E-cadherin loss promotes the initiation of squamous cell carcinoma invasion through modulation of integrin-mediated adhesion

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Summary

Much remains to be learned about how cell-cell and cell-matrix interactions are coordinated to influence the earliest development of neoplasia. We used novel 3D human tissue reconstructs that mimic premalignant disease in normal epidermis, to directly investigate how loss of E-cadherin function directs conversion to malignant disease. We used a genetically tagged variant of Ha-Ras-transformed human keratinocytes (II-4) expressing dominant-interfering E-cadherin fusion protein (H-2k^d-Ecad). These cells were admixed with normal human keratinocytes and tumor cell fate was monitored in 3D reconstructed epidermis upon transplantation to immunodeficient mice. Tumor initiation was suppressed in tissues harboring control- and mock-infected II-4 cells that lost contact with the stromal interface. By contrast, H-2k^d-Ecad-expressing cells persisted at this interface, thus enabling incipient tumor cell invasion upon in vivo transplantation. Loss of intercellular adhesion was linked to elevated cell surface expression of α2, α3 and β1 integrins and increased adhesion to laminin-1 and Types I and IV collagen that was blocked with β1-integrin antibodies, suggesting that invasion was linked to initial II-4 cell attachment at the stromal interface. Collectively, these results outline a novel aspect to loss of E-cadherin function that is linked to the mutually interdependent regulation of cell-cell and cell-matrix adhesion and has significant consequences for the conversion of premalignancy to cancer.

Key words: E-cadherin, Integrin, Squamous cell carcinoma, 3D cultures, Premalignant

Introduction

Cell fate decisions in whole organisms are largely controlled by signals imparted by the tissue microenvironment. This occurs through a complex network of molecular signals that is directed either by interactions between neighboring cells or with extracellular matrix components or by stimulation of cell surface receptors by soluble factors. This multitude of environmental signals provides an efficient system to control cell behavior and maintain tissue homeostasis. Conversely, cancer development may be considered as a breakdown of microenvironmental control of cell behavior (Bissell and Radisky, 2001). Consistent with this notion, both integrin-mediated cell-matrix adhesion and cadherin-mediated cell-cell adhesion are frequently altered in tumor cells as a result of loss or gain of specific adhesion receptors. For example, the cell-cell adhesion receptor E-cadherin is frequently lost during advanced stages of epithelial cancer progression (Perl et al., 1998; Birchmeier and Behrens, 1994) and this is considered to be an important step in invasion and metastasis of epithelial tumor cells (Bissell and Radisky, 2001; Cavallaro and Cristofori, 2004; Cano et al., 2000). However, it is not well understood how perturbation of cell adhesion networks can influence the initial stages of cancer progression as premalignant-to-malignant conversion occurs.

A complex epithelium such as the epidermis provides a model system that is well suited to study the networks of structural and contextual signals that govern the orderly execution of normal proliferation and differentiation programs (Jamora and Fuchs, 2002). During the earliest, intraepithelial stages of squamous cell carcinoma development, small nests of aberrant, dysplastic tumor cells are exposed to and affected by the same positional signals that govern normal epithelial cell behavior (Dlugosz et al., 2002). To better understand this interplay we have developed three-dimensional (3D) human tissue models that allow us to monitor premalignant, intraepithelial (IE) stages of squamous carcinoma progression in the context of an in-vivo-like stratified epithelium (Alt-Holland et al., 2005). We described previously that interactions between IE tumor cells and neighboring normal cells can lead to suppression of early neoplastic progression by inducing a state of 'intraepithelial dormancy' (Javaherian et al., 1998). This dormant state could be overcome by altered tissue dynamics in response to the tumor promoter TPA (Karen et al., 1999), ultraviolet (UV) irradiation (Mudgil et al., 2003), decreased adhesive interactions between tumor cells and adjacent epithelia

(Vaccariello et al., 1999) or by enabling tumor cells to interact with basement membrane (BM) proteins (Andriani et al., 2004). These observations implied that alterations in the ability of IE tumor cells to adhere to adjacent cells or in their capacity to persist at the stromal interface were of crucial importance to their invasive potential. Concurrently, other studies performed in conventional two-dimensional (2D) cultures, provided evidence for mutual interdependence between cadherin- and integrin-mediated adhesion events in cancer cells (Avizienyte et al., 2002; Zhang et al., 2003; Yano et al., 2004). However, the functional consequences of such crosstalk between these distinct adhesive pathways during the earliest stages of cancer development in 3D tissues or in vivo remain largely unknown.

The present study provides new insights into the dynamic equilibrium between cell-cell and cell-matrix adhesive events in early epithelial tumor progression. Specifically, ablation of E-cadherin function in cells with malignant potential imparted an invasive phenotype to these cells by allowing them to overcome microenvironmental constraints on their progression. This phenomenon was associated with retention of E-cadherin-deficient cells at the epithelial-stromal interface in epidermal reconstructs. Furthermore, increased retention at this interface co-segregated with enhanced adhesion of E-cadherin-deficient cells to extracellular matrix components including laminin, fibronectin and Type IV collagen in vitro. Finally, we describe increased expression of integrin subunits likely to mediate adhesion in E-cadherin-deficient cells to collagen Types I and IV at this interface. Collectively, our results point to a novel aspect of E-cadherin loss in epithelial tumor progression that is not only related to severing cell-cell adhesion but also is associated with increased cell-matrix adhesion of these cells. The functional consequence of enhanced cell-matrix adhesion is the initial attachment and retention of these cells at the epithelial-stromal interface, thus providing the appropriate microenvironmental conditions for incipient tumor cell invasion.

Results

Loss of E-cadherin enables premalignant-to-malignant conversion upon incipient tumor cell invasion

Our previous studies showed that II-4 cells were non-tumorigenic when placed in epidermal reconstructs with human epidermal keratinocytes (HEK) because of their microenvironmental growth suppression and were shed from the epithelial surface (Javaherian et al., 1998). Based on this earlier observation, we first determined if loss of E-cadherin function could circumvent this constraint by altering the dynamics of II-4 cell behavior when grown in the context of normal, differentiating epidermal cells in 3D reconstructs. Epidermal reconstructs were established in vitro with either control II-4 cells expressing pBabe or H-2k^{d}-EcadC25 or with II-4 cells expressing a dominant-interfering E-cadherin (H-2k^{d}-Ecad). The H-2k^{d}-Ecad transgene has previously been described to interfere with E-cadherin function in normal keratinocytes (Zhu and Watt, 1996) and II-4 cells (Margulis et al. 2005a). This occurs as overexpression of this fusion protein leads to destabilization of endogenous E-cadherin-β-catenin complexes upon the cytoplasmic sequestration of β-catenin by the dominant-interfering transgene and to loss of E-cadherin-mediated intercellular adhesion. By contrast, the H-2k^{d}-EcadC25 used as a control, lacks the β-catenin binding region in the intracellular domain of E-cadherin and has no discernible effect on E-cadherin function. To model a specific IE tumor cell load, HEK were mixed at a 4:1 ratio with II-4 cell variants expressing either of these vectors in 3D tissue constructs and were transplanted as surface grafts to the dorsum of nude mice for 4 weeks. Upon transplantation, only grafts containing HEK/H-2k^{d}-Ecad II-4 mixtures generated clinically apparent lesions (Fig. 1C) whereas reconstructs harboring pBabe and H-2k^{d}-Ecad-C25 gave rise to normal human epidermis (Fig. 1A,B respectively). HEK/H-2k^{d}-Ecad-II-4 mixtures gave rise to raised, nodular lesions with focal areas of erythema (Fig. 1C, arrow). H-2k^{d}-Ecad-II-4 tumor

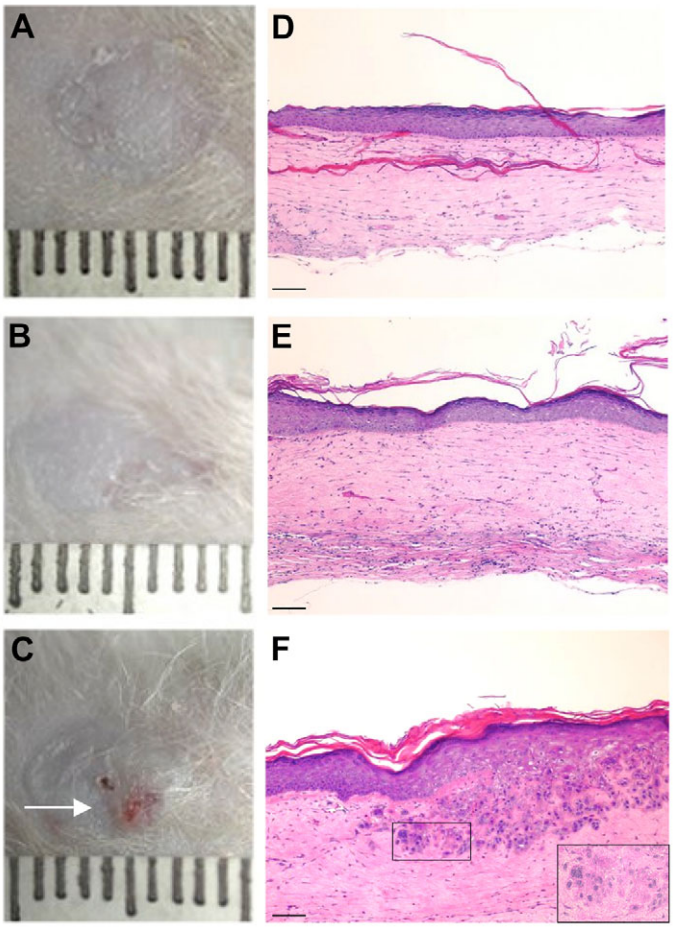

Fig. 1. Invasive tumor formation after in vivo transplantation was dependent upon loss of E-cadherin. (A-C) Clinical appearance of 3D surface transplants (left panel): 3D tissues were engineered in vitro as 4:1 mixtures of HEK and either pBabe-, H-2k^{d}-EcadC25- or H-2k^{d}-Ecad-II-4-expressing cells and transplanted to the dorsum of nude mice for 4 weeks. Grafts harboring E-cadherin-deficient, H-2k^{d}-Ecad-expressing II-4 cells generated nodular tumors with focal areas of erythema (C, arrow). By contrast, grafts containing II-4-pBabe (A) or H-2k^{d}-EcadC25-expressing cells (B) generated normal grafts without any evidence of tumor formation. (D-F) Histological appearance of 3D surface transplants. Tumors generated in transplants of 4:1 mixtures of HEK and H2k^{d}-Ecad-expressing II-4 cells demonstrated invasion of individual cells (F, inset) and small clusters of poorly differentiated tumor cells (F). By contrast, grafted mixtures of HEK with either pBabe- (D) or H-2k^{d}-EcadC25-expressing II-4 cells (E) generated normal epithelia without any evidence of residual tumor cells. Bars, 20 μm.

cells invaded into the dermis as small clusters or as individual cells (Fig. 1F, inset). By contrast, mixtures of control HEK/pBabe-II-4 (Fig. 1A,D) or HEK/H-2k^d-EcadC25-II4 (Fig. 1B,E) generated normal epidermal grafts with intact epithelial architecture in which no tumor cells were visible. The fate and phenotype of H-2k^d-Ecad-expressing II-4 cells was further studied by double immunostaining for the β-gal marker gene and β-catenin to determine that transgene expression was maintained in vivo and led to loss of adherens junctions from cell-cell borders (Fig. 2). β-gal-positive II-4 cells were absent from tissues that originally harbored mixtures of HEK with either the control pBabe- (Fig. 2A) or H-2k^d-EcadC25-expressing II-4 cells (Fig. 2B). In these transplants, β-catenin was detected only at cell-cell borders of HEK (Fig. 2A,B). By contrast, large groups of β-gal-positive, II-4 cells were apparent beneath the dermal/epidermal junction in HEK/H-2k^d-Ecad-II-4 grafts (Fig. 2C) indicating that these cells had traversed the BM and had invaded into the dermal compartment. Furthermore, these grafts showed cytoplasmic localization of β-gal (Fig. 2C) and β-catenin (Fig. 2D) in tumor cells indicating that loss of E-cadherin function was associated with redistribution of β-catenin from cell junctions in invading cells. Moreover, the majority of large groups of invading β-gal-positive tumor cells localized beneath the dermal/epidermal junction presented increased expression of the α2 integrin subunit (Fig. 2E). By contrast, in transplants that originally harbored the mixture of HEK with either the control pBabe (Fig. 2F) or H-2k^d-EcadC25-expressing II-4 cells (not shown), α2 integrin expression was restricted only to the basal HEK that were layered on the epithelial-stromal interface. This suggested that altered integrin-mediated adhesion may play a role in the invasive properties of these tumor cells. In summary, these results underscore that invasion of II-4 cells was contingent upon loss of E-cadherin function and required the capacity of these cells to persist in the tissue despite loss of adhesion with adjacent cells. This result raised the question as to how H-2k^d-Ecad-II-4 cells were retained at the BM interface as a precondition for tumor cell invasion.

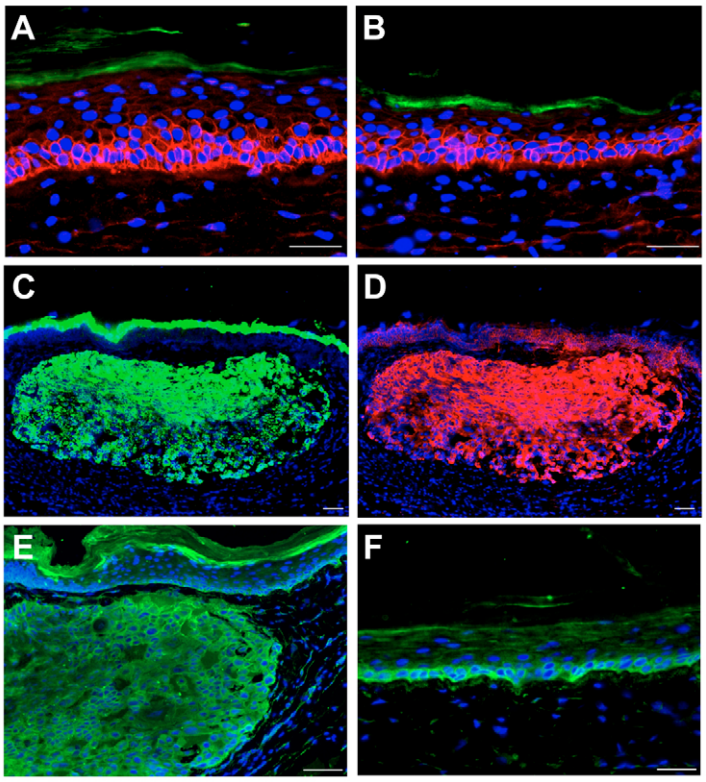

Fig. 2. Tumor cell invasion was linked to the cytoplasmic redistribution of β-catenin and increased expression of α2 integrin subunit. (A-F) Four weeks after in vivo transplantation of 4:1 mixtures, excised tissues were stained by double immunofluorescence for β-gal (green) and β-catenin (red). In transplanted mixtures comprised of HEK and control pBabe- (A) or EcadC25-expressing II-4 cells (B), β-gal-positive II-4 cells were not present and β-catenin was localized at cell-cell borders of the HEK cells. By contrast, as can be seen by the same immunostaining pattern, mixtures of HEK and H2k^d-Ecad-expressing II-4 cells demonstrated islands of invasive tumor cells that showed cytoplasmic co-localization of β-gal (C, green) and β-catenin (D, red), indicating that II-4 cell invasion was associated with loss of β-catenin from cell junctions and abrogation of cell-cell adhesion. The invading clusters of tumor cells showed increased expression of α2 integrin subunit (E, green), whereas in control grafts comprised of HEK and control pBabe-II-4 cells, α2 integrin expression was limited to the basal HEK at the epithelial-stromal interface (F, green). Bars, 10 μm.

Loss of E-cadherin function enhances attachment of II-4 cells to the epithelial-stromal interface and is a prerequisite for tumor cell invasion

To address this issue, we assessed the distribution of II-4 cell variants in cell mixtures in 3D in vitro constructs just prior to transplantation. Mixtures of HEK with any of the three types of II-4 cells were cultured at 4:1 ratios at an air-liquid interface for 7 days. The distribution of tumor cells in reconstructs was then analyzed by double immunofluorescent staining for β-gal and Type IV collagen to identify the II-4 cells and the BM interface, respectively. Reconstructs harboring H-2k^d-Ecad-expressing cells demonstrated individual β-gal-positive, II-4 cells in the basal layer of the epithelium, adjacent to Type IV collagen seen at the epithelial-stromal interface (Fig. 3C, arrow). By contrast, no β-gal-positive cells were seen at this interface in tissues containing the HEK/pBabe-II-4 (Fig. 3A) or HEK/H-2k^{d}EcadC25-II-4 mixtures (Fig. 3B). Consistent with their eventual loss by shedding from the epithelial surface, these control cells were exclusively observed in the suprabasal cell layers of the epithelium and had lost contact with the stromal interface (Fig. 3A,B, arrows). These findings suggested that loss of E-cadherin altered the adhesive repertoire of these cells, thus creating permissive conditions for their attachment and persistence in the basal layer. Moreover, these results identify two separate but mutually interdependent mechanisms by which loss of E-cadherin function may support initiation of squamous cell carcinoma progression. First, absence of E-cadherin function allowed these cells to evade tumor suppression mediated by cell-cell contact that could have prevented their persistence after in vivo transplantation. Secondly, loss of E-cadherin function enabled small numbers of II-4 cells to adhere to the stromal interface in vitro and, thus, to manifest their invasive potential after in vivo transplantation.

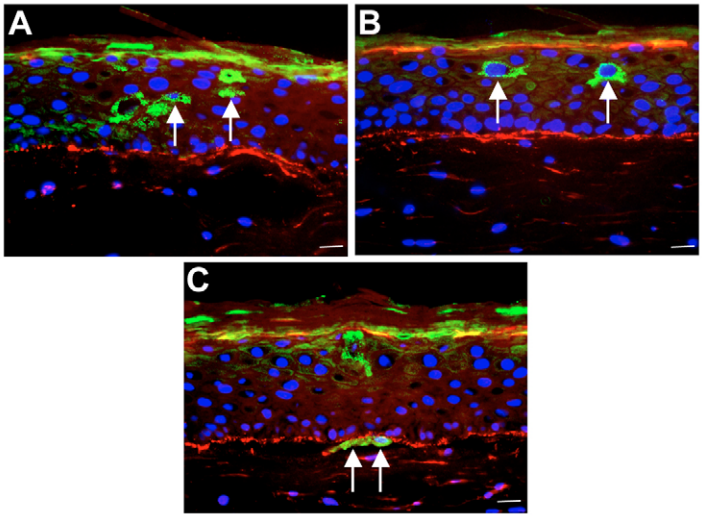

Fig. 3. Invasion of cell-cell adhesion-deficient II-4 cells was preceded by their attachment and localization at the epithelial-stromal interface in 3D in vitro tissues. In vitro 3D cultures were constructed as 4:1 mixtures of HEK with either pBabe-, H-2k^{d}-EcadC25- or H-2k^{d}-Ecad-II-4 cells and were grown at an air-liquid interface for 7 days. Tissues were stained by double immunofluorescence for the basement membrane component Type IV collagen (red) and β-gal (green). Constructs harboring mixtures of HEK with either pBabe- (A, arrows) or H-2k^{d}-EcadC25-expressing II-4 cells (B, arrows) demonstrated tumor cells that were limited to the suprabasal layers of the epithelium. By contrast, tissues harboring H-2k^{d}-Ecad-II-4 cells demonstrated β-gal-positive cells in the basal layer of the epithelium, adjacent to the Type IV collagen present at the epithelial-stromal interface (C, arrows). Bars, 10 μm.

Loss of E-cadherin function is associated with enhanced cell-matrix adhesion

To determine whether the attachment of E-cadherin-deficient cells to the stromal interface prior to tumor cell invasion was due to changes in their adhesion to extracellular matrix components, short-term attachment assays were performed in monolayer cultures. H-2k^{d}-Ecad-expressing II-4 cells demonstrated a significant increase in adhesion to surfaces coated with ECM substrates when compared with control pBabe- and H-2k^{d}EcadC25-expressing II-4 cells (Fig. 4). This effect was most pronounced when H-2k^{d}-Ecad-expressing cells were seeded on the BM constituents Type IV collagen (3.5-fold increase; Fig. 4B) and laminin (twofold increase; Fig. 4C). In addition, H-2k^{d}-Ecad-expressing II-4 cells demonstrated elevated adhesion to Type I collagen-coated (Fig. 4A) and fibronectin-coated (Fig. 4D) plates when compared with the control II-4 cells. This demonstrates that loss of E-cadherin-mediated adhesion was associated with a general increase in the repertoire of matrix-adhesive receptors on the surface of these cells.

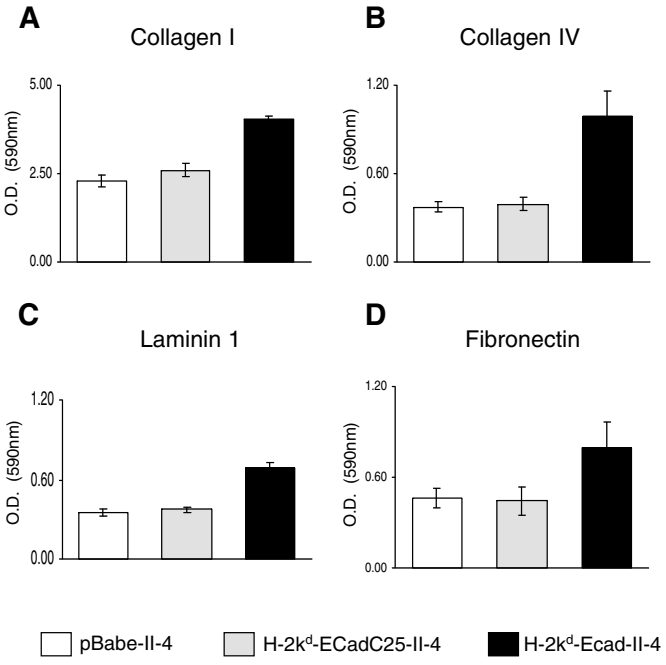

Fig. 4. Loss of E-cadherin increases II-4 cell attachment to extracellular matrix proteins. 2D cultures of pBabe-, H-2k^{d}-EcadC25- or H-2k^{d}-Ecad-II-4 cells were trypsinized briefly and replated onto plates coated with either Type I collagen (A), Type IV collagen (B), laminin-1 (C) or fibronectin (D) for 20 minutes. Attached cells were quantified by optical density determined at 590 nm. Results are calculated as the mean ± s.d. of four replicates and experiments were repeated four times. P<0.01 for H-2k^{d}-Ecad-II-4 cells vs control cells in adherence to Types I and IV collagen substrates. P<0.05 for H-2k^{d}-Ecad-II-4 cells vs control cells in adherence to laminin-1 and fibronectin substrates.

Loss of E-cadherin function in II-4 cells is associated with enhanced expression of α2, α3 and β1 integrin subunits

To address the mechanism underlying augmented matrix adhesion of H-2k^{d}-Ecad-II-4 cells, we compared the levels of specific integrin subunits expressed on the surface of the three II-4 cell lines in 2D monolayer cultures. Immunoblotting of membrane extracts revealed a marked increase in the level of α3 integrin expression in E-cadherin-deficient, H-2k^{d}-Ecad-II-4 cells, when compared with the relatively low α3 expression level present in the control pBabe-II-4 and in the H-2k^{d}EcadC25-expressing II-4 cultures (Fig. 5A). As in invading tumor cells in nude mice, a similar increase in expression was observed for the α2 integrin subunit, in H-2k^{d}-Ecad-expressing cells, when compared with control pBabe-II-4 cells and with H-2k^{d}EcadC25-expressing II-4 cells (Fig. 5A). However, in both control cultures, the expression level of α2 subunit was higher than the expression level of the α3 integrin subunit. As expected, the increased expression level of both α3 and α2 subunits was accompanied by an increased expression of the complementary β1 integrin subunit in the H-2k^{d}-Ecad-expressing cells, in comparison to β1 levels present in both control cultures.

These findings were supported by flow cytometric analysis of cell surface integrin expression of the three II-4 cell lines under investigation. H-2k^{d}-Ecad-II-4 and the control immunolabeled

pBabe-II-4 cells, and H-2k^{d}EcadC25-expressing II-4 cells were labeled with anti-α2, α3 or β1 integrin antibodies. The levels of cell surface integrin subunits α2, α3 and β1 were considerably increased in the E-cadherin-deficient, H-2k^d-Ecad-II-4 cells, when compared with levels of these cell surface integrin subunits in the control pBabe-II-4 cells (Fig. 5B, right panel). By contrast, no significant differences in cell surface integrin levels of α2, α3 and β1 subunits were identified between control pBabe-II-4 cells and H-2k^{d}EcadC25-II-4 cells (Fig. 5B, left panel).

Finally, to determine the functional significance of the increased expression of the integrin subunits in H-2k^d-Ecad-II-4 cells, short-term adherence assays to Type I and IV collagen and to laminin 1 substrates were performed in the presence or absence of β1 integrin blocking antibodies. As seen in Fig. 5C, the presence of specific β1-blocking antibodies dramatically reduced the ability of H-2k^d-Ecad-II-4 cells to adhere to Type I and IV collagens (80% and 55% decrease, respectively). By contrast, cells adhered to a lesser extent to laminin 1 and were mildly affected by the presence of β1 blocking antibodies (Fig. 5C, 20% decrease). Taken together these results demonstrate that loss of E-cadherin-mediated cell-cell adhesion was associated with increased expression of functioning cell surface α2, α3 and β1 integrin subunits. Furthermore, this increase in integrin expression is correlated with the ability of these cells to adhere rapidly to Type I and IV collagens, that are present at the epithelial-stromal interface.

Discussion

Microenvironmental cues inherent in normal tissue architecture are known to limit the malignant potential of precancerous tumor cells through an intrinsic, tissue-based control of early cancer progression (Bissell and Radisky, 2001; Alt-Holland et al., 2005). Consistent with this notion, we have previously established that interactions between tumor cells and adjacent normal keratinocytes induce a state of 'intraepithelial dormancy' in which premalignant tumor cells embedded in the stratifying epidermis withdraw from the cell cycle, lose attachment to the basement membrane and are eventually lost along with terminally differentiating keratinocytes (Javaherian et al., 1998). Here we report that this barrier to early squamous carcinoma progression can be overcome through previously unknown molecular events incurred upon loss of E-cadherin function in epithelial cells with malignant potential. Specifically, disruption of E-cadherin function in H-Ras-transformed human cell line was associated with an increase in integrin-mediated adhesion that enabled intraepithelial (IE) tumor cells to adhere to the epithelial-stromal interface in 3D tissue reconstructs. This increased adhesion set the stage for invasion of tumor cells that was observed after transplantation of skin reconstructs to immunodeficient mice. Collectively, these results suggest a novel role for E-cadherin loss in the initiation of epithelial tumor progression that arose from linkage between loss of cell-cell adhesion and gain of cell-matrix adhesion.

Both integrin-mediated cell-matrix adhesion and cadherin-mediated cell-cell adhesion are known to play central roles in tumor cell adhesion, migration, invasion, and metastasis (Cavallaro and Christofori, 2004; Friedl and Wolf, 2003). Recent studies using monolayer cultures of epithelial cancer cells revealed a complex network of molecular pathways linking cell-cell and cell-matrix adhesion. For example, integrin-mediated loss of E-cadherin from cell-cell junctions is associated with overexpression of *src* in colon carcinoma cells. In addition, integrin-linked kinase has been shown to control E-cadherin expression (Wu et al., 1999) and elements of the integrin signaling complex regulate N-cadherin-mediated adhesion (Gotzman et al., 2004). Furthermore, abrogation of E-cadherin function in breast cancer cells resulted in increased activity of the αv integrin subunit and increased cell migration (van Schliepp et al., 2000). We extend these earlier observations into 3D human tissue constructs by describing increased surface localization and function of β1 integrins upon loss of E-cadherin in II-4 cells. Upregulation of α2, α3 and β1 integrin subunits was paralleled by increased adhesion to extracellular matrix components, particularly laminin and Type IV collagen. Furthermore, only IE tumor cells that had lost functional adherens junctions, as seen by the cytoplasmic redistribution of β-catenin, were retained at the stromal interface in vitro. Thus, the present investigation demonstrates for the first time, that the mutually interdependent regulation of cell-cell and cell-matrix adhesion has significant consequences for the initial stages of epithelial cancer progression in 3D human tissues that mimic their in vivo counterparts.

The ability of E-cadherin-deficient II-4 cells to initiate invasion in the context of NHK is due specifically to increased integrin-mediated attachment to Types I and IV collagen at the epithelial-stromal interface. We have performed function-blocking studies using antibody directed against β1 integrin to show that this adhesion can be significantly limited when these cells are exposed to this blocking antibodies. Thus, increased expression and function of α2, α3 and β1 integrin subunits seen upon loss of E-cadherin function, enables adhesion of E-cadherin-deficient cells that is specific to these ECM proteins. These findings now enable us to hypothesize how the link between loss of E-cadherin and gain of specific integrin subunits allow intraepithelial tumor cells to manifest their invasive behavior. First, loss of E-cadherin and increased integrin function enables adhesion specifically to Type I collagen, which was the predominant protein present at the stromal interface when 3D cultures were seeded onto Type I collagen gels. Type IV collagen may also play a role in the retention of E-cadherin-deficient cells, as it is subsequently deposited by II-4 cells in the basal layer several days after cells are seeded into 3D tissues (Andriani et al., 2004). Interestingly, integrin-mediated binding to laminin-1 does not appear to play a role during these events, as integrin-blocking antibodies do not significantly decrease the adhesion of E-cad-deficient cells. It is possible that this laminin may play a role in adhesion through non-integrin receptors (Kim et al., 1999). Thus, only after attachment of E-cadherin-deficient cells to Type I and IV collagen at the stromal interface will these cells realize their malignant potential by overcoming local, microenvironmental constraints. This points to a crucial role for the loss of E-cadherin and acquisition of specific ECM attachment in the initiation of tumor cell invasion.

Acquisition of an invasive phenotype is a crucial event in early tumor progression, as this property is a prerequisite for connective tissue infiltration and metastatic dissemination associated with poor clinical outcomes. A molecular mechanism recognized to guard against invasion and metastasis is apoptosis or anoikis that is incurred upon loss of integrin-mediated ECM adhesion (Meredith et al., 1993; Frisch

and Francis, 1994). Anoikis has been described for both immortalized human keratinocytes with low malignant potential (Jost et al., 2001) and for SCC cell lines at a more advanced stage of tumor progression (Kim et al., 1999; Janes and Watt, 2004). Recently, anoikis was shown to be induced by the forced expression of αv integrin in an αv-negative SCC line,

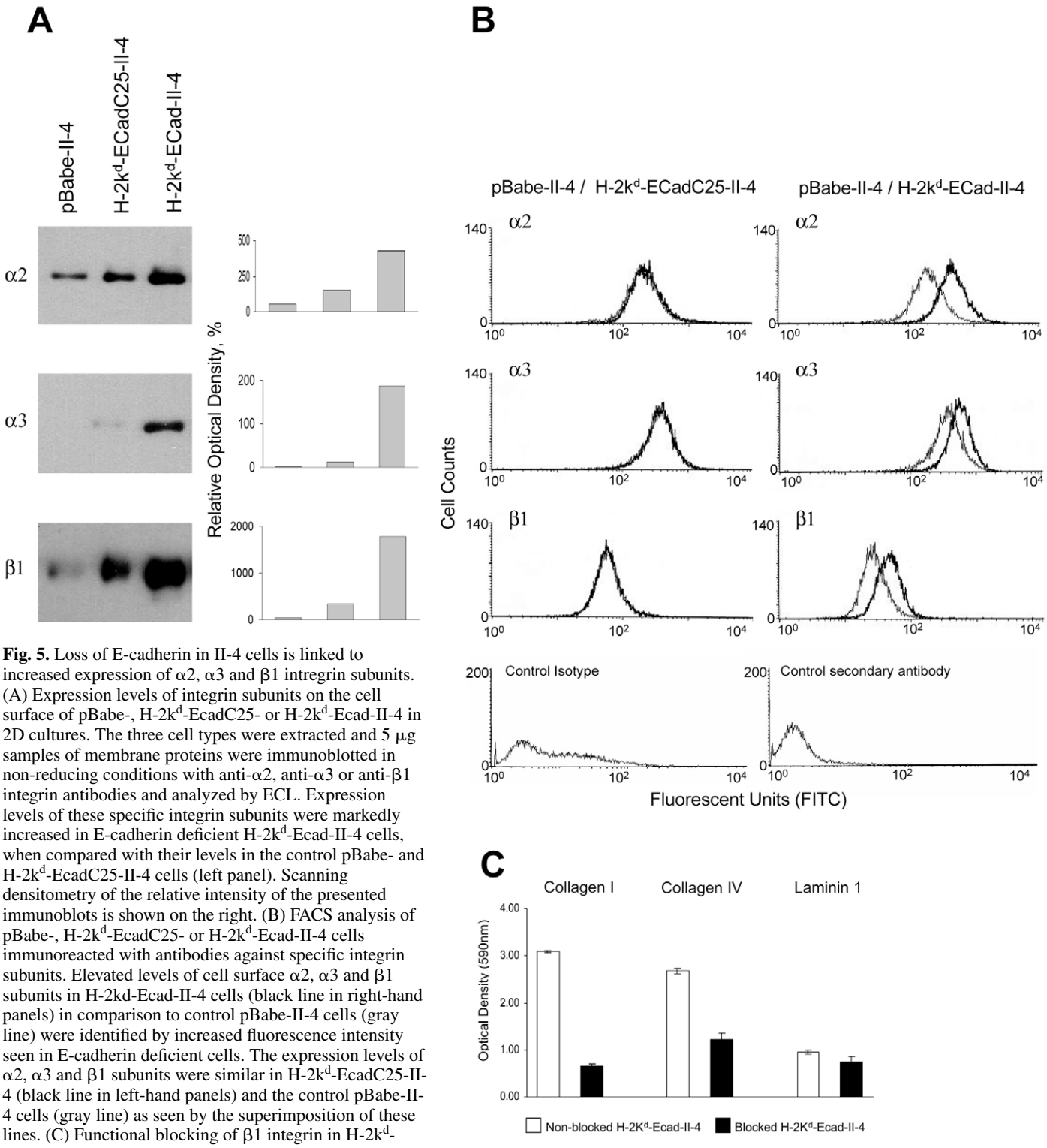

Fig. 5. Loss of E-cadherin in II-4 cells is linked to increased expression of α2, α3 and β1 intregrin subunits. (A) Expression levels of integrin subunits on the cell surface of pBabe-, H-2k^d-EcadC25- or H-2k^d-Ecad-II-4 in 2D cultures. The three cell types were extracted and 5 μg samples of membrane proteins were immunoblotted in non-reducing conditions with anti-α2, anti-α3 or anti-β1 integrin antibodies and analyzed by ECL. Expression levels of these specific integrin subunits were markedly increased in E-cadherin deficient H-2k^d-Ecad-II-4 cells, when compared with their levels in the control pBabe- and H-2k^d-EcadC25-II-4 cells (left panel). Scanning densitometry of the relative intensity of the presented immunoblots is shown on the right. (B) FACS analysis of pBabe-, H-2k^d-EcadC25- or H-2k^d-Ecad-II-4 cells immunoreacted with antibodies against specific integrin subunits. Elevated levels of cell surface α2, α3 and β1 subunits in H-2kd-Ecad-II-4 cells (black line in right-hand panels) in comparison to control pBabe-II-4 cells (gray line) were identified by increased fluorescence intensity seen in E-cadherin deficient cells. The expression levels of α2, α3 and β1 subunits were similar in H-2k^d-EcadC25-II-4 (black line in left-hand panels) and the control pBabe-II-4 cells (gray line) as seen by the superimposition of these lines. (C) Functional blocking of β1 integrin in H-2k^d-Ecad-II-4 cells. Two-dimensional cultures of Ecad-deficient H-2k^d-Ecad-II-4 cells were trypsinized briefly and replated for 15 minutes onto Type I and IV collagens or laminin-1 substrates, in the presence or absence of β1-integrin-blocking antibodies. Attached cells were quantified by optical density determined at 590 nm. Results are calculated as the mean ± s.d. of four replicates and experiments were repeated three times. $P<0.001$ for cell adherence to Type I collagen, Type IV collagen and laminin-1 substrates compared with levels in blocked controls.

demonstrating that levels of integrin subunit expression could modulate anoikis susceptibility and survival signaling (Janes and Watt, 2004). Interestingly, previous work also demonstrated that E-cadherin-mediated adhesion can counteract the requirement for matrix adhesion for epithelial cell survival thus enabling cells to circumvent anoikis (Kim et al., 1999; Green et al., 2004). These observations raise the question as to how loss of E-cadherin function could actually enhance survival and invasion of tumor cells in 3D tissue context as described in the present study. To reconcile these seemingly conflicting results we propose a dual role for E-cadherin in epithelial cell survival. When present in normal stratified squamous epithelial cells, E-cadherin may provide a physiological mechanism to counteract apoptosis induced by loss of ECM attachment as cells transit through suprabasal strata. However, when E-cadherin expression and/or function is lost during the early stages of epithelial neoplasia, small numbers of IE tumor cells are able to gain increased adhesion to extracellular matrix components at the BM, thus offsetting the loss of E-cadherin-mediated survival signals. Increased adhesion to the BM thus leads to intraepithelial retention of tumor cells at a site in the tissue that can subsequently facilitate initiation of tumor cell invasion.

The II-4 cell line used in our studies has been well characterized and represents an early stage of the malignant transformation process (Boukamp et al., 1990). Since II-4 cells harbor many of the important genetic hallmarks of the premalignant and early, invasive stages of SCC, such as mutations in the p53 gene and activation of Ha-Ras, this cell line is optimal for incorporation into models of early carcinoma progression. It is also important to understand if this genetic background contributes to the acquisition of adhesive and invasive properties seen upon loss of E-cadherin function in these cells. It is known that oncogenic Ha-Ras signaling cooperates with TGF-β to cause epithelial-mesenchymal transition (EMT) (Janda et al., 2002), that is a crucial event in late-stage tumorigenesis of transformed epithelial cells (Mercer et al., 2000). However, in early-stage tumor cells such as II-4, it is thought that mutation of the Ha-Ras gene may be more directly related to growth-regulatory pathways (Delhedde, 1999) rather than altering cell adhesion. As recently shown, E-cadherin-deficient-II-4 cells do not show all features of EMT (Margulis et al., 2005a), suggesting that the combinatorial effect of Ha-Ras activation, p53 loss and E-cadherin suppression are insufficient to fully induce EMT in these early-stage tumor cells.

While highlighting a novel aspect of E-cadherin loss in epithelial tumor progression, the current study extends our previous observations that loss of E-cadherin induces a highly aggressive tumor behavior in pure cultures of E-cadherin deficient cells (Margulis et al., 2005b) to mixtures of these cells with HEK. Only when the behavior of these cells was studied in the context of HEK has it been possible to determine that suppression of E-cadherin expression can induce loss of microenvironmental control of the malignant phenotype in II-4 cells in 3D tissues that mimic human premalignant disease. Similar impediments to tumor development inherent in 3D tissue context have been described in other tissue types, such as breast and prostate. For example, β1-integrin-mediated interactions between a premalignant breast epithelial cell line and adjacent ECM proteins have been shown to revert the malignant phenotype in a 3D model of early breast cancer progression (Miranti and Brugge, 2002; Wang et al., 1998; Schmeichel et al., 1998) by normalizing the distribution of E-cadherin upon reversion of malignant mammary epithelial cells to a normal phenotype (Weaver et al., 1997). Collectively, these observations demonstrate that genetic alterations present in individual initiated cells with malignant potential can have important functional consequences beyond tumor-autonomous roles in regulating cell cycle progression or cell survival. Rather, as in the case of E-cadherin loss, they significantly affect the complex interplay of tumor cells with the environment they find themselves in (Bissell and Radisky, 2001).

Our findings show that the integration of changes in cell-cell and cell-matrix adhesion are central to the conversion from premalignant lesions to early invasive carcinoma. These findings offer insight into incipient cancer invasion by demonstrating that microenvironmental, selective pressure drives the progression of precancer to fully-invasive tumors (Alt-Holland et al., 2005). This may help to explain why premalignant lesions such as actinic keratosis of skin, cervical dysplasia, oral leukoplakia and lobular carcinoma of the breast may contain numerous clones of initiated or dysplastic cells, yet have an unpredictable biological behavior that does not always advance to invasive cancer. By further understanding the progression of precancer to malignancy and by exploring signaling pathways associated with this transition in human 3D in-vivo-like tissue constructs, new therapeutic modalities designed to abrogate these events may be formulated to block early cancer invasion and thus prevent cancer occurrence.

Materials and Methods

2D cell culture

Human epidermal keratinocytes (HEK) were cultured from infant foreskin and were grown on an irradiated 3T3 fibroblast feeder layer, in DMEM containing 10% fetal calf serum (FCS). HaCaT-ras-II-4 (II-4) keratinocytes (Boukamp et al., 1990) that constitutively expressed the β-galactosidase gene to allow their identification in the context of HEK, were grown in DMEM containing 5% FCS. Two-dimensional, monolayer cultures of human dermal fibroblasts used for organotypic cultures were derived from infant foreskin and grown in media containing 10% FCS.

Retroviral infection

The dominant-negative retroviral E-cadherin vector (H-2k^{d}-Ecad) was a chimeric protein bearing the extracellular domain of mouse MHC class I antigen H-2K^{d} linked to the transmembrane domain of mouse E-cadherin (Zhu and Watt, 1996). Control vectors included H-2K^{d}-EcadC25, constructed from the H-2k^{d}-E-cad vector with a 25 amino acid deletion in the β-catenin-binding domain and the empty retroviral vector pBabe. The 293 Phoenix retroviral producer cells maintained in DMEM containing 10% bovine calf serum were transfected with pBabe, H-2K^{d}-Ecad and H-2K^{d}-EcadC25 plasmids (courtesy of F. Watt, Imperial Cancer Research Center, London, United Kingdom) by the calcium phosphate method.

3D cell culture and construction of premalignant tissues

Human tissue 3D constructs were prepared as previously described (Kolodka et al., 1998). HEK and II-4 cells that expressed each of the three retroviral constructs were mixed at 4:1 HEK:II-4 and 5×10^{5} cells from each cell mixture were seeded onto contracted collagen gels. Cultures were maintained submerged in low-calcium epidermal growth media for 2 days, submerged for 2 days in normal calcium epidermal growth media and raised to an air liquid interface for 5 days.

Transplantation of 3D cultures to nude mice

Three-dimensional tissue constructs were transplanted to the dorsum of 6-week-old male Swiss nude mice (N:NIHS-nuf DF; Taconic farms, Germantown, NY) and animals were sacrificed 4 weeks after transplantation. For routine light microscopy, tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin and 4 μm sections were stained with Hematoxylin and Eosin (H&E). Animal experiments were performed with the approval of the State University of New York (SUNY) Stony Brook Institutional Animal Care and Use Committees (IACUC).

Immunofluorescence staining

Tissue specimens were frozen in liquid nitrogen vapor and 6 μm serial sections were

mounted onto glass slides. Double immunofluorescent stains were performed using rabbit anti-β-gal (Cortex pharmaceuticals, CA) to detect II-4 cells and mouse anti-β-catenin (Zymed, CA) or mouse anti-Type IV collagen (Sigma) antibodies and detected using FITC-conjugated goat anti-rabbit IgG (Vector laboratories, CA) and Alexa Fluor 594-conjugated goat anti-mouse IgG (Molecular Probes, OR) antibodies. Fluorescence was visualized using a Nikon OptiPhot microscope.

Cell-matrix adhesion and functional blocking assays

Twenty-four-well plates coated with either Type I collagen, Type IV collagen, laminin, or fibronectin (Becton Dickinson, MA) were rinsed with PBS, blocked with 2% heat-denatured BSA at room temperature for 1 hour and rinsed again with PBS. II-4 cells expressing either of the E-cadherin vectors, or containing the pBabe-control vector, were trypsinized and resuspended in serum-free DMEM. Cells (5×10^4) were plated into each well, and plates were incubated at 37°C for 20 minutes. Cells were fixed at room temperature in 2% glutaraldehyde and non-adherent cells were removed by rinsing the plates several times in double-distilled H_2O. Plates were air-dried and adherent cells were stained with 0.1% crystal violet in 200 mM boric acid for 20 minutes. Plates were rinsed, air-dried and 10% acetic acid was added to each well. Optical density was determined at 590 nm.

For functional antibody blocking studies, cells were trypsinized, washed, resuspended in serum-free DMEM and preincubated for 45 minutes at 37°C in the absence (control, untreated cells) or the presence of 20 μg/ml of mouse anti-human β1-integrin function-blocking mAb (clone P5D2, Chemicon, Temecula, CA). Untreated II-4 cells or β1 integrin blocked II-4 cells (5×10^4 of each) were plated into each well and incubated at 37°C for 15 minutes. Thereafter, the procedure for fixing the cells, staining adherent cells and determination of optical density was the same as described above for adhesion assay.

Cell membrane fractionation and western blot analysis

Monolayer 2D cultures were extracted on ice into 400 μl cold PBS buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM NaF, 10 μg/ml aprotinin and leupeptin, 2 μg/ml pepstatin, 1 mM PMSF and 200 μl $NaVO_4$ (Sigma, St. Louis, MO). Cell lysates were subjected to five freeze-thaw cycles, centrifuged at 16,000 ***g*** for 25 minutes at 4°C and pellets were resuspended in 60 μl PBS containing 1% Triton X-100 as well as proteinase and phosphatase inhibitors. Pellets were homogenized four times over a 30 minute incubation on ice and centrifuged at 16,000 ***g*** for 25 minutes at 4°C. Protein concentration of the supernatant membrane fraction was measured using a modified Lowry assay (Bio-Rad DC Protein Assay Kit). For integrin analysis, 5 μg protein samples were boiled in Laemmli sample buffer without 2-β-Me, loaded onto 7.5% SDS-PAGE gel and separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). Immunoblotting was performed using rabbit anti-human integrin α2, rabbit anti-human integrin α3 or mouse anti-human β1 integrin antibodies (Chemicon) following by HRP-anti-rabbit or HRP-anti-mouse IgG antibodies (Amersham, Piscataway, NJ). Protein bands were visualized by ECL, utilizing Pierce SuperSignal Kit (Rockford, IL).

Flow cytometry analysis of cell surface integrins

II-4 cells expressing either of the E-cadherin vectors or the pBabe-control vector were trypsinized, washed in serum-containing medium, and rinsed twice in cold PBS. Cells were resuspended and incubated for 30 minutes on ice in cold PBS with either mouse anti-human integrin α2, integrin α3 or integrin β1 antibodies (Chemicon). Cells were then washed twice in cold PBS and incubated on ice for 30 minutes with goat-anti mouse FITC-conjugated secondary antibody. Following extensive washing with cold PBS, cells were resuspended in PBS containing propidium iodide and live cells analyzed by the cell-sorter FACS Vantage (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Control cell samples for analyzing the background levels of immunostaining were incubated with either secondary antibody alone or unrelated mouse IgG and were used as negative controls.

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