1,25(OH)$_2$D$_3$/VDR attenuates high glucose-induced epithelial-mesenchymal transition in human peritoneal mesothelial cells via the TGFβ/Smad3 pathway

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Abstract Epithelial-mesenchymal transition (EMT) has been recognized to accelerate peritoneal membrane dysfunction. 1,25(OH)$_2$D$_3$/vitamin D receptor (VDR) is important for preventing various types of EMT *in vivo*. However, its function on EMT and inflammation of human peritoneal mesothelial cells (HPMCs) remains to be elucidated. Therefore, the present study investigated the effects of 1,25(OH)$_2$D$_3$/VDR on high glucose (HG)-induced EMT and inflammation in HPMCs and the underlying molecular mechanism. It was determined that HG reduced VDR expression, increased inflammatory cytokine expression, including transforming growth factor β (TGFβ) and interleukin-6 (IL-6) and phosphorylated-SMAD family member 3 (p-Smad3) expression. EMT was promoted as the expression level of the epithelial marker E-cadherin was reduced, whereas expression levels of the mesenchymal markers α-SMA and FN were increased. 1,25(OH)$_2$D$_3$ pretreatment inhibited the expression of inflammatory cytokines in HPMCs and attenuated HG-induced EMT, possibly through inhibition of the TGFβ/Smad pathway by binding to its receptor VDR.

Introduction

Peritoneal dialysis (PD) is one of the most common renal replacement therapies for end stage renal disease (ESRD). Long-term PD is limited due to the morphological and functional changes of the peritoneum induced by PD fluids, which contain high concentrations of glucose that eventually lead to ultrafiltration failure (1). Epithelial-mesenchymal transition (EMT) is involved in the fibrosis of various organs, including renal fibrosis, liver fibrosis and peritoneal fibrosis (2-4). High glucose (HG)-induced EMT of human peritoneal mesothelial cells (HPMCs) may be a key process in the fibrosis and dysfunction of the peritoneal membrane (5). EMT is a pathological phenomenon where normal epithelial cells lose their characteristics, such as cell polarity and adhesion and gain characteristics of mesenchymal cells, such as migration and invasion (6). Biomarkers for the occurrence of EMT may include the loss of the epithelial adhesion protein E-cadherin and upregulation of the mesenchymal markers α-smooth muscle actin (α-SMA) and fibronectin (FN) (7). Preventing EMT may ameliorate peritoneal fibrosis, preserving the mesothelial cells during PD (8). Additionally, high glucose treatment was demonstrated to induce EMT-mediated inflammation in peritoneal mesothelial cells and the kidney fibroblasts (9,10).

Vitamin D, beyond its role in the regulation of calcium, phosphorus and the bone metabolism, has been demonstrated to serve an important role in the regulation of cell differentiation, cell proliferation and immunomodulation (11). 1,25(OH)$_2$D$_3$ is the active form of vitamin D and regulates bone, calcium and phosphate metabolism via the vitamin D receptor (VDR). The VDR forms a heterodimer with the retinoid X receptor and regulates gene expression in the nucleus. Vitamin D has received more attention in EMT and inflammation. Previous studies have determined that vitamin D attenuated renal tubular cell injury by suppressing EMT process and inflammation through inhibition of the nuclear factor-κB (NF-κB), transforming growth factor β (TGFβ)/SMAD family member 3 (Smad3) and β-catenin signaling pathways (12), vitamin D also inhibited migration, invasion and EMT induced by TGFβ in human airway epithelial cells (13). However, the effect of 1,25(OH)$_2$D$_3$ on HG-induced EMT and inflammation in HPMCs and the underlying molecular mechanism remain to be elucidated.

The present study determined whether 1,25(OH)$_2$D$_3$ protected HPMCs from HG-induced EMT and inflammation and whether this effect occurs through the modulation of TGFβ/Smad signaling pathway by binding to its receptor VDR.

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Materials and methods

Reagents. Fetal bovine serum (FBS), RPMI 1640 and penicillin streptomycin were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). $1,25(OH)_2D_3$ and bovine serum albumin (BSA) were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Rabbit monoclonal VDR (cat. no. ab109234), rabbit monoclonal α-SMA (cat. no. ab32575) and rabbit monoclonal E-cadherin (cat. no. ab133597) antibodies were purchased from Abcam (Cambridge, UK), rabbit monoclonal Smad3 (cat. no. 9523) and rabbit monoclonal phospho-Smad3 (cat. no. 9520) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), mouse monoclonal FN (cat. no. sc-53285) and mouse monoclonal β-actin (cat. no. sc-47778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The dilution used for all primary antibodies was 1:1,000 for western blot analysis and 1:200 for α-SMA in immunofluorescence staining. An enhanced chemiluminescence (ECL) kit was obtained from Pierce; Thermo Fisher Scientific, Inc. Human TGFβ and IL-6 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

HPMC culture. HPMCs were provided by Professor Di Na and Professor Huimian Xu (First Affiliated Hospital of China Medical University, Shenyang, China) and were routinely grown in RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 100 UI/ml penicillin and 100 μg/ml streptomycin. HPMCs were incubated in an atmosphere of 5% CO_2 at 37˚C and every 2-3 days the culture medium was changed. HPMCs were harvested with trypsin-EDTA at a subcultivation ratio of 1:3 to 1:4. Cells at passages 5-10 were used for all subsequent experiments. Cells were treated with 4 different concentrations of HG (control, 1.5%, 76 mM; 2.5%, 126 mM; 4.25%, 214 mM) for 24 h, and for 5 different durations (0, 6, 12, 24 and 48 h) of 2.5% HG (126 mM). Additionally, cells were pretreated with 10^{-7} mol/l $1,25(OH)_2D_3$ (14) for 2 h and followed by 2.5% HG (126 mM) for the 24 h.

Transfection. The VDR-short hairpin RNA (shRNA)-GV248 plasmids that recognize human VDR and negative control plasmids were purchased from GeneChem Co., Ltd. (Shanghai, China) were used for the transient transfections. The transfections were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Treatment groups. HPMCs were exposed to 7 different treatments: Control, cells were untreated; D, treatment with 10^{-7} mol/l $1,25(OH)_2D_3$; Negative control plasmids; VDR-shRNA; HG, treatment with 126 mM HG; HG+D, pretreatment with 10^{-7} mol/l $1,25(OH)_2D_3$ followed by 126 mM HG; and HG+D+shVDR pretreatment with 10^{-7} mol/l $1,25(OH)_2D_3$ followed by 126 mM HG and VDR-shRNA.

Western blot analysis. Western blot analysis was performed as previously described (9). All of the experiments were repeated at least 3 times. The dilution used for primary antibodies was 1:1,000, and 1:5,000 for the goat anti-rabbit/mouse horseradish peroxidase-conjugated secondary antibodies (cat. no. A0208 and A0216; Beyotime Institute of Biotechnology, Haimen, China). The blots were developed using a ECL kit and the images were captured with UVP (G:BOX EF, Chemi HR16; Syngene, Frederick, MD USA). The intensity of each band was measured with Image J software (Java v1.6.0_20; https://imagej.nih.gov/ij/) and the results were normalized against the reference gene β-actin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HPMCs using TRIzol reagent in accordance with the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, first-strand cDNA synthesis was performed using a Reverse Transcription kit in accordance with the manufacturer's protocol (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using ABI 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.) and the SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd.). Briefly, RT-qPCR was prepared in triplicates at a volume of 25 μl reaction mixture as follows: 12.5 μl SYBR Premix Ex Taq II, 1.0 μl forward and reverse specific primers and 2 μl cDNA template and 8.5 μl RNase-free water. The mRNA expression of the respective genes was calculated after normalizing to GAPDH. The specific primers were as follows: TGFβ sense 5'-CCCTTC ATGGTGGCTTTCTT-3' and antisense 5'-CTGGTTCTT GGGCGTCTTG-3'; IL-6 sense 5'-GCCAGAGCTGTGCAG ATGAG-3' and antisense 5'-TCAGCAGGCTGGCATTTG-3'; VDR sense 5'-ATGCCATCTGCATCGTCTC-3' and antisense 5'-GCACCGCACAGGCTGTCCTA-3'; and GAPDH sense 5'-GCACCGTCAAGGCTGAGAAC-3' and antisense 5'-TGGTGAACACGCCAGTGGA-3'.

Immunofluorescence staining with α-SMA. HPMCs were fixed in 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.25% Triton X-100 for 10 min, then washed with phosphate buffered saline (PBS), and blocked for 30 min at room temperature with 5% BSA (Sigma-Aldrich; Merck Millipore). The primary antibody rabbit anti-α-SMA (dilution, 1:200) was incubated with the cells overnight at 4˚C in a humidified chamber. Following 3 washes with PBS for 5 min, fluorescein isothiocyanate-conjugated secondary antibody anti-rabbit IgG (dilution, 1:50; cat. no. A0562; Beyotime Institute of Biotechnology) was incubated for 1 h at room temperature. In order to identify nuclei, cells were counterstained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 min. Stained cells were visualized using a fluorescence microscope (Nikon ECLIPSE Ti; Nikon Corporation, Tokyo, Japan).

ELISA. HPMCs were seeded at a density of 10^5 cells/well into 12-well plates, subsequently the cells were cultured under the conditions aforementioned, the supernatants were collected by centrifugation for 10 min at 1,500 x *g*, 4˚C, and the TGFβ and IL-6 proteins from the supernatants was detected using human TGFβ and human IL-6 ELISA kits according to the manufacturer's protocol. All procedures were performed at room temperature. The TGFβ and IL-6 levels were then expressed as pg/ml.

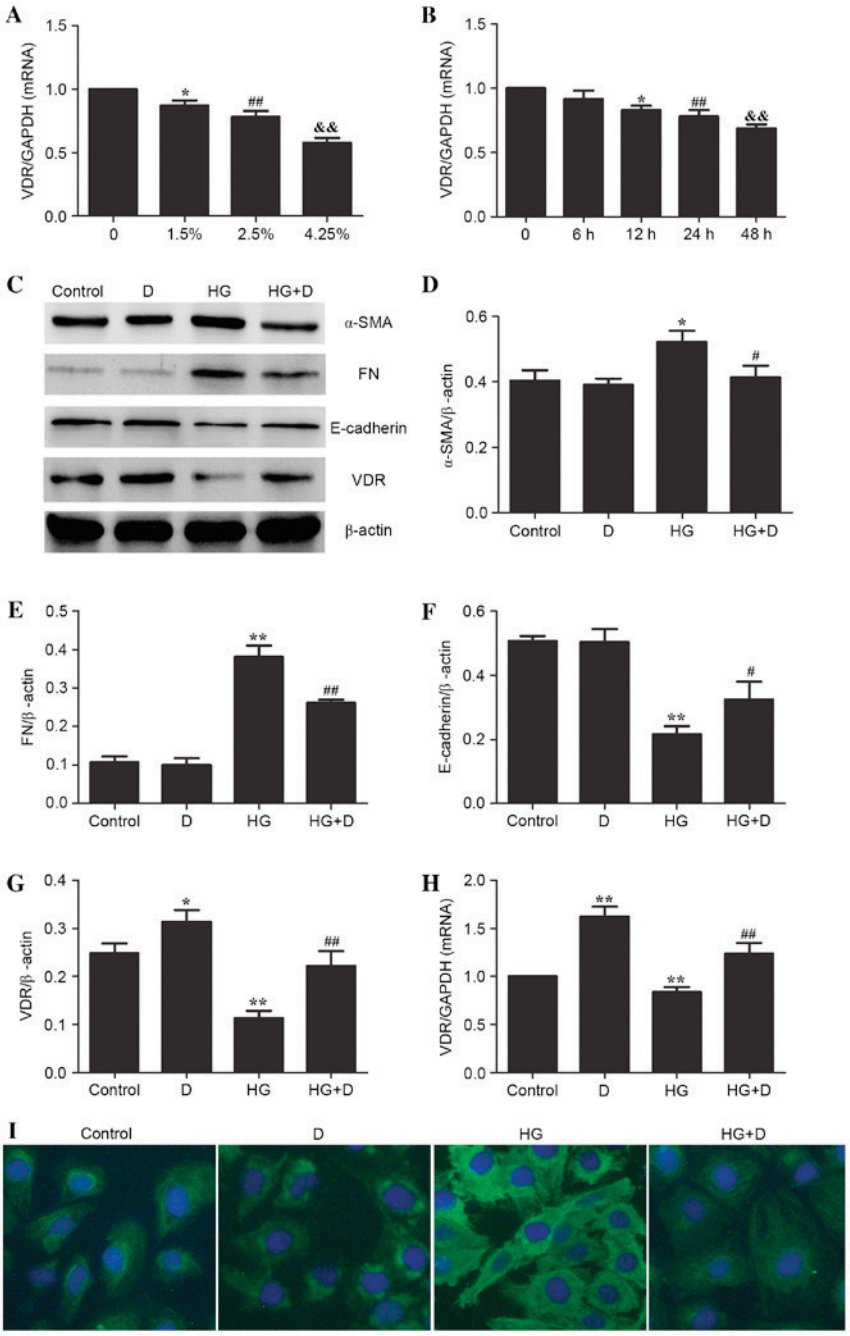

Figure 1. Effect of $1,25(OH)_2D_3$ on EMT biomarkers and VDR in HG-treated HPMCs. HPMCs were treated with (A) 4 different concentrations of HG (control; 1.5%, 76 mM; 2.5%, 126 mM; 4.25%, 214 mM) for 24 h and for (B) 5 different durations (0, 6, 12, 24 and 48 h) of 2.5% HG (126 mM). Cells were pretreated with 10^{-7}mol/l $1,25(OH)_2D_3$ and followed by 2.5% HG (126 mM) for 24 h. Reverse transcription-quantitative polymerase chain reaction was performed to detect the relative gene expression of VDR in the different HPMCs treatment groups. Data are presented as the mean ± standard error (n=3). *P<0.05; $^{\#\#}$P<0.01; $^{\&\&}$P<0.01 vs. control. (C) Western blotting was used to determine protein expression levels. Protein levels of (D) α-SMA, (E) FN, (F) E-cadherin and (G) VDR were assessed using densitometry and were expressed as relative intensities. Data are presented as the mean ± standard error (n=3). *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. HG group. (H) Relative gene expression of VDR in HPMCs following 24 h of HG stimulation in the presence or absence of $1,25(OH)_2D_3$. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. HG group. (I) Immunofluorescence staining of α-SMA demonstrated that $1,25(OH)_2D_3$ prevented expression of α-SMA in HPMCs. Magnification, x200. EMT, epithelial-mesenchymal transition; VDR, vitamin D receptor; HG, high glucose; HPMCs, human peritoneal mesothelial cells; α-SMA, α-smooth muscle actin; FN, fibronectin; D, $1,25(OH)_2D_3$.

Statistical analysis. Statistical analysis was performed using SPSS version 18 (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± standard error of the mean. Multiple comparisons were performed using analysis of variance. Differences between two variables were assessed using an unpaired Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Influence of $1,25(OH)_2D_3$ on VDR and HG-induced EMT in HPMCs. The present study analyzed the expression levels of VDR and EMT biomarkers, including α-SMA, FN and E-cadherin in order to determine the effect of $1,25(OH)_2D_3$ on HG-treated HPMCs. As presented in Fig. 1A and B, it was

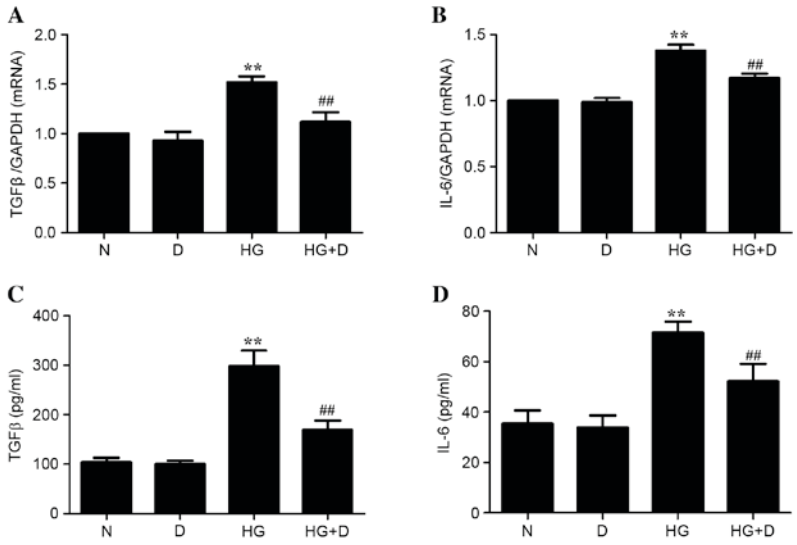

Figure 2. Effects of $1,25(OH)_2D_3$ on inflammatory cytokines in HG-treated HPMCs. HPMCs were exposed to 126 mM HG following pretreatment with 10^{-7} mol/l $1,25(OH)_2D_3$. Reverse transcription-quantitative polymerase chain reaction was performed to detect the relative gene expression of (A) TGFβ and (B) IL-6 in HPMCs (n=3). Protein levels of (C) TGFβ and (D) IL-6 were assessed using ELISA kits (n=6). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. HG group. HG, high glucose; HPMCs, human peritoneal mesothelial cells; TGFβ, transforming growth factor β; IL-6, interleukin-6; N, control; D, $1,25(OH)_2D_3$.

determined that HG significantly downregulated the mRNA expression of VDR in a dose- and time-dependent manner (P<0.05). Fig. 1C-F indicated that HG significantly upregulated the expression of α-SMA (P<0.05) and FN (P<0.01) and downregulated the expression levels of E-cadherin in HPMCs (P<0.01), which indicated that EMT may have occurred following HG treatment. Additionally, protein and mRNA expression of VDR were downregulated following HG treatment (Fig. 1C, G and H). However, HG-induced EMT was attenuated by 10^{-7} mol/l $1,25(OH)_2D_3$ pretreatment in HPMCs (Fig. 1C-F) which led to increased expression levels of VDR (Fig. 1C, G and H). In addition, immunofluorescence staining demonstrated that $1,25(OH)_2D_3$ attenuated HG-induced α-SMA in HPMCs (Fig. 1I).

Effect of $1,25(OH)_2D_3$ on inflammatory cytokines in HG-treated HPMCs. The mRNA and protein expression levels of inflammatory cytokines TGFβ and IL-6 were examined to determine the effect of $1,25(OH)_2D_3$ on inflammation in HG-treated HPMCs. As presented in Fig. 2, HG treatment significantly upregulated the mRNA and protein expression of inflammation cytokines TGFβ and IL-6 (P<0.01). However, this effect was prevented by 10^{-7} mol/l $1,25(OH)_2D_3$ pretreatment in HPMCs (P<0.01). Therefore, it is possible that $1,25(OH)_2D_3$ attenuated the HG-induced TGFβ and IL-6 expression in HPMCs.

Effect of VDR on HG-induced EMT and inflammation in HPMCs. The VDR-shRNA plasmid was used to observe the association of VDR, EMT markers and inflammatory cytokines in order to determine the effect of VDR on HG-induced EMT and inflammation. As presented in Fig. 3, it was determined that 126 mM HG treatment upregulated the expression levels of α-SMA and FN and downregulated the expression levels of E-cadherin and VDR (P<0.01). The expression levels were reversed by $1,25(OH)_2D_3$ pretreatment in HPMCs. Additionally, these effects were diminished when cells were transfected with VDR-shRNA. As presented in Fig. 4, it was demonstrated that 126 mM HG treatment upregulated the expression of TGFβ and IL-6, whereas $1,25(OH)_2D_3$ pretreatment was able to prevent the expression of inflammatory cytokines in HPMCs. The protective effect of $1,25(OH)_2D_3$ was diminished when transfected with VDR-shRNA. Therefore, the present study indicated that $1,25(OH)_2D_3$ may attenuate HG-induced EMT and inflammation in HPMCs by binding to its receptor.

Effects of $1,25(OH)_2D_3$ on the TGFβ/Smad pathway in HG-treated HPMCs. TGFβ/Smad signaling pathway has been previously reported to be involved in EMT (15). However, it remains to be elucidated whether $1,25(OH)_2D_3$ attenuated EMT in HPMCs through TGFβ/Smad pathway. Therefore, the present study examined the effect of $1,25(OH)_2D_3$ supplementation on the TGFβ/Smad pathway in the HPMCs. It was indicated that HG treatment increased the expression of TGFβ. Smad activation was further analyzed by western blotting with a p-Smad3 antibody. As presented in Fig. 5A and B, when the cells were exposed to HG alone, Smad3 phosphorylation was increased compared with the control (P<0.01), whereas it was significantly decreased when $1,25(OH)_2D_3$ pretreatment was administered (P<0.01). These observations suggest that $1,25(OH)_2D_3$ protects HG-stimulated HPMCs via the TGFβ/Smad pathway.

The VDR-shRNA plasmid was used to observe the association of VDR and the TGFβ/Smad pathway. As presented in Fig. 5C and D, it was determined that 126 mM HG upregulated the expression level of p-Smad3, which was reversed by $1,25(OH)_2D_3$ pretreatment. These effects were diminished when cells were transfected with VDR-shRNA.

Discussion

Peritoneal fibrosis is a serious complication in patients with ESRD, especially those undergoing long-term PD therapy. Vitamin D deficiency is highly prevalent in patients undergoing

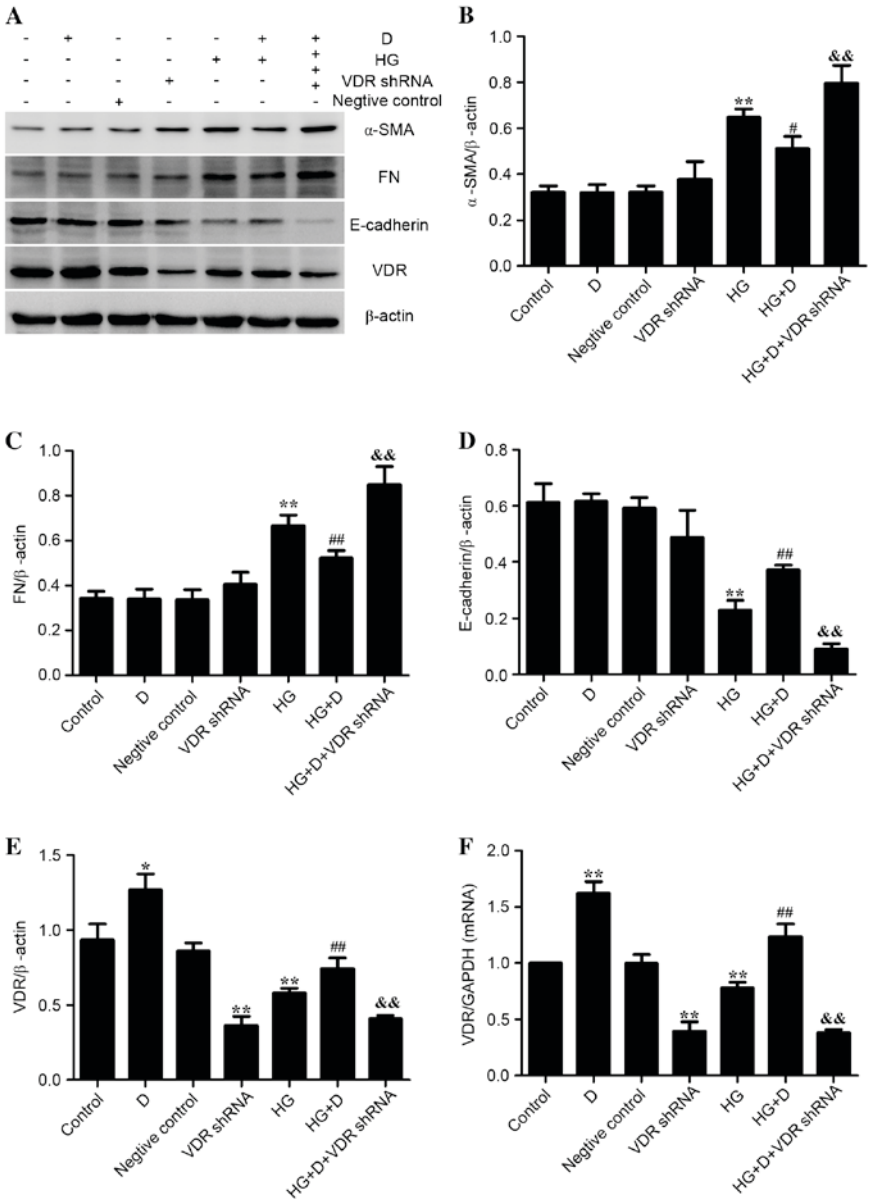

Figure 3. Effect of VDR on epithelial-mesenchymal transition biomarkers in HG-treated HPMCs. (A) Western blotting was used to determine protein expression levels. Relative expression levels of (B) α-SMA, (C) FN, (D) E-cadherin and (E) VDR were calculated and normalized to the loading control. Corresponding protein levels were assessed using densitometry and are expressed as relative intensities. Data are presented as the mean ± standard error (n=3). (F) Reverse transcription-quantitative polymerase chain reaction was performed to detect the relative gene expression of VDR in HPMCs. Data are presented as the mean ± standard error (n=6). *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. HG group; $^{\&\&}$P<0.01 vs. HG+D group. D, $1,25(OH)_2D_3$; HG, high glucose; shRNA, short hairpin RNA; VDR, vitamin D receptor; α-SMA, α-smooth muscle actin; FN, fibronectin; HPMCs, human peritoneal mesothelial cells.

dialysis. Previous studies suggested that $1,25(OH)_2D_3$ may affect organ fibrosis and exhibit anti-inflammatory capabilities (16-18). EMT is a key process leading to the subsequent development of peritoneal fibrosis and peritoneal failure associated with PD (19). A previous study indicated that the low expression of VDR in chronic kidney diseases was likely mediated by proinflammatory TNF-α, and late administration of active vitamin D was effective in restoring VDR expression and inhibited EMT in the mouse unilateral ureter obstruction model (20). Additionally, $1,25(OH)_2D_3$ has been identified to prevent lung and pancreatic cancer progression by inhibiting EMT (21,22). Previous studies demonstrated that EMT in HPMCs was associated with the recurrent use of HG treatment and has been linked to a decline of peritoneal function due to peritoneal fibrosis (23,24). Therefore, it is possible that $1,25(OH)_2D_3$ may affect fibrosis via inhibition of EMT in HPMCs. The present study used HG stimuli to reproduce the damage of peritoneal EMT *in vitro* and examined the effect of $1,25(OH)_2D_3$ on the EMT of HPMCs. The findings of the current study indicated that HG reduced VDR expression, altered HPMC morphology and EMT markers, decreased E-cadherin expression levels and increased of α-SMA and FN expression. Additionally, it was determined that $1,25(OH)_2D_3$ served an important role in protecting HG-treated HPMCs against EMT by binding to VDR.

Previous studies suggested that prolonged and chronic inflammation may lead to the occurrence of peritoneal fibrosis (25,26). However, how to prevent peritoneal inflammation and EMT

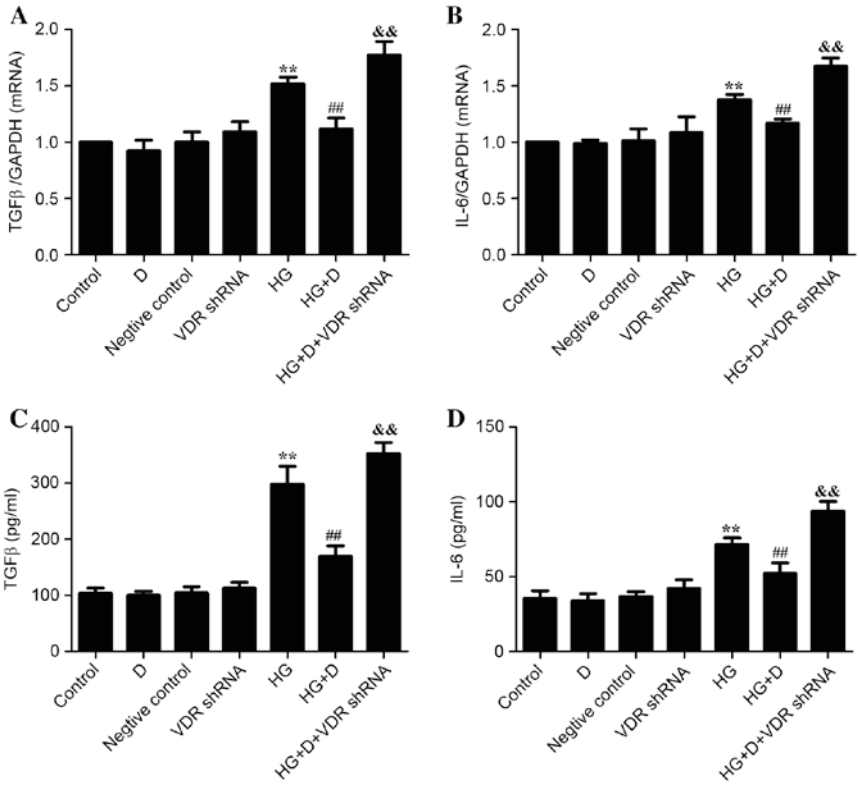

Figure 4. Effects of VDR on inflammatory cytokines in HG-treated HPMCs. Reverse transcription-quantitative polymerase chain reaction was performed to detect the relative gene expression of (A) TGFβ and (B) IL-6 in HPMCs (n=3). Protein expression levels of (C) TGFβ and (D) IL-6 were assessed using ELISA kits. Data are presented as the mean ± standard error (n=6). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. HG group; $^{\&\&}$P<0.01 vs. HG+D group. VDR, vitamin D receptor; HG, high glucose; HPMCs, human peritoneal mesothelial cells; TGFβ, transforming growth factor β; IL-6, interleukin-6; D, 1,25(OH)$_2$D$_3$; shRNA, short hairpin RNA.

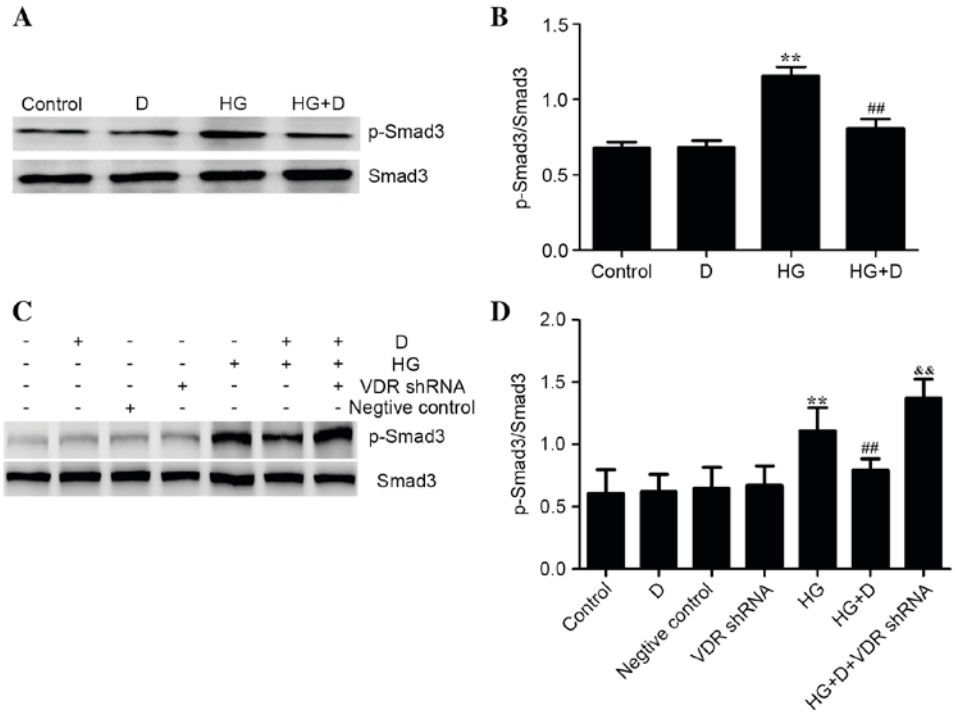

Figure 5. Effects of 1,25(OH)$_2$D$_3$/VDR on the TGFβ/Smad pathway in HG-treated HPMCs. HPMCs were exposed to 126 mM HG following pretreatment with 10^{-7} mol/l 1,25(OH)$_2$D$_3$. (A) Western blotting was used to determine protein expression. (B) p-Smad3/Smad3 protein expression levels were assessed using densitometry and were expressed as relative intensities. Data are presented as the mean ± standard error (n=3). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. HG group. (C) Western blotting was used to determine protein expression levels following VDR downregulation. (D) Protein expression levels of p-Smad3/Smad3 were assessed using densitometry and were expressed as relative intensities. Data are presented as the mean ± standard error (n=3). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. HG group, $^{\&\&}$P<0.01 vs. HG+D group. VDR, vitamin D receptor; TGFβ, transforming growth factor β; Smad3, SMAD family member 3; HG, high glucose; HPMCs, human peritoneal mesothelial cells; D, 1,25(OH)$_2$D$_3$; p-Smad3, phosphorylated-Smad3; shRNA, short hairpin RNA.

remains to be elucidated. The present study determined that HG treatment promoted EMT, increased the expression levels of inflammatory cytokines such as TGFβ and IL-6. Additionally, it was identified that $1,25(OH)_2D_3$ decreased TGFβ and IL-6 expression levels and reversed HG-induced EMT.

The TGFβ/Smad pathway has been thoroughly studied in terms of EMT and inflammation. A previous study demonstrated that vitamin D attenuated renal tubular cell injury by suppressing inflammation and EMT processes through inhibition of the NF-κB, TGFβ/Smad and β-catenin signaling pathways (12). Lee *et al* (27) determined that vitamin D reduced fibrosis, which may be due to its modulation of the anti-inflammatory potentials. The present study determined the association between TGFβ/Smad signaling, EMT and inflammation of HPMCs following HG treatment. The current observations demonstrated that treatment with HG activated the TGFβ/Smad signaling, and these changes were attenuated by $1,25(OH)_2D_3$ pretreatment.

In conclusion, the present study provided novel evidence on the association between $1,25(OH)_2D_3$/VDR and EMT in HPMCs. It was determined that $1,25(OH)_2D_3$ inhibited HG-induced EMT and inflammatory cytokines in HPMCs by binding to its receptor, VDR. In addition, $1,25(OH)_2D_3$ may exert its function via the TGFβ/Smad pathway. Understanding the role of $1,25(OH)_2D_3$/VDR in EMT and inflammation may improve the understanding of the subsequent EMT-mediated fibrosis and peritoneal injury in the development of PD. However, future studies should use animal models *in vivo* in order to determine efficacy.

Acknowledgements

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