EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

Twist Contributes to Proliferation and Epithelial-to-Mesenchymal Transition—Induced Fibrosis by Regulating YB-1 in Human Peritoneal Mesothelial Cells

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Twist is overexpressed in high glucose (HG) damage of human peritoneal mesothelial cells (HPMCs) *in vitro*. Herein, we further identified its precise function related to fibrosis of peritoneal membranes (PMs). The overexpression and activation of Twist and YB-1 (official name, YBX1) and a transformed fibroblastic phenotype of HPMCs were found to be positively related to epithelial-mesenchymal transition progress and PM fibrosis *ex vivo* in 93 patients who underwent continuous ambulatory peritoneal dialysis (PD), and also in HG-induced immortal HPMCs and an animal model of PD. Evidence from chromatin immunoprecipitation and luciferase reporter assays supported that *YBX1* is transcriptionally regulated by the direct binding of Twist to E-box. Overexpression of Twist and YB-1 led to an increase in epithelial-mesenchymal transition, proliferation, and cell cycle progress of HPMCs, which might contribute to PM fibrosis. In contrast, the silencing of Twist or YB-1 inhibited HG-induced growth and cell cycle progression of HPMCs; this led to a down-regulation in the expression of cyclin Ds and cyclin-dependent kinases, finally inhibiting PM fibrosis. Twist contributes to PM fibrosis during PD treatment, mainly through regulation of YB-1. (*Am J Pathol* 2015, 185: 2181–2193; <http://dx.doi.org/10.1016/j.ajpath.2015.04.008>)

Peritoneal dialysis (PD) is a common life-sustaining treatment in patients with end-stage renal disease, which uses the single-cell peritoneal membrane (PM) as a semipermeable membrane.¹ Sometimes, this dialysis is broken off by PM injury and fibrosis, which are involved in the morphological alterations of the peritoneum in patients who undergo chronic dialysis. It has been increasingly recognized that the phenotypic transition of the human peritoneal mesothelial cells (HPMCs) is a key step in PM early injury and fibrosis. Previous work also showed that a high glucose (HG) injury was actually responsible for fibrosis of the PM.²

An early process in peritoneal fibrosis is epithelial-mesenchymal transition (EMT) of HPMCs,³ which is a complex, step-wise phenomenon detrimental in fibrogenic tissues.^{4,5} It was thought that soon after dialysis is initiated,

peritoneal mesothelial cells undergo a transition from an epithelial phenotype to a mesenchymal phenotype, with EMT markers changed after 48 hours of HG stimulation *in vitro*. HG-induced phenotypic transition was the earliest phenomenon of peritoneal damage and could be observed earlier than significant morphological changes of HPMC cells. Although peritoneal fibrosis was an intractable process, a significant reversal of EMT of HPMCs at early damage stage would be found.

Therefore, preventing EMT can ameliorate peritoneal fibrosis, preserving the PM during PD. Some researchers

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have found that the growth and proliferation of HPMCs significantly changes when fibrosis develops in the PM after HG stimulation, followed by the cell cycle progression.⁶ Cell proliferation could not be affected or even be inhibited at the initial stimulation time for 48 hours, and then had an abnormal proliferation and cell cycle progress at the following HG stimulation days. Thus, the inhibition of growth and the induction of cell cycle arrest of HPMCs might contribute to protection against PM fibrosis.

Twist, a highly conserved helix-loop-helix transcription factor, was newly identified as a key regulator of embryogenesis, pulmonary fibrosis, and cancer metastasis.^{7–9} Our previous study found that Twist was involved in hypoxia-induced EMT of human tubular epithelial cells and might contribute to the pathogenesis of progressive renal fibrosis.¹⁰ Previous work in our laboratory also initially revealed the role of Twist in peritoneal fibrosis *in vitro*.¹¹ It implied that HG stimulation could activate Twist, and promote EMT of HPMCs *in vitro*.¹² However, the precise role of Twist in peritoneal fibrosis *in vivo* and its underlying mechanism are not fully understood.

YB-1, a member of the Y-box-binding protein family, contains a highly conserved nucleic acid binding domain and has several E-boxes located in the core promoter.¹³ YB-1 has been proposed to possess various biological activities in both nucleus and cytoplasm.¹⁴ Thus, YB-1, which is up-regulated in both proliferating adult tissues and cancer cells, may be closely correlated with the proliferation and phenotypic transition of HPMCs. Previous work in tumor showed that YB-1 might be a main target of Twist, and found that YB-1 reexpression almost completely rescued Twist siRNA-induced growth arrest, but cotransfection of Twist expression plasmid could not rescue YB-1 siRNA-induced growth arrest.¹⁵ Although YB-1 is thought to be a major downstream target of Twist and necessary for tumor cell proliferation and cell cycle progress, the mechanism of Twist and YB-1 in proliferation of HPMCs and EMT-induced peritoneal fibrosis were still unclear. In this study, we continue to uncover the role of Twist and YB-1 in proliferation and cell cycle progress of HPMCs and further investigate the exact mechanism of PM fibrosis.

Materials and Methods

Ethics Statement

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of Xijing Hospital (Xi'an, Shaanxi, People's Republic of China). Written informed consent was obtained from each patient. The Ethics Committee for Animal Experiments of the Fourth Military Medical University (Xi'an, Shaanxi, People's Republic of China) approved all animal work (permit number 20120023), and the experimental protocols strictly complied with the institutional guidelines and the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*.^{16,17}

HPMC Isolation and Culture

HPMCs, which were obtained from the effluents of 93 patients undergoing continuous ambulatory PD (CAPD) at our PD center in the Department of Nephrology, Xijing Hospital, from January 1, 2013, to December 31, 2013, were collected and cultured according to a previously described method.^{16,18–20} The use of human peritoneal fluid in this study was approved by the Institutional Review Board of the Fourth Military Medical University. This study was conducted in accordance with international guidelines for the use of human tissues. The inclusion criteria were as follows: <65 years of age, duration of CAPD >1 month, no peritonitis within the past 6 months, the use of 1.5% glucose dialysis solution, and no history of abdominal surgery or long-term use of an immunosuppressant. The CAPD patients were sorted into four groups (G) according to the duration of dialysis (G₁ was 1 to 6 months; G₂, 7 to 24 months; G₃, 25 to 48 months; and G₄, >48 months) (Supplemental Table S1). The human immortal peritoneal mesothelial cell line HPMC (American Type Culture Collection, Rockville, MD) is an untransformed HPMC line that yields data similar to the results obtained with primary cells. The immortal and primary HPMC cell lines were cultured at 37°C with 5% CO₂ in Earle's M199 medium²¹ supplemented with 10% fetal calf serum (Gibco, Grand Island, New York), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. EMT was induced in immortal HPMCs with HG (60 mmol/L; R&D Systems, Minneapolis, MN), as described previously.^{14,20,22}

Plasmid Construction and Cell Transfection

The pcDNA3.1-Twist plasmid was a gift from Prof. Carlotta A. Glackin (National Medical Center and Beckman Research Institute, Durate, CA).¹⁰ Targeted siRNA was determined with the siRNA design tool (Invitrogen, Carlsbad, CA). The Twist siRNA-specific targeting sequence was 5'-AAGATGGCAAGCTGCAGCTAT-3'; a scramble sequence, 5'-TCAGCAAGGTGACTGAGAC-3', was used as control. The YB-1 siRNA-specific target sequence was 5'-GGCGAAGGUUCCACCUUATT-3'; 5'-UUCUCCGAACGUGUCACGUdTdT-3' was used as a control. The transfections were performed with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Real-time PCR or Western blot analysis was used to assess the transfection efficiency in cells harvested 48 hours for mRNA and 72 hours for protein after siRNA transfection.

Generation of Twist Overexpression, Twist-shRNA, and YB-1-shRNA Lentivirus

The Twist overexpression, Twist-shRNA, or YB-1 shRNA-specific targeting sequence and the control sequence were described as above. Human embryonic kidney 293 T cells (Invitrogen) were transfected with the two expression vectors PLVX-IRES-mCherry and PLVX-shRNA2 and two packaging plasmids PMD and psPAX2, at 4, 1, and 3 mg of

DNA per 150-mm plate. After 6 hours, Dulbecco's modified Eagle's medium was changed. Forty-eight hours later, supernatants from four plates were pooled, filtered, and stored at 4°C short-term. Lentivirus titers were determined by transfecting human embryonic kidney 293 T cells with a dilution series of the viral suspension, and lentivirus samples with a titer of 4×10^8 transfection units/mL were stored at -80°C.^{23,24}

Peritoneal Exposure Model in Rats

A chronic infusion model of nonuremic animal PD was used, as described previously.^{6,14} Briefly, 16 male Sprague-Dawley rats (weight, 250 to 300 g) were divided into two groups with eight rats in each group. The control group was injected with normal saline (100 mL/kg per day) each day for 6 weeks; the HG-PD fluid (PDF; pH 4.5 to 6.5; GDP, 4.25%; Baxter, Chicago, IL) group was injected with 4.25% glucose dialysis solution (100 mL/kg per day) each day for 6 weeks. Another 24 HG-PDF male Sprague-Dawley rats were divided into three groups, which were separately treated with empty lentivirus vector (control-shRNA), lentivirus vector containing Twist-shRNA (Twist-shRNA), or YB-1 shRNA (YB-1-shRNA) i.p. via the peritoneal access port at a dose of 4×10^8 transfection units every 5 days, three times during HDF-PD injection.²⁵ Rats were manipulated and cared for according to NIH Animal Care and Use Committee guidelines in the Experiment Animal Center of the Fourth Military Medical University (Xi'an, Shanxi, Province, People's Republic of China).

Cell Proliferation Assay (MTT Assay)

HPMCs were plated onto 96-well plates (Corning, NY) at a density of 2.5×10^4 cells per well. Viable cells were assayed at 1, 2, 3, 4, and 5 days. After incubation with MTT assay (Sigma, San Francisco, CA), the cells were lysed in 150 μ L of 100% dimethyl sulfoxide and the absorbance was tested at 490 nm with a 96-well plate reader (Dynex Technologies, Chantilly, VA).²⁴ Each experiment was performed in triplicate.

Flow Cytometry

Flow cytometric analysis was performed as previously described.²⁴ The transfected cells were harvested when they were 70% to 80% confluent. After washing in ice-cold phosphate-buffered saline, the cells were suspended in approximately 0.5 mL of 70% alcohol and kept at 4°C overnight. The DNA content of stained nuclei was analyzed by a flow cytometer (EPICS XL; Coulter, Miami, FL). The cell cycle was analyzed with FACSscan software version 1.4 (Becton Dickinson, San Jose, CA). The proliferous indices (PIs) were calculated with the following equation:

$$PI = (S + G_2)/(S + G_2 + G_1) \quad (1)$$

Each experiment was performed in triplicate.

RT-PCR

Total RNA was extracted with the RNeasy kit (Qiagen GmbH, Hilden, Germany), and cDNA was obtained from 500 ng of total RNA with an Omniscript RT kit (Qiagen GmbH). Quantitative PCR was performed in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) with a SYBR Green kit (Roche Diagnostics GmbH). The following specific primer sets were used: glyceraldehyde-3-phosphate dehydrogenase, 5'-CCTCAAGATCATCAGCAAT-3' (forward) and 5'-CCATCCACAGTCTTCTGGGT-3' (reverse; used for normalization); Twist, 5'-CGGGAGTCCGCAGTCTTA-3' (forward) and 5'-ACCGGGTTGGTTTGTATCTG-3' (reverse); CDH1 (E-cadherin), 5'-TTCCCTCGACACCCGATTTC-3' (forward) and 5'-TAGGTGGAGTCCCAGGCGTA-3' (reverse); α -smooth muscle actin (α -SMA), 5'-AAGATGACCCAGATCATGTT-3' (forward) and 5'-TCATAGATGGGACATTGT-3' (reverse); and YB-1, 5'-AAGTGATGGAGGGTGCTGAC-3' (forward) and 5'-TTCTTCATTGCCGTCTCTC-3' (reverse). Experiments were performed in duplicate. After amplification, the PCR products were confirmed by melt curve analysis and gel electrophoresis.

Western Blot Analysis

Total protein extracts from the cultured cells were loaded onto SDS-PAGE gels, and Western blot analysis was performed according to a standard protocol. The antibodies used were as follows: anti-Twist, diluted 1:100 (Sigma); anti-E-cadherin, anti- α -SMA, and anti-YB-1, diluted 1:50 (Santa Cruz Biotechnology, Dallas, TX); anti-cyclin D1, anti-cyclin E, anti-cyclin-dependent kinase (CDK) 2, anti-CDK4, anti-proliferating cell nuclear antigen (PCNA), anti-matrix metalloproteinase (MMP)-2, anti-MMP-9, and anti-p27, diluted 1:150 (Santa Cruz Biotechnology); and anti- β -actin (Sigma), diluted 1:4000. The staining was performed as described previously.²⁴

Immunofluorescence and Immunohistochemistry

Cultured cells were grown on 0.8 $\times$ 0.8-cm slides, rinsed with ice-cold phosphate-buffered saline, and fixed in 4% paraformaldehyde. Immunofluorescence or immunohistochemistry staining was performed on cultured cells or paraffin-embedded sections, as described previously.¹² The samples were incubated with Ms/Rb polyclonal anti-Twist (Abcam, Cambridge, England; dilution 1:100), anti- α -SMA, anti-PCNA, and anti-E-cadherin (Santa Cruz Biotechnology; dilution 1:200), anti-fibronectin (FN), or anti-YB-1 antibodies (Santa Cruz Biotechnology; dilution 1:100). Then, they were counterstained with fluorescein isothiocyanate-labeled IgG secondary antibodies (Invitrogen) or poly-horseradish peroxidase anti-Ms or Rb IgG secondary antibody (ZSBIo, Beijing, People's Republic of China) and were stained with diaminobenzidine. Images were obtained with a Leica TCS microscope and imaging software (Leica software version SP8; Leica

Figure 1 Twist and YB-1 expression in high glucose (HG)-induced immortal human peritoneal mesothelial cells (HPMCs) *in vitro*. **A**: Western blot analysis shows the induction of Twist, YB-1, pYB-1, E-cadherin, and α -smooth muscle actin (α -SMA) expression in immortal HPMCs, which were exposed to HG for 48 hours *in vitro*. **B**: Fluorescence microscopy illustrates the location and expression of Twist, YB-1, E-cadherin, and α -SMA in HG-induced immortal HPMCs exposed to HG for 48 hours. **C**: Real-time PCR analysis shows the level of Twist, YB-1, E-cadherin, and α -SMA induced by HG stimulation for 48 hours in immortal HPMCs. Bars represent the fold induction over untreated cells. **D**: Staining for the Twist, YB-1, fibronectin (FN), proliferating cell nuclear antigen (PCNA), and Masson in peritoneal samples by immunohistochemistry reveals that peritoneal dialysis (PD) fluid containing HG exposure (HG-PDF) induces the up-regulation of Twist, YB-1, and PD-induced peritoneal membrane (PM) fibrosis *in vivo*. **E**: The histogram shows the thickness for hematoxylin and eosin (H&E) staining in the submesothelial compact zone in HG-PDF rats ($130.63 \pm 11.26 \mu\text{m}$) compared with control rats ($15.25 \pm 2.43 \mu\text{m}$). **F**: The histogram shows the average of cell cycle analysis in omentum-derived HPMCs from HG-PDF rat ($64.82\% \pm 2.41\%$) compared with the omentum-derived HPMCs from control rats ($47.15\% \pm 5.00\%$). Each was conducted in triplicate. **G**: Western blot analysis shows the expression of cyclin D1, cyclin E, p27, cyclin-dependent kinase (CDK) 2, CDK4, and PCNA in omentum-derived mesothelial cells from HG-PDF compared with the cells from control rats. Data are given as means $\pm$ SEM (**C** and **E**). $n = 3$ independent experiments conducted in duplicate (**C**); $n = 8$ (**E**). * $P < 0.05$ versus controls. Original magnifications: $\times 200$ (**B**); $\times 100$ (**D**, top panels); $\times 400$ (**D**, bottom panels).

Microsystems, Wetzlar, Germany). The images were processed with Photoshop CS (Adobe, San Jose, CA). The positive expression was graded from 0 to 3+ (0, no staining; 1+, 1% to 25%; 2+, 26% to 50%; 3+, >50% staining).

ChIP

Chromatin immunoprecipitation (ChIP) was performed as previously described.^{10,25} Chromatin fragments were

Table 1 Expression and Location of Twist in Primary HPMCs

	CAPD times (months)				Fisher's χ^2	P value
	1–6 (n = 37)	7–24 (n = 35)	25–48 (n = 18)	>48 (n = 3)		
Morphological features of primary HPMCs						
Cobblestone	28	6	1	0	64.950	<0.001
Mixed	9	22	3	0		
Fibroblast-like	0	7	14	3		

CAPD, continuous ambulatory peritoneal dialysis; HPMC, human peritoneal mesothelial cell.

co-immunoprecipitated from HG-treated HPMCs and control lysates with the appropriate amount of Twist antibody or an equivalent amount of rabbit IgG (8 μ g) as a control. The purified DNA fragments were used as templates for PCR amplification. Real-time PCR was performed with an ABI 7900 Sequence Detection System (Applied Biosystems, Carlsbad, California) and a SYBR Green PCR core reagent kit (Applied Biosystems). The specific primers were as follows: YB-1 ChIPF1, 5'-AGAACTGACTCGCGAACTATTC-3'; YB-1 ChIPR1, 5'-CAACTCACATTATTGAACAAG-3'. The values were normalized to the input and expressed as a fold increase relative to the IgG control.

Transient Transfection Luciferase Assay

The YB-1 promoter region from –1 to –1000 was cloned into the pGL3 luciferase vector (Promega, Madison, WI). The primers used to generate the mutant reporter were as follows: mutYB-1, 5'-GTGGATAGTAATCCCTCTATTGTAGAGCTGTTGCAGGAATAAAGTG-3' (forward) and 5'-CACTTATTCCTGCAACAGCTCTACAATAGAGGGATTATATCCAC-3' (reverse). For the transient transfection assays, HG-treated HPMCs or control cells were seeded 48 hours before transfection. Then, pGL3-basic was transfected with the PRLsv40 plasmid into cells in the presence of Lipofectamine 2000. The luciferase activity in the cell lysates that were collected 72 hours after transfection was measured as relative light units with the Fluoroscan Ascent FL (Labsystems, Franklin, MA) and the Dual-Luciferase Assay System (Promega). The relative luciferase activity was defined as the ratio of the firefly luciferase activity/mean Renilla luciferase activity for each construct relative to the pGL3 control vector (Promega).

Statistical Analysis

The bands from the Western blot or real-time PCR were quantified by Quantity One software version 4.62 (Bio-Rad, Hercules, CA). The numerical data are presented as the means $\pm$ SEM, and SPSS software version 12.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. The differences between the means were assessed using *t*-test, an analysis of variance, or the χ^2 test. Significant differences were determined by an analysis of variance, followed by post hoc comparisons with the Fisher protected least-significant-difference test. *P* < 0.05 was considered statistically significant.

Results

The Up-Regulation of Twist and YB-1 Occurs in Immortal HPMCs and in Animal Models

In our previous *in vitro* work, we found that Twist was overexpressed in immortal HPMC cell lines.¹¹ To obtain direct evidence of the effects of Twist and YB-1 expression on HG-induced EMT, we tested the expression of Twist and YB-1 genes in HG cultured immortal HPMCs *in vitro* by Western blot analysis and real-time PCR. When HPMCs were incubated with HG medium (60 mmol/L glucose) for 48 hours, the cells lost intercellular junctions, became scattered, and prepared to transform into spindle fibroblastic-shaped cells. Concordant with our *ex vivo* results, HPMCs demonstrated a loss of E-cadherin expression, a gain in α -SMA expression, translocation of Twist from the cytoplasm to the nucleus, and an increase in Twist and YB-1 expression at both the protein and mRNA levels in response to HG stimulation (Figure 1, A–C). Twist translocated into nucleus, but YB-1 only partly translocated into the nucleus after HG stimulation.

We also established an animal model of PD in which rats were injected with HG-PDF (4.25% glucose dialysis solution) or normal saline solution over a period of 6 weeks (Figure 1, D–F). The loss of the mesothelial cell monolayer with a thicker PM (130.63 $\pm$ 11.26) was found in the parietal peritoneum from the untreated PD group compared with the saline-treated control group (15.25 $\pm$ 2.43). An analysis of Twist, YB-1, E-cadherin, α -SMA, FN, and PCNA by immunohistochemistry indicated that HG-PDF up-regulated Twist, YB-1, α -SMA, FN, and PCNA and down-regulated E-cadherin in the PM of our animal model of PD. Masson staining and overexpression of FN, PCNA, and EMT markers α -SMA showed that HG-PD fluid induced PM fibrosis, rapid proliferation, and EMT of HPMCs in the PD animal model. Cell cycle analysis was used to test the cell cycle progress of omentum-derived mesothelial cells from the PD animal model by laser-scanning flow cytometry (Figure 1, E–G). HPMCs from HG-PDF rats showed a significant decrease in the proportion of HPMCs in the G₁ phase from 64.82% $\pm$ 2.41% to 47.15% $\pm$ 5.00%. PI increased from 35.18% to 52.85%. A substantial increase in the proportion of cells in the S fraction was also observed in HPMCs from HG-PDF rats. These data showed that both HG-induced HPMCs and omentum-derived HPMCs from HG-PDF rats overexpressed Twist and YB-1 on increased cell proliferation, progressed to the G₁/S cell cycle, and induced fibrosis.

Figure 2 *Ex vivo* expression of Twist and YB-1 in peritoneal dialysis (PD) effluent-derived human peritoneal mesothelial cells (HPMCs). **A** and **B**: Real-time PCR showing the mRNA levels of Twist and YB-1 in effluent-derived HPMCs from 93 patients from the different groups (G₁ to G₄) that underwent PD treatment. **C**: The correlation analysis of Twist and YB-1 in effluent-derived HPMCs from patients from different groups (G₁ to G₄) that underwent PD treatment.

The Up-Regulation of Twist and YB-1 Occurs *ex Vivo* in HPMCs from Patients with CAPD

To better understand the role of Twist and YB-1 in HPMCs induced by HG injury *in vivo*, we investigated the expression levels of Twist and YB-1 by real-time PCR in effluent-derived HPMCs *ex vivo* from effluents of 93 patients with CAPD who were followed up during a return visit from January 1 to December 31, 2013. After PD treatment, the morphological features of HPMCs were markedly variable and ranged from a cobblestone-like appearance to mixed cell populations and fibroblast-like cells (Table 1), which is consistent with previous findings.^{5,17,20} We assessed Twist and YB-1 expression in all 93 PD patients who were grouped into G₁, G₂, G₃, and G₄ groups according to the interval of PD treatment time. We found that Twist expression significantly increased in HPMCs from patients with CAPD in G₂, G₃, and G₄, compared with patients in G₁; this was consistent with previous *in vitro* studies (Figure 2A).¹¹ We also found the highest YB-1 expression in

patients in G₂, G₃, and G₄ who underwent more than half a year of PD compared with patients with CAPD who underwent PD for a shorter period of time (G₁) (Figure 2B). The YB-1 gene, which positively correlated with Twist, may be related to HG-induced injury of the PM during long-term PD, just like Twist ($r = 0.686$, $P < 0.01$) (Figure 2C). Thus, Twist and YB-1 may be related to the transition of HPMCs after the injury of PMs by exposure to HG during long-term PD.

Twist Transcriptionally Regulates YB-1 Expression by Directly Binding the E-Box in YB-1 Promoter

Structural analysis has revealed the existence of several E-boxes within the YB-1 promoter, suggesting a direct regulation of YB-1 transcription by Twist. To address this hypothesis, ChIP was performed to examine the binding of Twist to the E-box element in the YB-1 promoter in HPMCs. One E-box site (CACGTG, -654 to -649) in the promoter region of the YB-1 gene was predicted to be a candidate binding site of Twist. A 50-fold enrichment was set as the threshold for positive Twist binding. Our results demonstrated strong binding of Twist at this E-box site in HG-induced HPMCs, but weak binding in normal HPMCs. These data suggested that the greatly increased binding signal in the HG-induced HPMCs might be in agreement with the higher Twist expression (Figure 3A).

To further confirm the binding of Twist to the E-box element in the YB-1 promoter, a luciferase reporter assay was performed. Transfected full-length YB-1 plasmids containing an E-box site into HPMCs could stimulate luciferase activity either in normal medium ($P = 0.006$) (Figure 3B) or high-glucose medium ($P = 0.007$) (Figure 3C) compared with control vectors. The effect was more significant in HG-stimulated HPMCs (Figure 3C). Mutation or deletion of the E-box moderately affected their response, which was statistically significant compared with the YB-1 plasmids, especially in HG-HPMCs (mutation, $P = 0.006$; deletion, $P = 0.005$). These results suggested that the activation of Twist–YB-1 signaling occurs via the binding of Twist to YB-1, predominantly at the E-box site in its promoter region.

The Down-Regulation of Twist Causes Growth Retardation and G₁/S Arrest in HG-Induced HPMCs

To obtain further insight into the biological relevance of both Twist and YB-1, we analyzed the effect of Twist on the fibrosis, proliferation, and cell cycle progression of immortal HPMCs after HG stimulation. Cells were HG stimulated and cultured after transfection, which mimicked the damage and treatment of PDF in PD patients. Western blot analysis and immunofluorescence showed that Twist, YB-1, and α -SMA protein levels significantly decreased, whereas E-cadherin protein levels significantly increased, after transfection of siRNA against Twist in HG-induced HPMCs or in transfected HPMCs, which were stimulated by HG (Figure 4, A and B). But Twist and YB-1 were not rescued by HG after knockout of Twist in HPMCs (Supplemental Figure S1). Real-time PCR

Figure 3 Twist directly induces the expression and transcriptional activation of YB-1 in human peritoneal mesothelial cells (HPMCs). **A:** The identification of an E-box (CACGTG) in the YB-1 promoter from -654 to -649. Chromatin immunoprecipitation was used to examine the binding of Twist to the YB-1 promoter in high glucose (HG)-induced HPMCs and in control cells. Reaction controls included immunoprecipitation performed with a non-specific IgG monoclonal antibody; PCR was performed with whole-cell genomic DNA (input). A representative example of three independent experiments is shown. **B and C:** For the competition assays with the E-box, 200-fold molar excess of the YB-1 plasmid, mutant E-box, and deleted E-box were used in HPMCs (**B**) and HG-induced HPMCs (**C**). The average relative luciferase activity data in normal HPMCs transfected with control plasmid, YB-1 with normal E-box, mutant E-box, and E-box deleted separately are 0.015 ± 0.003 , 0.044 ± 0.003 , 0.018 ± 0.005 , and 0.015 ± 0.004 , respectively. All results shown are representative of at least three independent experiments. The average data in HG-induced HPMCs transfected with control plasmid, YB-1 with normal E-box, mutant E-box, and E-box deleted separately are 0.029 ± 0.005 , 0.037 ± 0.053 , 0.045 ± 0.009 , and 0.031 ± 0.006 , respectively. * $P < 0.05$ versus YB-1 (E-box) plasmid; † $P < 0.05$ versus control plasmid.

results were consistent with the immunofluorescence and Western blot analysis (Figure 4C). These experiments demonstrated that the down-regulation of Twist could inhibit EMT in HG-induced HPMCs. MTT assays showed the apparent growth retardation of HG-induced HPMCs after Twist expression was knocked down in HG-induced HPMCs (Figure 4D), which means that down-regulated Twist could hinder the proliferation of HG-induced HPMCs. To understand the implications on the cell cycle, HG-HPMCs were treated with Twist-siRNA at 72 hours after transfection and assayed for cell cycle analysis by laser-scanning flow cytometry (Figure 4, E–G). The silencing of Twist expression led to a significant increase in the proportion of HPMCs in the G₁ phase, from $57.22\% \pm 1.99\%$ to $82.03\% \pm 1.63\%$. PI

decreased from 42.78% to 17.97%. Furthermore, we observed a substantial decrease in the proportion of cells in the S fraction. All of these results showed that the knockdown of Twist also down-regulated the expression of YB-1, which resulted in the inhibition of cellular proliferation, G₁/S cell cycle arrest, and the delay of EMT-induced fibrosis. Thus, Twist might be an essential mediator of regulation of growth and proliferation in HG-induced HPMCs.

We also established PD animal model treatment with Twist shRNA lentivirus compared with control shRNA lentivirus (Figure 4, H and I). A thinner PM was found, with $61.50 \pm 23.57 \mu\text{m}$ in the parietal peritoneum in Twist-shRNA PD rats compared with $121.50 \pm 14.34 \mu\text{m}$ in the control-shRNA group (Figure 4H). An analysis of Twist, YB-1, E-cadherin, α -SMA, FN, and PCNA by immunohistochemistry indicated that treatment of Twist-shRNA lentivirus in PD rats caused down-regulation of YB-1, α -SMA, FN, and PCNA, but up-regulation of E-cadherin in the PM. Decreased expression of FN, PCNA, and EMT marker α -SMA showed that the down-regulation of Twist contributed to the alleviation of PM fibrosis, slower peritoneal transport rate [dialysate-to-plasma rate of urea (D/P urea) and end-to-initial dialysate (D/D₀) glucose] for lower molecular weight (Supplemental Figure S2), and growth retardation of HPMCs in the studied PD animal model.

The Up-Regulation of Twist Increases the Expression of YB-1, Promotes HG-Induced Fibrosis, and Leads to Abnormally Rapid Growth

We further induced the up-regulation of Twist expression to study the role of Twist in the proliferation and fibrosis of HPMCs. Western blot analysis and immunofluorescence showed that the overexpression of Twist after transfection of the full-length human Twist plasmid into immortal HPMCs led to significant down-regulation of E-cadherin and up-regulation of Twist, YB-1, and α -SMA protein (Figure 5, A and B) as well as mRNA (Figure 5C) levels. This is consistent with the results from HG-induced primary cultured HPMCs. These data suggested that the overexpression of Twist could up-regulate YB-1 expression and prompt EMT-induced fibrosis. The results of the MTT assays showed that when Twist was up-regulated by transfection of a plasmid, significant and rapid growth of HPMCs was observed from the third day compared with the cells that were transfected with a control plasmid (Figure 5D). To understand the implications of this increase in growth rate, the cells were treated with pcDNA3.1-Twist and assayed for DNA content at 72 hours after transfection by laser-scanning flow cytometry (Figure 5, E–G). The up-regulation of Twist expression also led to a significant and reproducible decrease in the proportion of HPMCs in the G₁/S phase; in addition, the percentage of cells in G₁ phase decreased from $56.64\% \pm 2.16\%$ to $42.18\% \pm 2.19\%$. Furthermore, we observed a substantial increase in the proportion of cells in the S phase. PI staining also increased from 43.36% to 57.82%. Therefore, Twist regulated YB-1 expression by promoting

Figure 4 The effect of Twist knockdown by Twist-siRNA on the ability of human peritoneal mesothelial cells (HPMCs) to undergo epithelial-mesenchymal transition—induced fibrosis, growth, and cell cycle progression *in vitro*. **A:** Western blot analysis of Twist, YB-1, pYB-1, E-cadherin, and α -smooth muscle actin (α -SMA) in HPMCs transfected with Twist-siRNA (siTwist) or control vector (cont), compared with high glucose (HG)—induced HPMCs (HG-HPMCs). **B:** Fluorescence microscopy shows the location and expression of Twist, YB-1, E-cadherin, and α -SMA in HG-induced Twist-siRNA-treated HPMCs (siTwist) and control cells (HG-HPMCs cont). **C:** Real-time PCR shows the mRNA levels of Twist, YB-1, E-cadherin, and α -SMA in HPMCs transfected with Twist-siRNA (siTwist), cont, and HG-induced HPMCs for 48 hours (HG-HPMCs). Bars represent the fold induction over untreated cells. **D:** The MTT assay shows the effect of Twist in the regulation of proliferation of HPMCs that were transfected with Twist-siRNA (siTwist), compared with cont in HG-induced HPMCs (HG-HPMCs cont). The monolayer growth rates of cells were determined by MTT assays. **E:** The histogram shows the average of cell cycle analysis in HG-induced HPMCs transfected with Twist-siRNA (siTwist), compared with the cont. Each experiment was conducted in triplicate. **F and G:** Cell cycle analysis shows that the inductions of G₁ phase of HG-HPMCs transfected with cont (HG-HPMCs cont) and HG-HPMCs transfected with Twist-siRNA (siTwist) are 55.24% (F) and 83.5% (G), respectively. **H:** The histogram shows the thickness for hematoxylin and eosin staining in the submesothelial compact zone in Twist shRNA HG-PDF rats (61.50 ± 23.57) compared with cont-shRNA HG-PDF rats (121.50 ± 14.34). **I:** Staining for the Twist, YB-1, fibronectin, E-cadherin, α -SMA, and proliferating cell nuclear antigen (PCNA) in peritoneal samples by immunohistochemistry in Twist shRNA HG—peritoneal dialysis fluid (PDF) rats compared with cont-shRNA HG-PDF rats *in vivo*. Data are given as means $\pm$ SEM (C, D, and H). $n = 3$ independent experiments conducted in duplicate (C); $n = 3$ or more separate experiments (D); $n = 8$ (H). * $P < 0.05$. Original magnifications: $\times 200$ (B); $\times 100$ (I, top panels); $\times 400$ (I, bottom panels).

fibrosis and cell proliferation, decreasing cell cycle arrest, and preventing HG-induced alterations.

The Down-Regulation of YB-1 Decreases HG-Induced Fibrosis of HPMCs and Inhibits Cell Proliferation and Cell Cycle Progress by the Down-Regulation of Cyclin D and CDK

To obtain further insight into the biological relevance of YB-1, we analyzed its effect on fibrosis, proliferation, cell cycle progression, and changes in the expression of cell cycle

proteins in immortal HPMCs after HG stimulation. Western blot analysis showed that YB-1 and α -SMA protein levels significantly decreased and that E-cadherin protein levels significantly increased after transfection of siRNA against YB-1 (Figure 6A). MMP-2, which was overexpressed after HG stimulation, also significantly decreased in HG-HPMCs after transfection with YB-1-shRNA, compared with control (transfected with control-shRNA) (Supplemental Figure S3). But MMP-9 expression did not change after HG stimulation and transfection with YB-1-shRNA (Supplemental Figure S3). The results of

Figure 5 Up-regulation of Twist by a Twist plasmid affects the epithelial-mesenchymal transition—induced fibrosis, proliferation, and cell cycle arrest in human peritoneal mesothelial cells (HPMCs) *in vitro*. **A:** Fluorescence microscopy illustrates the location and expression of the Twist, YB-1, E-cadherin, and α -smooth muscle actin (α -SMA) proteins in HPMCs transfected with a Twist plasmid (pcDNA3.1-Twist) or a control plasmid (HPMC pcDNA3.1). **B:** Western blot analysis of Twist, YB-1, pYB-1, E-cadherin, and α -SMA in HPMCs transfected with a Twist plasmid (pcDNA3.1-Twist) or a control plasmid (HPMC pcDNA3.1). **C:** Real-time PCR shows the mRNA levels of Twist, YB-1, E-cadherin, and α -SMA in HPMCs transfected with a Twist plasmid (pcDNA3.1-Twist) or a control plasmid (HPMC pcDNA3.1). **D:** Effect of Twist in the regulation of proliferation of HPMCs transfected with a Twist plasmid (pcDNA3.1-Twist) or a control plasmid (HPMC pcDNA3.1). Monolayer growth rates of cells were determined by MTT assays. **E:** The histogram shows the average of cell cycle analysis in HPMCs transfected with a Twist plasmid (pcDNA3.1-Twist) compared with a control plasmid (HPMC pcDNA3.1). Each experiment was conducted in triplicate. **F and G:** Cell cycle analysis in HPMCs transfected with pcDNA3.1-Twist or a control plasmid (pcDNA3.1) shows the proliferation induction of HPMCs that were transfected with control plasmid (HPMCs pcDNA3.1) or Twist plasmid (pcDNA3.1-Twist) as 54.17% (**F**) and 44.57% (**G**), respectively. Representative experiments of three were performed, with similar results. Data are given as means $\pm$ SEM (**D**). $n = 3$ or more separate experiments (**D**). $*P < 0.05$. Original magnification, $\times 200$ (**A**).

the real-time PCR experiment and immunohistochemistry were similar to those of the Western blot analysis (Figure 6, B and C). These data show that the down-regulation of YB-1 can inhibit EMT-induced fibrosis. The results of the MTT assays illustrated that the growth arrest was induced by YB-1—specific siRNA in HPMCs compared with the control HG-induced cells (Figure 6D). The down-regulation of YB-1 expression led to a significant and reproducible increase in the proportion of HPMCs in the G₁ phase (from $47.25\% \pm 2.75\%$ to $80.93\% \pm 2.98\%$), and PI staining decreased from 52.75% to 19.09% (Figure 6, E–G). To explore the underlying molecular mechanism of YB-1—induced cell cycle arrest, we detected the expression of cell cycle—related molecules after transfection (Figure 6H and Supplemental Figure S4). Our results indicated that the down-regulation of YB-1 protein expression was associated with a reduction in cyclin D1 and CDK4 and cyclin E and CDK2 expression, but was associated with an increase in p27Kip1 expression. Therefore, we infer that the down-regulation of YB-1 might also result in a decrease in cellular proliferation, G₁/S cell cycle arrest, and enhanced fibrosis after changes in various cell cycle proteins.

We further established PD animal model treatment with YB-1—shRNA lentivirus compared with control-shRNA lentivirus (Figure 6, I and J). A thinner PM was found as $69.00 \pm 25.38 \mu\text{m}$ in the parietal peritoneum in YB-1—shRNA PD rats compared with the control shRNA group (Figure 6J). An analysis of Twist, YB-1,

E-cadherin, α -SMA, FN, and PCNA by immunohistochemistry indicated that injection of YB-1—shRNA in PD rats caused down-regulation of YB-1, α -SMA, FN, and PCNA, but up-regulation of E-cadherin in the PM. The expression of Twist did not change after YB-1 down-regulation. The down-regulation of FN, PCNA, and EMT marker α -SMA showed that down-regulation of YB-1 caused alleviation of PM fibrosis and a slower peritoneal transport rate for lower molecular weight (Supplemental Figure S2) and growth retardation of HPMCs in PD rats.

Discussion

The peritoneal mesothelium plays a pivotal role in the control of peritoneal homeostasis. In a series of experiments, the loss of the mesothelium over time and the disruption of the interstitium after chronic exposure to glucose-containing PDF were demonstrated.^{26,27} Previous studies have also shown that exposure to HG could damage HPMCs both *in vivo* and *in vitro*,^{28,29} and it was considered to be the primary cause of peritoneal fibrosis. The inhibitory effect of HG concentrations on the proliferation of HPMCs is not solely related to some property of glucose itself, but rather it is related to its metabolic effects.^{30,31}

We sought to use HG to reproduce the damage caused by the PD effluent and the profibrotic stimuli that are induced during PM fibrosis. HG can induce EMT in HPMCs, and

Figure 6 The knockdown of YB-1 by siRNA affected the ability of human peritoneal mesothelial cells (HPMCs) to undergo epithelial-mesenchymal transition—induced fibrosis, growth, and cell cycle progression *in vitro*. **A:** Western blot analysis of Twist, YB-1, E-cadherin, and α -smooth muscle actin (α -SMA) in HPMCs transfected with YB-1-siRNA (siYB-1) compared with control vector (cont) in high glucose (HG)—induced HPMCs (HG-HPMCs). **B:** Real-time PCR shows the mRNA levels of YB-1, E-cadherin, and α -SMA in HPMCs transfected with siYB-1 and cont in HG-induced HPMCs for 48 hours (HG-HPMCs). Bars represent the fold induction over untreated cells. **C:** Fluorescence microscopy shows the location and expression of Twist, YB-1, E-cadherin, and α -SMA in HG-HPMCs transfected with YB-1—shRNA lentivirus compared with transfected control-shRNA cells. **D:** The effect of YB-1 in the regulation of the proliferation of HPMCs transfected with siYB-1, compared with cont in HG-induced HPMCs. Monolayer growth rates of the cells were determined by MTT assays. **E:** The histogram shows the average of cell cycle analysis in HG-HPMCs transfected with siYB-1, compared with cont. Each experiment was conducted in triplicate. **F and G:** Cell cycle analysis in HG-induced HPMC cells transfected with siYB-1 or a cont shows the proliferation induction of HPMCs that were transfected with siYB-1 vector as 84.18% (**F**) compared with transfected control plasmid for 44.17% (**G**), respectively. Representative experiment of three with similar results. **H:** The effect of YB-1 on the expression of cell cycle effectors by Western blot analysis. All examined gene expression levels were quantitatively analyzed and are expressed as ratios over β -actin. This is a representative experiment of three separate experiments with similar results. The expression of cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 2, CDK4, and p27 proteins was evaluated after infection with cont siRNA or siYB-1 in HG-induced HPMCs. **I:** Staining for Twist, YB-1, E-cadherin, α -SMA, fibronectin, and proliferating cell nuclear antigen (PCNA) in peritoneal membrane by immunohistochemistry in YB-1—shRNA HG—peritoneal dialysis fluid (PDF) rats compared with cont-shRNA HG-PDF rats *in vivo*. **J:** The histogram shows the thickness for hematoxylin and eosin staining in the submesothelial compact zone in YB-1 shRNA HG-PDF rats (69.00 ± 25.38) compared with cont-shRNA HG-PDF rats (121.50 ± 14.34). Data are given as means $\pm$ SEM (**B**, **D**, and **J**). $n = 3$ independent experiments conducted in duplicate (**B**); $n = 3$ or more separate experiments (**D**). $*P < 0.05$. Original magnifications: $\times 200$ (**C**); $\times 100$ (**I**, top panels); $\times 400$ (**I**, bottom panels).

HG exerts additional effects on cell morphology, which finally leads to PM fibrosis.³² It was thought that soon after dialysis is initiated, peritoneal mesothelial cells undergo a transition from an epithelial phenotype of cobblestone-like morphology to a mesenchymal phenotype of fibroblast-like morphology. Some studies have shown that even much early in CAPD, a loss of cuboid morphology and expression of E-cadherin are observed both *in vivo* and *ex vivo*, even when cells retain an epithelioid appearance. Continued PD results in long-term exposure to mechanical

denudation.²⁰ *In vitro* data suggested that EMT is induced after HG stimulation for 2 days, and could be reversed after changing the HG cell culture fluid to the normal glucose culture medium in <7 days. However, EMT reversion does not happen after HG stimulation for >7 days. So, HG-stimulated EMT of HPMC can be reversed at an early time stage but becomes irreversible at a later stage.^{5,16} A time dependence of EMT reversal was also found when using rapamycin, to induce EMT of mesothelial cells at early time points, but was unable to inhibit the fibroblast-like

phenotypic transformation on day 7 of transforming growth factor- β stimulation. Moreover, HG-induced phenotypic transition was observed as early as 48 hours of stimulation before any significant morphological changes of cells, which suggests that HG-induced alteration in cell phenotype may be one of the earliest phenomena of peritoneal damage. Although peritoneal fibrosis was regarded as a relentless process once it started, HG stimulation for 48 hours, followed by a removal, resulted in a significant reversal of EMT of HPMCs with epithelial redifferentiation to prestimulation level.

Some studies have shown that HG can alter the proliferation of HPMCs, following the changes in the cell cycle.³³ We observed that cell proliferation is not affected, or even inhibited, at the initial stimulation time for 48 hours; proliferation increases and cell cycle progresses rapidly after several days of HG stimulation. It is possible that at the initial HG stimulation time, cells encounter stress damage because of HG, and later HPMCs are rescued but show abnormal proliferation.

Transcription factors, both Twist and YB-1, were proto-oncogenes and are known to regulate the invasive and metastatic characteristics of cancer cells.^{7,34,35} In bladder cancer, Twist regulates YB-1 and is involved in cell growth, invasion, motility, and resistance to cisplatin and doxorubicin.³⁶ Twist also regulates EMT and is involved in tumor metastasis through YB-1. Inhibition of Twist can decrease the expression of YB-1 in highly metastatic cells and finally suppresses the metastatic ability of tumor cells.⁷

Twist induces EMT by HG stimulation of HPMCs *in vitro*, and might even promote PM fibrosis, according to our previous studies.¹¹ Our earlier study was only an initial exploration into the role of Twist in PM fibrosis, which contributed data about the molecular mechanism of Twist *in vitro*, but not *in vivo*. Thus, we continued to study the effects of Twist *in vivo* to identify its possible targets in PM fibrosis. Our data showed that Twist and YB-1 positively correlated and that both were up-regulated in deciduous HPMCs from the effluents of patients who underwent long-term PD.

YB-1 is expressed in cells and is the core EMT regulator that participated in tumor metastasis and growth.¹³ YB-1 promotes cell proliferation through the transactivation of target genes, such as proliferating cell nuclear antigen.³⁷ On the basis of these reports, we proposed that Twist might regulate the expression of YB-1 and might be involved in the promotion of cell proliferation and EMT-induced migration.^{7,15,38} Structural analysis of the YB-1 promoter reveals that several E-boxes may participate in the regulation of YB-1 expression. YB-1-null mice and Twist-null embryos both exhibit failure of neural tube closure,^{15,38,39} which suggests that YB-1 might be a major target of Twist. YB-1 inhibits the translation of the mRNAs encoding CDH1 by snail and other factors, which often appears to involve EMT and contributes to motile tumor cells. YB-1 appears to promote tumor activities by controlling cyclin D1, CDK2, cyclin E, and CDK4; both these processes promote cell cycle progression. YB-1 protein, as a RNA binding protein, can bind to the promoter of *PCNA*, and controls PCNA expression

in non-small cell lung cancer cells.⁴⁰ So, the Twist–YB-1 pathway likely plays an active role in tumor cell EMT and motility, proliferation and growth, cell cycle progression, and malignancy.

Twist overexpression in gastric cancer cells promotes the expression of cyclin D1 and MMP-2, whereas its suppression reduces cyclin D1 expression and MMP-2 activity. YB-1 transcriptionally regulates several MMPs, including MMP-2. Whether YB-1 activates or represses MMPs depends on the cellular context. MMP-2 and MMP-9 are fundamental enzymes in extracellular matrix homeostasis. MMP-2 is specifically implicated in EMT, and inhibition of MMP-2 prevents EMT in renal tubular cell culture.^{40–42} We also found that MMP-2, but not MMP-9, was overexpressed in HG-HPMCs compared with normal HPMCs. We also showed a significant decrease in the expression of MMP-2, but not MMP-9, after transfection with YB-1 shRNA in HG-HPMCs (Supplemental Figure S3).

We conclude that the subcellular localization of Twist and YB-1 might affect the changes in the cellular morphology and behavior of HPMCs, which might be associated with altered signaling pathways, subsequent growth, cell cycle changes, and fibrosis. The silencing of both Twist and YB-1 induced G₁/S arrest, and decelerated the growth of HPMCs. In addition, the decrease in growth of HPMCs might be partially regulated by various cell cycle proteins, such as cyclin and CDK complexes that function in the control of G₁ to S progression. Furthermore, our study also suggested that YB-1 is a major downstream target gene of Twist in HPMCs. This functional link between YB-1 and Twist suggested that Twist and its downstream effector, YB-1, might serve as promising molecular targets for the treatment of peritoneal fibrosis. Additional knockout animal models of chronic PD might provide more answers. This study is the first extensive characterization of a signaling pathway that controls cell proliferation and EMT-induced fibrosis in a nontumor primary cell culture model and provides basic knowledge and clinical relevance. These results could form the rationale for the development of drugs that can counteract the progressive deterioration of the PM that occurs in patients with CAPD.

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L.H. conceived and designed the experiments; M.C., J.H., S.L., Z.J., and W.L. performed the experiments; L.H. and H.W. analyzed the data; J.Y., C.L., S.S., H.W., X.C., and L.H. contributed reagents, materials, and analysis tools; and L.H. wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.04.008>.

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