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Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin

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Loss of E-cadherin expression or function in tumors leads to a more invasive phenotype. In this study, we investigated whether the invasion suppressor activity of E-cadherin is mediated directly by tighter physical cell adhesion, indirectly by sequestering β-catenin and thus antagonizing β-catenin/T cell factor (TCF) signaling, or by other signaling pathways. To distinguish mechanisms, we expressed wild-type E-cadherin and various E-cadherin mutants in invasive E-cadherin–negative human breast (MDA-MB-231) and prostate (TSU-Pr1) epithelial carcinoma cell lines using a tetracycline-inducible system. Our data confirm that E-cadherin inhibits human mammary and prostate tumor cell invasion. We find that adhesion is neither necessary nor sufficient for suppressing cancer invasion. Rather, the invasion suppressor signal is mediated through the β-catenin–binding domain of the E-cadherin cytoplasmic tail but not through the $p120^{ctn}$-binding domain. β-catenin depletion also results in invasion suppression. However, alteration in the β-catenin/TCF transcriptional regulation of target genes is not required for the invasion suppressor activity of E-cadherin, suggesting the involvement of other β-catenin–binding proteins.

Introduction

A disturbance in epithelial cell adhesion, which leads to a more invasive and metastatic phenotype, is a hallmark of tumor progression. E-cadherin, which has a widely acknowledged role in cell–cell adhesion, also functions as an invasion/tumor suppressor protein. Several immunohistochemical studies have reported a strong correlation between E-cadherin loss and the initiation and progression of tumors. This down-regulation is generally due to transcriptional repression. Somatic E-cadherin mutations have also been observed in a variety of human epithelial cancers (Berx et al., 1998). A direct role for E-cadherin in the suppression of tumor invasion has been demonstrated by the reversion of undifferentiated, invasive tumors to a differentiated phenotype after the transfection of E-cadherin cDNA in cell culture models (Vleminckx et al., 1991; Takeichi 1993). On the contrary, abrogation of E-cadherin–mediated cell adhesion in the transfected cells by function-perturbing antibodies and antisense RNA restores invasion (Behrens et al., 1989; Frixen et al., 1991; Vleminckx et al., 1991; Takeichi 1993). In a study using a transgenic mouse model of pancreatic β tumorigenesis, it has been demonstrated that E-cadherin–mediated cell adhesion is crucial in preventing the progression from well-differentiated adenoma to invasive carcinoma (Perl et al., 1998). These data emphasize the importance of E-cadherin in inhibiting tumor invasion and metastasis, which occur at later stages of tumor development.

The cadherin-associated protein β-catenin also has the potential to regulate cell motility or invasion. Although β-catenin was originally identified as an integral component of the cadherin adhesion protein complex, it is also an essential intracellular mediator for the Wnt growth factor signal transduction pathway through its interaction with the leukocyte enhancer factor (LEF)*/T cell factor (TCF) family to regulate transcription of target genes (for review see Peifer and Polakis 2000). The matrix metalloproteinase matrilysin and fibronectin have been identified as target genes of the β-catenin/TCF signaling pathway (Crawford et al., 1999; Gradl et al., 1999), and β-catenin could regulate invasion through these or other invasion-related genes.

Although the role of E-cadherin in the regulation of tumor invasion is well established, the exact mechanism of its invasion suppressor activity is less well defined. One possible mechanism is that the adhesive function of E-cadherin simply prevents cells from dissociating from one another and migrating into

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Key words: E-cadherin; b-catenin; invasion; adhesion; signaling

*Abbreviations used in this paper: LEF, leukocyte enhancer factor; NS, nonspecific; rtTA, reverse tetracycline-responsive transcriptional activator; siRNA, small interfering RNA; TCF, T cell factor.

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Table I. **Analysis of E-cadherin expression and in vitro behaviors of different human epithelial cancers**

Cancer cell lines	Type of tumor[a]	E-cadherin expression[b]	Matrigel invasion[c]	Soft agar growth[d]
MDA-MB-231	Breast	No	+++++	−
MDA-MB-435S	Breast	No	+	+++++
SKBr3	Breast	No	+/−	+++
Hs578T	Breast	No	+	−
TSU-Pr1	Prostate	No	++++++	+
PC-3	Prostate	Yes (lack α-cat)	+/−	+/−
MIA-PaCa-2	Pancreatic	No	+	+++++
AGS	Gastric	No	+/−	++++++
RF-1	Gastric	No	+/−	++++
SW480	Colon	Yes (APC mutation)	−	++++++

[a]The cell lines were from human carcinomas as described in American Type Culture Collection.
[b]E-cadherin expression was determined by Western blot analysis.
[c]In vitro invasion assay on Matrigel filters was performed as described in Materials and methods.
[d]Anchorage-independent assay on soft agar.

adjacent tissue. In favor of this hypothesis, it has been shown that adhesion-blocking E-cadherin antibodies increase the invasive behavior of cells (Behrens et al., 1989; Frixen et al., 1991). Alternatively, the interaction between E-cadherin and β-catenin at the adherens junction raises the intriguing possibility that the loss of E-cadherin function may actively regulate the levels of free β-catenin, thereby altering its ability to regulate target genes that support tumor invasion. This hypothesis is supported by previous findings demonstrating that cadherin expression strongly antagonizes β-catenin signaling in *Drosophila* and *Xenopus* embryos by binding and sequestering it in cadherin–catenin complexes at the plasma membrane (Heasman et al., 1994; Funayama et al., 1995; Fagotto et al., 1996; Sanson et al., 1996). It is also possible that E-cadherin could control invasion properties by altering cytoskeletal and junctional organization. One possible mechanism could be through $p120^{ctn}$ via its activity on Rho GTPases (Noren et al., 2000; Grosheva et al., 2001). $p120^{ctn}$ also enters the nucleus and interacts with the putative transcription factor Kaiso (Daniel and Reynolds 1999), and although its function is not yet known, it could potentially mediate effects of cadherins. E-cadherin could also control invasion by facilitating juxtacrine signaling via other receptor systems, since E-cadherin is fundamental for the establishment and maintenance of numerous other kinds of cell–cell interactions, for example, gap junctions, tight junctions, and juxtacrine ligand–receptor interactions. Thus, there are multiple ways that a reduction in E-cadherin expression could lead to enhanced tumor cell invasion.

E-cadherin expression was found to suppress the rate of tumor cell growth by inhibiting β-catenin/TCF nuclear signaling in the noninvasive human SW480 colorectal tumor cell line (Gottardi et al., 2001). To explore the invasion suppressor activity of E-cadherin, we express wild-type E-cadherin and various E-cadherin chimeras in invasive E-cadherin–negative human breast and prostate epithelial carcinoma cell lines using a tetracycline-inducible system.

Results

Inducible expression of E-cadherin in human cancer cell lines

We first screened for human tumor cell lines derived from various tissues that express little or no endogenous E-cad-

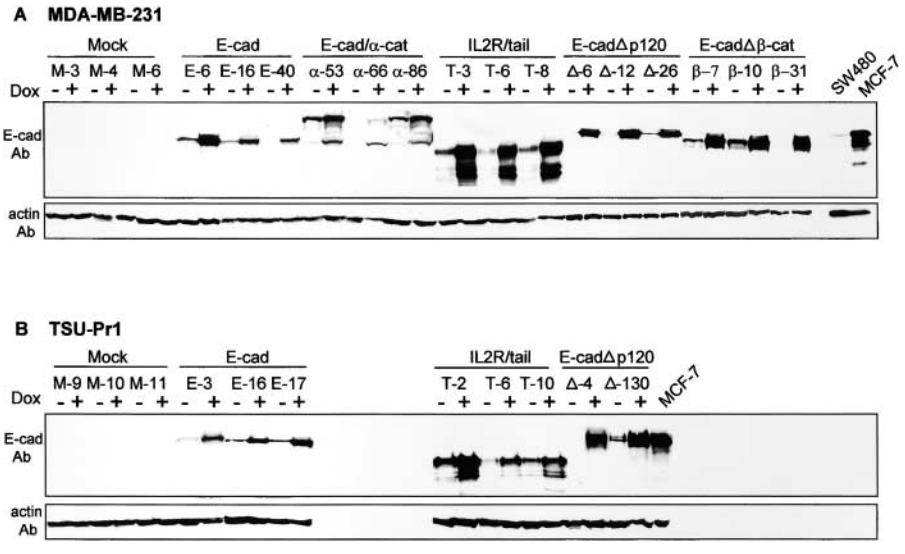

Figure 1. **A tet-on inducible E-cadherin expression system.** (A) MDA-MB-231 and (B) TSU-Pr1 cells. Equivalent micrograms of lysates from stable clones expressing the empty vector control, E-cadherin, E-cadherin–α-catenin, IL2R–cytoplasmic tail, E-cadherinΔp120, and E-cadherinΔβ-catenin; each untreated (−) or treated (+) with 1 μg/ml doxycycline were analyzed by Western blot using a mouse anti–human E-cadherin mAb (Transduction Laboratory). The blot was reprobed with an antiactin antibody as a loading control.

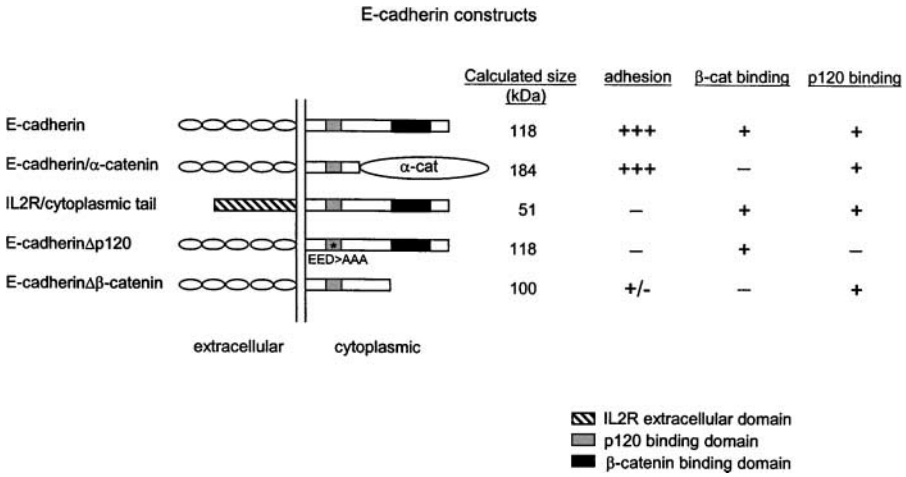

Figure 2. **Schematic diagram of E-cadherin constructs used in this study (adopted from Gottardi et al. [2001] with some modifications).** Wild-type E-cadherin is shown at the top. The E-cadherin–α-catenin fusion chimera was designed to mediate adhesion without interacting with β-catenin. Conversely, the IL2R–cytoplasmic tail chimera binds β-catenin but is defective in adhesion. The E-cadherinΔp120 mutant contains point mutations (EED > AAA) that abolish $p120^{ctn}$ binding to E-cadherin and abolish adhesion. The E-cadherinΔβ-catenin mutant has the β-catenin–binding region truncated, it lacks the ability to bind β-catenin, and exhibits little or no adhesive activity.

herin and have an invasive phenotype (Table I). The MDA-MB-231 breast cancer line and TSU-Pr1 prostate cancer line, which lack E-cadherin and are very invasive as measured by an in vitro Matrigel invasion assay (Table I), were selected. Because high levels of E-cadherin are sometimes inhibitory to cell growth (St. Croix et al., 1998; Sasaki et al., 2000; Gottardi et al., 2001; Stockinger et al., 2001), we chose to restore E-cadherin expression in a tetracycline-inducible (tet-on) expression system. This allowed us to select and grow the clones with no or minimal levels of E-cadherin expression.

We first expressed wild-type E-cadherin in MDA-MB-231 (Fig. 1 A) and TSU-Pr1 (Fig. 1 B) cell lines and obtained at least three independent clones for each construct. Parental and empty vector controls did not express E-cadherin (Fig. 1). Transfected clones expressed high levels of wild-type E-cadherin after doxycycline induction but had only a minimal basal level of expression as determined by Western blot analysis with an anti–E-cadherin antibody (Fig. 1). It is important to note that levels of E-cadherin in these stable cell lines were less than those detected in E-cadherin–expressing human epithelial cell lines, including MCF-7, indicating that expression was within normal levels. Fluorescence microscopy revealed that the E-cadherin protein was localized to areas of cell to cell contacts (see Fig. 9).

We then expressed other mutant E-cadherin constructs (e.g., the E-cadherin–α-catenin fusion, the IL2R–E-cadherin cytoplasmic tail chimera, the E-cadherinΔp120 mutant, and the E-cadherinΔβ-catenin) (Fig. 2) in MDA-MB-231 (Fig. 1 A) and TSU-Pr1 (Fig. 1 B) cells and obtained multiple clones for each construct. Western blot analysis revealed high levels of expression of these constructs after doxycycline induction and little or no expression when unin-

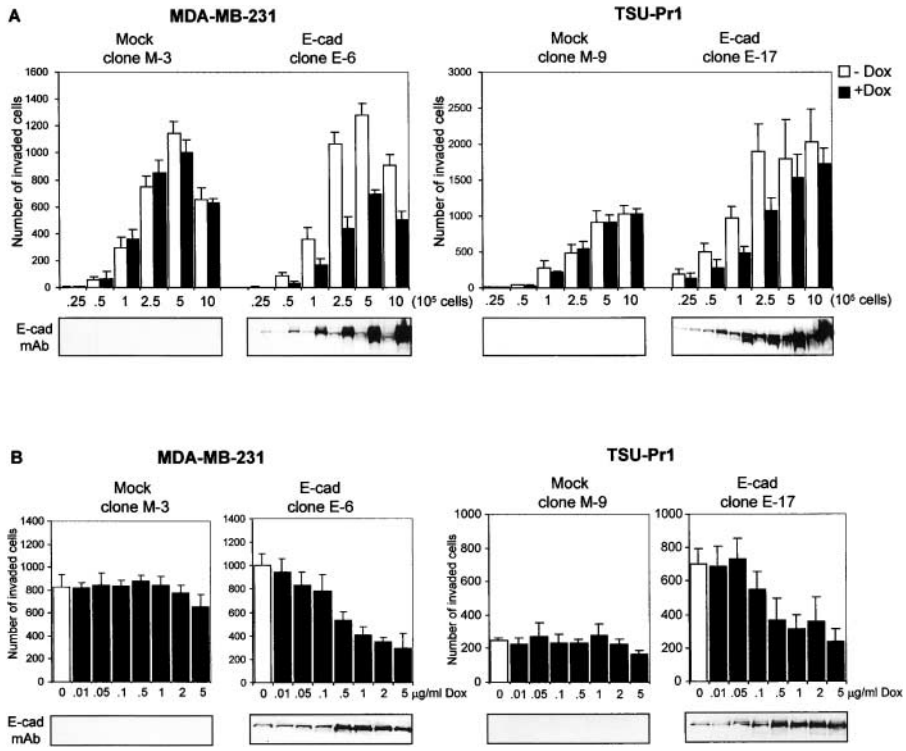

Figure 3. **Inhibition of invasion by E-cadherin induction.** In vitro Matrigel invasion of tetracycline-regulated MDA-MB-231 and TSU-Pr1 cells was determined as a function of increasing cell numbers and concentration of doxycycline. (A) An increasing number of cells was added to the top compartment of Matrigel filters in the absence (white bars) or presence (black bars) of 1 μg/ml doxycycline. (B) 2×10^5 MDA-MB-231 cells and 10^5 TUS-Pr1 cells were added onto Matrigel filters in the presence of varying amount of doxycycline (indicated). Values shown are the mean number of cells counted in four fields for replicate transwells from one experiment. Bars represent SD.

duced (Fig. 1). Although we did not obtain stable transfection of TSU-Pr1 cells expressing the E-cadherin–α-catenin chimera and the E-cadherinΔβ-catenin mutant, we were able to analyze the effect of these constructs and several others in a transient transfection and invasion assay system (see Fig. 4).

Tetracycline-regulated E-cadherin expression suppresses the invasive phenotype

To investigate the invasion suppression effect of E-cadherin, we performed an in vitro invasion assay on Matrigel filters, which is hypothesized to mimic the three-step process of invasion; adhesion, proteolytic dissolution of the extracellular matrix, and migration (Albini, 1998). The number of cells that transverse the filters reached a maximum at 5 to 10 × 10^5 cells; so we chose to use 2 and 1 × 10^5 cells for MDA-MB-231 and TSU-Pr1, respectively, in all future invasion assays (Fig. 3 A).

Induction of E-cadherin expression caused a significant reduction in invasive activity for both cell lines compared with their uninduced controls without doxycycline (Fig. 3 A). Suppression occurred over the entire range of cell densities, suggesting the invasion suppressor activity of E-cadherin is not strongly dependent on the density of cell contacts (Fig. 3 A). Parental and empty vector controls exhibited no difference in invasive activity in the absence or presence of doxycycline, indicating that transfection and expression of the tetracycline regulator had no significant effects on the behavior of these cells (Fig. 3 A). The data also suggest that the low levels of E-cadherin in the uninduced state were not sufficient to alter invasiveness. In general, there is no consistent difference in the invasiveness of E-cadherin–transfected cell lines in the uninduced state (Fig. 4 A, E-cad) compared with mock transfectants (Fig. 4 A, Mock) for both cell lines.

E-cadherin expression resulted in a dose-dependent decrease in tumor invasion (Fig. 3 B). At concentrations (0.1–2 μg/ml of doxycycline) over a range of increasing E-cadherin expression, there was a corresponding decrease in invasiveness, and after the maximum level of E-cadherin was at-

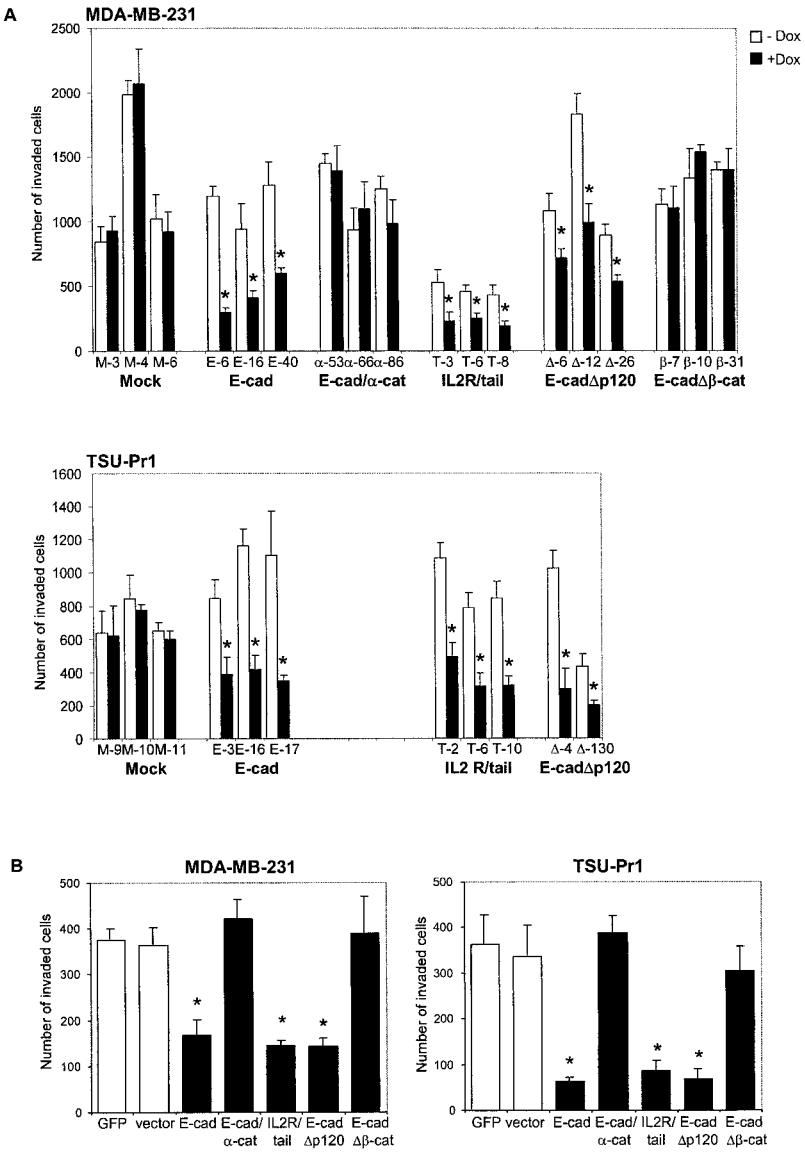

Figure 4. **Inhibition of invasion due to expression of the E-cadherin cytoplasmic tail but not increased cell adhesion.** (A) Induction of construct expression in stable lines by tet-on system. Stable clones expressing the empty vector control, E-cadherin, E-cadherin–α-catenin, IL2R–cytoplasmic tail, E-cadherinΔp120, or E-cadherinΔβ-catenin were added to the top compartment of Matrigel filters in the absence (white bars) or presence (black bars) of 1 μg/ml doxycycline. (B) Cells were transiently transfected with constructs along with GFP vector to sort expressing cells by flow cytometry. 10^5 sorted cells were then employed for in vitro Matrigel invasion assays. Values shown are the mean number of cells counted in four fields for replicate transwells from one experiment. Bars represent SD. *$P < 0.05$, which is considered statistically different from uninduced (white bars) cells.

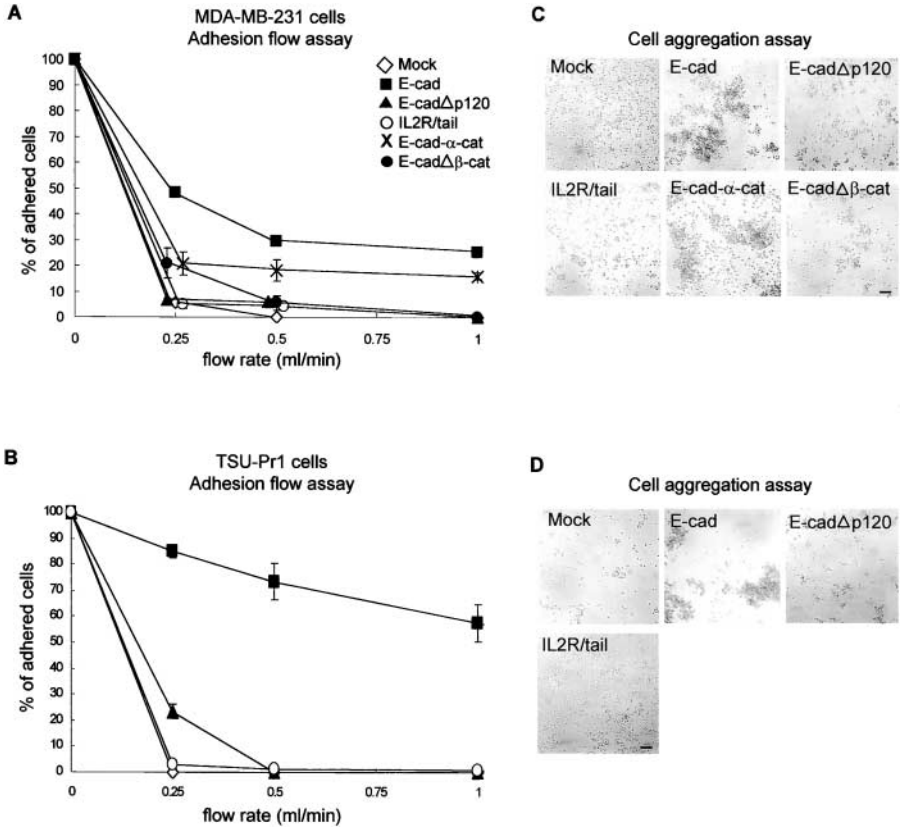

Figure 5. **Adhesive properties of cadherin-expressing cell lines.** (A and B) Use of a laminar flow adhesion assay and (C and D) use of a cell aggregation assay. (A) MDA-MB-231 and (B) TSU-Pr1 cells expressing E-cadherin and mutant E-cadherin constructs. Micrographs of (C) MDA-MB-231 and (D) TSU-Pr1 cells expressing various constructs in aggregation assays. All cadherin cell lines were induced with 1 μg/ml doxycycline to express a transgene, and the mock control cells were treated with the same level of doxycycline. Bar, 20 μm.

tained at 2 μg/ml of doxycycline, there was no further effect on invasion (Fig. 3 B). Therefore, we selected 1 μg/ml of doxycycline for all future experiments. Together, these data confirm that expression of E-cadherin at near normal level can function as an invasion suppressor in the cases of human breast (MDA-MB-231) and prostate (TSU-Pr1) carcinoma cell lines.

Invasion suppressor activity is independent of adhesion and maps to the cytoplasmic domain of E-cadherin

We asked whether the invasion suppressor activity of E-cadherin results from increases in physical cell adhesion or from antagonism of β-catenin nuclear signaling because both of these factors have been strongly implicated in tumorigenesis. Additional mechanisms are also possible, such as the p120-cadherin interaction, which has been implicated in cell motility, or other potential signal pathways. To address these possibilities, various mutant E-cadherin constructs were expressed in cells (Fig. 2). The cytoplasmic domain of E-cadherin fused to the extracellular domain of the interleukin-2 receptor α subunit (IL2R–E-cadherin cytoplasmic tail chimera), binds β-catenin and $p120^{ctn}$ and potentially other signaling molecules but is defective in adhesion (Gottardi et al., 2001). The E-cadherinΔ$p120^{ctn}$ construct has an EED to AAA substitution and is incapable of binding $p120^{ctn}$, and although it still interacts with other catenins, it is defective in cell adhesion (Thoreson et al., 2000; unpublished data; Fig. 5). E-cadherin fused directly to α-catenin (E-cadherin–α-catenin fusion chimera) lacks the β-catenin–binding region but still mediates strong homophilic adhesive activity and contains the juxtamembrane region for $p120^{ctn}$ binding. The E-cadherinΔβ-catenin mutant lacks the ability to bind β-catenin, and it has been found to exhibit little or no adhesive activity in some cell systems (Stappert and Kemler, 1994; Lickert et al., 2000; Kaplan et al., 2001) but mediates adhesion in others (Yap et al., 1998).

Stable transfection of the IL2R–E-cadherin cytoplasmic tail chimera and the E-cadherinΔ$p120^{ctn}$ construct significantly suppressed invasion, indicating that the adhesive function of E-cadherin is not necessary for its invasion suppressor effect in tumor cells (Fig. 4 A). Rather, the cytoplasmic tail of cadherin is more important. E-cadherin–α-catenin fusion chimera designed to rescue adhesion failed to suppress invasion in MDA-MB-231 cells (Fig. 4 A), indicating that increased adhesion is not sufficient to suppress invasion. We were unable to generate stable transfectants of TSU-Pr1 cells expressing the E-cadherin–α-catenin fusion and the E-cadherinΔβ-catenin mutant. Therefore, we turned to a transient transfection and invasion assay system which entails enriching transfected cells by sorting for coexpressed GFP (Fig. 4 B). This was a valid measure of the effect of E-cadherin on invasion, since the transient transfection assay yielded the same result for E-cadherin and the mutant constructs that were expressed stably (Fig. 4, A and B). Expression of the E-cadherin–α-catenin fusion in this transient assay did not suppress invasion in TSU-Pr1 cells (Fig. 4 B). Nor did the expression of E-cadherinΔβ-catenin mutant suppress invasion in the TSU-Pr1 carcinoma cell line (Fig. 4 B). These data suggest that increased cell adhesion does not account for the invasion suppressor activity of E-cadherin; rather, it is likely occurs as a result of signaling through the cytoplasmic tail.

The cytoplasmic domain of the cadherin can bind directly to p120^{ctn} and β-catenin, and either protein could play a role in invasion suppression. The construct that is defective in p120^{ctn} binding, the E-cadherinΔp120 mutant, was still able to suppress invasion. However, constructs with deletions of the β-catenin–binding domain, the E-cadherin–α-catenin chimera and the E-cadherinΔβ-catenin mutant, did not suppress invasion (Fig. 4, A and B). Thus, the inhibition of invasiveness by E-cadherin is associated with the β-catenin–binding domain but not the p120^{ctn} binding domain.

Our interpretation of the data that adhesion is neither necessary nor sufficient for suppressing cancer invasion depends on the use of various E-cadherin constructs with previously tested adhesion activities. However, these cadherin constructs have not been tested for adhesive function in MDA-MB-231 and TSU-Pr1 cells. In particular, the E-cadherinΔp120 mutant that lacks p120^{ctn} binding should be examined, since the role of p120^{ctn} or the binding site in adhesion has been variable depending on the system (Ozawa and Kemler, 1998; Yap et al., 1998; Aono et al., 1999; Thoreson et al., 2000; Ireton et al., 2002; Myster et al., 2003; Pacquelet et al., 2003). Therefore, we examined their relative adhesive activities using an adhesion flow assay to measure the strength of cell attachment to purified ectodomain of E-cadherin (Brieher et al., 1996; Gottardi et al., 2001) and an aggregation assay (Shimoyama et al., 1992). All cells were treated with 1 μg/ml doxycycline to induce transgene expression; the mock cells were treated with the same level of doxycycline. The E-cadherin–α-catenin chimera construct adhered strongly to cadherin-coated substrates and exhibited adhesive activity almost as good as the wild-type E-cadherin (Fig. 5 A). By contrast, cells expressing the IL2R–E-cadherin cytoplasmic tail and the E-cadherinΔp120 construct exhibited only background adhesive activity, which was indistinguishable from E-cadherin–negative mock-transfected MDA-MB-231 and TSU-Pr1 controls (Fig. 5, A and B). The E-cadherinΔβ-catenin construct had little or no adhesive activity, presumably due to the lack of interactions with the cytoskeleton which are important for strong cell–cell adhesion (Fig. 5 A). These results were consistent with those of aggregation assays; prominent aggregates were observed exclusively in cells expressing wild-type E-cadherin and E-cadherin–α-catenin but not other cadherin constructs (Fig. 5, C and D). We did not perform adhesion and aggregation assays on E-cadherin–α-catenin chimera fusion and E-cadherinΔβ-catenin mutant constructs in TSU-Pr1 cells because we did not obtain stable transfectants for these two constructs.

We also examined whether an adhesion blocking anti–E-cadherin antibody, HECD-1, could revert the suppression of tumor cell invasion in the MDA-MB-231 and TSU-Pr1 cell lines. HECD-1 did not revert the invasion suppression mediated by E-cadherin expression in either cell line (Fig. 6 A). For a positive control, HECD-1 caused cells expressing E-cadherin, but not in E-cadherin–negative mock-transfected cells, to dissociate and scatter (Fig. 6 B). Thus, treatment with an antibody that disrupts the adhesive function of E-cadherin did not abrogate the invasion suppression activity of E-cadherin.

Suppression of invasiveness involves β-catenin but is independent of TCF-mediated transcription

Since these data suggest roles for the β-catenin–binding domain, and by implication β-catenin, in the invasion suppres-

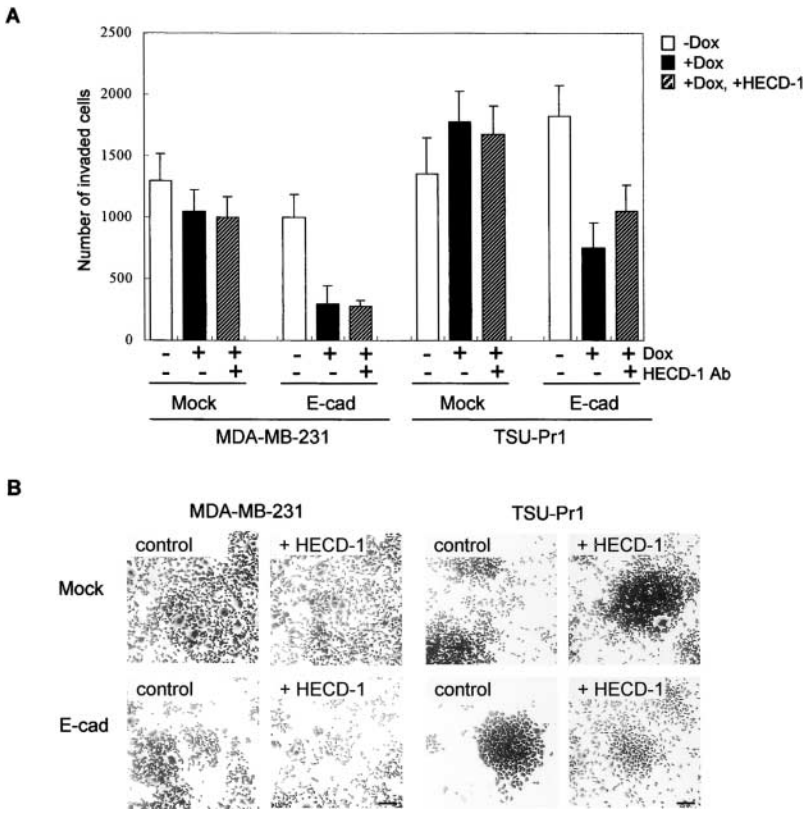

Figure 6. **Treatment with anti–E-cadherin mAb HECD-1 does not revert E-cadherin–mediated invasion suppression.** (A) Doxycycline-untreated (white bars) and treated (black bars). Mock-transfected control cells and cells stably expressing E-cadherin were plated on Matrigel filters for invasion assays in the presence (hatched bars) or absence (black bars) of HECD-1 antibody (50 μg/ml). After 24 h, the number of cells traversing the filter was determined. Data shown are the mean number of cells counted in four fields for replicate transwells from one experiment. Bars represent SD. (B) Corresponding control cell dissociation experiment (phase–contrast micrographs) showing inhibition of cell to cell adhesion by HECD-1 (50 μg/ml). Bar, 50 μm.

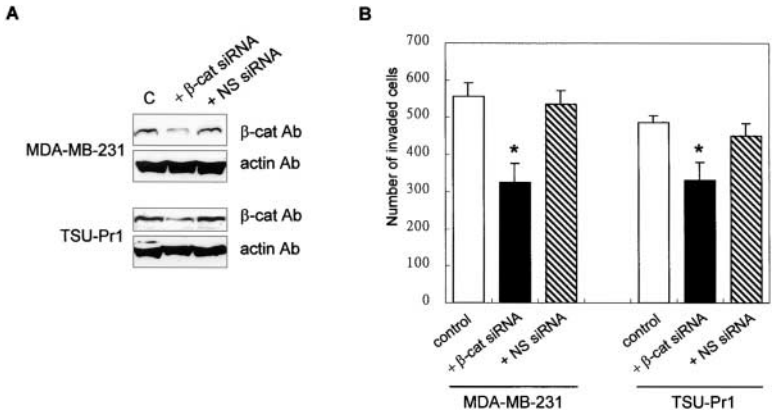

Figure 7. **siRNA-mediated depletion of β-catenin suppresses invasion.** (A) Western blot of cell lysates from equal protein of corresponding samples with antibodies as indicated. (B) MDA-MB-231 and TSU-Pr1 cells were transfected with β-catenin-siRNA (black bars) or a NS-siRNA (hatched bars) for 1 d and then harvested for invasion assays. Data are expressed as the mean number of cells counted in four fields for replicate transwells from one experiment. Bars represent SD. *$P < 0.05$, which is considered statistically different from untreated (white bars) cells.

sor function of E-cadherin we examined whether reduction or loss of β-catenin could suppress invasion in MDA-MB-231 and TSU-Pr1 cells. To do so, we chose to deplete β-catenin using small interfering RNA (siRNA) with a specific siRNA shown previously to deplete β-catenin (Deng et al., 2002). Treatment of cells with the β-catenin–siRNA, but not nonspecific (NS)-siRNA, resulted in a significant decrease in β-catenin levels (Fig. 7 A). Importantly, treatment

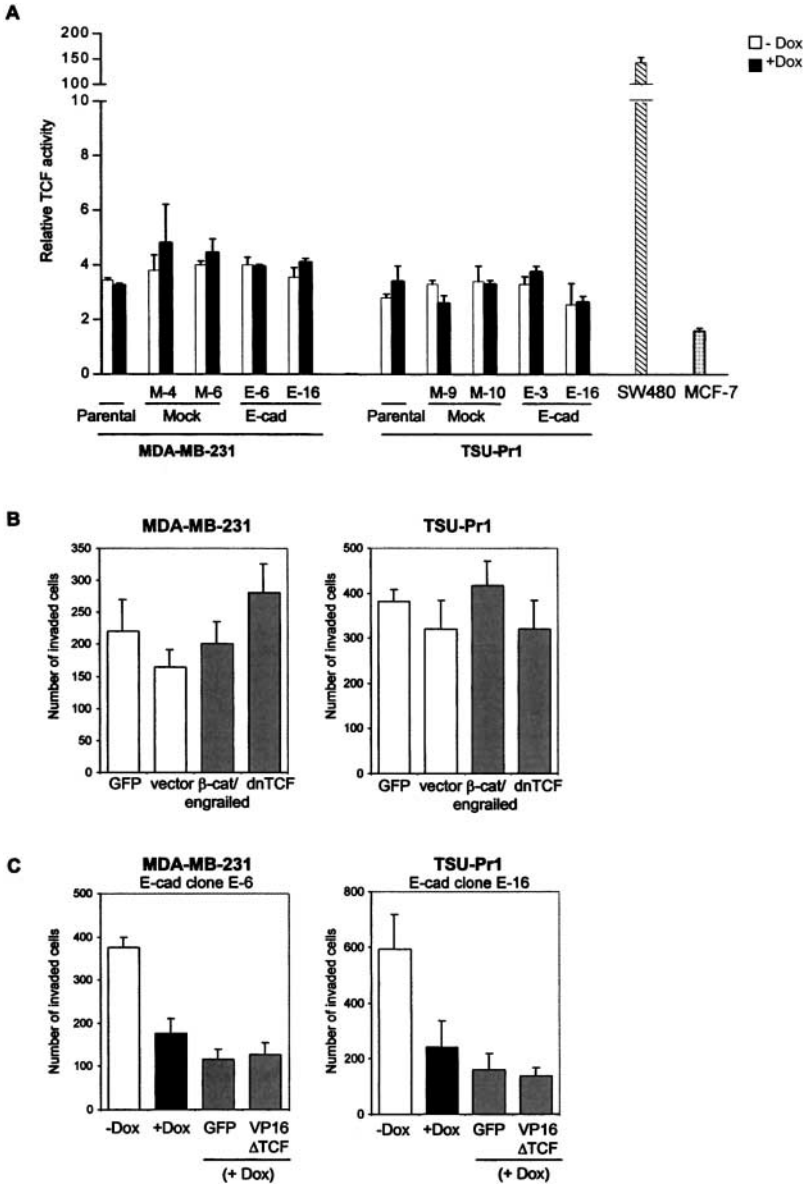

Figure 8. **No role for β-catenin/TCF transcriptional regulation in invasive activity of MDA-MB-231 and TSU-Pr1 cells.** (A) No detectable changes in β-catenin/TCF signaling by E-cadherin. Analysis of TCF reporter gene activation in various cell lines. β-catenin/TCF–mediated transcriptional activation was determined by transient transfection of TOPFLASH or FOPFLASH reporter constructs, which contain wild-type or mutant TCF-binding motifs upstream of the firefly luciferase cDNA. APC mutant colorectal cancer cell line SW480 served as a positive control. Stable clones expressing the empty vector control and E-cadherin in the absence (white bars) or presence (black bars) of 1 μg/ml doxycycline were assessed for their ability to modulate TCF-dependent transcription. TCF-mediated transcriptional activity was defined as the ratio of TOPFLASH:FOPFLASH luciferase activities, each corrected internally for Renilla luciferase activities and where no transactivation equals 1. (B) Inhibition of β-catenin/TCF signaling pathway does not suppress invasion. Cells were transiently transfected with GFP alone, an empty vector, a β-catenin/engrailed repressor, and a dominant-negative (dn) TCF and were assessed for in vitro invasion. (C) Constitutively active form of TCF (VP16ΔTCF) does not rescue E-cadherin–mediated invasion suppression. Values shown are the mean number of cells counted in four fields for replicate transwells from one experiment. Bars represent SD.

of parental MDA-MB-231 and TSU-Pr1 cells with β-catenin–siRNA suppressed invasion, but no inhibition was observed for a NS-siRNA (Fig. 7 B). These results indicate that β-catenin contributes to the invasive behavior of MDA-MB-231 and TSU-Pr1 cells and suggest that the invasion suppression activity of E-cadherin could be mediated through the sequestration of β-catenin.

Since the invasion suppressor activity of E-cadherin is associated with β-catenin, it could occur through changes in β-catenin signaling in the nucleus in association with LEF/TCF to regulate transcription. The β-catenin/TCF–dependent transcription was assayed using a TCF-responsive reporter, TOPFLASH (Korinek et al., 1997). Both MDA-MB-231 and TSU-Pr1 cell lines displayed low TCF-dependent transcriptional activities, with mean relative TCF activities between three and six (Fig. 8 A). As a positive control, the SW480 colorectal cancer cell line, which harbors mutations in the *APC* tumor suppressor protein, had a 137-fold enhanced transcriptional activity. In no case did E-cadherin induction alter TCF-dependent transcription activity compared with their uninduced controls or the mock transfectants (Fig. 8 A). Thus, the invasion inhibition activity of E-cadherin does not seem to be attributable to changes in β-catenin/TCF–dependent nuclear signaling.

To further test the possibility that the suppression of cell invasiveness may be mediated through a β-catenin/TCF–dependent signaling pathway, we asked whether expression of constructs known to inhibit β-catenin/TCF nuclear signaling at the level of target genes could inhibit cell invasion in these cell lines. β-catenin fused to the engrailed repressor domain, and a dominant-negative TCF-3 construct lacking a β-catenin–binding site in its NH_2 terminus were expressed transiently together with GFP, and cells were sorted by FACS® analysis and tested in the invasion assay. Neither the β-catenin/engrailed repressor nor the dominant-negative TCF construct caused a reduction in invasion through Matrigel filters (Fig. 8 B). Conversely, we asked whether invasiveness that was suppressed by E-cadherin could be rescued by expression of a downstream activator of the β-catenin signaling pathway, a constitutively active form of TCF. Suppression of invasion by E-cadherin expression was not reversed by VP16ΔTCF, a TCF-3 construct has its NH_2-terminal β-catenin–binding region replaced with the potent herpes simplex virus VP16 transactivation domain (Fig. 8 C). Together, these observations indicate that modulation of the β-catenin/TCF signaling pathway does not contribute significantly to the invasion suppression activity of E-cadherin in these cells.

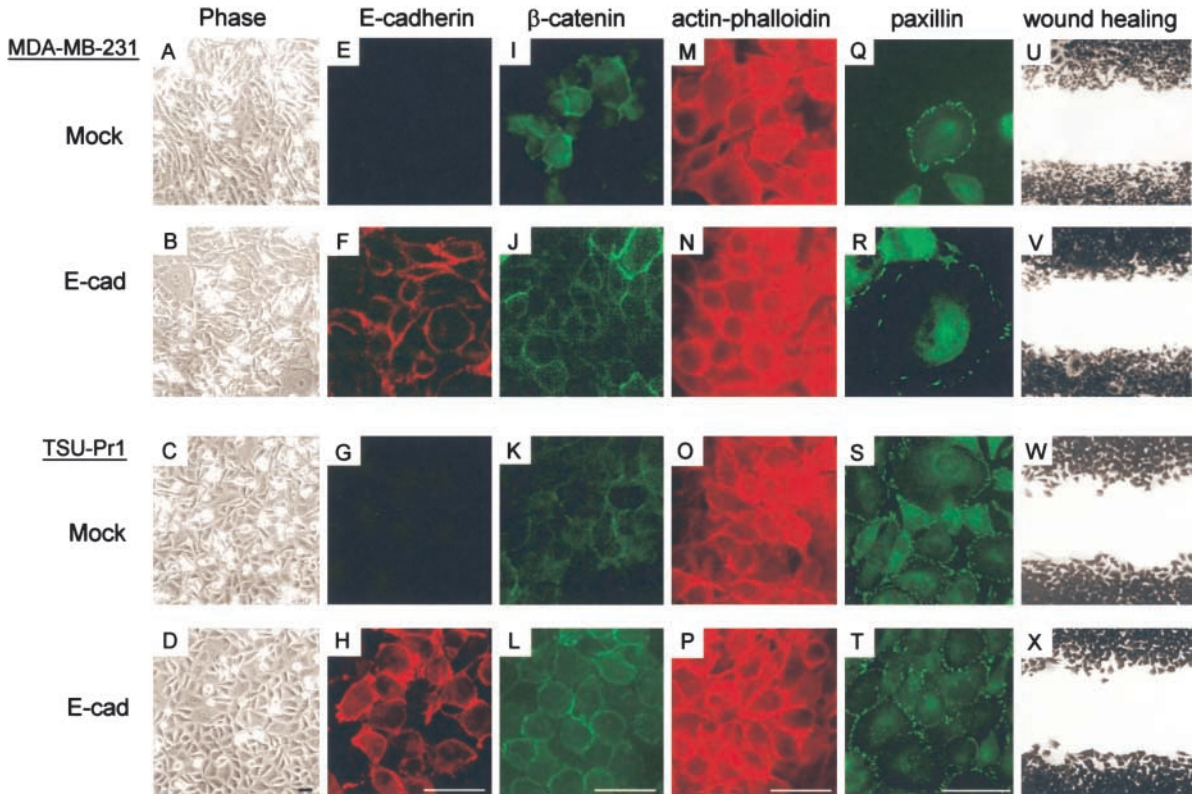

Figure 9. **Expression of E-cadherin has no obvious effect on morphology, cytoskeletal organization, or migration of MDA-MB-231 and TSU-Pr1 cells.** (A–D) Phase microscopy and indirect immunofluorescence staining of (E–H) E-cadherin (CY3), (I–L) β-catenin (FITC), and (Q–T) paxillin (FITC) in mock and E-cadherin stable cell lines. E-cadherin–transfected cells were treated with 1 μg/ml doxycycline to induce E-cadherin expression, and the control cells were treated with the same level of doxycycline. (M–P) F-actin was visualized after the binding of Texas red–conjugated phalloidin. (U–X) Effect of E-cadherin expression on cell migration. Confluent monolayers of MDA-MB-231 and TSU-Pr1 cultures of cells treated with 1 μg/ml doxycycline to induce expression of E-cadherin were scraped with a pipet tip to create a wound. The mock controls were treated with the same level of doxycycline. Cells were given fresh medium containing doxycycline in the presence of roscovitine (20 μM, to reduce cell growth), cultured for 24 h, and photographed. Bar, 10 μm.

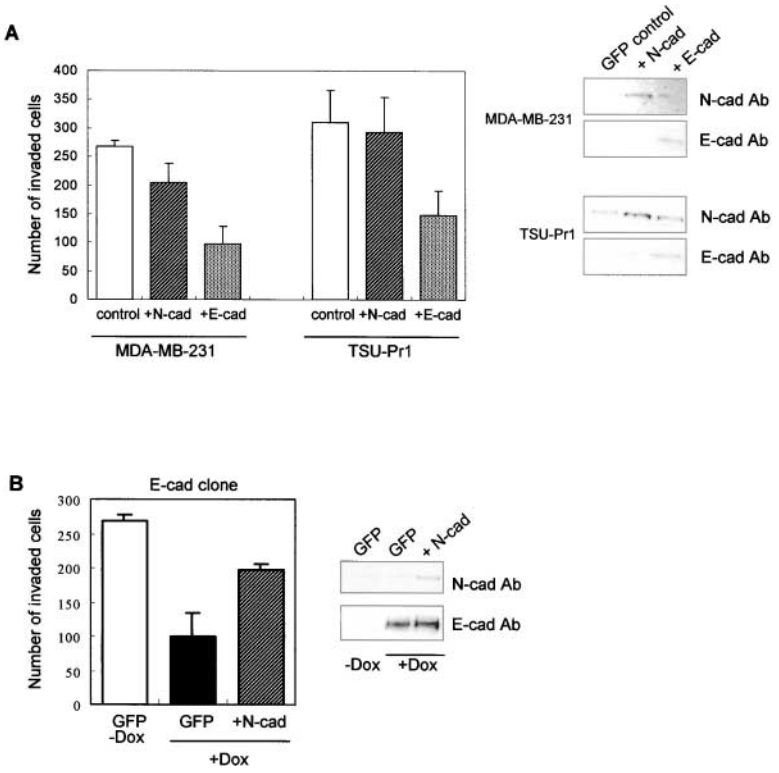

Figure 10. **Effect of N-cadherin on invasion.** (A) N-cadherin does not suppress invasion as well as E-cadherin. Parental cells transiently transfected with either N-cadherin or E-cadherin were compared with GFP control cells in invasion assays. (B) N-cadherin promotes invasion of E-cadherin–expressing cells. Doxycycline-induced E-cadherin cells transiently transfected with N-cadherin was compared with GFP controls. Values shown are the mean number of cells counted in four fields for replicate transwells from one experiment. Bars represent SD. Western blot of cell lysates from transient transfection samples before analyzed by FACS® were examined for cadherin expression with antibodies as indicated.

Roles of cell morphology, cytoskeletal organization, cell motility, and N-cadherin in invasion suppression by E-cadherin

Previous studies showed that expression of E-cadherin can alter cell morphology, cytoskeletal organization, and motility of some tumor cell types (Frixen et al., 1991; Luo et al., 1999). In the case of MDA-MB-231 and TSU-Pr1 cells, however, stable expression of E-cadherin did not significantly alter cell morphology compared with the mock-transfected controls (Fig. 9, A–D). Immunofluorescence microscopy revealed E-cadherin staining was enriched at the intercellular boundaries of E-cadherin transfectants only (Fig. 9, E–H). Even without expression of E-cadherin, β-catenin was present at low levels at cell contacts, probably due to its association with endogenous N-cadherin or other cadherins such as cadherin-11 (Fig. 10; Bussemakers et al., 1994; Wang et al., 2002). Expression of E-cadherin resulted in increased recruitment of β-catenin at the cell–cell contacts (Fig. 9, I–L). F-actin organization, visualized by Texas red–labeled phalloidin, appeared not to be significantly altered in E-cadherin–expressing MDA-MB-231 and TSU-Pr1 cells (Fig. 9, M–P). Similarly, E-cadherin–transfected cells and mock-transfected cells displayed similar staining patterns for the focal adhesion protein paxillin (Fig. 9, Q–T). We also examined whether E-cadherin altered cell motility using a classical wound-healing assay (Fig. 9, U–X). E-cadherin had no obvious effect on the motility of these cell monolayers.

Unlike E-cadherin, N-cadherin has been found to promote cell motility and invasion (Islam et al., 1996; Hazan et al., 2000). Nonetheless, we asked whether increased N-cadherin expression, which also binds β-catenin strongly, had any effect on cell invasion (Fig. 10). N-cadherin or E-cadherin was expressed transiently together with GFP and sorted by FACS® before testing in invasion assays. As before, expression of E-cadherin potently inhibited invasion (Fig. 10 A). In contrast, expression of N-cadherin did not result in significant suppression of invasion even though it was expressed (Fig. 10 A), indicating the invasion suppressing activity is unique to E-cadherin. In fact, N-cadherin expression promoted cell invasion when expressed in cells already expressing E-cadherin (Fig. 10 B), consistent with its reported invasion promoting activity (Islam et al., 1996; Hazan et al., 2000).

Discussion

Although the ability of E-cadherin to suppress tumor invasion has been known for many years, the mechanisms required for this inhibition have been less well defined. We have examined the mechanism by which E-cadherin expression suppresses tumor cell invasion in light of different aspects of cadherin function: the adhesive function and the ability to bind β-catenin and influence its signaling and transcriptional activity in the nucleus. Additional mechanisms are also possible, such as the p120-cadherin interaction, which has been implicated in cell motility (Grosheva et al., 2001; Noren et al., 2000), or even other potential signal pathways (Carmeliet et al., 1999; Xu and Carpenter 1999; Kaplan et al., 2001; Xu et al., 2002). We find that E-cadherin constructs that exhibit adhesive activity but do not bind β-catenin are unable to inhibit cell invasion. In contrast, using constructs that can bind β-catenin but are defective in adhesion suppresses invasion. Thus, the homophilic adhesive activity of E-cadherin is neither necessary nor sufficient to mediate tumor cell invasion suppression in MDA-

MB-231 breast and TSU-Pr1 prostate carcinoma cells. Since the adhesive function of cadherin is not important in suppressing tumor invasion, we also rule out the possibility that E-cadherin controls invasion by facilitating juxtacrine signaling via other receptor systems through the establishment and maintenance of other kinds of cell–cell interactions.

Previous studies using adhesion-blocking E-cadherin antibodies on MDCK (Behrens et al., 1985, 1989) and carcinoma cells (Frixen et al., 1991) or by modulating cell densities (Chen and Obrink 1991) suggested that decreased adhesion leads to increased invasion. In contrast, we did not observe a density independence or an increase in cell invasiveness using the HECD-1 mAb that blocks the adhesive function of human E-cadherin. Although it is not entirely clear why our findings differ in this regard from the previous reports, the role of E-cadherin may differ depending on the type of tumor or cell context. For example, Sommers et al. (1991) has found that treatment of E-cadherin–positive MCF-7 breast cancer cells with an adhesion-blocking antibody caused the cells to detach from one another but did not induce invasiveness in these cells. Furthermore, blocking antibodies can potentially also change E-cadherin conformation and signaling. For the two human carcinoma lines in our studies, the combined evidence from expression of mutant constructs, antibody treatment, and density independence lead us to conclude that E-cadherin suppresses invasion in an adhesion-independent manner.

$p120^{ctn}$ has the potential to regulate cell motility and invasion through its activity on Rho family GTPases and its interaction with a putative transcription factor (Daniel and Reynolds 1999). However, the mechanism by which E-cadherin suppresses tumor cell invasion is not likely mediated through $p120^{ctn}$, since mutation of the $p120^{ctn}$-binding region has no effect. These findings may appear to differ somewhat from those of Chen et al. (1997) who used an E-cadherin construct that lacks the juxtamembrane region (aa 595–617), which removes $p120^{ctn}$ binding (and perhaps other functions), to show that this region is essential for suppressing motility independent of adhesion. However, the work by Chen et al. (1997) was done in L-cell fibroblasts and astrocyte-like WC-5 cells, and the juxtamembrane domain might have a different role in those cell types compared with the MDA-MB-231 breast and TSU-Pr1 prostate cells. Furthermore, the roles of the $p120^{ctn}$-binding domain and $p120^{ctn}$ in regulating adhesion appear to differ significantly among various cell types and organisms (for review see Myster et al., 2003). Another possibility is that $p120^{ctn}$ could be more important in regulating cytoskeletal organization and cell motility in some cell types than invasion, since $p120^{ctn}$ has been suggested to be a regulator of the actin cytoskeleton via Rho (Daniel and Reynolds 1999; Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). Our findings indicate that E-cadherin functions as an invasion suppressor in MDA-MB-231 breast and TSU-Pr1 prostate carcinoma cells without any obvious effects on cytoskeletal organization or cell motility. It is likely that E-cadherin suppresses invasion through a different mechanism involving signaling through the cytoplasmic domain. For example, E-cadherin has been proposed to suppress prostate cancer invasiveness by modulating matrix metalloproteinase activity (Luo et al., 1999), which could help tumor cells to invade through basement membrane into surrounding tissue.

We provide evidence that both the β-catenin–binding domain of E-cadherin and β-catenin levels play roles in cell invasion. Nonetheless, on the basis of several different criteria, we conclude that the invasion suppressor activity of E-cadherin is not related to a change in the well-studied pathway involving the β-catenin/TCF regulation of target gene expression. E-cadherin expression is unable to change TCF-dependent transcriptional activity as measured by TOPFLASH reporter assay. Reduction in the activity of β-catenin/TCF signaling using dominant-negative TCF and β-catenin/engrailed repressor does not suppress invasion. Nor does the expression of a constitutively active form of TCF revert the invasion suppressor activity of E-cadherin. These findings differ from those obtained with embryos and cancer cells that have a strongly activated Wnt pathway in which expression of E-cadherin antagonizes β-catenin/TCF signaling (Heasman et al., 1994; Funayama et al., 1995; Fagotto et al., 1996; Sanson et al., 1996; Gottardi et al., 2001). In breast and prostate carcinomas, the Wnt pathway is not thought to be active, and components involved in mediating β-catenin/TCF–dependent gene regulation may not be expressed or active in MDA-MB-231 and TSU-Pr1 cells. Although these cells exhibit low TCF-dependent reporter gene activity, it is possible that the TOPFLASH reporter responds to other factors in these cells.

Our findings that the invasion suppressor activity of E-cadherin is mediated through the β-catenin–binding region, but not a β-catenin/TCF–dependent nuclear signaling mechanism, suggest the involvement of another signaling pathway. Since β-catenin depletion by siRNA treatment also suppresses invasion in both cell lines, the most likely explanation is that E-cadherin binds and sequesters β-catenin away from a target protein other than TCF. For example, several proteins that have the potential to regulate tumor invasion, MUC1 (Li et al., 1998; Schroeder et al., 2003), NF-kB (Deng et al., 2002), Nr-CAM (Conacci-Sorrell et al., 2002), MITF (Widlund et al., 2002), and IQGAP (Kuroda et al., 1998), have been shown to interact with β-catenin, and it is possible that E-cadherin expression titrates β-catenin away from one or more of them. We cannot rule out the possibility, however, that another signaling factor could also bind to the β-catenin–binding region. For example, Shc (Xu and Carpenter 1999), Gα12 (Kaplan et al., 2001), and PTP1B (Xu et al., 2002) have reported to bind to a partially overlapping region of the β-catenin–binding domain, although the functional significance of these interactions is not yet known. Another formal possibility is that the association of β-catenin with the cytoplasmic tail of cadherin could recruit other signaling molecules. For example, β-catenin associated with VE-cadherin recruits VEGF receptor-2/phosphatidylinositide 3-kinase, thus activating phosphatidylinositide 3-kinase and AKT (Carmeliet et al., 1999), and β-catenin has also been found to mediate the interaction of E-cadherin with EGF receptor (Hoschuetzky et al., 1994).

N-cadherin also binds β-catenin but stimulates tumor cell invasion (Islam et al., 1996; Hazan et al., 2000). Our data suggest that the invasion suppressor activity is specific to E-cadherin, despite the high homology between E- and

N-cadherin sequences in the cytoplasmic region. This finding is not really surprising; others have shown that N-cadherin has a dominant effect over E-cadherin (Islam et al., 1996; Nieman et al., 1999; Hazan et al., 2000), which we have confirmed in our experiments. This could be due to the activation of other pathways that can overcome the suppressive signals mediated by the β-catenin–binding domain. Indeed, there is considerable evidence that the invasion-promoting activity of N-cadherin resides primarily in the extracellular domain (Kim et al., 2000; Suyama et al., 2002), perhaps via its interaction with the FGF receptor (Suyama et al., 2002).

Materials and methods

Plasmids

cDNAs for wild-type E-cadherin, an E-cadherin–α-catenin chimera, an IL2R–E-cadherin cytoplasmic tail chimera, an E-cadherinΔp120 (a gift from A. Reynolds, Vanderbilt University, Nashville, TN) (Thoreson et al., 2000), an E-cadherinΔβ-catenin (a gift from D. Rimm, Yale University, New Haven, CT) were excised from plasmid pcDNA3 (Fig. 2; Gottardi et al., 2001) and subcloned into the XbaI site of the reverse tetracycline-responsive transcriptional activator (rtTA)-responsive plasmid pUHD10.3, a hygromycin-resistant pTRE vector (a gift from J. Massague, Memorial Sloan-Kettering Cancer Center, New York, NY). All constructs were verified by restriction endonuclease digestion and DNA sequencing. The β-catenin–engrailed repressor fusion construct was provided by U. Mueller and P. McCrea (University of Texas, Houston, TX) (Montross et al., 2000); dominant-negative *Xenopus* TCF-3 and activated TCF-3 were described elsewhere (Vonica et al., 2000). Mouse N-cadherin (Miyatani et al., 1992) was provided by R. Brackenbury (University of Cincinnati, Cincinnati, OH). pEGFP vector was purchased from CLONTECH Laboratories, Inc.

Cell culture and stable transfections

The E-cadherin–negative MDA-MB-231 human breast cancer cell line (a gift from N. Rosen, Memorial Sloan-Kettering Cancer Center, New York, NY) was maintained in DME and the TSU-Pr1 human prostate cancer cell line (a gift from P. Pandolfi, Memorial Sloan-Kettering Cancer Center, New York, NY) in RPMI 1640; both media contain 10% FBS, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. Cells were transfected with Lipofectamine reagent (Invitrogen). Stable transfections were performed first to establish clonal lines constitutively expressing rtTA encoded by the pTet-ON regulator plasmid, which contains a neomycin resistance gene, allowing cells to be selected in G418 (Gossen et al., 1995) (CLONTECH Laboratories, Inc.). One of these rtTA clones was selected for secondary stable transfection with rtTA-responsive pUHD10.3 hygromycin plasmids containing a wild-type E-cadherin, an E-cadherin–α-catenin chimera, an IL2R–E-cadherin cytoplasmic tail chimera, an E-cadherinΔp120, an E-cadherinΔβ-catenin (Fig. 2), or an empty vector alone, and colonies were selected in 400 μg/ml of G418 and 800 μg/ml hygromycin B (Invitrogen). Stable clones were screened for inducible expression by Western blotting after 24 h induction with 1 μg/ml of the doxycycline (CLONTECH Laboratories, Inc.). Positives were then subcloned one more time by limiting dilution and expanded into cell lines that were maintained in the selection medium. At least three independent clones were selected per cadherin construct based solely on expression of E-cadherin as determined by Western blot analysis.

Transient transfection and flow cytometry cell sorting for invasion assay

For some mutant constructs, we were unable to generate stable transfectants with sufficient levels of expression. Therefore, these constructs were transiently cotransfected along with pEGFP vector (as fluorescence marker) using Fugene 6 (Boehringer) and subjected to FACS® 48 h posttransfection (Vantage Sorter; Becton Dickinson). 10^5 sorted cells were immediately employed for Matrigel invasion assays as described below. Each experiment was repeated at least three times.

Matrigel invasion assay

Cells were grown in the absence or presence of 1 μg/ml of doxycycline for 48 h, harvested in 0.1% trypsin in PBS supplemented with 1 mM calcium and 0.5 mM magnesium, washed, suspended in DME containing 0.1% BSA in the presence and absence of doxycycline and counted. In some experiments, anti–E-cadherin antibody HECD-1 (50 μg/ml) (a gift from M. Takeichi, Kyoto University) was added to the medium. 1–2 × 10^5 cells (unless otherwise specified) were seeded on the upper well of Biocoat Matrigel chambers (8 μm pore size; Becton Dickinson). Conditioned cell culture medium of 3T3 fibroblasts containing 20 μg/ml of human plasma fibronectin (Invitrogen) in the absence or presence of 1 μg/ml of doxycycline filled the lower well as chemoattractant. The invasion assay was performed at 37°C under 5% CO_2/95% air atmosphere. After 24 h, medium was aspirated, and cells on the upper side of the membrane were removed with a cotton swab. The invading cells on the underside of the filters were stained with DAPI, and four fields were counted for each of the replicate membranes. We used SW480 colon cancer cell line as a negative control for invasion (Gottardi et al., 2001). Each experiment was repeated at least three times, although the results of one representative experiment were shown. Data were expressed as mean ± SD. Statistical significance was determined by unpaired Student's *t* test, and differences between groups were analyzed using the ANOVA; $P < 0.05$ was considered significant.

siRNA transfection

siRNA duplex oligo (Dharmacon) (13.5 μg/100 mm plate) targeting β-catenin mRNA (5′-AAGUCCUGUAUGAGUGGGAAC-3′) (Deng et al., 2002) or a nonspecific RNA (5′-GGCTACGTCCAGGAGCGCACC-3′) as a negative control were transfected using Lipofectamine (Invitrogen). Matrigel invasion assay was performed 1 d after transfection.

Adhesion flow and aggregation assays

Adhesion flow assays were performed as previously described by Brieher et al. (1996) except glass capillary tubes were coated with a human E-cadherin–Fc recombinant fusion protein as an adhesive substrate. Aggregation assays were done as described by Shimoyama et al. (1992) with only minor modifications.

β-catenin–LEF/TCF reporter gene (TOPFLASH) assay

In 24-well plates, cells were transiently transfected with 0.5 μg of the TOPLASH or FOPFLASH reporter plasmid (H. Clevers, University of Utrecht, Utrecht, Netherlands) using Fugene-6 (Boehringer). Transfection efficiencies were determined by cotransfection of the pRL-TK reporter construct (a gift from J. Massague, Memorial Sloan-Kettering Cancer Center, New York, NY) that contained the Renilla luciferase cDNA. Activities of Firefly and Renilla luciferases were measured sequentially from a single sample using the Dual-Luciferase Reporter Assay System (Promega).

Immunofluorescence

Cells were grown on glass coverslips, fixed in cold (−20°C) methanol, and then incubated with antibodies. F-actin was visualized after the binding of Texas red–conjugated phalloidin (Molecular Probes).

Wound healing

Monolayers of cells were wounded by scraping with a plastic pipet-tip, rinsed several times with media to remove dislodged cells, and placed back in medium with 10% FBS. Cells were given 20 μM roscovitine (Calbiochem) to reduce cell growth, cultured for 24 h, stained by 0.1% crystal violet, and photographed.

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