Fut8 group vs. HG group: 7.6 ± 0.4 vs. 15.1 ± 0.6 , respectively, $P < 0.05$). In PDGF/ERK signaling, Fut8 siRNA did not suppress the protein expression of PDGF receptors and ERK, but it significantly suppressed the phosphorylation of ERK (relative expression folds of HG + Fut8 group vs. HG group: 8.7 ± 0.9 vs. 15.6 ± 1.2 , respectively, $P < 0.05$). Blocking CF inactivated the activities of TGF- β and PDGF signaling pathways, and subsequently blocked EMT.

Conclusions: These results demonstrate that CF contributes to rat PMC EMT, and that blocking it attenuates EMT. CF regulation is a potential therapeutic target of peritoneal fibrosis.

Key words: α -1,6 Fucosyltransferase; Core Fucosylation; Epithelial-Mesenchymal Transition; Peritoneal Fibrosis

INTRODUCTION

Peritoneal dialysis (PD) is an effective and beneficial treatment for patients with end-stage renal disease,^[1,2] however, peritoneal fibrosis remains a serious complication of long-term PD patients, and a major cause of PD failure.^[3-5] Peritoneal fibrosis usually occurs in response to a variety of insults (including bioincompatible glucose dialysate fluids, peritonitis, uremic toxins, and chronic inflammation).^[6]

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Epithelial–mesenchymal transition (EMT) has been identified as a key mechanism of peritoneal fibrosis *in vitro* and in PD patients.^[5,7,8] Various studies have suggested that drugs and peptides could be useful for inhibiting EMT; however, these studies are limited as they block only one signaling pathway. Since multiple signaling pathways are involved in the process of EMT, blocking one signaling pathway may not be enough to prevent EMT. Unfortunately, there are no inhibitors currently available that can simultaneously block two or more signaling pathways that contribute to EMT.

In the past decades, alteration of protein expression was a regularly used strategy for regulating the functions of a protein. More recently, emerging studies have suggested that posttranslational glycosylation modification of proteins plays a key role in altering protein functions, exerting profound effects on many important physiological and pathological processes, including cell growth, migration, and differentiation.^[9-12] Core fucosylation (CF), which is catalyzed by α -1,6 fucosyltransferase (Fut8) in mammals,^[13] is an important posttranslational glycosylation found to play a crucial role in pathological processes, including emphysema,^[14-16] schizophrenia,^[17,18] and hepatocellular carcinoma.^[19] We recently demonstrated that diminishing the CF of transforming growth factor- β (TGF- β) receptors (TGF- β Rs) blocked renal tubular EMT in cultured human renal proximal tubular epithelial cells *in vitro* and alleviated renal interstitial fibrosis in rats with unilateral ureteral obstruction.^[20,21] However, it is currently unknown how CF may affect EMT in peritoneal fibrosis.

TGF- β is the strongest profibrotic factor in EMT of peritoneal mesothelial cells (PMCs) and a key mediator in dialysis-related peritoneal fibrosis.^[5,22] Platelet-derived growth factor (PDGF) also induced partial EMT *in vivo* and stimulated human PMC proliferation *in vitro*.^[23,24] As TGF- β R and PDGF receptors (PDGFR) are both glycosylated, and CF is their common posttranslational modification,^[25] we believed that blocking CF may simultaneously inhibit TGF- β and PDGF signaling pathways, resulting in a synergistic protective effect in ameliorating EMT.

In this study, we first hypothesized that CF participated in EMT. With this in mind, we investigated CF expression in rat PMCs cultured in high-glucose (HG) solution. Next, we hypothesized that blocking CF would prevent rat PMC EMT. For this purpose, we established a knockdown model of rat PMCs *in vitro* to observe the effect. Finally, we examined the activation of TGF- β /Smad2/3 signaling and PDGF/extracellular signal-regulated kinase (ERK) signaling after blocking CF. Our results suggest that CF plays a crucial role during EMT and that blocking it attenuates EMT by simultaneously suppressing the activation of TGF- β and PDGF signaling pathways.

METHODS

Cell culture and treatments

Rat PMCs were isolated and cultured according to a previously described method.^[5] Briefly, rat PMCs were obtained by

infusing 30 ml 0.25% trypsinase–0.2% EDTA–Na₂ into the rat abdominal cavity; after 2 h, the fluid was collected under sterile conditions. Next, the cellular components were isolated by centrifugation at 1400 rpm for 5 min, washed with Dulbecco's modified Eagle's medium (DMEM)/F12 medium, and suspended in culture medium supplemented with 15% (v/v) fetal bovine serum (FBS). The rat PMCs used in the experiments were derived from the second to fourth passages, and were incubated with serum-free medium for 24 h to arrest and synchronize the cell growth before each experiment. Next, the medium was changed to fresh 2% FBS–DMEM/F12 medium containing normal glucose (5.6 mmol/L) or HG (2.5% solution, 126 mmol/L) for 3 and 7 days with an exchange of medium every 2–3 days.

The rat PMCs were randomly divided into six groups: control (Con) group: cultured in DMEM/F12 medium; mock (M) group: subjected to 8-h transient transfection with 30 nmol/L scrambled small interfering RNA (siRNA) before incubation in DMEM/F12 medium; Fut8 group: subjected to 8-h transfection with 30 nmol/L Fut8 siRNA before incubation in DMEM/F12 medium; 2.5% HG group: cultured in HG solution for 72 h; 2.5% HG + M group (2.5% + M): subjected to 8-h transfection with 30 nmol/L scrambled siRNA before treatment in HG solution; and 2.5% HG + Fut8 group (2.5% + F): subjected to 8-h transfection with 30 nmol/L Fut8 siRNA before treatment in HG solution. The cells were less hurt by 8-h transfection with siRNA in our preliminary experiment.

Detection of fucosylation

Lens culinaris agglutinin–fluorescein isothiocyanate (LCA-FITC; Vector Laboratories, Burlingame, USA) was used to detect the expression of core fucose; and staining was performed as described previously.^[10,20]

Immunofluorescence

Rat PMCs were fixed in 4% paraformaldehyde, blocked by incubation in 1% (wt/vol) goat serum for 30 min, incubated with primary antibodies (polyclonal goat anti-rat Fut8 [1:100]; Santa Cruz Biotechnologies, CA, USA), LCA-FITC (1:1000; Vector Laboratories, Burlingame, USA), E-cadherin (1:200; Santa Cruz Biotechnologies, CA, USA), α -smooth muscle actin (α -SMA, 1:200; Santa Cruz Biotechnologies, CA, USA) at 4°C overnight, followed by incubation with the appropriate secondary antibodies at 37°C for 1 h. The cells were counterstained with 4,6 diamidino-2 phenylindole and examined under fluorescence microscopy. Quantitation was conducted by scanning and analyzing the fluorescence intensity using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, USA).

Design, preparation, and transfection of the α -1,6 fucosyltransferase small interfering RNA

The siRNAs targeting rat Fut8 and a scrambled siRNA were synthesized by GenePharma (Shanghai, China). The siRNA sequences were validated using BLAST and the rat genome database to evaluate possible cross-reactivity, as described in our previous study.^[10,20] Four siRNAs were

synthesized and pooled, and dried siRNA pools were reconstituted in diethyl pyrocarbonate-treated water to a final concentration of 30 nmol/L and stored at -20°C until further use. For transfection, cells were incubated for 24 h to allow multiplication. Then, the siRNAs and transfection reagent were complexed as recommended by the manufacturer, and added to the cell culture.

Real-time reverse transcription polymerase chain reaction

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed according to our protocol as detailed previously.^[20] During analysis of the results, gene expression levels of the target sequence were normalized in relation to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase to control variations in the amount of DNA available for PCR in different samples. All samples were analyzed in triplicate.

Immunoprecipitation

Immunoprecipitation was conducted according to our previously detailed protocol.^[20] Briefly, the rat PMCs were lysed in cold radioimmunoprecipitation assay (RIPA) lysis buffer and precleared using Protein G PLUS-Agarose (Santa Cruz Biotechnologies, CA, USA), and then the whole cell lysates were incubated with 2 μg anti-activin receptor-like kinase-5 (ALK-5) (TGF- β RI), anti-TGF- β RII antibody, 2 μg anti-PDGFR α , and anti-PDGFR β antibody at 4°C for 2 h on a rocker platform (30 rocks/min).

Lectin blotting

Lectin blotting of the immunoprecipitates of ALK-5 (TGF- β RI), TGF- β RII, PDGFR α , and PDGFR β was performed according to our protocol, as detailed previously.^[20]

Western blotting

After rat PMCs were harvested and lysed in RIPA buffer, the lysates were centrifuged at $12,000 \times g$ for 20 min at 4°C , and then the supernatant was collected. A bicinchoninic acid protein assay kit from Pierce (Madison, WI, USA) was used to determine the protein concentrations. Protein samples were denatured at 100°C for 5 min, separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes (Bio-Rad). Next, the blots were probed with the appropriate primary antibodies at 4°C overnight to detect the expression of ALK-5, TGF- β RII, Smad2/3, phosphorylated (p)-Smad2/3, Fut8, PDGFR α and PDGFR β , ERK, and phosphorylated (p)-ERK. The blots were then incubated with horseradish peroxidase-labeled secondary antibody for 1 h at 25°C , followed by detection with electrochemiluminescence. Band intensity was quantified for analysis using LabWorksTM Image Analysis software (UVP, Upland, USA).

Statistical analysis

All data are expressed as the mean \pm standard deviation (SD). Statistical analysis was performed using Student's *t*-test with SPSS version 13.0 software (SPSS, Chicago, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Core fucosylation was upregulated during rat peritoneal mesothelial cell epithelial–mesenchymal transition *in vitro*

In rats, PMC EMT is a key event leading to peritoneal fibrosis.^[26-28] Rat PMCs were treated with a HG solution to mimic the *in vitro* EMT process, and investigated whether CF was upregulated. A HG solution (2.5%) was used to establish the HG-induced EMT model after the effect of high osmotic pressure on the cells was eliminated using mannitol as a control [Figure 1a]. In rat PMCs incubated in HG solution for 3 and 7 days [Figure 1b and 1c], the expression of the epithelial marker E-cadherin was decreased (relative expression folds of HG group vs. Con group: 4.9 ± 0.3 vs. 7.7 ± 0.4 , respectively, $P = 0.033$ on day 3, and 4.1 ± 0.2 vs. 8.1 ± 0.3 , respectively, $P = 0.027$ on day 7), and the expression of α -SMA was increased (relative expression folds of HG group vs. Con group: 29.8 ± 2.1 vs. 9.2 ± 0.5 , respectively, $P = 0.024$ on day 3, and 27.9 ± 1.6 vs. 8.7 ± 0.3 , respectively, $P = 0.026$ on day 7). Furthermore, rat PMCs became elongated, developing a spindle shape and losing their cobblestone monolayer arrangement, and became disordered [Figure 1a], suggesting successful establishment of the EMT model by incubation with HG solution. Next, we investigated Fut8 messenger RNA (mRNA) expression [Figure 2a] and observed significantly increased expression on days 3 and 7 (relative expression folds of HG group vs. Con group: 1.5 ± 0.2 vs. 1.0 ± 0.1 , respectively, $P = 0.037$ on day 3, and 1.6 ± 0.3 vs. 1.0 ± 0.1 , respectively, $P = 0.031$, on day 7). Immunofluorescence staining showed that Fut8 and LCA were markedly upregulated during rat PMC EMT [Figure 2b]. As shown in Figure 2c, Western blotting also demonstrated that Fut8 was markedly upregulated during EMT (relative expression folds of HG group vs. Con group: 2.4 ± 0.3 vs. 1.0 ± 0.1 , respectively, $P = 0.030$ on day 3, and 2.1 ± 0.3 vs. 1.0 ± 0.1 , respectively, $P = 0.033$ on day 7). This *in vitro* experiment suggests that CF is upregulated during rat PMC EMT.

Inhibiting core fucosylation alleviated rat peritoneal mesothelial cell epithelial–mesenchymal transition *in vitro*

Next, we investigated the effect of CF on EMT by knocking down Fut8 with Fut8 siRNA *in vitro*. As CF is specifically catalyzed by Fut8,^[13] we designed and synthesized four Fut8 siRNAs and transfected them into rat PMCs to knock down Fut8 expression and to eliminate CF in the cells. As CF was similarly upregulated on days 3 and 7 in our initial experiments [Figure 2], rat PMCs were cultured in HG solution for 3 days for the remaining *in vitro* experiments. Before HG stimulation, Fut8 siRNAs were transfected into rat PMCs using Lipofectamine[®] 2000 from Invitrogen (Carlsbad, CA, USA) as previously described.^[10,20] Real-time RT-PCR [Figure 3a] and Western blotting [Figure 3b] analysis confirmed that Fut8 siRNAs effectively decreased Fut8 mRNA (relative expression folds of HG + Fut8 group vs. HG group: 0.8 ± 0.1 vs. 1.5 ± 0.2 , respectively, $P = 0.031$) and protein expression (relative expression folds of HG + Fut8 group vs. HG group: 3.6 ± 0.3 vs. 30.2 ± 1.3 , respectively, $P = 0.006$).

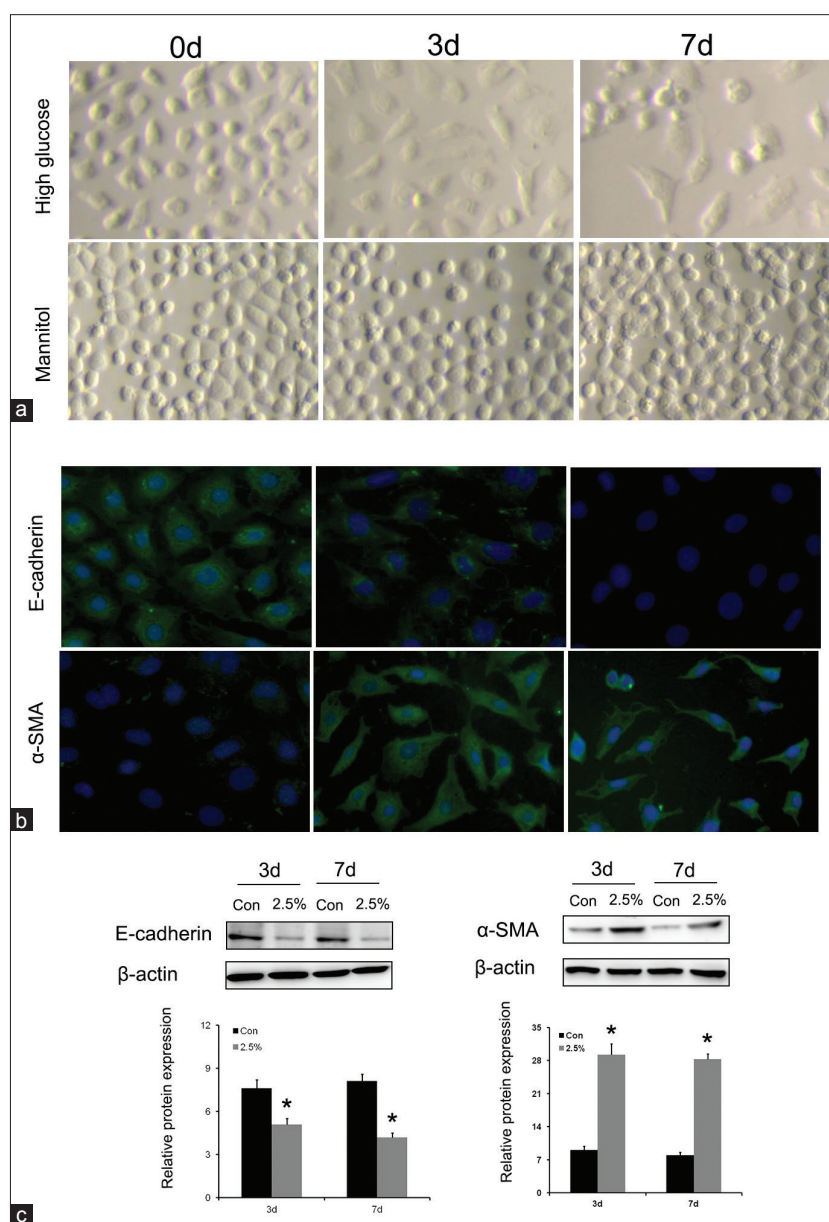


Figure 1: The establishment of epithelial–mesenchymal transition model in rat peritoneal mesothelial cells through HG stimulation. (a) Representative pictures of normal PMCs and fibroblast-like morphology after HG stimulation for 3 and 7 days. There was no cell shape change with mannitol (as a high osmosis control with 2.5% HG solution) for 3 and 7 days (original magnification, $\times 100$). (b) Parallel to the morphological changes, HG culture also decreased the expression of E-cadherin and increased α -SMA expression on days 3 and 7 (original magnification, $\times 100$). (c) Representative images and quantification of Western blotting expression levels of E-cadherin and α -SMA after continuous stimulation with HG culture for 3 and 7 days. Results are the mean \pm SD of three independent experiments. The 2.5% indicates HG solution (126 mmol/L). * $P < 0.05$ versus control group. Con: Control; PMC: Peritoneal mesothelial cell; HG: High glucose; α -SMA: α -smooth muscle actin; SD: Standard deviation.

We then investigated the EMT status by HG stimulation after blockade of CF. In the HG group, α -SMA expression was significantly increased on day 3, while E-cadherin expression was markedly decreased [Figure 3c and 3d]. Pretransfection of rat PMCs with Fut8 siRNA clearly attenuated the EMT changes in the HG + Fut8-siRNA group on day 3 (relative expression folds of HG + Fut8 group vs. HG group: E-cadherin: 9.2 ± 0.2 vs. 7.4 ± 0.2 , respectively, $P = 0.041$, and α -SMA: 6.5 ± 0.4 vs. 23.2 ± 2.4 , respectively, $P = 0.007$). In the HG group, the PMCs became elongated and spindle shaped, losing their cobblestone monolayer arrangement, and

also became disordered. However, pretransfection of the cells with Fut8 siRNA attenuated the phenotypic changes, and most cells retained their normal morphology [Figure 3e].

α -1,6 fucosyltransferase small interfering RNA suppressed transforming growth factor- β RII and activin receptor-like kinase-5 core fucosylation and inactivated transforming growth factor- β /Smad2/3 signaling

As TGF- β is the strongest profibrotic factor in PMC EMT, we examined TGF- β signaling activity after inhibiting CF. Parallel with rat PMC EMT, ALK-5 (TGF- β RI) and

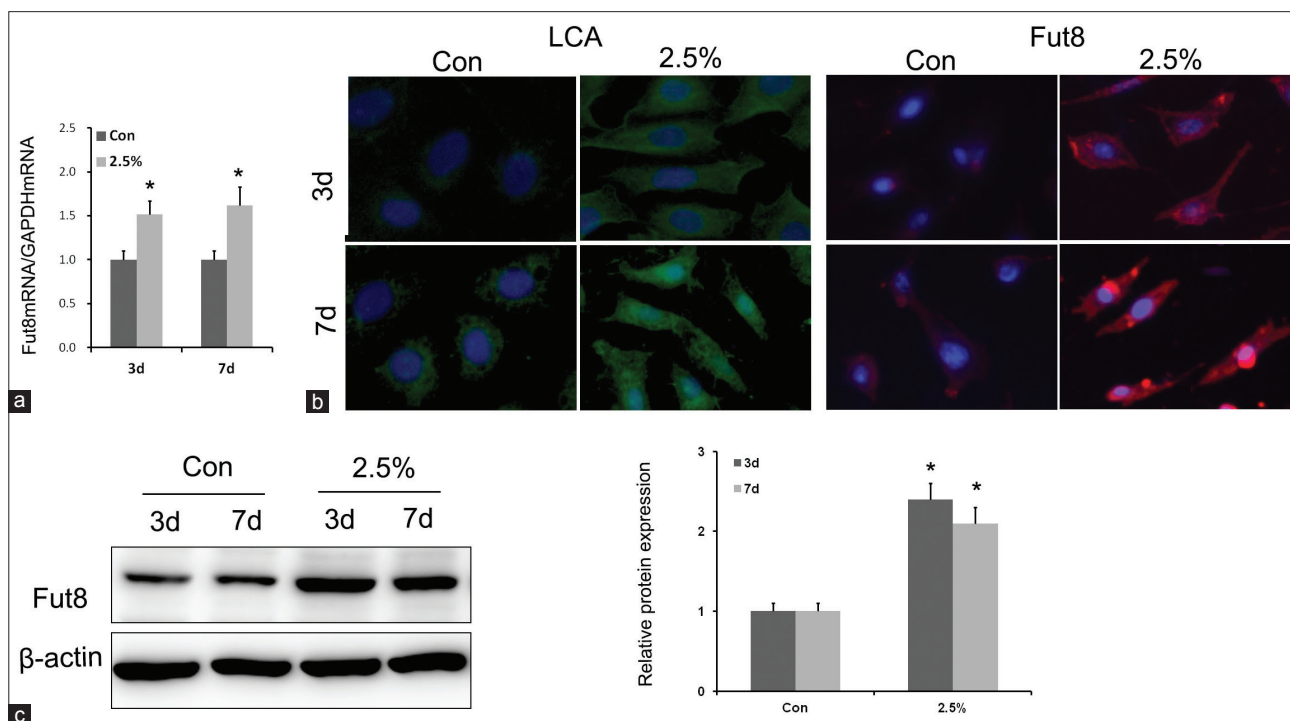


Figure 2: Core fucosylation was upregulated during rat peritoneal mesothelial cell epithelial–mesenchymal transition. (a) Graph of Fut8 messenger RNA expression; there was an increasing trend on days 3 and 7 after HG incubation. (b) Representative immunofluorescence images of increased Fut8 and LCA expression after HG incubation for 3 and 7 days (original magnification, $\times 400$). (c) Representative Western blotting image and quantification analysis of Fut8 expression after HG incubation for 3 and 7 days. Results are the mean \pm SD of three independent experiments. The 2.5% indicates HG solution (126 mmol/L). * $P < 0.05$ versus control group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Con: Control; HG: High glucose; LCA: Lens culinaris agglutinin; Fut8: α -1,6 fucosyltransferase; SD: Standard deviation.

TGF- β RII protein expression were increased following their posttranslational CF upregulation [Figure 4]. However, Fut8 siRNA significantly inhibited the increased CF of ALK-5 and TGF- β RII in the HG + Fut8-siRNA group compared with the HG group (relative expression folds of HG + Fut8 group vs. HG group: ALK-5: 7.4 ± 0.2 vs. 15.5 ± 0.4 , respectively, $P = 0.013$, and TGF- β RII: 7.6 ± 0.4 vs. 15.7 ± 1.4 , respectively, $P = 0.009$). We next examined the activation of the TGF- β /Smad2/3 signaling pathway. Western blotting analysis [Figure 4] showed that Fut8 siRNA downregulation of TGF- β R CF decreased p-Smad2/3 expression after HG stimulation (relative expression folds of HG + Fut8 group vs. HG group: 7.6 ± 0.4 vs. 15.1 ± 0.6 , respectively, $P = 0.020$). These results show that inhibiting CF decreased TGF- β /Smad2/3 signaling pathway activity, ameliorating rat PMC EMT.

α -1,6 fucosyltransferase small interfering RNA suppressed platelet-derived growth factor receptor core fucosylation and inactivated platelet-derived growth factor/extracellular signal-regulated kinase signaling

PDGFRs are also modified by CF. PDGF induces partial EMT *in vivo* and stimulates human PMC proliferation through increased PDGF/ERK signaling. Therefore, we investigated PDGF/ERK signaling activity after inhibiting CF. We first examined whether PDGFRs were target proteins modified by CF. Positive bands of core fucose were present in the PDGFR α and PDGFR β immunoprecipitates [Figure 5], indicating

their modification by CF. Western blotting analysis showed that PDGFR α and PDGFR β were both upregulated with increased CF in rat PMCs following HG stimulation; Fut8 siRNA suppressed their CF level (relative expression folds of HG + Fut8 group vs. HG group: PDGFR α : 6.3 ± 0.2 vs. 13.3 ± 0.4 , respectively, $P = 0.024$, PDGFR β : 9.2 ± 0.4 vs. 27.7 ± 1.5 , respectively, $P = 0.007$), but did not suppress their protein expression. We further studied PDGF/ERK signaling pathway activation. Western blotting analysis showed that p-ERK expression was decreased after HG stimulation [Figure 5] due to Fut8 siRNA downregulation of PDGFR CF (relative expression folds of HG + Fut8 group vs. HG group: 8.7 ± 0.9 vs. 15.6 ± 1.2 , respectively, $P = 0.029$). The results suggest that inhibiting CF could inhibit PDGF/ERK pathway activation, consequently ameliorating EMT.

DISCUSSION

The inhibition of EMT of mesothelial cells is an effective method to ameliorate peritoneal fibrosis; however, past studies focused on blocking one signaling pathway to inhibit EMT. CF, catalyzed by Fut8, is a common posttranslational modification in mammalian *N*-glycans^[13] that regulates protein functions and has profound effects on physiological or pathological processes. TGF- β R and PDGFRs both involve glycosylation and are fucosylated. Inhibiting CF may interfere with the function of aforementioned receptors and suppress the activities of the two signaling pathways.

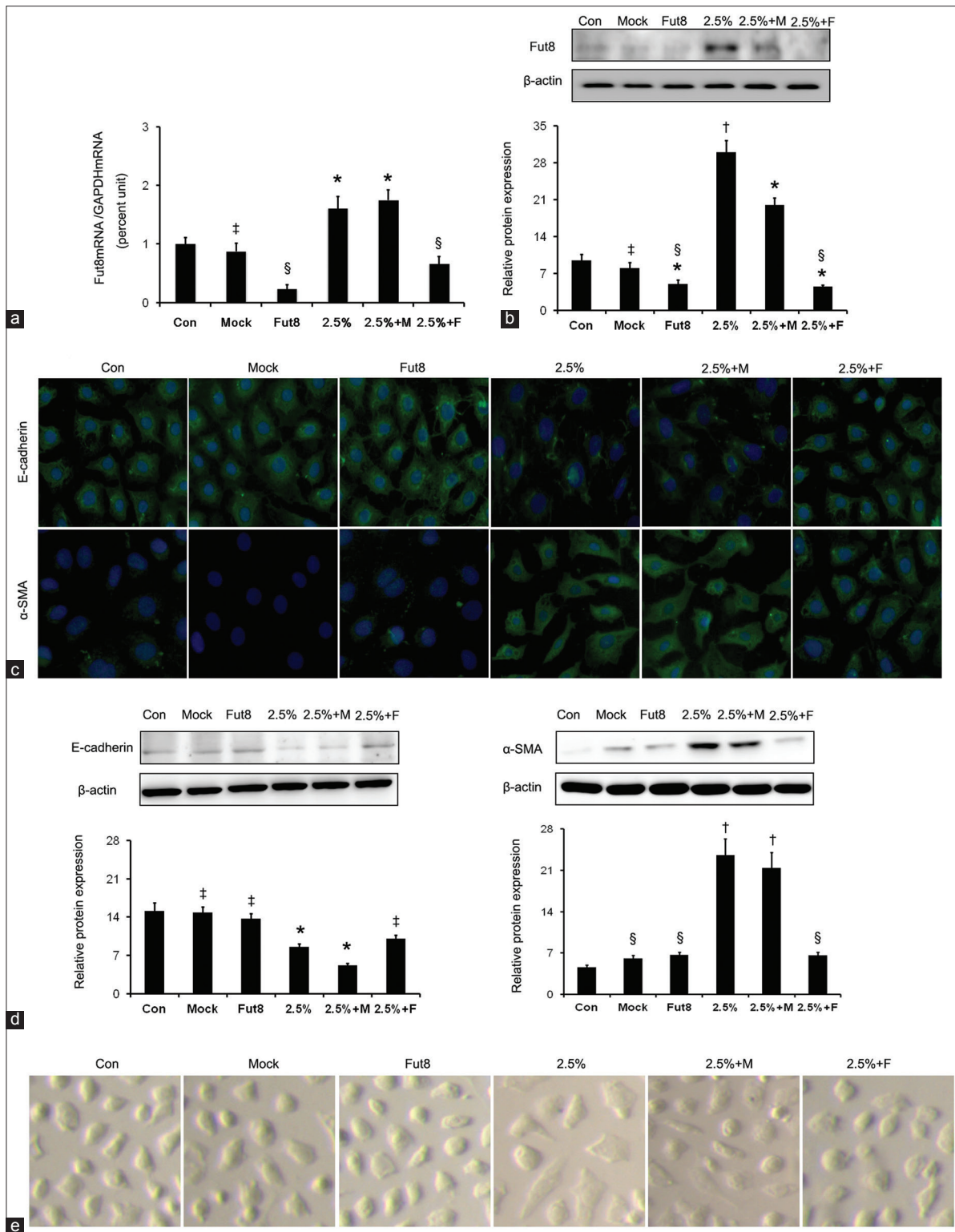


Figure 3: Fut8 siRNA alleviated rat PMC epithelial-mesenchymal transition. Real-time RT-PCR (a) and Western blotting (b) show that Fut8 siRNA inhibited both mRNA and protein expression, respectively, of endogenous Fut8. The Fut8 mRNA expression in each sample was normalized to GAPDH expression. The Fut8/GAPDH mRNA ratio for the normal group is taken as 1 unit. β -actin was used as the loading control in the Western blotting. (c) Representative photographs of E-cadherin and α -SMA immunofluorescence staining. (d) Representative images of Western blotting of E-cadherin and α -SMA. (e) Representative photographs of rat PMC morphological changes by Fut8 siRNA following HG stimulation. The 2.5% indicates HG solution (126 mmol/L). * $P < 0.05$; † $P < 0.01$ versus control group. ‡ $P < 0.05$; § $P < 0.01$ each group (except the normal group) versus the HG group. Results are the mean \pm SD of three independent experiments. (c and e) Original magnification, $\times 100$. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Con: Control; Fut8: α -1,6 fucosyltransferase; siRNA: Small interfering RNA; PMC: Peritoneal mesothelial cell; RT-PCR: Reverse transcription-polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; α -SMA: α -smooth muscle actin; HG: High glucose; SD: Standard deviation; M: Mock; F: Fut8 siRNA; mRNA: Messenger RNA.

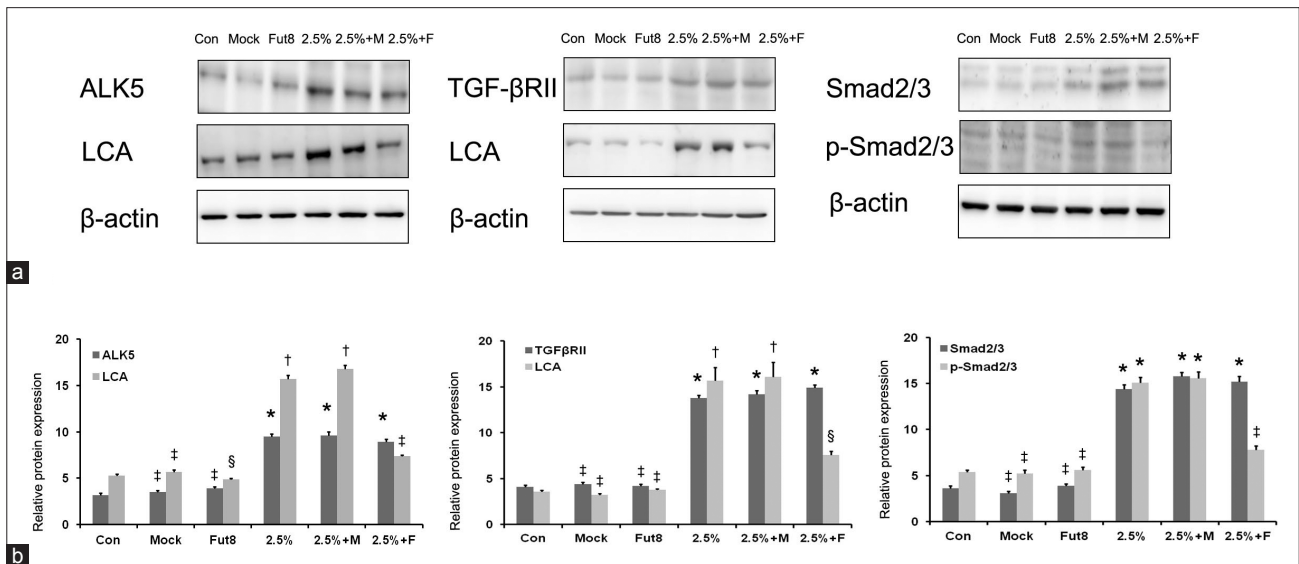


Figure 4: Inhibiting core fucosylation abolished TGF- β /Smad2/3 signaling pathway activation. (a and b) Representative images and quantification of the Western blotting, respectively, of ALK-5, TGF- β RII, LCA, Smad2/3, and p-Smad2/3. Results are the mean \pm SD of three independent experiments. The 2.5% indicates HG solution (126 mmol/L). * P < 0.05; † P < 0.01 versus control group. ‡ P < 0.05; § P < 0.01 for each group (except the normal group) versus the HG group *in vitro*. Con: Control; TGF- β : Transforming growth factor- β ; ALK-5: Activin receptor-like kinase 5; TGF- β RII: Transforming growth factor- β receptors II; LCA: Lens culinaris agglutinin; p-Smad: Phosphorylated-Smad; HG: High glucose; SD: Standard deviation; M: Mock; F: Fut8 siRNA. Fut8: α -1,6 fucosyltransferase; siRNA: Small interfering RNA.

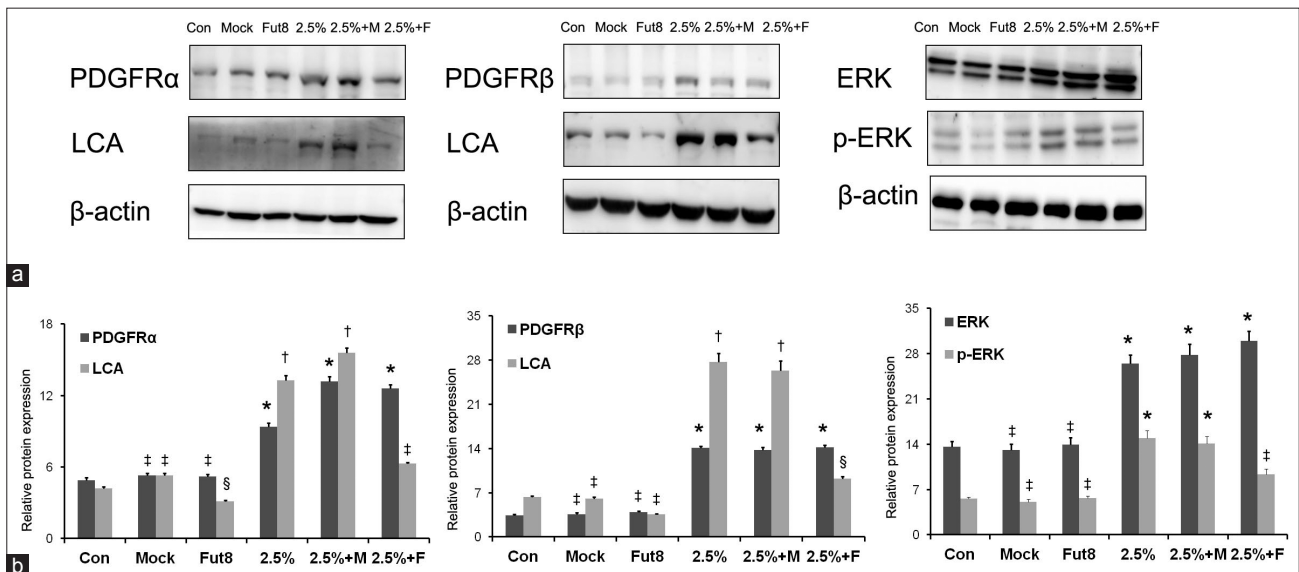


Figure 5: PDGFRs were modified by core fucosylation and inhibiting core fucosylation abolished PDGF/ERK pathway activation. (a and b) Representative images and quantification of the Western blotting, respectively, of PDGFR α , PDGFR β , LCA, ERK, and p-ERK *in vitro*. Core fucosylation modified PDGFR α and PDGFR β ; Fut8 siRNA disrupted this effect without affecting PDGFR α and PDGFR β expression. Results are the mean \pm SD of three independent experiments. The 2.5% indicates HG solution (126 mmol/L). * P < 0.05; † P < 0.01 versus control group. ‡ P < 0.05; § P < 0.01 for each group (except the normal group) versus the HG group *in vitro*. Con: Control; PDGFR: Platelet-derived growth factor receptor; LCA: Lens culinaris agglutinin; ERK: Extracellular signal-regulated kinase; p-ERK: Phosphorylated-ERK; Fut8: α -1,6 fucosyltransferase; siRNA: Small interfering RNA; HG: High glucose; SD: Standard deviation; M: Mock; F: Fut8 siRNA.

However, it is not clear whether core fucose exists in PMCs, so we first examined it by immunofluorescence staining and found that it existed in rat PMCs. We next investigated the role of CF during rat PMC EMT. The results showed that CF expression was upregulated during rat PMC EMT induced by HG stimulation, and inhibiting it with Fut8 siRNA attenuated EMT, suggesting that CF plays a role in EMT.

Furthermore, we explored the underlying mechanism. Since CF can regulate the function of cell signaling receptors, we then examined the activities of key cell signaling pathways. TGF- β has long been considered a key mediator of EMT and peritoneal fibrosis; TGF- β signals through receptor complexes consisting of TGF- β RI and TGF- β RII, and the activated receptors phosphorylate and activate the Smad

proteins. Previously, we confirmed that CF modified TGF- β Rs, and that diminishing their CF had protective effects in renal tubular cell EMT *in vitro*.^[20] In the present study, we designed and synthesized four Fut8 siRNAs and transfected them into rat PMCs to knock down Fut8 expression. Using this approach, we blocked the TGF- β R CF [Figure 4], and successfully restored α -SMA and E-cadherin expression, which also prevented the typical fibrotic morphological changes [Figure 3]. Suppressing TGF- β /Smad2/3 signaling activation might be an effective and specific route for therapeutic intervention against EMT; therefore, we examined Smad2/3 phosphorylation, the markers of TGF- β /Smad2/3 signaling activation. We found that inhibiting TGF- β R CF blocked Smad2/3 phosphorylation *in vitro*, causing the loss of TGF- β /Smad2/3 signaling activation [Figure 4]. The results indicate that inhibiting CF blocks EMT by suppressing TGF- β signaling.

As specifically blocking TGF- β R CF is very difficult, we cannot exclude the possibility that other target proteins of Fut8 fucosylation (such as PDGFR) might be dysfunctional after Fut8 knockdown. That means that the actions of Fut8 siRNA on these signaling molecules could have contributed to its protective effect in EMT. Therefore, we examined PDGFR and found that the PDGFRs were also upregulated, with increased CF in rat PMCs induced by HG stimulation [Figure 5]. Fut8 siRNA effectively inhibited their CF levels [Figure 5], with successful restoration of EMT. The results suggest that PDGFRs play a key role in EMT, and that blocking their CF is beneficial. We next examined ERK phosphorylation, the PDGFR/ERK signaling activation marker, and found that inhibiting PDGFR CF also blocked ERK phosphorylation [Figure 5]. Our results indicate that inhibiting CF blocked EMT by simultaneously suppressing TGF- β and PDGF signaling pathways. As CF is a common posttranslational modification of many glycoproteins, we will explore other glycoproteins involved in peritoneal fibrosis in the future studies to clarify whether they are also affected by the inhibition of CF. In addition, the expression of α -SMA is less in 7 days after cultured in HG solution than in 3 days. We think the reason may be that rat PMCs were in apoptosis process, resulting in smaller cells and more significant spindle shape and less expression of α -SMA. Therefore, this is another reason that we treated rat PMCs with HG solution for 3 days in the remaining *in vitro* experiments.

In summary, the present study shows that inhibiting CF successfully suppresses the activation of TGF- β /Smad and PDGF/ERK signaling pathways and attenuates rat PMC EMT *in vitro*. Our results suggest that regulating CF is a potential therapeutic target in peritoneal fibrosis.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Jain AK, Blake P, Cordy P, Garg AX. Global trends in rates of peritoneal dialysis. *J Am Soc Nephrol* 2012;23:533-44. doi: 10.1681/ASN.2011060607.
- Mehrotra R, Devuyst O, Davies SJ, Johnson DW. The current state of peritoneal dialysis. *J Am Soc Nephrol* 2016;27:3238-52. doi: 10.1681/ASN.2016010112.
- Busnadiego O, Loureiro-Álvarez J, Sandoval P, Lagares D, Dotor J, Pérez-Lozano ML, *et al*. A pathogenetic role for endothelin-1 in peritoneal dialysis-associated fibrosis. *J Am Soc Nephrol* 2015;26:173-82. doi: 10.1681/ASN.2013070799.
- de Lima SM, Otoni A, Sabino Ade P, Dusse LM, Gomes KB, Pinto SW, *et al*. Inflammation, neoangiogenesis and fibrosis in peritoneal dialysis. *Clin Chim Acta* 2013;421:46-50. doi: 10.1016/j.cca.2013.02.027.
- Nie J, Dou X, Hao W, Wang X, Peng W, Jia Z, *et al*. Smad7 gene transfer inhibits peritoneal fibrosis. *Kidney Int* 2007;72:1336-44. doi: 10.1038/sj.ki.5002533.
- Ueno T, Nakashima A, Doi S, Kawamoto T, Honda K, Yokoyama Y, *et al*. Mesenchymal stem cells ameliorate experimental peritoneal fibrosis by suppressing inflammation and inhibiting TGF- β 1 signaling. *Kidney Int* 2013;84:297-307. doi: 10.1038/ki.2013.81.
- Aroeira LS, Aguilera A, Sánchez-Tomero JA, Bajo MA, del Peso G, Jiménez-Heffernan JA, *et al*. Epithelial to mesenchymal transition and peritoneal membrane failure in peritoneal dialysis patients: Pathologic significance and potential therapeutic interventions. *J Am Soc Nephrol* 2007;18:2004-13. doi: 10.1681/ASN.2006111292.
- Liappas G, González-Mateo GT, Sánchez-Díaz R, Lazcano JJ, Lasarte S, Matesanz-Marín A, *et al*. Immune-Regulatory Molecule CD69 Controls Peritoneal Fibrosis. *J Am Soc Nephrol* 2016;27:3561-3576. doi: 10.1681/ASN.2015080909.
- Takahashi M, Kuroki Y, Ohtsubo K, Taniguchi N. Core fucose and bisecting GlcNAc, the direct modifiers of the N-glycan core: Their functions and target proteins. *Carbohydr Res* 2009;344:1387-90. doi: 10.1016/j.carres.2009.04.031.
- Wen X, Liu A, Yu C, Wang L, Zhou M, Wang N, *et al*. Inhibiting post-translational core fucosylation prevents vascular calcification in the model of uremia. *Int J Biochem Cell Biol* 2016;79:69-79. doi: 10.1016/j.biocel.2016.08.015.
- Shao K, Chen ZY, Gautam S, Deng NH, Zhou Y, Wu XZ. Posttranslational modification of E-cadherin by core fucosylation regulates Src activation and induces epithelial-mesenchymal transition-like process in lung cancer cells. *Glycobiology* 2016;26:142-54. doi: 10.1093/glycob/cwv089.
- Gu W, Fukuda T, Isaji T, Hashimoto H, Wang Y, Gu J. α 1,6-Fucosylation regulates neurite formation via the activin/phospho-Smad2 pathway in PC12 cells: The implicated dual effects of Fut8 for TGF- β /activin-mediated signaling. *FASEB J* 2013;27:3947-58. doi: 10.1096/fj.12-225805.
- Li W, Yu R, Ma B, Yang Y, Jiao X, Liu Y, *et al*. Core fucosylation of IgG B cell receptor is required for antigen recognition and antibody production. *J Immunol* 2015;194:2596-606. doi: 10.4049/jimmunol.1402678.
- Gao C, Maeno T, Ota F, Ueno M, Korekane H, Takamatsu S, *et al*. Sensitivity of heterozygous α 1,6-fucosyltransferase knock-out mice to cigarette smoke-induced emphysema: Implication of aberrant transforming growth factor- β signaling and matrix metalloproteinase gene expression. *J Biol Chem* 2012;287:16699-708. doi: 10.1074/jbc.M111.315333.
- Wang X, Inoue S, Gu J, Miyoshi E, Noda K, Li W, *et al*. Dysregulation of TGF- β 1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. *Proc Natl Acad Sci U S A* 2005;102:15791-6. doi: 10.1073/pnas.0507375102.
- Wang X, Gu J, Miyoshi E, Honke K, Taniguchi N. Phenotype changes of Fut8 knockout mouse: Core fucosylation is crucial for the function of growth factor receptor(s). *Methods Enzymol* 2006;417:11-22. doi: 10.1016/S0076-6879(06)17002-0.
- Kippe JM, Mueller TM, Haroutunian V, Meador-Woodruff JH. Abnormal N-acetylglucosaminyltransferase expression in prefrontal

- cortex in schizophrenia. *Schizophr Res* 2015;166:219-24. doi: 10.1016/j.schres.2015.06.002.
18. Mueller TM, Yates SD, Haroutunian V, Meador-Woodruff JH. Altered fucosyltransferase expression in the superior temporal gyrus of elderly patients with schizophrenia. *Schizophr Res* 2017;182:66-73. doi: 10.1016/j.schres.2016.10.024.
 19. Wang Y, Fukuda T, Isaji T, Lu J, Im S, Hang Q, *et al.* Loss of α 1,6-fucosyltransferase inhibits chemical-induced hepatocellular carcinoma and tumorigenesis by down-regulating several cell signaling pathways. *FASEB J* 2015;29:3217-27. doi: 10.1096/fj.15-270710.
 20. Lin H, Wang D, Wu T, Dong C, Shen N, Sun Y, *et al.* Blocking core fucosylation of TGF- β 1 receptors downregulates their functions and attenuates the epithelial-mesenchymal transition of renal tubular cells. *Am J Physiol Ren Physiol* 2011;300:3733-42. doi: 10.1152/ajprenal.00426.2010.
 21. Shen N, Lin H, Wu T, Wang D, Wang W, Xie H, *et al.* Inhibition of TGF- β 1-receptor posttranslational core fucosylation attenuates rat renal interstitial fibrosis. *Kidney Int* 2013;84:64-77. doi: 10.1038/ki.2013.82.
 22. Loureiro J, Aguilera A, Selgas R, Sandoval P, Albar-Vizcaíno P, Pérez-Lozano ML, *et al.* Blocking TGF- β 1 protects the peritoneal membrane from dialysate-induced damage. *J Am Soc Nephrol* 2011;22:1682-95. doi: 10.1681/ASN.2010111197.
 23. Hung KY, Chen CT, Yen CJ, Lee PH, Tsai TJ, Hsieh BS. Dipyridamole inhibits PDGF-stimulated human peritoneal mesothelial cell proliferation. *Kidney Int* 2001;60:872-81. doi: 10.1046/j.1523-1755.2001.060003872.x.
 24. Patel P, West-Mays J, Kolb M, Rodrigues JC, Hoff CM, Margetts PJ. Platelet derived growth factor B and epithelial mesenchymal transition of peritoneal mesothelial cells. *Matrix Biol* 2010;29:97-106. doi: 10.1016/j.matbio.2009.10.004.
 25. Venkatachalam MA, Weinberg JM. New wrinkles in old receptors: Core fucosylation is yet another target to inhibit TGF- β signaling. *Kidney Int* 2013;84:11-4. doi: 10.1038/ki.2013.95.
 26. Devuyt O, Margetts PJ, Topley N. The pathophysiology of the peritoneal membrane. *J Am Soc Nephrol* 2010;21:1077-85. doi: 10.1681/ASN.2009070694.
 27. Liu Y, Dong Z, Liu H, Zhu J, Liu F, Chen G. Transition of mesothelial cell to fibroblast in peritoneal dialysis: EMT, stem cell or bystander? *Perit Dial Int* 2015-Feb; 35:14-25. doi: 10.3747/pdi.2014.00188.
 28. Strippoli R, Moreno-Vicente R, Battistelli C, Cicchini C, Noce V, Amicone L, *et al.* Molecular mechanisms underlying peritoneal EMT and fibrosis. *Stem Cells Int* 2016;2016:3543678. doi: 10.1155/2016/3543678.