N-cadherin Gene Expression in Prostate Carcinoma Is Modulated by Integrin-Dependent Nuclear Translocation of Twist1

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Abstract

The gain of *N-cadherin* expression in carcinomas has been shown to be important in the regulation of cell migration, invasion, and survival. Here, we show that *N-cadherin* mRNA expression in PC-3 prostate carcinoma cells is dependent on β_1 integrin–mediated cell adhesion to fibronectin and the basic helix-loop-helix transcription factor Twist1. Depletion of *Twist1* mRNA by small interfering RNA resulted in decreased expression of both Twist1 and N-cadherin and the inhibition of cell migration. Whereas *Twist1* gene expression was independent of β_1 integrin–mediated adhesion, Twist1 protein failed to accumulate in the nuclei of cells cultured in anchorage-independent conditions. The increased nuclear accumulation of Twist1 following cell attachment was suppressed by treatment with an inhibitor of Rho kinase or a β_1 integrin neutralizing antibody. The effect of Twist1 on induction of *N-cadherin* mRNA required an E-box *cis*-element located within the first intron (+2,627) of the *N-cadherin* gene. These data raise the possibility that integrin-mediated adhesion to interstitial matrix proteins during metastasis differentially regulates the nuclear/cytoplasmic translocation and DNA binding of Twist1, activating *N-cadherin* transcription. (Cancer Res 2006; 66(7): 3365-9)

Introduction

The *E-cadherin* gene is a common target for transcriptional repression in epithelial malignancies and its regulation is considered a key step in the metastasis of carcinomas, including breast, and prostate (1). With the silencing of *E-cadherin* transcription, adhesion molecules, such as N-cadherin, are induced during metastatic progression (2). Aberrant N-cadherin expression in carcinomas has been shown to mediate antiapoptotic signaling pathways (3), the sustained signaling of the fibroblast growth factor receptor (4), and is required for cell migration during transforming growth factor β1 (TGFβ1)–stimulated epithelial-to-mesenchymal transformation (5). Although the mechanisms regulating the aberrant expression of N-cadherin in carcinoma progression remain unknown, the signaling of the GTPase RhoA has been shown to be necessary for N-cadherin induction by TGFβ1 (6). Recent evidence implicates Twist1, a basic helix-loop-helix transcription factor that is up-regulated in breast and prostate carcinomas, in the regulation of *E-cadherin* gene expression and the enhanced expression of mesenchymal genes (7, 8). Twist has been shown to induce the expression of *N-cadherin* mRNA in human breast epithelial cells (7) but it is not known whether the expression of *Twist1* directly activates *N-cadherin* transcription in carcinomas.

Prostate tumor cells undergo dynamic changes in integrin adhesion structures as they invade the interstitial extracellular matrix to metastasize. Integrins are a family of heterodimeric adhesion that bind to matrix proteins and activate specific intracellular signal transduction pathways, reorganize the actin cytoskeleton, and regulate the nuclear/cytoplasmic shuttling of transcription factors and kinases, such as Snail and extracellular signal–regulated kinase (9, 10). A dynamic regulation of *E-cadherin* transcription by integrin-mediated cell adhesion has been shown through the downstream signaling of integrin-linked kinase (11). In this study, we document a β_1 integrin–mediated induction of *N-cadherin* mRNA in PC-3 cells following adhesion to fibronectin. We show that *Twist1* is necessary for *N-cadherin* transcriptional activation and that Twist1 nuclear accumulation is dependent on β_1 integrin–mediated adhesion. The regulation of *N-cadherin* expression by Twist1 is through a direct interaction with an E-box regulatory element located within the first intron of the *N-cadherin* gene.

Materials and Methods

Cell culture. PC-3 and MCF-7 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). For anchorage-independent culture, PC-3 cells were detached with 5 mmol/L EDTA in PBS and cultured for 2 to 3 days in serum-free medium at 10^5cells/cm^2 in dishes coated with poly-2-hydroxyethylmethacrylate (Sigma, St. Louis, MO). The wound closure assay was carried out as previously described (5) with minor modifications.

Immunoblotting. Nuclei were isolated by swelling cells in hypotonic buffer [10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L $MgCl_2$, 1 mmol/L EDTA] for 30 minutes on ice and shearing cells in a Dounce homogenizer. Nuclei were pelleted and lysed in Laemmli SDS sample buffer with inhibitors. Western blotting was carried out as previously described (3) with antibodies to N-cadherin, E-cadherin, and β-catenin (Transduction Laboratories, San Diego, CA). Polyclonal anti-Twist (H-81 and N-19) and anti-Lamin A were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Densitometry was analyzed using Scion Image.

***Twist1* small interfering RNA and overexpression.** Small interfering RNA (siRNA) against the *Twist1* target sequence 5′-ACUCCAAGAUGG-CAAGCUG-3′ (nucleotides 857-875; NM000474) was purchased from Dharmacon (Lafayette, CO). siRNAs were added to cells at a final concentration of 50 nmol/L using the Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA). A siRNA against the firefly luciferase target sequence 5′-CTTACGCTGAGTACTTCGA-3′ was used as a negative control. The *Twist1* expression vector contains the entire coding sequence of human *Twist1* in pcDNA3.1 (12).

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

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Analysis of *E-cadherin* and *N-cadherin* mRNA levels. Quantitative real-time PCR primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in Supplementary data. For each experimental sample, transcript levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. Transcript levels were normalized to *RPL19* transcripts and were analyzed in triplicate. Northern blot analysis was completed as previously reported (13).

Gel shift analysis. Electrophoretic mobility shift assays were done as previously described (14). Nuclear extracts (10 μg) were incubated with ^{32}P-labeled double-stranded oligonucleotides for 15 minutes at room temperature in binding buffer [25 mmol/L HEPES (pH 7.5), 3 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.5% Nonidet P40, 10% glycerol] with 1 μg of poly(dI-dC). The *N-cadherin* first intron E-box sequence (+2,619 to +2,647) oligonucleotide was 5′-GGTTAAGTGCACCATGTGGATTGTACAACT-3′ whereas the mutant sequence was 5′-GGTTAAGTGCACTTTGTGGATTGTACAACT-3′. All unlabeled competitor oligonucleotides were added before incubation with labeled oligonucleotides whereas Twist1 antibodies were added 15 minutes after the labeled oligonucleotide. The *Twist1* coding sequence was subcloned in-frame into pGEX2T and the expressed protein was affinity purified. DNA protein complexes were resolved on a 5% nondenaturing polyacrylamide gel.

***N-cadherin* promoter luciferase reporter assays.** *N-cadherin* promoter activity was determined using constructs encoding the human *N-cadherin* 5′ promoter (−860 to +20) in pGL3basic (Promega, Madison, WI), creating NP-860pGL3, or the 5′ promoter (−860 to +20) and a region of the first intron from the human *N-cadherin* gene (+373 to +2,822 bp) downstream of luciferase in pGL3 (Supplementary data). Cells were transiently transfected in triplicate using the FuGENE 6 (Roche, Indianapolis, IN). The Dual Luciferase Kit (Promega) was used in luciferase assays according to the protocol of the manufacturer.

Results and Discussion

Reciprocal regulation of *E-cadherin* and *N-cadherin*. We used the prostate adenocarcinoma cell line PC-3, which expresses both E-cadherin and N-cadherin (3), to investigate the molecular events that contribute to the induction of N-cadherin. To examine whether the relative cadherin gene expression was reversible, total RNA was isolated from cells cultured in suspension as multicellular carcinoids, or from single cells attached to fibronectin. *E-cadherin* mRNA was high in suspended cells and decreased when the cells attached to a fibronectin substrate (Fig. 1*A*). In contrast, *N-cadherin* mRNA was 4-fold lower when cells were cultured in suspension as compared with attached cells. The protein levels of E-cadherin and N-cadherin in cells cultured in both conditions recapitulated the cadherin transcript levels (Fig. 1*B*). These results suggest matrix-dependent cell attachment and spreading are necessary for the dynamic regulation of *E-cadherin* and *N-cadherin* gene expression.

When suspended PC-3 carcinoids (Fig. 1*C* and *D*) were dissociated and replated onto a fibronectin substrate, the induction of *N-cadherin* mRNA level is 2-fold at 6 hours and maximal at 12 hours (Fig. 1*E*). β_1 integrin colocalization with F-actin was a rapid event, visible at 30 minutes following attachment and spreading on fibronectin (Fig. 1*F*). Activation of *N-cadherin* gene expression was dependent on β_1 integrin–mediated cell adhesion, as preincubation with a β_1 integrin function-blocking antibody, AIIB2 (15), before adhesion abrogated the increase in *N-cadherin* mRNA (Fig. 1*E*). The reexpression of β_1 integrin in β_1 integrin–null epithelial cells was shown to modulate cadherin gene expression and induce an epithelial-to-mesenchymal transformation (16). Thus, β_1 integrin–mediated signaling following cell attachment and spreading may be a mechanism regulating *N-cadherin* mRNA expression in epithelial tumors.

***Twist1* loss affects *N-cadherin* expression.** We next examined whether Twist1 was necessary for N-cadherin expression in PC-3 cells attached to fibronectin. Depletion of *Twist1* mRNA with siRNA resulted in decreased expression of both *Twist1* and *N-cadherin* transcripts (Fig. 2*A*) and protein levels (Fig. 2*B*). Transfection of *Twist1* siRNAs reduced the *Twist1* mRNA levels to ~60% and *N-cadherin* transcript levels to 50%. Twist1 protein levels were reproducibly reduced nearly 90% in each experiment and N-cadherin levels were 50% of the control samples. In contrast,

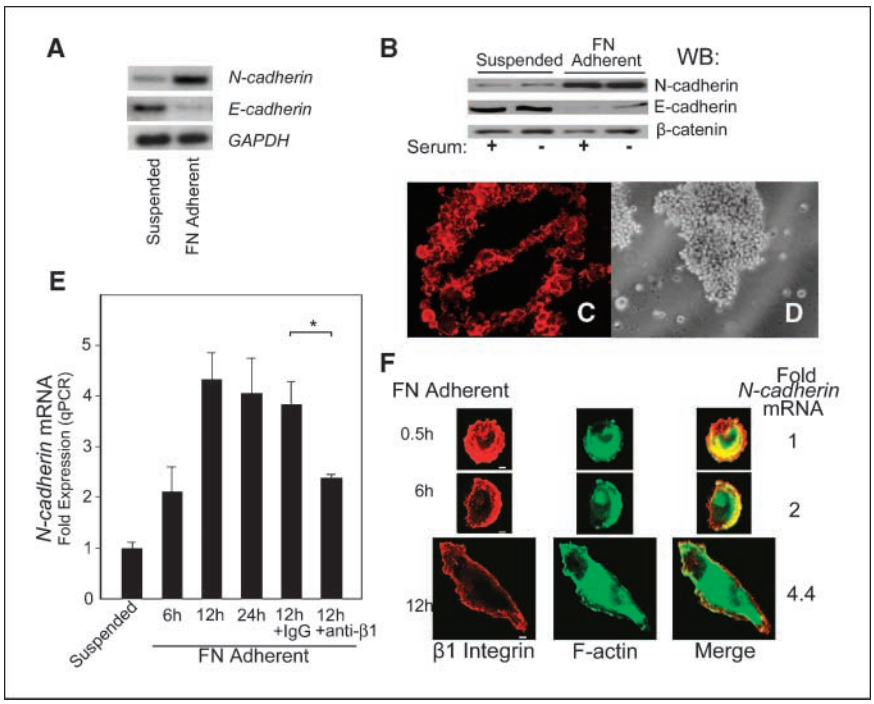

Figure 1. Dynamic regulation of *E-cadherin* and *N-cadherin* by cell adhesion to fibronectin. *A,* Northern blot analysis from total RNA for *E-cadherin, N-cadherin*, and *GAPDH* from PC-3 cells grown in suspension for 2 days or cells adherent to fibronectin for 24 hours (10^4 cells/cm^2). *B,* Western blot analysis of E-cadherin, N-cadherin, and β-catenin from cells with or without the addition of serum. *C,* β-catenin immunofluorescence in a cross section of a multicellular carcinoid. *D,* phase photomicrograph of a multicellular carcinoid. *E,* time course of induction of *N-cadherin* transcripts following adherence to fibronectin by quantitative real-time PCR analysis. Nonimmune mouse immunoglobulin G (IgG) or the function-blocking β_1 integrin antibody (AIIB2) was added at 5 μg/mL before adherence. *Columns,* mean fold increase over *N-cadherin* expression in suspended cells; *bars,* SD. *, $P < 0.05$. *F,* immunofluorescence of β_1 integrin and F-actin in adherent cells at the indicated times following attachment.

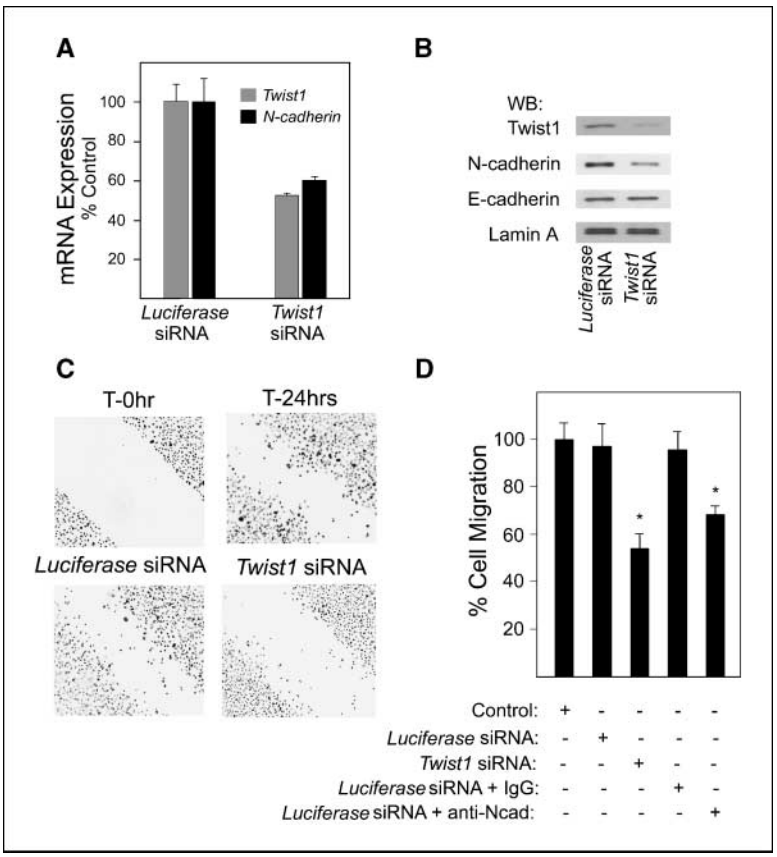

Figure 2. *N-cadherin* transcript levels and cell migration are dependent on *Twist1* gene expression. *A,* quantitative real-time PCR analysis of *N-cadherin* and *Twist1* mRNA levels following 24-hour treatment of PC-3 cells plated on fibronectin (10 μg/mL) with siRNA against *Twist1* or a control *luciferase* siRNA. *B,* Western blot analysis of N-cadherin, E-cadherin, and TWIST1 protein levels following siRNA treatment of cells plated on fibronectin. *C,* representative images of 4′,6-diamidino-2-phenylindole–stained nuclei of *luciferase* siRNA– and *Twist1* siRNA–treated cells following wound closure migration assay on fibronectin immediately following wounding (*T-0hr*) and 24 hours following wounding (*T-24hrs*). *D,* quantification of cell migration in wound closure assay for 24 hours. In some experiments, an irrelevant IgG or a function-blocking anti-N-cadherin monoclonal antibody (GC4) was added. Percent cell migration into wound space where the migration of control cells has been set at 100%; *columns,* mean of three independent experiments. *, $P < 0.05$.

treatment with a control *luciferase* siRNA did not alter levels of Twist1 or N-cadherin. These findings, together with previous studies, suggest that Twist1 is necessary for the expression of *N-cadherin* in carcinoma cells (7).

Twist1 has been shown to control the migration of cancer cell lines through its ability to regulate mesenchymal gene expression (7, 8). We were interested in whether Twist1 regulation of *N-cadherin* expression contributed to cell motility in wound closure assays. As shown in Fig. 2*C*, control PC-3 cells or cells treated with *luciferase* siRNA efficiently migrate into the wound. In contrast, cells treated with *Twist1* siRNA have reduced migration into the wound, and cell migration is decreased by 50% (Fig. 2*C* and *D*). N-cadherin has been shown to enhance cell migration during epithelial-to-mesenchymal transformation (5). To determine the contribution of N-cadherin to PC-3 cell migration, an N-cadherin adhesion-blocking monoclonal antibody was added to cells following wounding. This resulted in a statistically significant 30% decrease in cell migration. These data are in agreement with previous studies of Twist1 expression and function in metastatic cancer cells (7, 8) and suggest that regulation of cell migration by Twist1 is, in part, through its role in induction of *N-cadherin* gene expression.

Twist1 nuclear accumulation is integrin-mediated. Because inhibition of β_1 integrin engagement decreased *N-cadherin* transcript levels, we determined whether changes in *Twist1* transcript levels in anchorage-independent cells could account for the decrease in *N-cadherin* mRNA. Analysis of *Twist1* mRNA levels showed no change in the transcript level in suspended cells or in adherent cells whereas *N-cadherin* expression was up-regulated in adherent cells (Fig. 3*A*). In comparison, the zinc finger transcriptional repressor *Slug* showed increased mRNA expression in adherent cells, which is consistent with its role in silencing *E-cadherin* gene expression in attached cells (17). We next examined whether Twist1 was present in the nuclei of anchorage-independent cells by subcellular fractionation. Although the level of *Twist1* mRNA did not change in suspended or adherent cells, Twist1 protein was absent from the nucleus in cells cultured in suspension (Fig. 3*B*). When suspended cells were replated onto a fibronectin substrate, there was a rapid accumulation of Twist1 into the nucleus, which was maximal at 30 minutes. This translocation was inhibited by addition of the β_1 integrin neutralizing antibody, AIIB2 (Fig. 3*B* and *C*). To examine the adhesion-dependent signaling pathways downstream of the β_1 integrin that regulate Twist1 nuclear accumulation, cells were pretreated with inhibitors before cell attachment to fibronectin. An inhibitor of Rho-associated kinase, Y-27632, and the actin-destabilizing drugs latrunculin B and jasplakinolide inhibited the nuclear accumulation of Twist1 (Fig. 3*C*). Consistent with the inhibition of Twist1 nuclear accumulation by Y-27632, latrunculin B, and jasplakinolide, the levels of N-cadherin were also reduced in attached and spread cells (Fig. 3*D*). Together these results suggest that the regulation of *N-cadherin* transcript levels by cell adhesion is due to β_1 integrin–mediated adhesion through increased nuclear Twist1 accumulation. β_1 integrin activation of Rho-associated kinase and actin dynamics link the extracellular microenvironment with nuclear signaling. Twist1 may therefore be a transcriptional activator modulating activity downstream of β_1 integrin during invasion and metastasis.

Twist1 controls *N-cadherin* promoter activity. To determine whether the induction of *N-cadherin* mRNA by Twist1 in PC-3 cells

involves direct binding of Twist1 to a *cis*-element in the promoter of *N-cadherin*, we generated a luciferase reporter construct containing the −860 to +20 bp region of the human *N–cadherin* 5′ proximal promoter (Fig. 4*A*). This includes the previously described minimal basal core promoter region −335 to −18 bp (18). Cotransfection of the 5′ *N-cadherin* promoter construct NP860 (+860 to −20) and *Twist1* showed no increase in promoter activity in PC-3 cells nor in the *N-cadherin*- and *Twist1*-negative breast cancer cell line MCF-7 (Fig. 4*B*). This suggests that Twist1 has little direct or indirect effect on activity of the *N-cadherin* 5′ proximal promoter. Of the two documented *Drosophila* Twist1 E-box *cis*-element sequences (CATATG or CATGTG; ref. 14), we found a potential E-box sequence CATGTG located within the first intron of the human *N-cadherin* gene at +2,627 to +2,632 bp. Incorporation of the +373 to +2,822 region of the *N-cadherin* first intron downstream of luciferase in the 5′ *N-cadherin* promoter reporter construct resulted in Twist1-mediated increases in *N-cadherin* promoter activity (Fig. 4*B*). Mutagenesis of the E-box sequence at +2,627 eliminated the ability of Twist1 to induce *N-cadherin* promoter activity.

Electrophoretic mobility shift assays were then done to determine the ability of Twist1 to bind the putative Twist1 E-box element in the *N-cadherin* first intron. Nuclear extracts from PC-3 cells were used to determine whether endogenously expressed Twist1 could associate with the *N-cadherin* first intron E-box sequence. Three DNA-protein complexes (Fig. 4*C, lane 2, C1, C2*, and *C3*) were formed when nuclear extracts from adherent cells were incubated with labeled *N-cadherin* E-box oligonucleotides. Addition of excess unlabeled wild-type E-box oligonucleotides efficiently competed for DNA-protein complex formation whereas excess mutant oligonucleotides (TTTGTG) did not inhibit complex formation (*lanes 3* and *4*, respectively). To define Twist1 as the nuclear protein associating with the putative Twist1 E-box sequence, Twist1 antibody was added to the binding reactions which resulted in a supershifted DNA-protein band (*lane 5*). As expected, nuclear extracts from PC-3 cells in suspension failed to bind to the Twist1 E-box oligonucleotides (*lane 6*). A glutathione *S*-transferase (GST)-Twist1 fusion protein formed a distinct DNA-protein complex when incubated with radiolabeled *N-cadherin* E-box oligonucleotides (Fig. 4*C, lane 8*). This DNA-protein complex was eliminated when an excess of unlabeled wild-type oligonucleotides was added to binding reactions (Fig. 4*C, lane 9*). In contrast, addition of an excess of mutant oligonucleotides (TTTGTG) did not inhibit complex formation (Fig. 4*C, lane 10*), suggesting that Twist1 binds to the E-box sequence at +2,627 bp in the *N-cadherin* first intron. The tissue-specific regulation of *E-cadherin* gene expression was shown to involve both the first and second introns *in vivo* (19). Whereas the sequences of the first and second introns of the *E-cadherin* and *N-cadherin* genes are quite dissimilar, it seems that the intronic regulation of gene expression has been conserved between these two type I cadherin genes.

Concurrent with the loss of *E-cadherin* mRNA expression, the gain of *N-cadherin* expression in epithelial malignancies has been shown to be important in the regulation of cell migration, invasion, and survival. We provide evidence of transcriptional regulation of *N-cadherin* mRNA expression in epithelial cancer cells through an E-box sequence located within the first intron of the *N-cadherin* gene. In gastric carcinoma progression, a correlation was also described between *N-cadherin* and *Twist1* mRNA expression (20). Twist1 immunolocalization correlates with an increased Gleason score in prostate cancer specimens and displays both nuclear and cytoplasmic accumulation (8). Our work documents the β_1 integrin–mediated control of Twist1 nuclear accumulation and

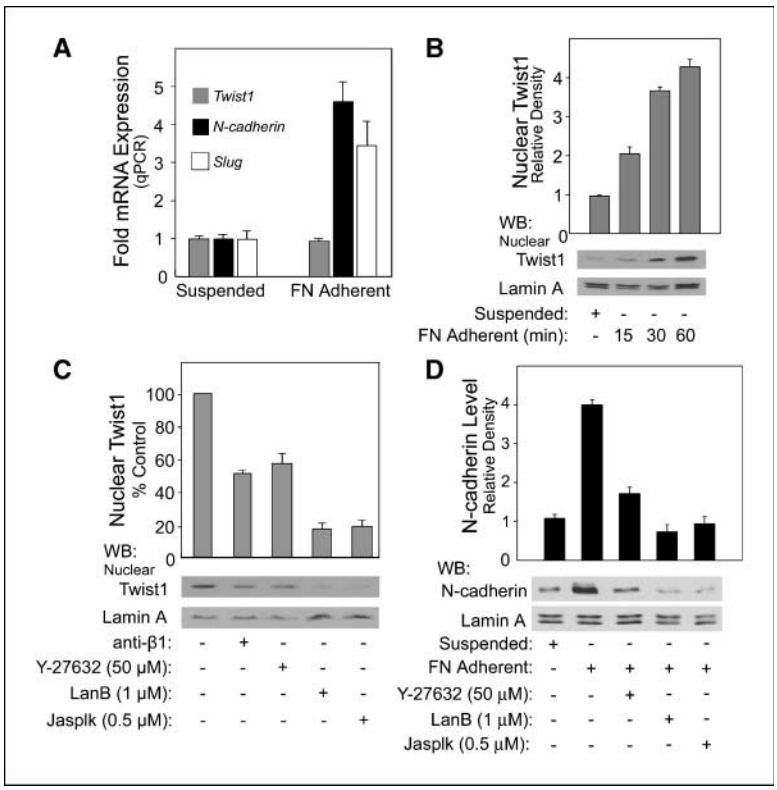

Figure 3. Cell adhesion to fibronectin regulates Twist1 nuclear accumulation. *A,* quantitative real-time PCR analysis of *Twist1, N-cadherin*, and *Slug* mRNA in adherent and nonadherent PC-3 cells. *B,* Western blot analysis of Twist1 nuclear accumulation following adherence of dissociated PC-3 carcinoids to fibronectin. *C,* Western blot analysis of Twist1 nuclear accumulation following adherence of dissociated PC-3 carcinoids to fibronectin for 30 minutes following preincubation with β_1 integrin function-blocking antibody AIIB2 (5 μg/mL) and the inhibitors Y-27632 (50 μmol/L), latrunculin B (1 μmol/L), and jasplakinolide (0.5 μmol/L). *D,* N-cadherin expression following adherence of dissociated carcinoids to fibronectin for 24 hours and treatment with Y-27632 (50 μmol/L), latrunculin B (1 μmol/L), and jasplakinolide (0.5 μmol/L). *Columns,* mean relative densities of protein bands from three independent experiments; *bars,* SD.

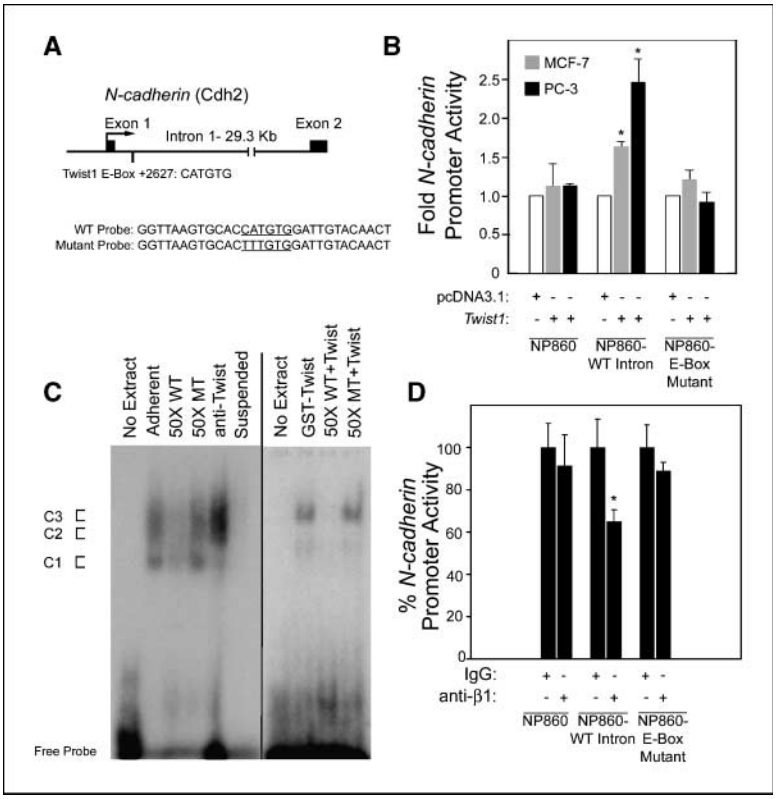

Figure 4. Twist1 induces *N-cadherin* transcriptional activation through binding to an E-box regulatory element within the *N-cadherin* gene. *A,* schematic showing the potential E-box *cis*-element in intron 1 of the *N-cadherin* 5′ promoter region. The ATG is designated as +1 (18). *B, N-cadherin* promoter activity in MCF-7 and PC-3 cells cotransfected with empty vector (pcDNA3.1) or human *Twist1*. Luciferase reporter constructs carrying the human *N-cadherin* promoter sequences: 5′ proximal promoter (*NP860*), 5′ proximal promoter with a wild-type region of the first intron of *N-cadherin* (*NP860-WT Intron*), or 5′ proximal promoter and the segment of the *N-cadherin* first intron with a mutated E-box sequence (*NP860-E-box Mutant*). Luciferase activity was normalized to the activity of the pTK*Renilla* control and represented as fold over control (pcDNA3.1) for each cell line. *, $P < 0.005$, MCF-7 and PC-3, respectively. *C,* electrophoretic mobility shift assay analysis with the *N-cadherin* first intron E-box oligonucleotides (*WT*) and nuclear extracts from adherent cells (*lanes 2-5*) or suspended cells (*lane 6*) and GST-Twist (*lanes 8-10*). *Lanes 1* and *7,* probe only; *lane 2,* adherent cell nuclear extract; *lane 3,* 50× WT competitor; *lane 4,* 50× mutant (MT) competitor (CATGTG to TTTGTG); *lane 5,* supershift with Twist antibody; *lane 6,* suspended PC-3 cell nuclear extract; *lane 8,* GST-Twist1 (100 ng); *lane 9,* 50× WT competitor + GST-Twist1; *lane 10,* 50× MT + GST-Twist1. *D,* β_1 integrin–mediated adhesion regulates *N-cadherin* promoter activation. Cells transiently transfected with *N-cadherin* luciferase constructs were treated with irrelevant IgG or β_1 integrin function-blocking monoclonal antibody (AIIB2). *Columns,* percent control where IgG was set as 100%.

the transcriptional induction of *N-cadherin mRNA*. Moreover, blocking β_1 integrin cell adhesion was found to inhibit *N-cadherin* promoter activity when the Twist1 E-box *cis*-element is present (Fig. 4*D*). Together these data indicate that Twist1 is a pivotal transcription factor that regulates the gene expression of *N-cadherin* during cancer metastasis through multiple mechanisms, including the direct transcriptional regulation of *N-cadherin* and the regulation of cell migration.

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