[CANCER RESEARCH 59, 3552–3556, August 1, 1999]

*Advances in Brief*

# Suppression of Prostate Cancer Invasive Potential and Matrix Metalloproteinase Activity by E-Cadherin Transfection[1]

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## Abstract

**Our previous studies have demonstrated the heterogeneous expression of E-cadherin in a Dunning rat prostate tumor model. From this model, cloned E-cadherin-negative cells exhibited enhanced invasive and metastatic potential when compared with E-cadherin-positive cells. In this report, we examined the invasion suppressor function of E-cadherin in these prostate tumor cell clones. The E-cadherin gene was stably transfected into E-cadherin-negative Dunning clones. E-cadherin transfection resulted in the up-regulation of the three major catenins ($\alpha$-, $\beta$-, and $\gamma$-catenin) and enhanced $Ca^{2+}$-dependent cellular cohesiveness. Morphological analyses of E-cadherin transfectants revealed a reversion from a fibroblastic, motile phenotype to a more stationary epithelial phenotype. Matrix metalloproteinase 2, an important marker associated with invasive and metastatic potential, was reduced in all six stable transfected lines. A concomitant decrease in cellular invasiveness was observed, as assessed *in vitro* by the ability of the transfected cells to invade biological matrices. These results lend further support to the hypothesis that in this experimental system, E-cadherin plays a central role in reducing the cellular invasiveness of prostatic adenocarcinoma, due in part to the down-regulation of matrix metalloproteinase 2 activity. Moreover, the data shed additional light on the possible mechanisms involved in E-cadherin-dependent modulation of invasion.**

## Introduction

Prostate cancer is the most commonly diagnosed cancer and is the second leading cause of cancer mortality in American males. Although the majority of the early-stage tumors are not life threatening, a subset of these tumors will progress to give rise to metastasis and cancer-related death. Tumor metastasis involves a series of sequential steps, which include the acquisition of cellular invasiveness (1). Accordingly, transition from the noninvasive to the invasive phenotype is a crucial event in prostate cancer progression. This transition involves a number of molecular alterations, including those leading to altered cell-substrate attachment, decreased cell cohesion, and increased cell motility. It is imperative to identify these molecular alterations and explore their relative importance in the progression of prostate cancer invasion and metastasis.

E-cadherin, a transmembrane glycoprotein responsible for intercellular adhesion in epithelial cells, has been identified as a key player in the progression of carcinoma and the acquisition of an invasive phenotype (2). In normal epithelial tissues, E-cadherin complexes with actin cytoskeleton via cytoplasmic catenins ($\alpha$-, $\beta$-, and $\gamma$-catenin) to maintain the functional characteristics of epithelia (3). Disruption of this complex, due primarily to loss or decreased expression of E-cadherin, is common to many advanced, poorly differentiated carcinomas (4, 5). In some prostate cancer cell lines, E-cadherin gene promoter hypermethylation and $\alpha$-catenin gene deletion may be the cause of the defective E-cadherin/catenin pathway (6, 7). Both experimental and clinicopathological studies of prostate cancer have indirectly correlated the decreased E-cadherin and $\alpha$-catenin expression with an invasive and metastatic phenotype (8–12). Therapeutic strategies aimed at rescue of the defective E-cadherin/catenin function have been explored in tumor models using demethylating agents or gene transfer into PC-3 cells with deletion of the $\alpha$-catenin gene (6, 7). However, there is no direct evidence supporting the functional significance of E-cadherin in suppressing prostate cancer invasion.

Our previous studies have demonstrated the heterogeneous expression of E-cadherin/catenin in a Dunning rat prostate tumor model (13). Clonal cell lines were derived from a morphologically heterogeneous primary Dunning culture. Cloned E-cadherin-negative cells exhibit enhanced invasive and metastatic potential when compared with E-cadherin-positive cells. This system offers an excellent model to study the functional significance of E-cadherin in prostate cancer invasion and metastasis. In this study, we investigated whether restoration of a functional E-cadherin can reverse the invasive phenotype in the E-cadherin-negative Dunning cell clones, and we have identified changes in catenins and MMP-2[3] activity that are directly associated with the restoration of E-cadherin expression.

## Materials and Methods

**Cell Cultures and DNA Transfection.** The Dunning rat prostatic adenocarcinoma lines used in this study have been described previously and characterized by our laboratory (13). Briefly, R3327-5 is a poorly invasive cell line derived from a slow-growing, androgen-dependent subline of the Dunning tumor R3327/132. R3327-5 cells were inoculated s.c. into male Copenhagen rats to yield tumors. Clonal cell lines were generated from a primary culture of a Dunning rat prostate tumor (R3327-5′) demonstrating heterogeneous expression of E-cadherin. Both E-cadherin-positive and E-cadherin-negative cells were cloned and characterized with respect to their invasive phenotype and metastatic potential. Clones 5′A, 5′C, and 5′D are E-cadherin negative and demonstrated dispersed, fibroblastic morphology, whereas the E-cadherin positive clone 5′B grew in tight clusters. All of the E-cadherin-negative clones (clones 5′A, 5′C, and 5′D) exhibited enhanced *in vitro* invasiveness and *in vivo* metastasis when compared with the E-cadherin-positive 5′B cells. The distinctive features exhibited by the cell clones can be maintained during long-term *in vitro* culture. For DNA transfection, 5′C cells were grown to 80% confluence in a 6-well culture dish and then transfected with a total of 2 $\mu$g of DNA using the LipofectAMINE protocol (Life Technologies, Inc., Gaithersburg, MD). E-cadherin expression vector pBATEM-2 (14) and the neomycin resistance selection marker pCDNA3.1 (Invitrogen, San Diego, CA) at a ratio of 20:1 were cotransfected to facilitate the selection. Sham transfections were performed with pBAT (pBATEM-2 with E-cadheirn cDNA deleted) and

Received 4/26/99; accepted 6/16/99.

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[1] Supported in part by NIH Grant R21 CA/ES 69838 (to D. M. L.) and The University of Iowa Foundation (M. J. C. H.).

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[3] The abbreviations used are: MMP, matrix metalloproteinase; MICS, membrane invasion culture system.

pCDNA 3.1 at a ratio of 20:1. Stable transfectants were selected with 700 μg/ml G418 and tested for transgene expression.

**Slot Blot Analysis.** Copy numbers of the integrated E-cadherin gene in the E-cadherin transfectants were examined using the Minifold II Slot Blot System (Schleicher & Schuell, Keene, NH). Purified genomic DNA and standard samples were immobilized on a Schleicher & Schuell nitrocellulose membrane and prehybridized for 2 h at 68°C. The 2.5-kb *Eco*RI fragment of pBATEM-2 was nick-labeled using the Nick Translation System (Life Technologies, Paisley, PA) and used as a probe for the integrated E-cadherin gene. Hybridization was carried out overnight at 42°C under standard conditions, according to the manufacturer's instructions.

**Northern Blot Analysis.** Total RNA was isolated using the Trizol system (Life Technologies, Inc.), according to the manufacturer's instructions. Total RNA (20 μg) was separated on formamide/formaldehyde gels and transferred onto a GeneScreen Plus membrane (NEN Life Science Products, Boston, MA). The radiolabeled 2.5-kb *Eco*RI fragment of pBATEM-2 was used to probe the E-cadherin mRNA.

**Western Blot Analysis.** For immunoblot analysis of E-cadherin and catenins, cells were grown to confluence and treated with lysis buffer containing 125 mM Tris-HCl, 2% SDS, 5% glycerol, 0.003% bromphenol blue, and 1% β-mercaptoethanol. The protein concentrations were measured by the dotMETRIC protein quantification kit (GenoTechnology, St. Louis, MO). Twenty μg of protein/lane were separated by 10% SDS-PAGE and transblotted onto Imobilon-P membrane (Millipore, Bedford, MA). Membranes were stained with a recommended dilution of primary antibodies against E-cadherin and α-, β-, and γ-catenins (Transduction Laboratories, Lexington, KY). After extensive washes, blots were incubated with horseradish peroxidase-conjugated goat antimouse IgG diluted at 1:10,000 (ICN, Costa Mesa, CA), followed by development with an enhanced chemiluminescence Western blot detection kit (Amersham, Buckinghamshire, United Kingdom).

**Immunofluorescence Localization of E-Cadherin.** Cells were grown to confluence on glass coverslips in 24-well tissue plates and washed three times with PBS, followed by fixation in 3.7% formaldehyde for 7 min. After extensive washing, the coverslips were blocked for 30 min with PBS containing 1% BSA and 0.1% Triton X-100. The cells were then incubated with anti-E-cadherin antibody (Transduction Laboratories) diluted 1:100 in PBS. Coverslips were washed again and incubated for 30 min with FITC-conjugated goat antimouse secondary antibody (Zymed, San Francisco, CA) diluted 1:100 in PBS. After additional washing, coverslips were mounted using the Prolong antifade kit (Molecular Probes, Eugene, OR) and visualized using a Zeiss fluorescence microscope.

**Aggregation Assay.** Cell aggregation assays were performed according to the method described by Nagafuchi *et al.* (14). Briefly, cells grown to confluence were detached by incubating with 0.01% crystalline trypsin (type I; Sigma, St. Louis, MO) in PBS in the presence of 1 mM $CaCl_2$. Under these conditions, E-cadherin can be protected from proteolytic digestion. One million cells suspended in 3 ml of PBS in the presence of 10 mM $CaCl_2$ were seeded into wells coated with BSA and incubated at 37°C on a gyratory shaker at 80 rpm for 30 min. Cell aggregation was measured by calculating the ratio of the total particle number at the end of the assay to the initial particle number, with the latter being identical to the total number of cells added.

**Invasion Assay.** The MICS assay was performed as described previously (15). Random invasion and stimulated invasion assays were performed. Briefly, a polycarbonate membrane with 10-μm pores (Osmonics, Livermore, CA) was coated with a uniform thickness of defined basement membrane matrix containing laminin/type IV collagen/gelatin and placed inside the MICS chambers (which are modified Boyden chambers). Both the upper and lower chambers were filled with RPMI 1640 Mito$^+$ serum-free media (Collaborative Research, Bedford, MA) for random invasion analysis. In the case of stimulated invasion assays, the lower chambers were filled with 50% NIH3T3 conditioned media in RPMI 1640 Mito$^+$ serum-free media (Collaborative Research). Single cell suspensions were prepared as described for the aggregation assay to protect the E-cadherin molecules from proteolytic digestion. Cells were seeded at a concentration of $1 \times 10^5$ cells/well in the upper chambers. After incubation for 24 h, the cells that had invaded the basement membrane matrix were collected and stained for microscopic counting. The invasion rate was calculated as the percentage of cells capable of invading the matrix-coated membrane compared to the total number of cells seeded (in the absence of cell proliferation). Data were analyzed by two-way repeated ANOVA, followed by the Student-Newmann-Keuls method using SigmaStat statistical software.

**Zymographic Assay.** Cells were cultured on 6-well tissue culture plates precoated with the defined matrix as used in the invasion assay. Zymographic analysis of MMP activity was performed as described previously (16). Briefly, conditioned media were collected 1 day after replacing the complete media with RPMI 1640 Mito$^+$ serum-free media (Collaborative Research). After normalizing for cell number and medium volume, the samples were mixed with Laemmli's sample buffer (minus β-mercaptoethanol and without boiling) and resolved on a 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gels were washed in $50 \times 10^{-3}$ M Tris-HCl (pH 7.5) containing 2.5% Triton X-100 for 30 min and then incubated at 37°C for 24 h in $50 \times 10^{-3}$ M Tris-HCl (pH 7.5) containing $20 \times 10^{-3}$ $CaCl_2$ and 0.02% sodium azide. The gels were stained with Coomassie Brilliant Blue R-250 and destained.

## Results

**Stable Transfection of E-Cadherin.** Six individual cloned transfectants and two sham transfectants were established by ring cloning. Stable expression of both E-cadherin mRNA and the associated protein was confirmed by Northern blot (data not shown) and Western blot assays (Fig. 1), respectively. Genomic integration was confirmed by slot blot analysis (data not shown). The integrated copy number ranged from 2–8/cell. However, there was no definitive correlation between copy number and the expression levels of mRNA or the associated protein within different transfectants. All of the stable transfectants showed markedly increased levels of α-, β-, and γ-catenin when compared with the corresponding sham transfectants (Fig. 1). This regulation is believed to be the result of the increased half-life of the catenins complexing with E-cadherin (17). Immunofluorescence analysis revealed E-cadherin at the intercellular boundaries of the transfected cell clones (Fig. 2). Cell aggregation assays were performed under conditions favoring cadherin-mediated adhesion to confirm the functionality of the E-cadherin molecule. As illustrated in

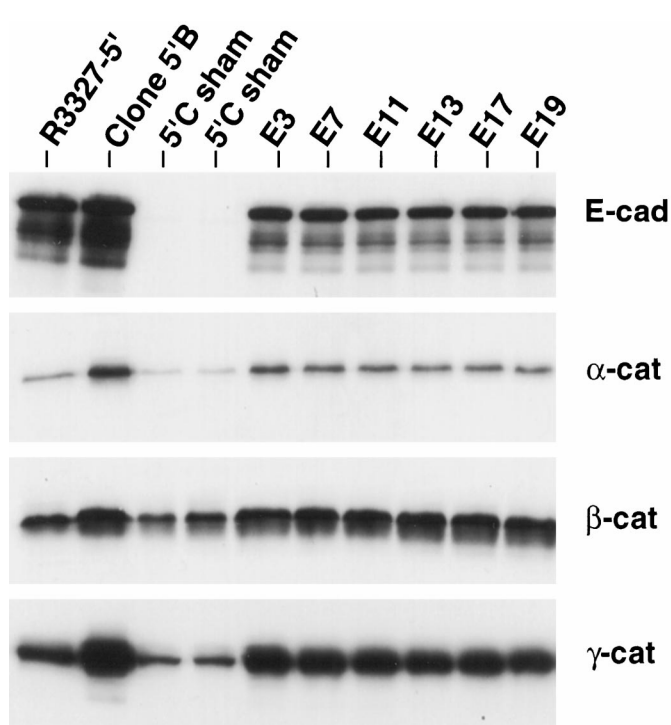


Fig. 1. Western blot analysis of E-cadherin/catenin after the transfection of E-cadherin cDNA into clone 5′C cells. The R3327-5′ clones and sham-transfected cells were used as controls. E-cadherin transfection significantly up-regulated the levels of the three major catenins. *5′C sham*, sham-transfected clone 5′C; *E3–E19*, stable E-cadherin transfectants of clone 5′C.

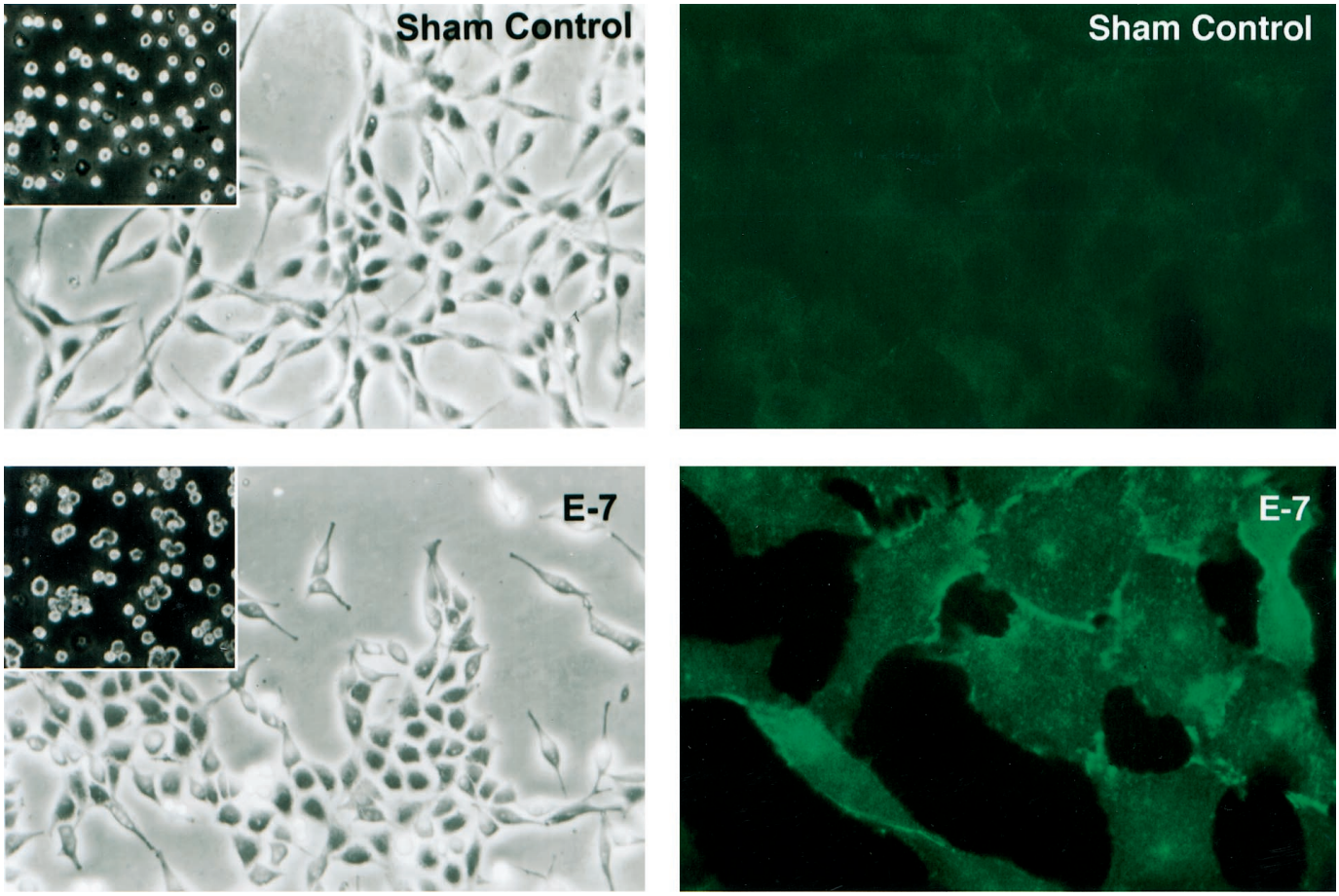


Fig. 2. E-cadherin transfection induces morphological changes and cellular cohesion. The transfected cells (E-7, *bottom left panel*) tend to adhere to each other and form compact, epithelial-like clusters, whereas sham-transfected cells (sham control; *top left panel*) exhibit a dispersed fibroblastic phenotype. Increased $Ca^{2+}$-dependent cellular cohesion was observed in the transfectants (E-7; *bottom left panel, inset*) when compared with the sham control (*top left panel, inset*). Immunofluorescence staining demonstrates the expression of E-cadherin in the intercellular contacts between transfected cells (E-7; *bottom right panel*). No staining was found in the sham control cells, even when the cells were confluent (*top right panel*).

the Fig. 2 *insets*, we detected a significant enhancement of $Ca^{2+}$-dependent cell-cell adhesion in the stable transfectants.

**Morphological Changes Induced by E-Cadherin Expression.** Morphological conversions after E-cadherin transfection were observed in the transfected cell lines (Fig. 2). E-cadherin-transfected 5′C cells were more adherent to each other and formed compact clusters, whereas the sham-transfected cells continued to exhibit a dispersed, fibroblastic morphology. However, complete conversion to the clone 5′B phenotype, an E-cadherin-positive cell line derived from the same primary culture, was not observed.

**E-Cadherin Transfection Suppresses *in Vitro* Invasion.** For the analysis of tumor cell invasiveness, the MICS assay was performed to test the ability of the cells to invade through biological matrices *in vitro*. The relevance of this assay for other invasion assays and for *in vivo* malignancy has been documented extensively (13, 15). Under conditions in which E-cadherin was protected from proteolytic digestion, E-cadherin transfectants and the control cell lines were tested for both nonstimulated and stimulated invasive potential. Results from representative cell lines are shown in Fig. 3*A*. Under nonstimulated conditions, the E-cadherin-positive clone 5′B cells showed invasion rates below 3.3%, whereas 13.4–20.1% invasion rates were attained by the E-cadherin-negative clone 5′A and sham-transfected clone 5′C cells. The parental R3327-5′ cells, which demonstrate heterogeneous expression of E-cadherin, exhibited an intermediate level of invasive potential. Stable expression of E-cadherin by transfection suppressed the invasive potential of the original E-cadherin-negative clone 5′C cells. As shown in Fig. 3*A*, all of the E-cadherin transfectants yielded invasion profiles ranging from 6.0–9.4%, which were significantly lower than those in sham-transfected cells ($P < 0.01$). When stimulated by 50% conditioned media from NIH3T3 cells, significant increases in invasive potential were observed in all of the cell lines tested, with the exception of the endogenously E-cadherin-positive clone 5′B. The inverse correlation between invasive potential and E-cadherin expression was maintained under stimulated conditions.

**E-Cadherin Transfection Down-Regulates MMP-2 Activity.** MMPs, especially MMP-2 and MMP-9, are molecules indicative of the invasive and metastatic potential of prostate cancer cells (18, 19). To ascertain whether suppression of the *in vitro* invasiveness by E-cadherin was accompanied by changes in MMP activity, we analyzed the gelatinolytic activity of conditioned media from E-cadherin-transfected and sham-transfected cells by the zymogram assay. As illustrated in Fig. 3*B*, the $M_r$ 72,000 MMP-2 represents the major gelatinolytic activity from all of the collected samples in the zymogram assay. MMP-2 activity was down-regulated in all of the E-cadherin transfectants when compared to the original E-cadherin-negative cells. An inverse correlation of E-cadherin expression levels and MMP-2 activity was observed in transfectants expressing variable levels of exogeneous E-cadherin molecule in another independent experiment.

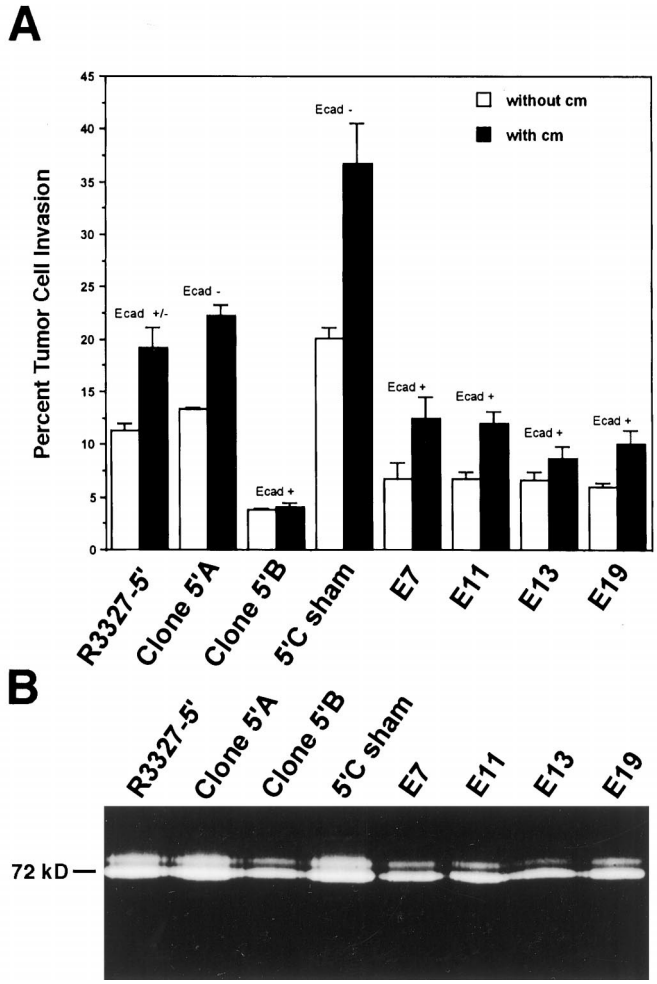


Fig. 3. Reduced invasive ability and MMP activity in the E-cadherin-transfected cells. *A*, transfected cell lines (*E-cad* +) showed significantly reduced random invasiveness (□) through laminin/collagen IV/gelatin matrix *in vitro* as compared to the sham controls ($P < 0.01$). When stimulated by 50% NIH3T3 conditioned media, all cells exhibited a significantly higher ($P < 0.05$) invasive potential (■), with the exception of clone 5′B. The inverse correlation between E-cadherin levels and invasive potential was maintained under both nonstimulated and stimulated conditions. *B*, the gelatinolytic activity of MMP-2 ($M_r$ 72,000) was down-regulated as a result of E-cadherin transfection (*E7, E11, E13,* and *E19*). Results from the other experimental and control transfectants were consistent. *Error bar,* SE.

## Discussion

The present study represents the first report indicating that E-cadherin plays a central role in suppressing the invasive phenotype of prostate cancer cells. Loss or decreased expression of E-cadherin has been detected in more than 50% of prostate cancer patients (8–12), although the true percentage may be underestimated because of the heterogeneity of E-cadherin expression in a single tumor. In some cases, the restoration of the E-cadherin function may be an important therapeutic consideration (20). However, no direct evidence was previously available with respect to the invasion suppressor function of E-cadherin in prostate cancer models. In our experimental system, E-cadherin transfection can drastically enhance cellular cohesion, possibly through concurrent up-regulation of the associated catenins. Although restoration of E-cadherin function did not render a complete epithelial conversion of the cells tested, a preponderance of epithelial-like clusters accompanied by a less invasive phenotype was observed. Furthermore, important parameters for cellular invasiveness, including random and stimulated *in vitro* invasive ability and MMP-2 expression, were suppressed by E-cadherin expression. Although it is not clear whether a defective E-cadherin/catenin complex is the sole factor that determines the invasive phenotype of prostate cancer cells, it is proposed, based on these observations, that the prostate cancer invasive phenotype can be rescued by E-cadherin transfection, possibly through the down-regulation of MMP-2 activity. These results suggest that E-cadherin should be thoroughly examined for its putative role in preventing prostate cancer invasion and ultimately metastasis *in vivo*, possibly through the strategy of targeted gene delivery.

Changes in cell-to-cell adhesion have been implicated in the earliest step of tumor formation, and there is emerging evidence that these changes in adhesive properties are permissive for genomic instability and the accumulation of multiple mutations (21). Although overwhelming evidence supports the hypothesis that down-regulation of E-cadherin resulting from either genetic alterations or epigenetic effects is a determining factor in the progression of many cancers, the mechanism by which E-cadherin exerts its invasion suppressor function is not known. The best interpretation seems to be that physical interaction between tumor cells mediated by E-cadherin acts to restrain cell migration. It is then conceivable that a loss of E-cadherin would favor the migration of tumor cells from the local site. However, other than cellular cohesion, E-cadherin may regulate additional aspects of the tumor phenotype, including MMP activity, as revealed in our studies. It is not clear whether these additional effects are a sequential result of the loss of cell cohesion or, alternatively, an independent process involving other cellular signaling events. Recent findings revealed cross-talk between the E-cadherin/catenin complex and the catalytic signaling pathways (22). Also, β-catenin was recently identified as an oncoprotein able to transduce signals to the nucleus under pathological conditions (23). Thus, it is possible that perturbation of E-cadherin-mediated cell adhesion may modulate gene expression and hence tumor phenotype. Indeed, our observations provide a direct connection between the re-expression of E-cadherin, the acquisition of an epithelial phenotype, and the down-regulation of MMP-2 activity, important biological targets for therapeutic intervention.

## Acknowledgments

We gratefully acknowledge the valuable scientific guidance from Elisabeth Seftor (The University of Iowa, Iowa City, IA) for the invasion assays. The E-cadherin expression vector pBATEM-2 was a generous gift from Dr. M. Takeichi (Kyoto University, Kyoto, Japan).

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*Cancer Res* 1999;59:3552-3556.