Restoration of E-cadherin Cell-Cell Junctions Requires Both Expression of E-cadherin and Suppression of ERK MAP Kinase Activation in Ras-Transformed Breast Epithelial Cells

Abstract

E-cadherin is a main component of the cell-cell adhesion junctions that play a principal role in maintaining normal breast epithelial cell morphology. Breast and other cancers that have up-regulated activity of Ras are often found to have down-regulated or mislocalized E-cadherin expression. Disruption of E-cadherin junctions and consequent gain of cell motility contribute to the process known as epithelial-to-mesenchymal transition (EMT). Enforced expression of E-cadherin or inhibition of Ras-signal transduction pathway has been shown to be effective in causing reversion of EMT in several oncogene-transformed and cancer-derived cell lines. In this study, we investigated MCF10A human breast epithelial cells and derivatives that were transformed with either activated H-Ras or N-Ras to test for the reversion of EMT by inhibition of Ras-driven signaling pathways. Our results demonstrated that inhibition of mitogen-activated protein kinase (MAPK) kinase, but not PI3-kinase, Rac, or myosin light chain kinase, was able to completely restore E-cadherin cell-cell junctions and epithelial morphology in cell lines with moderate H-Ras expression. In MCF10A cells transformed by a high-level expression of activated H-Ras or N-Ras, restoration of E-cadherin junction required both the enforced reexpression of E-cadherin and suppression of MAPK kinase. Enforced expression of E-cadherin alone did not induce reversion from the mesenchymal phenotype. Our results suggest that Ras transformation has at least two independent actions to disrupt E-cadherin junctions, with effects to cause both mislocalization of E-cadherin away from the cell surface and profound decrease in the expression of E-cadherin.

Introduction

In normal breast tissue, the epithelial cells form polarized, tubuloductal structures that can further differentiate into alveoli that are functional for lactation. The organization of the mammary epithelium is dependent on maintenance of cell-cell adherens junctions that are mediated by homotypic interactions between E-cadherin glycoproteins on adjacent cells [1]. As the prototypical classic cadherin, E-cadherin is a calcium-dependent intercellular adhesion molecule that couples to the actin cytoskeleton through β-catenin, which binds to its cytoplasmic tail [2]. In addition to its structural role in directing cellular adhesions, E-cadherin also controls the ability of β-catenin to operate as a transcription factor by regulating its subcellular localization [3].

There is a complex relationship between E-cadherin and the development and progression of breast cancer [1]. E-cadherin may function as a tumor suppressor in lobular breast cancer, as there is commonly early loss of E-cadherin expression in this disease, including by germ line mutation [1,4]. In ductal breast carcinomas, E-cadherin expression is more variable [1], but there is evidence that it may be lost from metastases even if maintained in the primary...
tumor [5]. Loss of functional E-cadherin contributes to a process called epithelial-to-mesenchymal transition (EMT), in which epithelial cells undergo loss of cell-cell adhesion and contact inhibition of proliferation and gain of function in motility and invasiveness [6]. Loss of functional E-cadherin expression can result not only from mutation but also more commonly from reversible transcriptional suppression [7,8] or mislocalization from the cell surface [9,10].

The localization of E-cadherin is determined by polarized trafficking of E-cadherin to the cell surface and by subsequent internalization and recycling [11]. Both arms of this pathway are regulated by several factors, including stimulation of cell surface receptors [12], interaction with catenins [13], and activation of Rho small GTPases, including RhoA, Rac, and Cdc42 [14–17]. The actions of Rho may be dependent on the downstream phosphorylation of myosin light chain, because ML-7, an inhibitor of myosin light chain kinase (MLCK), can restore E-cadherin cell-cell junctions that have been disrupted after transformation by Ras or Src [14,18].

There is a reciprocal relationship between E-cadherin function and the activity of receptor tyrosine kinases (RTKs). The formation of E-cadherin junctions reduces ligand-dependent activation of several RTKs, including the epidermal growth factor receptor (EGFR) [19], whereas mutation of E-cadherin increases EGF-induced Ras activation through a mechanism that may include stabilization of the EGFR at the cell surface [20]. Conversely, EGFR activation destabilizes the functional E-cadherin complex [21]. This inhibitory effect of RTK activation on E-cadherin function may be caused by a direct phosphorylation of components of the E-cadherin complex [21,22]. In addition to these direct effects, Ras-dependent signal transduction downstream of RTK activation may also contribute to down-regulation of E-cadherin. For example, inhibition of Ras can restore E-cadherin expression in a variety of human cancer cells [23], whereas inhibition of the extracellular signal–regulated kinase (ERK) mitogen-activated protein kinase (MAPK) cascade that is downstream of Ras blocks the ability of EGF to disrupt adherens junctions [24]. Ras signaling can also lead to regulation of Rho small GTPases and thus control of EMT [25]. In intestinal epithelial cells, for example, expression of activated Ras is sufficient to disrupt E-cadherin through an ERK-dependent pathway [9], which results in both suppression of its transcription [26] and mislocalization from the cell surface [27]. Enhanced overexpression of E-cadherin is not able to restore epithelial morphology to Ras-transformed intestinal epithelial cells as cell surface localization of E-cadherin is not restored [27]. Nevertheless, expression of E-cadherin in MDA-MB-231 breast cancer cells, which contain an activating mutation in K-Ras [28], is sufficient to promote reversion to an epithelial morphology [26]. Further, overexpression on E-cadherin suppresses invasiveness in a mouse mammary tumor cell model [29].

In this study, we tested the ability of E-cadherin reexpression, in combination with inhibitors of Ras-dependent signal transduction pathways, to restore E-cadherin adherens junctions in models of Ras-transformed breast epithelial cells. Our data showed that in cells with moderate levels of activated Ras, expression of E-cadherin was retained but mislocalized and that inhibition of the ERK MAPK cascade was sufficient to restore adherens junctions and epithelial morphology. In highly Ras-transformed cells, E-cadherin expression was greatly reduced and not restored by inhibition of ERK MAPK. Reexpression of E-cadherin did not restore adherens junctions unless these cells were also treated with inhibitors of ERK MAPK activation but not with inhibitors of other pathways downstream of Ras. When both E-cadherin was reexpressed and ERK MAPK was inhibited, the Ras-transformed cells showed restoration of E-cadherin cell-cell junctions and reversion to an epithelial morphology.

Materials and Methods

Reagents

Dulbecco’s modified Eagle’s medium/F12, horse serum, PBS, human EGF, sodium pyruvate solution, l-glutamine solution, Lipofectamine 2000, and antifade reagent were purchased from Invitrogen (Carlsbad, CA). Dulbecco’s modified Eagle’s medium, trypsin/EDTA solution, and penicillin-streptomycin were obtained from Cellgro (Herndon, VA). Fetal bovine serum (FBS) was from HyClone (Logan, UT). Protease inhibitor cocktail tablets were obtained from Roche (Indianapolis, IN). The MAPK kinase inhibitor PD184352 (CI-1040) was generously supplied by Pfizer, Inc (New York, NY). The MAPK kinase inhibitor U0126, Rac1 inhibitor NSC23766, and MLCK inhibitor ML-7 were purchased from Calbiochem (La Jolla, CA). Western blot analysis detection reagents were purchased from GE Healthcare (Amersham, UK). Glutathione Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden). 4’,6-Diamidino-2-phenylindole was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA), dithiothreitol (DTT), hexadimethrine bromide (polybrene), hydrocortisone, FITC-conjugated phalloidin, the PI3 kinase inhibitor wortmannin, and other chemicals not otherwise listed were obtained from Sigma (St. Louis, MO). Mouse anti-E-cadherin, mouse anti-N-cadherin, Alexa Fluor 555 goat antirabbit antibodies were purchased from BD Biosciences (Bedford, MA). Rabbit anti-phospho-Akt(Ser473) and rabbit anti-β-catenin antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-H-Ras antibody was from Calbiochem. Mouse anti-N-Ras, mouse anti-RhoA, rabbit anti-RhoB, and rabbit anti-Cdc42 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Rac antibody was from Upstate (Lake Placid, NY). Mouse anti–phospho-Erk1/2 antibody was from Sigma. Mouse antitubulin antibody was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). Normal donkey serum, normal goat serum, horseradish peroxidase (HRP)–conjugated donkey antimouse IgG, and HRP-conjugated donkey antirabbit antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell Lines and Culture

MCF10A human breast epithelial progression series of cells (MCF10A, NeoT, AT1, and DCIS) were obtained from the Cell Lines Resource (Karmanos Center Institute, Detroit, MI). MCF10A cell lines stably expressing constitutively active H-Ras.D12 (H-Ras MCF10A) or active N-Ras.D12 (N-Ras MCF10A) have been previously described [30] and were kind gifts from Dr. Hyeong-Reh Choi Kim (Wayne State University, Detroit, MI). The MCF10A series of cells and the retrovirus packaging cell line Hek293T were cultured as previously described [31].

Retrovirus Production and Infection

The retrovirus construct LZRS-GFP-hEcad that expresses human E-cadherin and green fluorescent protein (GFP) and the control construct LZRS-MS-GFP that only expresses GFP are derived from those
previously described [32] and were generous gifts from Dr. Albert B. Reynolds (Vanderbilt University, Nashville, TN). Packaging of retroviruses and infection of target cells were performed as described [31]. Briefly, Hek293T packaging cells were triply transfected with the virus construct and the two packaging plasmids, pVPack-GP and pVPack-VSV-G (Stratagene, La Jolla, CA). The viral broth was collected as batches at time points of 36, 48, and 72 hours. The MCF10A and derivative cells were infected at a low cell confluence of approximately 5% in the presence of 12 μg/ml polybrene, with approximately two to three runs of infection every 24 hours to produce a final infection efficiency that was approximately 95% by monitoring for GFP-positive cells under fluorescent microscopy.

**Immunocytochemistry**

Cells were cultured on 12-mm round coverslips in 24-well plates with 0.5 ml of culture medium per well. For immunocytochemical staining, cells were subjected to the following treatments: fixation with 4% paraformaldehyde for 20 minutes; permeabilization with 0.5% Triton X-100 for 10 minutes; further fixation with 95% ice-cold EtOH for 10 minutes; quenching and washing with PBS containing 0.75% glycine for three times, 10 minutes each time; and blocking for overnight at 4°C with immunofluorescent buffer (IF buffer; 130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20, pH 7.5). Then, the cells were stained for 2 hours with the first antibody diluted in IF buffer, washed with PBS for three times, and stained for 1.5 hours with the fluorescently conjugated secondary antibody diluted in IF buffer containing 5% normal serum from the same species of the animal as used for raising the second antibody. As needed, FITC-phalloidin and 4′,6-diamidino-2-phenylindole were included in the second antibody mixture to stain the actin cytoskeleton and nuclei, respectively. The coverslips were mounted onto slides with antifade reagent. Cells were imaged using LSM 510 confocal microscope (Zeiss, Gottingen, Germany).

**Western Blot Assays**

Cells were cultured in growth medium on 35-mm tissue culture plates to approximately 80% confluence. Then, fresh medium was added with or without the presence of drugs, and the cells were cultured for 24 hours. After briefly rinsing with PBS, the cells were lysed in buffer: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM sodium pyrophosphate, 2 mM EDTA, 1% Nonidet P-40, 1% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 0.005% bromophenol blue, and supplemented with protease inhibitor cocktail according to the manufacturer's instructions. The cell lysates were subjected to brief sonication and heated for 5 minutes at 100°C. After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes, which were blocked with 2% milk for 1 hour in TBS-T buffer: 25 mM Tris-HCl, pH 7.4, 135 mM NaCl, 3 mM KCl, and 0.05% Tween 20. This blocking buffer was also used for the dilution of antibodies.

**Small GTPase Activity Assay**

GST-fusion proteins that bind to the active form of small GTPases were used in this study to assay the activation state of Ras and Rho-family proteins. GST-Raf-RBD was used to isolate GTP-bound forms of H-Ras and N-Ras largely as previously described [33]. GST-PAK-PBD was used to isolate GTP-bound forms of Rac and Cdc42 largely as previously described [34]. GST-Rhotekin-RBD, which was a generous gift from Dr. Martin Schwartz (University of Virginia), was used to isolate the GTP-bound forms of RhoA and RhoB. Briefly, cells were cultured in growth medium with 10 μM U0126, 1 μM PD184352, or vehicle (dimethylsulfoxide, DMSO) for 24 hours and were lysed in 25 mM HEPES, pH 7.5, 100 mM NaCl, 15 mM MgCl2, 0.5 mM EDTA, 5% glycerol, 1% Nonidet P-40, 0.25% deoxycholate supplemented with 1 mM DTT, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride before use. Lysates were collected, briefly sonicated, centrifuged for 5 minutes at 10,000g, and standardized for protein content. Equal amounts of the GST-fusion protein beads were mixed with each of the cell lysates and incubated with rotation at 4°C for 1.5 hours to let the active form of GTPases to bind to the beads. The beads were then collected by centrifugation and washed three times with lysis buffer. The retained proteins were then subjected to SDS-PAGE and Western blot assays.

**Results**

**Ras-Driven Down-regulation of E-cadherin Expression in Derivatives of MCF10A Breast Epithelial Cells**

MCF10A cells are a spontaneously immortalized human breast epithelial cell line that was developed from a patient who underwent a reduction mammoplasty [35]. MCF10A cells are pseudodiploid, form typical cobblestone epithelial sheets in monolayer culture (Figure 1A), and are used as a model of normal human breast epithelium [36]. Stable transfection of MCF10A cells with an activated H-Ras-V12 construct produced the MCF10AneoT (NeoT) line [37]. NeoT cells retain an epithelial morphology in culture (Figure 1A) but exhibit some aspects of the malignant phenotype in that they can form xenograft lesions [38]. The validity of this model is based on the widespread functional activation of Ras in human breast cancers [28] owing to overexpressed growth factors and their receptors [39], and the evidence that Ras-directed therapeutics may be useful in breast cancer treatment [40], although Ras mutations per se are not common. The relevance of this model is further supported by study of gene expression signatures that define Ras pathway activation, which confirm that Ras activation is prevalent in breast cancer and predict sensitivity to therapeutics that target Ras signaling [41]. By serial passaging of NeoT xenograft lesions in vivo, a more advanced cell line was isolated and named MCF10.AT1 (AT1). AT1 cells produce xenograft lesions with characteristics of atypical (grade 3) hyperplasia [38] but show only a mild derangement of epithelial morphology in vitro (Figure 1A). After further serial passages in vivo, MCF10.DCIS.com (DCIS) cells were isolated. As xenografts, DCIS cells initially form lesions with the characteristics of comedo ductal carcinoma in situ, which may progress to invasive carcinoma over time [42]. The DCIS cells exhibit a clear loss of epithelial organization in vitro (Figure 1A) with a more fibroblast-like phenotype that is characterized by less distinct cell-cell contacts.

In addition to the premalignant progression series of MCF10A, NeoT, AT1, and DCIS cells, we also tested two further variants of MCF10A cells that were made by retroviral-mediated overexpression of activated H-Ras.D12 or N-Ras.D12 constructs [30]. Both of these cell lines showed complete loss of epithelial morphology, with decreased cell-cell and cell-substrate attachment (the latter particularly notable in N-Ras MCF10A cells), and an overall conversion to a phenotype that is consistent with Ras transformation (Figure 1A).
To test the relationship between Ras transformation and E-cadherin in the MCF10A-derived cell lines, we assayed the expression of E- and N-cadherin, small GTPases, and markers of Ras activation by Western blot analysis (Figure 2A). The results show that there is a progressive increase in the expression of H-Ras from MCF10A to DCIS cells that is correlated with a modest reduction in the level of E-cadherin. The very high levels of activated H-Ras and N-Ras expression in the H-Ras MCF10A and N-Ras MCF10A cells are correlated with much more profound loss of E-cadherin expression, although a low level of expression can be detected with prolonged exposures (Figure 2A). MCF10A cells have a low level of N-cadherin expression, which is consistent with previous reports [43] and is increased in H-Ras and N-Ras MCF10A cells (Figure 2A). A switch from E-cadherin to N-cadherin expression has been described as a feature of EMT and transformation in several cancers [44].

The effect of activated Ras to down-regulate E-cadherin may be through mechanisms that could involve activation of classic signaling pathways downstream of Ras, such as ERK MAPK or PI 3-kinase/Akt [45], and could also be mediated through regulation of Rho and Rac proteins [17,25]. The activation level of ERK MAPK, which was
highest in the DCIS, H-Ras, and N-Ras MCF10A lines, did correlate well with both the increase in Ras expression and the decrease in E-cadherin in the MCF10A derivatives (Figure 2B). The activation level of Akt did increase in the progression series from MCF10A to DCIS cells, but was not elevated in the H-Ras and N-Ras MCF10A lines, and also correlated less strongly with the reduction in E-cadherin (Figure 2B). The expression levels of RhoA and Rac1 did not consistently change with Ras transformation status or correlate with down-regulation of E-cadherin (Figure 2B).

**Restoration of E-cadherin Localization in DCIS Cells Treated with an Inhibitor of ERK MAPK Activation**

To determine whether alterations in epithelial morphology in the MCF10A derivatives were correlated with changes in the cell surface expression of E-cadherin, we performed confocal immunofluorescence to localize E-cadherin (Figure 1B). The results show that prominent cell surface localization of E-cadherin is maintained during premalignant progression from MCF10A cells through NeoT and AT1 variants, which correlates with the maintenance of epithelial morphology (Figure 1). In DCIS cells, which have somewhat reduced total E-cadherin expression (Figure 2), there is markedly less E-cadherin at the cell surface but an apparent increase in intracellular E-cadherin (Figure 1B, top row). In the H-Ras and N-Ras MCF10A lines, there is much less E-cadherin expression (Figure 2), and what remains is not at the cell surface but is localized intracellularly (Figure 1B, top row).

To begin investigation of which pathways downstream of Ras activation may be required for the changes in E-cadherin in the MCF10A derivative cell lines, we compared the localization of E-cadherin in control (vehicle-treated) cells with those treated with selective inhibitors of MAPK kinase (U0126), PI 3-kinase (wortmannin), activation of Rac (NSC23766), or MLCK (ML-7). None of these treatments had any effect on the low levels of E-cadherin in H-Ras and N-Ras MCF10A cells (Figure 1B). Similarly, these treatments had little observable effect on the cell surface expression of E-cadherin that was present in MCF10A, NeoT, or AT1 cells, although ML-7 did reproducibly induce an additional increase in E-cadherin at a site that appeared to be perinuclear. The most striking effects occurred in DCIS cells, where U0126 in particular, and to a lesser extent ML-7, induced an increase in the cell surface localization of E-cadherin.

**Restoration of Epithelial Morphology in DCIS Cells Treated with Inhibitors of ERK MAPK Activation**

To investigate the effects of ERK MAPK inhibition on cellular morphology in more detail, we compared the effects of U0126 to a second, more potent inhibitor, PD184352 [33] on E-cadherin localization and the actin cytoskeleton (Figure 3). MCF10A cells have an organized actin cytoskeleton that is characterized by a cortical ring, and that structure is maintained and perhaps intensified by inhibition of ERK MAPK activation (Figure 3). In addition, both U0126 and PD184352 induce an apparent increase in size of the MCF10A cells that is likely caused by increased cell spreading. DCIS cells do not show the same organization of the actin cytoskeleton under control conditions, and there is a limited effect of U0126 on this parameter, but PD184352 is able to completely restore the cortical actin organization and cell-cell localization of E-cadherin and produces cells that have a clearly epithelial morphology (Figure 3). Although there is no effect of either U0126 or PD184352 on E-cadherin localization in the H-Ras and N-Ras MCF10A cells, inhibition of ERK MAPK activation does produce a striking change in the actin cytoskeleton. The cells are clearly more spread and have prominent actin stress fibers after inhibition of MAPK kinase, with again a stronger effect induced by PD184352 (Figure 3). Interestingly, the H-Ras and N-Ras MCF10A cells seem to make more cell-cell contacts after inhibition of ERK MAPK activation, although E-cadherin localization is not restored (Figure 3).

To further investigate the effects of the MAPK kinase inhibitors on cell spreading, we performed experiments on subconfluent cells (Figure 4). The results show that both U0126 and PD184352 greatly

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**Figure 2.** (A, B) Overexpression of active H- and N-Ras decreases E-cadherin and activates ERK MAPK. MCF10A and derivative cells were cultured in growth medium. Cell lysates were subjected to Western blot assays as indicated. Two exposures are shown for the E-cadherin blots to reveal both the variation that occurs during progression from MCF10A to DCIS cells and the low level of E-cadherin expression that remains in the H-Ras and N-Ras MCF10A cells. Tubulin was probed to verify equal loading.
increase the spreading of the H-Ras and N-Ras MCF10A cells and induce prominent increases in actin stress fibers. Notably, these data also show that the increased stress fibers do not depend on cell-cell contacts because they are found even in isolated cells (Figure 4).

**Enforced Expression of Exogenous E-cadherin Correlated with Up-regulated Expression of Endogenous β-catenin**

Another potential method to restore epithelial morphology to transformed cells is to reintroduce expression of E-cadherin [27,46]. In the...
present study, we used retroviral transduction of the MCF10A cells and derivatives and observed overexpression of E-cadherin in all cell lines to approximately the same level (Figure 5). The retroviral construct used, LZRS-GFP-hEcad, has both E-cadherin and GFP coding sequences with an internal ribosome entry site between them. This arrangement allows bicistronic expression of both E-cadherin and GFP, with the latter being a reporter gene for E-cadherin expression. Cells infected with a GFP-only construct were used as control. The degree of overexpression of E-cadherin obtained was approximately two-fold over that in control MCF10A cells. Interestingly, overexpression of E-cadherin correlated with up-regulation of β-catenin expression, but had no effect on the activation of ERK MAPK (Figure 5).

In MCF10A, NeoT, AT1, and DCIS cells, the increase in β-catenin seems modest compared to the substantial levels of protein in control, GFP-infected cells. In H-Ras and N-Ras MCF10A cells, the increase in β-catenin induced by reexpression of E-cadherin, although of a similar magnitude, produces a much greater relative change over the minimal levels present in control, GFP-infected cells (Figure 5).

Both Reexpression of E-cadherin and Suppression of ERK MAPK Kinase Activity Were Required to Restore E-cadherin Cell-cell Junctions in H-Ras and N-Ras MCF10A Cells

In our study, the H-Ras and N-Ras MCF10A cell lines have very low expression levels of E-cadherin (Figure 2), and, although inhibitors of ERK MAPK activation cause increased cell spreading (Figures 3 and 4), they do not restore E-cadherin cell-cell junctions (Figure 4). We therefore tested whether reexpression of E-cadherin would reverse the mesenchymal phenotype back to an epithelial phenotype, either as a single intervention or in combination with MAPK kinase inhibition (Figure 6). The results show that reexpression of E-cadherin restored neither the E-cadherin junctions nor the epithelial morphology to H-Ras and N-Ras MCF10A cells (Figure 6, top row). The E-cadherin protein remained predominantly intracellular after overexpression. However, when these cells were induced to express E-cadherin and treated with MAPK kinase inhibitors U0126 or PD184352, the E-cadherin protein was targeted to cell-cell junctions, with less remaining localized intracellularly (Figure 6).

Figure 4. Inhibition of MAPK kinase induces cell spreading in Ras-transformed MCF10A cells. H-Ras and N-Ras MCF10A cells were cultured on coverslips for 3 days in the presence of U0126 (10 μM), PD184352 (1 μM), or vehicle (DMSO). Then, the cells were fixed and stained for actin cytoskeleton (green), nuclei (blue), and processed for immunofluorescent detection of E-cadherin (pink). Confocal fluorescent images are shown. Scale bar, 30 μm.
Figure 6. Both reexpression of E-cadherin and inhibition of MAPK kinase are required to restore E-cadherin cell-cell junction in H-Ras and N-Ras MCF10A cells. H-Ras and N-Ras MCF10A cells were infected with retroviruses to reexpress E-cadherin plus GFP or the expression of only GFP as the control. The cells were cultured on coverslips in the presence of U0126 (10 μM), PD184352 (1 μM), or vehicle (DMSO) for 3 days. The cells were then fixed and stained for nuclei (blue) and processed for immunofluorescent detection of E-cadherin (pink). The infected cells are green. Confocal fluorescent images are shown. Scale bar, 30 μm.

Figure 5. Enforced expression of E-cadherin in MCF10A and derivative cells. MCF10A and derivative cells were infected with retroviruses encoding E-cadherin plus GFP (label: Ecad) or GFP alone (label: GFP). After 5 days of infection, cell lysates were subjected to Western blot assay and probed for E-cadherin, β-catenin, and phospho-ERK1/2. Longer exposures of the E-cadherin blot reveal low levels of expression in the GFP-control condition for H-Ras and N-Ras MCF10A cells that are consistent with the levels shown in untransfected H-Ras and N-Ras MCF10A cells in Figure 2 (not shown). Tubulin was probed to verify equal loading.
To further investigate the pathways downstream of Ras that may contribute to the regulation of E-cadherin localization, we tested whether wortmannin, NSC23766, or ML-7 could induce exogenous E-cadherin to localize at cell-cell junctions (Figure 7). The results show that exogenous E-cadherin is strongly localized to the cell surface only in MCF10A cells, with a mix of cell surface and intracellular localization in DCIS cells. The peripheral localization of exogenous E-cadherin is somewhat reduced by the pathway inhibitors, which tend to produce an increase in the intracellular localization of E-cadherin (Figure 7, left columns). Similarly, but in contrast to the effects of U0126 and PD184352 (Figure 6), none of these inhibitors caused the localization of expressed E-cadherin to the cell surface of H-Ras and N-Ras MCF10A cells (Figure 7).

Inhibition of ERK MAPK Activation Affects a Broad Panel of Cell Adhesion-related Proteins and Pathways

Our results showed that inhibition of ERK MAPK kinase activation promotes the reversion to epithelial morphology in Ras-transformed MCF10A cells. We confirmed that the two MAPK kinase inhibitors efficiently down-regulated ERK activation under the conditions used in this study (Figure 8A). Because ERK MAPKs control many downstream events, we further characterized the treated cells to find effects...
that may contribute to the reversion of mesenchymal morphology. One possibility could be that MAPK activity may up-regulate the expression level of the zinc-finger family transcription factor Slug that suppresses E-cadherin expression [47,48], and thus, inhibition of MAPK kinase could enhance the level of E-cadherin. Such an effect has been seen for U0126 or PD98059 treatment of colon cancer cells, for example [47]. Our results, however, show that treatment with U0126 led to only a minimal increase in the expression of E-cadherin in H-Ras MCF10A cells but with no effect in MCF10A, DCIS, or N-Ras MCF10A cells (Figure 8B). PD184352, which is more effective than U0126 in the reversion to normal epithelial morphology (Figure 3), did not increase the expression of E-cadherin (Figure 8B).
These data show that MAPK kinase inhibitors are effective in restoring E-cadherin localization to cell-cell junctions (Figures 3 and 6) in the absence of a significant effect on E-cadherin expression levels (Figure 8B). Another potential mechanism for phenotypic reversion could be the loss of expression of mesenchymal markers. In this case, however, there was little effect of the MAPK kinase inhibitors on the expression of N-cadherin in the Ras-transformed cells (Figure 8B). Although there was no consistent effect of the MAPK kinase inhibitors on the expression of the cadherins, there was a consistent but modest increase in the expression of β-catenin in H-Ras and N-Ras MCF10A cells (Figure 8C). Quantification of these results showed that both U0126 and PD184352 caused an approximate doubling of the level of β-catenin in the H-Ras and N-Ras MCF10A cells but no change in the expression of β-catenin in MCF10A or DCIS cells (Figure 8D).

Another direct, albeit unlikely, possibility for how a treatment could reverse the effects of Ras transformation would be if the activation level of Ras was reduced. We therefore tested whether inhibition of ERK MAPK activity affected the level of active Ras-GTP in the MCF10A and derivative cells (Figure 9A). We found no effect of PD184352 to reduce the level of H-Ras-GTP in NeoT, AT1, DCIS, or H-Ras MCF10A cells or of N-Ras-GTP in N-Ras MCF10A cells. There was, however, an opposite and modest effect of PD184352 to increase the very low level endogenous H-Ras-GTP in MCF10A cells. This result may suggest that PD184352 blocks a feedback inhibitory pathway that acts to limit Ras activation in this cell type.

Inhibition of MAPK kinase induced cell spreading and an increase in actin stress fibers in H-Ras and N-Ras MCF10A cells (Figures 3 and 4). Because these results implicated changes in the actin cytoskeleton, we further tested for effects on the Rho family of small GTPases that have been well-documented to be regulators of epithelial cell adhesion, shape, polarity, and motility [49–52]. Our results show that the activation levels of Rac and Cdc42 are generally consistent across the whole series of cells tested and also are insensitive to inhibition of MAPK kinase (Figure 9A). Active levels of RhoA were only detectable in H-Ras and N-Ras MCF10A cells and consistently declined after PD184352 treatment (Figure 9, A and B). Active levels...
of RhoB were only detectable in N-Ras MCF10A cells and also declined after PD184352 treatment (Figure 9, A and C).

Discussion

Because E-cadherin plays a central role in the maintenance of the architecture and function of normal breast epithelial tissue, engineering the expression of E-cadherin in breast cancers that have lost its functional expression could be a new approach to the disease. For example, there could be mechanisms to induce reexpression of the endogenous E-cadherin. E-cadherin expression can be controlled at the transcriptional level by enhancing transcription factors AML1, Sp1, p300, and HNF3β [46] and by transcriptional repressors Snail, Slug, SIP-1, and Twist [26,53–55]. Slug, for example, can suppress E-cadherin expression in Ras transformation of intestinal epithelial cells [26] and in several breast cancer cell lines [7]. Knock-down of Slug by siRNA in Ras-transformed intestinal epithelial cells [26] and expression of the E-cadherin–enhancing transcription factor HNF3β or inhibition of DNA methylation in breast cancer cell lines [46] lead to restored E-cadherin expression and promote near-complete rescue of the epithelial morphology. Similar reversion of EMT in Ras-transformed breast epithelial cells can be induced by inhibition of NF-κB [56]. Another approach has been to directly reexpress E-cadherin. In MDA-MB-231 breast cancer cells, reexpression of E-cadherin leads to restoration of E-cadherin cell-cell junctions and reversion to epithelial morphology [26], impaired EGF and IGF-1 signaling [19], and more organized growth in three-dimensional culture [57]. In the current study, we found that reexpression of E-cadherin was not effective in reversing EMT in MCF10A cells that had been transformed by the high-level expression of activated H-Ras or N-Ras. This result is consistent with that reported in H-Ras–transformed intestinal epithelial cells [27]. In that report and in the current study, reexpressed E-cadherin did not relocalize to the cell surface but remained predominantly intracellular. The current study may be the first to demonstrate the effects of N-Ras transformation on E-cadherin junctions in human breast epithelial cells but is consistent with a report that activated N-Ras can cause EMT in a mouse mammary cell line [58].

In this study, higher levels of activated Ras produced more dramatic transformation of breast epithelial cells that was manifested at both morphologic and molecular levels. It is likely that the activation of the multiple effector pathways downstream of Ras may require different threshold levels of Ras activation, and thus that changes in the expression level of active Ras may trigger varying panels of downstream signaling events. For example, in keratinocytes, a lower level of v-Ha-Ras expression produces benign tumors, whereas a higher level of v-Ha-Ras causes malignant transformation [59]. In cells transformed by oncogenic N-Ras, the level of N-Ras expression determines activation of specific downstream pathways [60]. Lower levels of N-Ras expression couple to the Ras-GDS and PI 3-kinase pathways to trigger the mislocalization of the CDK inhibitor p27kip1, leading to CDK activation, but do not affect the TGF-β/Smad2/3 pathway. In contrast, higher levels of N-Ras expression not only induce the CDK pathway but also couple to the activation of MEK/ERK MAPK signaling that leads to inactivation of the TGF-β/Smad2/3 pathway [60]. These reports and our data suggest that a higher level of activated Ras expression may trigger more downstream pathways than a lower level of Ras expression and lead to stronger and more malignant transformation. Thus, the therapeutic reversion of cells that have undergone malignant transformation caused by higher levels of oncogene expression may require effective block of more than one pathway. For example, in the case of the present study, both inhibition of MAPK kinase and enforced expression of E-cadherin were required to restore adherens junctions in the H-Ras and N-Ras MCF10A cells.

The results of the current study and published work [27] suggest that Ras transformation not only can lead to the suppression of E-cadherin expression but may also prevent the formation of functional E-cadherin junctions by preventing the appropriate subcellular localization of E-cadherin. We have found that inhibition of ERK MAPK activation is effective in restoration of E-cadherin junctions in DCIS cells, which have a modest level of active Ras expression. Similarly, inhibition of ERK MAPK activation can induce reexpressed E-cadherin to locate to cell-cell junctions in H-Ras and N-Ras MCF10A cells. These results are again consistent with the effect of U0126 to cause relocalization of E-cadherin to the cell surface in Ras-transformed intestinal epithelial cells [9]. In both DCIS cells, where significant E-cadherin expression remains, albeit at an intracellular location, and in H-Ras and N-Ras MCF10A cells infected with retroviruses encoding E-cadherin, the effect of MAPK kinase inhibition is predominantly on the relocalization of E-cadherin. We have investigated several potential mechanisms that could drive this effect. For example, inhibition of ERK MAPK activation caused a modest increase in the expression of β-catenin, which, because it can stabilize functional E-cadherin complexes at the cell surface [13], could contribute to the response. Whereas this could be a factor in the DCIS cells, it seems unlikely to explain the relocalization of expressed E-cadherin in the H-Ras and N-Ras MCF10A cells, as the reexpression of E-cadherin itself produces a larger increase in expression of β-catenin but does not restore the adherens junctions. Another potential and related mechanism could be through a reduction in N-cadherin expression so that the β-catenin could be restored to complexes with E-cadherin, but N-cadherin expression was not reduced in the MAPK kinase inhibitor–treated cells.

E-cadherin localization is also regulated by the activation of Rho family small GTPases. RhoA, Rac, and Cdc42 have roles in the establishment and maintenance of E-cadherin cell junctions [15–17] and also in the dysregulation of E-cadherin localization in Ras-transformed cells [14,25]. The connection between Ras and the Rho GTPases seems to be cell type–dependent. For example, in colon carcinoma cells, Ras–stimulated ERK activity promotes Rac activation but inhibits Rho activation [61]. In contrast, in Madin–Darby canine kidney (MDCK) cells, Ras–stimulated ERK activity inhibits Rho activation but promotes Rho activation [25]. Our results in MCF10A cell derivatives do not reveal any changes in Rac1 expression or activation in response to either Ras transformation or inhibition of ERK MAPK activation. Further, an inhibitor of Rac activation had no effect on relocalization of E-cadherin. Thus, Rac1 is not likely to be a mediator of either the dysregulated or the restored E-cadherin localization in this study. Similarly, we did not record any changes in Cdc42 activation in response to Ras transformation or inhibition of ERK MAPK activation.

In contrast to the lack of evidence for a role for Rac or Cdc42 in the relocalization of E-cadherin, we found several effects that are related to Rho, including activation of RhoA in H-Ras MCF10A cells and activation of both RhoA and RhoB in N-Ras MCF10A cells. This may be the first report of activation of RhoB in response to activated N-Ras and provides some contrast to a previous report that activated N-Ras suppresses the expression of RhoB [62]. It is not likely that RhoB activation plays a specific role in the localization of
E-cadherin, because the dysregulation and restoration of E-cadherin junctions occur similarly in H-Ras and N-Ras MCF10A cells that are distinct in their RhoB activation status. There are, however, differences in the invasiveness of H-Ras and N-Ras MCF10A ([30] and our unpublished observations) that may be related to RhoB. Activation of both RhoA and RhoB was reduced by inhibition of ERK MAPK activation. Thus, increased Rho activation in the Ras-transformed cells correlates with profound disruption of E-cadherin junctions, whereas reduced Rho activation after inhibition of MAPK kinase correlates with the relocalization of reexpressed E-cadherin to the cell surface. Inhibition of Rho function and downstream signaling, e.g., by inhibition of MLCK with ML-7 [65], has previously been shown to restore E-cadherin localization in MCF10A cells transfected with activated H-Ras [14]. Although we did not find any such effect of ML-7 in the H-Ras and N-Ras MCF10A cells, ML-7 did have some modest ability to restore E-cadherin localization in DCIS cells. It may be that the ability of ML-7 to restore E-cadherin localization is dependent on a relatively low level of expression of activated Ras (such as is found in DCIS cells). Notably, even when E-cadherin was reexpressed in H-Ras and N-Ras MCF10A, ML-7 was still not able to restore its localization to the cell surface.

The effects of Rho activation on epithelial morphology vary with the cell models studied. For example, in another model of MCF10A cells transfected with activated H-Ras, Rho activity is required for the mesenchymal contractility and motility, and inhibition of Rho by a dominant-negative mutant of RhoA partially restores normal epithelial phenotype [14]. However, in the same cell model, Rho activity is also required for normal cell-cell adhesion junction formation and maintenance, and blockage of Rho activity by C3 exotransferrase completely disrupts E-cadherin junction [14]. In human keratinocytes and MDCK cells, Rho is also required for the establishment of cadherin-dependent cell-cell junction [15,64]. In contrast, activation of Rho in MDCK cells can also result in EMT [65]. The discrepancies between these reports may come from methodological issues, e.g., C3 exotransferrase, which is a potent Rho inhibitor, inhibits several Rho isoforms (RhoA, RhoB, and RhoC), and dominant-negative RhoA may also be able to diminish the activation of other isoforms. RhoB, for example, clearly has distinct cellular functions [49,66,67]. In the H-Ras and N-Ras MCF10A cell models in this study, increased RhoA activity may contribute to the fibroblastoid phenotype. Mitogen-activated protein kinase kinase inhibition induced a flattened cell morphology with thick, dense stress fibers that were independent of cell confluency. These thick stress fibers are quite different from the fine actin mesh in normal MCF10A cells. This observation is consistent with results from U0126-treated human colon carcinoma cells, which also display flattened morphology and remarkable stress fibers [61]. The difference between these results is that in the colon carcinoma cell lines, the stress fibers correlate with enhanced Rho activity after MAPK kinase inhibition. In the current study, MAPK kinase inhibition induced a decrease in activation of RhoA (and also RhoB). However, even after MAPK kinase inhibition, there was still a substantial level of Rho activity remaining compared to the parental MCF10A cells. It is possible that this remaining Rho activity will be required for the formation of the observed stress fibers.

Overall, our results suggest that the ability to restore epithelial morphology and E-cadherin adherens junctions in Ras-transformed breast epithelial cells is dependent on the degree of expression of activated Ras. In cells that express lower levels of activated Ras and retain some E-cadherin expression, the relocalization of that E-cadherin by inhibition of the ERK MAPK pathway is very effective in restoring the adherens junctions. In cells with a more marked Ras-transformed phenotype that is characterized by a strong activation of RhoA, both reexpression of E-cadherin and inhibition of ERK MAPK are required to restore epithelial morphology and E-cadherin cell-cell junctions. The ability to restore adherens junctions has been proposed to be of potential significance as a cancer therapy, but evidence suggests that ductal breast carcinomas, which often retain E-cadherin expression, have a worse prognosis than lobular breast carcinomas that typically lack E-cadherin expression [1]. Further, although loss of E-cadherin can occur in the metastasis of ductal carcinoma to the pancreas, expression can be maintained in metastases to other sites [5], and it has been reported that increased E-cadherin expression is associated with metastasis to bone [68]. Overall, it is likely that there is a complex relationship between E-cadherin expression and breast cancer, with dynamic changes at different stages of tumor progression [1]. Because inhibition of MAPK kinase has been suggested as a potential therapeutic approach to breast cancer [69], it will be important to determine whether such changes in E-cadherin occur in animal models and patient samples.

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References
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Li and Mattingly


