Clusterin Mediates TGF-β–Induced Epithelial–Mesenchymal Transition and Metastasis via Twist1 in Prostate Cancer Cells

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Abstract

TGF-β promotes epithelial–mesenchymal transition (EMT) and induces clusterin (CLU) expression, linking these genes to cancer metastasis. CLU is a pleiotropic molecular chaperone that confers survival and proliferative advantage to cancer cells. However, the molecular mechanisms by which TGF-β regulates CLU expression and CLU affects metastasis remain unknown. In this study, we report that the transcription factor Twist1 mediates TGF-β–induced CLU expression. By binding to E-boxes in the distal promoter region of CLU gene, Twist1 regulates basal and TGF-β–induced CLU transcription. In addition, CLU reduction reduced TGF-β induction of the mesenchymal markers, N-cadherin and fibronectin, thereby inhibiting the migratory and invasive properties induced by TGF-β. Targeted inhibition of CLU also suppressed metastasis in an in vivo model. Taken together, our findings indicate that CLU is an important mediator of TGF-β–induced EMT, and suggest that CLU suppression may represent a promising therapeutic option for suppressing prostate cancer metastatic progression. Cancer Res; 72(20); 1–12. ©2012 AACR.

Introduction

Prostate cancer is the most common nonskin cancer and the second leading cause of cancer-related death among males in developed countries. The incidence of prostate cancer has been increasing as a result of an aging population and prevalence of high-fat diets (1). Men with prostate cancer die predominantly from metastatic disease predisposed to become resistant to androgen-deprivation therapy, termed castration-resistant prostate cancer (CRPC). Acquisition of migratory properties is a prerequisite for cancer invasion into surrounding tissue. In cancer, acquisition of invasiveness requires a dramatic morphologic alteration, termed epithelial–mesenchymal transition (EMT), wherein cancer cells lose their epithelial characteristics of cell polarity and cell–cell adhesion, and switch to a mesenchymal phenotype (2). EMT is a characteristic of cancer cell invasation and metastasis and is closely associated with CRPC. E-cadherin is a cell-to-cell adhesion molecule in which loss of expression is a hallmark of EMT, leading to increased cell motility and invasion (3). On the other hand, N-cadherin and fibronectin are mesenchymal markers (4) in which expression is regulated by several transcription factors including a basic helix–loop–helix (bHLH) transcription factor Twist1, Slug, and Snail1 (5–7).

Clusterin (CLU) is a stress-activated and apoptosis-associated molecular chaperone functioning to protect cells from various stressors and is highly expressed in many human cancers. In prostate cancer, CLU expression levels are low in low-grade tissues but increase with higher Gleason score (8). Moreover, CLU expression is shown to be enhanced following castration in CRPC models (9, 10), wherein overexpression of CLU in prostate cancer accelerates progression after androgen-deprivation therapy or chemotherapy, identifying CLU as an antiapoptotic gene upregulated by treatment stress that confers therapeutic resistance when overexpressed (9, 11, 12). Previously, regulation of CLU expression by Egr-1 (13), androgen receptor (14), and Y-box binding protein-1 (15) has been reported. Interestingly, CLU overexpression enhances invasive and metastatic abilities in renal cancer (16) and hepatocellular carcinoma (17), whereas CLU silencing suppresses EMT via inhibition of Slug (18).

TGF-β is a multifunctional cytokine that inhibits the proliferation of normal prostate epithelial cells and premalignant lesions. Increased levels of TGF-β in patients’ serum have significant prognostic value for highly aggressive metastatic disease and are considered a poor prognosis marker (19–23). One mechanism by which TGF-β contributes to cancer progression is through induction of EMT. Upon TGF-β treatment, epithelial cells changed from a cuboidal to an elongated spindle shape with decreased expression of epithelial markers and enhanced expression of mesenchymal markers, such as E-cadherin and fibronectin. TGF-β induces...
both Twist1 (24, 25) as well as CLU expression in various cells (26–28). In addition, both Twist1 and CLU are associated with EMT characteristics. However, the mechanisms by which they drive progression through EMT remain obscure. Here, we define molecular mechanism linking Twist1-regulation of CLU expression by TGF-β, which are important regulators of EMT. Moreover, we investigate the therapeutic effects of CLU knockdown in suppressing EMT and prostate cancer metastasis.

**Materials and Methods**

**Cell culture and transfection**

PC-3 and 22Rv1 cells, which express the TGF-β receptor, were obtained from and authenticated by American Type Culture Collection and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Scientific) and RPMI-1640 (Thermo Scientific) supplemented with 5% FBS, respectively. PC-3M-luc (C6) cells, which are stably expressing luciferase protein, were obtained from Caliper Life Sciences and maintained according to the manufacturer’s instruction. All cell lines within 6 months after resuscitation were used. Cells were transfected with the indicated siRNA or the indicated plasmid as previously described (29, 30).

**Antibodies and reagents**

Antibodies against PCNA (sc-56), c-Myc (sc-815), CLU (sc-6419), and Twist1 (sc-81417) were purchased from Santa Cruz Biotechnology. Anti-Snail1 and anti-Slug antibodies were obtained from Cell Signaling. Antibodies against E-cadherin, N-cadherin, and fibronectin were purchased from BD Biosciences. Anti-β-actin antibody was purchased from Sigma. Human TGF-β1 recombinant protein was obtained from R&D Systems.

**Plasmids**

Twist1-Myc-Flag-plasmid expressing C-terminally Myc-Flag-tagged Twist1 protein and Egr-1-Myc-Flag plasmid expressing C-terminally Myc-Flag-tagged Egr-1 protein were purchased from OriGene. The Twist1 reporter plasmid (Twist–Luc) cloned the Twist1 promoter region (~969 bp) was kindly provided from Dr. L.H. Wang (Mount Sinai School of Medicine, New York, NY; ref. 31). Various lengths of CLU reporter plasmids (CLU–Luc –1,998/+254, –1,998/+702, –1,116/+702, and –707/+254) containing the promoter and partial first exon of the wild-type CLU gene were constructed as previously described (15). Mutated CLU reporter plasmid (CLU–Luc E1MT and E2MT) were constructed by introducing mutations into the CLU–Luc –1,998/+702 plasmid using a QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies) with the following primer pairs: 5'-CGCAATTCCGGTGGATGGGAGTG-3' and 5'-CCTCACCCACGACCATCTGACACCAGGTTGTCATCCCTGCC-3' for CLU–Luc E1MT; 5'-GATCTGCTTAGAATGAAAACCTCTGGT-GATGATTGTATGGGCC-3' and 5'-GGCCCATATATGGCATCACCCAGGATTCTATATTTCCACGATC-3' for CLU–Luc E2MT. The underlined nucleotides indicate the mutated sequences.

**siRNAs and antisense oligonucleotides**

The following double-stranded 25-bp siRNA oligonucleotides were commercially generated (Invitrogen Life Technologies, Inc.): 5’-CUCCUCGGCUGUUGCAACGGUCUC-3’ for Twist1 #1; 5’-UGAGGGCGUCAAUUGCUCAGCAUC-3’ for Twist1 #2; 5’-CCGUAUCCUAUGAGAGUUAUCUCA-3’ for Slug #1; 5’-GGCUACUUGCAAGCCAAUUCUGAU-3’ for Slug #2; 5’-UCCCAUGUAGCAUUGCCAGGAGG-3’ for Snail1 #1; 5’-UUUCAGCUCGGAGAUAUCUCCGCU-3’ for Snail1 #2. The sequence of siRNA corresponding to the human CLU initiation site, which was supplied by Dharmacon Research Inc., was 5’-ACACCTGTCATTCTCTGATAGA-3’. The sequence of siRNA targeting the 3’-untranslated region of Twist1 gene (Twist1 siRNA #3), which was supplied by Qiagen was 5’-CATCTGTCATTCTCTGATAGA-3’. The sequence of siRNA targeting the 3’-untranslated region of Twist1 gene (Twist1 siRNA #3), which was supplied by Qiagen was 5’-CATCTGTCATTCTCTGATAGA-3’. The sequence of siRNA targeting the 3’-untranslated region of Twist1 gene (Twist1 siRNA #3), which was supplied by Qiagen was 5’-CATCTGTCATTCTCTGATAGA-3’. The sequence of siRNA targeting the 3’-untranslated region of Twist1 gene (Twist1 siRNA #3), which was supplied by Qiagen was 5’-CATCTGTCATTCTCTGATAGA-3’.

**Quantitative reverse transcription PCR**

RNA extraction and reverse transcription PCR (RT-PCR) were conducted as previously described (32, 33). Real-time monitoring of PCR amplification of cDNA was conducted using the following primer pairs and probes, Twist1 (Hs00361186_m1), CLU (Hs0156548_m1), and GAPDH (Hs03929097_g1; Applied Biosystems) on ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with TaqMan Gene Expression Master Mix (Applied Biosystems). Target gene expression was normalized to GAPDH levels in respective samples as an internal control. The results are representative of at least 3 independent experiments.

**Western blot analysis**

Whole-cell extracts were obtained by lysis of cells in an appropriate volume of ice-cold radioimmunoprecipitation assay (RIPA) buffer composed of 50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS containing 1 mmol/L Na2VO4, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets (Complete, Roche Applied Science). Cellular extracts were clarified by centrifugation at 13,000 × g for 10 minutes, and protein concentrations of the extracts determined by a BCA protein assay kit (Thermo Scientific). Whole-cell extracts (30 μg) were boiled for 5 minutes in SDS sample buffer and separated by SDS–PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were probed with dilutions of primary antibodies followed by incubation with horseradish peroxidase–conjugated secondary antibodies. After extensive washing, proteins were visualized by a chemiluminescent detection system (GE Healthcare).

**Luciferase reporter assay**

PC-3 cells were transfected with the indicated reporter plasmid, expression plasmid, siRNA, and pRL-TK as an internal
control. In case using TGF-β, media were changed to serum-free media with or without TGF-β. The luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and a microplate luminometer (EG&G Berthold). The Firefly luciferase activities were corrected by the corresponding Renilla luciferase activities. The results are representative of at least 3 independent experiments.

DNA pull-down assay

The following 5'-biotinylated oligonucleotides were used; 5'-ACCGGGGTGCAGATGGATGCTGGGGAT-3' and 5'-ATCCCCAGCAGTCACCGGTG-3' for E1MT; 5'-ATATGAAACATCTGGTGATGCA-3' and 5'-TGCAATCACCAGAGTTTTCATAT-3' for E2MT; 5'-GCCCTTAGCCAGCAGC-3' and 5'-GGCTTTCAGCAGCTAAGGG-3' for E3MT; 5'-CCCTAATTGCGTGTGGCTAAGGC-3' and 5'-GCCCTTAGCCAGCTAAGGG-3' for E3WT; 5'-ATATGAAAACATCTGGTGATGCA-3' and 5'-TGCAATCACCAGAGTTTTCATAT-3' for E2MT; 5'-GCCCTTAGCCAGCAGC-3' and 5'-GGCTTTCAGCAGCTAAGGG-3' for E3MT; 5'-TCATCTGCAGCAGCTAAGGG-3' for E3WT; and 5'-TGCAATCACCAGAGTTTTCATAT-3' and 5'-GCCCTTCTCCATCTGGAGATGA-3' for E4WT. The primer pairs for Wnt3a, Wnt5a, CLU were obtained from Qiagen. The primer pairs for Wnt3a, Wnt5a, and CLU were previously described (15). Biotinylated oligonucleotides (4 μg of nuclear extract in immunoprecipitation extraction, the primer pairs (described later), and RT2 Real-Time SYBR Green/Rox PCR master mix (Qiagen) was conducted with paraformaldehyde and digested with micrococcal nuclease overnight with 30% methanol and 10% glacial acetic acid. Coomassie blue R250 (Sigma) for 2 hours, and destained in 500 mL 90% ethanol) and optical density (OD) was measured using a Dual-Luciferase Reporter Assay System (Promega). The luciferase activities were corrected by the corresponding Renilla luciferase activities. The results are representative of at least 3 independent experiments.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChiP) assay was conducted as previously described (15). Briefly, PC-3 cells were cross-linked with paraformaldehyde and digested with micrococcal nuclease to achieve a DNA smear of 200 to 1,000 bp. ChiP assay was conducted using SimpleChiP Enzymatic Chromatin IP Kit (Agarose Beads) according to the manufacturer’s protocol (Cell Signaling Technology) on the CLU and RPL30 genes. Then, obtained samples were treated with RNase (Qiagen). The quantitative RT-PCR (qRT-PCR) assay with 2 μL of 20 μL DNA extraction, the primer pairs (described later), and RT2 Real-Time SYBR Green/Rox PCR master mix (Qiagen) was conducted using ABI 7900HT System (Applied Biosystems). The results are representative of at least 3 independent experiments. The primer pairs for CLU = 0.02 (GPH025704(−02A) targeting around −1,403 bp, CLU = 01 (GPH025704(−01A) targeting around −417 bp, and CLU = 07 (GPH025704(+07A) targeting around +6,603 bp were obtained from Qiagen. The primer pairs for RPL30 gene (exon 3) and antihistone H3 antibody were included in Simple-Chip Enzymatic Chromatin IP Kit.

Cell migration and invasion assay

Cell migration was determined by a wound-healing assay. After PC-3 cells transfected with the indicated siRNA reached confluency, a wound was made in the monolayer by pressing a pipette tip down on the plate. The debris was removed by washing the monolayer twice with serum-free media, and the cells were cultured in serum-free media with or without 0.1 ng/mL of TGF-β for an additional 48 hours. Cell migration was recorded in 6 different microscopic fields. The percentage of wound healing was calculated by the equation: 

\[
\text{percentage of wound healing} = \frac{\text{average of [(gap area: 0 hours) - (gap area: 48 hours) / (gap area: 0 hours)]}}{3}
\]

Cell invasion was administered using Matrigel invasion chambers (BD Biosciences). PC-3 cells (5 × 10⁴ cells) transfected with the indicated siRNA were applied on the upper compartment with 500 μL of serum-free medium, and the lower compartment was filled in 500 μL of DMEM with or without 0.1 ng/mL of TGF-β. After 24 hours of incubation, noninvaded cells on the upper surface of the filter were removed carefully with a cotton swab, and cells were fixed with 100% methanol for 2 minutes. Invading cells on the lower side of the filter were stained with 0.5% crystal violet for 5 minutes, and eluted the dye with Sorensen’s solution (9 mg trisodium citrate, 305 mL distilled water, 195 mL 0.1 N HCl, 500 mL 90% ethanol) and optical density (OD) was measured in 595 nm.

Zymography

PC-3 cells transfected with the indicated siRNA were incubated in serum-free media with or without 0.1 ng/mL of TGF-β for 24 hours, and the proteins in the conditioned medium were concentrated with Amicon Ultra-4 (Millipore) at 13,000 × g for 30 minutes at 4°C. Proteins (20 μg) were loaded in nonreducing conditions on a 10% polyacrylamide gel containing 0.1% gelatin (Sigma). After electrophoresis, gels were incubated in Triton X-100 exchange buffer composed of 20 mM/L Tris–HCl (pH 8.0), 150 mM/L NaCl, 5 mM/L CaCl₂, and 2.5% Triton X-100, for 30 minutes followed by a 10 minutes wash 3 times with the incubation buffer (same buffer without Triton X-100). Gels were incubated in buffer overnight at 37°C, stained with 0.5% Coomassie blue R250 (Sigma) for 2 hours, and destained overnight with 30% methanol and 10% glacial acetic acid. Gelatinolytic activity was shown as clear areas in the gel.

In vivo tumor metastasis model

PC-3M-luc cells (2 × 10⁶ cells) were injected into tail vein of 6- to 8-week-old male athymic nude mice (Harlan Sprague-Dawley, Inc.) via a 30-gauge needle. The following day, mice were then randomly divided into 2 groups for treatment with Scr ASO or OGX-011. Each experimental group consisted of 10 mice. After randomization, 12.5 mg/kg of Scr ASO or OGX-011 was injected intraperitoneally (i.p.) once daily for 7 days followed by 3 weekly treatments thereafter. To quantify in vivo tumor burden, mice were then imaged on weeks 2, 4, 6, 8, and 10 in an IVIS200 Imaging System (Caliper Life Sciences; see later). Animal procedures were conducted according to the guidelines of the Canadian Council on Animal Care.

Bioluminescence imaging

Tumors in mice were imaged using an IVIS200 camera (Caliper Life Sciences) as previously described (34). Briefly, mice were injected i.p. with 150 mg/kg ν-luciferin (Caliper Lifesciences). Animal procedures were conducted according to the guidelines of the Canadian Council on Animal Care.
Figure 1. TGF-β mediated induction of Twist1 regulates CLU expression. A, PC-3 cells were treated with 0.1 ng/mL of TGF-β for the indicated duration and qRT-PCR was conducted using the primer pairs and probes for Twist1, CLU, and GAPDH. Each transcript level from nontreated cells was set as 1. Boxes, mean; bars, ±SD; *, P < 0.05 (compared with no treatment). Whole-cell extracts were analyzed by SDS–PAGE and analyzed by Western blot analysis with specified
Twist1 regulates CLU transcription by binding to distal promoter region of CLU

Because Twist1 knockdown reduces CLU expression at both mRNA and protein levels, we explored whether Twist1 is a transcription factor for CLU gene. To investigate mechanisms by which Twist1 regulates CLU transcription, the CLU promoter was analyzed, and 8 E-boxes were found that are known to be Twist1 binding sites (6, 35). We then used various lengths of CLU reporter plasmids (CLU–Luc −1,998/+254, −1,998/−702, −1,116/−702, and −707/+254) to identify regions associated with transcriptional activity, as shown in Fig. 2A. CLU–Luc −1,998/−702 exhibited most luciferase activity in PC-3 cells compared with other CLU–Luc plasmids. Because CLU–Luc −1,116/−702 plasmid did not exhibit much activity, the CLU promoter region between −1,998 bp and −1,116 bp seems to be most responsible for Twist1-activated CLU transcription (Fig. 2B). When Twist1 was overexpressed, the reporter plasmids containing the region between −1,998 bp and −1,116 bp were again most active (Fig. 2C). Also, Egr-1 overexpression increased luciferase activity by CLU reporter plasmid as previously reported (Supplementary Fig. S2; ref. 13). DNA pull-down assays were conducted to investigate which E-box is functional in CLU promoter region between −1,998 bp and −1,116 bp; Fig. 2D indicates that Twist1 preferentially bound to E1 (E1WT) and E2 (E2WT) compared with E3 (E3WT) and E4 (E4WT), and not to mutated E-box (E1MT, E2MT, E3MT, and E4MT). To further confirm the regulation of CLU transcription via Twist1-binding to E-box, we introduced mutations in 2 E-boxes (E1 and E2) into CLU–Luc −1,998/−702 plasmid, and produced CLU–Luc E1MT and E2MT reporter plasmids as shown in Fig. 2A. We then examined Twist1-activated CLU transcription by using these E-box–mutated reporter plasmids. As shown in Fig. 2E, mutations in E1 and E2 reduced CLU transcription and blunted activation of CLU transcription by Twist1 overexpression. ChIP assays were then used to further confirm Twist1 binding to CLU promoter region around −1,400 bp from transcription start site represented by CLU −02. No binding was apparent in the CLU gene around +6,600 bp from transcription start site and PRL30 gene (Fig. 2F).

TGF-β induces CLU expression by enhancing Twist1 binding to CLU promoter region

To further define mechanisms by which TGF-β regulates Twist1 and CLU, we examined the Twist1 and CLU transcription activity using luciferase reporter plasmids. As shown in Fig. 3A and B, both Twist1 and CLU transcription were...
Figure 2. Twist1 regulates CLU transcription by binding to distal promoter region of CLU. A, schematic representation of the promoter region and 5' end of the CLU gene. Black boxes, E-boxes (5'-CANNTG-3'); gray box, HSF1-binding site; white box, AP-1-binding site; rail, primer pairs used in ChIP assays. CLU–Luc plasmids (−1,998/+254, −1,998−/−702, −1,116−/−702, −707−/−254, E1MT and E2MT) used in B, C, and E are also shown. B, PC-3 cells were transfected with 0.5 μg/mL of the various CLU–Luc plasmids shown in A and 0.05 μg/mL of pRL-TK. The luciferase activity of CLU–Luc −1,998/+254 was set as 1. Boxes, mean; bars, ±SD; *P < 0.05 (compared with CLU–Luc −1,998/+254). C, PC-3 cells were cotransfected with 0.5 μg/mL of the indicated expression plasmid, and 0.05 μg/mL of pRL-TK. The luciferase activity of each CLU–Luc with mock plasmid (Myc-Flag) was set as 1. Boxes, mean; bars, ±SD; *P < 0.05 (compared with mock). D, nuclear extracts from PC-3 cells were incubated with specified 5'-biotinylated oligonucleotides and input and pulled-down samples analyzed by SDS–PAGE and Western blot analysis with specific antibodies. E, PC-3 cells were cotransfected with 0.5 μg/mL of the various CLU–Luc plasmids shown in A, 0.5 μg/mL of the indicated expression plasmid, and 0.05 μg/mL of pRL-TK. The luciferase activity of each CLU–Luc with mock plasmid (Myc-Flag) was set as 1. Boxes, mean; bars, ±SD; *P < 0.05 (compared with mock). D, nuclear extracts from PC-3 cells were incubated with specified 5'-biotinylated oligonucleotides and input and pulled-down samples analyzed by SDS–PAGE and Western blot analysis with specific antibodies. E, PC-3 cells were cotransfected with 0.5 μg/mL of the various CLU–Luc plasmids shown in A, 0.5 μg/mL of the indicated expression plasmid, and 0.05 μg/mL of pRL-TK. The luciferase activity of CLU–Luc −1,998/+254 with mock plasmid (Myc-Flag) was set as 1. Boxes, mean; bars, ±SD; *P < 0.05 (compared with CLU–Luc −1,998/+254). F, ChIP assays were conducted on nuclear extracts from PC-3 cells using 2.0 μg of the indicated antibodies and 20 μL of Protein G agarose. The qRT-PCR was conducted using immunoprecipitated DNAs, soluble chromatin and specific primers pairs for the CLU and RPL30 genes. The results of immunoprecipitated samples were corrected for the results of the corresponding soluble chromatin samples. Boxes, mean; bars, ±SD; *P < 0.05 (compared with IgG).
increased by TGF-β. However, luciferase activity of CLU–Luc E1MT and E2MT was significantly reduced and less affected by TGF-β (Fig. 3C). These findings were supported by DNA pull-down assay, which suggests that Twist1 binding to wild-type E-box (E1WT and E2WT) was augmented by TGF-β (Fig. 3D). To further confirm that TGF-β induced CLU via increases in Twist1, we conducted ChIP assays after TGF-β treatment. As shown in Fig. 3E, Twist1 binding to CLU promoters (CLU−02) were enhanced after TGF-β treatment in PC-3 cells, indicating increased Twist1 binding to CLU promoter after TGF-β treatment, leading to induction of CLU transcription.

**TGF-β induces EMT via Twist1-CLU pathway**

To further evaluate the role of Twist1 in TGF-β–induced CLU and EMT, we examined effects of Twist1 silencing on TGF-β–induced CLU expression. As shown in Fig. 4A, TGF-β–induced stimulation of Twist1 as well as CLU transcript expression was potently suppressed by Twist1 silencing. In addition, luciferase reporter assays using CLU–Luc showed that Twist1 knockdown repressed TGF-β–induced CLU transcription (Fig. 4B). Twist1 knockdown also repressed TGF-β–induced increases in CLU protein levels in PC-3 cells (Fig. 4C). Moreover, Twist1, as well as CLU,
silencing suppressed the TGF-β induction of EMT markers, such as N-cadherin and fibronectin (Fig. 4C). Similarly, using in 22Rv1 prostate cancer cells, Twist1 or CLU knockdown reduced N-cadherin and fibronectin, whereas increasing E-cadherin expression confirms in another cell line that CLU silencing can suppress TGF-β induction of EMT (Fig. 4D).

**Twist1 and CLU suppression inhibits TGF-β–induced migration and invasive**

The biologic consequences of the role of Twist1 and CLU in TGF-β regulation of invasion and migration were examined using cell biology assays. Scratch assays showed that TGF-β increased cell migration, which was almost abolished by Twist1 and CLU knockdown (Fig. 5A, Supplemental Fig. S3).
Moreover, we found that Twist1 or CLU silencing significantly reduced basal and TGF-β–induced matrix metalloproteinase-9 (MMP-9) activity (Fig. 5B) as well as TGF-β–induced increases in cell invasion (Fig. 5C).

**CLU knockdown using OGX-011 suppresses prostate cancer metastasis in vivo**

On the basis of the previous findings that CLU inhibition reduced TGF-β induction of markers of EMT as well as cell invasion and migration, we next investigated the effects of CLU suppression on prostate cancer metastatic ability in vivo using the CLU inhibitor, OGX-011, which has been previously shown to suppress CLU expression in vivo in xenograft tissue (32, 33, 36), as well as in human prostate tissue (37) and sera (38). To establish a metastatic cancer model in vivo, highly metastatic PC-3M-luc cells stably expressing luciferase were injected into tail vein of male nude mice. Beginning the next day, mice were treated i.p. with 12.5 mg/kg of OGX-011 or scramble control,

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**Figure 5.** Suppression of Twist1 or CLU inhibits TGF-β–induced migration and invasion. A, PC-3 cells were transfected with 40 nmol/L of indicated siRNA. The scratch widths were measured after 48 hours of additional incubation at 0.1 ng/mL TGF-β. Boxes, mean; bars, ±SD; *, *P < 0.05 (compared with control siRNA). Representative figures of each experiment are shown in the right part. B, PC-3 cells were transfected with 40 nmol/L of the indicated siRNA. For zymography, the media was changed to serum-free media at 0.1 ng/mL TGF-β 48 hours after transfection. Twenty-four hours later, conditional media was concentrated and subjected to zymography. The MMP-9 activity when transfected with control siRNA and treated without TGF-β was set as 1. Boxes, mean; bars, ±SD; *, *P < 0.05 (compared with control siRNA). Representative figures of each experiment are shown in the right part. C, PC-3 cells were transfected with 40 nmol/L of the indicated siRNA. Invasion assay was conducted as described in Material and Methods. The invasive cell count when transfected with control siRNA and treated without TGF-β was set as 1. Boxes, mean; bars, ±SD; *, *P < 0.05 (compared with control siRNA). Representative figures of each experiment are shown in the right part.
and a bioluminescent signal was monitored every 2 weeks. A bioluminescent signal first became detectable at 6 week after tail vein injection. At 6 weeks, bioluminescent signals were lower in OGX-011–treated group compared with those in control group. Moreover, bioluminescent signals in control group increased over time, whereas those in OGX-011–treated group remained suppressed (Fig. 6A and B), suggesting that OGX-011 can inhibit prostate cancer metastasis in this preclinical study.

Discussion

Promoter sequences of CLU are conserved during evolution and include several stress-associated sites. For example, heat shock factor-1 (HSF-1) is a well-known regulator of CLU expression by binding CLE sites in CLU promoter regions (39). In addition, AP-1 and Egr-1 also regulate CLU expression (13, 40). Furthermore, we recently reported that Y-box binding protein-1 is a key transcriptional factor that induces CLU expression during endoplasmic reticulum stress (15). In this study, we report that, in addition to these stress-related transcription factors, the EMT-related transcription factor Twist1 also regulates CLU expression in response to TGF-β. While both Twist1 and CLU are known to be induced by TGF-β (24–28), any functional interaction between Twist1 and CLU in regulating TGF-β–induced EMT is undefined. This study indicates that CLU is an important downstream mediator of TGF-β and Twist1–induced EMT. To date, many studies have shown that Twist1 plays a critical role in EMT of both normal and cancer cells, mediated by EMT regulators, such as E-cadherin, N-cadherin, and fibronectin (41). Consistent with these prior reports, this study confirms that Twist1 silencing reduces TGF-β induction of N-cadherin and fibronectin along with biologic actions, such as migration and invasion in prostate cancer cells. Moreover, CLU was identified as a Twist1-induced chaperone that also regulates these EMT-related markers and phenotype in prostate cancer cells. This finding is consistent with, and adds mechanistic insight into, previous reports of CLU in EMT and cancer. For example, Chou and colleagues reported CLU involvement in EMT through extracellular signal-regulated kinase (ERK)/Slug pathway in lung cancer cells (18). Furthermore, in monocyes and macrophages, CLU was shown to regulate MMP-9 expression via ERK1/2 and PI3K/Akt/NF-kB pathways, which may contribute to the tissue reorganization by serving as a modulator for extracellular matrix degradation (42). CLU overexpression augmented metastatic potential of renal cancer cells as indicated by increased lung metastasis in a mice tail vein model (16). Similarly, CLU overexpression in hepatocellular carcinoma increased migratory and metastatic abilities in vitro and in vivo (17). recently, orthotopic primary tumors derived from CLU-overexpressing breast cancer cells grew more rapidly and metastasized more often than tumors derived from parental cells (43). Collectively, these findings link CLU overexpression to promotion of metastases in several types of cancer.

CLU has been shown to interact with the C-terminus of TGF-β receptors (44), and to regulate Smad2/3 stability (45). Because TGF-β–Smad2/3 signaling is a critical regulator of EMT, CLU may regulate EMT and metastasis through TGF-β–Smad2/3 signaling. In addition, we and others (18, 42) showed that TGF-β and CLU regulated MMP-9 activity, which also contributes to cancer invasiveness. Either dependent on, or in addition to, these mechanisms described earlier, CLU knockdown suppresses EMT-related molecules N-cadherin and fibronectin. N-cadherin plays a critical role in EMT and castration resistance of prostate cancer, and N-cadherin–specific monoclonal antibodies can suppress metastasis and castration resistance in preclinical model (46). Because CLU (9, 47), N-cadherin (46), and EMT (48) are functionally associated with progression to castration resistance, this study identifies and defines Twist1-activated CLU as a mediator of TGF-β–induced N-cadherin, EMT, and metastases in CRPC.

Recently, anti-CLU antibody therapy was shown to repress metastasis in breast cancer models (28). While the
antimetastatic activity using anti-CLU antibodies has not been directly compared withCLU knockdown using OGX-011, anti-CLU antibody therapy targets only secretory CLU, which is unlikely to affect TGF-β–Smad2/3 signaling because cytoplasmic CLU interacts with TGF-β receptors (44). While anti-N-cadherin antibody therapy is another promising approach to target EMT, metastasis, and treatment resistance, N-cadherin is more downstream than CLU. For example, CLU inhibition decreases both N-cadherin as well as fibronectin expression in addition to TGF-β signaling and MMP-9 activity. Therefore, CLU may be more attractive “node” to target because of its many clients and pleiotropic actions. Indeed, many preclinical studies report that inhibition of CLU enhances treatment-induced cancer cell death, inhibits metastases, and delays progression in prostate and other cancer models. Clinical studies reported successful inhibition of CLU in human prostate cancer tissues (37) and 7-month survival benefit in randomized trials when OGX-011 was combined with docetaxel in patients with CRPC (38, 49). OGX-011 is now in phase III trials (50) in metastatic CRPC.

In conclusion, we found that TGF-β–regulated CLU expression via Twist1 transcription factor, which induced a mesenchymal phenotype represented by increased N-cadherin and fibronectin expression in prostate cancer cells. Targeted inhibition of Twist1 or CLU reversed EMT markers, and reduced invasive and metastatic abilities in prostate cancer cells. These data support targeting CLU using OGX-011 as a rational strategy for suppressing the role of EMT in progression of metastatic CRPC.

References


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