Overexpression of the integrin-linked kinase mesenchymally transforms mammary epithelial cells

Aruna Somasiri¹, Andrew Howarth¹, Duna Goswami¹, Shoukat Dedhar² and Calvin D. Roskelley¹,³,*

¹Dept of Anatomy, University of British Columbia, Vancouver B.C., Canada, V6T 1Z3
²B.C. Cancer Agency, Jack Bell Research Center and Dept of Biochemistry, University of British Columbia, Vancouver B.C. Canada, V6H 3Z6
³Dept of Ob/Gyn, University of British Columbia, Vancouver B.C., Canada, V6H 3V5

*Author for correspondence (e-mail: roskelley@interchange.ubc.ca)

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SUMMARY

Signals generated by the interaction of β1 integrins with laminin in the basement membrane contribute to mammary epithelial cell morphogenesis and differentiation. The integrin-linked kinase (ILK) is one of the signaling moieties that associates with the cytoplasmic domain of β1 integrin subunits with some specificity. Forced expression of a dominant negative, kinase-dead form of ILK subtly altered mouse mammary epithelial cell morphogenesis but it did not prevent differentiative milk protein expression. In contrast, forced overexpression of wild-type ILK strongly inhibited both morphogenesis and differentiation. Overexpression of wild-type ILK also caused the cells to lose the cell-cell adhesion molecule E-cadherin, become invasive, reorganize cortical actin into cytoplasmic stress fibers, and switch from an epithelial cytokeratin to a mesenchymal vimentin intermediate filament phenotype. Forced expression of E-cadherin in the latter mesenchymal cells rescued epithelial cytokeratin expression and it partially restored the ability of the cells to differentiate and undergo morphogenesis. These data demonstrate that ILK, which responds to interactions between cells and the extracellular matrix, induces a mesenchymal transformation in mammary epithelial cells, at least in part, by disrupting cell-cell junctions.

Key words: Integrin-linked kinase, Mammary epithelium, Mesenchymal transformation

INTRODUCTION

Most, if not all, cellular functions are modulated by the extracellular matrix (ECM; Bissell et al., 1982; Adams and Watt, 1993; Giancotti and Roushlahti, 1999). This is particularly evident in the mammary gland where interactions between mammary epithelial cells and the basement membrane ECM profoundly influence proliferation, differentiation, morphogenesis and apoptosis during the developmental cycles of pregnancy, lactation and involution (Schmeichel et al., 1998). Many of these phenotypes can be modeled in vitro using 3-dimensional culture systems (Somasiri and Roskelley, 1999). For example, when mammary epithelial cells derived from mid-pregnant mice are placed on reconstituted basement membrane gels they round-up, aggregate and polarize. This morphogenic process is completed when the resulting spherical structures cavitate to form a central lumen (Aggeler et al., 1991). The cells within these structures express milk proteins and secrete them vectorially into the central lumen (Barcellos-Hoff et al., 1989). Differentiative expression of one of these milk proteins, β-casein, is transcriptionally regulated (Schmidhauser et al., 1990) and this regulation requires an interaction between β1 subunit-containing integrins on the cell surface and laminin in the basement membrane (Streuli et al., 1991; Streuli et al., 1995; Muschler et al., 1999). In addition, when these integrin-laminin interactions are functionally blocked the cells no longer respond appropriately to soluble factors in the microenvironment and they ultimately undergo apoptosis (Boudreau et al., 1995; Pullan et al., 1996).

Integrins have overlapping and competing affinities for individual ECM ligands (Hynes, 1992). In response to ECM ligation, integrins cluster in the plane of the membrane and recruit large, proteinaceous plaques that associate with the cytoskeleton and initiate numerous signaling cascades (Juliano and Haskill, 1993; Yamada and Geiger, 1997). Components of the MAP kinase signaling pathway are often associated with these plaques and, in some cases, this pathway is stimulated by the integrin-mediated activation of the focal adhesion kinase (FAK; Giancotti and Rousslahti, 1999). FAK is activated when mammary epithelial cells contact laminin (Roskelley and Bissell, 1995) but the functional consequences of this activation are not clear as inhibition of the MAP kinase pathway does not prevent differentiative milk protein gene induction (Wartmann et al., 1996). Integrin ligation also activates phosphatidylinositol-3-OH kinase signaling (PI3-kinase; Khwaja et al., 1997; King et al., 1997) and we have shown that PI3-kinase is required for both basement membrane-dependent morphogenesis and differentiative β-casein expression in mammary epithelial cells (Somasiri et al., 2000). Basement membrane-dependent integrin signaling also potentiates the ability of insulin-like growth factor to activate PI3-kinase signaling and prevent apoptosis in these cells (Farrelly et al., 1999). One integrin-associated signaling moiety that has been implicated in PI3-kinase signaling is the integrin-linked kinase (ILK).
ILK associates with the cytoplasmic domain of β1, β2 and β3 integrin subunits and the kinase activity of the molecule, which is serine/threonine directed, is modulated by integrin ligation in a PI3-kinase dependent manner (Hannigan et al., 1996; Delcomenne et al., 1998). ILK also acts downstream of PI3-kinase by stimulating the phosphorylation of protein kinase B (PKB) on serine 473 (Lynch et al., 1999; Persad et al., 2000). This potentiation of PKB plays a role in the ability of ILK to downregulate glycogen synthase kinase-B which in turn influences both β-catenin and AP-1 signaling (Novak et al., 1998; Troussard et al., 1999). ILK also contains four non-catalytic ankyrin repeats which help target the molecule to focal adhesion complexes and link it to growth factor receptor tyrosine kinase signaling via the adapter protein Pinch/Vn97 (Tu et al., 1998; Hobert et al., 1999; Li et al., 1999). In epithelial cells, forced overexpression of wild-type ILK suppresses suspension-mediated apoptosis and stimulates anchorage-independent growth (Radeva et al., 1997). Thus, ILK overexpression appears to constitutively activate integrin signaling pathways such that the the anchored state is mimicked in the absence of cell-ECM interactions (Attwell et al., 2000).

In this report we investigated the role of ILK in the basement membrane-dependent differentiation and morphogenesis of the scp2 mouse mammary epithelial cell line. We found that ILK activity is increased by cellular interactions with either a reconstituted basement membrane gel or by purified laminin. Forced expression of a dominant negative form of ILK did not prevent differentiative β-casein induction but it did subtly affect spheroidal morphogenesis. In contrast, forced overexpression of wild-type ILK inhibited differentiation, prevented morphogenesis and it initiated a mesenchymal transformation of the cells that was associated with a loss of cell-cell junctions.

### MATERIALS AND METHODS

#### Cell culture

scp2 cells represent a homogeneous, functional mouse mammary epithelial line that was isolated from the heterogeneous CID-9 line (Schmidhauser et al., 1990) by limited dilution cloning (Desprez et al., 1993). For routine 2-dimensional (2-D) monolayer culture, the cells were maintained in growth medium consisting of DMEM/F12 medium (1:1 mixture) supplemented with 5% FBS, insulin (5 μg/ml) and gentamycin (50 μg/ml; all Sigma, St Louis MO). For 3-dimensional (3-D) differentiative culture, the cells were plated on reconstituted basement membrane gels (Matrigel; Becton-Dickenson, Bedford MA) in serum-free DMEM/F12 medium supplemented with a full complement of lactogenic hormones (insulin 1 μg/ml hydrocortisone, 3 μg/ml prolactin). Alternatively, cell monolayers were overlaid with purified laminin (Sigma, 50 μg/ml) diluted in the presence of lactogenic hormones (Streuli et al., 1995).

#### Viral infection and transfection

Wild-type (wt) ILK and dominant negative kinase-dead (kd) ILK cDNAs were epitope-tagged in the pcDNA3.1 V5-His expression vector (Invitrogen, Carlsbad CA; Persad et al., 2000) and subcloned into the MCV-pac retroviral packaging vector. Replication-defective ecotropic retrovirus was produced by transient transfection of the latter construct into BOSC-29 packaging cells (Pear et al., 1993). 48 hours after retroviral infection, scp2 cells were either lysed for an initial analysis of transgene expression or they were put under selection for 5 days (puromycin, 2 μg/ml) and then pooled. Empty MCV-pac containing virus was used as the control in these experiments.

scp2 cells were also transfected using lipofectamine (Gibco-BRL; Gaithersburg MD) with either a control antisense (ILK-14) or a sense wtILK cDNA (ILK-13) under the constitutive control of the CMV promoter (Hannigan et al., 1996). Stable transfectants were produced by selection under G-418 (400 μg/ml) and clones were isolated by limiting dilution. From a total of twelve ILK-13 clones isolated, three were chosen for further study based on their varying levels of ILK overexpression (Novak et al., 1998; also see Table 1). The highest overexpressing clone, ILK-13-8, was co-transfected with a mouse E-cadherin cDNA under the constitutive control of the β-actin promoter (Nose et al., 1988) and a hygromycin selection plasmid (Strategene, La Jolla CA). Stable double-transfectants were produced under hygromycin selection (100 μg/ml) and clones were isolated by limiting dilution. Hygromycin vector alone was stably transfected into ILK-13-8 cells as a double-transfectant control.

#### Immunofluorescence

Cells were grown on glass coverslips, fixed in cold (~20°C) methanol and then permeabilized in cold methanol-acetone (1:1) for all antigens except ILK and β-catenin, for which cells were fixed in paraformaldehyde (4%, 15 minutes in PBS) followed by permeabilization with Triton X-100 (0.1%, 10 minutes) at room temperature. Fixed and permeabilized cells were then incubated with antibodies against ILK (mouse monoclonal; Li et al., 1999), E-cadherin and β-catenin (mouse monoclonals, Transduction Labs, Lexington KY), pan-cytokeratin (rabbit polyclonal, Dako, Carpenteria, CA) or vimentin (mouse monoclonal, Sigma). Binding of primary antibodies was visualized by epifluorescence microscopy after incubation with fluorescently labelled anti-species-specific second antibodies. f-actin was visualized after the binding of rhodamine phalloidin (Molecular Probes, Eugene, OR) to paraformaldehyde-fixed, acetone-permeabilized cells.

#### Western blotting

For ILK and epitope-tag immunoblotting, 5 μg of whole cell NP-40 lysates (1% NP-40, 0.5% deoxycholate) were separated by 10% SDS-PAGE, transferred to nylon membranes (PVDF, Bio-Rad, Hercules, CA) and probed with either an affinity-purified rabbit polyclonal antibody against ILK (91-3; Hannigan et al., 1996) or a mouse monoclonal antibody against the 14 amino acid V5 epitope (Invitrogen, Carlsbad CA). For intermediate filament immunoblotting, 30 μg of solubilized cytoskeletal protein obtained after high salt extraction (Achtstaetter et al., 1986) was separated by 8.5% SDS-PAGE, transferred and probed with either a 1:1 mixture of AE1/AE3 monoclonal antibodies which recognize the great majority of cytokeratins (Sun et al., 1985) or a goat polyclonal antibody against

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scp2 mouse mammary epithelial cells were stably transfected with a mammalian expression vector containing either a control antisense ILK cDNA (ILK14) or a wild-type ILK sense cDNA (ILK13) and clones were isolated by limited dilution. The levels of ILK overexpression were assessed by western blotting and β-catenin localization was determined by immunofluorescence (see Novak et al., 1998).
vimentin (Accurate Chemical, Westbury, NY). For E-cadherin and β-catenin immunoblotting, 50 μg and 20 μg of whole cell RIPA (0.1% SDS, 1% deoxycholate, 1% NP-40) lysates respectively were separated by 10% SDS-PAGE, transferred and probed with the antibodies described above for immunofluorescence. To assess mammary-specific differentiation, cells were maintained either on tissue culture plastic in the presence or absence of a laminin overlay or on Matrigel, in all the presence of lactogenic hormones for up to five days which results in maximal differentiation of the parent scp2 cell line (Roskelley et al., 1994). On the days indicated, cells were liberated from the matrix using a neutral protease (Dispase, Collaborative Res, Bedford, MA), lysed in RIPA buffer and 10 μg of total protein was separated by 10% SDS-PAGE, transferred and probed with a mouse monoclonal against the milk protein β-casein (Kaetzel and Ray, 1984). In all cases, primary antibody binding was visualized using horseradish peroxidase-labelled, species-specific second antibodies followed by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**ILK kinase activity**

scp2 parent cells were maintained in lactogenic hormones as monolayers, as laminin-overlaid clusters, or as spherical structures on Matrigel for 5 days. The cells were then lysed in NP-40 buffer and 200 μg of total protein was immunoprecipitated using the 91-3 anti-ILK polyclonal antibody. Protein kinase assays were performed in 50 μl kinase reaction buffer (50 mM HEPES, pH 7.0, 10 mM MgCl2, 2 mM NaF, 1 mM Na3VO4) containing 10 μCi [γ-32P]ATP and 5 μg myelin basic protein as substrate (Upstate Biotechnologies, Lake Placid NY). Reactions were incubated at 30°C for 30 minutes, stopped by the addition of SDS-PAGE sample buffer and the products were resolved by 15% SDS-PAGE and visualized by fluorography (Hannigan et al., 1996).

**RT-PCR**

5 μg total RNA was subjected to first strand cDNA synthesis kit (Pharmacia, Morgan, Canada) in a total volume of 33 μl. 10 μl of cDNA was then mixed with 200 mM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl2, 0.001% gelatin, 2.5 U Taq polymerase (Gibco, Grand Island NY) in a final volume of 100 μl and subjected to 35 cycles of amplification using 0.5 pmoles of the appropriate primers. Primers used, which span introns to detect specific mRNA sequences, were 5'-GGGTGACTACAAAA TC-3' and 5'-GGGGGACATAGGGGCTCTTTP-3', which amplifies a 252 bp fragment of the transmembrane domain of E-cadherin (Risinger et al., 1995), or, 5'-TGATCCACATCTGCTGAAAGTTG-3' and 5'-GGACCTGACTGACTACCTC-ATGAA-3' which amplifies a 510 bp fragment of β-actin. Each amplification cycle consisted of denaturation at 94°C for 60 seconds, primer annealing at 58°C for 90 seconds, extension at 72°C for 120°C, and a final extension at 72°C for 15 minutes.

**RESULTS**

**Endogenous ILK kinase activity in mammary epithelial cells is upregulated by interactions with the basement membrane**

scp2 mouse mammary epithelial cells undergo ECM-dependent morphogenesis and differentiation (Desprez et al., 1993; Roskelley et al., 1994). Laminin in the basement membrane matrix is sufficient to induce β-casein expression and this induction requires functional β1 integrins (Streuli et al., 1995; Muschler et al., 1999). In the current study we found that cellular interactions with laminin and basement membrane gels slightly decreased the amount of immunoprecipitable ILK activity from NP-40 whole cell lysates but at the same time increased the kinase activity of this material (Fig. 1). These observations led us to ask whether ILK plays a role in the basement membrane-dependent morphogenesis and differentiation of scp2 mammary epithelial cells.

**Overexpression of wtILK inhibits basement membrane-dependent morphogenesis and differentiation**

Forced expression of a kinase dead (kd) mutant form of ILK blocks endogenous ILK kinase activity in a dominant negative fashion while overexpression of wild-type (wt) ILK leads to a constitutive increase in ILK kinase activity (Persad et al., 2000). Thus, we used a retroviral delivery system to express these two forms of ILK in scp2 cells. Retroviral infection caused an appreciable expression of the ILK transgenes two days after infection (Fig. 2A). A 5 day genetic selection period (puromycin, 2 μg/ml) removed about half of the cells (data not shown) and further increased the expression of both kdILK and wtILK on a population basis (Fig. 2A). Infected, selected and pooled cells were then used rapidly, within two passages, for further experiments. It is important to note that these populations were not cloned, and thus are heterogeneous.

In 3-dimensional basement membrane culture, virally-infected kdILK expressing cell populations aggregated and formed spheres. However, these structures were much smaller than those that formed in the vector-infected controls (Fig. 2B). Despite these morphological effects, induction of β-casein expression was not affected by forced kdILK expression (Fig. 2C). In contrast, virally-infected wtILK overexpressing cell
populations did not express β-casein (Fig. 2B), basement membrane-dependent sphere formation was prevented, and the cells migrated into the gel (Fig. 2C).

wtILK overexpression disrupts adherens junctions

scp2 mammary epithelial cells form classical cobblestone epithelial monolayers in 2-dimensional culture (Desprez et al., 1993). In the current study, the same was true of the virally-infected vector controls and kdILK expressing cell populations, although a subtle cell rounding was observed in the latter population (Fig. 3A). In contrast, virally-infected wtILK overexpressing cell populations were morphologically heterogeneous; some cells remained close-packed and epithelial but many others detached from each other and became bipolar and fibroblastic (Fig. 3A).

scp2 mammary epithelial cells form E-cadherin-mediated adherens junctions in both 2-dimensional monolayers and in 3-dimensional basement membrane gel culture (Lochter et al., 1997; Somasiri et al., 2000). The localization of E-cadherin and β-catenin to areas of cell-cell interaction in 2-dimensional monolayers indicated that adherens junctions formed in the

Fig. 3. wtILK overexpression in viral infectants disrupts cell-cell junctions. Pooled heterogeneous cell populations were maintained as 2-dimensional monolayers on tissue culture plastic. In A, morphology was assessed by phase microscopy and E-cadherin and β-catenin localization were visualized by immunofluorescence microscopy; arrows indicate the position of E-cadherin negative wtILK-infected cells (bar, 25 µm phase; 15 µm immunofluorescence). In B, steady state levels of E-cadherin and β-catenin in whole cell lysates were determined by western blotting.
ILK-mediated mesenchymal transformation

virally-infected vector controls and kdILK expressing cell populations (Fig. 3A). In contrast, E-cadherin localization was discontinuous or the protein was completely absent from the majority of the cells in the virally-infected wtILK overexpressing population (Fig. 3A). The latter observation was reflected by an overall decrease in steady state E-cadherin levels (Fig. 3B), an observation that has been made previously in wtILK overexpressing intestinal epithelial cells (Wu et al., 1998). Junctional β-catenin localization was also disrupted in a significant number of the cells of the wtILK overexpressing population. In some of the latter cells β-catenin was observed in the nucleus (Fig. 3A). Steady-state β-catenin levels were similar in all of the virally-infected populations (Fig. 3B).

wtilK overexpression initiates an epithelial to mesenchymal transformation

The disruption of adherens junctions in the virally-infected wtILK overexpressing cell population was associated with changes to the actin cytoskeleton. In vector control and kdILK-infected cells most of the filamentous (f-) actin was localized to cell-cell junctions. As such, the f-actin formed honeycomb patterns in 2-dimensional monolayers in both of these pooled cell populations (Fig. 4). In contrast, many of the fibroblastic cells in the wtILK overexpressing population contained prominent cytoplasmic actin stress fibers. The combination of a fibroblastic morphology, disruption of adherens junctions and the acquisition of stress fibers suggested to us that some of the cells in this heterogeneous wtILK overexpressing population may have undergone an epithelial to mesenchymal transformation (EMT). To address this possibility we examined the intermediate filament cytoskeleton. As expected (Desprez et al., 1993), the great majority of the virally-infected control cells contained epithelial cytokeratins and very few cells contained mesenchymal vimentin intermediate filaments (Fig. 4). The same was true of virally-infected kdILK expressing population. In contrast, approximately half of the wtILK-infected cells had lost cytokeratin filaments and a considerable number of the cells had acquired vimentin filaments.

To determine if wtILK overexpression truly leads to intermediate filament switching and mesenchymal transformation we also examined homogeneous populations of wtILK overexpressing cells. Previously, we stably transfected scp2 cells with a wtILK expression vector and isolated clones in which the transgene is overexpressed to varying degrees (Novak et al., 1998). In this earlier study we demonstrated that increasing stable wtILK overexpression decreased steady state E-cadherin levels, caused nuclear translocation of β-catenin and stimulated invasion into basement membrane gels (see Table 1 for summary and Fig. 9 below). We now demonstrate that increasing wtILK overexpression in these homogeneous clones caused the cells to become fibroblastic in monolayer culture and it decreased cytokeratin intermediate filaments and increased vimentin intermediate filaments in each of these clones (Fig. 5). In the highest wtILK overexpressing clone, ILK13-8, all of the cells contained only mesenchymal vimentin filaments. This wtILK-induced intermediate filament protein switching was confirmed by western blotting (Fig. 6A,B). In agreement with the findings described above for the heterogeneous viral wtILK overexpressing populations, increasing wtILK overexpression in the homogeneous transfected clones also inhibited β-casein induction (Fig. 6C). Therefore, high levels of stable wtILK overexpression initiated a mesenchymal transformation of scp2 cells that was incompatible with basement membrane-dependent morphogenesis and differentiation.

Subcellular ILK localization is altered in mesenchymally transformed cells

At first glance it was somewhat surprising that wtILK overexpression prevented basement membrane-dependent β-casein expression given the fact that differentiative contact with laminin upregulated endogenous ILK activity in the parental scp2 cells. However, subtle, but potentially significant, alterations in ILK subcellular localization may provide an

Fig. 4. wtILK overexpression in viral infectants alters the cytoskeleton. Pooled heterogeneous cell populations were maintained as 2-dimensional monolayers on tissue culture plastic. f-actin was then visualized after rhodamine phalloidin staining while epithelial cytokeratin and mesenchymal vimentin intermediate filaments were visualized by immunofluorescence microscopy (different fields for each staining; bar, 15 μm).
explanation. Specifically, the ILK protein was often localized to elongated streaks at the cell-substratum interface in the mesenchymal (i.e. keratin negative) wtILK overexpressing cells (Fig. 7). Morphologically these streaks, which resemble focal adhesions, were very different from the discrete punctate spots of endogenous ILK staining observed in control populations. Therefore, ILK function may differ in the mesenchymal overexpressors and the epithelial parental cell line.

**Forced E-cadherin expression causes an epithelial reversion in mesenchymal wtILK-overexpressing cells**

Steady state E-cadherin levels were reduced in the heterogeneous virally-infected wtILK overexpressing cell population (see Fig. 2 above). In addition, the homogenous ILK13-8 clone did not contain any observable E-cadherin protein or mRNA (Fig. 8A). Given the importance of E-cadherin in regulating mammary epithelial cell function (Gilles and Thompson, 1996), we next asked if forced expression of this cell-cell adhesion molecule would rescue the epithelial phenotype in the mesenchymal ILK13-8 clone. As such, we stably transfected ILK13-8 cells with a mouse E-cadherin cDNA under the control of the constitutively active β-actin promoter (Nose et al., 1988) and selected a second set of doubly-transfected clones by limiting dilution.

ILK13-8 cells sham-transfected with a second selection vector only were designated ILK13-8Vect. Like the original ILK13-8 cells these controls did not express E-cadherin and they did not undergo basement membrane-dependent differentiation (Fig. 8). Five doubly-transfected clones expressing increasing amounts of E-cadherin were designated ILK13-8EC-a to ILK13-8EC-e. Two of these clones (EC-a and EC-c) differentiated and expressed β-casein when cultured on basement membrane gels (Fig. 8). However, there was no correlation between the steady-state levels of residual wtILK overexpression or E-cadherin and the restoration of the differentiated phenotype.

ILK13-8Vect control cells did not contain epithelial cytokeratin filaments and they remained invasive in 3-D basement membrane culture (Fig. 9). In contrast, the majority of the cells in all of the ILK13-8EC clonal lines were cytokeratin positive and non-invasive. Interestingly, however, only 2 of the 5 clones underwent complete spherical morphogenesis in 3-D culture and these were the same two clones that expressed β-casein under the same conditions (EC-a and EC-c). Therefore, while forced E-cadherin expression re-epithelialized the cells it only weakly restored differentiative and morphogenic potential.

**DISCUSSION**

The products of many oncogenes and tumor suppressor genes function by impinging upon the cell cycle (Hunter, 1997).
and the interactions between cells and the ECM (Gumbiner, 1996). Mutation, changes in expression or localization, and functional alteration of these molecules has been observed in many cancers. Thus, adhesion molecules are being increasingly viewed as architectural oncogenes and tumor suppressors (Boudreau and Bissell, 1998).

In carcinomas, which are the most prevalent of all human solid tumors, an inappropriate epithelial to mesenchymal transformation (EMT) is a key architectural event that contributes to invasion and metastasis (Birchmeier et al., 1996). In breast carcinomas this inappropriate EMT is associated with alterations in both cell-cell and cell-ECM interactions (Gilles and Thompson, 1996). Experimental evidence suggests that these alterations are functionally interrelated. For example, forced expression of an activated form of the metalloprotease stromelysin-1 in normal mammary epithelial cells degrades the ECM as well as the extracellular domain of E-cadherin, and both changes contribute to mesenchymal transformation (Lochter et al., 1997). In addition, a phenotypic switching from the anchoring α6β4 integrin to migratory β1 integrins disrupts junctional E-cadherin localization and epithelial architecture during breast tumor cell progression (Weaver et al., 1997). The latter also constitutively upregulates endogenous ILK kinase activity (Wang et al., 1998). In this report, we demonstrate that the overexpression of wtILK caused functional mouse mammary epithelial scp2 cells to become non-differentiative, mesenchymal and invasive.

When they are placed upon a basement membrane gel, scp2 cells undergo cell-cell junction-dependent spheroidal morphogenesis (Lochter et al., 1997; Somasiri et al., 2000). Under these conditions the cells also express the milk protein β-casein, a differentiative event that is regulated, at least in part, by interactions between laminin in the basement membrane and β1 integrins on the cell surface (Streuli et al., 1991; Streuli et al., 1995; Muschler et al., 1999). ILK interacts with the cytoplasmic tail of β1 integrin subunits and the kinase activity of the molecule is activated when intestinal epithelial cells adhere to ECM in a β1 integrin-dependent manner (Hannigan et al., 1996). Thus, it was not unexpected that adhesion of mammary epithelial cells to a basement membrane ECM also increased ILK activity. However, this adhesion also decreased the amount of ILK that could be immunoprecipitated from NP-40 cell lysates. The latter result may have occurred because of a steady-state decrease in the ILK protein, or it may indicate ECM-mediated changes in cytoplasmic ILK solubility, perhaps due to changes in association with the cytoskeleton. If the latter were the case it would suggest that the ILK not associated with the cytoskeleton (i.e. the NP-40 soluble pool) has a significantly elevated kinase activity. Regardless, the finding that both the basement membrane and purified laminin altered ILK kinase activity led us to examine the role of ILK in scp2 cell morphogenesis and differentiation.

Forced expression of a dominant negative kdILK mutant did not prevent basement membrane-dependent β-casein expression or the formation of spheres on basement membrane gels. However, the latter structures were considerably smaller than those formed by the control cells. While it is possible that cell-cell aggregation, which is a pre-requisite for spheroidal morphogenesis, was decreased in the kdILK expressing cells, we did not find any evidence that adherens junctions were disrupted in this condition. However, we have yet to examine
other junctions. Interestingly, parental scp2 cells do not form functional tight junctions in monolayer culture (Woo et al., 2000) but these junctions do form in an apical location in fully polarized spheres on basement membrane gels (A.S. and C.D.R., unpublished observations). Thus, it will be interesting to determine if tight junction assembly and localization are ILK-dependent, especially given the fact that these junctions also contribute to mammary morphogenesis in vivo and appear to be disrupted during the progression of ductal carcinoma in situ into truly malignant tumors (Nguyen and Neville, 1998).

ILK plays a role in anchorage-dependent growth and survival (Radeva et al., 1997) and wtILK overexpression in scp2 cells prevents anoikic apoptosis (Attwell et al., 2000). Therefore, it is also possible that dominant negative kdILK expression reduced sphere size by impinging upon the basement membrane’s ability to protect mammary epithelial cells from undergoing apoptosis (Boudreau et al., 1995). If this is indeed the case, it will be important to determine the relative contribution of both integrins and growth factor receptors as ILK also augments insulin-mediated signaling (Delcomenne et al., 1998; Tu et al., 1998). Interestingly, the latter pathway has been shown to inhibit mammary epithelial cell apoptosis in a

Fig. 7. ILK localization is altered in wtILK overexpressing, mesenchymally transformed cells. Virally-infected, heterogeneous cell populations (a, vector control; b, wt ILK) and homogenous stable transfectants (c, ILK-14-1 control; d, ILK13-8 overexpressor) were maintained as 2-D monolayers. Virally-infected cells were co-immunostained for epithelial cytokeratin (red) and ILK (green) and epifluorescent photomicrographs were taken at the plane of the cell/substratum interface. ILK staining (yellow) was confined to small, discrete circular dots in the vector control cells (a) and in those wtILK infected cells that remained epithelial (i.e. contained cytokeratin, b). In contrast, a significant amount of the ILK protein (green) localized to elongated streaks in the keratin-negative (i.e. mesenchymal) cells (b). Stable transfectants were stained for ILK only (white). ILK was localized in small punctate spots in the epithelial ILK14-1 control cells (c) and in larger elongated streaks in the mesenchymal ILK13-8 overexpressing cells (d), none of which contain cytokeratin (see Figs 5 and 6 above). Bar, 8 μm (a,b); 5 μm (c,d).

Fig. 8. wtILK overexpression downregulates E-cadherin. In A, the steady state levels of ILK and E-cadherin protein as well as E-cadherin mRNA in epithelial scp2 cells (lane 1) and mesenchymal wtILK13-8 stable transfectants (lane 2) were assessed by western blotting and RT-PCR, respectively. For the PCR, the quality of the cDNA was assessed by amplifying β-actin. In B, mesenchymal ILK13-8 cells were transfected with either a second selection vector alone (Vect) or with an E-cadherin c-DNA (13-8EC). After genetic selection, the vector control (Vect) and five E-cadherin transfected clones (13-8ECa-e) were assessed for steady state ILK and E-Cad protein and the ability to express β-casein in basement membrane culture by western blotting of whole cell lysates.
Forced overexpression of wtILK did not cause scp2 cells to express β-casein in the absence of basement membrane matrix. On the contrary, stable wt ILK overexpression caused scp2 cells to become completely non-differentiative, even in the presence of basement membrane. These findings do not completely rule out the possibility that some degree of ILK-mediated signaling may be compatible with mammary morphogenesis and differentiation. For example, differences in the kinetics of activation between matrix-stimulated parental cells and chronically wtILK overexpressing cells could be responsible for the differences in the phenotypic endpoints observed. This is certainly the case for the erb-B2 receptor where a precisely controlled developmental activation of this tyrosine kinase contributes to late-stage mammary alveolar morphogenesis and differentiation (Yang et al., 1995) while chronic stimulation causes a loss of differentiation and mammary tumor formation (Muller et al., 1988). In a similar vein, ILK could act to either promote or inhibit the differentiation of the mammary epithelium in a developmentally stage-specific manner, as is the case during myogenic differentiation (Huang et al., 2000).

Non-differentiative wtILK overexpressing scp2 cells lost cytokeratins, became fibroblastic and they invaded basement membrane gels. These fibroblastic cells also acquired vimentin, cytoplasmic stress fibers and they no longer expressed E-cadherin. Thus, stable overexpression of wtILK caused a complete EMT in scp2 cells. When E-cadherin is force-expressed in true fibroblasts, cell-cell junctions partially form and the production of fibronectin, a mesenchymal ECM protein, is downregulated (Nagafuchi et al., 1987; Finneman et al., 1995; Yonemura et al., 1995). Forced E-cadherin expression also causes an epithelialization of metastatic breast carcinoma cells (Meiners et al., 1998) and of invasive, mesenchymal-like ovarian surface epithelial cells (Auersperg et al., 1999). Taken together, these data led us to tentatively conclude that the loss of E-cadherin might be a critical component of the wtILK-induced EMT in scp2 cells. We directly tested this hypothesis by force-expressing E-cadherin in the completely mesenchymal wtILK-overexpressing clone ILK13-8. This caused the reappearance of cytokeratin and it prevented cellular invasion of the basement membrane in all of the clones examined. Therefore, E-cadherin appeared to restore a modicum of the epithelial phenotype. In contrast, only two of five of these clones underwent basement membrane-dependent

![Fig. 9. Forced E-cadherin expression rescues the epithelial phenotype in wtILK overexpressing cells. Mesenchymal ILK13-8 cells transfected with vector alone (Vect) and the five E-cadherin expressing clones (ECa-e) were maintained as 2-D monolayers and E-cadherin and cytokeratin localization were assessed by immunofluorescence microscopy. The cells were also maintained in 3-D basement membrane culture and the ability of the cells to undergo spheroidal morphogenesis was assessed by phase microscopy. Bar, 15 μm immunofluorescence; 50 μm phase.](image-url)
differentiation. There was no correlation between the levels of wtILK overexpression or E-cadherin expression and this differentiative rescue. However, those clones that did differentiate underwent the most complete speriferal morphogenesis. Therefore, a combined morphological and differentiative rescue by E-cadherin may rely upon the complete restoration of adhesive and tight junctions between the cells. This is a possibility that we are currently investigating.

wtILK overexpression promotes the phosphorylation and activation of protein kinase B (PKB) which then phosphorylates glycogen synthase kinase-3 (GSK-3) and decreases its activity (Lynch et al., 1999; Persad et al., 2000). During development, wnt/wingless signaling also inhibits GSK-3 activity which prevents it from phosphorylating β-catenin. As a result, free β-catenin escapes degradation and can be transported to the nucleus where it interacts with the Tcf/LEF-1 family of architectural transcription factors to regulate the expression of genes involved in regulating the cell cycle and developmental mesenchymal transformation (McCartney and Peifer, 2000). While it has been suggested that PKB-mediated decreases in GSK-3 activity alone, in the absence of wnt/wingless signaling, are not be sufficient to translocate β-catenin to the nucleus (Yuan et al., 1999), we did observe nuclear β-catenin in wtILK overexpressing scp2 mammary epithelial cells and the same occurs in wtILK overexpressing intestinal epithelial cells (Novak et al., 1998). Therefore, the ability of ILK overexpression to induce an EMT in mammary epithelial cells could be mediated by its ability to upregulate nuclear β-catenin signaling.

β-Catenin/LEF-1-mediated transactivation can be reversed by the forced expression of cadherins or cadherin cytoplasmic domains (Fagotto et al., 1996; Miller and Moon, 1997; Sadot et al., 1998, Orsulic et al., 1999). Presumably this effect is mediated by the binding and sequestering of β-catenin. Thus, it is possible that the forced expression of E-cadherin in the mesenchymal wtILK overexpressing scp2 cells caused a re-epithelialization by reversing β-catenin signaling. One of the candidate targets of ILK-mediated β-catenin signaling that could initiate the mesenchymal transformation candidate is Snail, a transcription factor that acts to repress E-cadherin

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