E-cadherin Suppression Accelerates Squamous Cell Carcinoma Progression in Three-Dimensional, Human Tissue Constructs

Alexander Margulis,1 Weiitan Zhang,1 Addy Alt-Holland,1 Howard C. Crawford,2 Norbert E. Fusenig,3 and Jonathan A. Garlick†

1Division of Cancer Biology and Tissue Engineering, Department of Oral and Maxillofacial Pathology, School of Dental Medicine, Tufts University, Boston, Massachusetts; 2Department of Pharmacology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York; and 3German Cancer Research Center, Heidelberg, Germany

Abstract
We studied the link between loss of E-cadherin-mediated adhesion and acquisition of malignant properties in three-dimensional, human tissue constructs that mimicked the initial stages of squamous cell cancer progression. Suppression of E-cadherin expression in early-stage, skin-derived tumor cells (HaCaT-II-4) was induced by cytoplasmic sequestration of β-catenin upon stable expression of a dominant-negative E-cadherin fusion protein (H-2Kd-Ecad). In monolayer cultures, expression of H-2Kd-Ecad resulted in decreased levels of E-cadherin, redistribution of β-catenin to the cytoplasm, and complete loss of intercellular adhesion when compared with control II-4 cells. This was accompanied by a 7-fold decrease in β-catenin-mediated transcription and a 12-fold increase in cell migration. In three-dimensional constructs, E-cadherin-deficient tissues showed disruption of architecture, loss of adherens junctional proteins from cell contacts, and focal tumor cell invasion. Invasion was linked to activation of matrix metalloproteinase (MMP)–mediated degradation of basement membrane in H-2Kd-Ecad–expressing tissue constructs that was blocked by MMP inhibition (GM6001). Quantitative reverse transcription–PCR showed a 2.5-fold increase in MMP-2 and an 8-fold increase in MMP-9 in cells expressing the H-2Kd-Ecad fusion protein when compared with controls, and gel zymography showed increased MMP protein levels. Following surface transplantation of three-dimensional tissues, suppression of E-cadherin expression greatly accelerated tumorigenesis in vivo by inducing a switch to high-grade carcinomas that resulted in a 5-fold increase in tumor size after 4 weeks. Suppression of E-cadherin expression and loss of its function fundamentally modified squamous cell carcinoma progression by activating a highly invasive, aggressive tumor phenotype, whereas maintenance of E-cadherin prevented invasion in vitro and limited tumor progression in vivo. (Cancer Res 2005; 65(5): 1783-91)

Introduction
Squamous cell carcinoma originates as a premalignant process in which foci of abnormal cells selectively expand within a stratified squamous epithelium to dominate the tissue before connective tissue invasion (1). This is followed by tumor cell invasion through the basement membrane barrier that marks the point of transition from premalignant to malignant disease (2). In human cancers, acquisition of early invasive cellular properties is associated with the proteolytic degradation of basement membrane proteins and increased cell motility (3). However, the role played by alterations in intercellular adhesion in the transition from premalignant disease to early invasive carcinoma remain unclear. Due to its ability to integrate cell-cell adhesion with growth signaling (4), altered E-cadherin is known to play a significant role in the advanced stages of carcinoma progression (5, 6) and has been associated with the poor clinical prognosis of human cancers (7, 8). Studies done using two-dimensional, monolayer cultures have shown that abrogation of E-cadherin–mediated adhesion induced tumor cell invasion (9), whereas restoration of E-cadherin function resulted in growth retardation and inhibition of invasive properties (10). Consequently, E-cadherin has been defined as a tumor suppressor, whose loss is associated with the advanced stages of cancer progression (11, 12). However, the role that loss of E-cadherin function may play in early stages of carcinoma development are not well understood insasmuch as studies have shown either a decrease (13, 14) or increase (15, 16) in E-cadherin function during early cancer progression.

Further elucidation of the effect of altered cell-cell adhesion on early events in cancer progression has been limited by the nature of two-dimensional, monolayer culture, which lacks proper tissue architecture and microenvironmental context to mimic the earliest stages of tumorigenesis as they occur in vivo. Two-dimensional tissue culture systems cannot fully replicate the biologically meaningful pathways that couple cell-cell adhesion and growth that occur in three-dimensional tissues (17). Because cell-cell adhesion is intimately linked to tissue organization, the effect of the abrogation of E-cadherin–mediated adhesion on the early stages of cancer progression needs to be studied in three-dimensional tissue context to more accurately represent these initial events as they occur in vivo. To accomplish this, we have previously developed three-dimensional, human tissue constructs that mimic premalignant disease of stratified squamous epithelium as it occurs in human tissues (18). These three-dimensional tissues have been generated in the presence of structured basement membrane (19) with a well-characterized cell line (HaCaT-II-4) that represents an early stage in the malignant transformation process in human skin (20). Generating three-dimensional tissues that mimic the essential features of human, premalignant disease and inhibition of invasive properties (10). Consequently, E-cadherin has been defined as a tumor suppressor, whose loss is associated with the advanced stages of cancer progression (11, 12). However, the role that loss of E-cadherin function may play in early stages of carcinoma development are not well understood insasmuch as studies have shown either a decrease (13, 14) or increase (15, 16) in E-cadherin function during early cancer progression.

Further elucidation of the effect of altered cell-cell adhesion on early events in cancer progression has been limited by the nature of two-dimensional, monolayer culture, which lacks proper tissue architecture and microenvironmental context to mimic the earliest stages of tumorigenesis as they occur in vivo. Two-dimensional tissue culture systems cannot fully replicate the biologically meaningful pathways that couple cell-cell adhesion and growth that occur in three-dimensional tissues (17). Because cell-cell adhesion is intimately linked to tissue organization, the effect of the abrogation of E-cadherin–mediated adhesion on the early stages of cancer progression needs to be studied in three-dimensional tissue context to more accurately represent these initial events as they occur in vivo. To accomplish this, we have previously developed three-dimensional, human tissue constructs that mimic premalignant disease of stratified squamous epithelium as it occurs in human tissues (18). These three-dimensional tissues have been generated in the presence of structured basement membrane (19) with a well-characterized cell line (HaCaT-II-4) that represents an early stage in the malignant transformation process in human skin (20). Generating three-dimensional tissues that mimic the essential features of human, premalignant disease and inhibition of invasive properties (10). Consequently, E-cadherin has been defined as a tumor suppressor, whose loss is associated with the advanced stages of cancer progression (11, 12). However, the role that loss of E-cadherin function may play in early stages of carcinoma development are not well understood insasmuch as studies have shown either a decrease (13, 14) or increase (15, 16) in E-cadherin function during early cancer progression.

In the current study, we have characterized how suppression of E-cadherin expression and loss of E-cadherin function affects the behavior and phenotype of early-stage, epithelial tumor cells. We have shown that loss of E-cadherin–mediated adhesion was
associated with cytoplasmic sequestration of β-catenin, abrogation of adherens junctions and increased migration of II-4 cells in two-dimensional cultures. In three-dimensional, in vitro tissue constructs, cells that had lost E-cadherin function underwent tumor cell invasion upon activation of matrix metalloproteinase (MMP)–dependent, basement membrane degradation. When these three-dimensional tissues were transplanted to nude mice, in vivo tumorigenesis was significantly accelerated through the induction of a switch from a low-grade squamous cell carcinoma (SCC) to an aggressive and highly infiltrative SCC. Because control tumors that retained E-cadherin function did not undergo invasion in vitro and maintained their low-grade behavior after in vitro transplantation, our findings support the known role of E-cadherin as a tumor suppressor of early stages of cancer progression in human stratified squamous epithelium. We show that loss of E-cadherin–mediated adhesion is a critical step in the initiation of early tumor cell invasion in SCC and directs a switch to a highly aggressive form of this disease after invasion.

**Materials and Methods**

### Two-Dimensional, Monolayer Cultures

The HaCaT-ras-II-4 (II-4) cell line (20) was grown in DMEM containing 5% fetal bovine serum. Human dermal fibroblasts used for three-dimensional cultures were derived from newborn foreskins and grown in medium containing DMEM and 10% fetal bovine serum. 293 Phoenix cells used for retroviral production were maintained in DMEM containing 10% bovine calf serum. All cells were grown at 37°C in 5% CO₂.

**Retroviral Infection to Generate II-4 Cells with Altered Adhesion.** 293 Phoenix producer cells were transfected with pBABE, pBABE-H-2K-D, and pBABE-H-2K-D-ecdαC25 plasmids (ref. 22; gifts from Dr. F. Watt, Imperial Cancer Research Center, London, United Kingdom) by calcium phosphate method and transfected cells were grown at 32°C. Viral supernatants were collected 48 hours later and used to infect II-4 cells in the presence of 4 μg/mL polybrene for 3 hours at 32°C. Cells were maintained under puromycin selection (5 μg/mL) starting 2 days post infection.

### Three-Dimensional, Organotypic Constructs

Organotypic constructs with intact basement membrane were prepared as previously described (19). Early-passage human dermal fibroblasts were added to neutralized bovine type I collagen (Organogenesis, Canton, MA) to a final concentration of 2.5 × 10⁴ cells/mL. Three milliliters of this mixture were added to each 35-mm well insert of a six-well plate and incubated for 4 to 6 days in medium containing DMEM and 10% FCS until the collagen matrix showed no further shrinkage. To grow cells in three-dimensional organotypic cultures in the presence of basement membrane, cells were cultured on a deepidermalized dermis derived from human skin (AlloDerm, LifeCell Corp., Branchburg, NJ), which was rehydrated in 1× PBS for 1 hour at 37°C and layered on the contracted collagen gel described above. A total of 5 × 10⁵ cells was seeded on the surface of the AlloDerm. Cultures were maintained submerged for 2 days in low-calcium epithelial growth medium that contained the 3:1 mixture of modified DMEM (JRL Inc, Lenexa, KS) + 8 × 10⁻⁴ mol/L MgSO₄ (JRL Inc) and Ham’s F12 plus 0.4 mmol/L L-glutamine, 0.54 μg/mL hydrocortisone, ITES (mixture of insulin, transferrin, ethanalamine, and selenium, 10 ng/mL final concentration each, Cambrex, Walkersville, MD), 2 × 10⁻⁵ mol/L triiodothyronine (Sigma, St. Louis, MO), 0.1 mmol/L L-phenylalanine (Sigma), 0.18 mmol/L adenine (Sigma), 3.8 mmol/L calcium chloride, 4 × 10⁻⁵ mol/L L-progesterone (Sigma), and 0.2% chelated fetal bovine serum (HyClone, Logan, UT). Cultures were submerged for 2 days in normal-calcium epithelial growth medium and raised to the air-liquid interface for 5 days. While grown at this interface, tissues were fed from below with a 1:1 mixture of modified DMEM and Han’s F12 plus 2% fetal bovine serum with all of the above additives except progesterone. For MMP-blocking experiments, 10 mmol/L of the MMP inhibitor GM6001 (EMD Bioscience, La Jolla, CA) was added to three-dimensional cultures on day 4 and cultures maintained for 5 days with this agent. Cultures were done in triplicate for three independent experiments.

**Immunofluorescence.** Two-dimensional, monolayer cultures grown on glass coverslips were fixed in 2% paraformaldehyde for 15 minutes at room temperature. Three-dimensional constructs and in vitro tissues were frozen in embedding medium (Triangle Biomedical, Durham, NC) in liquid nitrogen vapor after being fixed in 2% paraformaldehyde for 30 minutes at 4°C and placed in 2 mol/L sucrose for 2 hours at 4°C. Immunocytochemistry or immunohistochemistry were done with mouse anti-E-cadherin (anti-cytoplasmic domain, BD PharMingen Transduction Labs, Lexington, KY), mouse anti-H-2K-D (BD PharMingen Transduction Labs), mouse anti-E-cadherin (anti-extracellular domain, HECD-1), rabbit anti-β-catenin (Zymed, South San Francisco, CA), and rabbit anti-type IV collagen (Sigma) antibodies. Immunoreactive proteins were detected using Alexa 488-conjugated goat anti-rabbit or Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Fluorescence was visualized using a Nikon Optiphot microscope and single- or double-exposure photomicroscopy was done using either FITC or Texas Red filters, or both, at 40× magnification. For routine light microscopy, tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and 4-μm sections were stained with H&E. Ki-67 staining was done using paraffin-embedded sections that were deparaffinized and stained with a mouse anti-Ki-67 antibody after antigen retrieval with sodium citrate. Sections were visualized at either 10× or 40× magnification, as indicated.

**Immunoprecipitation and Immunoblotting.** Two-dimensional monolayer cultures were extracted in radioimmunoprecipitation assay buffer [1% NP40, 0.2% SDS, 1% sodium deoxycholate, 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl] supplemented with protease and phosphatase inhibitors to generate total cell lysates and protein concentration was normalized by DC Bradford reagent (Bio-Rad) (Bio-Rad, Hercules, CA). Lysates were generated from three-dimensional organotypic constructs, the collagen matrices and their dermal fibroblasts were physically separated with forceps, and tissues that contained the epithelium and deepidermalized human dermis were homogenized in the above lysis buffer using glass homogenizer. To determine relative levels of E-cadherin, α-catenin, and β-catenin, 15 μg lysate were boiled in 2× sample buffer, resolved on 7.5% SDS-PAGE, and immunoblotted onto nitrocellulose membrane (Osmonics, Westminster, MA). The blot was probed with mouse anti-E-cadherin, anti-β-catenin, anti-γ-catenin (BD PharMingen Transduction Labs), and rabbit anti-α-catenin (Zymed). Immunoreactive proteins were visualized by horseradish peroxidase–linked anti-mouse, anti-rabbit (Amersham, Piscataway, NJ), and anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies. Blots were developed in enhanced chemiluminescence reagent (ECL, Amersham) and exposed to autoradiography. For immunoprecipitation experiments, 200 μg of the radioimmunoprecipitation assay buffer cell lysate was subjected to immunoprecipitation with the above anti-β-catenin or H-2K-D antibodies (gift from Dr. B. Arnold, German Cancer Research Center, Heidelberg, Germany). The immunocomplex was immobilized by γ-avidin beads (Amersham) and Western blot was done.

**Matrigel Migration Assay.** A total of 2.5 × 10⁵ II-4 cells expressing the retroviral constructs were seeded onto growth factor–reduced Matrigel invasion chamber inserts in 24-well plates (BD Biosciences, Bedford, MA) in serum-free DMEM-0.1% bovine serum albumin and were incubated for 36 hours at 37°C. DMEM with 5% fetal bovine serum was used as a chemoattractant. Matrigel and cells that did not pass through
the membrane were scraped from the upper surface of the chamber using a cotton swab. To view the cells that had transmigrated, the inserts were fixed in 2% paraformaldehyde for 15 minutes at room temperature and stained with 0.2% crystal violet in PBS for 30 minutes. Cells on the bottom of each insert were visualized by light microscopy and counted at 10× magnification.

β-Catenin-LEFT/TCF Reporter Gene (TOPFLASH) Assay. In 24-well plates, 5 × 10^5 cells were seeded in each well and transiently transfected with 300 ng of either TOPFLASH (4X wild-type TCF site) or FOPFLASH (4X mutant TCF site) firefly luciferase reporter and cotransfected with 10 ng of RL-CMV Renilla luciferase (Promega, Madison, WI) with transfection reagent (Promega). Each combination was done in triplicate for three separate experiments. Cells were lysed and analyzed using the Dual-Luciferase Reporter Assay System (Promega) and the Veritas luminometer (Turner Biosystems, Sunnyvale, CA). Firefly luciferase values were normalized to Renilla luciferase values. Data were expressed as the ratio of normalized TOPFLASH to normalized FOPFLASH.

MMP Analysis: Real-time Reverse Transcription–PCR and Gel Zymography. For MMP analysis, 2 × 10^5 cells were seeded in two-dimensional cultures on type IV collagen-coated, six-well plates (BD Biosciences). Cells were grown for 24 hours in medium containing DMEM and 5% fetal bovine serum, washed twice with serum-free medium, and maintained in serum-free medium for an additional 24 hours for collection of conditioned medium and for RNA analysis. RNA isolation was done as described previously (23). First-strand cDNA synthesis was done with 2 μg total RNA using SuperScript II Reverse Transcriptase and oligo(dT) 12 to 18 primers (Invitrogen), as recommended by the manufacturer. Real-time PCR was carried out with 2 μL of the cDNA using LightCycler FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN) on LightCycler 2.0 instrument (Roche) as recommended by the manufacturer. Forward and reverse primers for MMP-2 and MMP-9 were designed as follows: MMP-2 primers (forward 5'-ATGACAGCTGCACCACTGAG-3' and reverse 5'-TTTGTTTCGACGAAGGAAATGG-3'), MMP-9 primers (forward 5'-CTCGAACTTGGACAGCAGCA-3' and reverse 5'-GCCATTCACGTCGTCCTTAT-3'), glyceraldehyde-3-phosphate dehydrogenase primers (forward 5'-TGGTGCCATCAATGACCCC-3' and reverse 5'-GCCATTCACGTCGTCCTTAT-3'). Product sizes for MMP-2, MMP-9, and glyceraldehyde-3-phosphate dehydrogenase were 174, 178, and 450 bp, respectively. For gel zymography, an equal amount of conditioned medium (1.5 mL) was concentrated 10-fold using Centricon YM-30 columns. The volume of the retrograde buffer was measured, and a normalized volume of it (9–10 μL) was mixed with an equal amount of zymography buffer (Bio-Rad) and subjected to electrophoresis on a 10% premade SDS-PAGE containing gelatin (Bio-Rad). After electrophoresis, gels were incubated in renaturing buffer (Invitrogen) for 30 minutes at room temperature. The gels were then incubated in zymography developing buffer (Invitrogen) for 30 minutes at room temperature and 48 hours at 37°C. After incubation, gels were stained with Coomassie Brilliant Blue R-250.

Results

H-2K^d-Ecad Expression Induces the Dominant-Negative Loss of Intercellular Adhesion through Cytoplasmic Sequestration of β-Catenin and Redistribution of Adherens Junction Proteins in II-4 Cells. II-4 cells were transduced with two mutant E-cadherin retroviral constructs expressed in a pBabe-puro retroviral vector. The H-2K^d-Ecad vector contains the cytoplasmic and transmembrane portion of E-cadherin and the extracellular domain of II-4 and was previously shown to abrogate cell adhesion in primary keratinocytes due to loss of E-cadherin-mediated adhesion (22). As a control vector, we used H-2K^d-EcadΔC25, a fusion protein identical to H-2K^d-Ecad except for a 25-amino-acid deletion in its β-catenin-binding domain. This mutant form of E-cadherin has previously been shown to allow normal cell adhesion due to its inability to interact with β-catenin (22). The empty pBabe-puro vector was also used as a control in II-4 cells. Expression of these fusion proteins was first verified by immunoblot analysis from total cell lysates of both two-dimensional, monolayer and three-dimensional cultures of pBabe-, H-2K^d-Ecad-, and H-2K^d-EcadΔC25–expressing II-4 cells using an antibody against the cytoplasmic domain of E-cadherin (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A). Protein analysis revealed elevated expression of both H-2K^d-Ecad and H-2K^d-EcadΔC25 fusion proteins (66- and 62-kDa bands, respectively) in both two- and three-dimensional cultures. Levels of endogenous E-cadherin were substantially reduced in H-2K^d-Ecad–expressing II-4 cells (Fig. 1 A).
or β-catenin expression. Levels of β-catenin were not changed by expression of any of the three vectors (Fig. 1A).

We next determined the ability of H-2Kd-Ecad to associate with β-catenin (Fig. 1B). Lysates from two- and three-dimensional cultures of pBabe-, H-2Kd-Ecad-, and H-2Kd-EcadΔC25-expressing II-4 cells were immunoprecipitated with antibodies against either H-2Kd (Fig. 1B) or β-catenin (Fig. 1C) and both immunoprecipitates were immunoblotted with anti-E-cadherin or anti-β-catenin antibodies. In H-2Kd immunoprecipitates, β-catenin was detected only in H-2Kd-Ecad-expressing, but not in H-2Kd-EcadΔC25-expressing II-4 cells (Fig. 1B), due to the absence of the β-catenin-binding site in the latter construct. β-Catenin immunoprecipitates showed a large increase in the amount of exogenous E-cadherin when compared with endogenous E-cadherin (Fig. 1C), showing that the exogenous form of E-cadherin was highly efficient in its capacity to compete with endogenous E-cadherin for β-catenin binding in H-2Kd-Ecad-expressing II-4 cells.

We then studied if H-2Kd-Ecad fusion protein could induce a dominant-negative effect on E-cadherin adhesive function in two-dimensional, monolayer cultures by determining the effect of E-cadherin fusion protein expression on cell morphology, intercellular adhesion, and subcellular distribution of adherens junction components (Fig. 2A). Phase-contrast microscopy showed confluent groups of pBabe- (Fig. 2A, a) and H-2Kd-EcadΔC25-expressing II-4 cells (Fig. 2A, c) with normal intercellular contacts. In contrast, cell-cell adhesion was completely disrupted in H-2Kd-Ecad-expressing cells that appeared as isolated cells with no intercellular contact (Fig. 2A, b). Upon immunohistochemical stain, these cells showed loss of endogenous E-cadherin and redistribution of β-catenin to the cytoplasm (Fig. 2A, e), where this protein colocalized with the H-2Kd fusion protein (Fig. 2A, h, yellow). In contrast, β-catenin (green) and E-cadherin (red) remained colocalized at cell borders in pBabe-infected controls (Fig. 2A, d and g) and in H-2Kd-EcadΔC25-expressing cells (Fig. 2A, f). H-2Kd-EcadΔC25 fusion protein was distributed to the cytoplasm (red), whereas β-catenin remained at intercellular borders (Fig. 2A, i, green).

To determine if cytoplasmic sequestration of β-catenin altered LEF/TCF-mediated transcriptional regulation, II-4 cells expressing each one of the three vectors were transfected with β-catenin-LEF/TCF-responsive reporter constructs. II-4 cells that expressed H-2Kd-Ecad showed a 7-fold reduction in TCF-dependent promoter activity compared with pBabe and H-2Kd-EcadΔC25 controls (Fig. 2B). These results showed that loss of E-cadherin function was associated with a dramatic reduction of β-catenin-mediated transcriptional signaling upon cytoplasmic sequestration of β-catenin. Taken together, these results showed that H-2Kd-Ecad expression induced a dominant-negative effect on II-4 cell adhesion due to the sequestration of β-catenin and the decreased availability of β-catenin to associate with endogenous E-cadherin. We asked whether this dominant-negative effect on cell-cell adhesion induced by H-2Kd-Ecad altered the migratory capacity of II-4 cells in two-dimensional cultures. Using a reconstituted basement membrane (Matrigel) migration assay, II-4 cells expressing H-2Kd-Ecad showed a 10- to 15-fold greater migration through Matrigel-coated membranes compared with II-4 cells expressing either pBabe or H-2Kd-EcadΔC25 (Fig. 2C). This showed that abrogation of E-cadherin-mediated adhesion greatly augmented the migratory capacity of II-4 cells in monolayer culture.

**Suppression of E-cadherin Expression and Abrogation of E-cadherin Function Initiates Tumor Cell Invasion in Three-Dimensional Human Tissue Constructs.** We next studied
whether loss of intercellular adhesion and increased migration seen in two-dimensional cultures of H-2K^d-Ecad–expressing II-4 cells were associated with altered tumor cell behavior in three-dimensional tissues. Morphologic analysis of H&E-stained tissues expressing H-2K^d-Ecad showed cells in the basal layer with reduced cell-cell adhesion characterized by widened intercellular spaces between them (Fig. 3A, b, short arrows). Individual cells had separated from the basal layer and invaded into the superficial connective tissue beneath the basement membrane (Fig. 3A, b, long arrows). When numbers of invading cells were counted in multiple sections it was found that roughly 0.1% of basal cells had undergone invasion. In contrast, II-4 cells expressing pBabe and H-2K^d-EcadΔC25 formed well-organized tissues containing basal cells with normal intercellular adhesion that did not undergo invasion (Fig. 3A, a and c).

Double immunohistochemical staining was done on three-dimensional constructs to determine if the distribution of adherens junction proteins was altered in tissues undergoing invasion. This was done by using an anti-β-catenin antibody and an antibody directed against the cytoplasmic portion of E-cadherin to detect both endogenous and exogenous forms of this protein to determine if fusion protein expression was maintained in the tissue. Three-dimensional tissues constructed with H-2K^d-Ecad–expressing II-4 cells showed loss of β-catenin from cell borders and its redistribution to the cytoplasm, where it colocalized with E-cadherin (Fig. 3A, e, yellow cells). Cells with this staining pattern detached from surrounding cells in the basal layer and were superficially invasive (Fig. 3A, e, arrow) as invading cells were seen below the basement membrane interface when double immunohistochemical stains were done for type IV collagen and E-cadherin (Fig. 3B, b). This showed that loss of intercellular adhesion upon redistribution of adherens junction proteins from cell-cell junctions and the disruption of tissue architecture were associated with tumor cell invasion. In contrast, β-catenin remained at cell borders, where it colocalized with E-cadherin in tissues constructed with II-4 cells transduced with the pBabe control vector (Fig. 3A, d). Similarly, II-4 cells expressing the H-2K^d-EcadΔC25 vector showed β-catenin at cell borders where it colocalized with endogenous

Figure 3. Expression of H-2K^d-Ecad fusion protein induced invasion of II-4 cells in three-dimensional constructs that was associated with MMP-dependent loss of type IV collagen and increased MMP-2 and MMP-9 expression. A, three-dimensional cultures expressing pBabe (a and d), H-2K^d-Ecad (b and e), and H-2K^d-EcadΔC25 (c and f) were analyzed by H&E stain (a-c) and double immunofluorescence staining for cytoplasmic portion of E-cadherin (Texas Red) and β-catenin (FITC; d and f). Individual H-2K^d-Ecad–expressing cells invaded into the connective tissue (b, long arrows) and exhibited cytoplasmic redistribution of β-catenin (a) where it colocalized with exogenous E-cadherin (e, yellow). In addition, some basal cells showed widened intercellular spaces (b, short arrows). In contrast, controls did not exhibit this invasive phenotype (a, c) and staining for these proteins was limited to cell borders (d and f, yellow). B, double immunofluorescence staining for extracellular (EC) domain of E-cadherin (Texas Red) and type IV collagen (green) revealed nearly complete loss of type IV collagen in three-dimensional constructs of H-2K^d-Ecad–expressing cells that lacked endogenous E-cadherin in most cells (b). In contrast, pBabe (a) and H-2K^d-EcadΔC25 (c) controls, as well as H-2K^d-Ecad–expressing cultures treated with GM6001 (d), showed linear and continuous type IV collagen staining (c and d). II-4 cells expressing the three vectors were grown on type IV collagen-coated plates and reverse transcription–PCR analysis (C) revealed a significant increase of MMP-9 and MMP-2 mRNA levels in H-2K^d-Ecad–expressing cells relative to controls (P < 0.005). Results are presented as the fold induction relative to pBabe-expressing II-4 cells and SD was calculated from three independent experiments done in triplicate. D, increase in MMP RNA production was confirmed by gelatin zymography. Lane 1, conditioned medium from pBabe–, lane 2 from H-2K^d-Ecad–, and lane 3 from H-2K^d-EcadΔC25–expressing cells.
E-cadherin (Fig. 3A, f, yellow), whereas the distribution of E-cadherin fusion protein was cytoplasmic (Fig. 3A, i, red), showing that transgene expression was sustained without altering the distribution of adherens junction proteins or cell adhesion. Thus, maintenance of E-cadherin–mediated adhesion preserved integrity of tissue architecture and was not permissive for tumor cell invasion.

**Loss of E-cadherin–Mediated Adhesion Increases Expression of MMP-2 and MMP-9 and Activates MMP-Dependent Basement Membrane Degradation.** We then investigated whether tumor cell invasion seen in three-dimensional tissue constructs was associated with the proteolytic degradation and loss of basement membrane integrity immediately preceding and during early tumor cell invasion. Tissues generated with II-4 cells expressing each one of the three vectors were analyzed by double immunohistochemical stains for the extracellular domain of E-cadherin and the basement membrane component type IV collagen (Fig. 3B). Constructs generated with H-2K<sup>d</sup>-EcadΔC25–expressing II-4 cells and pBabe controls showed no degradation of type IV collagen as seen by the intact, linear staining pattern seen for this protein that was accompanied by localization of endogenous E-cadherin at cell-cell borders (Fig. 3B, a and c). In contrast, H-2K<sup>d</sup>-Ecad–expressing II-4 constructs generated tissues that showed a faint, patchy staining of endogenous E-cadherin at cell junctions and nearly complete loss of type IV collagen staining along the basement membrane zone (Fig. 3B, b, short arrows). Loss of basement membrane integrity was associated with initiation of tumor cell invasion, as seen by cells that had completely lost endogenous E-cadherin, separated from the basal layer, and traversed the epidermal-stromal interface (Fig. 3B, b, long arrow). These results showed that loss of basement membrane proteins was a very early event that preceded tumor cell invasion during the transition of adhesion-deficient tissues from a preinvasive to an early invasive stage.

To determine whether loss of basement membrane integrity seen upon invasion of H-2K<sup>d</sup>-Ecad–expressing cells occurred through MMP-mediated, proteolytic pathways, three-dimensional constructs were generated with these cells and exposed for 5 days to the MMP inhibitor GM6001 and analyzed by immunohistochemical stain for the extracellular domain of E-cadherin and for type IV collagen. Type IV collagen staining was linear and continuous in GM6001-treated H-2K<sup>d</sup>-Ecad–expressing tissues (Fig. 3B, d), demonstrating that type IV collagen could be restored and loss of basement membrane integrity could be reversed upon MMP inhibition. Because GM6001 is a broad-spectrum inhibitor of MMPs, we next did analyses to determine which MMPs were specifically increased in tumor cells that had lost E-cadherin function. To accomplish this, II-4 cells transduced with each of the three vectors were grown as two-dimensional cultures on plates coated with type IV collagen. We examined mRNA levels of MMP-2 and MMP-9, the MMPs most often associated with basement membrane invasion of early SCC (24), using real-time PCR for quantitative analysis of gene expression. II-4 cells that expressed H-2K<sup>d</sup>-Ecad showed a statistically significant elevation in transcription that was 8-fold greater for MMP-9 and 2.5-fold greater for MMP-2 when compared with control cells after being normalized for glyceraldehyde-3-phosphate dehydrogenase mRNA levels (Fig. 3C). These results were confirmed by gelatin zymography (Fig. 3D), that revealed an increase in protein levels of these MMPs in H-2K<sup>d</sup>-Ecad–expressing II-4 cells when compared with control II-4 cells. These results showed that loss of E-cadherin function led to an increase in MMP expression that was associated with the initiation of tumor cell invasion in three-dimensional tissue constructs.

**Abrogation of E-cadherin Function Dramatically Accelerated the Tumorigenic and Invasive Potential of II-4 Cells after In vivo Surface Transplantation.** We next studied whether loss of E-cadherin–mediated adhesion could directly alter tumor progression in three-dimensional tissue constructs in vivo. Tissues constructed with H-2K<sup>d</sup>-EcadΔC25-, H-2K<sup>d</sup>-Ecad-, and pBabe-expressing II-4 cells were transplanted as surface grafts to the dorsal fascia of nude mice and the fate and phenotype of tumor cells were followed 4 weeks later. Four weeks after grafting, excised tumors composed of H-2K<sup>d</sup>-Ecad–expressing cells showed a 5-fold increase in tumor weight compared with control tumors (Fig. 4B). These large tumors showed a nodular, erythematous, and exophytic surface (Fig. 4A, b and inset) and were indurated at tumor margins with normal mouse skin. Grafts harboring pBabe and H-2K<sup>d</sup>-EcadΔC25–expressing II-4 cells appeared as hyperkeratotic plaques that were firm and slightly raised above the surface (Fig. 4A, a and c). At both 2 weeks (data not shown) and 4 weeks (Fig. 4C, b), tumors composed of H-2K<sup>d</sup>-Ecad–expressing cells showed sheets of poorly differentiated, pleomorphic tumor cells showing areas with widened intercellular spaces (Fig. 4C, b, circle). These cells infiltrated throughout the stroma and under adjacent normal mouse epithelium with an aggressive pattern of invasion and showed no evidence of cellular differentiation. The advancing edge of these tumor cell sheets showed cells that migrated into adjacent stroma as individual cells (Fig. 4C, b, inset arrows). In contrast, surface grafts composed of pBabe– (Fig. 4C, a) and H-2K<sup>d</sup>-EcadΔC25–expressing (Fig. 4C, c) II-4 cells invaded into the underlying connective tissue as large islands of tumor cells that were well demarcated from the surrounding connective tissue and well differentiated as seen by keratin pearls.

Loss of E-cadherin–mediated adhesion in these aggressively invading, H-2K<sup>d</sup>-Ecad–expressing tumor cells was associated with a diffuse pattern of tumor cell proliferation, as seen by Ki-67 staining. In contrast, pBabe (Fig. 4C, d) and H-2K<sup>d</sup>-EcadΔC25–expressing (Fig. 4C, f) tumors revealed Ki-67–positive, proliferating cells that were generally restricted to basal cells at the periphery of tumor cell islands. In contrast, tumors generated with H-2K<sup>d</sup>-Ecad–expressing II-4 cells showed Ki-67–positive cells throughout the highly infiltrative sheets of tumor cells, as well in individual cells at their advancing edge (Fig. 4C, e and inset). These results directly implicate loss of E-cadherin–mediated adhesion in the switch from a low-grade to a high-grade SCC that resulted in increased tumor cell proliferation and accelerated tumorigenesis in vivo.

**Discussion**

Tumor progression is responsive to evolving architectural and contextual changes in the tissue microenvironment that may alter tumor cell growth, survival, and differentiation (11, 18, 25–27). The role that alterations in E-cadherin–mediated adhesion may play in the development of incipient invasive carcinoma has remained unclear, as previous studies have shown conflicting findings regarding how loss of E-cadherin alters the early preinvasive stages of cancer progression in stratified squamous epithelium (13–16). In the current study, we therefore asked how a fundamental alteration in tissue architecture,
namely, the abrogation of intercellular adhesion mediated by E-cadherin, would affect the fate of early-stage SCC cells in three-dimensional constructs that closely mimic this stage of cancer in humans. We have shown that loss of E-cadherin–mediated adhesion triggered tumor cell invasion through the MMP-dependent degradation of basement membrane in vitro and activated a switch from a slow-growing, low-grade SCC to an aggressive, high-grade SCC in vivo. Loss of E-cadherin function was associated with the dramatic acceleration of SCC progression in tumor cells that already harbored phenotypic properties characteristic of the early stages of neoplastic progression.

Invasion of IE tumor cells through the basement membrane barrier marks the point of transition from premalignancy to malignancy (3). Acquisition of this invasive behavior in vivo is associated with degradation of proteins along the basement membrane zone and increased cell motility through the activation of MMP-2 and MMP-9 (2, 24). We have shown that loss of E-cadherin activates MMP-mediated, basement membrane degradation and initial tumor cell invasion in constructs of stratified squamous epithelium. This supports the view that incipient tumor cell invasion in vitro and rapid tumor cell dissemination seen after in vivo transplantation were due to the linkage between loss of cell-cell adhesion and the concomitant activation or dysregulation of pathways that control MMP activity. It has previously been shown that decreased expression of E-cadherin was associated with up-regulation of MMP 9 in mouse keratinocytes (28). Furthermore, induction of E-cadherin function has decreased synthesis of MMP-9 in premalignant human oral keratinocytes (29), down-regulated MMP-2 and MMP-9 in bronchial tumor cells (30), and decreased activity of MMP-2 in prostate carcinoma cells (31). It has previously been shown that H-2Kd-Ecad expression in breast cancer cells resulted in an increase of their integrin-mediated migration (32). However, our findings mark the first time that the relationship between loss

Figure 4. Loss of E-cadherin function in H-2Kd-Ecad–expressing II-4 cells triggers a switch to a highly aggressive SCC after in vivo surface transplantation. Three-dimensional constructs of II-4 cells transduced with pBabe or fusion proteins were grafted to the dorsum of nude mice. A, clinical appearance of representative tumors seen 4 weeks later. Transplants composed of pBabe- (a) and H-2Kd-EcadΔC25–expressing II-4 cells (c) generated flat, hyperkeratotic lesions with a roughened surface. In contrast, H-2Kd-Ecad–expressing transplants formed large, erythematous nodular tumors (b) that were exophytic in nature (b, inset). B, H-2Kd-Ecad–expressing tumors weighed 5-fold more than tumors generated with pBabe and H-2Kd-EcadΔC25 (P < 0.005). There was no difference in tumor mass between these controls (P > 0.05). The experiment was done three times in triplicate. Columns, mean values for a representative experiment; bars, SD. C, histologic analysis of tumors generated with H-2Kd-Ecad–expressing II-4 cells 4 weeks after transplantation (b) revealed sheets of cells without cellular differentiation and with widened intercellular spaces (b, circle). These cells underwent proliferation throughout the tumor mass as seen by the random pattern of Ki-67 staining (e). The infiltrating edge of these tumors showed stromal invasion of actively proliferating, single cells (e, inset, arrows). In contrast II-4 cells expressing pBabe (a and d) and H-2Kd-EcadΔC25 (c and f) invaded as large, well-differentiated tumor islands beneath mouse epithelium (a and c) in which proliferation was restricted to cells at the periphery of these tumor islands (d and f).
of E-cadherin function and MMP activation has been shown in human, stratified, squamous epithelial tissue constructs that closely mimic the architectural features of the in vivo tissues.

In vivo, we found that the loss of intercellular adhesion mediated by E-cadherin was sufficient to switch the biological behavior of II-4 cells from low-grade SCC, composed of islands of well-differentiated tumor cells, to high-grade SCC demonstrating aggressively infiltrating tumor cells, some of which invaded as single cells devoid of intercellular contact. This pattern of single-cell invasion was similar to those described for aggressive forms of breast carcinoma that have a poor clinical prognosis (33). The induction of this high-grade carcinoma was associated with the rapid growth and dissemination of tumors that showed a 5-fold increase in the size of tumors within 4 weeks after grafting when compared with control transplants. These highly invasive tumors showed a large increase in tumor cell proliferation when compared with grafted controls. H-2K<sup>d</sup>-Ecad–expressing tumors also showed proliferative, Ki-67–stained cells that were present in a random pattern when compared with the distribution of Ki-67–positive cells seen in control tumors, in which positive cells were limited to the periphery of cell clusters. The random distribution of Ki-67–positive tumor cells has been associated with a considerably poorer survival rate when compared with SCC that displayed an organized, restricted pattern of Ki-67 staining (34). Similarly, in vivo studies in humans have shown that highly infiltrative SCC with a decreased expression of adherens junction proteins has been associated with a poor clinical prognosis (8). Importantly, similar findings have been shown in an in vivo experimental system in which invasive tumors, which were identical to human SCC, developed from engineered, normal, human epidermal tissues upon coexpression of oncogenic ras and CDK4 (35). These invasive tumors were very similar to those seen in the current study as characterized by decreased E-cadherin expression, lack of differentiation, and elevated tumor cell proliferation.

Although pathways for the in vivo activation of tumor phenotype remain to be elucidated, we have shown that the dominant-negative abrogation of intercellular adhesion in II-4 cells was likely due to the ability of the H-2K<sup>d</sup>-Ecad fusion protein to sequester β-catenin and limit its availability to form functional complexes with the endogenous form of E-cadherin. In the absence of its association with β-catenin, the cytoplasmic tail of E-cadherin is unstable, leading to the proteolytic degradation of E-cadherin (36). Thus, it seems that the dominant-negative reduction in levels of endogenous E-cadherin are due the inability of β-catenin to associate with it and lead to the suppression of E-cadherin function reported. Additional strategies that could directly suppress levels of E-cadherin, such as siRNA, need to be explored to determine if loss of cell adhesion and induction of tumor phenotypes are similar to the dominant negative suppression described above. The cytoplasmic sequestration of β-catenin was associated with a reduction in transcriptional activity of β-catenin in the TOP-FLASH reporter assay in these cells. This finding was similar to the decrease in β-catenin–mediated transcriptional activation shown upon overexpression of the E-cadherin cytoplasmic domain in colon carcinoma cells (37). Furthermore, it was recently shown that levels of E-cadherin expression do not control activity of β-catenin signaling in breast and prostate carcinoma cells (38), leading to the conclusion that the invasion-modulating activity of E-cadherin is independent of β-catenin regulation of target gene expression. The acquisition of the highly aggressive pattern of tumor cell dissemination supports the view that the loss of E-cadherin is an important prognostic risk factor for the rapid progression of SCC in humans whose activation pathways remain to be elucidated.

The II-4 cell line used in our studies has been well characterized as representing an early stage of the malignant transformation process (19, 20). Because II-4 cells exhibit low-grade malignant behavior in vivo and harbor many of the important genetic hallmarks of the premalignant and early, invasive stages of SCC (1), such as mutations in p53 and ras, this cell line is optimal for incorporation into models of early carcinoma progression. Other cell lines representing such an early stage of SCC progression have yet to be studied in this way. We have previously shown that II-4 cells maintain their sensitivity to microenvironmental control signals from adjacent normal cells that could suppress their neoplastic potential and that II-4 cells formed dysplastic tissues without tumor cell invasion (18, 19).

Complete loss of cell adhesion in these cells fully activates their invasive potential. However, activation of tumor cell invasion was seen only in a relatively small number of H-2K<sup>d</sup>-Ecad–expressing II-4 cells in three-dimensional tissues in vitro. This may be because only a fraction of cells in the tissue were in contact with basement membrane and thus in a position to undergo invasion upon MMP activation. It is also possible that invasion only occurred in II-4 cells that expressed levels of H-2K<sup>d</sup>-Ecad that were sufficient to induce the complete loss of E-cadherin–mediated adhesion. Because such tumor cell heterogeneity is known to exist during the early stage of SCC progression in humans (1), the emergence of a relatively small number of cells with features that can enhance tumor progression may be a critical initial step in early tumor development.

It is critical to study the biology of human cancers using cell and tissue culture systems that mimic the organizational complexity and structural features of human tissues (11, 17, 21). In the current study, we have generated tissues that contain well-structured basement membrane as it occurs in human tissues to study early tumor cell invasion in three-dimensional, in vitro tissue constructs. This extends our previous report using adenoviral vectors (39) that was limited by the transient nature of adenoviral gene expression that did not allow study of the effects of loss of tumor cell adhesion in long-term studies. In the current study, we have used retroviral gene transfer to achieve stable, long-term transgene expression to directly show that loss of E-cadherin initiates tumor cell invasion in vitro and is associated with a switch of SCC from a low-grade to high-grade biological behavior in three-dimensional, human tissue constructs. Disruption of tissue organization mediated by E-cadherin is therefore a critical microenvironmental factor in the promotion and dissemination of human SCC.

Acknowledgments


Grant support: National Institutes of Dental and Craniofacial Research grant 2R01DE011250-06.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dres. E. Watt for the gift of the chimeric E-cadherin retroviral vectors and B. Arnold for the H-2K<sup>d</sup> antibody, Sujata Pawagi, Ning Lin, Padmaja Prabhhu, Jennifer Landmann, Laura Bertolotti, and Larry Pfeiffer for their technical assistance, Dr. Michael Frohman for assistance with real-time PCR analyses, and Dres. Dafina Bar Sagi, Valerie Weaver, Martha Furie, Soosan Ghazizadeh, and Lorne Taichman for critical comments.
References

E-cadherin Suppression Accelerates Squamous Cell Carcinoma Progression in Three-Dimensional, Human Tissue Constructs


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/5/1783

Cited articles
This article cites 37 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/5/1783.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/65/5/1783.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.