ILEI: A cytokine essential for EMT, tumor formation, and late events in metastasis in epithelial cells

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Summary

Erk/MAPK and TGFβ signaling cause epithelial to mesenchymal transition (EMT) and metastasis in mouse mammary epithelial cells (EpH4) transformed with oncogenic Ras (EpRas). In trials to unravel underlying mechanisms, expression profiling for EMT-specific genes identified a secreted interleukin-related protein (ILEI), upregulated exclusively at the translational level. Stable overexpression of ILEI in EpH4 and EpRas cells caused EMT, tumor growth, and metastasis, independent of TGFβ-R signaling and enhanced by Bcl2. RNAi-mediated knockdown of ILEI in EpRas cells before and after EMT (EpRasXT) prevented and reverted TGFβ-dependent EMT, also abrogating metastasis formation. ILEI is overexpressed and/or altered in intracellular localization in multiple human tumors, an event strongly correlated to invasion/EMT, metastasis formation, and survival in human colon and breast cancer.

Introduction

During generation, progression, and metastasis of epithelial tumors (carcinomas), oncogenes and tumor suppressor genes cause multiple cell-autonomous alterations, e.g., aberrant control of proliferation, apoptosis, angiogenesis, and cellular life span (Hahn and Weinberg, 2002). Particularly during late-stage carcinoma progression, crossstalk between the often heterogeneous tumor cells and different cell types of the “tumor stroma” is essential (for review, see Mueller and Fusenig, 2004). Besides cell-cell interactions and interaction with the (often modified) extracellular matrix (ECM), this crossstalk involves multiple secreted proteins acting in an autocrine or paracrine fashion (e.g., chemokines, cytokines, tyrosine-serine kinase ligands, developmental regulators; Balkwill, 2004; Coussens and Werb, 2002). Epithelial-mesenchymal transition (EMT), a process governed by these heterotypic cell interactions, has emerged as a central process during embryonic development, cancer progression, metastasis, and chronic inflammation/fibrosis (Balkwill, 2004; Huber et al., 2005; Thiery, 2003).

EMT has been intensely studied in tissue culture models of epithelial cells—including nontumorigenic, polarized mammary gland epithelial cells (EpH4; Grünert et al., 2003; Janda et al., 2002a)—and transgenic mouse tumor models (Siegel et al., 2003). EpH4 cells transformed with oncogenic Ras (EpRas) are tumorigenic. Both in vitro and in mice, EpRas cells undergo EMT in response to TGFβ, stabilized by an autocrine TGFβ loop (Oft et al., 1996). Also, in other cellular models, sustained TGFβR signaling maintains EMT and is important for metastasis in vivo (Oft et al., 1998; Yang et al., 2002).

EpRas cells fail to metastasize from primary tumors but readily do so upon tail vein injection (Jechlinger et al., 2006), thus being able to perform late steps in metastasis. Using EpH4 cells expressing Ras effector mutants, we showed that Ras-dependent MAPK pathway hyperactivation is required for both EMT and metastasis (EpS35 cells). In contrast, a Ras-induced, hyperactive PI3K pathway caused reversible “scattering” but not EMT and was essential for apoptosis protection and accelerated tumor growth (EpC40 cells; Janda et al., 2002a, 2002b). Besides the Ras-TGFβ cooperation, numerous other signaling pathways have been implicated in metastasis; however, little is known about the complex interplay of these pathways in metastasis to the bone, liver, and lung. Observations in our and other laboratories indicate that crossstalk between tumor cells and stromal cells plays a critical role in these metastatic processes (Janda et al., 2002a, 2002b). Nevertheless, the identification of the mechanisms underlying EMT and metastasis has only revealed the tip of the iceberg. In the following, we describe the discovery of a cytokine-like protein, ILEI, which is necessary and sufficient for EMT, tumor formation, and metastasis, and thus represents a key player in the metastatic process.

Significance

Metastatic progression of carcinomas causes >80% of cancer deaths and involves crossstalk of carcinoma cells with multiple other cell types, frequently involving secreted cytokines/chemokines often overlooked in expression profiling studies due to exclusive translational control. Expression profiling of polysome bound mRNA identified ILEI, a cytokine-like protein both necessary and sufficient for EMT, tumorigenesis, and metastasis of normal epithelial cells. Discovery of ILEI thus added a key player to the growing list of mechanisms governing metastasis. Abnormal ILEI expression (cytoplasmic overexpression instead of vesicular localization) was clearly associated with EMT in human colon cancer and predictive for metastasis formation and poor prognosis in human breast cancer, rendering ILEI a promising target for therapeutic intervention by humanized monoclonal antibodies.

pathways (e.g., hedgehog, Notch, and wnt/β-catenin signaling) and transcriptional regulators (e.g., Twist, NFκB, STAT3, Id2/3, and particularly the Snail superfamily) contribute to EMT in development and cancer (for review, see Huber et al., 2005). One possible reason why so many different, often crosstalk ing mechanisms cause EMT is that the term “EMT” comprises a wide spectrum of changes in epithelial plasticity. These can be reversible or metastable and may involve less or more severe gene expression changes toward a mesenchymal cell phenotype (reviewed in Grünert et al., 2003; Huber et al., 2005). “Complete EMT”—defined by a metastable, fibroblastoid phenotype plus loss of E-cadherin and gain of vimentin (Janda et al., 2002a)—was most closely correlated with local invasion and metastasis (Grünert et al., 2003). In an attempt to molecularly characterize this “complete EMT” phenotype, Eph4- and CT26-derived cell pairs showing scattering or complete EMT (Figure 1A; see the Supplemental Experimental Procedures) were employed for expression profiling of polysome bound mRNA, allowing additional detection of translationally regulated genes (Pradet-Balade et al., 2001; Jechlinger et al., 2003). A cluster of >30 genes specific for “complete EMT” and metastasis was identified, which served to identified a translationally regulated gene that belongs to a recently discovered gene family (FAM3A–D) with no sequence homology to known genes (Zhu et al., 2002). We show here that the FAM3C protein, referred to as interleukin-like EMT inducer (ILEI), is both necessary and sufficient to cause EMT, slow tumor growth, and late steps in metastasis. ILEI was also shown to be an important player in human cancer progression.

Results

ILEI mRNA translation and protein secretion is specifically upregulated during EMT

Polysome bound mRNA expression profiling of four Eph4-derived cell pairs undergoing complete EMT or scattering—and the colon carcinoma-derived EMT cell pair CKR2-CT26—revealed EMT/metastasis-specific ILEI expression (Figure 1A and Supplemental Experimental Procedures). Semiquantitative RT-PCR for ILEI showed that polysome bound but not total ILEI mRNA was strongly upregulated in mesenchymal EpRasXT cells as compared to epithelial EpRas cells (Figure 1B), clearly showing translational control of ILEI expression.

To verify these findings at the protein level, we raised and affinity purified peptide antibodies to ILEI in rabbits (see Experimental Procedures). One of these antibodies identified V5-tagged and untagged ILEI protein in Western blots after
Expression of exogenous ILEI induces EMT in normal and transformed epithelial cells

To determine if ILEI alters epithelial plasticity, EpH4 cells and their various derivatives were infected with a ILEI-IRES-GFP retroviral vector and sorted for strongly GFP-positive cell populations (see Experimental Procedures). Western blot analysis of these cell populations using anti-ILEI antibodies showed that all EpH4/EpRas derivatives infected with the ILEI retrovirus showed the expected enhanced expression of secreted ILEI (Figure 2C, top panels).

Efficient EMT induction in the EpRas model strongly depends on culture conditions (plastic dishes, porous supports, or collagen gels; Grüner et al., 2003). This prompted us to analyze both ILEI-expressing cells and GFP retrovirus-infected control cells under these different conditions for morphology, motility, and expression of epithelial and mesenchymal markers. Both on plastic and in collagen gels, ILEI expression either did not affect or slightly reduced proliferation of these cells (Figure S1D and data not shown).

In collagen gels, all EpH4-derived cells expressing ILEI underwent complete EMT, forming unordered structures of fibroblastoid cells expressing vimentin but not E-cadherin (Figure 2A, Figures S1B and S1C), a phenotype that was stable during enlargement of the cell structures. On porous supports (still supporting complete epithelial polarity), ILEI induced complete EMT—persisting in confluent cultures—in EpRas and EpS35 cells, which undergo EMT in response to TGFβ1 and a hyperactive Erk/MAPK pathway (EpRas, EpS35). In contrast, EpH4-ILEI, EpBcl-2-ILEI, and EpC40-ILEI cells on filters underwent EMT in sparse cultures, which was reversed in confluent monolayers (Figure S1A), possibly due to blocked passage of apically secreted ILEI through the filter pores to reach the presumably basalateral ILEI receptors. Similarly, sparse cultures on plastic of ILEI-expressing EpH4, EpBcl-2, and EpC40 cells showed a fibroblastoid phenotype and EMT, which reverted to an epithelial phenotype in larger epithelial islands or cell sheets (Figure 2A and Figures S1A and S1B). In contrast, ILEI induced complete EMT at all cell densities in EpRas and EpS35 cells cultivated on plastic (see Discussion).

The above results on marker expression could be confirmed by Western blot analysis (Figure 2C), showing that EpRas-ILEI and EpS35-ILEI completely lost E-cadherin expression and gained vimentin expression while EpH4-ILEI, EpBcl-2-ILEI, and EpC40-ILEI cells (harvested from semi confluent cultures) showed only partial phenotypes with respect to epithelial/mesenchymal marker expression. In conclusion, stable expression of ILEI induces EMT in all EpH4 derivatives in collagen gels but requires a hyperactive Erk/MAPK pathway for EMT on plastic (Figure S1B).

Since TGFβ1-induced EMT in EpRas cells depends on an elevated Erk/MAPK pathway induced by oncogenic Ras, we tested whether stable ILEI expression had similar effects. In both EpH4 and EpC40 cells, which show basal Erk/MAPK activation, ILEI expression enhanced phospho-Erk levels (Figure 2C). Furthermore, a nontoxic Ras farnesylation inhibitor (L739.749; Janda et al., 2002a) reverted EMT in EpH4-ILEI and EpC40 ILEI cells (Figure 2F), suggesting that Ras function is important in ILEI-induced EMT. Finally, we tested whether stable ILEI expression would induce cell migration, employing in vitro wound healing assays. Indeed, EpH4-ILEI and EpC40-ILEI cells closed a “wound” scratched into a confluent epithelial monolayer much faster than respective control cells expressing the empty GFP vector (Figures 2D and 2E). Since these assays were done in serum-free media, preventing proliferation (see Experimental Procedures), the accelerated wound closure could not be due to effects of ILEI on proliferation.

ILEI induces tumorigenicity and late steps in metastasis formation

Next, we investigated whether stable ILEI expression would induce tumor formation in normal, nontumorigenic EpH4 and EpBcl-2 cells. We also asked whether ILEI would cause EMT in vivo and enhanced tumor growth in EpC40 cells, which form tumors but not metastases in nude mice (Janda et al., 2002a, 2002b). Accordingly, EpH4-ILEI, EpBcl-2-ILEI, and EpC40-ILEI cells were injected into mammary gland fat pads of nude mice, together with respective empty vector control cells.

EpH4-ILEI and EpBcl-2-ILEI cells induced small, slowly growing tumors in 8/10 and 4/6 mice, respectively (Figure 3A), while the respective GFP control cells only formed tiny, rapidly regressing nodules consisting of normal epithelial cells (data not shown; Oft et al., 1996). Interestingly, the GFP+ tumor cells from EpH4-ILEI lacked E-cadherin but expressed vimentin, showing that the cells undergo EMT in vivo (Figure 3B). Recultivated, GFP-positive tumor cells obtained by sorting retained their “reversible EMT” phenotype on plastic (Figure 3C; compare to Figure S1A).

Analysis of tumor formation by EpC40-ILEI cells and control EpC40 cells revealed that ILEI strongly enhanced tumor size (3.6-fold increase in mean tumor weight per mouse; Figure 3A). To analyze whether ILEI induced EMT in these tumors, we compared EpC40 and EpC40-ILEI tumors by immunohistochemistry (IHC), staining serial tumor sections for E-cadherin, vimentin, and ILEI expression. As expected (Janda et al., 2002a), EpC40-derived tumors showed strong, membrane-localized E-cadherin expression, while vimentin staining was predominantly detected in the E-cadherin/ILEI-negative areas presumably representing stroma (dashed lines, Figure 3D). In contrast, the ILEI-positive cells in the EpC40-ILEI tumors showed no or weak cytoplasmic E-cadherin staining but strong vimentin expression (Figure 3E). Interestingly, ILEI was restricted to cytoplasmic vesicles in the (nonmetastatic, epithelial) EpC40 cells but showed enhanced cytoplasmic expression in the EMT-prone, metastatic EpC40-ILEI cells (Figures 3D and 3E, bottom insets).

Importantly, the cells in both tumor types showed comparable proliferation rates, as analyzed by Ki67 staining (Figures S3A and S3B). Thus, ILEI might enhance tumor growth by facilitating local invasion as a consequence of EMT, rather than by accelerating cell proliferation. The ability of ILEI to induce tumors was not restricted to epithelial cells, since stable ILEI expression also induced tumor formation in nontumorigenic 3T3 fibroblasts (Figures S3C–S3E) and NMuMG mammary epithelial cells (A.G., K.H. Heider, and H.B., unpublished data).
Figure 2. Overexpressed exogenous ILIEI causes EMT and migration in EpH4-derived cells

A and B: EpH4 cells (A) and EpRas cells (B) overexpressing retrovirally transduced ILIEI (EpH4-ILEI, EpRas-ILEI) or empty GFP vector (EpH4, EpRas) were analyzed on plastic, porous supports and in collagen gels for EMT by phase contrast micrography and immunofluorescence for E-cadherin and vimentin expression (see Experimental Procedures).

C: Lysates from the EpH4-derived cells indicated, expressing or not expressing ILIEI, were analyzed for E-cadherin, vimentin, and phospho-Erk/total Erk expression by Western blot, verifying ILIEI overexpression by using anti-ILIEI antibodies (top panels). Loading control, Smad2–3 antibodies (bottom panels).

D and E: EpC40-ILEI and control EpC40 cells (EpC40-GFP) were analyzed for migratory activity in a wounding assay on plastic (see Experimental Procedures) by photography 72 hr after wounding; (D: red dotted lines) and quantification of wound closure (E).

F: EpH4-ILEI and EpC40-ILEI cells were cultivated in collagen gels for 5 days, treated with 10 μM Ras inhibitor L793949 for another 5 days, and photographed (representative data from one of three independent experiments). EpRas control cells treated similarly showed no evidence of inhibitor toxicity (right panels). Scale bars in A–D and F, 100 μM.
Since cells from the EpH4/EpRas model essentially fail to metastasize directly from primary tumors, their metastatic ability was analyzed by tail vein injection, allowing us to demonstrate that EMT is a faithful in vitro correlate of late steps in metastasis (see Huber et al., 2005). Accordingly, EpH4-ILEI, EpBcl-2-ILEI, and EpC40-ILEI cells were tested for metastatic behavior by tail vein injection of nude mice, in comparison to respective control cells. EpH4-ILEI and EpBcl-2-ILEI cells induced lung metastases in ~70% of the injected mice, the latter developing increased numbers of much larger metastases (Figures 4A and 4B). In line with this, ILEI expression also induced strong metastatic capacity in the tumorigenic but nonmetastatic EpC40 cells (Figures 4A and 4B; 100% of mice, large metastases). Immunohistochemical staining of EpBcl-2-ILEI-induced metastases revealed cytoplasmatic ILEI expression, weak E-cadherin staining, and distinct vimentin expression (Figure 4C). Thus, stable ILEI expression can enable normal EpH4 cells and nontumorigenic EpBcl-2 cells to cause tumors and late steps in metastasis.

Recombinant ILEI protein can induce EMT in EpH4 and EpRas cells

To study whether extracellularly added ILEI protein could induce similar readouts in EpH4 and EpRas cells as stable ILEI overexpression, we tried to express and affinity purify recombinant ILEI. Although several preparations of purified, biologically active ILEI could be obtained (see Figures S2A and S2B), a reproducible procedure to generate large amounts of recombinant ILEI with high and stable bioactivity remains to be developed.

Recombinant ILEI added to EpH4 or EpRas cells in collagen gels was able to induce complete EMT, as shown by formation of unorganized structures consisting of migratory cells that expressed vimentin and lacked E-cadherin, while a control preparation did not (Figures S2C and S2D). Interestingly, recombinant ILEI but not the control preparation also caused EMT in polarized epithelial tubules pregrown in collagen gels in the absence of ILEI (Figure S2E). As shown by time-lapse cinematography, recombinant ILEI also induced motility of EpH4 cells grown on plastic (Figure S2C and data not shown). For preliminary experiments suggesting that neutralizing ILEI antibodies might prevent EMT, see the Discussion and Figure S2F.

ILEI knockdown by RNAi in EpRasXT cells reverses EMT and prevents metastasis

We next investigated if endogenous ILEI is necessary for EMT and metastatic ability, also to rule out that the observed effects
of stable, exogenous ILEI expression represent overexpression artifacts. Transient and stable RNA interference (RNAi) were employed to specifically reduce ILEI protein expression in EpRas cells and EpRasXT cells, respectively. First, EpRasXT cells were infected with retroviruses (Paddison et al., 2002) expressing two different ILEI-sh-RNAs (“A” and “B”) or luciferase-sh-RNA as a control, together with red fluorescent protein (RFP) to allow sorting of infected cells (see Experimental Procedures). Using both ILEI constructs, strongly RFP-positive mass cultures and clones could be expanded, in which secreted ILEI protein was strongly reduced, as determined by Western blot (EpRasXT + siRNA-ILEI; Figure 5A). On plastic, these cells had regained epithelial morphology and membrane-localized E-cadherin expression but showed absent or strongly reduced vimentin expression, as detected by immunofluorescence (Figure 5B) and Western blot (Figure 5A). In contrast, EMT was not affected in control cells (Figure 5B; EpRasXT + siRNA-Luci). A similar reversal of EMT in EpRasXT cells by retroviral ILEI-RNAi was obtained in collagen gels (Figure 5C) and Western blot (Figure 5A). In contrast, ETM was not affected in control cells (Figure 5B; EpRasXT + siRNA-Luci). A similar reversal of ETM in EpRasXT cells by ILEI-RNAi was obtained in collagen gels (Figure 5C, left panels). Since ILEI probably functions as an extracellular, secreted protein, recombinant ILEI should reinduce EMT in the epithelial EpRasXT + siRNA-ILEI cells. Addition of recombinant ILEI to these cells in collagen gels indeed reinduced EMT, while a mock preparation had no such effects (Figure 5C). These findings confirm the specificity of the ILEI knockdown by RNAi.

Transient transfection with a pool of four different siRNA oligonucleotides against ILEI—or Luciferase as a control—was used to determine whether ILEI is required for TGFβ-mediated EMT induction in EpRas cells. Strong or even complete knockdown of ILEI protein expression was observed between 2 and 7 days after transfection. Accordingly, ILEI-siRNA-transfected cells seeded into collagen gels failed to become migratory or lose plasma membrane E-cadherin expression in response to TGFβ, while the control oligo-transfected cells became migratory and spindle shaped, showing strongly reduced, often cytoplasmic E-cadherin expression (Figure 5D and data not shown).

Finally, we analyzed EpRasXT clones showing stable reversal of EMT by retroviral ILEI-RNAi for tumorigenicity and metastatic ability in nude mice, using EpRasXT + siRNA-Luci cells as a control. Knockdown of ILEI still allowed tumor growth (Figure 5E) but strongly inhibited metastasis formation by EpRasXT cells, since EpRasXT + siRNA-ILEI cells induced either no metastases or a few tiny metastases after tail vein injection (Figures 5F and 5G).

ILEI can induce EMT independently of TGFβ signaling

So far, our results did not exclude the possibility that ILEI acted through TGFβ, either by induction of TGFβ secretion or by cross-talk to the TGFβ receptor. Consequently, knockdown of ILEI might abolish autocrine TGFβ production in EpRasXT cells—shown earlier to cause slow reversal of EMT (Oft et al., 1996). To test whether ILEI induces TGFβ secretion, supernatants from EpH4 and EpRas cells expressing or not expressing exogenous ILEI were analyzed in TGFβ-ELISA assays. Secreted TGFβ was not detectably upregulated in EpH4-ILEI or EpRas-ILEI cells, as compared to EpH4/EpRas control cells.
We then added high doses (5 ng/ml) of TGFβ to epithelial EpRasXT-siRNA-ILEI cells, using EpRasXT + siRNA-Luci cells as positive controls. As expected, TGFβ readily induced EMT in the control cells but failed to do so in the EpRasXT cells in which ILEI was knocked down (Figure 6B). Thus, exogenous TGFβ seems to require enhanced ILEI expression in order to induce EMT, raising the possibility that TGFβ might induce ILEI expression. Indeed, addition of TGFβ to EpH4 and EpRas cells clearly enhanced both intracellular and secreted ILEI protein levels in these cells (Figure 6C).

We then addressed whether or not ILEI-induced EMT would require signaling through the TGFβ receptor, using the specific TGFβ-RI kinase inhibitor SB 431.542 (Inman et al., 2002). This inhibitor prevented TGFβ-induced EMT in EpRas cells (Figure 6D, top panels) but did not detectably interfere with EMT induction by recombinant ILEI in the same EpRas cells (Figure 6D, bottom panels). In conclusion, ILEI may represent an essential and important downstream effector of TGFβ during EMT induction.

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Delocalized ILEI expression in human carcinomas is associated with EMT, metastasis formation, and poor prognosis

Finally, we asked whether ILEI expression was induced, enhanced, or altered in human carcinomas as compared to respective normal epithelia. Since measuring ILEI mRNA levels appeared to be meaningless due to translational control of ILEI expression, we analyzed ILEI protein expression in sections of human normal and tumor tissues by IHC, using ILEI peptide antibodies (see Experimental Procedures). Many epithelial tissues were negative (e.g., larynx, bladder, uterus, stomach, prostate, lung; Figure 7C and data not shown). Other—predominantly secretory—epithelia (e.g., salivary gland, pancreas, colon, duodenum, mammary gland) showed ILEI expression restricted to small dot-like structures apical of the nucleus (which could represent storage vesicles or Golgi-like structures; Figures 7A and 7B, green arrowheads). Except isolated positive cells of possibly hematopoietic origin (Figures 7A–7C; red

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arrows) in the interstitial mesenchyme of these epithelia, connective tissues, endothelia as well as smooth and striated muscle were negative, as were neuronal tissues with the exception of Purkinje cells (data not shown).

Interestingly, staining of various human carcinomas showed enhanced cytoplasmic ILEI expression, sometimes in addition to vesicle staining (Figures 7D–7H). In many tumor types (e.g., breast, colon, prostate, lung, liver, head, and neck) variable percentages of cases (13 to >50%) showed this strong ILEI staining (Figure 7I and data not shown). Tumors induced by human carcinoma cell lines in mouse xenograft assays showed a similar enhanced staining for ILEI (Figure S3F). To determine whether strong cytoplasmic ILEI expression is associated with EMT in human tumors, we employed the recent finding that epithelial cells in the invasion front of human colon carcinomas undergo transient EMT. These migratory cells show loss of E-cadherin, induction of vimentin, and nuclear β-catenin expression (reviewed in Brabletz et al., 2005). While epithelial tumor cells in the primary colon carcinoma (detected by basal cytokeratin staining) show cytoplasmic β-catenin and granular staining for ILEI (Figures S4A–S4C, middle panels), cells in the invasion front combined nuclear β-catenin expression with strong cytoplasmic ILEI staining (Figures S4A–S4C, bottom panels). Thus, cytoplasmic ILEI expression correlates with EMT and metastasis (see also Figures 3E, 3F, and 4C).

Finally, we evaluated ILEI staining patterns in a human breast cancer array containing tissue samples from 56 breast cancer patients with known case histories covering up to 15 years, in comparison to normal mammary gland. As expected, both murine and human mammary gland tissue showed ILEI expression in granules apical of the nucleus (Figures 8A and 8B). In mammary carcinomas, some tumors retained granular ILEI staining, while others showed cytoplasmic staining (Figure 8C, red insets of left and right panels, respectively). ILEI staining data from 43 patients were then correlated with the frequency of metastasis-free and overall survival in Kaplan-Meyer plots. Cytoplasmic ILEI expression was clearly correlated with a huge, statistically highly significant decrease in metastasis-free and overall survival (Figure 8D, Kaplan-Meyer plots; see Discussion). Interestingly, KI67 staining of tumors with cytoplasmic ILEI expression showed unchanged or reduced proliferation rates in comparison to tumors with granular ILEI expression (Figure 8C, blue insets) For definitions and details, see the Supplemental Experimental Procedures.

Discussion

In this paper, we identify and functionally characterize ILEI as a key player in both EMT and late-stage carcinoma progression. ILEI is a member of a group of secreted proteins with largely unknown function (FAM3A-D; Pilipenko et al., 2004; Zhu et al., 2002). Polysome bound mRNA expression profiling (Pradet-Balade et al., 2001) was instrumental to the identification of ILEI, since expression of this gene was exclusively controlled at the
Thus, in the EpH4 system, ILEI is able to cause EMT (reviewed in Grünert et al., 2003). Similarly, the Wnt/β-catenin, Notch, and hedgehog signaling pathways induced EMT induction and tumor progression when cooperating with other oncogenic events but sometimes acted as tumor suppressors on their own (reviewed in Huber et al., 2005). To some extent, ILEI function is mirrored by the transcription factor Twist, sufficient for EMT induction in polarized human mammary epithelial cells and necessary for metastasis in murine mammary carcinoma cell lines (Yang et al., 2004).

Due to the limitations of the EpH4/EpRas model—metastasis formation upon tail vein injection but no metastasis from orthotopic primary tumor sites—we can only conclude that ILEI is sufficient for late steps in metastasis such as extravasation, invasion, and proliferation of tumor cells at distant sites. Clearly, EMT itself seems to be necessary but not sufficient for metastasis, since weakly metastatic cells and their highly metastatic variants can both exhibit EMT (reviewed in Huber et al., 2005). Based on encouraging results from pilot experiments (data not shown), we are currently investigating stable ILEI knockdown in CT26 cells (Figure 5), which metastasize from orthotopic tumor sites (Oft et al., 1998) and show enhanced ILEI secretion (Figure 1D).

It needs to be mentioned that EMT induced by ILEI alone is reversible on plastic and porous supports at high cell density (Figure S1A), in contrast to EMT induced by Ras plus TGFβ. We speculate that ILEI-induced loss of epithelial polarity might be reversible if the adherent cells in dense 2D cultures are forced to undergo cell-cell contacts. This reestablishment of epithelial polarity would then block ILEI function by preventing access of apically secreted ILEI to the (basolaterally expressed) ILEI receptors. In collagen gels, EpH4-ILEI cells can maintain stable EMT, since cell-cell adhesion is not enforced by adhesion to plastic. The fact that EpRas and EpS35 cells express N-Cadherin (A. von Bredow and H.B., unpublished data) and thus exhibit more "plastic" epithelial contacts might also explain why ILEI can cause stable EMT of these cells in 2D cultures (Figure S1B).

Endogenous ILEI is necessary for EMT, tumor formation, and metastasis and acts downstream of TGFβ receptor signaling

Endogenous ILEI expression, which is upregulated in EpRas cells after EMT (EpRasXT cells), is necessary for EMT and tumor progression, as demonstrated by transient and stable knockdown of ILEI via RNAi in EpRas and EpRasXT cells, respectively (Figure 5). This ruled out the possibility that the observations made with exogenously expressed ILEI were artifacts due to nonphysiological expression levels. The stable ILEI knockdown was specific for ILEI, since recombinant ILEI (Figure S2) induced EMT in epithelial EpRasXT-siRNA-ILEI cells (Figure 5C). First results with neutralizing ILEI antibodies also suggest that endogenous, secreted ILEI can cause EMT via an autocrine loop (Figure S2F). Our finding that ILEI acts independently of TGFβ signaling and downstream of TGFβ function (Figure 6) excludes the (somewhat trivial) possibility that ILEI employs TGFβ secretion or TGFβ-R signaling to induce EMT. Mouse tumors induced by EpRas cells initially undergo EMT in vivo in response to stromal TGFβ (Oft et al., 1996). Thus, TGFβ produced by the tumor stroma might induce ILEI both in the tumor cells and in the nonrelevant (low case numbers). Scale bars in A–H, 50 µM.

Figure 7. Enhanced and delocalized expression of LEI protein in human tumors
ILEI protein expression and localization in normal human tissues (A–C, mammary tissue; see Figures 8A and 8B) or colon (D and E, colorectal AC) and mammary carcinomas (F, IDC-G2; H, IDC-G3, G, AC) as analyzed by IHC staining. Green arrowheads, granular staining of epithelial cells; red arrows, single stained cells in interstitial mesenchyme. A: Abnormal ILEI expression detected by IHC in various types of human carcinomas (compare A and D). AC, adenocarcinoma; IDC, invasive ductal carcinoma; ILC, infiltrating lobular carcinoma; SCC, small cell carcinoma; BAC, benign adenocarcinoma; n.r., nonrelevant (low case numbers). Scale bars in A–H, 50 µM.

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<th>% ILEI pos.</th>
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<td>n.r.</td>
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<td>n.r.</td>
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<td>larynx</td>
<td>head and neck</td>
<td>1/8</td>
<td>13</td>
</tr>
</tbody>
</table>

level of translation (Figure 1B). This might also explain why ILEI was not identified earlier by total mRNA expression profiling studies (van’t Veer et al., 2002).

Exogenous ILEI expression: Sufficient for EMT, tumor formation, and metastasis?
Stable expression of ILEI in various EpH4-derived cells was sufficient to induce EMT, (slow) tumor growth, and lung metastasis upon subcutaneous and intravenous injection, respectively (Figures 2–4). Thus, in the EpH4 system, ILEI is able to cause EMT and tumor progression on its own. Oncogenic Ras alone only upon subcutaneous and intravenous injection, respectively (Figure 5). This ruled out the possibility that the observations made with exogenously expressed ILEI were artifacts due to nonphysiological expression levels. The stable ILEI knockdown was specific for ILEI, since recombinant ILEI (Figure S2) induced EMT in epithelial EpRasXT-siRNA-ILEI cells (Figure 5C). First results with neutralizing ILEI antibodies also suggest that endogenous, secreted ILEI can cause EMT via an autocrine loop (Figure S2F). Our finding that ILEI acts independently of TGFβ signaling and downstream of TGFβ function (Figure 6) excludes the (somewhat trivial) possibility that ILEI employs TGFβ secretion or TGFβ-R signaling to induce EMT. Mouse tumors induced by EpRas cells initially undergo EMT in vivo in response to stromal TGFβ (Oft et al., 1996). Thus, TGFβ produced by the tumor stroma might induce ILEI both in the tumor cells and in the nonrelevant (low case numbers). Scale bars in A–H, 50 µM.

Figure 7. Enhanced and delocalized expression of LEI protein in human tumors
ILEI protein expression and localization in normal human tissues (A–C, mammary tissue; see Figures 8A and 8B) or colon (D and E, colorectal AC) and mammary carcinomas (F, IDC-G2; H, IDC-G3, G, AC) as analyzed by IHC staining. Green arrowheads, granular staining of epithelial cells; red arrows, single stained cells in interstitial mesenchyme. A: Abnormal ILEI expression detected by IHC in various types of human carcinomas (compare A and D). AC, adenocarcinoma; IDC, invasive ductal carcinoma; ILC, infiltrating lobular carcinoma; SCC, small cell carcinoma; BAC, benign adenocarcinoma; n.r., nonrelevant (low case numbers). Scale bars in A–H, 50 µM.

<table>
<thead>
<tr>
<th>tissue</th>
<th>tumor type</th>
<th>No. ILEI pos.</th>
<th>% ILEI pos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>breast</td>
<td>IDC (G2–G3)</td>
<td>33/85</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>ILC (G2–G3)</td>
<td>3/16</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>2/2</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td>medullary AC</td>
<td>2/2</td>
<td>n.r.</td>
</tr>
<tr>
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<td>colorectal AC</td>
<td>17/24</td>
<td>71</td>
</tr>
<tr>
<td>prostate</td>
<td>hyperplasia</td>
<td>1/5</td>
<td>20</td>
</tr>
<tr>
<td>lung</td>
<td>AC (G2–G3)</td>
<td>7/26</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>SCC</td>
<td>11/31</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>BAC</td>
<td>2/3</td>
<td>n.r.</td>
</tr>
<tr>
<td>larynx</td>
<td>head and neck</td>
<td>1/8</td>
<td>13</td>
</tr>
</tbody>
</table>
stromal cells itself, thus contributing to tumor progression in a dual fashion (see below).

**How does ILEI function in altering epithelial plasticity?**

Since analysis of ILEI-dependent signal transduction or identification of the receptor has to await mass production of bioactive ILEI (see the Supplemental Experimental Procedures), little is known about its mechanisms of action. Interestingly, expression profiling of ILEI-expressing EpH4 and EpRas cells revealed ILEI-induced upregulation of multiple chemokines, cytokines, growth factor receptors, and cytoplasmic signaling intermediates (T.W. and H.B., unpublished data). In addition, preliminary data suggest that ILEI expression strongly induced upregulation of the PDGF receptor (PDGFRα and β; W. Mikulits and H.B., unpublished data), which is required in an autocrine PDGF/PDGFR loop for murine and human tumor progression (Jechlinger et al., 2006). This suggests that ILEI might be an inducer of multiple autocrine growth factor and chemokine loops, raising the question of whether ILEI-dependent signaling pathways that are necessary for ILEI function are induced via these secondary autocrine loops or directly via the unknown ILEI receptor. Examples for such pathways are the observed activation of the Erk/MAPK pathway in EpH4 and EpC40 cells (Figure 2C) and the requirement of Ras function for ILEI-induced EMT (Figure 2F). Since EMT induction by ILEI readily occurs only in collagen gels (where extensive biochemical analysis is difficult; Janda et al., 2002b), biochemical studies on ILEI function will be a challenge even when recombinant ILEI is available.

Does ILEI also function in other cellular systems? Stable ILEI expression causes epithelial plasticity changes and/or tumor formation in NMuMG mammary epithelial cells (A.G., K.H. Heider, and H.B., unpublished data) or hepatocytes (W. Mikulits and H.B., unpublished data). Furthermore, ILEI renders 3T3 cells tumorigenic (Figure S3). Finally, siRNA-mediated knockdown of human ILEI in the dedifferentiated, invasive human mammary carcinoma cell line CAMA-I interfered with its EMT phenotype (Figure S5A) in a fashion similar to that observed for PDGF-R tyrosine kinase inhibitors (Jechlinger et al., 2006). This shows that ILEI is also important in human tumor cells.

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**Figure 8.** ILEI expression in human breast cancer: Strong cytoplasmic ILEI expression correlates with increased metastasis and decreased survival

A and B: A human breast cancer tissue array derived from 56 patients with known case histories, containing 71 tumor (T) or lymph node metastasis (Lnm; L) tissue samples was analyzed for intracellular distribution of ILEI by IHC. Normal murine (A) and human mammary gland tissue (B; N; adjacent to T34 or L 59) show granular ILEI expression.

C: ILEI staining of tumors T44, T45, and T31 (from patients without metastasis) and T9, T30, and Lnm L14 (from patients with two or more types of metastases), yielding granular and cytoplasmic ILEI staining, respectively (red insets). Intensities of ILEI staining (0–6) and proliferation rates of these tumors as analyzed by Ki67 antibody staining (blue insets) are also indicated.

D: Intracellular ILEI distribution (see C) was analyzed in all available tumor samples and correlated in Kaplan-Meier plots to metastasis-free survival (top panel) and overall survival (bottom panel). Vertical ticks, censored patients. For definitions of these terms and further details, see the Supplemental Experimental Procedures.

Scale bars in A–C, 50 μM.
ILEI: Important for cancer progression in human carcinomas?

Expression of endogenous ILEI in human tumors and normal tissues had to be measured at the protein level, since this gene is translationally regulated and ubiquitously expressed at the mRNA level (Zhu et al., 2002). This also explained why respective analyses using the GeneLogic database (Dolzng et al., 2005) did not reveal significant upregulation of ILEI mRNA in human tumors. In both normal tissues and certain tumors, ILEI protein was localized to dot-like, suprabasal structures that could represent Golgi structures or storage vesicles (Figures 7A, 7B, and 8A–8C). In many other tumors, however, enhanced, often cytoplasmic staining for ILEI was observed (Figures 7D–7H and 8A–8C). Interestingly, this cytoplasmic staining was also observed in tumor cells undergoing EMT at the invasion front of colon carcinomas (Figure S4), suggested to represent migratory tumor stem cells (Brabletz et al., 2005). Furthermore, cytoplasmic ILEI staining was strongly correlated with a shortened metastasis-free and overall survival in human breast cancer patients (Figure 8D). Importantly, cytoplasmic ILEI staining did not correlate with multiple other clinical markers for a bad prognosis in breast cancer, with the possible exception of HER2 protein expression (Table S1). This suggests that ILEI can serve as an independent prognostic marker in breast cancer.

How could vesicular versus cytoplasmic localization of ILEI have an impact on tumor cell invasiveness? We speculate that granular IHC staining indicates vesicular storage of ILEI protein in normal and benign tumor cells that do not secrete ILEI except in response to specific stimuli. In line with this, polysome bound ILEI mRNA levels correlated with secreted but not total ILEI protein levels. This points to strict control of ILEI secretion in normal cells, which might be altered or lost during EMT or cancer progression, leading to autocrine secretion plus reuptake of ILEI into endocytic vesicles, which would appear as cytoplasmic staining at the low resolution of IHC. Similar results have been obtained for deposition of the EMT-specific ECM protein Tenascin C, which is likewise translationally controlled (Jechlinger et al., 2003).

Interestingly, single small cells localized in the interstitial mesenchyme of normal epithelia also showed strong ILEI staining, most likely representing hematopoietic cells, which were sometimes massively increased in carcinomas. In chronic inflammatory tissue (e.g., collagen-induced rheumatoid arthritis in rat paws), macrophages, mast cells, and lymphocytes were strongly ILEI positive (IHC; T.W. and H.B., unpublished data). During tumorigenesis, ILEI might thus be produced initially by chronic inflammatory cells (CousSENS and WErb, 2002), favoring epithelial plasticity, migration, EMT, and autocrine production of ILEI during tumor cell progression/dedifferentiation. Once potent neutralizing anti-ILEI antibodies are available, these will be tested for their ability to interfere with tumor growth and/or local invasion of human tumor cells in mouse xenografts, helping to decide whether ILEI might be a promising target for therapeutic, humanized antibodies to combat breast carcinoma progression.

Experimental procedures

Cells and culture conditions

The origin, culture conditions, and analysis of epithelial/mesenchymal markers in Eph4 mouse mammary epithelial cells and derivatives (for a detailed description, see the Supplemental Experimental Procedures) have been described earlier (Janda et al., 2002a; Oft et al., 1998). The breast ductal carcinoma cell line MDA-MB-435, the human epidermoid carcinoma line A431, and NIH 3T3 fibroblasts were purchased from ATCC (Manassas, VA).

Eph4/EpRas cells expressing retrovirally transduced ILEI cells were cultivated in F12/DMEM (Gibco) plus 10% fetal calf serum (FCS) and 20% conditioned medium (from confluent cultures, added every 48 hr). Three-dimensional collagen gel cultures of Eph4 cells and their derivatives were performed as described earlier (Janda et al., 2002a; Oft et al., 1996), and 0.1%–0.5% FCS was added for the first 2 days of culture.

Identification of ILEI as an EMT/metastasis-specific, translationally controlled gene

Gene expression profiling of polysome bound mRNA from the Eph4- and CT26-derived cell lines mentioned above (see Figure 1A) and identification of EMT-specific genes (including ILEI) by cluster analysis were performed as described earlier (Jechlinger et al., 2003). After cloning and sequencing of the full-length ILEI c-DNA, further searching of the database identified ILEI as a member (FAM3C; accession number AAR84605) of a little-characterized gene family (FAM3; Zhu et al., 2002).

Determination of total and polysome bound ILEI mRNA by semiquantitative RT-PCR

Polyosomal bound mRNA of EphRas and EpRASXT cells was isolated by sucrose gradient fractionation (Jechlinger et al., 2003) and mRNA isolation using RNeasy Mini Kit (Qiagen). One-tube RT-PCR was performed as described by the supplier (Titan, Roche Inc.) Either 15 (total RNA) or 19 PCR cycles (polysomal RNA) were performed to quantitify ILEI mRNA, using β-actin as a loading control. For primers used, see the Supplemental Data.

Stable cell lines expressing retrovirally transduced ILEI and ILEI-siRNA

The ILEI coding sequence (see Zhu et al., 2002) was PCR amplified using Gateway (Invitrogen)-compatible primers (see the Supplemental Data). Retroviral producer cell lines (NIH-3T3 gp + 86 cells) were transfected with the retroviral construct pMSCV-IRE-SGF; (Moriggl et al., 1999) using Fugene (Roche). Supernatants from FACS-sorted, strongly GFP+ producer cells were used to infect Eph4 derivatives and NIH3T3 cells. Cells were FACS sorted twice for stable GFP expression, generating mass cultures (>80% GFP+) or single cell clones. V5-tagged ILEI was generated by inserting the ILEI coding sequence into the modified pDEST 47 vector (Invitrogen) and used to transfect Cos 7 cells with Fugene 6 (Roche) according to the manufacturer’s protocols.

Two different oligos encoding ILEI sh-RNAs (“A” and “B”; for sequences, see the Supplemental Data) were inserted into the vector pSHAG according to protocols from Gregory Hannon (CSH laboratories, NY) and transferred to the Gateway modified pMSCV-IRE-SGRF vector (Clontech). Retroviral infection and selection of RFP-expressing cells were performed as described above. ILEI expression of all cell lines generated was determined by Western blot analysis. For transient knockdown of ILEI in EpRas cells, a Smart siRNA pool coding for four different ILEI regions (Dharmacon Inc.) was transfected with Dharmacon buffer 2 using 10 ng of siRNA according to the manufacturer’s instructions. As a control, scrambled oligos (Dharmacon) were used similarly (for sequences, see the Supplemental Data). ILEI levels were monitored by Western blot 2–7 days after transfection. Two days after transfection, cells were seeded into collagen gels and treated or not treated with TGFβ (Janda et al., 2002a).

Antibodies

Antibodies to ILEI were generated by immunization of rabbits (Gramsch Laboratories) with a KLH-coupled ILEI peptide or purified, full-length ILEI protein. For IHC, antibodies to crossreacting FAM3 family members were absorbed prior to IHC using peptides from FAM3A and FAM3D and used at 300 ng/ml for IHC and 15 ng/ml for Western blot analysis. For origin, preparation, description, and dilutions of all antibodies used for IHC, immunofluorescence, Western blots, or functional assays in this paper, see the Supplemental Experimental Procedures.
**Immunofluorescence and IHC**

Antibody staining of cells for immunofluorescence, performed on plastic (glass chamber slides; Nunc), on filter culture inserts (pore size 0.4 μm, Becton Dickinson), or in whole collagen gel structures was performed as described earlier (Janda et al., 2002a; Oft et al., 1996). Fluorescent staining for GFP, RFP, E-cadherin, and vimentin was visualized by conventional or confocal fluorescence microscopy (Axiovert 200M, Zeiss), using DAPI (Roche) for DNA counterstaining.

For IHC, the staining unit “Discovery” (Ventana Medical systems) was used according to the manufacturer’s instructions (for details, see the Supplemental Experimental Procedures).

**Western blot analysis**

All cell lysates were prepared and subjected to Western blot analysis as described earlier (Janda et al., 2002a); for antibodies see above. Lysates from cells with stable ILEI expression were prepared from cells cultivated for 24 hr with less than 30% final confluency. To determine secreted ILEI protein levels, 1 × 10⁶ cells were cultured on a 10 cm dish (NUNC) in 10 ml of serum-containing media for 18 hr, washed three times, and cultivated for 18 hr in 7 ml of serum-free media (DMEM + 0.1% BSA). Cleared supernatants were concentrated 5- to 7-fold by vacuum drying and analyzed in Western blots, after normalization to cell numbers. Total protein content was determined from cell pellets (Bradford assay, BioRad Inc.).

**Quantification of TGFβ1 by ELISA**

Cell culture supernatants of EpH4 derivatives (3 × 10⁵ cells, 35 mm dishes, 1 ml of serum-free medium, 5-fold concentration) were normalized as above (WB ILEI). They were analyzed for total secreted TGFβ1 by the TGFβ1 Emax Immuno Assay System (Promega, Madison), according to the manufacturer’s instructions, and independently determined by a contract laboratory (Perbio Science GmbH, Germany).

**Phenotypical analysis of EpH4-derived cells stably expressing ILEI**

ILEI-expressing and control EpH4 cells and derivatives (see Figure 1A) were analyzed on plastic and porous supports (using both sparse [<20% confluent] and dense [50%-80% confluent] cultures) and collagen gels for morphology, E-cadherin, and vimentin as described earlier (Janda et al., 2002a; Oft et al., 1996). For wound closure assay, a wound (~2 mm) was scratched into confluent cultures (10 cm dish, Nunc) of GFP- or ILEI-expressing EpH4 or EpC40 cells, which were preincubated with serum-free DMEM for 24 hr, and cultivated in serum-free DMEM containing glutamine (Gibco), 0.2% BSA, and human transferrin. Wound regions with an identical width were marked, and wound closure (percentage of closed scratch area) was measured in photographs of 15 randomly selected wound areas at the time points indicated.

**Experimental tumor and metastasis assay**

Tumor formation and metastatic ability of EpH4 cells and derivatives were determined by mammary gland fat pad or tail vein intravenous injection into 6- to 9-week-old BalbC athymic nude mice (nu/nu, Charles River Wega GmbH, Germany; Janda et al., 2002a), according to IMP-held animal experiment approvals from the Austrian Bundesministerium für Bildung, Wissenschaft und Kultur. After determination of tumor weight and/or photography, excised tumors and metastatic lungs were fixed with paraformaldehyde (4%, 18 hr, 4°C) and post fixed (70% ethanol, 16 hr) before dehydration and paraffin embedding. For frozen sections, fixed tissues were incubated in HEPES-buffered 20% sucrose (18 hr), embedded Tissue-Tek (Lab-Tek Products, Miles Lab.), and frozen (~40°C). Paraffin sections were stained with hematoxylin/eosin according to standard protocols or subjected to IHC staining. Lung metastasis was quantified by counting metastatic lesions in 50 sections (~0.3 mm apart, representing about 50% of lung volume). Data (corrected for large metastatic lesions spanning more than one section) were expressed as average numbers of metasteses from ten sections. For recultivation of cells from ILEI-induced mouse tumors, tumor tissues were dissociated as described elsewhere (Janda et al., 2002a). Tumor cells were expanded (media plus 30 mg/ml ciprofloxacin; Roche), and mass cultures (>90% GFP-positive cell populations) were selected by repeatedly FACS cell sorting.

**Human normal and carcinoma tissues**

The following paraffin-embedded human normal or tumor tissues were employed: prostate cancer array (no. CC 19-01-003), lung cancer array T8235724-5 (BioCat, Heidelberg, Germany), diverse human normal and cancer tissues (Oridis Biomed, Graz, Austria), and colon carcinomas (T.B.). A large breast cancer array (MS) was analyzed retrospectively by IHC staining, according to protocols approved by the institutional review board of the Medical University of Vienna (see the Supplemental Experimental Procedures).

**Supplemental data**

The Supplemental Data include Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/3/227/DC1/.

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