

Chronic hypoxia-induced slug promotes invasive behavior of prostate cancer cells by activating expression of ephrin-B1

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Advanced solid tumors are exposed to hypoxic conditions over longer periods of time as they grow. Tumor hypoxia is a major factor that induces malignant progression, but most previous studies on tumor hypoxia were performed under short-term hypoxia for up to 72 hours and few studies have focused on tumor response to chronic hypoxic conditions. Here we show a molecular mechanism by which chronic hypoxia promotes invasive behavior in prostate cancer cells. We found that an epithelial-mesenchymal transition (EMT)-driving transcription factor, slug, is specifically upregulated under chronic hypoxia and promotes tumor cell migration and invasion. Unexpectedly, processes associated with EMT, such as loss of E-cadherin, are not observed under chronic hypoxia. Instead, expression of ephrin-B1, a ligand of Eph-related receptor tyrosine kinases, is markedly induced by slug through E-box motifs and promotes cell migration and invasion. Furthermore, slug and ephrin-B1 are highly coexpressed in chronic hypoxic cells of human prostate adenocarcinoma tissues after androgen deprivation, which is known to cause tumor hypoxia. Taken together, these results indicate that chronic hypoxia-induced slug promotes invasive behavior of prostate cancer cells by activating the expression of ephrin-B1. In addition, ephrin-B1 may be a novel therapeutic target in combination with androgen deprivation therapy for aggressive prostate cancer.

KEYWORDS

androgen deprivation, chronic hypoxia, ephrin-B1, invasion, slug

1 | INTRODUCTION

The major cause of cancer deaths is metastasis.^{1,2} Once epithelial tumor cells spread from the primary site of origin to other parts of the body, patients often have limited treatment options for cure. Therefore, elucidating the molecular mechanisms of metastasis and devising ways to prevent this process are critical to combat cancer.

Metastasis is a multistep process including local tumor cell invasion, intravasation, extravasation and colonization.² At the earliest step

of metastasis, tumor cells become more motile and invade through the basement membrane.³ To acquire these properties, tumor cells often undergo epithelial-mesenchymal transition (EMT), a developmental process by which epithelial cells transdifferentiate into mesenchymal cells.^{4,5} Examples of EMT in development can be observed during gastrulation and somitogenesis, as well as at the invasive edges of tumors, where this process plays a crucial role in the initiation of metastasis.⁴⁻⁸

Epithelial-mesenchymal transition involves changes in gene expression profiles.⁹ During EMT, E-cadherin, an epithelial adherens

junction (AJ) protein, and cytokeratins are replaced by N-cadherin, a mesenchymal AJ protein, and vimentin.^{7,9} The replacement of E-cadherin with N-cadherin enables the epithelial cells to form homotypic interaction with stromal cells and it promotes their migration and invasion.¹⁰ Transcription factors of the snail family, snail and slug, and of the twist family, Twist1 and Twist2, are major drivers of EMT and are known to repress expression of the E-cadherin gene.⁹

Advanced solid tumors develop regions with low oxygen concentrations (hypoxia) as they grow.^{11,12} Oxygen delivery to solid tumors is reduced by deteriorating conditions for diffusion due to abnormal structure and function of the tumor microvessels and disturbed microcirculation. Tumor hypoxia is a major factor that induces malignant progression and is strongly associated with a poor clinical prognosis in a variety of cancers, including carcinoma of the head and neck,¹³ carcinoma of the uterine cervix,¹⁴ melanoma¹⁵ and prostate adenocarcinoma.^{16,17} Several studies demonstrated that hypoxia promotes EMT by inducing the expression of EMT-driving transcription factors by hypoxia-inducible factor-1 α .^{18–20} Tumor cells in these studies were cultured under hypoxia for 18–72 hours (acute hypoxic condition), but most solid tumors found in cancer patients are exposed to hypoxic conditions over longer periods of time (chronic hypoxic condition).^{11,12} Therefore, we have investigated how chronic hypoxia affects the progression of tumors using human prostate cancer cells.^{21–23}

In this study, we demonstrate that chronic hypoxia-induced slug, an EMT-driving transcription factor, promotes cell migration and invasion of prostate cancer cells by activating the expression of ephrin-B1, a ligand of Eph-related receptor tyrosine kinases. We further show that slug and ephrin-B1 are coexpressed in chronic hypoxic cells in clinical samples of prostate adenocarcinoma after androgen deprivation therapy (ADT), which causes disruption of tumor blood vessels and results in tumor hypoxia.^{24,25}

2 | MATERIAL AND METHODS

2.1 | Plasmids and mutagenesis

pLenti6.3/V5-DEST-ephrin-B1 and -slug were created by recombining the Gateway entry vectors containing the human ephrin-B1 cDNA (FLJ80318AAAF, NITE Biological Research Center, Chiba, Japan) and slug cDNA (FLJ93051AAAF) with the destination vector pLenti6.3/V5-DEST (Invitrogen, Carlsbad, CA, USA). The luciferase reporter plasmid pGL3-Basic containing the human ephrin-B1 promoter sequence from –1000 to +1 relative to the start site of transcription (pGL3-Basic-ephrin-B1 promoter-WT) was a gift from Professor Yoshimi Homma, Fukushima Medical University, Japan.²⁶ Mutagenesis of the E-box motifs, E-box1 and E-box2, in the ephrin-B1 promoter was performed using the KOD-Plus Mutagenesis Kit (TOYOBO, Osaka, Japan) and mutated reporter plasmids were designated as pGL3-Basic-ephrin-B1 promoter-E1m (mutation in E-box1), –E2m (mutation in E-box2), and –E1mE2m (mutations in both E-box1 and 2). The sequence of forward primers used for mutagenesis were as follows: E1m: 5'-gacaagaccCCCGGGGgcgaggccgg-3'; E2m: 5'-gactccgggtcCCCGGGGcaagcagacag-3'.

2.2 | Cell culture

The human prostate cancer cell line LNCaP (ATCC, Rockville, MD, USA) was maintained in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 15% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich), 50 IU/mL penicillin, and 50 μ g/mL streptomycin and cultured at 37°C in humidified cell incubators containing 5% CO₂ and 95% air (in normoxia), or 5% CO₂, 1% O₂, and 94% N₂ (in hypoxia). Acute hypoxia-conditioned LNCaP cells (LNCaP/AH) were cultured under hypoxia for 48 hours, and chronic hypoxia-conditioned LNCaP cells (LNCaP/CH1M and LNCaP/CH6M) were cultured for 1 and 6 months, respectively. The LNCaP/CH cells were passaged once a week with changing culture medium every 3–4 days. The human embryonic kidney cell line 293T (ATCC CRL-3216), the human breast cancer cell line MCF-7 (ATCC HTB-22), and the monkey kidney fibroblast cell line COS-7 (ATCC CRL-1651) were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NE, USA) supplemented with 10% heat-inactivated FCS, 50 IU/mL penicillin, and 50 μ g/mL streptomycin.

2.3 | Migration and invasion assays

Cell migration and invasion assays were performed under normoxic (for LNCaP/N) or hypoxic (for LNCaP/AH and LNCaP/CH) conditions using 24-well BD BioCoat Control Insert Chambers with 8.0- μ m pore size polyethylene terephthalate membranes and BD BioCoat Matrigel Invasion Chambers, respectively (BD Biosciences, San Jose, CA, USA). Cells (5×10^4 cells/well) were seeded in serum-free RPMI-1640 in the upper chamber and in RPMI-1640 containing 15% heat-inactivated FCS in the lower chamber. After 72 hours of incubation, cells that had migrated to the undersurface of the membranes were counted as previously described.²² The average number of cells was calculated from 3 independent experiments performed in triplicate.

2.4 | Whole genome microarray and data analysis

Genome-wide expression profiling was performed using the Whole Human Genome 4×44 K Microarray (Agilent Technologies, Tokyo, Japan) to identify differentially expressed genes among normoxia-conditioned LNCaP cells (LNCaP/N), LNCaP/AH, and LNCaP/CH6M.²³ Expression profiles of slug small interfering RNA (siRNA)-treated LNCaP/CH6M (siSlug LNCaP/CH6M) cells and control siRNA-treated LNCaP/CH6M (siScr LNCaP/CH6M) cells were compared to identify slug-regulating genes. Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood, CA, USA) was used for the data analysis, as previously described.²³

2.5 | Real-time quantitative RT-PCR

First-strand cDNA was synthesized from the total RNA using ThermoScript RT-PCR System (Roche, Indianapolis, IN, USA). PCR was performed on a LightCycler system (Roche) using LightCycler FastStart DNA Master SYBR Green I reaction mix (Roche) and QuantiTect

Primer Assays (QIAGEN, Hilden, Germany). The primers used were SNAI1 (snail; Hs_SNAI1_1_SG QuantiTect Primer Assay; QT00010010), SNAI2 (slug; Hs_SNAI2; QT00044128), EFN1 (ephrin-B1; Hs_EFN1; QT00024143), and β -actin (Hs_ACTB; QT00095431). β -Actin mRNA quantification was used for normalization.

2.6 | Western blotting

Cells were lysed in lysis buffer as described previously.²² Equal amounts of protein were loaded on each lane and separated on a sodium dodecylsulfate–polyacrylamide gel and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were probed with the appropriate antibodies followed by the enhanced chemiluminescence Prime Western blotting detection system (Amersham, Little Chalfont, UK). The following antibodies were used: anti-snail rabbit IgG (1:1000; Cell Signaling Technology, Beverly, MA, USA); anti-slug rabbit IgG (1:1000; Cell Signaling); anti-E-cadherin rabbit IgG (1:1000; Cell Signaling); anti-N-cadherin (1:1000; Cell Signaling); anti-vimentin (1:1000; Cell Signaling); anti- β -tubulin mouse monoclonal IgG (1:5000; Millipore); and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG as a secondary antibody (1:2000; Promega, Madison, WI, USA).

2.7 | RNAi experiments

The LNCaP/CH6M cells were transfected with siRNAs (20 nmol/L final concentration) using Lipofectamine RNAiMAX (Invitrogen). siRNA duplexes (Dharmacon, Little Chalfont, UK) used to target slug and ephrin-B1 mRNAs were as follows: slug, 5'-UCUCUCCUCUUUCCGGAUA-3', 5'-GCGAUGCCCAGUCUAGAAA-3', 5'-ACAGCGAACUGGACACACA-3', 5'-GAAUGUCUCUCCUGCACAA-3'; ephrin-B1, 5'-CGACAUCAUUAUCCCUUA-3', 5'-GAAUACGCUUUACCAUCA-3', 5'-UCAAGAAGCACCAUGAUUA-3', 5'-UCAAGAAGCACCAUGAUUA-3', 5'-GAGACAAGCUGGACAUCAU-3'. The negative control siRNA (siScr, Stealth RNAi Negative Control Duplexes) was from Invitrogen.

2.8 | Immunofluorescence analysis

The LNCaP cells grown on chamber slides were fixed with 4% paraformaldehyde followed by permeabilization with PBS plus 0.1% Triton X-100. The cells were incubated with anti-E-cadherin rabbit IgG (1:200; Cell Signaling) and anti-vimentin mouse monoclonal IgG (1:50; Santa Cruz, Dallas, TX, USA), and subsequently stained with secondary antibodies conjugated to Alexa 488 or 594 (1:1000; Invitrogen). The nuclei were stained with DAPI. Images were photographed with a Carl Zeiss LSM-710 laser scanning microscope. Clinical samples of prostate adenocarcinoma and healthy prostate tissues were obtained from the Department of Urology, Oita University Hospital, under approval from the Ethics Committee of Oita University Faculty of Medicine (Permission number: 1340). To analyze effects of androgen deprivation on normal prostate tissues, normal glands adjacent to the cancerous lesions were studied. The

tissues were fixed with 10% buffered formalin, and paraffin-embedded tissue sections were deparaffinized and incubated with 10% normal goat serum in PBS for 10 min, followed by incubation with anti-ephrin-B1 rabbit IgG (1:50; Santa Cruz), anti-slug mouse monoclonal IgG (1:50; Santa Cruz), and anti-carbonic anhydrase IX (CAIX) mouse monoclonal IgG (1:200; Abcam, Cambridge, MA, USA). Double staining was performed as described herein.

2.9 | Production of lentivirus and generation of stable cell lines

Lentiviruses carrying pLenti6.3/V5-DEST-ephrin-B1 or pLenti6.3/V5-DEST empty (control) were packaged with 293T cells using the ViraPower Lentiviral Expression System (Invitrogen). LNCaP cells were infected with the lentiviral particles and the ephrin-B1 control or overexpressing cell lines were selected using 10 μ g/mL blasticidin (Invitrogen).

2.10 | Cell transfection and luciferase reporter assays

The LNCaP cells were seeded into 6-well plates (4×10^5 cells/well) and transfected with pLenti6.3/V5-DEST-slug (0–1000 ng/well) using Lipofectamine 3000 (Invitrogen). Cells were harvested for total RNA extraction 72 hours after transfection. For luciferase reporter assays, LNCaP cells were seeded in 12-well plates (1×10^5 cells/well) and transfected with 1000 ng/well reporter constructs (pGL-Basic-ephrin-B1 promoter WT, E1m, E2m, or E1mE2m), 1 ng/well Renilla-luciferase vector (pRL-SV40, Promega), and pLenti6.3/V5-DEST-slug (0–1000 ng/well) using Lipofectamine 3000. The cells were harvested 72 hours after transfection and the activity of firefly and Renilla luciferases were measured using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity.

2.11 | Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was carried out using a ChIP-IT Express Enzymatic kit (Active Motif, Tokyo, Japan) according to manufacturer's instructions. DNA-bound proteins extracted from the LNCaP/CH6M cells were immunoprecipitated with anti-slug mouse monoclonal IgG (Santa Cruz) or control mouse IgG (Santa Cruz). The DNA pulled down by ChIP was subjected to PCR amplification of the ephrin-B1 promoter regions containing E-box1 or E-box2. The primer sequences were as follows: E-box1-containing region: forward, 5'-GGTCTGAGCTATGGGATCGAG-3'; reverse, 5'-GCATCCTGGACCGGAGAAG-3'; E-box2-containing region: forward, 5'-TCCCCCTCCCACTGTTCTCAC-3'; reverse, 5'-CCTCGTCCAGTCCCACGTAA-3'.

2.12 | Statistical analysis

Data are expressed as mean \pm SD. Statistical analysis was performed using Student's *t* test. *P* < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Chronic hypoxia promotes prostate cancer cell migration and invasion

Chronic hypoxia has been shown to promote invasive behavior of human prostate cancer cells, LNCaP.²¹⁻²³ Here, we confirmed that cell migration and invasion are increased under chronic hypoxic conditions by performing migration and invasion assays (Figure 1). Cell invasion under chronic hypoxia for >6 months (LNCaP/CH6M) was significantly increased by 24-fold compared with normoxia (LNCaP/N), and 4-fold compared with acute hypoxia (LNCaP/AH).

3.2 | Chronic hypoxia specifically upregulates the expression of an EMT-driving transcription factor, slug

Given that EMT has been implicated in cell migration, invasion and initiation of metastasis,⁴⁻⁸ we next analyzed the expression of major EMT-driving genes of the snail family, snail, slug, and Smuc; and of the twist family, Twist1 and Twist2.⁹ In our previous study, we performed the genome-wide expression profiling to identify differentially expressed genes among LNCaP/N, LNCaP/AH, and LNCaP/CH6M.²³ Using these profiling data, we found that expression of slug was specifically and strongly upregulated under chronic hypoxia in LNCaP/CH6M by 30-fold compared with in LNCaP/N and LNCaP/AH (Figure 2A). We further confirmed that slug mRNA and protein levels were markedly enhanced in LNCaP/CH6M on quantitative RT-PCR and western blot analysis, respectively (Figure 2B,C).

3.3 | siRNA-mediated repression of slug strongly inhibits chronic hypoxia-induced cell migration and invasion

To demonstrate whether the upregulation of slug is required for chronic hypoxia-induced enhancement of cell migration and invasion, siRNA-mediated repression of slug was performed in LNCaP/CH6M cells. We first confirmed that there was reduced expression of slug in the slug siRNA-transfected LNCaP/CH6M cells (siSlug; Figure 3A). Knockdown of slug strongly inhibited migration and invasion of LNCaP/CH6M (siSlug) compared with non-targeting control siRNA-transfected cells (siScr; Figure 3B,C). This suggests that slug plays a crucial role in increasing cell migration and invasion, which is induced by chronic hypoxia.

3.4 | Neither loss of E-cadherin expression nor induction of mesenchymal markers is observed in the LNCaP/CH6M cells

As mentioned herein, slug is a well-known EMT-driving transcription factor.⁹ Therefore, to confirm that the EMT process was actually activated by chronic hypoxia-induced slug, we investigated whether there is loss of E-cadherin and increase in expression of N-cadherin, an event considered one of the hallmarks of EMT,^{4,5} on western blot analysis. Unexpectedly, we found that the expression of E-cadherin was almost unchanged between normoxic and hypoxic conditions and neither N-cadherin nor vimentin, a mesenchymal intermediate filament component,⁹ was induced by chronic hypoxia (Figure 4A). Furthermore, on light microscopy the LNCaP/CH6M cells were not

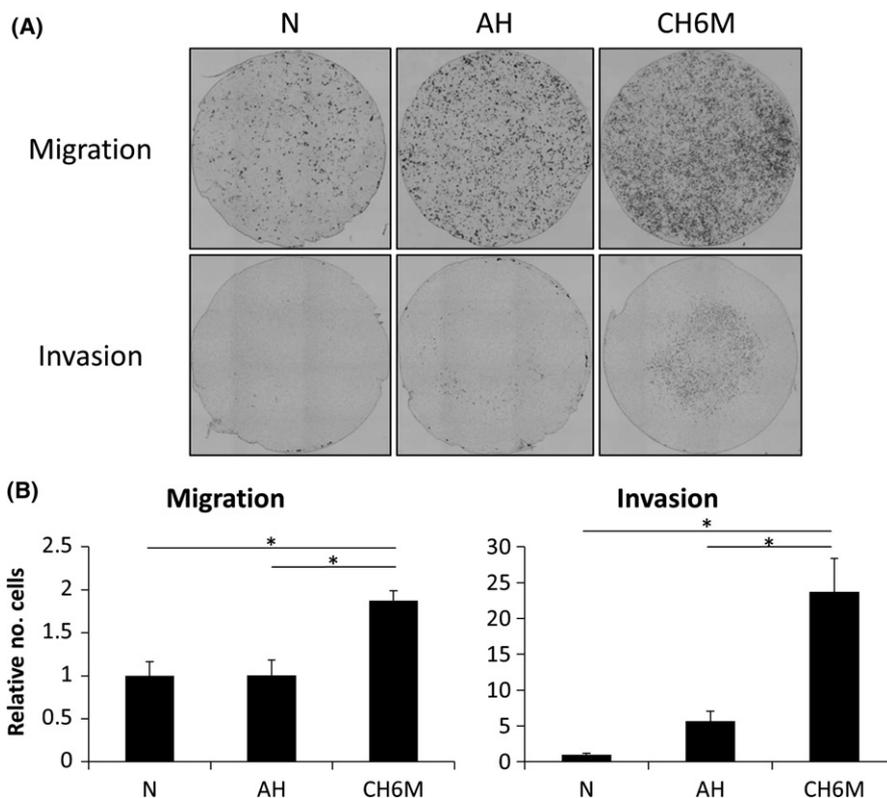
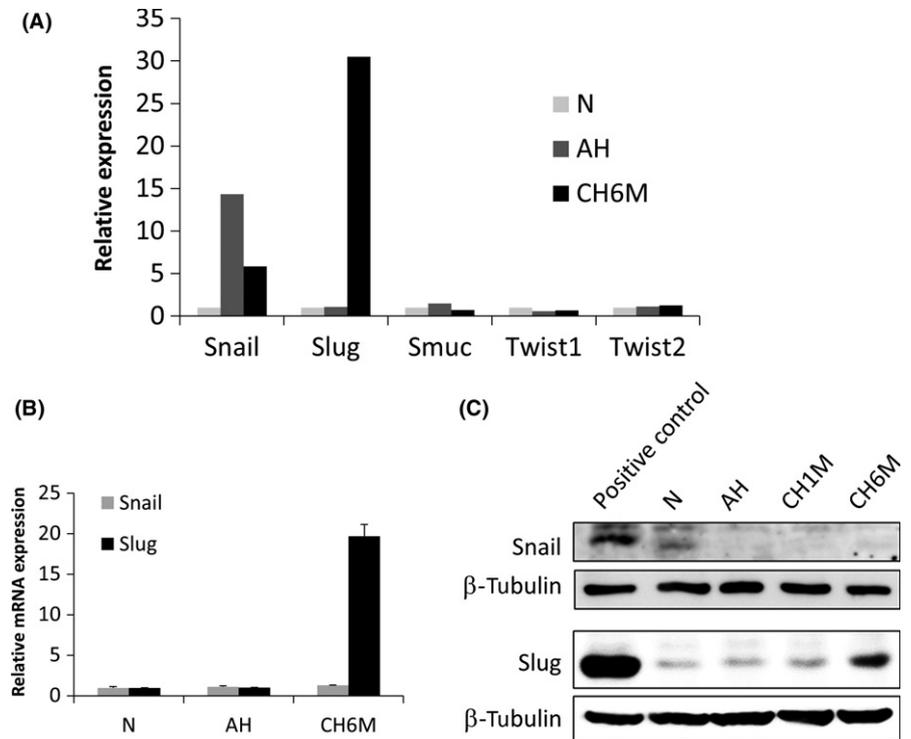


FIGURE 1 Chronic hypoxia promotes migration and invasion of the prostate cancer cell, LNCaP. A, Toluidine blue staining of cells that migrated or invaded to the undersurface of the membrane under normoxic (N), acute hypoxic (AH), and chronic hypoxic (CH6M) conditions. Cell migration (upper panels) and invasion (lower panels) were analyzed using Control Insert Chambers and Matrigel Invasion Chambers respectively. B, Fold change of the number of the cells that migrated or invaded to the undersurface of the membrane. Data given as mean \pm SD. * $P < 0.05$

FIGURE 2 Chronic hypoxia specifically upregulates expression of an epithelial-mesenchymal transition (EMT)-driving transcription factor slug. A, Fold change of expression levels of EMT-driving genes, snail, slug, Smuc, Twist1, and Twist2, in LNCaP under normoxic (N), acute hypoxic (AH), and chronic hypoxic (CH6M) conditions. B, Quantitative RT-PCR and C, western blot analysis of expression of snail and slug in LNCaP under the same conditions. B, Data given as mean \pm SD. C, Total cell lysates of COS-7 and 293T were used as positive controls for snail and slug expression, respectively. β -Tubulin was used as a loading control



dispersed and did not show fibroblast-like shapes that are characteristic of mesenchymal cells.^{4,5} In addition, on immunocytochemistry the E-cadherin expression was sustained and vimentin expression was not induced in LNCaP/CH6M, consistent with the western blot results (Figure 4B). This suggests that chronic hypoxia-induced slug enhances invasive behavior without inducing EMT-like features such as repression of E-cadherin, and it appears to be promoting cell migration and invasion through alternative pathways.

3.5 | Identification of chronic hypoxia-induced slug-upregulated genes associated with cell migration and invasion

We compared expression profiles of slug siRNA-treated LNCaP/CH6M (siSlug LNCaP/CH6M) with those of control siRNA-treated LNCaP/CH6M (siScr LNCaP/CH6M) and identified genes that had their expression significantly downregulated in siSlug LNCaP/CH6M (>50% reduction). In other words, these genes correspond to the genes upregulated by chronic hypoxia-induced slug. We also chose genes specifically upregulated by chronic hypoxia (>2-fold increase) by comparing the gene expression microarray data of LNCaP/N with that of LNCaP/CH6M.²³ From the list of the genes upregulated by slug as well as chronic hypoxia, we then selected genes known to be associated with cell migration and invasion using IPA (Table 1).

3.6 | Chronic hypoxia-induced ephrin-B1 promotes cell migration and invasion

Given that elevated expression of B-type ephrin (a ligand of Eph-related receptor tyrosine kinases) has been known to

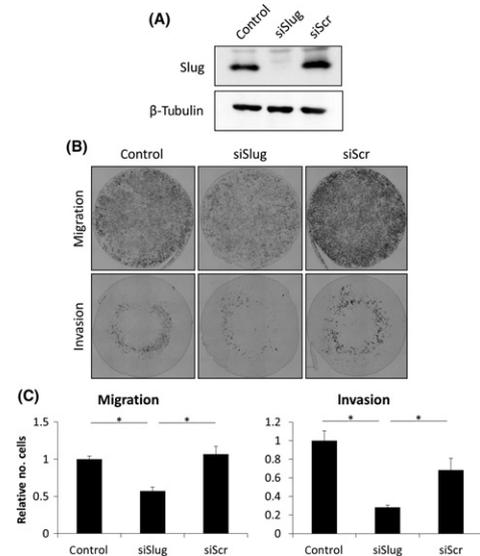


FIGURE 3 Knockdown of slug inhibits chronic hypoxia-induced cell migration and invasion. A, Western blot analysis of slug expression in the non-transfected (control), slug siRNA-transfected (siSlug), and control siRNA-transfected (siScr) LNCaP/CH6M cells. β -Tubulin was used as a loading control. B, Toluidine blue staining of the siRNA-transfected (control, siSlug, and siScr) LNCaP/CH6M cells that migrated to the undersurface of the membrane. C, Fold change of the number of cells that migrated or invaded to the undersurface of the membrane (n = 4). Data given as mean \pm SD. * P < 0.05

correlate with progression and metastasis in various cancers²⁷⁻²⁹ and recent work suggests that the B-type ephrin-mediated signaling acts as a regulator of prostate development,³⁰ we decided to focus on EFNB1, which encodes an ephrin-B1 ligand

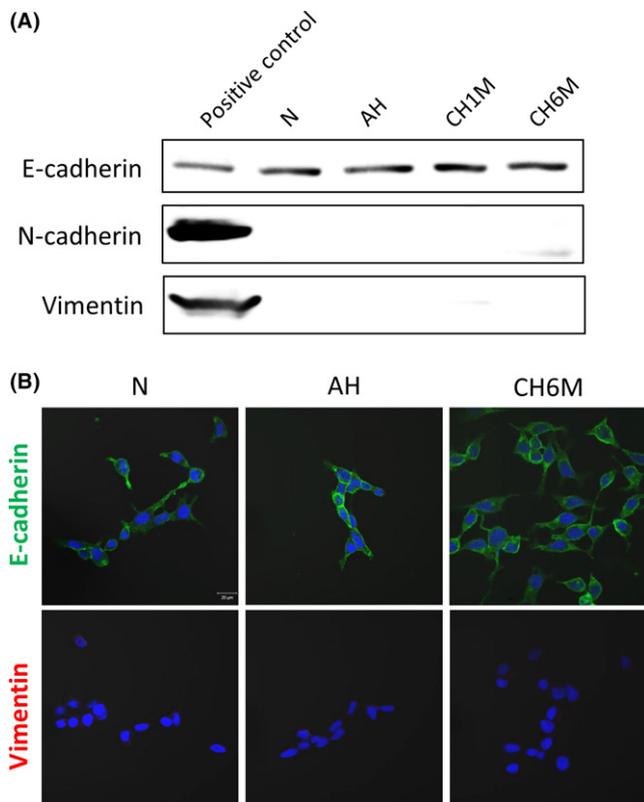


FIGURE 4 Chronic hypoxia does not show epithelial-mesenchymal transition-like features such as loss of E-cadherin. A, Western blot analysis of expression of E-cadherin, N-cadherin and vimentin in LNCaP cells under normoxic (N), acute hypoxic (AH), and chronic hypoxic (CH1M and CH6M) conditions. Total cell lysates of MCF7, 293T, and COS-7 were used as positive controls for E-cadherin, N-cadherin, and vimentin expression, respectively. B, Immunofluorescence staining of E-cadherin (green) and vimentin (red) in LNCaP cells under the same conditions. DAPI was used to stain nuclei (blue)

(Table 1). First we confirmed that expression of ephrin-B1 is strongly upregulated under chronic hypoxia in LNCaP/CH6M (Figure 5A). EphB1, 2, 3, 4, and 6 are known to work as ephrin-B1 receptors.³¹ We found that EphB2, 3, and 4 are preferentially expressed in LNCaP/CH6M and that expression of EphB4 is markedly induced under chronic hypoxia (Figure S1). Second, to investigate whether the upregulation of ephrin-B1 promotes cell migration and invasion under chronic hypoxia, siRNA-mediated repression of ephrin-B1 was performed in LNCaP/CH6M. We confirmed that there was reduced expression of ephrin-B1 in the ephrin-B1 siRNA-transfected LNCaP/CH6M cells (siEphrin-B1; Figure 5B). Knockdown of ephrin-B1 strongly inhibited migration and invasion of LNCaP/CH6M (siEphrin-B1 LNCaP/CH6M) (>75% reduction) compared with control siRNA-transfected cells (siScr LNCaP/CH6M; Figure 5C,D). Furthermore, forced expression of ephrin-B1 in LNCaP/N significantly enhanced cell migration and invasion (Figure 5E,F,G), indicating that chronic hypoxia-induced ephrin-B1 promotes cell migration and invasion.

TABLE 1 Chronic hypoxia-induced slug-upregulated genes associated with cell migration and invasion

Gene symbol	Gene name
PGC	Progastricsin (pepsinogen C)
PLEKHA6	Pleckstrin homology domain containing, family A member 6
LAMC1	Laminin, gamma 1 (formerly LAMB2)
RNF182	Ring finger protein 182 (RNF182)
C2orf72	Chromosome 2 open reading frame 72
APOL3	Apolipoprotein L, 3
ZNF577	Zinc finger protein 577
CA12	Carbonic anhydrase XII
KRT19	Keratin 19
ASB2	Ankyrin repeat and SOCS box-containing 2
TLR3	Toll-like receptor 3
C1orf21	Chromosome 1 open reading frame 21
NLGN4X	Neuroigin 4, X-linked
DPYSL2	Dihydropyrimidinase-like 2
FAM189A2	Family with sequence similarity 189, member A2
KIAA1683	KIAA1683
GPR162	G protein-coupled receptor 162
TNNT1	Troponin T type 1 (skeletal, slow)
PPFIA4	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4
LHFPL2	Lipoma HMGIC fusion partner-like 2
TGFBR3	Transforming growth factor, beta receptor III
FHL2	Four and a half LIM domains 2
TMEM45A	Transmembrane protein 45A
SLC24A3	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
CLEC3B	C-type lectin domain family 3, member B
SDC3	Syndecan 3
HES2	Hairy and enhancer of split 2 (Drosophila)
EFNB1	Ephrin-B1
AP1S2	Adaptor-related protein complex 1, sigma 2 subunit
RASSF2 SCNN1G	Ras association (RalGDS/AF-6) domain family member 2 sodium channel, non-voltage-gated 1, gamma
STMN4	Stathmin-like 4
HTR5A	5-hydroxytryptamine (serotonin) receptor 5A
MDGA1	MAM domain containing glycosylphosphatidylinositol anchor 1
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
ATP11C	ATPase, class VI, type 11C
ANK1	Ankyrin 1, erythrocytic
TECPR2	Tectonin beta-propeller repeat containing 2
HSPA6	Heat shock 70 kDa protein 6 (HSP70B')

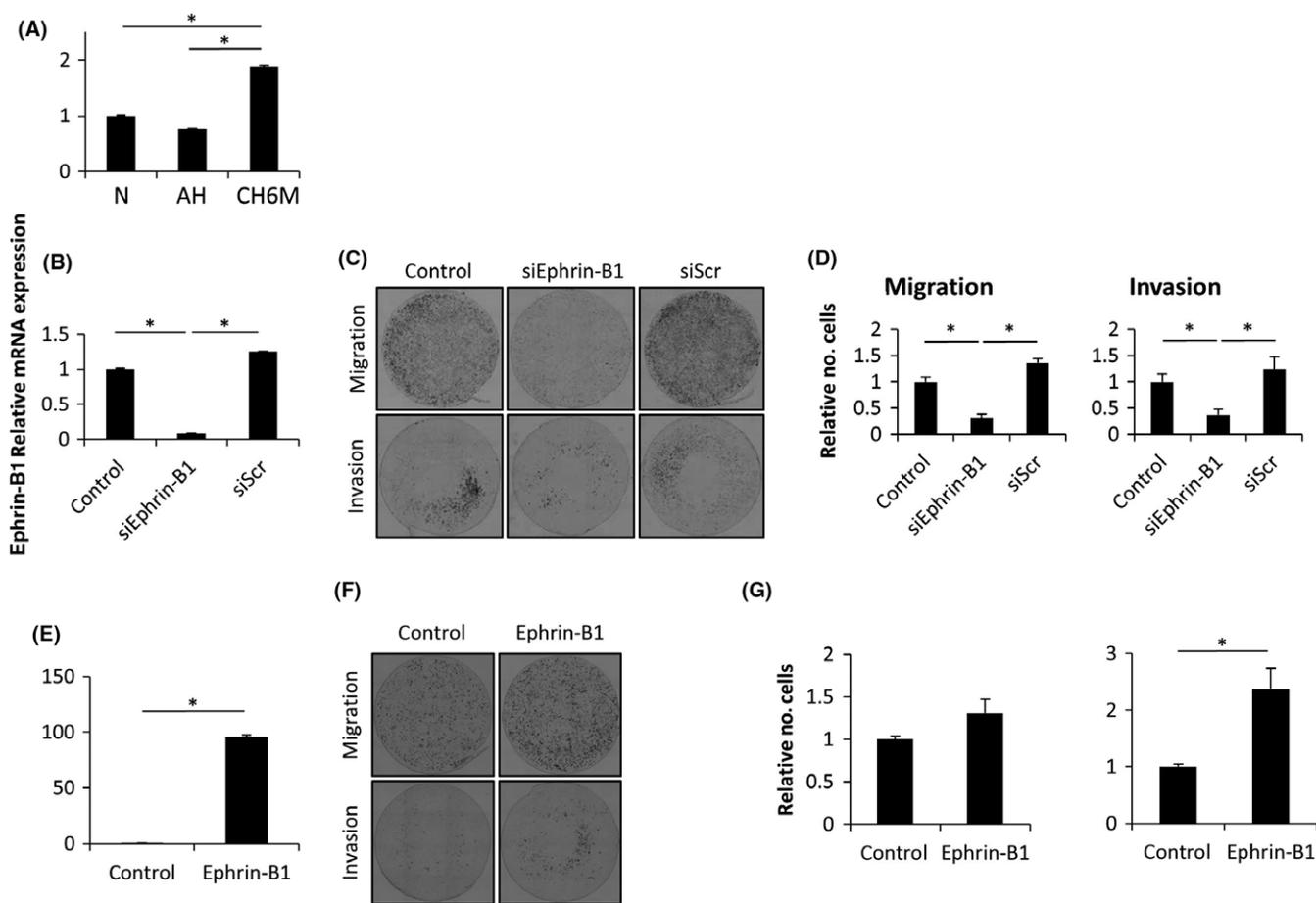


FIGURE 5 Chronic hypoxia-induced ephrin-B1 promotes cell migration and invasion. A, Relative expression of ephrin-B1 mRNA in LNCaP under normoxic (N), acute hypoxic (AH), and chronic hypoxic (CH6M) conditions. B, Relative expression of ephrin-B1 mRNA in non-transfected (control), ephrin-B1 siRNA-transfected (siEphrin-B1) and control siRNA-transfected (siScr) LNCaP/CH6M cells. C, Toluidine blue staining of the siRNA-transfected (control, siEphrin-B1, and siScr) LNCaP/CH6M cells that migrated to the undersurface of the membrane. D, Fold change of the number of the cells that migrated or invaded to the undersurface of the membrane. E, Relative expression of ephrin-B1 mRNA in the stably ephrin-B1-overexpressing LNCaP/N cells (Ephrin-B1) and empty vector-overexpressing LNCaP/N cells (control). F, Toluidine blue staining of the ephrin-B1 control and overexpressing LNCaP/N cells that migrated or invaded to the undersurface of the membrane. G, Fold change of the number of the cells that migrated or invaded to the undersurface of the membrane. Data given as mean \pm SD. * $P < 0.05$

3.7 | Chronic hypoxia-induced slug activates expression of ephrin-B1 through E-box motifs

We next examined whether expression of the ephrin-B1 gene is actually activated by chronic hypoxia-induced slug in LNCaP/CH6M. Slug siRNA-transfected LNCaP/CH6M cells had a significantly decreased ephrin-B1 mRNA level (>50% reduction), while control siRNA had no detectable effect on ephrin-B1 mRNA level (Figure 6A). We further confirmed that ephrin-B1 mRNA was upregulated by forced expression of slug in a dose-dependent manner in LNCaP/N (Figure 6B). Slug functions either as an activator^{20,32–34} or a repressor^{34–37} by binding to an E-box, a sequence containing the CANNTG motif, in its target gene promoters.^{20,32,35–37} We found 2 E-boxes at positions at –877 (E1) and –483 (E2) relative to the transcription start site (+1) of the ephrin-B1 gene (Figure 6C). To confirm that the E-boxes in the ephrin-B1 promoter are essential for the slug-mediated transactivation of ephrin-B1,

we used the luciferase reporter plasmid containing the ephrin-B1 promoter sequence (from –1000 to +1 relative to the transcription start site, WT) and introduced mutations in either E-box1 or E-box2 as well as both E-box1 and 2 (E1m, E2m, and E1mE2m, respectively) in the promoter (Figure 6C).²⁶ The LNCaP/N cells were co-transfected with the WT reporter plasmid and various amounts of the slug expression vector. As expected, slug increased the ephrin-B1 promoter activity in a dose-dependent manner (Figure 6D). Mutations of E-boxes in the promoter, E1m, E2m, and E1mE2m, led to an almost complete loss of slug-mediated activation of the ephrin-B1 promoter (Figure 6E). Furthermore, we performed ChIP assays to investigate whether endogenous slug could be recruited to the E-box motifs in the ephrin-B1 promoter. As shown in Figure 6F, slug indeed bound to both E-box-containing regions, but predominantly bound to the E-box1. Taken together, these results demonstrated that chronic hypoxia-induced slug activates the expression of ephrin-B1 through E-box motifs.

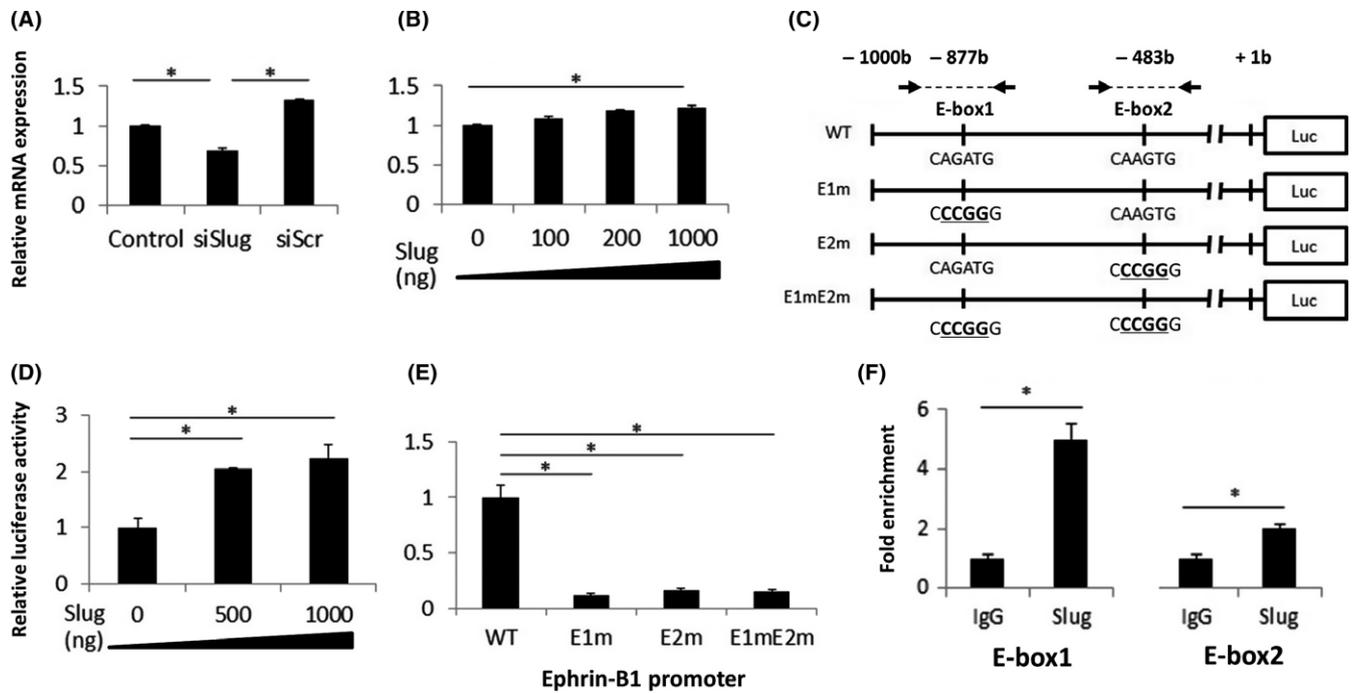


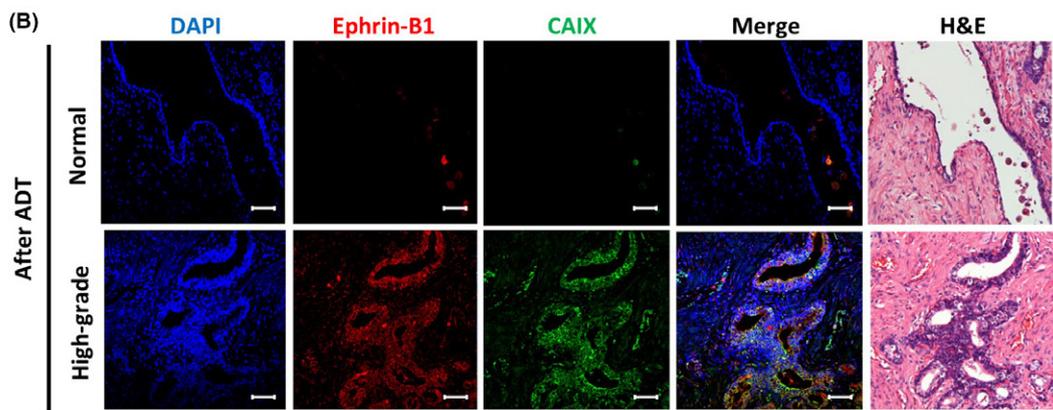
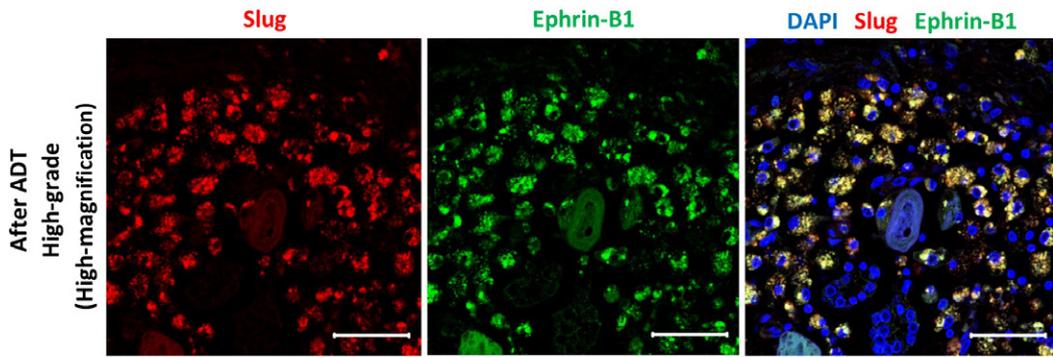
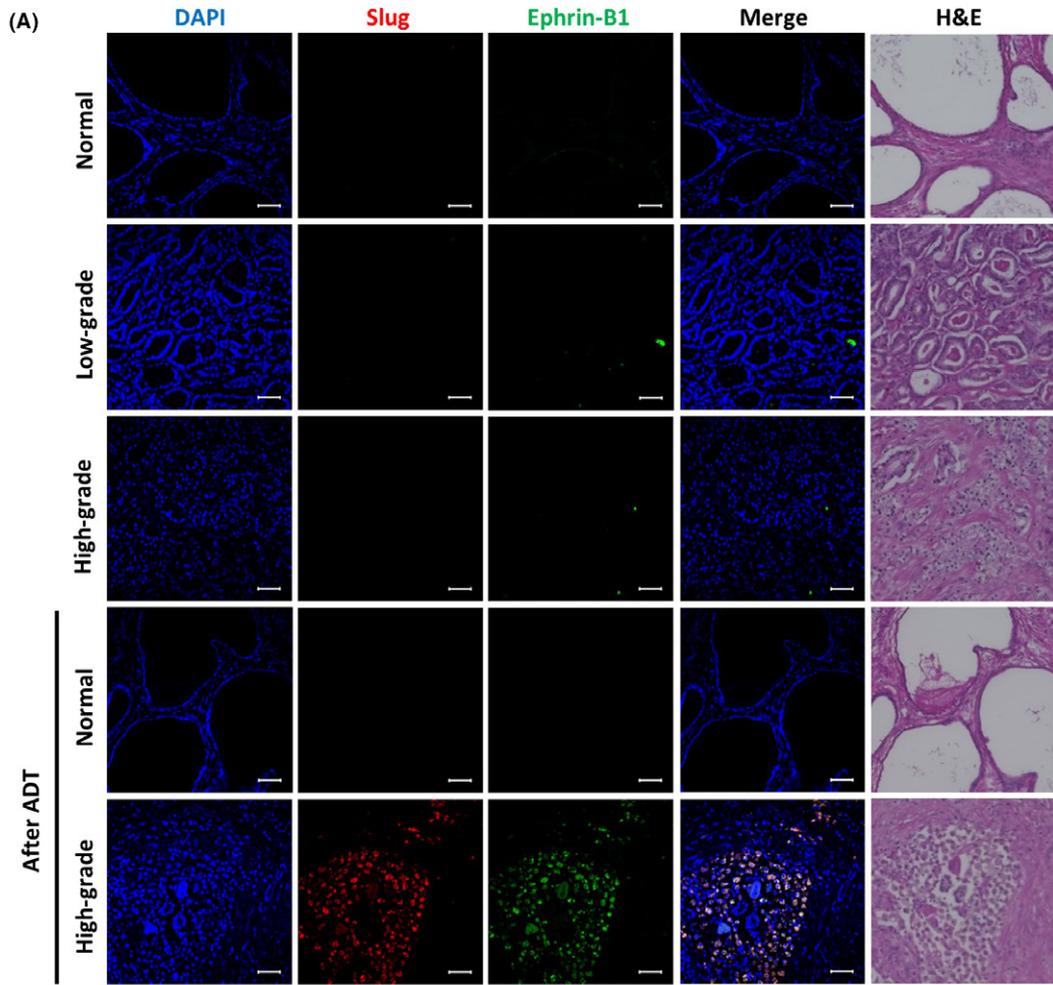
FIGURE 6 Chronic hypoxia-induced slug activates expression of ephrin-B1 through E-box motifs. A, Relative expression of ephrin-B1 mRNA in non-transfected (control), slug siRNA-transfected (siSlug) and control siRNA-transfected (siScr) LNCaP/CH6M cells. B, Relative expression of ephrin-B1 mRNA in the LNCaP/N cells transfected with various amounts of the slug-expression vector, pLenti6.3/V5-DEST-slug (0-1000 ng). C, Schematic diagram of luciferase reporter constructs containing wild-type human ephrin-B1 promoter (pGL-Basic-ephrin-B1 promoter WT), E-box1 (E1m) or E-box2 (E2m) mutations, or double mutations (E1mE2m). Arrows, primer pairs flanking E-box motifs used for chromatin immunoprecipitation (ChIP) analysis. D, Effect of slug overexpression on the wild-type ephrin-B1 promoter activity. LNCaP/N cells were transfected with 1000 ng pGL-Basic-ephrin-B1 promoter WT and various amounts of the slug-expression vector, pLenti6.3/V5-DEST-slug (0-1000 ng). Firefly luciferase activity was normalized to Renilla luciferase activity. E, Mutational analysis of the E-box motifs in the ephrin-B1 promoter. LNCaP/N cells were transfected with 1000 ng pLenti6.3/V5-DEST-slug, and 1000 ng pGL-Basic-ephrin-B1 promoter WT, E1m, E2m, or E1mE2m. F, ChIP analysis of slug on the E-box regions in the ephrin-B1 promoter. Soluble chromatin extracted from LNCaP/CH6M cells was immunoprecipitated with anti-slug and control IgG antibodies. ChIP was analyzed on quantitative PCR using primers flanking the E-box motifs, E-box1 and E-box2, shown in (C). Fold change compared with control IgG-enriched DNA fragments was measured. Data given as mean \pm SD. * $P < 0.05$

3.8 | Coexpression of slug and ephrin-B1 is strongly induced in human prostate adenocarcinoma cells by ADT

We next examined slug and ephrin-B1 expression levels and patterns in 24 prostate adenocarcinoma (12 cases of high-grade or poorly differentiated cancer with Gleason score ≥ 7 and 12 cases of low-grade or well-differentiated cancer with Gleason score < 7) and 12 healthy prostate tissues. As shown in Figure 7A, most of the healthy prostate tissue and prostate adenocarcinoma cells barely expressed slug and ephrin-B1. In some cases of prostate adenocarcinoma (1 of 12 low-grade cases and 1 of 12 high-grade cases), slug and ephrin-B1 were obviously coexpressed in the same

cells, but their expression levels did not correlate with tumor histological grade. Given that androgen deprivation has been shown to cause tumor hypoxia over a prolonged period of time by a marked loss of tumor-associated microvessels in the human prostate tumor xenograft models,^{24,25} we next investigated their expression levels and patterns in the adenocarcinoma and normal glands adjacent to the cancerous lesions after ADT. Intriguingly, a strong induction of both slug and ephrin-B1 expression was observed in the adenocarcinoma cells after ADT (7 of 12 high-grade cases), while ADT did not induce expression of slug or ephrin-B1 in most healthy prostate tissues (11 of 12 cases). Higher magnification view of the high-grade prostate cancer sample after ADT showed that slug and ephrin-B1 are preferentially accumulated in the cytoplasm. This

FIGURE 7 Slug and ephrin-B1 are coexpressed at a high level in human prostate adenocarcinoma cells after androgen deprivation therapy (ADT). A, Immunofluorescence of healthy and adenocarcinoma prostate tissues shows red staining for slug, green staining for ephrin-B1, and yellow fluorescence in the merged pictures for coexpression of slug and ephrin-B1. The tissues were counterstained with H&E and with DAPI. Representative immunofluorescence images of normal prostate tissues, low-grade and high-grade prostate adenocarcinomas, adenocarcinoma tissues and normal tissues adjacent to the cancerous region after ADT. Scale bar, 50 μ m. B, Immunofluorescence of high-grade prostate adenocarcinoma tissues and normal tissues adjacent to the cancerous region after ADT shows red staining for ephrin-B1, green staining for carbonic anhydrase IX (CAIX), and yellow fluorescence in the merged pictures for coexpression of ephrin-B1 and CAIX. Scale bars, 50 μ m



observation is consistent with previous reports on the cytoplasmic expression of slug and ephrin-B1 in various cancers with poor prognosis.^{28,38–40} Furthermore, the expression of ephrin-B1 was induced in the cells expressing an endogenous marker of prolonged chronic hypoxia, carbonic anhydrase IX (CAIX)^{41,42} in adenocarcinoma tissues after ADT (Figure 7B). This suggests that coexpression of slug and ephrin-B1 is markedly induced in human prostate adenocarcinoma cells under the chronic hypoxic conditions caused by androgen deprivation.

4 | DISCUSSION

Solid tumors are exposed to hypoxic conditions over longer periods of time as they grow.^{11,12} The hypoxic condition contributes to the acquisition of the malignant phenotype including cell invasive behavior, but the effects of hypoxia on malignant progression have been mainly studied using tumor cells cultured under acute hypoxic conditions.^{13–17} In this study, we demonstrated that chronic hypoxia promotes prostate cancer cell migration and invasion by upregulating the expression of an EMT-driving transcription factor, slug. Slug is widely known to work as a direct E-cadherin repressor,^{36,37} and its dysregulated expression correlates with poor clinical outcome in various types of cancers.^{43–46} We found that prostate cancer cells under chronic hypoxia shows neither loss of E-cadherin expression nor induction of mesenchymal markers; instead, expression of ephrin-B1 is markedly induced by slug and promotes cell migration and invasion.

Mechanisms through which slug is upregulated under chronic hypoxia and slug-overexpressing cells survive under chronic hypoxia by evading apoptosis have not been elucidated. Huang et al demonstrated that slug expression is activated by acute hypoxia-induced HIF-1 α .²⁰ Indeed, our genome-wide expression profiling showed that the expression of HIF-1 α is elevated under acute hypoxia, but it decreases to that observed under normoxia in the chronic hypoxic condition (unpublished data). Therefore, we speculate that upregulation of slug under chronic hypoxia may be controlled by transcription factors specifically induced under chronic hypoxia, rather than directly and solely by HIF-1 α . We have found that knockdown of slug in the LNCaP/CH6M cells decreases the expression of a mitochondrial antioxidant, peroxiredoxin-3 (Prdx3), suggesting that Prdx3 expression is upregulated by chronic hypoxia-induced slug (unpublished data). Overexpression of peroxiredoxins including Prdx3 has been reported in various cancers.⁴⁷ Furthermore, increased expression of Prdx3 has been known to protect cells from hypoxia-induced apoptosis.⁴⁸ Thus, it is presumed that overexpression of Prdx3 activated by chronic hypoxia-induced slug could confer anti-apoptotic properties to prostate cancer cells under chronic hypoxia.

Given that the growth of prostate cancer cells depends on androgens and androgen receptor (AR) signaling, ADT is widely used for the treatment of locally advanced and metastatic disease.

ADT initially controls the growth of androgen-dependent tumors, but eventually the majority of these tumors progress to more aggressive and metastatic castration-resistant tumors.^{49,50} Docetaxel has been used as the standard chemotherapeutic drug for metastatic castration-resistant cancers, but resistance eventually occurs through a number of mechanisms, including activation of pro-survival signaling, increased drug-efflux, and overexpression of β -tubulin isoforms and inflammation-associated molecules.^{51,52} Although many novel agents designed to overcome chemoresistance are currently under development, the prognosis of castration-resistant cancers remains poor.⁵³ It is noteworthy that ephrin-B1 expression is strongly induced after ADT of prostate adenocarcinoma cells and, perhaps more importantly, ADT does not induce ephrin-B1 expression in normal tissue adjacent to the cancerous region. This suggests that ephrin-B1 may be a novel promising therapeutic target with a low risk of local toxicity in healthy tissue, in combination with ADT or as adjuvant treatment after ADT for aggressive prostate cancers.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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