Up-Regulation of TWIST in Prostate Cancer and Its Implication as a Therapeutic Target

Wai Kei Kwok,[1] Ming-Tat Ling,[1] Tak-Wing Lee,[1] Tracy C.M. Lau,[1] Chun Zhou,[1] Xiaomeng Zhang,[1] Chee Wai Chua,[1] Kwok W. Chan,[2] Franky L. Chan,[3] Carlotta Glackin,[4] Yong-Chuan Wong,[1] and Xianghong Wang[1]

Departments of [1]Anatomy and [2]Pathology, Faculty of Medicine, The University of Hong Kong; [3]Department of Anatomy, The Chinese University of Hong Kong, Hong Kong, SAR, China; and [4]Division of Molecular Medicine, Beckman Research Institute of the City of Hope, Duarte, California

Abstract

Androgen-independent metastatic prostate cancer is the main obstacle in the treatment of this cancer. Unlike a majority of solid cancers, prostate cancer usually shows poor response to chemotherapeutic drugs. In this study, we have shown a potential novel target, TWIST, a highly conserved bHLH transcription factor, in the treatment of prostate cancer. Using malignant and nonmalignant prostate tissues, we found that TWIST expression was highly expressed in the majority (90%) of prostate cancer tissues but only in a small percentage (6.7%) of benign prostate hyperplasia. In addition, the TWIST expression levels were positively correlated with Gleason grading and metastasis, indicating its role in the development and progression of prostate cancer. Furthermore, down-regulation of TWIST through small interfering RNA in androgen-independent prostate cancer cell lines, DU145 and PC3, resulted in increased sensitivity to the anticancer drug taxol-induced cell death which was associated with decreased Bcl/Bax ratio, leading to activation of the apoptosis pathway. More importantly, inactivation of TWIST suppressed migration and invasion abilities of androgen-independent prostate cancer cells, which was correlated with induction of E-cadherin expression as well as morphologic and molecular changes associated with mesenchymal to epithelial transition. These results were further confirmed on the androgen-dependent LNCaP cells ectopically expressing the TWIST protein. Our results have identified TWIST as a critical regulator of prostate cancer cell growth and suggest a potential therapeutic approach to inhibit the growth and metastasis of androgen-independent prostate cancer through inactivation of the *TWIST* gene. (Cancer Res 2005; 65(12): 5153-62)

Introduction

Prostate cancer is the most commonly diagnosed cancer in American men representing one third of all new cancer cases each year. This means that one of six men is being diagnosed with prostate cancer over their lifetime. Thus, over 30,000 men die each year from prostate cancer–related illness in the United States (1). Early-stage androgen-sensitive prostate cancer is manageable with androgen depletion therapy. However, approximately one third of the prostate cancer patients have micrometastatic disease at the time of presentation, this group of patients will eventually progress to clinically detectable metastatic and androgen-independent diseases at which point the median survival is only 12 to 15 months (2). Therefore, metastatic prostate cancer remains the main cause of prostate cancer–related death in men. Identification of key factors that are specifically expressed in prostate cancer and essential for cancer cell growth is a crucial step towards understanding prostate cancer progression and developing of new anticancer therapies.

Recently, TWIST, a highly conserved basic helix-loop-helix transcription factor, is suggested as an oncogene (3, 4). For example, exogenous TWIST expression promotes colony formation of mouse embryonic fibroblasts in soft agar and this process is mediated through suppression of the ARF/MDM2/p53 pathway (4). Recently, using gene profiling analysis, up-regulation of *TWIST* is found associated with malignant transformation of melanoma and T-cell lymphoma (5, 6). TWIST is also shown to positively regulate the expression of the *GLI1* gene, which is overexpressed in many types of human carcinoma (7). In addition, increased TWIST expression is found in over 50% of the rhabdomyosarcoma and 40% of gastric carcinomas compared with nonmalignant tissues (4, 8). The oncogenic effect of TWIST is suggested mediated through its role as an antiapoptotic factor (4). Recently, evidence also indicates TWIST as a key factor responsible for metastasis of breast cancer by promoting epithelial-to-mesenchymal transition (EMT) in an *in vivo* system and down-regulation of TWIST is able to suppress metastatic ability by inducing mesenchymal-to-epithelial transition (MET; ref. 9). In addition, in primary melanoma patients, increased TWIST is correlated with poor outcome and shorter survival (5). These results implicate that TWIST may be a novel oncogene that induces tumorigenesis in nonmalignant cells and promotes tumor progression in malignant cells. Previously, we have found that *TWIST* gene amplification is associated with the development of acquired resistance to an anticancer drug (taxol), and ectopic expression of TWIST leads to resistance to microtubule disrupting agents (10). These lines of evidence further suggest that TWIST may also be a positive factor in promoting resistance to anticancer drugs, which is one of the characteristics of advanced cancers. In this study, we found up-regulation of TWIST in human prostate cancer and increased TWIST protein level was positively correlated with Gleason grading. Inactivation of TWIST in androgen-independent prostate cancer cells resulted in increased chemodrug-induced apoptosis and suppression of invasion ability. Our results identified a novel key

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Xianghong Wang or Yong-Chuan Wong, Department of Anatomy, Laboratory Block Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, SAR, China. Phone: 852-2819-2867; Fax: 852-2817-0857; E-mail: xhwang@hkucc.hku.hk or ycwong@hkucc.hku.hk.

factor in the growth of androgen-independent prostate cancer and provided a new target for improving the treatment efficiency of androgen-independent metastatic prostate cancer through inactivation of the *TWIST* gene.

Materials and Methods

Cell culture conditions. Human prostate adenocarcinoma cel1 lines (LNCaP, PC-3, and DU145) were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 5% FCS and penicillin/streptomycin.

Tissue specimens. All the formalin-fixed and paraffin-embedded prostate tissues were obtained from prostate tissue banks in the Department of Pathology, West China University of Medical Sciences (11) and the Institute of Urology, Peking University. All the tissues were either obtained from prostatic needle biopsies or radical prostatectomy. The histopathology of the specimens was examined and classified by pathologists in the Department of Pathology in the same hospitals. The mean patient age for benign prostate hyperplasia (BPH) tissues was 62 $\pm$ 2.5 years, who were diagnosed with BPH by both clinical and pathologic examinations. All BPH specimens (n = 45) showed histologically epithelial and/or stromal cell hyperplasia with no evidence of malignant cells. For the prostate cancer specimens (n = 46), the mean patient age at the time of surgery was 67 $\pm$ 8.7 years. All the patients were diagnosed with prostate cancer by histopathologic examination. The prostate cancer patients were divided into two groups: the Gleason score of <7 (n = 25) and $\geq$7 (n = 21). None of the cases have been treated with preoperative androgen ablation. The two cases of metastatic prostate cancer specimens were obtained from archival paraffin blocks in the Department of Pathology, Queen Mary Hospital, The University of Hong Kong and the Department of Anatomy, The Chinese University of Hong Kong, respectively.

Immunohistochemistry. Detailed experimental procedures were described previously (11). Briefly, tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer-thick sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. After antigen retrieval, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes followed by rehydration in PBS and incubation with 5% rabbit serum for 30 minutes to bind nonspecific antigens. The slides were then incubated overnight at 4°C with polyclonal antibodies against TWIST (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) in TBS containing 2% rabbit serum and 1% bovine serum albumin (BSA). This was followed by incubation with biotinylated anti-rabbit immunoglobulin G at dilution of 1:200 for 30 minutes at room temperature followed by peroxidase-conjugated avidin-biotin complexes and 3,3′-diaminobenzidine (DAKO, Glostrup, Denmark). The sections were then counterstained with Mayer's hematoxylin and analyzed by standard light microscopy. Sections were incubated with TBS containing 2% rabbit serum and 1% BSA without primary antibody as negative controls.

Evaluation of TWIST immunostaining. Staining intensity was semi-quantified in 10 randomly selected fields per tumor at 400× magnifications by computer scan and the intensity reading of each specimen was recorded. The average intensity reading of each slide was calculated and compared with the average staining intensity reading from BPH tissues which was assigned as 1. For each experiment, both BPH and prostate cancer specimens were included so that the relative staining intensity between these two groups could be justified and compared. Data presented in Fig. 1*B* represented the average fold increase in staining intensity in prostate cancer compared with BPH tissues and error bars indicated SD.

Statistical analysis. Prostate cancer specimens were grouped according to predetermined Gleason scores (<7 or $\geq$7). Statistical analysis was done on the staining intensity between BPH and cancer, Gleason score of $\geq$7 and Gleason score of <7 using the SPSS statistical software (SPSS, Inc., Chicago, IL). The analysis of the association between TWIST expression levels and Gleason scores for prostate cancer was done by χ^2 test for categorical variables and by Spearman correlation test for continuous variables. A level of $P < 0.05$ was considered statistically significant.

Generation of stable Si-TWIST transfectants. TWIST-siRNA vector was generated using the GeneSuppressor System kit (Imgenex, San Diego, CA) according to the manufacturer's instruction. Briefly, the primers containing the short hairpin RNA (shRNA) sequence targeting the *TWIST* gene-coding region were annealed and cloned into pSuppressor-Retro vector to generate the small interfering RNA (siRNA) expression vector. The sequences of the Si-TWIST primers were Si-TWIST-F, TCGAGCTGAGCAAGATTCAGACCCTGAAGCTTGAGGGTCTGAATCTTGCTCAGCTTTTT and Si1TWIST-R, CTAGAAAAAGCTGAGCAAGATTCAGACCCTCAAGCTTCAGGGTCTGAATCTTGCTCAGC. The control vector was generated using the same procedures as the siRNA vector except that the shRNA sequences were replaced with nonsense sequences that are not homologous to the human genome. The sequences of the control primers were Si-Con-F, TCGAGCGTATTGCCTAGCATTACGTGATGCTTGACGTAATGCTAGGCAATACGCTTTTT and Si-Con-R, CTAGAAAAAGCGTATTGCCTAGCATTSCGTCAAGCTTCACGTAATGCTAGGCAATACGC. The resulting vectors were then transfected into the packaging cell line 293HEK using Fugene 6 transfection reagent and the procedures was described by the manufacturer (Roche Diagnostics, Indianapolis, IN). The retroviruses were collected 48 hours later, filtered, and mixed with polybrene (8 μg/mL) and added directly into DU145 and PC3 cells for infection. Positive clones were then selected in Neomycin (400 μg/mL) 48 hours after infection. Stable Si-TWIST-1 transfectants were isolated after ~14 days drug selection. A vector control was generated from a pool of >20 individual clones transfected with the control vector.

Generation of stable TWIST transfectants. The pLXIN-TWIST vector was constructed by recloning the TWIST cDNA from pcDNA3-TWIST expression vector (a gift from Dr. C. Glackin, Division of Molecular Medicine, Beckman Research Institute of the City of Hope, CA; ref. 12) into the pLXIN vector. The pLXIN-TWIST and p-LXIN-neo were transfected into the PT67 packaging cell line (obtained from American Type Culture Collection) respectively using the Fugene 6 transfection reagent according to the procedures described by the manufacturer (Roche Diagnostics). The supernatant was harvested, filtered, and incubated with LNCaP cells together with 8 μg/mL polybrene. Neomycin (600 μg/mL) was added 48 hours later and stable transfectants were generated. Vector control was generated from a pool of >20 individual clones transfected with pLXIN.

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cell growth rate was measured using an 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay kit according to the manufacturer's instructions (Roche Diagnostics). Briefly, 3,000 cells were seeded in 96-well plates and cultured in 5% FCS for 24 hours. Taxol (0, 10, 20, 30, and 40 ng/mL) was added 24 hours after plating and cell viability was examined at 24 hours post-exposure time point. Before testing, 10 μL of MTT labeling reagent (5 mg/mL MTT in PBS) were added and the cells were incubated for a further 4 hours at 37°C. Then 100 μL of solubilizing reagent (10% SDS in 0.01 mol/L HCl) were added and the plate was incubated overnight at 37°C to dissolve the formazan crystals. The absorbance was measured at a wavelength of 570 nm on a Labsystem multiskan microplate reader (Merck Eurolab, Dietikon, Schweiz). Each time point was done in triplicate wells and each experiment was repeated at least twice. Each data point represented the mean and SD.

Bromodeoxyuridine staining. Detailed experimental procedures have been described previously (13). Briefly, monolayer DU145, PC3 and LNCaP cells and their transfectants were grown on 4-mm Chamber slides (ICN, Biomedicals, Aurora, OH) and the cells were incubated with bromodeoxyuridine (BrdUrd, 10 mmol/L) for 2 hours and washed once with PBS. The cells were then fixed in cold methanol for 5 minutes at room temperature and washed in PBS. The cells were incubated with mouse monoclonal antibody against BrdUrd (1:10, Roche Diagnostics) for 1 hour and with anti-mouse IgG-FITC for 1 hour at 37°C after washing with PBS. The percentages of FTIC-positive cells were evaluated after counting at least 1,000 cells. The error bars represent the SD generated from three independent experiments.

Cell cycle analysis. Detailed experimental procedures were described previously (13).

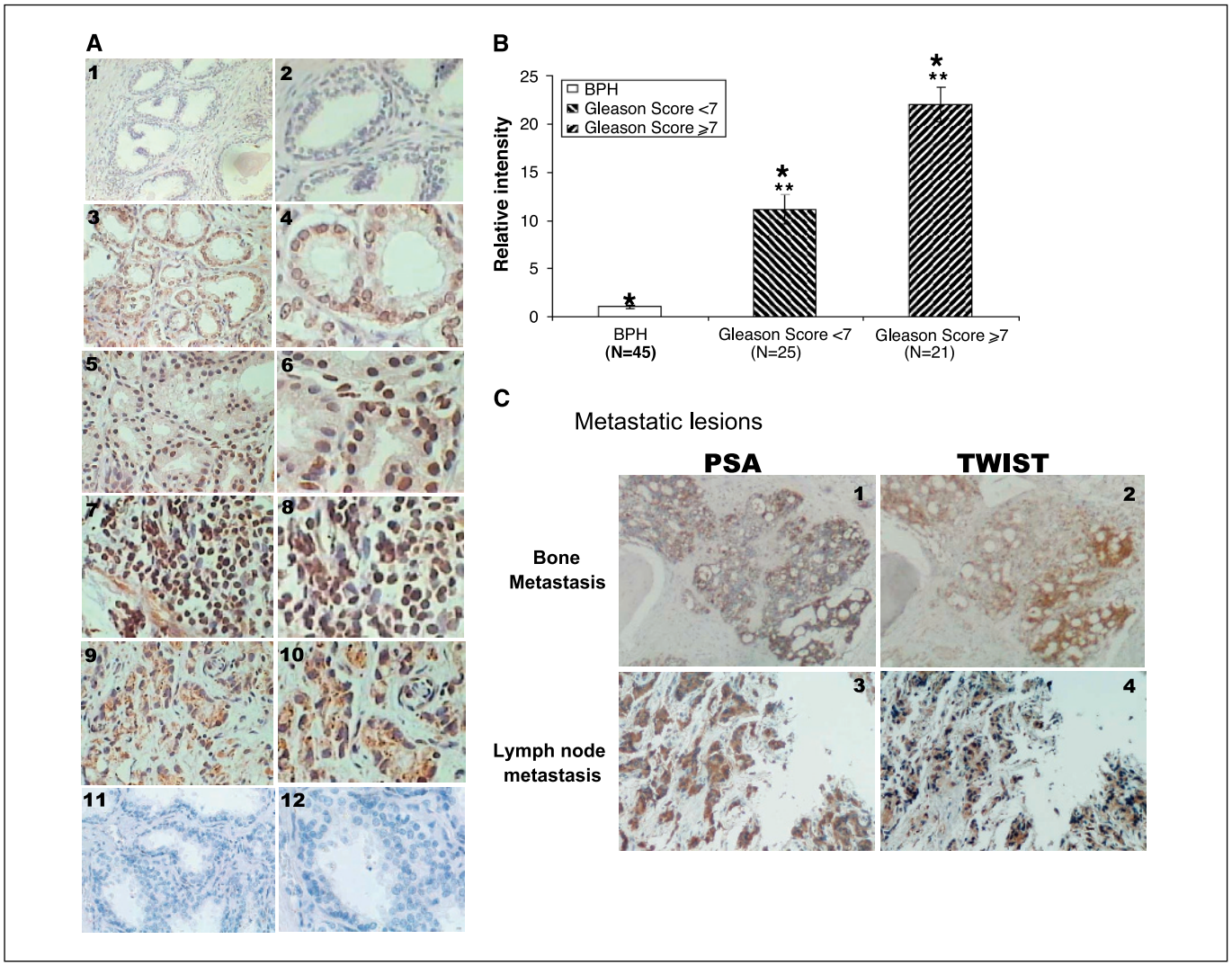

Figure 1. Differential expression of TWIST in malignant and nonmalignant prostate tissues. *A,* representative results of immunohistochemical staining of TWIST protein in BPH (*1* and *2*) and prostate cancer tissues (*3-10*). *11* and *12,* negative controls with primary antibody omitted. Photos were taken under 200× (*1, 3, 5, 7, 9, 11*) and 400× (*2, 4, 6, 8, 10, 12*) magnifications. Note that TWIST protein expression is positively correlated with increased Gleason grading. *B,* summary of differential TWIST expression between BPH and prostate cancer tissues (*, $P < 0.001$) and between prostate cancer Gleason grading <7 and ≥7 specimens (**, $P < 0.05$). Note that TWIST expression is high in prostate cancer tissues compared with BPH, and increased TWIST expression is associated with higher Gleason grading tumors. *C,* prostate-specific antigen (*PSA*) and TWIST expression in prostate cancer specimens derived from bone (*1-2*) and lymph node (*3-4*) metastatic lesions. Photos were taken under 100× magnifications.

Western blotting. Detailed experimental procedures have been described previously (14). Briefly, cell lysates were prepared by suspending the cell pellets in a modified radioimmunoprecipitation buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, not deoxycholic acid, 0.1% SDS] including proteinase inhibitors (1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride), and protein concentration was measured using the protein assay kit (Bio-Rad, Hercules, CA). Equal amount of protein (20 μg) was separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and blotted onto the nitrocellulose membrane (Amersham, Piscataway, NJ). After blocking with 5% nonfat dry milk/2% BSA in TBS-T for 1 hour, the membrane was incubated with primary antibodies for 1 hour at room temperature against TWIST (1:250, Santa Cruz Biotechnology), MDM2 (1:100, Santa Cruz Biotechnology), Bcl-2 (1:250, Santa Cruz Biotechnology), Bcl-xL (1:1,000, Cell Signaling Biotechnology, Beverly, MA), Bax (1:500, Cell Signaling Biotechnology), $p21^{Waf1}$(1:500, Santa Cruz Biotechnology), $p27^{Kip1}$(1:1,000, Santa Cruz biotechnology), p53 (1:2,000, Oncogene, San Diego, CA), α-catenin (1:3,000, Transduction Laboratories, Lexington, KY), β-catenin (1:3,000, Transduction Laboratories, Palo Alto, CA), γ-catenin (1:3000, Transduction Laboratories), E-cadherin (1:3,000, Transduction Laboratories), fibronectin (1: 250, BD Biosciences PharMingen, San Diego, CA), and vimentin (1:2000, Chemicon International, Temecula, CA). After washing with TBS-T, the membrane was incubated with secondary antibodies against rabbit immunoglobulin G or mouse immunoglobulin G and the signals were visualized by enhanced chemiluminescence Western blotting system (Amersham). Expression of actin was also measured as an internal loading control using a specific antibody (Santa Cruz Biotechnology). Results represent three independent experiments. Protein (50 μg) was examined for Bcl-2 expression. Protein (60 μg) was separated for MDM2 detection by electrophoresis on a 7.5% SDS-PAGE.

Detection of DNA ladder. Detailed experimental procedures were described previously (15).

Immunofluorescent staining of E-cadherin. LNCaP and DU145 cells (3,000 cells per well) were grown on Chamber slides (ICN, Biomedicals) in RPMI and 5% fetal bovine serum for 24 hours. The cells were then fixed with 4% paraformaldehyde in 1× PBS for 10 minutes at room temperature. After

washing with 1× PBS, the cells were permeabilized with 1% Triton X-100 (in 1× PBS) for 15 minutes at room temperature. The cells were then washed with 1× PBS and blocked with 1% BSA for 30 minutes at room temperature. After blocking, the cells were incubated with antibody against E-cadherin (BD Bioscience 610181, 1:100, in 0.1% BSA) for 1 hour at 37°C. The cells were washed thrice with 1× PBS followed by incubation with FITC-labeled secondary antibody (DakoCytomation, Carpinteria, CA, 1:40 in 0.1% BSA) for 1 hour at room temperature. After three washes with 1× PBS, the slides were mounted and covered with coverslip. Images of the cell signal were captured by a laser confocal microscope under 400× magnifications.

Three-dimensional collagen colony-forming assay. Two hundred microliters of cell suspension (3×10^4 cells/mL) were mixed with 200 μL of cold rat tail collagen, type I (3.60 mg/mL, BD Biosciences, Bedford, MA). The mixed cell solution in collagen was plated as droplets (at least three for each sample) in 60-mm Petri dish until set. RPMI 1640 containing 5% of FCS was added in each dish containing the semisolid collagen cell droplets and then the cells were cultured for 3 days (for PC3) or 5 days (for DU145). Cell morphology was observed under a phase-contrast microscope and pictures were captured under 200× magnifications. At least 500 colonies were counted from each sample and percentage of colonies that consisted of elongated or scattered morphology was calculated.

Wound closure assay. A wound was induced on the confluent monolayer cells by scraping a gap using a micropipette tip and the speed of wound closure was monitored every 24 hours. Photographs were taken under 100× magnifications using phase-contrast microscopy immediately after wound incision and at later time points.

Results

Up-regulation of TWIST in prostate cancer specimens. To investigate if there was a differential TWIST expression between benign and malignant prostate tissues, we did immunohistochemical staining on BPH ($n = 45$) and prostate cancer tissues ($n = 46$). As shown in Fig. 1*A* (*1* and *2*), we found that high percentage of BPH tissues (42 of 45, 93.3%) showed undetectable TWIST protein staining, whereas the majority of malignant prostate cancer tissues (40 of 46, 90%) were stained positive for TWIST protein (*3-10*). The staining pattern mainly agrees with a previous report on rhabdomyosarcoma tissues that TWIST protein was detected in the nucleus of majority of cancer cells (4). However, we also noted that a number of cases of high Gleason grading tumors showed both cytoplasmic and nuclear staining (e.g., see, Fig. 1*A, 9* and *10*). In addition, the staining intensity was also significantly increased with increased Gleason grading (Fig. 1*A, 3-10*). Statistical analysis showed (Fig. 1*B*) that overall TWIST expression level was significantly higher in prostate cancer specimens compared with the BPH tissues ($P < 0.001$). In addition, TWIST expression was much higher in the tissues with higher Gleason scores (≥ 7) than the ones with lower Gleason scores (<7; $P < 0.05$). Furthermore, immunostaining results on bone and lymph node metastatic prostate cancer tissues also showed high TWIST expression (both cytoplasmic and nuclear staining; Fig. 1*C, 2* and *4*). Prostate-specific antigen staining results were also shown to indicate prostate cancer cells (Fig. 1*C, 1* and *3*). These results indicate that TWIST protein expression is increased in prostate cancer tissues compared with nonmalignant tissues and its expression is positively correlated with Gleason grades.

Inactivation of TWIST leads to increased taxol-induced apoptosis in androgen-independent prostate cancer cells. To further confirm the significance of TWIST expression on prostate cancer cell growth, we transfected a retroviral vector containing the Si-TWIST into DU145 and PC3 cells which showed relatively high TWIST protein expression and generated stable transfectants (see Supplementary Fig. S1*A* and *B*). We then studied if down-regulation of TWIST could lead to any changes in the expression of p53 and MDM2 as well as apoptosis related proteins such as Bcl-2, Bax, and Bcl-xL. Down-regulation of MDM2 was observed in the Si-TWIST transfectants, whereas p53 protein was undetectable in PC3 (containing a deleted *p53* gene) and unchanged in DU145 (containing a mutated *p53* gene; refs. 16, 17; see Supplementary Fig. S1*B*). In addition, decreased Bcl-2 protein expression was found in the Si-TWIST transfectants but there were no significant alterations in the Bax and Bcl-XL levels between the cells with high and low levels of TWIST. The differential Bax protein expression between DU145 (barely detectable) and PC3 (relatively high expression) cells may be due to the presence of a mutated Bax gene in DU145 cells but a functional Bax in PC3 cells as reported previously (18). When we treated the cells with taxol, we found that the Si-TWIST transfected cells were much more sensitive to taxol-induced apoptosis shown by the increased percentage of sub-G_1 phase cells in the flow cytometric studies (see Supplementary Fig. S1*C, arrows*). In addition, the appearance of DNA ladder was also more evident in the Si-TWIST transfectants when the cells were treated with five doses of taxol (10, 20, 30, 40, and 50 ng/mL; representative results are shown in Supplementary Fig. S1*D*) compared with the vector controls. MTT assay also confirmed these results that Si-TWIST transfectants showed much lower cell viability (filled columns) compared with the vector controls (open columns) when both cell types were treated with same concentrations of taxol (Supplementary Fig. S1*E*). These results indicate that down-regulation of TWIST in androgen-independent prostate cancer cells has led to increased sensitivity to taxol through activation of drug induced apoptosis. Further Western blotting analysis showed (Supplementary Fig. S1*F*) that after exposure to taxol, both decreased TWIST and Bcl-2 expression was found in the Si-TWIST transfectants but alterations of Bax and Bcl-xL protein levels were less significant. In contrast, the relative amount of the cleaved PARP was higher in the Si-TWIST transfectants compared with the vector controls (Supplementary Fig. S1*G*). Because the presence of cleaved PARP is another indicator of activation of the apoptosis pathway (19), these results further indicate that inactivation of TWIST has led to activation of the apoptosis pathway in Si-TWIST transfectants in response to taxol. However, down-regulation of TWIST was not associated with decreased cell growth rate in the transfectants evidenced by the similar BrdUrd incorporation rates (Fig. 2*A*) and percentage of cell cycle S-phase fraction (Fig. 2*B*) between the Si-TWIST transfectants and the vector controls, which agrees with a recent study on breast cancer cells that inactivation of TWIST did not alter cell proliferation rate (9). These results suggest that the increased sensitivity to taxol in the Si-TWIST transfectants was not due to its effect on cell proliferation.

Down-regulation of TWIST leads to suppression of invasion ability through induction of mesenchymal-to-epithelial transition–like changes. Recently, it was reported that in a breast cancer animal model, high levels of TWIST were associated with increased tumor metastatic ability and ectopic TWIST expression in human epithelial cells led to epithelial-to-mesenchymal transition (EMT; ref. 9), which is thought to contribute to the invasion ability of cancer cells (20). Although few assay systems are available to directly show MET, several morphologic as well as molecular alterations have been implicated accompanied with EMT (20, 21). For example, when epithelial cells are undergoing EMT, their morphology changes from well organized cell-cell adhesion and cell polarity to loss of cell-cell contacts and cell scattering which is often

associated with loss of epithelial makers such as E-cadherin and catenins, and gain of mesenchymal markers such as vimentin and smooth muscle actin (Sm-actin; ref. 9). In this study, we found that inactivation of TWIST in DU145 cells resulted in morphologic changes from scattered and fibroblast-like growth to tightly packed colonies (Fig. 3*A*), which represents one of the characteristics of MET. To further determine if molecular alterations of an MET occurred in the Si-TWIST transfectants, we examined the expression of epithelial markers such as E-cadherin, α-catenin, and β-catenin as well as the expression of the fibroblast markers, vimentin and Sm-actin. As shown in Fig. 3*B*, increased E-cadherin, α-catenin, and β-catenin protein levels were observed in the Si-TWIST transfectants, whereas the levels of vimentin and Sm-actin were relatively low in both cell lines (22). Because loss of E-cadherin is one of the critical features of EMT (9), and its membrane localization indicates its biological function (23), we then did immunofluorescent staining on DU145 cells. As shown in Fig. 3*C*, whereas E-cadherin protein was mainly localized in the cytoplasm of the vector control cells, in the Si-TWIST transfectants, E-cadherin

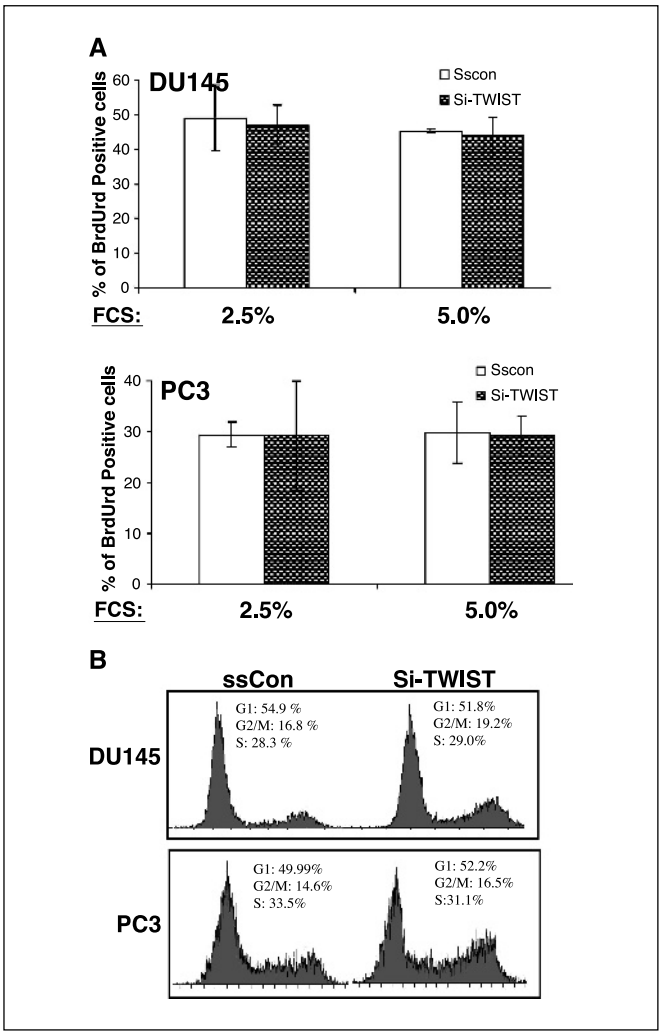

Figure 2. Effect of TWIST inactivation on prostate cancer cell proliferation. *A,* BrdUrd incorporation rate between the Si-TWIST transfectants (*filled columns*) and the vector controls (*open columns*). *B,* cell cycle distribution between the cells with high and low levels of TWIST. Note that downregulation of TWIST does not lead to alterations in BrdUrd incorporation rate and cell cycle S-phase fraction in PC3 and DU145 cells.

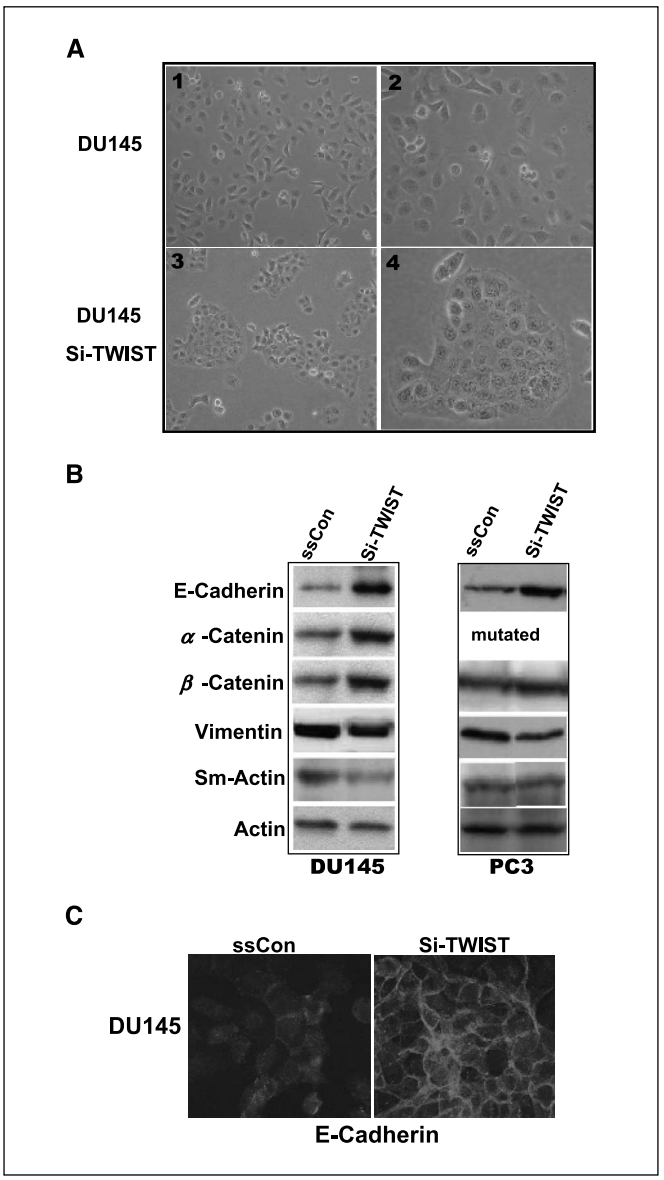

Figure 3. Effect of TWIST inactivation on mesenchymal to epithelial transition. *A,* morphologic changes induced by downregulation of TWIST in DU145 cells. Photos were taken under 100× (*1* and *3*) and 400× (*2* and *4*) magnifications. Note that inactivation of TWIST leads to morphological changes of MET. *B,* expression of epithelial proteins, E-cadherin, α-catenin, and β-catenin and mesenchymal proteins vimentin and Sm-actin in DU145 and PC3 cells with high and low levels of TWIST examined by Western blotting. *C,* immunofluorescent staining of E-cadherin protein in DU145 cells expressing high and low levels of TWIST. Note that E-cadherin protein is detected mainly along cell membrane in Si-TWIST cells while it is mainly located in the cytoplasm of the control cells.

protein was detected mainly in cell membrane. These results indicate that inactivation of the TWIST in these cells is associated with morphologic as well as molecular changes of MET. Because down-regulation of E-cadherin is one of the most frequently reported characteristics in metastatic prostate cancers (24) and increased membrane E-cadherin expression was found in the *TWIST* transfectants, we hypothesized that down-regulation of Si-TWIST may lead to suppression of the invasion ability of prostate cancer cells.

To test this hypothesis, we studied the invasion and migration ability of DU145 and PC3 cells expressing high or low levels of the TWIST protein. As shown in Fig. 4*A* and *B*, using three-dimensional

collagen invasion assay, we found that high percentage ($DU145_{ssCon}$ = 27 ± 4.5%; $PC3_{ssCon}$ = 36.6 ± 2.9%) of the vector control cells grew extensively inside the semisolid collagen gel and displayed an elongated or scattered morphology, showing their ability to invade into extracellular matrix. In contrast, majority of the Si-TWIST transfectants were unable to penetrate into collagen gel and grew into tight colonies, and only a small percentage ($DU145_{Si\text{-}TWIST}$ = 9.4 ± 1.2%; $PC3_{Si\text{-}TWIST}$ = 12 ± 1.9%) of them showed invasion ability in collagen ($P < 0.05$ compared with the vector control). These results suggest that inactivation of TWIST leads to suppression of invasion ability in these cells. Next, we studied cell migration rate using wound closure assay. As shown in Fig. 4*C*, the migration rate of the DU145-SiTWIST cells was much lower than the vector control (see *arrows*), indicating that down-regulation of TWIST is also associated with suppression of cell migration rate. Similar results were observed in PC3 cells (data not shown). These results indicate that down-regulation of TWIST has led to suppression of both invasion and migration abilities of prostate cancer cells, possibly through promoting MET.

Ectopic TWIST expression leads to suppression of apoptosis pathway and promotion of epithelial-to-mesenchymal transition. To further confirm the role of TWIST on prostate cancer cells, we transfected a *TWIST* expression vector into an androgen-sensitive prostate cancer cell line, LNCaP, which had low TWIST expression (see Supplementary Fig. S1*A*) and a wild-type p53 (16, 17), and studied the expression of p53, Bcl-2, and Bax as well as E-cadherin, α-catenins, β-catenins, fibronectin, and Sm-actin. As shown in Fig. 5*A*, the TWIST protein was high in the transfectants (a pool of over 20 stable transfectant clones) which was associated with decreased Bax expression. However, the p53 protein levels were unchanged. These results agree with previous reports that up-regulation of TWIST led to alterations of p53-responsive factors such as Bax, rather than p53 itself in an environment where p53 pathway is intact (4, 25, 26). However, unlike observed in DU145 and PC3 cells, we did not find any significant alterations of the Bcl-2 protein between the TWIST transfectants and the vector control. The fact that down-regulation of TWIST in DU145 and PC3 cells which contain nonfunctional p53 pathway also led to promotion of apoptosis suggests that the antiapoptotic role of TWIST may regulate through both p53-dependent and p53-independent pathways. It is also possible the increased Bcl-2/Bax ratio, rather than expression level of Bcl-2 and Bax proteins, is responsible for the protective role of TWIST against apoptosis in prostate cancer cells. To confirm this hypothesis, we then treated several LNCaP transfectant clones with taxol and examined the expression of both Bcl-2 and Bax proteins. As shown in Fig. 5*B*, after exposure to

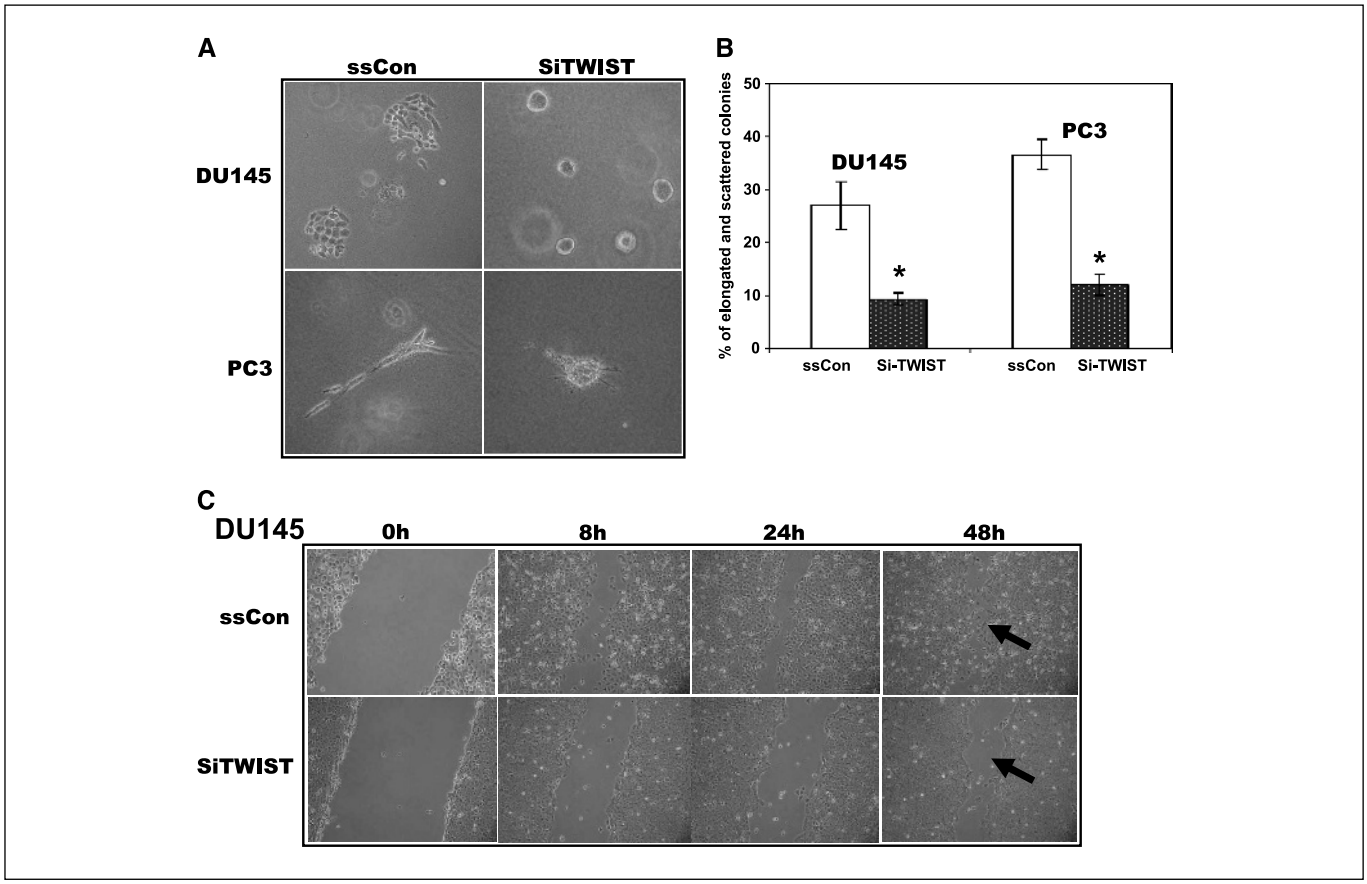

Figure 4. Effect of TWIST inactivation on prostate cancer cell invasion and migration. *A,* representative photos of three-dimensional collagen invasion assay. Morphologically different colonies were observed between the cells with high (scattered or elongated morphology) and low (packed morphology) levels of TWIST. *B,* summary of differential invasion ability between the cells with high and low levels of TWIST. Same number of cells was plated in semisolid collagen gel, and 3 days (for PC3) and 5 days (DU145) later, percentage of cells showing elongated and scattered morphology was calculated. *Columns,* mean of three experiments; *bars,* ±SE. *C,* representative results of the wound closure assay. A scraped wound was introduced on confluent monolayer DU145 cells and the speed of wound closure was monitored at 8, 24, and 48 hours time points. Arrows indicate the differential motility rate between the cells with high and low levels of TWIST. Note that down-regulation of TWIST leads to suppression of invasion and migration abilities of prostate cancer cells.

taxol (1 ng/mL), Bax expression remained low in TWIST transfectants, whereas Bcl-2 was constantly expressed. We also noted that clone 1 (C1) had relatively low TWIST expression but similar Bax levels as the other clones. It may be due to clonal variation during selection of stable transfectants. Nevertheless, these results support the hypothesis that decreased TWIST is associated with activation of the apoptosis pathway. However, as observed in DU145 and PC3 cells, using BrdUrd staining and MTT assay, we did not find any significant difference in cell proliferation rate between the cells with high and low levels of TWIST (Fig. 5*C* and *D*). When we treated the cells with two doses of taxol (1 and 2 ng/mL), the percentage of apoptosis cells was much lower in the TWIST transfectants compared with the controls in a time-dependent manner (Fig. 5*E*). These results further confirm previous evidence from PC3 and DU145 cells indicating that TWIST expression may provide a protection against apoptosis in prostate cancer cells.

To study if overexpression of TWIST could reverse the MET-like alterations observed in the Si-TWIST transfectants, we also examined the expression of E-cadherin, α-catenin, β-catenin as well as fibronection and Sm-actin in LNCaP cells. As shown in Fig. 6*A*, down-regulation of E-cadherin and α-catenin was found in the pool of LNCaP cells ectopically expressing the TWIST protein. In contrast, the expression of fibronectin and Sm-actin was increased. However, we did not observe any alterations of β-catenin expression between the transfectant and the vector control (γ-catenin and vimentin are not detectable in LNCaP cells; data not shown; ref. 27). To further confirm E-cadherin expression levels in the TWIST transfectants, we then did Western blotting on additional four individual transfectant clones. As shown in Fig. 6*B*, moderate decrease in E-cadherin protein level was found in all the transfectant clones compared with the vector control. To further confirm these results, we then did fluorescent immunostaining using an anti-E-cadherin antibody. As shown in Fig. 6*C*, the membrane localization of E-cadherin protein in the vector control almost disappeared completely in the TWIST transfectants, although the total staining intensity was also relatively low in the TWIST expressing cells. These results indicate that ectopic TWIST expression may have led to inactivation of the E-cadherin protein. To further study the association between TWIST expression and metastatic ability of LNCaP cells, we then tested the invasion and migration ability of the LNCaP cells expressing

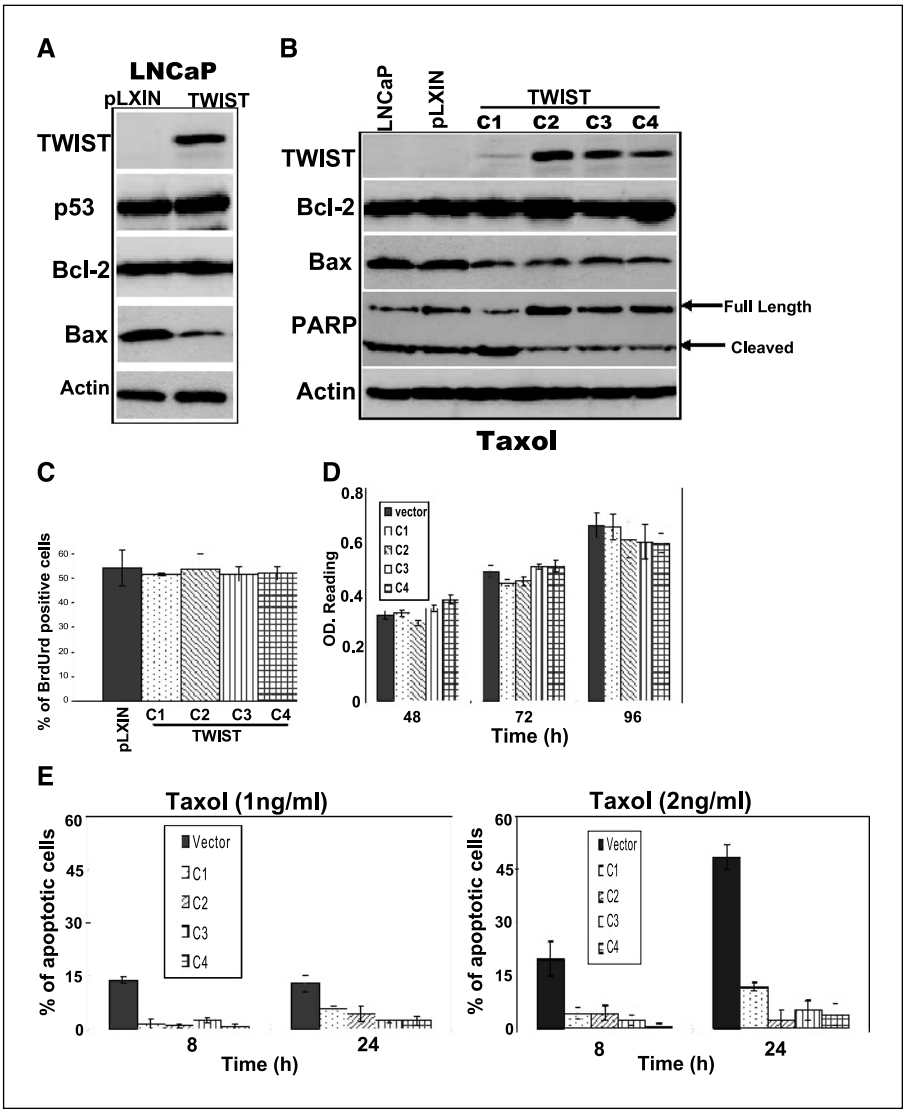

Figure 5. Effect of ectopic TWIST expression on taxol-induced apoptosis in LNCaP cells. *A,* expression of TWIST, p53, Bcl-2, and Bax between the TWIST transfectant (a pool of over 20 stable clones) and the vector control (pLXIN) examined by Western blotting. Note that ectopic TWIST expression leads to down-regulation of Bax. *B,* effect of ectopic TWIST expression on taxol-induced alterations in apoptosis-related proteins, Bcl-2, Bax, and PARP in individual stable transfectant clones (*C1-C4*). Cells were treated with taxol (5 ng/mL) for 24 hours and cell lysate was examined by Western blotting. Note that ectopic TWIST expression results in downregulation of Bax which is associated with decreased cleaved PARP protein. Effect of ectopic TWIST expression on DNA synthesis (*C*) and growth (*D*) rates by BrdUrd staining and MTT assay, respectively. Note that there is no difference in the BrdUrd synthesis and growth rates between the transfectants and the controls. *E,* taxol-induced apoptosis in LNCaP cells expressing high and low levels of TWIST examined by DAPI staining. Note that ectopic TWIST expression in LNCaP cells leads to protection against taxol-induced apoptosis.

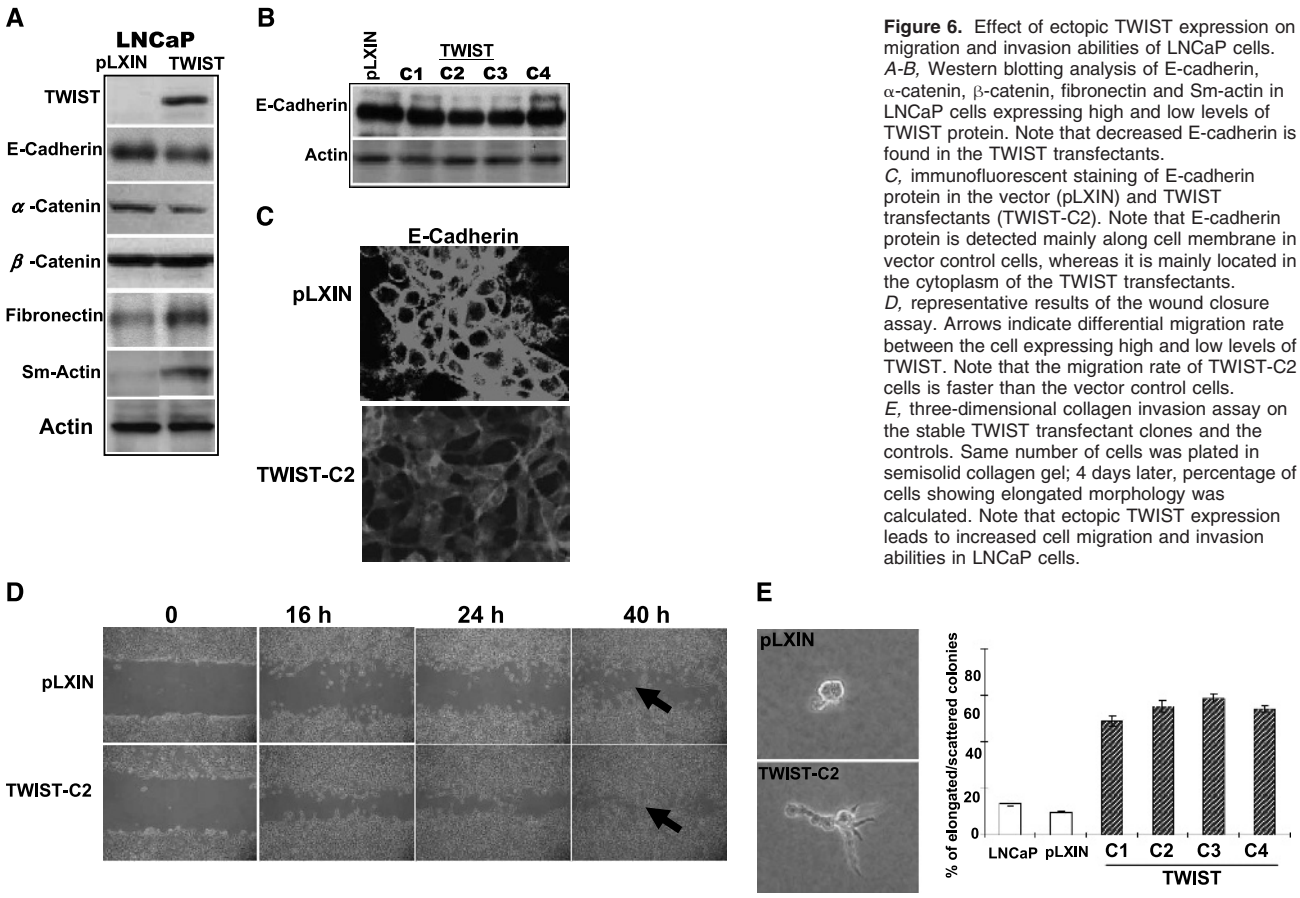

Figure 6. Effect of ectopic TWIST expression on migration and invasion abilities of LNCaP cells. *A-B,* Western blotting analysis of E-cadherin, α-catenin, β-catenin, fibronectin and Sm-actin in LNCaP cells expressing high and low levels of TWIST protein. Note that decreased E-cadherin is found in the TWIST transfectants. *C,* immunofluorescent staining of E-cadherin protein in the vector (pLXIN) and TWIST transfectants (TWIST-C2). Note that E-cadherin protein is detected mainly along cell membrane in vector control cells, whereas it is mainly located in the cytoplasm of the TWIST transfectants. *D,* representative results of the wound closure assay. Arrows indicate differential migration rate between the cell expressing high and low levels of TWIST. Note that the migration rate of TWIST-C2 cells is faster than the vector control cells. *E,* three-dimensional collagen invasion assay on the stable TWIST transfectant clones and the controls. Same number of cells was plated in semisolid collagen gel; 4 days later, percentage of cells showing elongated morphology was calculated. Note that ectopic TWIST expression leads to increased cell migration and invasion abilities in LNCaP cells.

high and low levels of the TWIST protein. As shown in Fig. 6*D* and *E*, using wound closure and three-dimensional collagen assays, we found that TWIST expression resulted in both increased cell migration and invasion abilities in LNCaP cells. These results further confirm results generated from DU145 and PC3 cells and suggest again a positive association between TWIST expression and invasion ability of prostate cancer cells.

Discussion

The evidence presented in this study suggests that TWIST may be a novel factor in the development and progression of prostate cancer. The fact that up-regulation of TWIST was only observed in prostate cancer tissues but absent in the nonmalignant tissues indicates that it may play a part in the development of prostate cancer. Previously, one of the functions of TWIST was reported to be the inhibition of cell differentiation (12, 28, 29); in this study, we found that increased TWIST protein levels were positively correlated with Gleason grading, suggesting its negative role in the differentiation of prostate cancer cells (Fig. 1). In addition, the results that down-regulation of TWIST in androgen-independent prostate cancer cells led to increased sensitivity to taxol (Supplementary Fig. S1), and suppression of cancer cell migration and invasion abilities (Fig. 4) further provide evidence to suggest that TWIST may be a novel target for improving treatment efficiency of androgen-independent metastatic prostate cancer.

Androgen-independent prostate cancer is always an obstacle in the treatment of prostate cancer because the conventional androgen depletion therapy is no longer effective at this stage. In this study, we found that down-regulation of TWIST in androgen-independent prostate cancer cells led to increased sensitivity to taxol, a commonly used anticancer drug. This chemosensitization effect was associated with down-regulation of Bcl-2/Bax ratio and subsequently activation of the apoptosis pathway (Supplementary Fig. S1) but not alterations of cell proliferation (Fig. 2). Previously, we reported that increased TWIST was responsible for the development of acquired taxol resistance in nasopharyngeal carcinoma cells and ectopic expression of TWIST conferred resistance to microtubule disrupting agents such as taxol and vincristine (10). It is possible that the increased TWIST in prostate cancer cells may contribute to its low response to chemotherapeutic drugs such as taxol, probably through its protective role against apoptosis. To support this hypothesis, a recent study on the expression of TWIST in clinical tissues of the melanoma patients reported that primary tumor patients with high TWIST expression showed much shorter survival than the ones with relatively low levels of TWIST (P = 0.0028; ref. 5), indicating the association of TWIST and poor treatment response. This hypothesis is also supported by studies on rodent cells. For example, TWIST knockout mice die before birth, which is associated with increased apoptosis in multiple tissues (30). Down-regulation of TWIST by antisense

technology in mouse fibroblasts increased sensitivity to etoposide (a DNA-damaging anticancer drug)– induced apoptosis (31). In addition, fibroblasts from TWIST2 knockout mice, which are highly homologous to human TWIST, are hypersensitive to tumor necrosis factor-α–induced apoptosis (32). These results in combination with the results presented in the current study further suggest that down-regulation of TWIST may be a target to increase cellular sensitivity to drug-induced apoptosis.

The first evidence on the role of TWIST in cancer metastasis was reported in a breast cancer animal model and it was suggested that the TWIST-induced EMT in the highly metastatic breast cancer cells played a key role in promoting tumor cell invasion (9), although the role of TWIST in EMT has been suggested previously during limb morphogenesis in mice (33). In this study, we found that inactivation of the *TWIST* gene conferred morphologic transition of prostate cancer cells from a fibroblastic to epithelial appearance which was accompanied by a gain of epithelial cell markers such as E-cadherin and catenins and loss of the mesenchymal markers such as vimentin, fibronectin, and/or Sm-actin (Fig. 3). In addition, these changes were accompanied by the translocation of the E-cadherin protein from cytoplasm to cell membrane (Fig. 3*C*), indicating its functional activation. These MET-like changes were correlated with decreased invasion and migration abilities (Fig. 4). These results were further confirmed on LNCaP cells ectopically expressing the TWIST protein (Fig. 6). Although similar results were reported in nonmalignant epithelial cells (9), the present study was the first to show the role of TWIST in metastasis of human cancer cells. Previously, increased TWIST was reported in the more invasive defused gastric carcinoma tissues, which was associated with decreased E-cadherin expression (8). In addition, in human breast cancer, elevated TWIST was found in 70% invasive lobular carcinomas, whereas a much smaller portion (30%) was found in less invasive tumors (9). In this study, we also found that the TWIST expression was high in tissues derived from metastatic lesions (Fig. 1*C*). These results further indicate the importance of TWIST in metastatic ability of human cancers. Although further studies are required to show the direct association of TWIST and metastasis of human cancer, these results indicate that TWIST may be a target for inhibition of metastatic ability of cancer cells.

In summary, in the past few years, increasing evidence has suggested that TWIST may be a new positive factor in the development and progression of human cancer. Its functions include promoting soft agar colony formation (4) and inhibiting cell differentiation (12, 28, 29) in nonmalignant cells and protecting against anticancer drug-induced apoptosis (10) and promoting metastasis (9) in malignant cells in *in vitro* systems. In addition, high levels of TWIST are found in gastric carcinoma (8), rhabdomyosarcoma (4), melanoma (5), breast cancer (9), Sezary Syndrome (a leukemia variant of T-cell lymphoma; ref. 6), and prostate cancer (present study). Furthermore, increased TWIST is correlated with decreased E-cadherin expression and poor survival and more aggressive phenotypes of cancer cells (refs. 5, 9 and present study). Although the molecular mechanisms responsible for the action of TWIST in human cancer require further investigation, the present study in combination with previous results suggest that it may be a novel oncogene. However, more recently, promoter hypermethylation of the TWIST gene has been reported in breast cancer, especially in the metastatic lesions (34). It is also possible that the oncogenic effect of TWIST may be cell type specific. Nevertheless, the role of TWIST in the development and progression of human cancer deserves further attention.

Acknowledgments

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Up-Regulation of TWIST in Prostate Cancer and Its Implication as a Therapeutic Target

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