1,25-Dihydroxyvitamin D3 Prevents Epithelial-Mesenchymal Transition of HMrSV5 Human Peritoneal Mesothelial Cells by Inhibiting Histone Deacetylase 3 (HDAC3) and Increasing Vitamin D Receptor (VDR) Expression Through the Wnt/β-Catenin Signaling Pathway

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Peritoneal dialysis is the most common treatment for end-stage renal disease. However, peritoneal fibrosis resulting from long-term peritoneal dialysis restricts peritoneal ultrafiltration. Previous studies have shown a role for 1,25-dihydroxyvitamin D3 (1,25[OH]$_2$D3) in preventing fibrosis, but the potential mechanisms remain unknown. This study aimed to investigate the role of 1,25(OH)$_2$D3 in epithelial-mesenchymal transition (EMT) and the downstream signaling pathway in HMrSV5 human peritoneal mesothelial cells in vitro.

Material/Methods: An in vitro cell model of peritoneal fibrosis was established using the HMrSV5 human peritoneal mesothelial cell line. High glucose and lipopolysaccharide (LPS) culture conditions, with or without 1,25(OH)$_2$D3, were used. Wnt agonist 1, a Wnt signaling pathway activator, was applied. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot were used to measure the vitamin D receptor (VDR) and histone deacetylase 3 (HDAC3) gene and protein expression levels, β-catenin, and EMT-associated biomarkers.

Results: High glucose plus LPS culture medium inhibited cell proliferation, induced cell apoptosis and promoted EMT in HMrSV5 cells, which was reversed by 1,25(OH)$_2$D3 by down-regulation of HDAC3 and upregulation of VDR. HDAC3 inhibited VDR gene expression. The expression of EMT-associated biomarkers was increased by Wnt agonist 1 and inhibited by 1,25(OH)$_2$D3.

Conclusions: In HMrSV5 human peritoneal mesothelial cells, 1,25(OH)$_2$D3 reversed EMT by inhibiting the expression of HDAC3 and upregulating VDR gene expression via the Wnt/β-catenin signaling pathway.

MeSH Keywords: Complement Inactivating Agents • Peritoneal Fibrosis • Wnt Signaling Pathway

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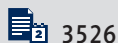
Background

Worldwide, end-stage renal disease leading to kidney failure has become an important public health concern. In 2010, it has been reported that in China there were more than 100,000 patients suffering from end-stage renal disease treated with peritoneal dialysis or maintenance hemodialysis [1]. Peritoneal dialysis is the most widely used treatment for end-stage renal disease. However, the chronic exposure of the peritoneal membrane to the peritoneal dialysis solution during long-term peritoneal dialysis can result in several complications [2]. Peritoneal fibrosis can occur in patients undergoing peritoneal dialysis and is also one of the reasons for patients with end-stage renal disease to withdraw from peritoneal dialysis treatment [3]. Therefore, it is important to investigate the underlying mechanisms of peritoneal fibrosis.

Peritoneal fibrosis is accompanied by loss of the mesothelial cell layer and proliferation of cells in the submesothelial layer [4,5]. The morphological changes in the peritoneal membrane ultimately lead to changes in the submesothelial subcompact zone and blood vessels, which results in failure of ultrafiltration [6,7]. Previous studies have shown that epithelial-mesenchymal transition (EMT) of peritoneal mesothelial cells is one of the key factors contributing to the development of peritoneal fibrosis [8,9]. Although novel types of peritoneal dialysis solutions and anti-fibrotic agents have been developed, their therapeutic effects on peritoneal fibrosis have remained unsatisfactory due to the characteristics of the fluid composition that includes high glucose, low pH, increased lactate concentration and osmolality, and the presence of glucose degradation products [10,11]. The mechanisms of anti-fibrotic drugs, including tamoxifen, remain unknown [12]. Therefore, the mechanisms associated with peritoneal fibrosis require further study.

1,25-dihydroxyvitamin D3 (1,25[OH]₂D3) is the bioactive metabolite of vitamin D, which plays a crucial role in many biological mechanisms through binding the vitamin D receptor (VDR). Early studies demonstrated the key roles of 1,25(OH)₂D3 in calcium homeostasis, cell proliferation and cell differentiation [13]. Recent studies have also indicated the regulatory functions of 1,25(OH)₂D3 in inflammation and fibrosis [14,15]. Vitamin D insufficiency is common among patients with chronic kidney disease, as a reduced glomerular filtration rate (GFR) can result in decreased production of 1,25(OH)₂D3 [16,17]. Treatment with 1,25(OH)₂D3 has been shown to significantly reduce the development of fibrotic lesions in experimental models of chronic kidney diseases [18]. Both of *in vivo* and *in vitro* studies have also shown the role of 1,25(OH)₂D3 in inhibiting the process of epithelial-mesenchymal transition (EMT) in renal interstitial fibrosis [18]. Also, 1,25(OH)₂D3 has been shown to reverse the reduced cell viability and apoptosis of mesothelial cells, which was correlated with the duration of peritoneal dialysis in the

peritoneum in a rat model [19]. Therefore, further studies are needed to determine the role of 1,25(OH)₂D3 in the prevention of the progression of peritoneal fibrosis.

The regulatory role of histone deacetylases (HDACs) in the development of fibrosis has been shown in several disease models, including cardiac fibrosis, the activity of hepatic stellate cells or hepatic pericytes, and renal fibroblasts [20,21]. HDACs are enzymes that catalyze the removal of acetyl groups from lysine residues in histones and proteins and are associated with decreased proliferation and morphological changes of fibroblasts induced by TGF-β signaling [22]. However, whether there is a correlation between 1,25(OH)₂D3 and HDACs remains unknown.

Therefore, this study aimed to investigate the role of 1,25(OH)₂D3 in EMT and the downstream signaling pathway in HMrSV5 human peritoneal mesothelial cells *in vitro*. The study also aimed to investigate the regulatory signaling network of 1,25(OH)₂D3 on HDAC3 and VDR through Wnt/β-catenin signaling pathway.

Material and Methods

Cell culture and treatment

Human peritoneal mesothelial cell line HMrSV5 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HMrSV5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermofisher Scientific, Waltham, MA, USA) with adding 10% fetal bovine serum (FBS) (Gibco, Thermofisher Scientific, Waltham, MA, USA), 100 ul/ml streptomycin and 100 U/ml penicillin (Life Technologies, Carlsbad, CA, USA). The cells were incubated at 37°C humidified atmosphere with 5% CO₂ until 50–70% confluence before used. Cells were transferred to serum-free DMEM medium 12 h prior to each experiment. Cells in the control group were maintained in serum-free DMEM medium. For the experimental groups, HMrSV5 cells were treated with high glucose (25 μM) and lipopolysaccharide (LPS0, 910 μg/ml) for 24 h. As for the role of 1,25(OH)₂D3, HMrSV5 cells were pre-incubated with 1,25(OH)₂D3 (10^{-6} mol/L) for 2 h and subsequently treated with high glucose (25 μM) and LPS (10 μg/ml) for 24 h. Wnt agonist 1, 0.7 μM for 12 h (MedChem Express, Monmouth Junction, NJ, USA) was also used, as previously described [23]. Glucose, LPS, and 1,25(OH)₂D3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The concentrations of glucose and LPS were determined from the findings of preliminary experiments.

MTT assay

To determine the optimized concentrations of glucose and LPS used in the subsequent experiments, HMrSV5 cells were seeded

in 96-well plate and cultured after exposure to solutions containing gradients of glucose and LPS (glucose: 0, 10, 25, 40, 65, 80 µM; LPS: 0, 1, 5, 10, 20, 30 µg/ml). Cells were then incubated with 50 µg/ml MTT at a dilution of 1: 10 for 3 h at 37°C. At the end of treatment, the MTT solution was removed and 150 µl of dimethyl sulfoxide (DMSO) was added to each well. The dark-blue formazan crystals that formed in each well were stirred to until they dissolved. The proportion of viable cells was measured at a wavelength of 450 nm with a spectrophotometer. The experiments were performed in triplicate and the mean values of each group of cultures were compared.

Cell Counting Kit-8 (CCK-8) cell proliferation assay

The proliferation of HMrSV5 cells was assessed with Cell Counting Kit-8 (CCK-8) assay (Vazyme Biotech Co., Nanjing, China), according to the manufacturer's instructions. HMrSV5 cells were seeded into a 96-well plate incubated with high glucose plus LPS for the indicated times. An enzyme-linked immunosorbent assay (ELISA) plate reader was used to measure the absorbance at 490 nm. Each experiment was performed in triplicate.

Flow cytometry

Cells were obtained after centrifugation for 5 min at 500×g at 4°C, and then resuspended to 1×10^6 cells/ml. An apoptosis detection kit (Nanjing KeyGen Biotech, Nanjing, China) was used, according to the manufacturer's instructions and measurements were performed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). All data were analyzed with BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA).

Cell transfection

The short-hairpin RNA (shRNA) sequences targeting human VDR and HDAC3 genes were obtained from GenePharma (Shanghai, China) and were transfected into cells with Lipofectamine 2000 (Life Technologies, USA), according to the manufacturer's instructions. For HDAC3 gene overexpression, transfection was performed with 2 µg of the plasmid into HMrSV5 cells. Wnt agonist 1, a Wnt signaling pathway activator, was used (MedChem Express, Monmouth Junction, NJ, USA).

RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and diluted to 200 ng/ml. The mRNA levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR), which was performed using OneStep SYBR® PrimeScript™ RT-PCR Kit II

(Takara, Minato-ku, Tokyo, Japan) according to manufacturer's protocol. The relative gene expression was calculated using the 2$^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal control. Primers used in qRT-PCR were as follows:
VDR: Forward: 5'-GCCCACCACAAGACCTA-3'.
Reverse: 5'-CCTTTTGGATGCTGTAACTG-3'.
GAPDH: Forward: 5'-GGACCAATACGACCAAATCCG-3'.
Reverse: 5'-AGCCACATCGCTCAGACAC-3'.

Western blot

Cells were harvested and homogenized with cell lysis buffer (Beyotime, Shanghai, China) on ice for 30 min. Lysed cells were centrifuged for 30 min at 4°C and the supernatants were taken. The total protein was measured using the Bradford method. An equal amount of protein samples was separated by denaturing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked in 5% dried skimmed milk powder in TBST solution at room temperature for 1 h. The membrane was then washed and incubated at 4°C overnight with specific primary antibodies to: histone deacetylase 3 (HDAC3) (1: 300) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), vitamin D receptor (VDR) (1: 1000) (Cell Signaling Technology, Danvers, MA, USA), β-catenin (1: 1000) (Cell Signaling Technology, Danvers, MA, USA), E-cadherin (1: 1000) (Abcam, Cambridge, MA, USA), N-cadherin (1: 1000, BD, USA), α-smooth muscle actin (α-SMA) (1: 1000) (Abcam, Cambridge, MA, USA), type I collagen (1: 1500) (Abcam, Cambridge, MA, USA), fibronectin (1: 2000) (Abcam, Cambridge, MA, USA), and β-actin (1: 1000) (Sigma-Aldrich, St. Louis MO, USA). The membrane was incubated in the secondary antibodies conjugated with horseradish peroxidase (HRP) (1: 1000) (Bio-Rad, Hercules, CA, USA) at room temperature for 2 h. Protein bands were visualized using an enhanced chemiluminescence (ECL) Western blotting detection system (GE Healthcare Life Sciences, Logan, UT, USA) according to the manufacturer's instruction. The intensity of each band was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistical significance was determined by Student's t-test. Multiple groups were analyzed using analysis of variance (ANOVA). A P-value <0.05 was considered to be significant.

Results

Culture medium containing high glucose and lipopolysaccharide (LPS) induced apoptosis and epithelial-mesenchymal transition (EMT) of HMrSV5 human peritoneal mesothelial cells

HMrSV5 cells were exposed to increasing concentrations of glucose and lipopolysaccharide (LPS). The findings of the MTT assay were used to determine the optimal concentration levels for the subsequent experiments. As shown in Figure 1A and 1B, the optimal concentration of glucose was between 25–40 μM, while the optimal concentration of LPS was between 10–20 μg/ml. The final concentration of glucose (25 μM) and LPS (10 μg/ml) were determined and used in subsequent experiments.

The Cell Counting Kit-8 (CCK-8) assay was also performed to determine the effects of high glucose plus LPS on the inhibition of cell growth, and the results showed that high glucose combined with LPS significantly reduced cell viability when compared with the controls (Figure 1C). Flow cytometry showed that the apoptotic cell rate was also significantly increased in HMrSV5 cells stimulating with high glucose plus LPS (Figure 1D), A variety of morphological features of HMrSV5 mesothelial cells were observed (Figure 1E), with a trend towards fibroblast-like cell differentiation in the high glucose plus LPS group when compared with the control group. Western blot showed that the expression levels of the EMT-associated biomarker E-cadherin were significantly decreased, while the expression of β-catenin, N-cadherin, α-SMA, collagen I, and fibronectin were significantly increased in high glucose plus LPS treated cells when compared with the controls (Figure 1F). These data suggested that culture medium containing high glucose combined with LPS could induce apoptosis and EMT, which are changes associated with peritoneal fibrosis seen *in vivo*.

The effects of 1,25(OH)₂D3 on cell apoptosis and EMT by high glucose plus LPS

To further investigate the role of 1,25(OH)₂D3 in apoptosis and EMT, the HMrSV5 cells were pre-incubated with 1,25(OH)₂D3 before exposure to high glucose plus LPS. The Cell Counting Kit-8 (CCK-8) assay was performed to assess the cell proliferation and showed that high glucose plus LPS significantly inhibited cell proliferation, which was reversed by 1,25(OH)₂D3 (Figure 2A).

Flow cytometry showed that the increase in apoptosis induced by high glucose plus LPS was also significantly reduced in the presence of 1,25(OH)₂D3 (Figure 2B). Also, cells exposed to high glucose plus LPS showed a trend for the morphological change to fibroblast-like cells, but 1,25(OH)₂D3 converted the

morphology of HMrSV5 to more typical mesothelial cells with a cobblestone-like growth pattern, which was similar to that in control group (Figure 2C). Western blot analysis also showed that the protein expression levels of the EMT-associated biomarker E-cadherin was significantly increased and other biomarkers, including β-catenin, N-cadherin, α-SMA, collagen I, and fibronectin were reduced in the presence of 1,25(OH)₂D3 (Figure 2D). These findings indicated that 1,25(OH)₂D3 may have a protective role to prevent the inhibition of cell growth and the process of EMT stimulated by high glucose combined with LPS.

1,25(OH)₂D3 regulated EMT by inhibiting HDAC3 and upregulating VDR expression

To further explore the underlying mechanism responsible for regulatory functions of 1,25(OH)₂D3 on the process of EMT, quantitative real-time polymerase chain reaction (qRT-PCR) and western blot were performed to detect the expression pattern of HDAC3 and VDR genes and protein in HMrSV5 cells exposed to high glucose plus LPS in the presence or absence of 1,25(OH)₂D3. HDAC3 was significantly upregulated when HMrSV5 cells were exposed to high glucose plus LPS, while this effect was reversed with 1,25(OH)₂D3 (Figure 3A, 3B). The expression of VDR was down-regulated when HMrSV5 cells were cultured in media containing high glucose plus LPS, but VDR expression increased in the presence of 1,25(OH)₂D3 (Figure 3C, 3D).

Based on the findings from previous studies, a short-hairpin RNA (shRNA) plasmid was used to specifically target VDR and HDAC3 to induce overexpression and used for cell transfection. Western blot showed that the expression of the EMT-associated biomarker E-cadherin was increased and the expression levels of other biomarkers, β-catenin, N-cadherin, α-SMA, collagen I, and fibronectin, were reduced in the absence of 1,25(OH)₂D3. Knockdown of VDR or overexpression of HDAC3 both blocked the effects of 1,25(OH)₂D3 (Figure 3E). These data suggested that 1,25(OH)₂D3 might prevent EMT in mesothelial cells by modifying VDR and HDAC3 expression.

HDAC3 negatively regulated VDR expression

To further explore the relationship between HDAC3 and VDR in cells cultured in high levels of glucose with LPS, or with treatment with 1,25(OH)₂D3, knockdown of HDAC3 by shRNA was used. As shown in Figure 4A, 4B, the mRNA and protein levels of VDR were significantly down-regulated or upregulated in cells transfected with HDAC3 or shHDAC3, compared with the control group. Analysis of cell apoptosis, detected using the flow cytometry, showed that the increased apoptotic cell rate induced by high glucose plus LPS was significantly reduced by HDAC3 knockdown or VDR overexpression (Figure 4C, 4D).
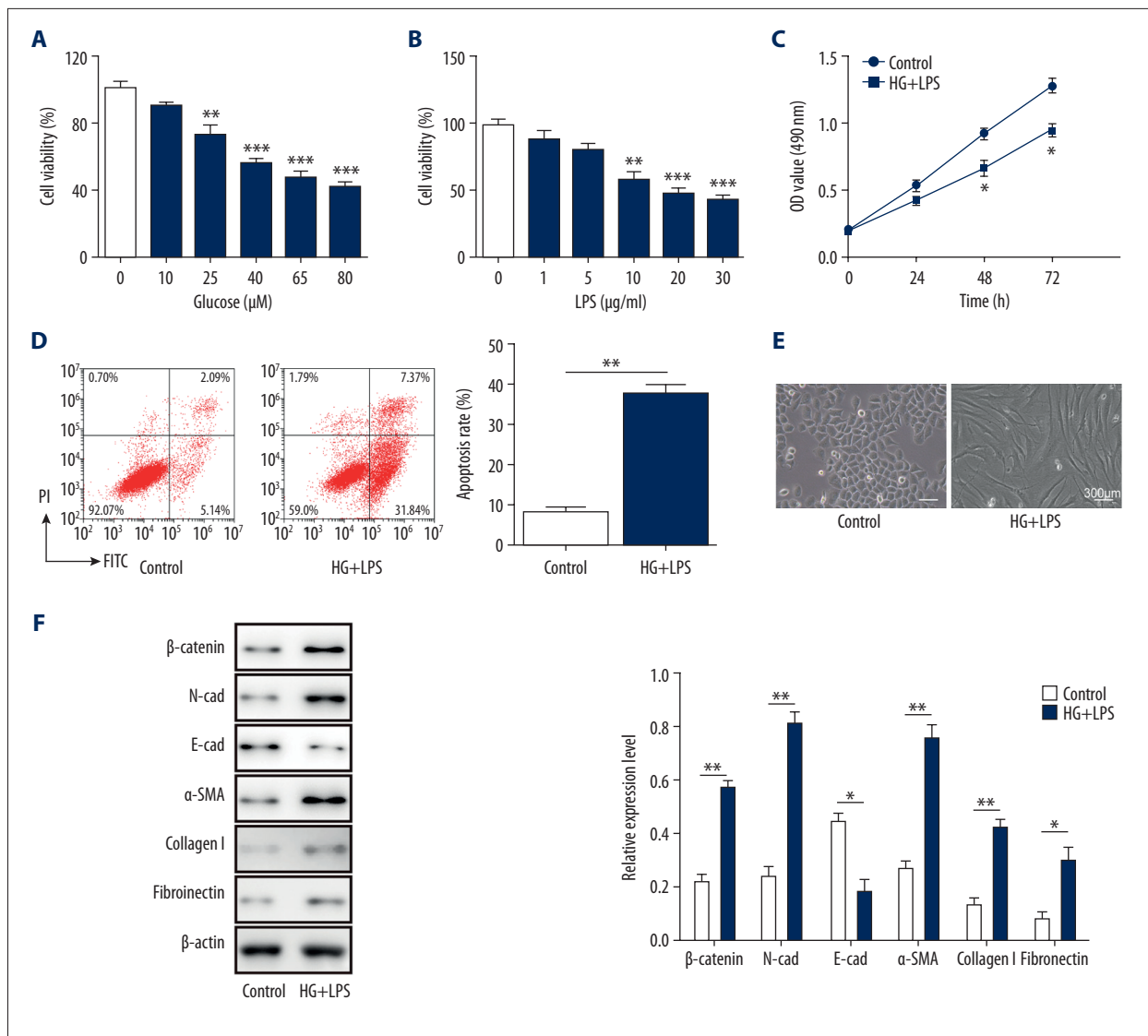
Figure 1. The effects of high glucose and lipopolysaccharide (LPS) on HMrSV5 human peritoneal mesothelial cells *in vitro*. (**A**) HMrSV5 cells were treated with glucose (0, 10, 25, 40, 65, and 80 μM) for 24 h, and cell viability was measured using the MTT assay. ** P<0.01 and *** P<0.001 *vs.* control (0 μM). (**B**) HMrSV5 cells were treated with LPS (0, 1, 5, 10, 20, 30 μg/ml) for 24h, and the cell viability was analyzed by the MTT assay. ** P<0.01 and *** P<0.001 *vs.* control (0 μg/mL). (**C**) The Cell Counting Kit-8 (CCK-8) assay was performed to detect the cell proliferation of HMrSV5 cells cultured with high glucose plus LPS. * P<0.05 *vs.* control. (**D**) Flow cytometry was performed to detect cell apoptosis of HMrSV5 cells treated with 25 μM high glucose plus 10 μg/ml LPS. ** P<0.01 *vs.* control. (**E**) Phase-contrast microscopy showed different morphological characteristics of HMrSV5 cells treated with 25 μM high glucose plus 10 μg/ml LPS compared with the control group. Scale bar, 300 μm. (**F**) The expression of epithelial-mesenchymal transition (EMT) markers, E-cadherin, N-cadherin, α-smooth muscle actin (α-SMA), collagen I, fibronectin, and β-catenin were detected by Western blot, and β-actin was used as an internal control. * P<0.05 and ** P<0.01 *vs.* control. Values represent the mean ± standard deviation (SD) of three independent experiments. HG – high glucose; LPS – lipopolysaccharide.

1,25(OH)₂D3 mediated EMT in human peritoneal mesothelial cells through the inhibition of Wnt/β-catenin signaling by regulating HDAC3/VDR

To further investigate whether the Wnt/β-catenin signaling pathway was involved in 1,25(OH)₂D3-mediated EMT induced by high glucose combined with LPS, Wnt agonist 1 was used. The morphological changes in HMrSV5 cells showed fibroblast-like cells in HMrSV5 cells stimulating by high glucose plus LPS treatment, and these changes were prevented by the administration of 1,25(OH)₂D3. However, cells converted back again to a fibroblast-like morphological appearance after pre-culture
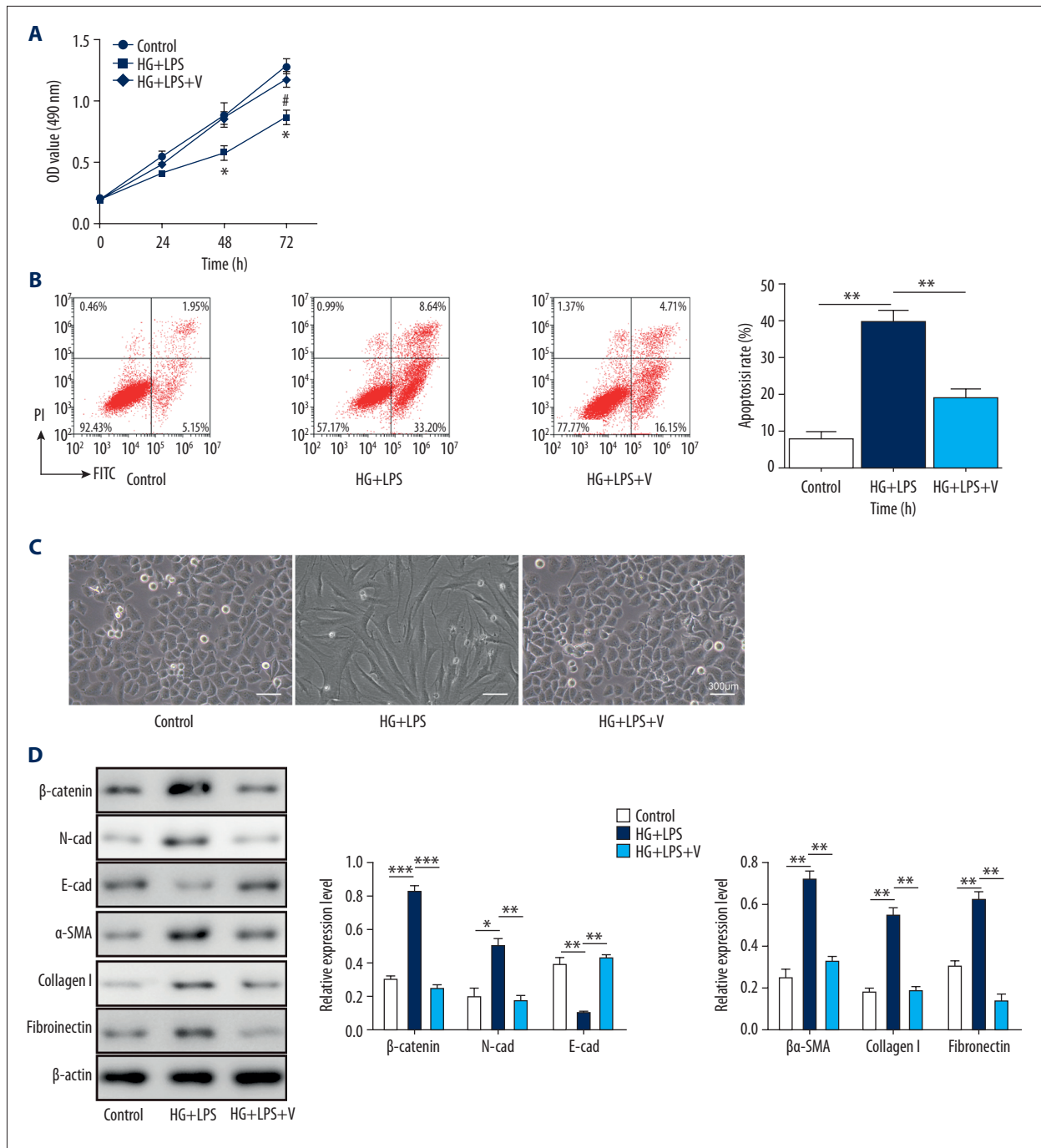
Figure 2. The effects of 1,25-dihydroxyvitamin D3 (1,25[OH]₂D3) on HMrSV5 human peritoneal mesothelial cells induced by high glucose plus lipopolysaccharide (LPS). (**A**) The cell proliferation of HMrSV5 cells under high glucose (HG) plus lipopolysaccharide (LPS) culture conditions with or without 1,25(OH)₂D3 pretreatment (10⁻⁶ mol/L) was determined by the Cell Counting Kit-8 (CCK-8) assay. * P<0.05 *vs.* control, # P<0.05 *vs.* HG+LPS. (**B**) The flow cytometry assay was performed to measure cell apoptosis under high glucose plus LPS condition or pretreatment 1,25(OH)₂D3 (10⁻⁶ mol/L). ** P<0.01 *vs.* control and ** P<0.01 *vs.* HG+LPS. (**C**) Phase-contrast microscopy shows the different morphological characteristics of HMrSV5 cells treated with normal culture medium, 25 μM high glucose plus 10 μg/ml LPS, 25 μM high glucose plus 10 μg/ml LPS supplemented with 1,25(OH)₂D3 (10⁻⁶ mol/L). Scale bar, 300 μm. (**D**) Western blot analysis to determine the expression of epithelial-mesenchymal transition (EMT) markers E-cadherin, N-cadherin, α-smooth muscle actin (α-SMA), collagen I, fibronectin and β-catenin. β-actin was used as an internal control. HG – high glucose; V – 1,25(OH)₂D3; LPS – lipopolysaccharide.
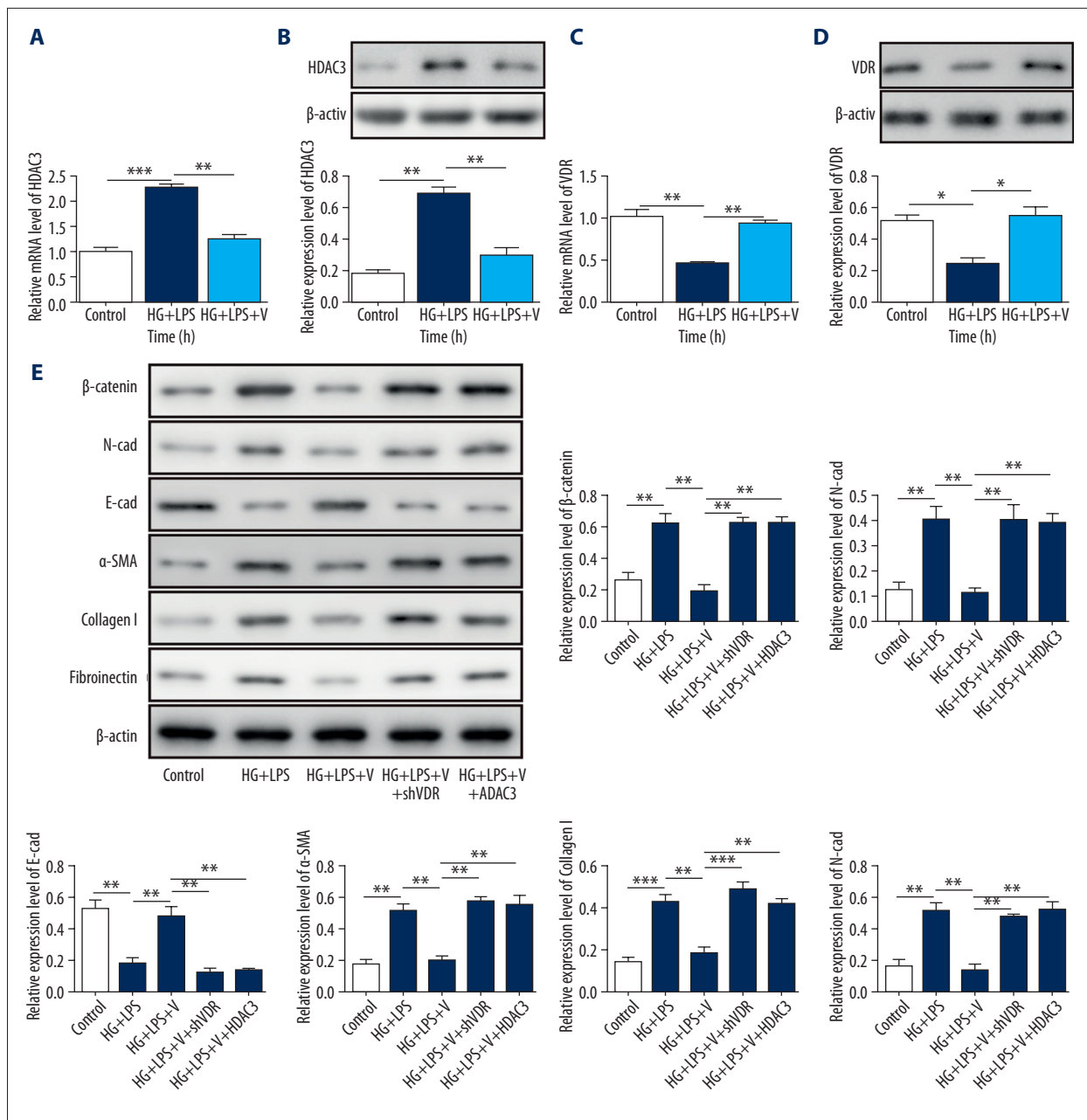
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Figure 3. 1,25-dihydroxyvitamin D3 (1,25[OH]₂D3) regulated HDAC3 and VDR expression in epithelial-mesenchymal transition (EMT). (**A, B**) Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot assays of HDAC3 mRNA and protein expression levels in HMrSV5 cells. β-actin was used as an internal control. ** P<0.01 and *** P<0.001 *vs.* control. ** P<0.01 *vs.* high glucose (HG)+lipopolysaccharide (LPS). (**C, D**) qRT-PCR and Western blot analysis of VDR mRNA and protein expression levels in HMrSV5 cells. β-actin was used as an internal control. * P<0.05 and ** P<0.01 *vs.* control. * P<0.05 and ** P<0.01 *vs.* HG+LPS. (**E**) The expression of epithelial-mesenchymal transition (EMT) markers E-cadherin, N-cadherin, α-smooth muscle actin (α-SMA), collagen I, fibronectin, and β-catenin were detected by Western blot. β-actin was used as an internal control. ** P<0.01 and *** P<0.001 *vs.* control. ** P<0.01 *vs.* HG+LPS. ** P<0.01 and *** P<0.001 *vs.* HG+LPS+V. HG – high glucose; LPS – lipopolysaccharide; V – 1,25(OH)₂D3.

with Wnt agonist 1 (Figure 5A). Western blot showed that the regulatory effects of 1,25(OH)₂D3 on the down-regulation of HDAC3 and the upregulation of VDR were also reversed by Wnt agonist 1 (Figure 5B).

EMT-associated biomarkers were also measured. As shown in Figure 5C, 1,25(OH)₂D3 reversed the inhibition of E-cadherin expression and inhibited the upregulation of β-catenin, N-cadherin, α-SMA, collagen I and fibronectin associated with
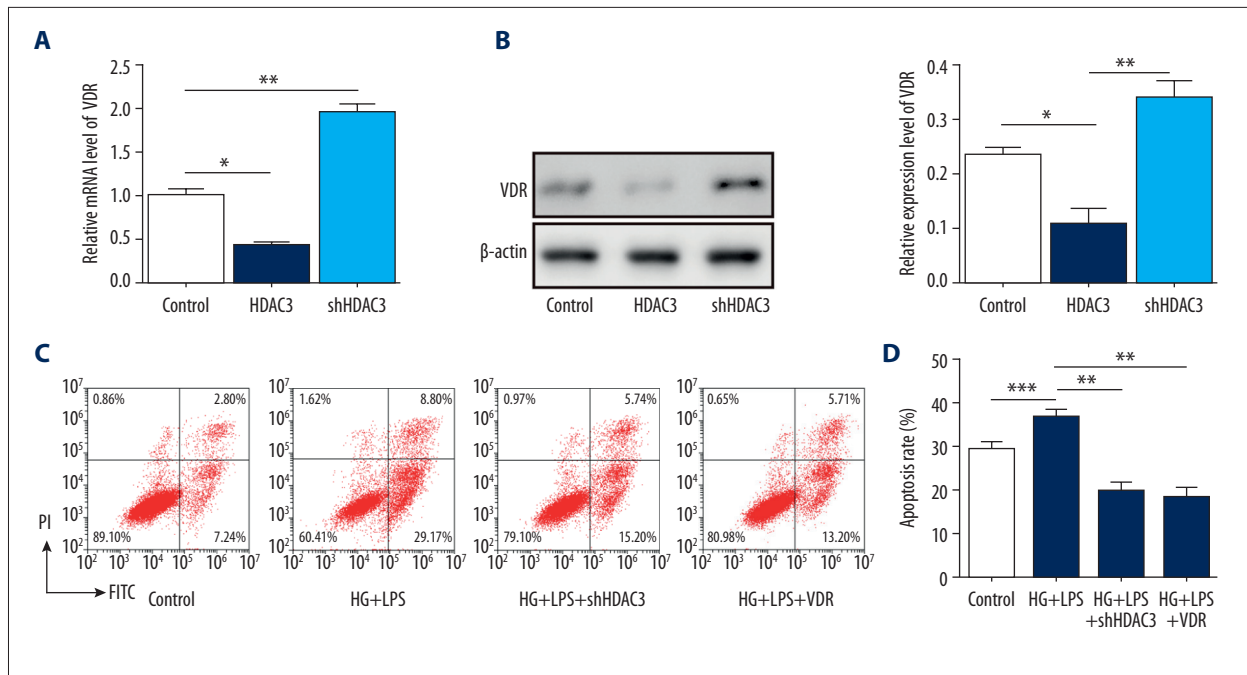
Figure 4. The negative correlation between HDAC3 and VDR expression. (**A**) A quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed to detect the mRNA expression of VDR in HMrSV5 cells with knocked down or overexpressed HDAC3. * P<0.05 and ** P<0.01 *vs.* control. (**B**) Western blot assay to measure the protein expression of vitamin D receptor (VDR) in HMrSV5 cells with knocked down or overexpressed HDAC3. β-actin was used as an internal control. * P<0.05 and ** P<0.01 *vs.* control. (**C, D**) Cell apoptosis of HMrSV5 cells transfected with short-hairpin HDAC3 (shHDAC3) or VDR following exposure with high glucose (HG) plus lipopolysaccharide (LPS). *** P<0.01 *vs.* control. ** P<0.01 *vs.* HG+LPS. Values represent the mean ± standard deviation (SD) of three independent experiments.
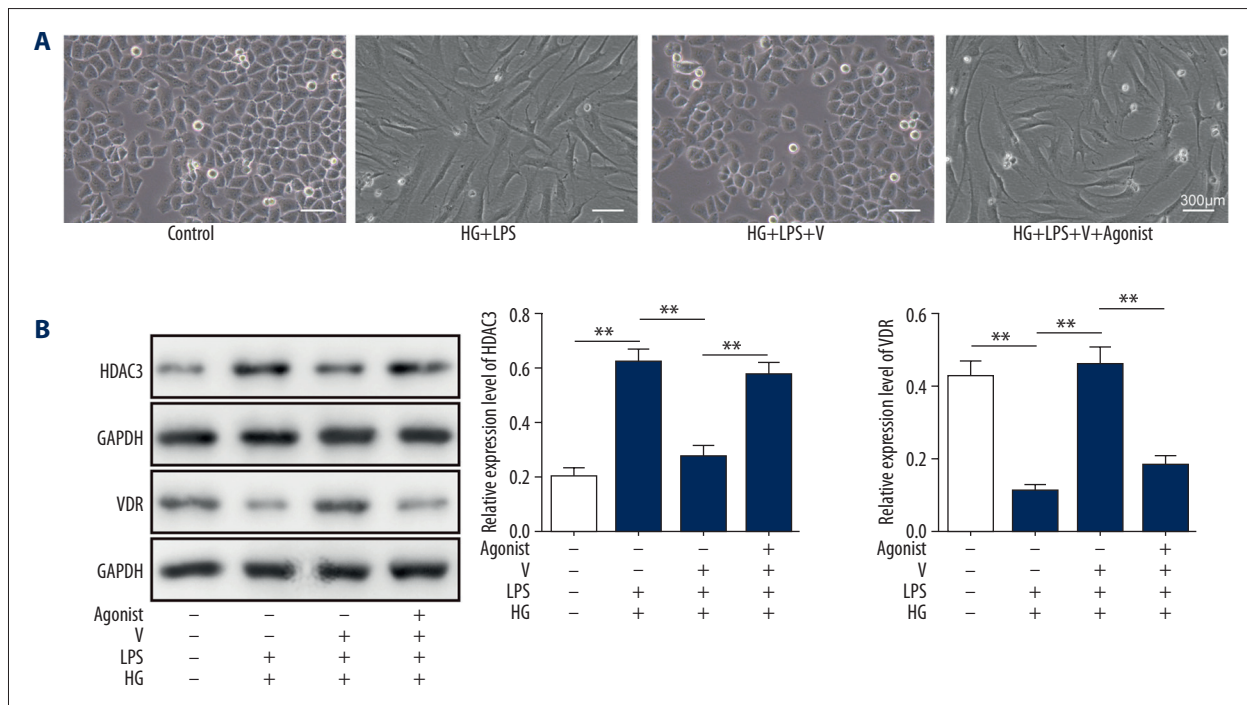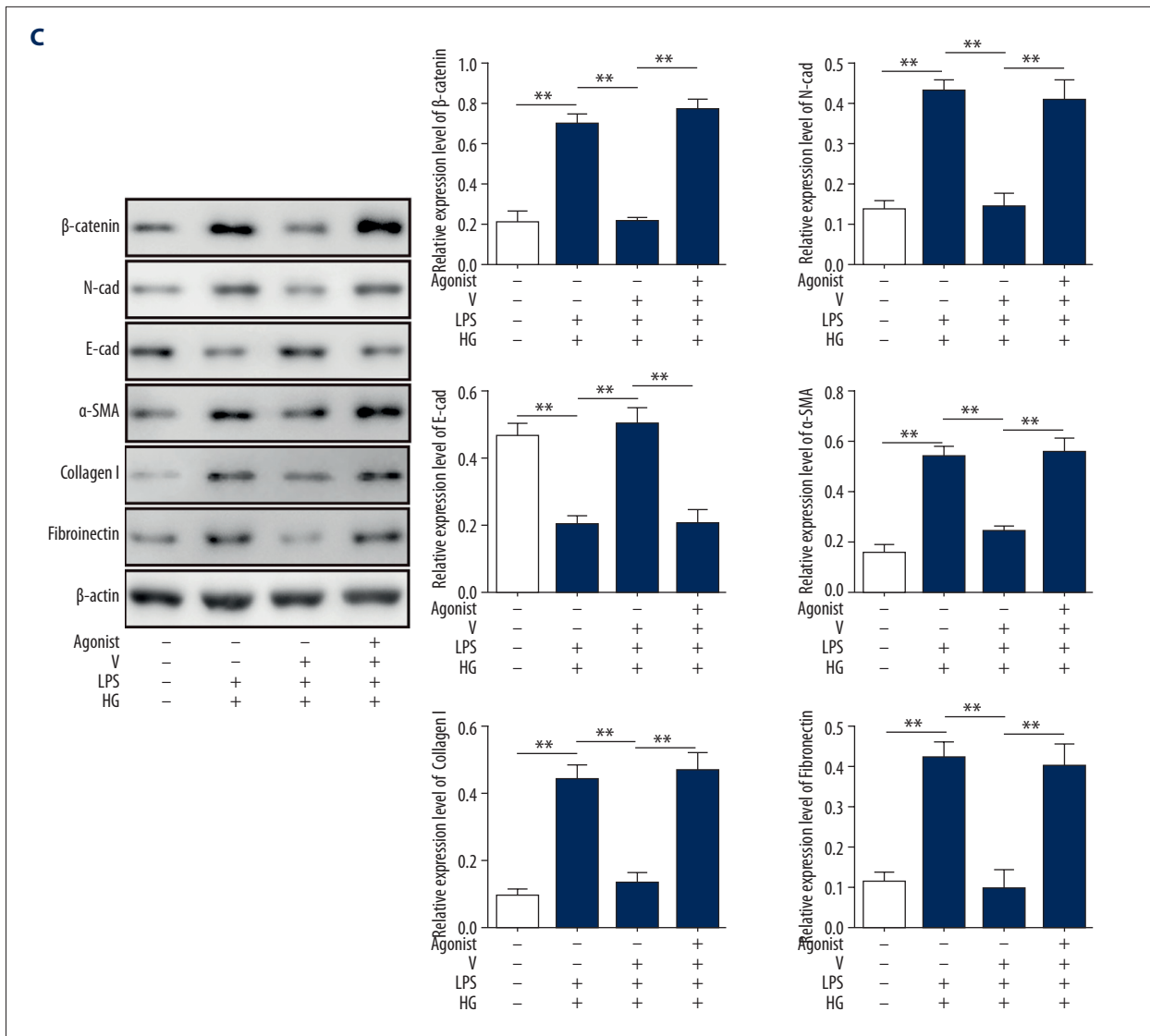
Figure 5. 1,25-dihydroxyvitamin D3 (1,25[OH]₂D3) mediated epithelial-mesenchymal transition through HDAC3 and VDR via Wnt/β-catenin signaling. Wnt agonist 1 was used to confirm the further molecular mechanisms of 1,25(OH)₂D3. HMrSV5 cells were incubated with the Wnt agonist 1 in culture medium with 1,25(OH)₂D3, following culture with high glucose (HG) plus lipopolysaccharide (LPS). (**A**) Phase-contrast microscopy showed different morphological characteristics of HMrSV5 cells. Scale bar, 300 μm. (**B**) The expression of HDAC3 and VDR was detected by Western blot analysis. ** P<0.01 *vs.* control. ** P<0.01 *vs.* HG+LPS. ** P<0.01 *vs.* HG+LPS+V. (**C**). The expressions of epithelial-mesenchymal transition (EMT) markers E-cadherin, N-cadherin, α-smooth muscle actin (α-SMA), collagen I, fibronectin, and β-catenin were detected by Western blot. β-actin was used as an internal control. ** P<0.01 *vs.* control. ** P<0.01 *vs.* HG+LPS. ** P<0.01 *vs.* HG+LPS+V. HG – high glucose; LPS – lipopolysaccharide; V – 1,25(OH)₂D3; Agonist – Wnt agonist 1.

cell culture with high glucose plus LPS. However, these effects were reversed with pre-treatment with Wnt agonist 1. These findings supported the role of 1,25(OH)₂D3 in the inhibition of apoptosis and EMT of human peritoneal mesothelial cells *in vitro* by regulating HDAC3/VDR through Wnt/β-catenin signaling.

Discussion

The present study aimed to investigate the role of 1,25-dihydroxyvitamin D3 (1,25[OH]₂D3) in epithelial-mesenchymal transition (EMT) and the downstream signaling pathway in HMrSV5 human peritoneal mesothelial cells *in vitro*. The results demonstrated a protective role for 1,25(OH)₂D3 when HMrSV5 cells were cultured in medium containing high concentrations of glucose combined with lipopolysaccharide (LPS), in terms of

cell apoptosis and epithelial-mesenchymal transition (EMT). As the active metabolite of vitamin D3, $1,25(OH)_2D3$ was previously reported to play a vital role in maintaining mineral and skeletal homeostasis [24]. A recently published study has also demonstrated the role of $1,25(OH)_2D3$ in modulating cell proliferation and differentiation, inflammation, and fibrosis [18]. The protective role of $1,25(OH)_2D3$ has been reported in liver fibrosis, muscle fibrosis and renal fibrosis [25–27]. Among multiple signals involved in the metabolism of $1,25(OH)_2D3$, its specific receptor, vitamin D receptor (VDR), and its interaction with $1,25(OH)_2D3$ have been reported to mediate the expression of transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) in peritoneal mesothelial cells, which is associated with the progression of EMT during the pathogenesis of peritoneal fibrosis [27].

EMT is considered to be a critical step in the development of peritoneal fibrosis progress and is characterized by the loss of the expression of the associated biomarker E-cadherin, accompanied by upregulation of N-cadherin, β-catenin, α-smooth muscle actin (α-SMA), type 1 collagen, and fibronectin. Therefore, in this study, the impact of $1,25(OH)_2D3$ on EMT was also evaluated in this study and was shown to reduce apoptosis induced by high glucose plus LPS in human peritoneal mesothelial cells and to inhibit the progression of EMT, as demonstrated by the expression levels of associated biomarkers and changes in cell morphology. Also, the regulatory network of $1,25(OH)_2D3$ during EMT was identified by inhibiting histone deacetylase 3 (HDAC3) and upregulating VDR, and was supported by the findings on Western blot.

Previously reported studies have shown that the inhibition of HDAC3 can reduce the degree of fibrosis in several chronic diseases [25–30]. VDR, via its co-repressor protein, can associate with HDACs in the absence of ligand and can act as a transcription factor [31]. HDAC3 has been previously shown to stimulate TGFβ transcription during EMT in human peritoneal mesothelial cells *in vitro* [3]. The inhibitor of HDAC3 has previously been shown to reverse EMT in human peritoneal mesothelial cells by blocking TGFβ/Smad3 pathway [3]. Given that the down-regulation of VDR in a mouse colon tumor model was previously shown to be partially driven by HDAC expression [32], VDR expression was stimulated by the administration of an HDAC inhibitor in a model of human small cell lung cancer [33]. The findings of the present study were consistent with previously published findings that HDAC3 negatively regulates expression of VDR, but is a novel finding in human peritoneal mesothelial cells.

In the present study, $1,25(OH)_2D3$ was shown to upregulate VDR gene expression by inhibiting HDAC3, which inhibited the progression of EMT by reducing Wnt/β-catenin signaling under high glucose plus LPS culture conditions. There has been published evidence to support that $1,25(OH)_2D3$ could reduce chlorhexidine gluconate-induced EMT *in vivo* in both rat and mouse models [34,35]. Previous studies focused on the pivotal role of TGFβ1 in peritoneal fibrosis, and have shown that the TGFβ/Smad signaling pathway might be one of the molecular mechanisms for the antifibrotic effect of $1,25(OH)_2D3$ on peritoneal fibrosis. Consistent with the findings of previous studies, the findings of the present study confirmed that the same effects of $1,25(OH)_2D3$ on the progression of EMT exists in a human peritoneal mesothelial cell line. However, unlike previous studies that focused on TGFβ signaling and its regulatory mechanisms during peritoneal fibrosis, the findings of the present study demonstrated the regulatory role of $1,25(OH)_2D3$ on Wnt/β-catenin signaling in a human peritoneal mesothelial cell line. This study also showed that the administration of Wnt agonist 1, a Wnt signaling pathway activator, reversed the regulatory effect of $1,25(OH)_2D3$ on HDAC3 and VDR gene expression and the process of EMT in peritoneal mesothelial cells.

TGFβ is known to be a key cytokine involved in EMT and is associated with peritoneal fibrosis [34]. Wnt signaling is essential to angiogenesis in the peritoneum [36], and increased expression of Wnt/β-catenin also contributes to mesothelial-mesenchymal transition and peritoneal fibrosis induce by peritoneal dialysis [37]. More importantly, hyperactivation of the Wnt/β-catenin pathway can result in cell death [38]. The findings of the present study indicate a potential therapeutic pathway for targeting apoptosis during the process of peritoneal fibrosis, other than that of the TGFβ-associated inflammation. Also, chlorhexidine gluconate, which was used in previous studies, is a chemical irritant and is not a dialysate that induces peritoneal fibrosis clinically. In previous studies using animal models, the dose of $1,25(OH)_2D3$ used was supraphysiological for humans, which may cause side effects, such as vascular calcification and hypercalcemia. Therefore, the findings of the present study provide some insight into the potential clinical therapeutic role of $1,25(OH)_2D3$ in peritoneal dialysis-induced peritoneal fibrosis.

However, this study had several limitations. This was an *in vitro* study that used a single cell line. Further *in vivo* studies are required to support the findings of this preliminary study. Also, peritoneal tissues from patients with uremia may be used in future studies to determine the effects of toxins on the peritoneum. Dialysate, rather than high glucose combined with LPS medium, could be used to evaluate the role of $1,25(OH)_2D3$ on the prevention or reduction of peritoneal dialysis-induced peritoneal fibrosis in future studies.

Conclusions

Worldwide, peritoneal dialysis remains the most widely used treatment for end-stage renal disease, but the complication

of peritoneal fibrosis can cause the patient to withdraw from peritoneal dialysis. Peritoneal dialysis fluid has a high glucose content, and lipopolysaccharide (LPS) is glycolipid secreted by Gram-negative bacteria, which are a common cause of dialysis-induced infection. By establishing an *in vitro* model of mesothelial cells grown in a high glucose combined with LPS, the findings of this study showed that 1,25-dihydroxyvitamin D3 (1,25[OH]₂D3) reverse the process of epithelial-mesenchymal transition (EMT) in human peritoneal mesothelial cells by inhibiting the expression of histone deacetylase 3 (HDAC3), while increasing the expression of vitamin D receptor (VDR) by regulating the Wnt/β-catenin signaling pathway. These findings require further study, but provide some insight into peritoneal fibrosis caused peritoneal dialysis.

Conflict of interest

None.

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