

# E-Cadherin–dependent Growth Suppression is Mediated by the Cyclin-dependent Kinase Inhibitor p27<sup>KIP1</sup>

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**Abstract.** Recent studies have demonstrated the importance of E-cadherin, a homophilic cell–cell adhesion molecule, in contact inhibition of growth of normal epithelial cells. Many tumor cells also maintain strong intercellular adhesion, and are growth-inhibited by cell–cell contact, especially when grown in three-dimensional culture. To determine if E-cadherin could mediate contact-dependent growth inhibition of nonadherent EMT/6 mouse mammary carcinoma cells that lack E-cadherin, we transfected these cells with an exogenous E-cadherin expression vector. E-cadherin expression in EMT/6 cells resulted in tighter adhesion of multicellular spheroids and a reduced proliferative fraction in three-dimensional culture. In addition to increased cell–cell adhesion, E-cadherin expression also resulted in dephosphorylation of the retinoblastoma protein, an increase in the level of the cyclin-dependent kinase inhibitor p27<sup>kip1</sup> and a late reduction in cyclin D1 protein. Tightly adherent spheroids also showed increased levels of p27 bound to the cyclin E-cdk2 complex, and a

reduction in cyclin E-cdk2 activity. Exposure to E-cadherin–neutralizing antibodies in three-dimensional culture simultaneously prevented adhesion and stimulated proliferation of E-cadherin transfectants as well as a panel of human colon, breast, and lung carcinoma cell lines that express functional E-cadherin. To test the importance of p27 in E-cadherin–dependent growth inhibition, we engineered E-cadherin–positive cells to express inducible p27. By forcing expression of p27 levels similar to those observed in aggregated cells, the stimulatory effect of E-cadherin–neutralizing antibodies on proliferation could be inhibited. This study demonstrates that E-cadherin, classically described as an invasion suppressor, is also a major growth suppressor, and its ability to inhibit proliferation involves upregulation of the cyclin-dependent kinase inhibitor p27.

**Key words:** E-cadherin • p27 • kip1 • cyclin D • epidermal growth factor receptor

**I**NTERCELLULAR adhesion molecules and extracellular matrix (ECM),<sup>1</sup> once viewed simply as cellular glue and mechanical scaffolding, are now known to have profound effects on cell behavior (Juliano and Haskill, 1993). For example, interactions between integrins and ECM promote the growth and survival of many cell lines in culture (Meredith and Schwartz, 1997; Bottazzi and As-

soian, 1997). In addition, intercellular adhesion molecules have been shown to be necessary for nontransformed cells growing in monolayer tissue culture to become quiescent when they reach confluence by a process known as contact inhibition (Aoki et al., 1991; Takahashi and Suzuki, 1996; Kandikonda et al., 1996). Although much has been learned recently about the mechanisms by which cell–ECM interactions promote growth (Assoian, 1997; Bottazzi and Assoian, 1997), much less is known about the molecular pathways by which homophilic cell–cell interactions inhibit cell cycle progression.

Many tumor cell lines are subject to contact-dependent growth inhibition, although not to the same degree as normal cells. Because solid tumors usually grow as three-dimensional masses, our laboratory has been interested in understanding the impact of cellular aggregation on the growth properties of cells. By growing cells in three-dimensional culture, we observed a greater degree of con-

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1. *Abbreviations used in this paper:* cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitors; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; HA, hyaluronic acid; PI3K, phosphatidylinositol-3-kinase; RTK, receptor tyrosine kinase.

tact-dependent growth inhibition than could be observed in confluent vs. subconfluent monolayer cultures. We also observed, using clonally derived variants of the EMT/6 mouse mammary carcinoma cell line, that the rate of proliferation in three-dimensional culture depended largely on the degree of intercellular adhesion, since spontaneously nonadherent variant cell lines incorporated significantly more bromodeoxyuridine than did tightly-adherent cell lines (St. Croix et al., 1996b). Although the precise mechanism controlling intercellular adhesion in tightly-adherent EMT/6 variant cell lines is yet unknown, adding hyaluronidase could abolish such adhesion; we therefore refer to this adhesion mechanism as hyaluronic acid (HA)-dependent. Furthermore, tightly-adherent variants dispersed with hyaluronidase grew at a rate similar to that observed for loosely-adherent EMT/6 variants.

The recent identification of several key regulators of cell cycle progression provides a valuable new avenue for delineating the molecular mechanisms underlying contact inhibition. The cell cycle is governed by sequential activation and inactivation of a family of cyclin-dependent kinases (cdks; Morgan, 1995). Cdk activation requires cyclin association, and cyclin levels typically oscillate throughout the cell cycle. Because contact-inhibited cells enter quiescence or the G1/G0 phase of the cell cycle, molecules regulating G0/G1-phase are likely to be particularly important for understanding contact inhibition. Progression through G1 into S-phase is regulated by D-type cyclins associated with cdk4 or cdk6, and by cyclin E-cdk2. Subsequently, as cells enter S phase, cyclin A-cdk2 is activated. Recently two families of cyclin-dependent kinase inhibitors (CKIs) have been identified that bind to and inhibit the activity of cdks (Sherr and Roberts, 1995). The INK4 family is comprised of p15<sup>INK4B</sup>, p16<sup>INK4A</sup>, p18<sup>INK4C</sup>, and p19<sup>INK4D</sup>. INK4 family members share four ankyrin motifs and specifically inhibit cyclin D-dependent cdks (cdk4 and cdk6). Three KIP family members have also been identified, and include p21<sup>CIP1/WAF1</sup>, p27<sup>KIP1</sup>, and p57<sup>KIP2</sup>. Members of this family show broader substrate specificity, and inhibit the activity of both cyclin D-cdk4/6 and cyclin E-cdk2. Because levels and/or activity of p27 are elevated upon cell-cell contact of nontransformed fibroblasts or epithelial cells (Hengst et al., 1994; Slingerland et al., 1994; Polyak et al., 1994; Winston et al., 1996; Kato et al., 1997), this inhibitor may be of particular importance in regulating contact inhibition. Similarly, our recent studies demonstrate that p27 levels increase when breast, colon, and ovarian carcinoma cell lines are transferred from two- to three-dimensional culture. Importantly, depleting p27 from tumor cells using an antisense strategy resulted in significant growth stimulation, but only in three-dimensional culture (St. Croix et al., 1996a). Therefore, p27 is at least in part responsible for contact-dependent growth inhibition of carcinoma cells. However, normal fibroblasts from p27<sup>-/-</sup> mice undergo contact inhibition (Nakayama et al., 1996) suggesting that there may be cell type specificity or redundancy in the regulation of contact inhibition at the molecular level.

Although p27 levels have been temporally correlated with cell-cell contact, the ability of homophilic cell adhesion molecules to regulate signaling pathways that impinge upon p27 levels has not been fully investigated. In this

study, we analyzed the effect of E-cadherin on both proliferation and p27 levels. E-cadherin is a major calcium-dependent homophilic cell adhesion molecule found on normal epithelial cells. Its intracellular domain binds directly to  $\beta$ -catenin which, along with  $\alpha$ -catenin, links E-cadherin to the underlying actin cytoskeleton (Aberle et al., 1996). In addition to its role in adhesion,  $\beta$ -catenin has also been implicated in *Wnt* signal transduction, and interacts with the APC tumor suppressor protein as well as transcription factors of the LEF/TCF family (Behrens et al., 1996; Molenaar et al., 1996; Huber et al., 1996; Su et al., 1993; Rubinfeld et al., 1993). By sequestering  $\beta$ -catenin at the cell surface, cadherins have been shown to antagonize  $\beta$ -catenin signaling pathways which are required for normal dorsoventral axis formation in *xenopus* (Fagotto et al., 1996). In many carcinomas, cadherins or catenins are lost or downregulated, resulting in a reduced level of intercellular adhesion (Becker et al., 1994; Berx et al., 1996). Loss of E-cadherin function may endow tumor cells with a relative growth advantage over normal contact-inhibited cells. Indeed, several studies have suggested a potential role for E-cadherin as a growth suppressor (Hermiston and Gordon, 1995b; Miyaki et al., 1995; Navarro et al., 1991; Takahashi and Suzuki, 1996; Kandikonda et al., 1996; Watabe et al., 1994). However, the mechanism(s) by which E-cadherin affects the cell cycle machinery have not been elucidated.

In the present study we investigated the role of E-cadherin in contact-dependent growth inhibition of carcinoma cells, and analyzed potential cell cycle molecules regulated by cadherin-mediated adhesion. Carcinoma cells that maintain E-cadherin function were growth inhibited by cell-cell contact when grown in a three-dimensional context. Importantly, the cyclin-dependent kinase inhibitor p27 was increased in E-cadherin-arrested cellular aggregates to levels sufficient to saturate and inhibit cyclin E-cdk2 complexes. One mechanism by which E-cadherin can elevate p27 is through inhibition of mitogenic signaling pathways initiated from receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR). Our results suggest that E-cadherin is not only an invasion suppressor (Vlemingckx et al., 1991), but also a proliferation suppressor, and its ability to suppress proliferation depends on its ability to prevent growth factor induced reduction in p27 protein levels.

## Materials and Methods

### Cell Lines and Culture Conditions

The human MCF-7 breast carcinoma cell line and the murine EMT/6 mammary carcinoma cell line were a gift from Dr. Beverly Teicher (Dan-Farber Cancer Institute, Boston, MA). Pc5-T, Pc7-T, Pc10-L, and Pc11-L are clones of the parent EMT/6 cell line that grow as either tightly (T) or loosely (L) adherent aggregates in three-dimensional culture, and were described previously (St. Croix et al., 1996b). Stable E-cadherin-expressing clones of EMT/6 were obtained by lipofectin-mediated (Gibco Laboratories, Grand Island, NY) cotransfection of 0.5  $\mu$ g of the plasmid pSV2neo along with 10  $\mu$ g of the expression vector pBATEM2 containing the mouse E-cadherin gene under the control of a  $\beta$ -actin promoter (M. Takeichi, Kyoto University, Kyoto, Japan). After selection in G418-containing medium, G418-resistant clones were screened for E-cadherin expression by immunoblotting. All EMT/6 cells were cultured in Waymouth's MB 752/1 medium supplemented with 10% FBS. To prevent HA-

mediated intercellular adhesion of Pc5-T and Pc7-T cells, 1,000 U/ml of bovine testicular hyaluronidase (Worthington Biochemical Corp., Freehold, NJ) was added to three-dimensional cultures at the time of cell plating. EMT/6-E-cadherin transfectants were also grown in the presence of hyaluronidase to ensure the absence of HA-mediated adhesion. Intercellular adhesion of EMT/6-E-cadherin transfectants or E-cadherin-positive human cell lines was prevented by treatment with 2  $\mu$ g/ml of either anti-E-cadherin antibody, DECMA-1 (Sigma Chemical Co., St. Louis, MO) or SHE78-7 anti-E-cadherin antibody (Zymed Labs, Inc., S. San Francisco, CA), respectively, at the time of cell plating. As a control, GoH3 anti- $\alpha$ 6 integrin antibody (Bio/can Scientific, Mississauga, ON) was used. For antibodies used in functional studies, sodium azide was removed using dialysis cassettes (Pierce Chemical Co., Rockford, IL). Cells were collected and assayed 48 h after treatment with hyaluronidase or E-cadherin-neutralizing antibodies, unless otherwise stated.

Human L23 large cell lung and BT-20 breast carcinoma cell lines were maintained in RPMI 1640 containing 10% FCS. HT29 and DLD-1 colon and MCF-7 breast carcinoma cell lines were maintained in DMEM medium supplemented with 10% FCS. HBL-100 breast carcinoma cells were grown in McCoy's 5A medium (Gibco Laboratories) with 10% FCS. The DLD-1 cell line was a gift from T. Sasazuki (Kyushu University, Fukuoka, Japan) and the L23 cell line was a gift of P. Twentyman (Lincoln's Inn Fields, London, United Kingdom). All other cell lines were purchased from the American Type Culture Collection (Rockville, MD). Multicellular aggregates were prepared using the liquid overlay method as previously described (Kobayashi et al., 1993). In brief, a 4% stock solution of Seaplaque agarose (FMC Bioproducts, Rockland, Maine) was microwaved, diluted to 1% with heated (56°C) serum-free medium, and coated (0.25 ml) into each well of 24-well plates.  $10^5$  tumor cells in 1 ml of complete medium were then plated on top of the solidified agarose.

### Generation of Cell Lines with Inducible p27

A metallothionein-inducible p27 expression vector, pMT27, was constructed by excising the BglIII-EcoRV fragment containing the entire human p27 cDNA from the plasmid pET-28, (a gift from L. Hengst and S. Reed, Scripps Research Institute, La Jolla, CA), which was then ligated between BamHI and EcoRV of the multiple cloning site of the plasmid pCDNA3-MT. pCDNA3-MT, a gift from J. Filmus (Sunnybrook Health Science Center, Toronto, ON), was generated by replacing the CMV promoter of the plasmid pCDNA3 (Invitrogen Corp., Carlsbad, CA) with the rat metallothionein promoter. Stable clones expressing inducible p27 were obtained by transfecting HT29 or DLD-1 cells with 5  $\mu$ g of pMT27 using standard lipofectin procedures followed by selection in G418-containing medium. G418-resistant clones were screened for inducible p27 by immunoblotting. For induction of the metallothionein promoter, media was supplemented with either 1 $\times$  ZnCd (100  $\mu$ M ZnSO<sub>4</sub> and 2  $\mu$ M CdCl<sub>2</sub>) or 2 $\times$  ZnCd (200  $\mu$ M ZnSO<sub>4</sub> and 4  $\mu$ M CdCl<sub>2</sub>).

### Bromodeoxyuridine (BrdU) Incorporation Assay

EMT/6 cells growing in either three-dimensional culture for 24 h or in monolayer culture were pulsed with 5  $\mu$ M BrdU for a further 24 h, after which cells were immediately rinsed with PBS, trypsinized, rinsed again, and fixed with 70% ethanol (4°C) for at least 1 h. Cells were rinsed with PBS, pelleted, and resuspended in 1 ml of cold 0.1 N HCl/0.7% Triton X-100 and left for 10 min on ice. After rinsing once with PBS, cells were resuspended in 0.5 ml of distilled water and transferred into a 0.5-ml Eppendorf tube already containing 16  $\mu$ l of 0.1 N HCl. Next, Eppendorf tubes were heated in a PCR machine for 8 min at 95°C and placed immediately on ice. Cells were then transferred to a 5-ml tube, rinsed two times with HNFN buffer (i.e. 10 mM Hepes [pH 7.4], 150 mM NaCl, 4% FCS, 0.1% NaN<sub>3</sub>) containing 0.5% Tween 20, and once with HNFN alone. Pellets were then resuspended in a 1/40 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Cedarlane Labs. Ltd., Hornby, Ontario, Canada) and left in the dark on ice for 45 min. Stained cells were rinsed twice with HNFN Tween 20, once with HNFN, pelleted, resuspended in 1 ml of PI solution (50  $\mu$ g/ml propidium iodide, 10  $\mu$ g/ml Ribonuclease A in PBS), and left for 1 h (4°C) in the dark before analyzing on an Epics Elite V flow cytometer (Coulter Electronics, Burlington, Ontario, Canada).

### [<sup>3</sup>H]Thymidine Incorporation Assay

Cells growing in monolayer culture were harvested, and 10,000 cells/well in a final volume of 100  $\mu$ l of medium were added to 96-well U-bottom

Nunc plates (Canadian Life Technologies, Burlington, Ontario, Canada). To prevent attachment of cells to the bottom of 96-well plates, the wells had been previously treated with a heated (56°C) solution of 2% poly(2-hydroxyethylmethacrylate) (polyhema; Aldrich Chemical Co., Milwaukee, WI) in ethanol, which was briefly added to and then removed from the plate, leaving behind a thin film in each well. Intercellular adhesion of E-cadherin-positive human cell lines was prevented by supplementing wells with various concentrations of SHE78-7 anti-E-cadherin antibody or isotype-matched control IgG antibodies (Sigma Chemical Co.). All cells were cultured in their normal growth medium containing 10% FCS, with the following exceptions: in the experiment that examined mitogen-dependence of growth stimulation by E-cadherin antibodies, cells were plated in serum-free medium or medium containing either 10% FCS or 50 ng/ml TGF- $\alpha$  (R&D Systems, Inc., Minneapolis, MN). TGF- $\alpha$  signaling was inhibited using 20  $\mu$ g/ml of the C225-neutralizing anti-EGFR monoclonal antibody (ImClone, New York, NY). To analyze potential pathways involved in TGF- $\alpha$  signaling, E-cadherin antibody-dispersed HT29 cells were plated in the presence of 50 ng/ml TGF- $\alpha$  along with either the (PI3K) inhibitor LY294002 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), or the antioxidant *N*-acetyl-L-cysteine (Sigma Chemical Co.). Cells in this experiment were labeled after 20 h of incubation in three-dimensional culture by adding 2  $\mu$ Ci of [<sup>3</sup>H]thymidine in 50  $\mu$ l of medium to each well. In all other experiments, labeling of cells began at 44 h. After a 4-h pulse (i.e., at 24 or 48 h), labeled cells were frozen at -20°C. Importantly, [<sup>3</sup>H]thymidine has been shown to penetrate large (400–600  $\mu$ m) spheroids rapidly, reaching full distribution in less than 1 min (Nederman et al., 1988). Next, cells were harvested onto filtermats using a Titertek cell harvester 530, and radioactive filtermats were counted using a 1205 beta plate liquid scintillation counter (Fisher Scientific Ltd., Nepean, Ontario, Canada). The rate of DNA synthesis of the groups treated with anti-E-cadherin antibodies was calculated as a fraction of the nontreated controls.

### Immunoblotting

Cells were lysed in ice-cold NP-40 lysis buffer (1% NP-40, 10% glycerol, 20mM Tris-HCl [pH 7.5], 137 mM NaCl, 100 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethyl sulphonyl fluoride, and 10  $\mu$ g/ml each of leupeptin and aprotinin). The lysates were sonicated and clarified by centrifugation, resolved by SDS-PAGE, blotted onto Immobilon-P-membranes (Millipore Corporation, Bedford, MA) and blocked in 10% dry milk in TBS-T (TBS [pH 7.6], 0.1% Tween 20). mAbs to p27 and E-cadherin, used for immunoblotting, were obtained from Transduction Laboratories (Lexington, KY), mAb against pRb was from PharMingen (Mississauga, Ontario, Canada), polyclonal antibody (pAb) to  $\beta$ -catenin was from Sigma Chemical Co. (Oakville, Ontario, Canada), pAb to  $\alpha$ -catenin was from Zymed Laboratories, Inc. (San Francisco, CA), pAbs to cyclin E, cdk2, cdk4, cdk6, p130, p16, p18, p19, p21, and mAb against mouse cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, CA). DCS-6 mAb, a gift from J. Bartek (Danish Cancer Society, Denmark) was used to detect human cyclin D1. After washing in TBS-T, the immunoreactive proteins were visualized using either HRP-conjugated anti-mouse- or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania), and the enhanced chemiluminescence Western blotting detection system (Amersham Canada Ltd., Oakville, Ontario).

### Immunoprecipitation

Cells were lysed in NP-40 lysis buffer and immunoprecipitated with antibodies to E-cadherin (Transduction Laboratories) overnight or to cyclin E for 90 min (4°C). In some cases, before cyclin E immunoprecipitation, samples were divided into two separate tubes, and one was serially immunodepleted three times with p27 antibodies. E-cadherin and cyclin E precipitates were collected for 1 h on either protein G or protein A Sepharose beads, respectively. After washing four times with lysis buffer, precipitates were resuspended in Laemmli SDS sample buffer and resolved by SDS-PAGE. The immunoprecipitated protein complexes were resolved and probed as for the immunoblots to detect associated proteins using antibodies against the p27 and cyclin E primary antibodies. In the case of p27, an HRP-conjugated anti-mouse IgG that only recognized the Fcy fragment (Jackson ImmunoResearch Laboratories) was used to avoid cross-reactivity with the light chain of the immunoprecipitating antibody that ran at a molecular weight similar to p27. Likewise, an HRP-conjugated anti-rabbit IgG specific for the F(ab')<sub>2</sub> fragment (Jackson ImmunoResearch Laboratories) was used to avoid cross-reactivity with the heavy

chain of the cyclin E immunoprecipitating antibody. Because some residual heavy chain cross-reactivity was still observed, a high cross-linking gel (7.5% acrylamide, 0.25% bis-acrylamide) was used to maximize separation of the bands.

### Cyclin-E-dependent Kinase Assay

To measure cyclin E-associated cdk2 activity, cells were lysed in ice-cold kinase lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.6], 0.1% NP40, 1 mM PMSF, 10  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin) and 100  $\mu$ g of total protein was immunoprecipitated with anti-cyclin E antibody. After washing the precipitates three times with kinase lysis buffer and twice with reaction buffer (22  $\mu$ M ATP, 7.5 mM MgCl<sub>2</sub>, and 20 mM Tris, pH 7.5), 0.25  $\mu$ g of Histone H1 (Boehringer Mannheim, Laval, Quebec, Canada) and 10  $\mu$ Ci of <sup>32</sup>P- $\gamma$  ATP were added to the reaction buffer for 30 min at 37°C. Reaction products were resolved by SDS-PAGE, and the gel was dried and exposed to x-ray film.

### p27 Immunodepletion

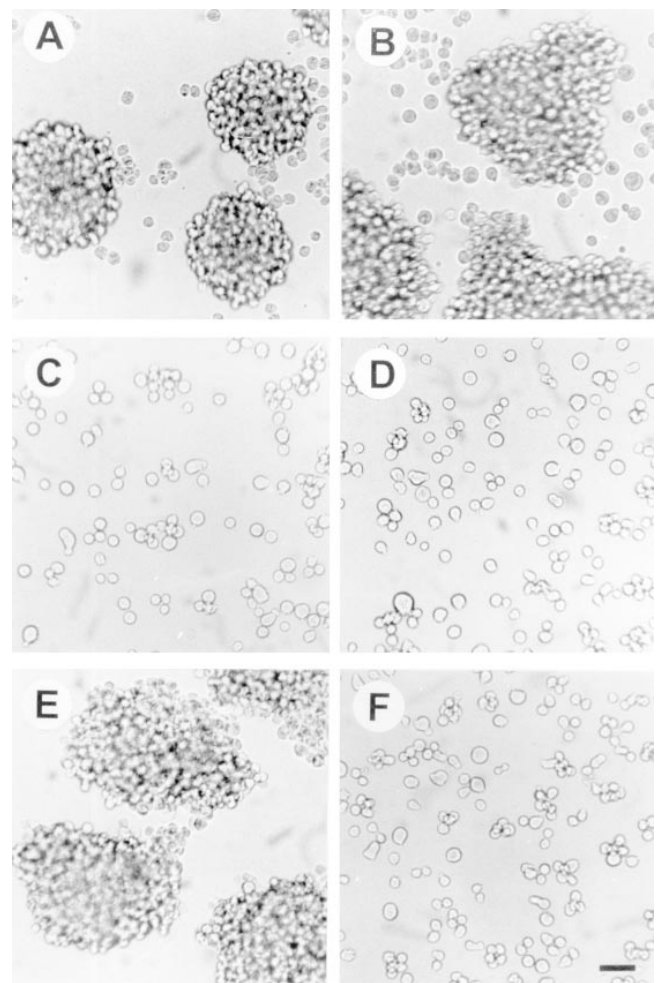
Cells lysed in kinase lysis buffer were immunodepleted of p27 by three sequential 60-min immunoprecipitations using p27 polyclonal antibodies (Santa Cruz Biotechnology). After each round of immunoprecipitation, complexes were collected for 30 min on protein A Sepharose beads, and were then washed extensively with lysis buffer. Beads from each round were resuspended in Laemmli SDS sample buffer, and eluted protein was resolved by SDS-PAGE and probed with anti-p27 antibodies by immunoblotting. Supernatants from the final round of immunodepletion were immunoprecipitated with cyclin E, and cyclin E-associated proteins and kinase activity were assayed.

## Results

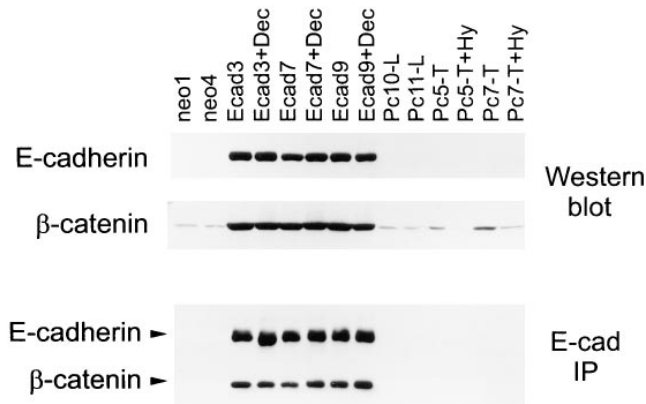
### E-cadherin Mediates Intercellular Adhesion and Growth Suppression of EMT/6 cells

To investigate the impact of E-cadherin on contact-dependent growth regulation, we transfected EMT/6 mouse mammary carcinoma cells with a murine E-cadherin expression vector under control of a constitutive  $\beta$ -actin promoter. Neomycin-resistant control transfectants like the EMT/6 parent cell line were loosely adherent in three-dimensional culture (Fig. 1), and lacked any detectable E-cadherin expression by Western blot analysis, although  $\alpha$ - and  $\beta$ -catenin were readily detectable in these cells (see Fig. 2 and data not shown). In contrast, within 24 h E-cadherin transfectants spontaneously formed compact multicellular aggregates, and expressed high levels of E-cadherin protein. To confirm that E-cadherin was responsible for the increased compaction of these clones, suspension cultures were treated with DECMA-1 antibody, which blocks mouse E-cadherin-mediated adhesion (Vestweber and Kemler, 1985). As shown in Fig. 1, DECMA-1 antibody completely prevented E-cadherin-dependent aggregation, while neutralizing anti- $\alpha$ 6 integrin antibody used as a control had no effect on adhesion, even though these cells express high levels of  $\alpha$ 6 $\beta$ 4 integrin (our unpublished observations). Furthermore, treatment with DECMA-1 did not block HA-dependent aggregation of EMT/6 cells. Conversely, hyaluronidase abolished HA-dependent adhesion, but failed to block E-cadherin-mediated adhesion. E-cadherin transfectants were also dispersed by adding EDTA; however, such treatment did not affect cells with HA-dependent adhesion (our unpublished observations). Thus, using EMT/6 cells we have generated a convenient model to study the effects of two distinct adhesion systems: one dependent on E-cadherin and the other dependent on HA.

Our previous studies demonstrated that HA-dependent aggregation of EMT/6 variants results in reduced proliferation of cells in three-dimensional culture (St. Croix et al., 1996b). To determine if E-cadherin also suppressed cell proliferation, we pulsed EMT/6-E-cadherin transfectants for 24 h with bromodeoxyuridine (BrdU), stained cells with FITC-tagged anti-BrdU antibody, and then analyzed BrdU incorporation into DNA by flow cytometry. As shown in Fig. 3 a and Table I, 49–70% of cells from E-cadherin-expressing clones incorporated BrdU after a 24-h pulse in three-dimensional culture. In contrast, under the same conditions, over 95% of loosely adherent neomycin control transfectants incorporated BrdU. E-cadherin-expressing cells that failed to incorporate BrdU were found to be primarily in the G1 phase of the cell cycle (~80–90%; see Fig. 3 a and data not shown). The remaining non-BrdU-labeled cells were found in the S- and G2/M-phase, consistent with other reports on cell cycle kinetics in spheroids



**Figure 1.** Morphology of EMT/6 cells displaying either E-cadherin or HA-dependent intercellular adhesion in three-dimensional culture. (a–c) EMT/6 cells transfected with E-cadherin (Ecad9); (d) with neomycin vector alone (neo8), or (e and f) Pc5-T cells with HA-dependent adhesion were cultured with 1,000 U/ml hyaluronidase (a–d, f), 10  $\mu$ g/ml DECMA-1 antibody (c and e), or 10  $\mu$ g/ml of GoH3 anti- $\alpha$ 6-integrin antibody (b). Cells were photographed after 24 h in three-dimensional culture. Bar, 50  $\mu$ m.



**Figure 2.** Immunoblot analysis of E-cadherin and  $\beta$ -catenin in EMT/6 cells. EMT/6 neomycin-resistant control transfectants (neo1, neo4), E-cadherin transfectants (Ecad3, Ecad7, or Ecad9), or EMT/6 loosely adherent (Pc10-L and Pc11-L) or tightly adherent (Pc5-T, Pc7-T) variants were grown for 48 h in three-dimensional culture. E-cadherin-dependent adhesion was prevented by treatment with DECMA-1 antibody (*Dec*), and HA-dependent adhesion was prevented with hyaluronidase (*Hy*). In the top panel, anti-E-cadherin and anti- $\beta$ -catenin antibodies detected proteins of 120 and 95 kD, respectively, by Western blot analysis. When cell lysates were immunoprecipitated with E-cadherin antibodies and then immunoblotted with anti-E-cadherin and anti- $\beta$ -catenin antibodies (*bottom*), again bands of 120 and 95 kD were detected.

(Allison et al., 1983; Freyer and Sutherland, 1980). It is unlikely that an insufficient BrdU pulse is responsible for nonlabeled S- and G2/M-phase cells, because the BrdU-labeling time chosen (24 h) exceeded the cell division time ( $\sim 16$  h; our unpublished observations). An independent pulse labeling with [ $^3$ H]thymidine over 4 h confirmed the BrdU results, and demonstrated that treatment of EMT/6-E-cadherin transfectants with 2  $\mu$ g/ml DECMA-1 neutralizing antibody restored [ $^3$ H]thymidine uptake to the level observed in neomycin-resistant control transfectants (see Fig. 3 *b*). Importantly, the observed growth inhibition mediated by E-cadherin in three-dimensional cultures was not detected in subconfluent monolayer cultures (see Table I). Likewise 2  $\mu$ g/ml DECMA-1 antibody had no effect on growth of subconfluent monolayers, and a very minor effect, if any, on confluent cultures (data not shown).

### **Growth Suppression by E-cadherin is Associated with pRb Hypophosphorylation, Reduced Cyclin D1, and Elevated p27 Levels in EMT/6 Cells**

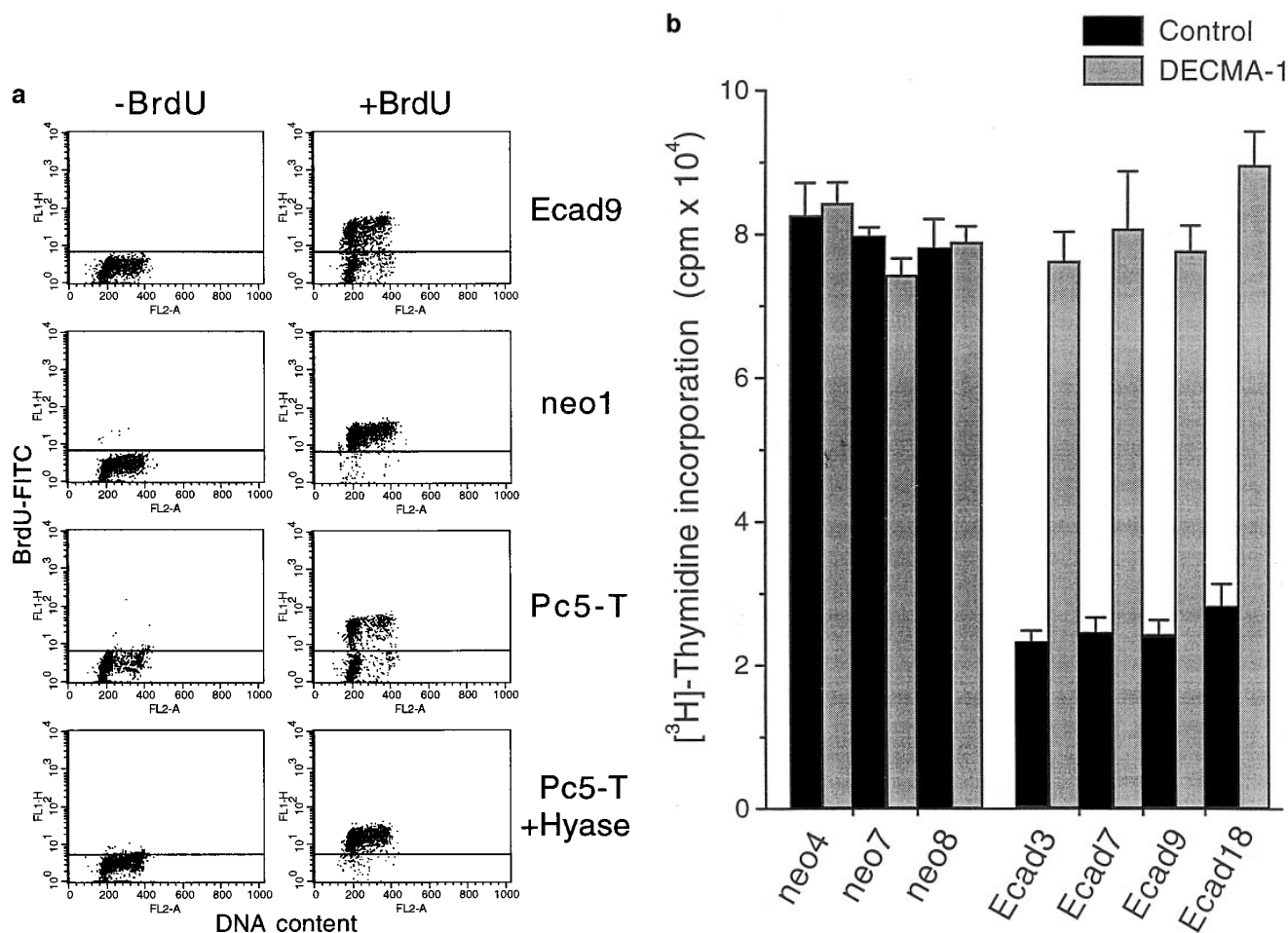
To elucidate the mechanism responsible for E-cadherin-dependent growth suppression, an analysis of cell cycle regulators was undertaken. As a control, we included an analysis of EMT/6 variants that display either a loosely adherent or HA-dependent tightly adherent morphology in three-dimensional culture. Since the majority of noncycling tightly adherent cells were found to be in the G1 phase of the cell cycle (Fig. 3 *a*), Western blots were probed for cell cycle regulators of G1 $\rightarrow$ S-phase progression. The pRb protein was almost exclusively hyperphosphorylated in all EMT/6 loosely adherent cell types (Fig.

4 *a*), including neomycin-resistant control transfectants (neo1 and neo4), DECMA-1-treated E-cadherin transfectants (Ecad3, Ecad7, and Ecad9), loosely adherent clones (Pc10-L and Pc11-L), and two tightly adherent clones (Pc5-T and Pc7-T) that had been dispersed with hyaluronidase. In contrast, the hypophosphorylated faster-migrating form of pRb was prominent only in tightly adherent variants displaying either E-cadherin or HA-dependent adhesion. Hypophosphorylation of the pocket protein p130 was also noted in tightly adherent clones (data not shown). For both pRb and p130, changes in phosphorylation status were only evident in cells grown in three-dimensional culture.

Next we analyzed the expression of cell cycle molecules of potential importance in mediating the E-cadherin-dependent growth suppression and the changes in pRb and p130 phosphorylation. After 48 h in three-dimensional culture, levels of cdk2, cdk4, and cdk6 were unchanged in all EMT/6 cells regardless of their capacity to aggregate. Likewise, cyclin E and A protein levels were unaffected by the degree of intercellular adhesion (Fig. 4 *a* and data not shown). In contrast, after 48 h in three-dimensional culture, levels of cyclin D1 were consistently higher in EMT/6 cells growing in loosely adherent clusters, as compared with tightly adherent cells. In monolayer culture, levels of cyclin D1 were unaffected 48 h after treatment with DECMA-1 or hyaluronidase (data not shown).

We also analyzed levels of known CKIs. Although p15 was not detected in EMT/6 cells, p16, p18, p19, and p21 were expressed, but levels of these inhibitors did not correlate with adhesion. (Fig. 4 *a* and data not shown). We were particularly interested in the effect of E-cadherin on p27 protein levels since previous studies have implicated this CKI in contact-dependent growth inhibition (Slingerland et al., 1994; Hengst et al., 1994; Polyak et al., 1994; St. Croix et al., 1996a). Although the base level of p27 varied somewhat between individual clones, cell lines with a tightly adherent morphology generally displayed higher p27 levels than did loosely adherent variants (see Fig. 4 *a*). Furthermore, high p27 levels in tightly adherent clones displaying either E-cadherin or HA-dependent adhesion were consistently reduced by treatment with either DECMA-1 or hyaluronidase, respectively. In contrast, this treatment did not affect loosely adherent control cells grown under the same conditions, or tightly adherent cells grown in monolayer (data not shown). Thus, increased p27 levels paralleled aggregate compaction, and an inverse pattern was observed for cyclin D1, but only in three-dimensional culture.

To determine the kinetics of changes in p27 and cyclin D1 in response to aggregation, levels of these proteins were assayed at various time points after plating cells into three-dimensional culture. As shown in Fig. 4 *b*, within 6 h of transferring cells to three-dimensional culture, p27 levels were increased in E-cadherin-expressing cells. The decrease in cyclin D1, however, occurred between 24 and 48 h, and its levels fell progressively thereafter. There was no increase in the association of p27 with cyclin D1 after transfer of E-cadherin-overexpressing clones into three-dimensional culture (data not shown). Thus, our data do not support a role for p27 as an inhibitor of cyclin D1-associated cdk activity after growth suppression by E-cadherin.



**Figure 3.** E-cadherin suppresses growth of EMT/6 cells in three-dimensional culture. (a) Proliferation of EMT/6 cells transfected with E-cadherin (*Ecad9*) or neomycin vector alone (*neo1*) as measured by BrdU incorporation into DNA. 24 h after plating in three-dimensional culture, cells were either untreated (–BrdU) or pulsed with BrdU (+BrdU) for a further 24 h. Cells were prepared as described in the Materials and Methods, and cell cycle profiles were analyzed by flow cytometry. Tightly adherent or hyaluronidase (*HYase*) dispersed Pc5-T cells were also included as an additional control. (b) Proliferation of neomycin-resistant control transfectants (*neo4*, *neo7*, *neo8*) or E-cadherin transfectants (*Ecad3*, *Ecad7*, *Ecad9*, and *Ecad18*) as measured by  $[^3\text{H}]$ thymidine incorporation into DNA. Intercellular adhesion of E-cadherin transfectants was prevented by treatment with 2  $\mu\text{g}/\text{ml}$  DECMA-1 antibody at the time of plating.

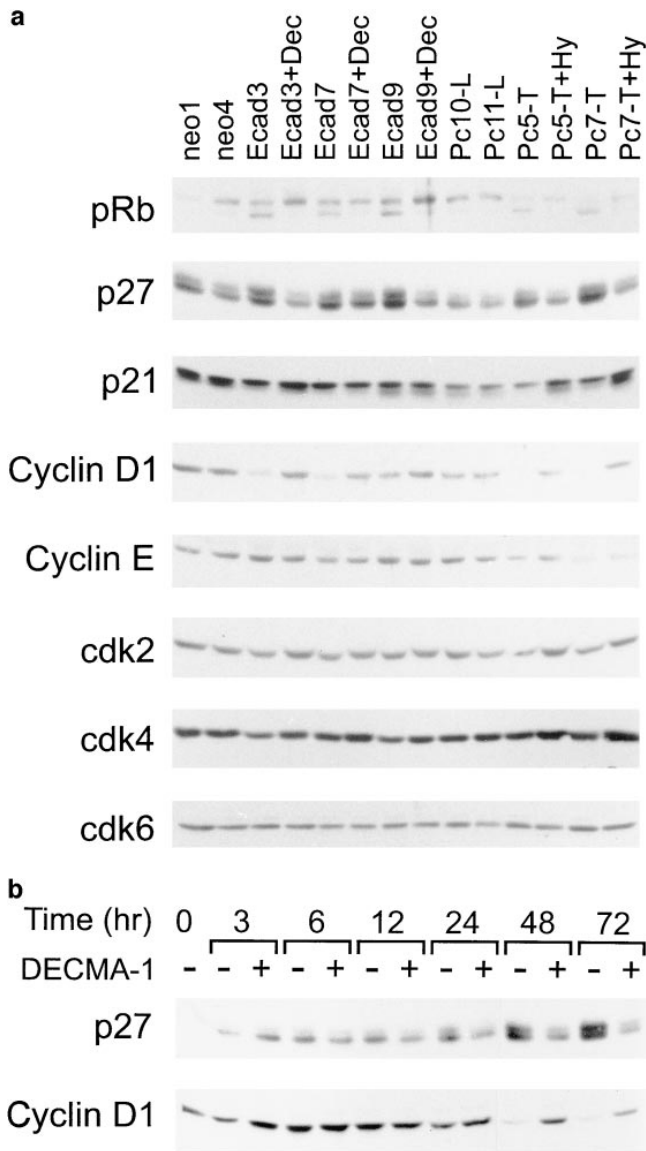
Because p27 upregulation by cell–cell contact occurred rapidly, and this CKI has been shown to inhibit cyclin E-cdk2 (Slingerland et al., 1994; Hengst et al., 1994; Polyak et al., 1994), we analyzed cyclin E-associated cdk2 activity. Cyclin E-cdk2 activity was reduced in E-cadherin transfectants compared with neomycin resistant controls (see Fig. 5 a). Likewise, tightly adherent Pc5-T cells displayed low cyclin E-associated kinase activity in three-dimensional culture. Kinase activity was increased when hyaluronidase

was added to the medium, or when cells were grown in monolayer culture. Immunoprecipitation blotting revealed higher levels of p27 bound to cyclin E in tightly adherent spheroids compared with their loosely adherent counterparts (see Fig. 5 b), while cyclin E levels were unchanged (not shown). To demonstrate that increased p27 binding was responsible for the decreased cyclin E-associated kinase activity, we immunodepleted p27 from cadherin-arrested cells (Fig. 5 c). As shown in Fig. 5 d, almost all of

**Table I.** Ability of Cells to Aggregate in Three-dimensional Culture Correlates with Fraction of Proliferating EMT/6 Cells According to Percentage of BrdU-labeled Cells After a 24-h Pulse

	neo1	neo4	neo7	neo8	Ecad3	Ecad7	Ecad9	Ecad18	Pc10-L	Pc5-T	Pc5-T + HYase <sup>l</sup>
Aggregates*	–	–	–	–	+	+	+	+	–	+	–
2-D <sup>‡</sup>	99.2	99.7	99.9	99.9	99.8	99.9	99.8	99.5	99.7	99.2	99.2
3-D <sup>§</sup>	98.1	97.1	95.1	96.7	49.8	53.8	59.9	70.3	97.1	53.5	95.8

\*Ability of cells to form aggregates in three-dimensional culture. (–) indicates loosely or nonadherent aggregates, and (+) indicates tightly adherent aggregates as shown in Fig. 1. <sup>‡</sup>Two-dimensional monolayer culture. <sup>§</sup>Three-dimensional culture. <sup>l</sup>Hyaluronidase-treated.



**Figure 4.** Changes in cell cycle molecules in response to intercellular adhesion of EMT/6 cells as detected by immunoblotting. (a) Three-dimensional cultures of EMT/6 neomycin-resistant control transfectants (*neo1*, *neo4*), E-cadherin transfectants (*Ecad3*, *Ecad7*, and *Ecad9*), EMT/6 loosely-adherent variants (*Pc10-L* and *Pc11-L*) or HA-dependent tightly adherent variants (*Pc5-T*, *Pc7-T*) were examined. Immunoblots were probed for the cell cycle molecules indicated. E-cadherin- or HA-dependent intercellular adhesion was prevented by treatment with either DECMA-1 antibody (*Dec*) or hyaluronidase (*Hy*), respectively. Note changes in pRb phosphorylation and levels of cyclin D1 and p27 in cells allowed to aggregate in three-dimensional culture. (b) Time course demonstrating changes in p27 and cyclin D1 protein levels as cells aggregate in three-dimensional culture. Cells transfected with E-cadherin (*Ecad3*) grown in the presence or absence of 2  $\mu$ g/ml DECMA-1 antibody for the indicated times were probed for p27 and cyclin D1 by immunoblotting. At time = 0 cells were transferred from two- to three-dimensional culture.

the detectable cyclin E was removed from the cell lysate by serial immunodepletion of p27. After p27 immunodepletion, the minor amount of residual free cyclin E-cdk2 remaining in the supernatant had a dramatically elevated

kinase activity (Fig. 5 e). This increase in kinase activity may occur because p27 is no longer available to block cyclin E-cdk2 activity directly or indirectly through a release of the steric inhibition of CAK phosphorylation imposed by p27 (Mandal et al., 1998; Kato et al., 1994). Taken together, these studies suggest that the inhibition of cyclin E-cdk2 by E-cadherin is mediated by p27.

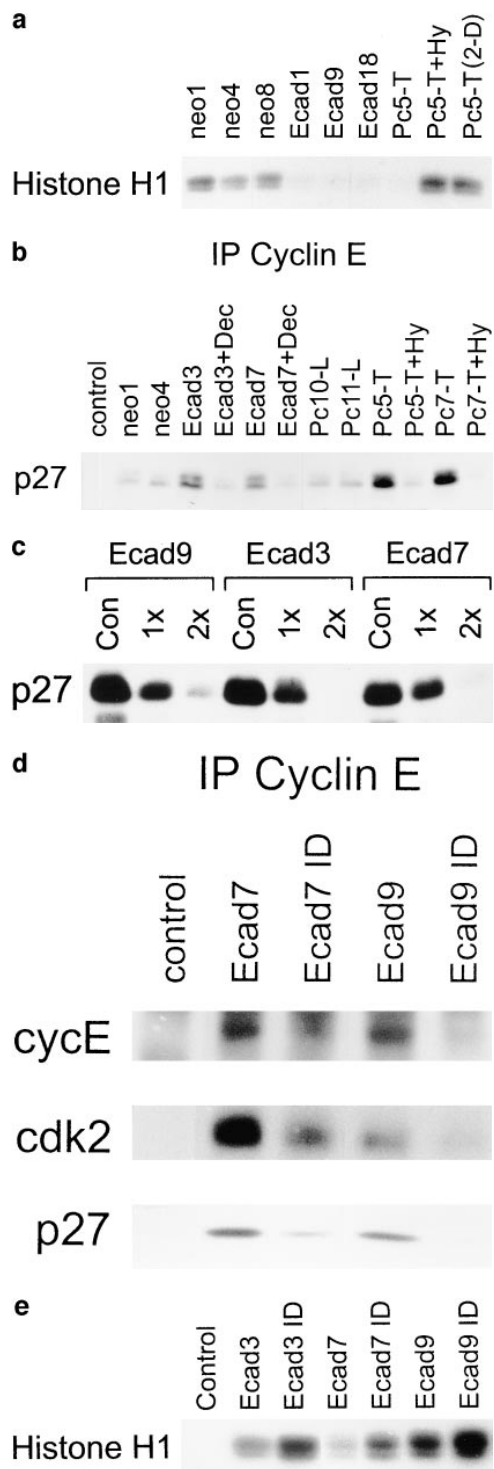
#### *E-Cadherin-neutralizing Antibodies Fail to Release $\beta$ -Catenin from E-Cadherin*

The mechanism by which E-cadherin regulates p27 is unclear. Because  $\beta$ -catenin has been implicated in signal transduction (Behrens et al., 1996; Molenaar et al., 1996; Huber et al., 1996), we investigated whether DECMA-1 induces growth of E-cadherin transfectants by stimulating the release of  $\beta$ -catenin bound to E-cadherin at the cell surface. As shown in Fig. 2, levels of endogenous  $\beta$ -catenin protein were elevated in all EMT/6-E-cadherin transfectants, an observation consistent with other reports (Papkoff, 1997); this is most likely due to stabilization of  $\beta$ -catenin bound to E-cadherin at the cell surface.  $\beta$ -catenin levels in E-cadherin immunocomplexes were unaffected by DECMA-1 antibody treatment (see Fig. 2). Likewise, levels of E-cadherin bound to immunoprecipitated  $\beta$ -catenin were unchanged by this antibody treatment (data not shown). These results suggest that changes in the association of  $\beta$ -catenin with E-cadherin are not involved in growth stimulation mediated by E-cadherin-neutralizing antibodies.

#### *Endogenous E-Cadherin Mediates Intercellular Adhesion, Growth Suppression, and Upregulation of p27 in Human Carcinoma Cell Lines*

To ascertain the role of E-cadherin in contact-dependent growth inhibition, we analyzed the effect of SHE78-7, an anti-human E-cadherin-neutralizing antibody (Watabe et al., 1994), on various human E-cadherin positive colon (HT29 and DLD-1), breast (MCF-7 and BT-20), and lung (L23) carcinoma cell lines. Similar to our observations with EMT/6-E-cadherin transfectants, SHE78-7 antibody was highly effective at preventing E-cadherin-mediated adhesion (Fig. 6 and data not shown). In contrast, intercellular adhesion of these human cells was unaffected by isotype-matched control IgG, and SHE78-7 failed to block adhesion of tightly adherent HBL-100 breast carcinoma cells that lack any detectable E-cadherin expression (see Figs. 6 and 8).

We next asked whether blocking E-cadherin function could stimulate the growth of these human cell lines. As shown in Fig. 7, adding SHE78-7 to the culture medium caused a dose-dependent increase in [ $^3$ H]thymidine uptake by each of the colon, breast, and lung cancer cell lines tested. The effect of SHE78-7 on growth paralleled its effect on intercellular adhesion. Interestingly, antibody stimulation reached a plateau at concentrations of 1–2  $\mu$ g/ml, presumably due to saturation of antibody-binding sites at the cell surface. The antibody was completely nontoxic, even at concentrations of 50  $\mu$ g/ml (data not shown). Again, E-cadherin-negative HBL-100 cells were unaffected by SHE78-7.



**Figure 5.** Increased p27 binding to cyclin E mediates reduced Cyclin E-associated kinase activity in aggregated cells. (a) Cyclin E-associated cdk2 kinase activity, as measured by phosphorylation of Histone H1 substrate in representative neomycin-resistant control transfectants (*neo1*, *neo4*, *neo8*) or E-cadherin transfectants (*Ecad1*, *Ecad9*, *Ecad18*) grown in three-dimensional culture. Also included are Pc5-T cells grown in monolayer (2-D) culture, or in three-dimensional culture in either the presence or absence of hyaluronidase (Hy). (b) Increased levels of p27 bound to cyclin E in tightly adherent EMT/6 spheroids. After immunoprecipitating cyclin E from tightly- or loosely adherent aggregates, levels of cyclin E-bound p27 were detected by immunoblotting. Cell-free

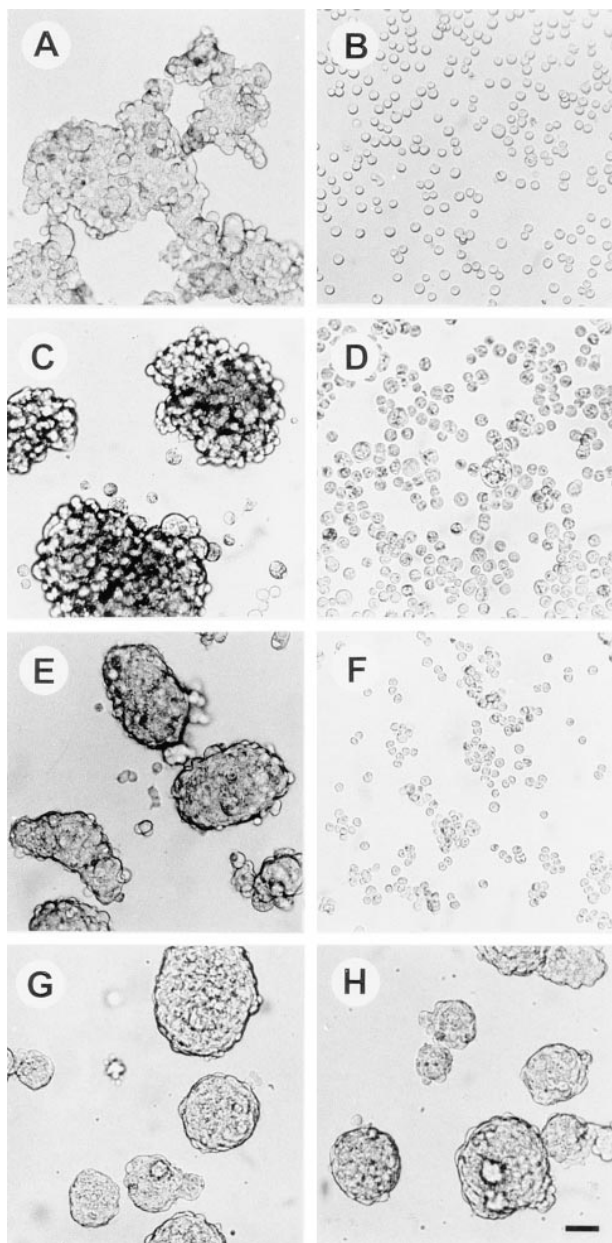
lysis buffer was used for the control sample. (c) Immunodepletion of p27 from aggregated E-cadherin transfectants. Cell extracts immunodepleted one (1×) or two (2×) times with antibodies against p27 were probed for p27 by immunoblotting. Controls (*Con*) represent extracts before immunodepletion (d). Cyclin E, cyclin E-associated cdk2, and p27 are markedly reduced in p27-immunodepleted (*ID*) supernatants from E-cadherin-aggregated cells. Cyclin E immunoprecipitates from lysates with or without prior immunodepletion with p27 antibodies were probed for cyclin E, cdk2, or p27 by immunoblotting. As a control, *Ecad9* lysates were immunoprecipitated with nonspecific rabbit IgG antibodies instead of cyclin E antibodies. (e) Cyclin E-associated cdk2 kinase activity is increased after p27-immunodepletion (*ID*) of E-cadherin-arrested cells. The representative control in this experiment was *Ecad7* lysate immunoprecipitated with nonspecific rabbit IgG antibodies instead of cyclin E antibodies. In each of these experiments cells were cultured for 48 h in three-dimensional culture.

### Enforced Upregulation of p27 Inhibits the Proliferation Induced by Anti-E-Cadherin Antibodies

To determine whether the effects of E-cadherin on pRb, p130, cyclin D1, and p27 in murine EMT/6 cells were similar in human cells, we analyzed levels of these proteins in colon (HT29 and DLD-1), lung (L23), and breast (BT20 and HBL100) carcinoma cell lines. Cells grown for 48 h in three-dimensional culture in either the presence or absence of SHE78-7 antibody were analyzed by Western blot analysis. Similar to our previous results with EMT/6-E-cadherin transfectants, when E-cadherin-mediated adhesion of human cells was abrogated by SHE78-7, there was a shift to increased phosphorylation of pRb and p130 (see Fig. 8). However, unlike in our initial experiments, adding E-cadherin-neutralizing antibodies did not result in a detectable increase in cyclin D1 by 48 h. In each of the E-cadherin-positive cell lines allowed to aggregate, p27 levels increased from ~2.5-fold to ~15-fold depending on the cell line, and this effect could be reversed by SHE78-7 antibody. Importantly, E-cadherin-negative HBL100 cells were unaffected by E-cadherin antibody treatment.

To assess further the role of p27 in E-cadherin-mediated growth suppression, we transfected HT29 cells with a metallothionein-inducible human p27 expression vector. One of the transfectants, H/MT27, which was found to produce high levels of p27 in response to treatment with zinc and cadmium, was plated into three-dimensional culture in the presence or absence of SHE78-7 E-cadherin-neutralizing antibody. Importantly, by titrating the concentration of zinc and cadmium, we were able to control the level of p27 in these cells accurately. Thus, in HT29 cells in which intercellular adhesion was prevented by SHE78-7 antibody treatment, we could enforce expression of the same amount of p27 that was present in tightly adherent cells grown in the absence of SHE78-7. As shown in Fig. 9, enforced elevation of p27 in SHE78-7-treated cells inhibited by ~50% the increase in proliferation mediated by blocking E-cadherin with SHE78-7. In contrast, neither the HT29 parent population or neomycin-resistant control transfectants were significantly affected by treatment with zinc and cadmium. We have observed similar results for pMT27 transfectants of DLD-1 cells (data not shown).



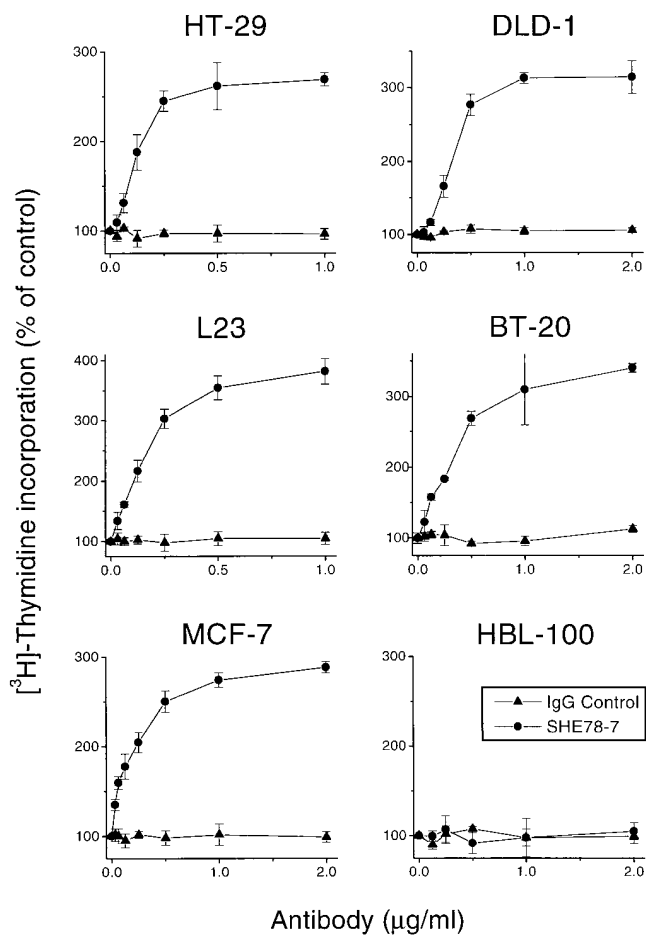


**Figure 6.** Morphology of human E-cadherin-positive cell lines in three-dimensional culture. E-cadherin positive HT29 colon (*a* and *b*), L23 lung (*c* and *d*), BT20 breast (*e* and *f*), or E-cadherin-negative HBL100 breast carcinoma cell lines (*g* and *h*) were cultured in the presence of 2  $\mu\text{g/ml}$  SHE78-7 anti-E-cadherin antibody (*b*, *d*, *f*, and *h*) or isotype-matched control IgG antibody (*a*, *c*, *e*, and *g*). Cells were photographed 24 h after plating. Bar, 50  $\mu\text{m}$ .

These results suggest that p27 levels are rate-limiting for the growth of cells that are loosely adherent, and support the conclusion that increased p27 levels mediate E-cadherin-dependent growth inhibition.

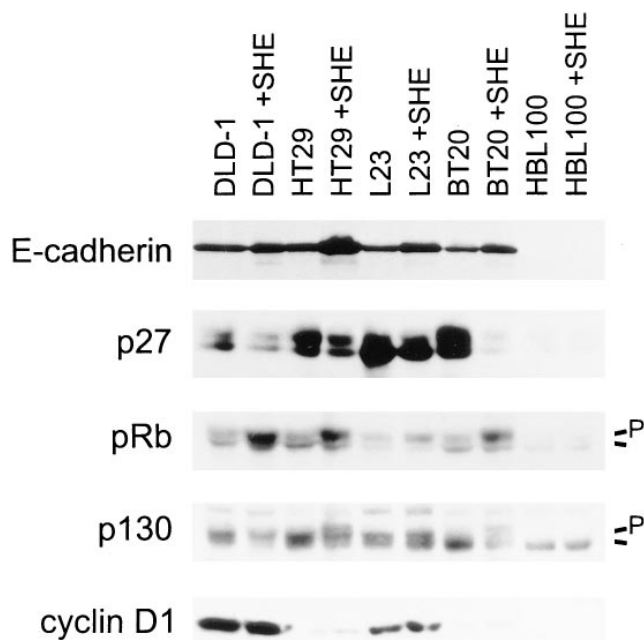
#### ***E-Cadherin Suppresses Growth by Inhibiting Downregulation of p27 by Growth Factors***

Recent studies have provided circumstantial evidence that E-cadherin-mediated adhesion may suppress growth by



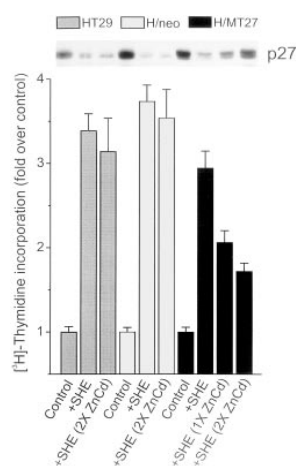
**Figure 7.** E-cadherin-neutralizing antibodies stimulate the growth of human E-cadherin positive cell lines in three-dimensional culture. Colon (*HT29*, *DLD-1*), lung (*L23*), and breast (*BT20*, *MCF-7*, *HBL100*) carcinoma cell lines were cultured for 48 h in the presence of SHE78-7 anti-E-cadherin antibodies (●) or isotype-matched control IgG antibodies (▲). After pulsing with [ $^3\text{H}$ ]thymidine, radioactivity incorporated into DNA was measured and expressed as a percentage of nontreated control cells. Note that E-cadherin antibodies only stimulate the growth of cell lines that display E-cadherin-mediated intercellular adhesion.

inactivating RTK activity. Before testing this directly, we asked whether or not E-cadherin-neutralizing antibodies could stimulate growth in the absence of mitogens in the culture medium. HT29 was used since this cell line is E-cadherin-positive and continues to grow, albeit more slowly, in the absence of FCS (see Fig. 10 *a*). When HT29 cells were treated with SHE78-7 in serum-free medium, p27 levels remained high, and [ $^3\text{H}$ ]thymidine uptake was unaffected, even though intercellular adhesion was prevented. When 10% FCS was added to the medium alone, only a twofold increase in [ $^3\text{H}$ ]thymidine uptake of compact aggregates was observed. Adding 50 ng/ml TGF- $\alpha$  was moderately more potent, causing an  $\sim$  threefold stimulation of [ $^3\text{H}$ ]thymidine uptake. These were optimal growth stimulatory concentrations of FCS and TGF- $\alpha$  in three-dimensional culture as determined by dose-response studies (data not shown). However, if E-cadherin-mediated adhesion was blocked by SHE78-7, growth stimulation by FCS



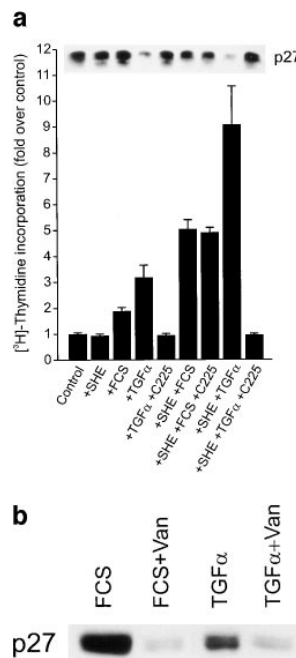
**Figure 8.** Changes in cell cycle regulators in response to E-cadherin-mediated adhesion of human carcinoma cell lines. Immunoblots of colon (HT29, DLD-1), lung (L23), and breast (BT20) carcinoma cell lines were probed for p27, pRb, p130, and cyclin D1. E-cadherin-dependent intercellular adhesion was prevented by treatment with 2  $\mu$ g/ml of SHE78-7 antibody (+SHE).

was even greater ( $\sim$  fivefold), while TGF- $\alpha$  stimulated growth almost tenfold compared with serum-free controls. These effects on [ $^3$ H]thymidine uptake were clearly synergistic because SHE78-7 antibody had no effect on proliferation by itself in the absence of mitogens. When TGF- $\alpha$ , FCS, and SHE78-7 were all added simultaneously, an even larger ( $\sim$ 14-fold) growth stimulation was noted (data not shown). Importantly, the growth stimulation observed after treatment with E-cadherin antibodies was associated



**Figure 9.** Enforced expression of p27 inhibits cell growth in the presence of E-cadherin neutralizing antibodies. HT29 cells transfected with a metallothionein-inducible p27 expression plasmid (*H/MT27*), neomycin vector alone (*H/neo*), or nontransfected HT29 cells (*HT29*) were grown in three-dimensional culture for 48 h in the absence (*Control*) or presence of SHE78-7 antibody (+SHE). For induction of the metallothionein promoter, cultures were supplemented with either a one (1 $\times$ ) or two times (2 $\times$ ) concentration of Zinc and Cadmium (*ZnCd*). After pulsing

with [ $^3$ H]thymidine, radioactivity incorporated into DNA was measured and expressed as fold increase in counts over nontreated control cells. The corresponding levels of p27 as determined by immunoblotting are shown in the inset.



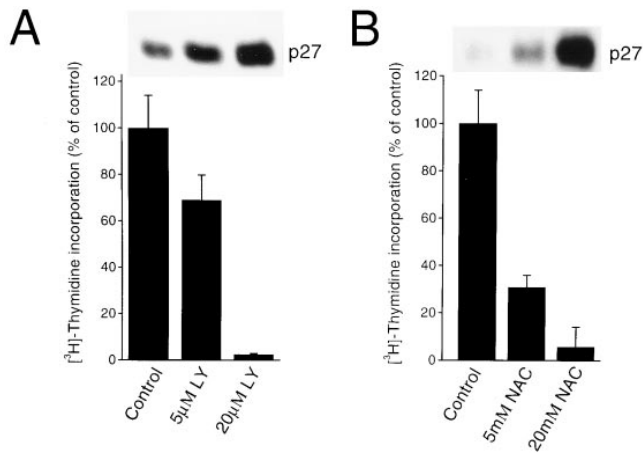
**Figure 10.** E-cadherin inhibits growth factor-mediated downregulation of p27 through a phosphatase-dependent mechanism. (a) Mitogens are required for growth stimulation in the presence of E-cadherin-neutralizing antibodies. Nontreated HT29 cells (*control*) were grown in serum-free medium for 48 h. Treated cells were grown in the presence of 2  $\mu$ g/ml SHE78-7 antibody (+SHE), 10% FCS (+FCS), or 50 ng/ml TGF- $\alpha$  (+TGF- $\alpha$ ), either alone or in various combinations. To prevent TGF- $\alpha$  activity, cultures were treated with c225-neutralizing anti-EGFR antibody. All factors were added to three-dimensional cell cultures at the time of cell plating. After pulsing with [ $^3$ H]thymidine, radioactivity incorporated into DNA was measured and expressed as fold increase in counts over nontreated control cells. The corresponding levels of p27 as determined by immunoblotting are shown in the inset. (b) Inhibition of phosphatase activity lowers p27 levels in aggregated HT29 cells. HT29 cells plated in three-dimensional culture in the presence of either FCS or TGF- $\alpha$  were treated with 50  $\mu$ M sodium orthovanadate (+Van). 48 h later, cells were collected and levels of p27 were determined by immunoblotting.

with a reduction in p27 protein. Furthermore, the ability of TGF- $\alpha$  to both stimulate growth and reduce p27 protein levels could be completely abrogated by adding C225 EGFR-blocking antibody to the culture medium. In contrast, treatment with C225 had no effect on FCS-induced growth stimulation. We conclude that E-cadherin inhibits growth of HT29 cells by inhibiting either TGF- $\alpha$  stimulation of EGFR kinase activity or serum stimulation of an uncharacterized mitogenic pathway.

One mechanism by which cell-cell adhesion may prevent kinase activity of growth factor receptors is by stimulating endogenous phosphatase activity. To determine if such a mechanism could be responsible for elevated p27 protein levels in aggregated HT29 cells, we treated these cells with the phosphatase inhibitor sodium orthovanadate. As shown in Fig. 10 *b*, vanadate treatment resulted in low p27 protein levels in adherent spheroids. Thus, endogenous phosphatase activity is necessary for p27 upregulation in compact spheroids.

### Intracellular Signaling Events Involved in Regulation of p27 Protein Levels

PI3K is activated by a number of growth factors including EGF, and is a direct downstream target of ras (Bjorge et al., 1990; Rodriguez-Viciana et al., 1994). To test whether PI3K signaling plays a role in downregulating p27 levels and stimulating proliferation of HT29 cells dispersed with SHE78-7 antibody, we treated these cells with LY294002,



**Figure 11.** Inhibiting PI3K activity or generating reactive oxygen species elevates p27 protein levels and reduces [<sup>3</sup>H]thymidine uptake of E-cadherin antibody-dispersed HT29 cells. Cells plated into three-dimensional culture in the presence of TGF- $\alpha$  and SHE78-7 antibody were treated with either (a) the PI3K inhibitor LY294002 (LY) or (b) the antioxidant *N*-acetyl-L-cysteine (NAC). [<sup>3</sup>H]thymidine incorporation was measured after 24 h. The inset for both a and b shows the corresponding levels of p27 protein as determined by immunoblotting.

a potent inhibitor of PI3-K. As shown in Fig. 11, treatment with LY294002 inhibited growth and increased p27 levels in these loosely adherent E-cadherin antibody-treated cells. A similar dose-dependent growth-inhibition was observed when these cells were treated with the antioxidant *N*-acetyl-L-cysteine (NAC). The effects of LY294002 and NAC on proliferation and p27 levels did not result from cytotoxicity, as both were reversed upon drug removal. Importantly, both PI3K and reactive oxygen species are thought to function in signaling pathways that operate downstream of ras, and that are separate from the raf/MAP kinase pathway (Rodriguez-Viciano et al., 1997; Irani et al., 1997). In agreement with this, the MEK inhibitor PD98059 did not affect growth and p27 levels in HT29 cells (data not shown).

## Discussion

It has long been recognized that nontransformed cell lines are contact-inhibited when they reach confluence in monolayer tissue culture. However, only recently has it been shown using normal fibroblasts and epithelial cells that intercellular adhesion molecules such as NCAM and E-cadherin, respectively, are involved in this process (Aoki et al., 1991; Takahashi and Suzuki, 1996; Kandikonda et al., 1996). Although loss of contact inhibition is one of the classical hallmarks of transformation, many tumor cell lines also appear to maintain a degree of contact inhibition that we refer to as contact-dependent growth inhibition/suppression. This phenomenon, which appears to be most pronounced when cells are cultured as three-dimensional masses, may reflect an incomplete loss of contact inhibition during the transformation process.

## E-Cadherin Mediates Contact-dependent Growth Inhibition of Tumor Cells

The present study suggests that, as for normal cells, E-cadherin is also involved in contact-dependent growth inhibition of carcinoma cell lines that maintain functional E-cadherin. This was shown using E-cadherin-neutralizing antibodies that simultaneously disrupted adhesion and stimulated proliferation of several E-cadherin-positive breast, colon, and lung carcinoma cell lines grown in three-dimensional culture. Similar effects of E-cadherin on proliferation were obtained by Watabe and coworkers, in this case using an  $\alpha$ -catenin-expressing variant of the F9 small cell lung carcinoma cell line that has an intact E-cadherin adhesion system (Watabe et al., 1994). Interestingly, this cell line displays very weak attachment to tissue culture flasks, growing mostly as free-floating multicellular spheroids. Furthermore, transfection of E-cadherin into carcinoma cell lines that have lost E-cadherin expression has been shown to cause morphological reversion to an adherent epithelial phenotype, and to decrease tumor growth rate when the cells are injected subcutaneously into mice (Miyaki et al., 1995; Navarro et al., 1991). Similar results were obtained after transfection of wild-type  $\alpha$ -catenin into an ovarian cell line with a mutant  $\alpha$ -catenin gene (Bullions et al., 1997). Hermiston and Gordon have shown that inactivation of endogenous E-cadherin in intestinal crypt cells using a dominant negative strategy leads to the formation of adenomas (Hermiston and Gordon, 1995b). Conversely, forced overexpression of E-cadherin suppresses proliferation in the crypt (Hermiston et al., 1996). These studies, in addition to our own, provide strong evidence that E-cadherin is not only a suppressor of invasion (Vleminckx et al., 1991), but also has the ability to suppress proliferation. Importantly, E-cadherin-dependent growth suppression appears to be undetectable in subconfluent monolayer culture, but can be observed in confluent monolayers or three-dimensional cell cultures.

## Increased Levels of p27 are Involved in E-Cadherin-mediated Growth Suppression

To elucidate the mechanism(s) responsible for E-cadherin-dependent growth suppression, we have investigated the effect of E-cadherin-mediated adhesion on cell cycle regulators. Our analysis revealed that the CDK inhibitor p27<sup>kip1</sup> was consistently elevated in a panel of cell lines that manifest E-cadherin-dependent adhesion compared with the same cell lines rendered loosely adherent by treatment with anti-E-cadherin antibodies. Further studies demonstrated that this increase in p27 is responsible, at least in part, for E-cadherin-dependent growth suppression through cyclin E-cdk2 inhibition. The E-cadherin-dependent increase in p27 was sufficient to saturate almost all cellular cyclin E-cdk2. Very little cyclin E was detectable after immunodepletion of p27 from E-cadherin-expressing clones, and loss of associated p27 led to a dramatic increase in the activity of residual cyclin E-cdk2 complexes remaining after p27-depletion. Thus, E-cadherin-mediated adhesion lead to suppression of cyclin E-associated kinase activity through increased p27 binding to the cyclinE-cdk2 complex. Furthermore, when cell spheroids disrupted by E-cadherin-neutralizing antibodies

were engineered to express p27 levels corresponding to those observed in adherent spheroids, antibody-stimulated proliferation was repressed by ~50%. Consistent with our results, when p27 activity was first identified it was found to mediate cyclin E-cdk2 inhibition in contact-inhibited monolayer cultures of MvLu epithelial cells (Slingerland et al., 1994; Polyak et al., 1994). Interestingly, MvLu cells express E-cadherin at sites of cell-cell contact (Brady-Kalnay et al., 1993). We have previously shown that p27 is an essential mediator of growth arrest in response to HA-dependent aggregation (St. Croix et al., 1996a). We conclude, therefore, that p27 plays an important role in both E-cadherin and HA-dependent growth arrest. Thus, distinct adhesion mechanisms may ultimately impinge on the same signal transduction pathways regulating the cell cycle machinery.

Enforced p27 overexpression in HT29 cells could only inhibit incompletely the proliferative effect of anti-E cadherin antibody treatment. Thus, other factors are likely to regulate E-cadherin-dependent growth inhibition. When E-cadherin-positive cell lines were allowed to aggregate, we also observed hypophosphorylation of pRb and p130, and in tightly adherent EMT/6 cells, cyclin D1 was reduced. The loss of pRb and p130 phosphorylation in adherent cells is most likely a consequence of reduced cyclin-cdk activity as cells arrest in G1/G0. However, the E-cadherin-dependent downregulation of cyclin D1 that occurs after cells have left the cell cycle may contribute to maintenance of the arrested state. Effects of adhesion on cyclin D1 appear to be cell type-dependent because, in our panel of E-cadherin-positive human tumor lines, cyclin D1 levels appeared unaffected by treatment with E-cadherin-neutralizing antibodies. It is possible, however, that other D-type cyclins are involved. In the EMT/6 cells, E-cadherin may affect cyclin D1 turnover through ubiquitin-mediated proteolysis (Diehl et al., 1997). While further studies are necessary to address these possibilities, a connection between cyclin D and E-cadherin is intriguing given the relationship between growth factor dependence of E-cadherin-mediated growth suppression and the known ability of growth factors to regulate cyclin D turnover (Sherr, 1995).

### ***Mitogenic Pathways are Required for E-Cadherin-dependent Growth Suppression and Elevation of p27 Protein Levels***

During the course of these studies, we became interested in understanding signal transduction pathway(s) by which E-cadherin regulates p27. Initially we hypothesized that E-cadherin may sequester  $\beta$ -catenin at the cell surface, thereby preventing its ability to translocate to the nucleus and alter transcription of growth-promoting genes. However, in preliminary experiments we could find no evidence to support this hypothesis, and our later results suggested that classical mitogenic pathways are necessary for growth stimulation in the presence of E-cadherin-neutralizing antibodies. Indeed, under serum-free conditions, E-cadherin-neutralizing antibodies were unable to stimulate growth or reduce p27 levels in HT29 cells, even though intercellular adhesion was completely prevented. However, E-cadherin-neutralizing antibodies synergized with

FCS or TGF- $\alpha$ , increasing their capacity to stimulate growth of HT29 cells by 2.5- and 3.2-fold, respectively. Importantly, the effect of TGF- $\alpha$  on both p27 levels and growth could be completely abolished by adding C225-neutralizing antibody against the EGFR to cell cultures. Thus, E-cadherin-dependent adhesion regulates p27 levels and proliferation indirectly by preventing the mitogenic activity of soluble growth factors.

It is unclear exactly how adhesion mediated by E-cadherin prevents signaling through RTKs such as the EGFR, which in turn leads to increased levels of p27 protein. Several reports have shown that ligand-induced phosphorylation of RTKs such as the EGFR or Erb-B2 is lost upon cell-cell contact, even in the presence of excess ligand (Mansbridge et al., 1992; Lichtner and Schirrmacher, 1990). In one study, E-cadherin antibodies were shown to prevent contact inhibition and restore EGFR phosphorylation in response to EGF (Takahashi and Suzuki, 1996). Because phosphorylation of RTKs can be rescued upon cell-cell contact by adding phosphatase inhibitors (Mansbridge et al., 1992; Sorby and Ostman, 1996), contact-dependent RTK inactivation is thought to be a consequence of endogenous phosphatase activity. Indeed, several receptor protein tyrosine phosphatases (RPTPs) that span the cellular membrane have been shown to be upregulated and activated by cell-cell contact (Pallen and Tong, 1991; Ostman et al., 1994; Gebbink et al., 1995; Gaits et al., 1995) and in some cases colocalize with cadherin-catenin complexes (Wang et al., 1996; Kypta et al., 1996; Balsamo et al., 1996). Tight adhesion mediated by E-cadherin or other adhesion mechanisms may serve to strengthen interactions between adjacent RPTPs, ensuring high continuous phosphatase activity. This in turn may lead to dephosphorylation of the EGFR, and a buildup of p27 levels (St. Croix and Kerbel, 1997). In support of this model, we found that the phosphatase inhibitor orthovanadate suppresses p27 induction in aggregating EMT6/E-cadherin transfectants.

Interestingly,  $\beta$ -catenin has been found by coimmunoprecipitation studies to link cadherin-catenin complexes to several receptor tyrosine kinases such as the EGFR, c-erbB2, and possibly *c-met* (Hoschuetzky et al., 1994; Shibata et al., 1996; Kanai et al., 1995). Whether or not adhesion mediated by E-cadherin can inhibit RTK activity through such an interaction is unclear. However, in at least some systems the opposite appears to be true, that is, ligand-induced activation of RTKs appears to destabilize adhesion (Shibamoto et al., 1994; Shiozaki et al., 1995). It has been proposed that this effect may be due to the phosphorylation of  $\beta$ -catenin by RTKs since phosphorylated  $\beta$ -catenin is not found in cadherin-catenin complexes bound to the actin cytoskeleton (Hoschuetzky et al., 1994). Although plausible, evidence for this hypothesis is mostly circumstantial, and one study demonstrated that phosphorylation of  $\beta$ -catenin is not required to shift cadherin-based adhesion from the strong to the weak state (Takeda et al., 1995). Furthermore, we and others have found that adhesion is unaffected by the presence of mitogens such as TGF- $\alpha$  and EGF, arguing against a role for RTK-induced destabilization of adhesion (Shibamoto et al., 1994). Instead, it has been proposed using sparse monolayer cultures of normal epithelial cells that RTK phosphorylation of  $\beta$ -catenin may inhibit binding of  $\beta$ -catenin to  $\alpha$ -catenin,

thereby preventing an inappropriate connection to the underlying cytoskeleton before cell–cell contact occurs (Takahashi and Suzuki, 1996). Once cell–cell contact occurs, dephosphorylation of RTKs by phosphatases may effectively block  $\beta$ -catenin phosphorylation.

The mechanisms by which mitogen-activated RTK signaling pathways regulate p27 is an area of active investigation. Several studies have demonstrated that p27 levels are reduced in epithelial cells, endothelial cells, fibroblasts, and T-cells in response to growth factors such as EGF, VEGF, PDGF, and IL2 (Sandhu et al., 1997; Peng et al., 1996; Watanabe et al., 1997; Agrawal et al., 1996; Shankland et al., 1997; Winston et al., 1996; Coats et al., 1996). Furthermore, depletion of p27 using antisense strategies partially prevents withdrawal from the cell cycle in response to mitogen deprivation (Coats et al., 1996; Rivard et al., 1996). Exit from quiescence after adding growth factors has been shown to decrease p27 protein levels due to increased proteasomal degradation of p27 (Pagano et al., 1995), but other translational mechanisms such as a decreased rate of p27 synthesis (Hengst and Reed, 1996; Agrawal et al., 1996) or a decrease in transcription (Peng et al., 1996) have also been described. In fibroblasts, ras signaling through the MAP kinase pathway has been implicated in enhanced p27 degradation (Takuwa and Takuwa, 1997). Interestingly, p27 is phosphorylated by MAP kinase *in vitro*, and phosphorylated p27 shows reduced binding to and inhibition of cdk2 (Kawada et al., 1997; Alessandrini et al., 1997). Phosphorylation of p27 by cyclin E-cdk2 may also regulate p27 destruction in response to mitogens (Sheaff et al., 1997; Vlach et al., 1997).

In our experiments, mitogenic stimulation through abrogation of the E-cadherin pathway by SHE78-7 antibody was dependent on TGF- $\alpha$  signaling. Stimulation by TGF- $\alpha$  leads to activation of both PI3K and MAPK pathways. In HT29 cells grown as spheroids, such treatment activates cellular mitogenesis only after the cells have been disaggregated with the neutralizing anti-E-cadherin antibody with a commensurate decrease in p27 levels. We observed that both thymidine incorporation and p27 downregulation induced by TGF- $\alpha$  can be blocked by the C225-neutralizing monoclonal anti-EGF receptor-neutralizing antibody, the PI3K inhibitor LY29002, and an inhibitor of superoxide-mediated signaling, NAC, but not by the MEK-1 inhibitor PD98059. These observations suggest that the effect of E-cadherin on p27 regulation is indirect, and possibly involves activation of PI3K and superoxide signaling via the EGF receptor.

Based on our studies of HT29 cells, it appears that signals from at least three independent sources impinge on the cell cycle machinery to regulate proliferation of tumor cells. First, unlike nontransformed cells, these cells are anchorage-independent and grow autonomously in the absence of exogenously added growth factors. Signaling in this case appears to be constitutive, and may be the result of genetic alterations in oncogenes or tumor-suppressor genes. Indeed, HT29 cells are known to have mutated p53 and APC genes (Huang et al., 1994). Second, adding mitogens such as FCS or TGF- $\alpha$  to the growth medium enhances the rate of proliferation of HT29 tumor cells. Finally, results of this study demonstrate that adhesion mediated by E-cadherin was also a powerful regulator of

growth, repressing mitogenic growth stimulation more than threefold.

### *Incomplete Suppression of Tumor Growth by E-Cadherin*

An important finding from these studies is that contact-dependent growth suppression of tightly adherent tumor cells, in contrast to normal cells, is incomplete. In fact, we found it difficult to detect any effect of E-cadherin–neutralizing antibodies on high cell density monolayer cultures of E-cadherin–positive tumor cells, unlike other studies that have used normal epithelial cells (Takahashi and Suzuki, 1996; Kandikonda et al., 1996). This discrepancy may be explained by a reduced sensitivity of tumor cells to undergoing contact-dependent growth inhibition. However, in a tumor-like three-dimensional context, all E-cadherin–positive tumor cell lines displayed a significant, albeit still incomplete, reduction in cell growth. The enhanced ability to detect contact-dependent growth inhibition in three-dimensional culture may be due to the fact that under such conditions tumor cells are exposed to a relatively greater area of cell surface contact, and consequently may receive a stronger growth inhibitory signal. Whatever the reasons, these results suggest a need for caution when studying tumor cell growth exclusively in monolayer culture.

Given the ability of E-cadherin to suppress both proliferation and invasion, carcinomas that lose E-cadherin–mediated adhesion either through direct loss of E-cadherin or indirectly through loss of  $\alpha$ - or  $\beta$ -catenin, may acquire a more aggressive behavior. In that regard, it will be of interest to understand what causes partial loss of contact inhibition in cell lines where E-cadherin-dependent adhesion is still observed (Miyaki et al., 1995; Navarro et al., 1991). One possibility is that transformation induces changes in the levels or activities of components of the E-cadherin system itself, resulting in weaker E-cadherin–mediated adhesion, for example through phosphorylation of E-cadherin (Stappert and Kemler, 1994) or  $\beta$ -catenin (Shibamoto et al., 1994). Alternatively, incomplete contact-dependent growth inhibition may result from loss of a secondary adhesion mechanism (such as HA-dependent adhesion), or from the acquisition of constitutive intracellular mitogenic signaling.

The fact that partial E-cadherin function is maintained in certain tumors and is not selected against despite its ability to inhibit growth, implies that some level of E-cadherin function may be advantageous to tumor cells. For example, E-cadherin has been shown to protect cells from undergoing apoptosis or programmed cell death (Hermiston and Gordon, 1995a; Hermiston and Gordon, 1995b). Thus, in addition to growing more slowly (Miyaki et al., 1995; Navarro et al., 1991), solid tumors with functional E-cadherin may also be resistant to apoptosis induced by environmental stress (Helmlinger et al., 1997) or cytotoxic anticancer therapy (Dimanche-Boitrel et al., 1994; St. Croix and Kerbel, 1997). Even when E-cadherin adhesion is completely disabled because of a loss of E-cadherin,  $\alpha$ -catenin, or  $\beta$ -catenin, tumor cells may still display partial contact-dependent growth inhibition due to the presence of other adhesion mechanisms such as the HA-

dependent mechanism observed in EMT/6 cells. Indeed, whereas E-cadherin expression is frequently downregulated with tumor progression, many other adhesion molecules are upregulated. These include CD44, CEA (carcinoembryonic antigen), and ICAM-1 (Jothy et al., 1995; Johnson, 1991).

In summary, we demonstrate using tumor cells that the cdk inhibitor p27 plays a significant role in E-cadherin-dependent growth inhibition. Growth inhibition by cell-cell adhesion may also help to explain why solid tumors, despite harboring multiple mutant oncogenes and tumor suppressor genes, often contain low proliferative fractions in vivo. E-cadherin can inhibit proliferation by inhibiting the activity of mitogenic pathways such as the EGFR, which in turn regulate the level of p27 in cells. E-cadherin-dependent upregulation of p27 may also help to explain the widely observed phenomenon of contact inhibition of normal epithelial cells.

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