Published in final edited form as:

Oncogene. 2017 June 29; 36(26): 3706–3717. doi:10.1038/onc.2016.504.

A double-negative feedback loop between EpCAM and ERK contributes to the regulation of epithelial-mesenchymal transition in cancer

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Abstract

Epithelial-mesenchymal transition (EMT) is an important biologic process that has been implicated in cancer metastasis. Epithelial cell adhesion molecule (EpCAM) is expressed at the basolateral membrane of most normal epithelial cells, but is overexpressed in many epithelial cancers. In our studies of the role of EpCAM in cancer biology, we observed that EpCAM expression is decreased in mesenchymal-like primary cancer specimens *in vivo*, and following induction of EMT in cancer cell lines *in vitro*. Extracellular signal-related kinase (ERK) is a key regulator of EMT. We observed that EpCAM expression is decreased with activation of the ERK pathway in primary cancer specimens *in vivo* and in cancer cell lines *in vitro*. In experimental models, growth factor stimulation and/or oncogene-induced ERK2 activation suppressed EpCAM expression, whereas genetic or pharmacologic inhibition of the ERK pathway restored EpCAM expression. In detailed studies of the EpCAM promoter region, we observed that ERK2 suppresses EpCAM transcription directly by binding to a consensus ERK2 binding site in the EpCAM promoter, and indirectly through activation of EMT-associated transcription factors SNAIL1, SNAIL2, TWIST1, and ZEB1, which bind to E-box sites in the EpCAM promoter. Surprisingly, EpCAM appears to modulate ERK activity. Using multiple cell lines, we demonstrated that specific ablation of EpCAM resulted in increased ERK pathway activity, SNAIL2 expression, migration and invasion, whereas forced expression of EpCAM resulted in decreased ERK pathway activity, decreased SNAIL2 expression, migration and invasion. These observations provide important insights into the regulation of EpCAM expression during EMT, demonstrate an unexpected role for EpCAM in the regulation of ERK, and define a novel double-negative feedback loop between EpCAM and ERK that contributes to the regulation of EMT. These studies have important translational implications as both EpCAM and ERK are currently being targeted in human clinical trials.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Keywords

EpCAM; ERK; cancer; epithelial-mesenchymal transition

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a developmental regulatory program that has recently been implicated as a key process by which transformed epithelial cells can acquire the abilities to invade, resist apoptosis, and disseminate in the body.^{21, 35, 53} By repurposing a process involved in embryonic morphogenesis and wound healing, epithelial cancers can acquire properties that enable tumor cell invasion and metastasis. Despite the importance of EMT in cancer biology, the molecular regulation of EMT in cancer remains to be fully elucidated. The mitogen-activated protein kinase/extracellular signaling-related kinase (MAPK/ERK) signaling pathway plays an important role in regulating EMT in cancer^{29, 44, 45} and multiple positive and negative feedback loops contribute to the regulation of EMT.^{3, 13}

EpCAM (TACSTD1/CD326) is expressed at the basolateral membrane of most normal epithelial cells, but is overexpressed in many epithelial cancers. EpCAM was the first cancer-associated protein to be targeted with monoclonal antibodies in human clinical trials, and it remains an attractive target for molecular therapy in many cancer types.^{1, 32} EpCAM is also one of the most widely utilized biomarkers in cancer biology. The CellSearch circulating tumor cell assay is based on magnetic enrichment of EpCAM-positive circulating tumor cells.¹⁰ Although initial studies focused on the role of EpCAM as an adhesion molecule, and biomarker in epithelial cancers, we and others have recently demonstrated that EpCAM plays an important role in cancer biology, either indirectly by modulating oncogenic signaling pathways,^{34, 39–41} or directly by nuclear translocation of the cytosolic short domain.²⁷

Extracellular-signal-regulated kinases (ERKs, or more specifically ERK1 and ERK2) are widely expressed protein kinase signaling molecules that are involved in the regulation of meiosis, mitosis, and post-mitotic function. However, when inappropriately activated, ERKs may contribute to malignant transformation. The MAPK/ERK signaling cascade is activated by a wide variety of growth factor receptors and oncogenes, and upon activation, ERKs are able to phosphorylate an array of target proteins, and/or translocate to the nucleus to directly regulate the transcription of target genes, including SNAIL^{8, 57} and other transcription factors responsible for the induction of EMT. There is strong evidence linking ERKs to the regulation of EMT in cancer. Oncogenic B-Raf and N-ras induce EMT in melanoma cells by activation of ERK1/2, and subsequent activation of ZEB1, TWIST1 and FRA1.⁶ In Ras activated cells, ERK2 induces EMT through ZEB1 upregulation.⁴⁵

Although the regulation of EpCAM during EMT has not been the focus of previous studies, global gene expression analyses demonstrate that EpCAM is decreased in mesenchymal-like cancers. For example, Byers et al.⁵ reported a 76-gene EMT signature in cancer, and EpCAM is one of the genes that is downregulated in this gene signature. Similarly, EpCAM expression is decreased following induction of EMT in cancer cell lines^{15, 42, 56}. In addition,

a negative correlation between the activity of EMT-associated transcription factors SNAI1^{26, 56}, and ZEB1^{16, 54} and EpCAM expression has been reported. Specific ablation of ZEB1 by ZEB1 shRNA, or forced expression of miR-200c or miR-205 (ZEB1 suppressors) was associated with increased EpCAM expression³⁰. These reports are consistent with the hypothesis that EpCAM expression is dynamically regulated during EMT.

Altogether, these reports suggest that EpCAM is decreased during EMT. However, the regulation of EpCAM expression during EMT has not been studied in depth. Similarly, the functional role of EpCAM during EMT has not been studied. To address these questions we studied the regulation of EpCAM expression in cancer cell lines undergoing EMT, and in primary human cancer specimens. We demonstrate that EpCAM expression is suppressed during EMT, and that ERK contributes to the regulation of EpCAM expression during EMT. We also explore the functional role of EpCAM in the regulation of EMT, demonstrating that EpCAM contributes to the regulation of EMT by suppressing ERK in a novel double-negative feedback loop. These studies significantly advance our understanding of EpCAM in the regulation of EMT and in cancer biology, with important translational implications.

RESULTS

EpCAM expression is significantly decreased in mesenchymal-like cancer cell lines, and primary human cancers

To investigate the relationship between EMT and EpCAM expression, we analyzed gene signatures of cancer cell lines, primary lung cancers, and primary breast cancers. Specifically, we analyzed existing microarray data obtained from the Gene Expression Omnibus data repository from 166 epithelial cancer cell lines,¹⁹ 129 primary lung cancers,³⁷ and 136 primary breast cancers.³⁶ We used a recently-published gene set of 76 EMT signature genes to classify the cancer cell lines and primary cancers into epithelial-like or mesenchymal-like cancers.⁵ We removed EpCAM from the 76 gene signature to eliminate any bias from our analysis. EpCAM expression is significantly higher in cancer cell lines and primary cancers classified as epithelial-like, and is significantly decreased (or absent) in cancers classified as mesenchymal-like (Figure 1a–c, $P < 0.001$). Vimentin expression is significantly higher in cancer cell lines and primary cancers classified as mesenchymal-like (Figure 1a–c, $P < 0.001$).

EpCAM expression is significantly decreased following induction of EMT *in vitro*

To directly investigate the impact of EMT on EpCAM expression, we treated a panel of well-characterized normal epithelial and epithelial cancer cell lines (MCF-10A, A549, HaCaT) *in vitro* with TGF β 1 and TNF α , a combination that is known to induce EMT.² Following 72 hours of cytokine treatment, the MCF-10A, A549 and HaCaT cell lines developed a mesenchymal morphology (Figure 2a). Immunofluorescence staining of cells shows decreased expression of the epithelial markers EpCAM and E-cadherin, and increased expression of the mesenchymal marker vimentin compared to control cells (Figure 2a). In parallel experiments, protein immunoblots of the cytokine-treated cells confirmed decreased expression of EpCAM and E-cadherin and elevated levels of vimentin, N-cadherin, SNAI1,

SNAI2, TWIST1 and ZEB1 (Figure 2b and Supplementary Figure S1a). Cytokine treatment was also associated with increased migration and invasion (Supplementary Figure S1b).

EpCAM expression is decreased in cancer cell lines and human cancer specimens with activated MEK/ERK pathway

To explore the relationship between EpCAM expression and signaling pathways known to be involved in the regulation of EMT, we examined EpCAM expression and signaling pathway activity in a panel of 31 epithelial cancer cell lines representing a diverse array of cancer types. Flow cytometry was used to quantify EpCAM expression, and results were normalized across experiments to the MCF-10A mammary epithelial cell line. EpCAM expression varied significantly between the cancer cell lines from less than 25 MFI to greater than 800 MFI (Figure 3a). All cell lines with EpCAM expression less than 25 MFI had no detectable EpCAM expression by protein immunoblot (data not shown). A panel of pathway profiling reporter assays (Clontech Laboratories, Inc.) was used to assess the activity of a variety of oncogenic signaling pathways including the activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), MEK/ERK, signal transducer and activator of transcription 3 (STAT3), Myc, and the retinoblastoma protein/E2F signaling pathways. The 31 cancer cell lines were transfected with reporter constructs and signaling activity was quantified by normalized luciferase reporter expression. We observed a strong inverse association between MEK/ERK pathway activity and EpCAM expression (Figure 3b and c, $P=0.01$). No other signaling pathways were significantly associated with EpCAM expression (data not shown). Cancer cell lines with high MEK/ERK signaling activity and low or absent EpCAM expression were highly invasive in matrigel invasion assays, consistent with a mesenchymal phenotype (data not shown). To confirm these results, we also assessed MEK/ERK signaling activity by measuring phospho-ERK1/2 levels by flow cytometry. There was a strong inverse correlation between EpCAM expression and phospho-ERK1/2 levels (Figure 3d, $P=0.01$).

To confirm the relevance of these *in vitro* findings, we analyzed gene signatures from primary breast cancers, primary lung cancers, and cancer cell lines using the publically available datasets obtained from the Gene Expression Omnibus data repository described previously (Figure 1). We used a recently published gene set of 18 genes known to be regulated by ERK to cluster the cancer cell lines and primary cancers into MEK/ERK pathway-high and MEK/ERK pathway-low clusters.¹¹ EpCAM expression is significantly higher in cancer cell lines classified as MEK/ERK pathway-low, compared to cancer cell lines classified as MEK/ERK pathway-high (Figure 3e and f, $P<0.001$). We observed similar results in primary breast and lung cancers (Supplementary Figure S2a, b, $P<0.001$).

Given that EpCAM expression is significantly decreased in mesenchymal-like cancers, and given the strong inverse association observed between MEK/ERK signaling pathway activity and EpCAM expression, we investigated whether ERK1 and/or ERK2 regulates EpCAM expression using loss-of-function and gain-of-function model systems. Using the MCF-10A mammary epithelial cell line we specifically ablated either ERK1 or ERK2 expression using lentiviral shRNA vectors. Specific ablation of ERK2, but not ERK1, prevented TGF β 1-mediated suppression of EpCAM (Figure 4a). Conversely, forced expression of ERK2 is

associated with decreased EpCAM expression (Figure 4b). To extend these findings, we treated the HaCaT and MCF-7 cancer cell lines with TGF β 1/TNF α or EGF to induce MAPK/ERK pathway activity, and/or pretreated the cells with U0126, a potent and specific MEK1/2 inhibitor (Figure 4c and d). After culture for the indicated times, cells were harvested and lysates were analyzed for ERK1/2 phosphorylation and EpCAM expression. Cytokine or growth factor treatment was associated with increased ERK1/2 activity and decreased EpCAM expression. Treatment with U0126 prevented TGF β 1/TNF α - or EGF-mediated suppression of EpCAM expression (Figure 4c and d). To extend these findings, we treated a panel of 18 cancer cell lines with low or absent EpCAM expression with U0126. Overnight culture with U0126 was associated with a significant increase in EpCAM expression in all of these cancer cell lines (Figure 4e). No other pharmacologic inhibitors were associated with a significant change in EpCAM expression, including inhibitors targeting the P38, JNK or PI3K signaling pathways (Figure 4f). Taken together, these results provide strong evidence that the MAPK/ERK pathway can suppress EpCAM expression across a range of cancer types, although it remains unclear if EpCAM expression is suppressed directly or indirectly.

EpCAM transcription is suppressed both directly and indirectly by ERK2

ERK2 is known to regulate the transcription of target genes both directly (by direct binding to the promoter region of the target gene),²⁰ and indirectly (by regulating the activity or expression levels of transcription factors such as SNAI2 and SNAI1).^{8, 17} To investigate the mechanisms of ERK2-mediated regulation of EpCAM transcription, we performed detailed analyses of the interaction between ERK2 or ERK2-regulated E-box binding transcription factors and the EpCAM promoter region. Sequence analysis of the EpCAM promoter identified a candidate ERK2 binding site (GAAAC).²⁰ To determine if ERK2 binds to this site and directly regulates EpCAM transcription, we used wildtype and mutant EpCAM promoter luciferase reporter constructs. Wildtype ERK2, or a constitutively active mutant form of ERK2 (ERK2^{E319N}, or ERK2-CA), were transfected into cell lines together with EpCAM promoter reporter constructs, and reporter activity was measured. Forced expression of wildtype ERK2 or ERK2^{E319N} results in suppression of EpCAM promoter reporter activity, and mutations in the ERK binding site abrogate this suppression (Figure 5a). In order to verify binding of ERK2 to the EpCAM promoter, we performed a chromatin immunoprecipitation (ChIP) assay. An ERK2-FLAG construct was transfected into HEK-293 cells. The cells were stimulated with serum, fixed, and DNA-protein complexes were immunoprecipitated with a FLAG antibody to assess promoter occupancy. The FLAG antibody immunoprecipitated the EpCAM promoter region (Figure 5b), confirming direct binding of ERK2 to the EpCAM promoter.

To determine if ERK2 regulates EpCAM transcription indirectly, we investigated the interaction between the ERK2-regulated transcription factors SNAI2, TWIST1, SNAI1 and ZEB1 and the EpCAM promoter region. E-box consensus domains (CANNTG) are commonly used by SNAI2, TWIST1, SNAI1 and ZEB1 to regulate target genes during EMT. Of note, the EpCAM promoter region contains three consensus E-Box domains close to the TATA-box. We created three mutant EpCAM promoter luciferase reporter constructs, each with a mutation in one of the E-Box domains. These constructs were transfected into

HEK-293 cells along with a SNAI2 expression vector. Forced expression of SNAI2 suppresses wildtype EpCAM promoter reporter activity, but a mutation in E-Box domain 3 abrogates this suppression, whereas mutations in E-Box domain 1 or 2 have minimal impact (Figure 5c). SNAI2 binding to E-Box domain 3 of the EpCAM promoter was confirmed by ChIP analysis (Figure 5d).

To test if other ERK2-regulated transcription factors bind to the EpCAM promoter and suppress EpCAM transcription, we transduced MCF-10A mammary epithelial cells with tamoxifen-inducible SNAI2, TWIST1 and SNAI1 constructs,^{18, 29} and induced expression of these EMT-associated transcription factors with 4-OHT treatment. Induction of either SNAI2, TWIST1 or SNAI1 for 72-hours was associated with the development of a mesenchymal phenotype (Figure 5e, top panels), with 80% loss of EpCAM expression observed following induction of SNAI2, and complete loss of EpCAM expression observed following induction of either TWIST1 or SNAI1 (Figure 5e, bottom panels). The EpCAM promoter luciferase reporter was also transfected into these cells, and induction of SNAI2, TWIST1 or SNAI1 suppressed EpCAM promoter reporter activity in a dose-dependent fashion (Figure 5e, bar graph, middle panels). TWIST1, SNAI1 and ZEB1 binding to the E-Box 3 region of the EpCAM promoter was confirmed by ChIP analysis (Figure 5f). These observations are consistent with publically available gene expression data demonstrating that induced EMT by TGF β 1 or forced expression of TWIST1 or SNAI1 in HMLE cells is associated with decreased EpCAM expression (Supplementary Figure S3a).⁵²

ZEB1 is also an ERK2-regulated transcription factor that is known to play an important role in the regulation of EMT.^{6, 45, 58} Specific ablation of ZEB1 in the mesenchymal-like breast cancer cell lines MDA-231 and WHIM 3 resulted in increased EpCAM and E-cadherin protein expression, and decreased vimentin protein expression (Supplementary Figure S3b). Specific ablation of ZEB1 also increased EpCAM gene expression up to five-fold, suggesting that ZEB1 regulates EpCAM expression at the level of transcription (Supplementary Figure S3c). Specific ablation of ZEB1 was associated with a change in morphology and decreased invasion in the MDA-231 and WHIM-3 breast cancer cell lines. (Supplementary Figure S3d and e). These data are consistent with gene expression data from 166 cancer cell lines demonstrating a strong inverse association between EpCAM and ZEB1 gene expression ($R > 0.78$, $P = 0.001$) (Supplementary Figure S3f).

EpCAM contributes to the regulation of EMT and ERK

Recent reports have highlighted the functional role of EpCAM in cancer biology. Specifically, EpCAM has been shown to modulate migration, invasion and other biologic processes in multiple different cancer types. To determine if EpCAM contributes to the regulation of EMT, we performed studies using the MCF-10A epithelial cell line. EGF treatment of MCF-10A cells is known to be an experimental model of EMT, predominantly through upregulation of SNAI2 expression.^{8, 22} For these studies, we specifically ablated and/or rescued EpCAM expression in MCF-10A cells, treated the cells with EGF to induce EMT, and measured protein and/or gene expression. Specific ablation of EpCAM followed by EGF treatment is associated with increased SNAI2 and vimentin protein expression, and decreased E-cadherin expression compared to control shRNA treatment (Figure 6a),

suggesting a potential role for EpCAM in modulating EMT. In these experiments shEP3 provides a dose response control, as shEP3 is associated with only partial EpCAM knockdown, and partial EpCAM knockdown is associated with only partial changes in SNAI2 and E-cadherin protein expression.

EpCAM has recently been shown to directly inhibit novel protein kinase C (PKC) and the MAPK/ERK signaling pathway in embryos and cancer cell lines with constitutive activity of PKC.²⁸ To determine if EpCAM can inhibit ERK activity more broadly in cancer cell lines undergoing EMT, we specifically ablated EpCAM, and then treated MCF-10A cells with EGF. Specific ablation of EpCAM results in increased ERK1/2 phosphorylation. Rescue of EpCAM expression using an expression construct resistant to RNA interference is associated with decreased ERK1/2 phosphorylation (Figure 6b). Previous studies have evaluated the gene expression profile of MCF-10A cells before and after EGF treatment, identifying key genes regulated by the MAPK/ERK pathway contributing to the induction of EMT.⁵¹ To determine the impact of EpCAM on these ERK1/2-regulated genes during EMT, we specifically ablated EpCAM and then performed quantitative RT-PCR and protein immunoblot to measure the expression of these genes and phospho-ERK1/2 at serial time points following EGF stimulation. As shown in Figure 6c, specific ablation of EpCAM results in the prolonged activation of phospho-ERK1/2 and upregulation of several key ERK target genes involved in EMT, including SNAI2, EGR1, c-fos, JUN, ATF3 and others.

To extend these findings, we specifically ablated EpCAM in five cancer cell lines with high endogenous EpCAM expression, and forced EpCAM expression in six cell lines with low endogenous EpCAM expression, and treated the cells with media containing EGF. Specific ablation of EpCAM was associated with increased ERK luciferase reporter activity, and increased phospho-ERK1/2 protein levels (Figure 6d, left panel). Forced expression of EpCAM was associated with decreased ERK luciferase reporter activity, and decreased phospho-ERK1/2 protein levels (Figure 6d, right panel).

These data suggest that EpCAM modulates ERK activity, and key genes downstream of ERK that contribute to the regulation of EMT. To test the functional impact of EpCAM expression we specifically ablated or rescued EpCAM expression in MCF-10A cells and then incubated the cells with EGF. Specific ablation of EpCAM is associated with increased migration and invasion, but EpCAM rescue abrogates this effect (Figure 6e).

DISCUSSION

Cancer metastasis is a multistep process involving invasion, intravasation, extravasation, and colonization at a distant site. EMT plays a critical role in this process. During EMT, epithelial cells lose epithelial characteristics, and acquire a mesenchymal phenotype^{14, 47, 59}. This important change in cell differentiation is mediated predominantly by key E-box binding transcription factors, including SNAI2, SNAI1, TWIST1/2, and ZEB1/2.²³ In this study, we show that EpCAM expression is dynamically regulated during EMT. Specifically, we demonstrate that ERK2 can suppress EpCAM expression directly (ERK2 binding to the EpCAM promoter region) and indirectly (following ERK2-dependent activation of the EMT-associated transcription factors SNAI1, SNAI2, and TWIST1). The fact that ERK2

suppresses EpCAM directly is of particular note as very few genes are directly regulated by nuclear ERK2 at the level of transcription.²⁰

ERK2 is a serine/threonine kinase that plays an important role in the mitogen-activated protein kinase (MAPK) signal transduction pathway. ERK2 can regulate downstream proteins/genes by at least three distinct mechanisms. First, ERK2 can phosphorylate proteins in the cytoplasm, and ERK2 is capable of phosphorylating over 100 distinct substrates.^{7, 9} Second, ERK2 can translocate to the nucleus and phosphorylate proteins in the nucleus, including EMT-associated transcription factors.³⁸ Third, ERK2 can translocate to the nucleus and act as a transcription factor by binding directly to the promoter of regulated genes.²⁰ Of note, ERK2 regulates relatively few genes as a transcription factor, and many of these genes are interferon-inducible genes.²⁰ Given the overlapping mechanisms that are being used by ERK2 to regulate EpCAM, and the consistency of regulation across a diverse panel of cancer cell lines, ERK2 regulation of EpCAM is likely to have an important biological role.

EpCAM has been associated with a number of diverse physiologic processes including cell adhesion, proliferation, migration, invasion, differentiation, and stem cell self-renewal. Several mechanisms of action have been proposed to explain EpCAM function. In a recent report, Maetzel et al. demonstrated that EpCAM undergoes regulated intra-membrane proteolysis, followed by nuclear localization of the intracellular domain of EpCAM (EpICD) with concomitant nuclear signaling, proliferation and invasion.²⁷ A recent study by Maghzal et al. proposes an alternate mechanism of action.²⁸ They recently reported that EpCAM acts as a potent inhibitor of novel protein kinase C (nPKC) and the MAPK/ERK signaling pathway in both embryos and cancer cells. They identified a short segment of the EpCAM cytoplasmic tail that can bind to and inhibit nPKC.

Other mechanisms of action have been proposed for EpCAM, including modulation of existing signaling pathways,³¹ and modulation of E-cadherin-mediated adhesions.¹² In normal cells, EMT induction using growth factor stimulation results in ERK1/2 activation for 4–6 hours. Our data shows that the loss of EpCAM can maintain ERK1/2 activation for a longer duration than observed with growth factor stimulation alone (data not shown). In addition to the longer duration of ERK1/2 activation, EpCAM loss leads to transcriptional activation required for EMT maintenance. Further studies will be required to determine how EpCAM modulates ERK2 activity during EMT. Our preliminary studies suggest that the ability of EpCAM to modulate ERK2 activity may not be dependent on the intracellular domain.

The impact of EpCAM expression on cancer prognosis and patient outcome is quite variable and appears to be dependent upon cancer type.⁴⁹ For example, EpCAM expression is associated with a favorable prognosis in colorectal cancer,⁴⁸ but is associated with an unfavorable prognosis in some breast cancers.⁵⁰ Of note, we recently demonstrated that the impact of EpCAM expression on cancer prognosis is variable even in breast cancer; EpCAM is associated with an unfavorable prognosis in the luminal and basal-like intrinsic subtypes, but with a favorable prognosis in the HER2 intrinsic subtype.⁴⁹ The context-dependence of EpCAM may be explained in part by the diversity of biological mechanisms of action

associated with EpCAM function. Specifically, in cancers where ERK activation is critical for EMT maintenance or an invasive phenotype, EpCAM expression may be associated with a favorable prognosis. In cancers where ERK activation does not contribute to the invasive phenotype, nuclear localization of EpCAM or other mechanisms of action may have a more dominant impact on cancer biology.

Double-negative feedback loops are common in biological systems and facilitate switch-like behavior between bistable states. Double-negative feedback loops may be important in regulating switch-like behavior in EMT. Several double-negative feedback loops have been described in the regulation of EMT, including feedback loops involving miR let-7 and LIN28,⁵⁵ miR15a/16-1 and AP4,⁴³ miR-34 and SNAI1,⁴⁶ and miR-200 and ZEB1.⁴ The double-negative feedback loop described here may be particularly important in the regulation of EMT in epithelial cancers as ERK has a central role in the regulation of EMT, and EpCAM is highly expressed in epithelia and epithelial cancers.

The loss of EpCAM expression during EMT has been observed in both embryonic tissues and tumor cells^{15, 25, 42, 56}. The current study confirms and extends these observations, demonstrating that EpCAM expression is regulated directly and indirectly by activated ERK1/2. EpCAM also has the ability to regulate ERK1/2 activity, defining a double-negative feedback loop. This is the first report that of a functional role for EpCAM in the regulation of ERK1/2 activity and EMT. Onder et al³³ have shown that specific ablation of E-cadherin can induce EMT in RAS-transduced HMLE cells grown in growth factor media. Our data shows that specific ablation of EpCAM can enhance induction of EMT following stimulation with TGFβ1 or EGF, and forced expression of EpCAM inhibits ERK and EMT. EMT is a complex process, and it is not clear whether EpCAM acts directly to inhibit ERK, or whether this is an indirect effect related to the ability of EpCAM to support cell junctions, promoting EMT with a secondary impact on ERK.

In conclusion, EpCAM is an important cancer biomarker, and is currently a target for molecular therapy in several epithelial cancers despite the lack of insight into its biologic function. We demonstrate here that EpCAM is dynamically regulated during EMT, and participates with ERK in an important double-negative feedback loop contributing to the regulation of EMT. These functional insights have important translational implications for the application of EpCAM-targeted therapies, and support ongoing investigations into the mechanisms of action of EpCAM biology in specific cancer types.

MATERIALS AND METHODS

Cell lines and reagents

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). WHIM 3 is a breast cancer cell line that was derived from a patient-derived xenograft.²⁴ WHIM 3 was provided by the HAMLET core at WUSM. All cell lines were maintained in RPMI or DMEM Supplemented with 10% FBS and antibiotics (GIBCO BRL, Grand Island, NY). MCF-10A cells were grown in MEGM Mammary Epithelial Cell Growth Medium (Lonza, Allendale, NJ) Supplemented with 5% Horse serum. Recombinant EGF, TGFβ and TNFα were purchased from R&D Systems (Minneapolis, MN). The MAP

kinase inhibitors U0126, PD98059, SB203580, SP600125, and LY29004 were purchased from Selleck Chemicals (Houston, TX).

RNA interference

Lentiviral-mediated RNA interference was performed as previously described.³⁹ shRNA sequences targeting EpCAM at the 3' UTR region (EpCAM-shRNA1), EpCAM at nucleotide 271 (EpCAM-shRNA2), ERK1, ERK2, and ZEB1 are detailed in Supplementary Table 1.

Plasmid constructs

The full-length open reading frame of EpCAM was amplified from the MCF-10A mammary epithelial cell line and sub-cloned into both pcDNA3 and the retroviral vector pBABE. Expression constructs for ERK2 were the kind gift of Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA). Retroviral constructs pBabePuro-hSNAIL-ER (19292), pBabePuro-hSNAIL2-ER (19293) and pWZL-Blast-Twist-ER (18799) were obtained from Addgene (Cambridge, MA).

The EpCAM promoter sequence (–1312 to +49) was cloned into pGL3-Basic and sequence-verified at the WUSM Protein and Nucleic Acid Laboratory. Site-directed mutagenesis of the E-box and ERK2 binding sites were carried out using the QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Mutations were sequence-verified.

Retroviral transduction

Phoenix-ECO packaging cells were transfected when nearly confluent with 2.5 µg of pBABE-hygro-EpCAM using FuGENE HD (Promega, Madison, WI). Twenty four hours after transfection the medium was replaced with 10% FBS. Forty eight hours after transfection viral supernatants were collected, filtered through 0.45 micron filters and then added to MCF-10A cells in media containing 8 µg/mL protamine sulfate. After two successive retroviral infections, cells were grown for 48 hours and selected in hygromycin for two weeks.

cDNA synthesis and quantitative RT-PCR analysis

RNA was purified from cell lines using RNeasy (Qiagen, Valencia, CA). Two micrograms of RNA was reverse transcribed using a cDNA synthesis kit (Ambion, Austin, TX). mRNA expression was quantified using SYBR green chemistry and an ABI Prism 7700 Sequence Detector (Life Technologies, Carlsbad, CA). Primer sequences are detailed in Supplementary Table 2. Each reaction was performed in triplicate, and the data is representative of two independent RNA preparations.

Protein immunoblots

For phosphoprotein immunoblots, stimulated cells were washed with ice-cold PBS and lysed in cell lysis buffer with a protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA). Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). 20–30 µg of protein was subjected to SDS-PAGE (NuPAGE, Life Technologies), and transferred by electrophoresis to a PVDF membrane. Signal detection was performed using

the SuperSignal West Pico chemiluminescent immunodetection system (Thermo Scientific, Rockford, IL). To quantify band density, immunoblots were developed on film, scanned, and pixels in each band were measured using Image J software. Plots of each lane were generated, and the area under the peak corresponding to the appropriate control sample was determined, and arbitrarily set at 1.0. Antibodies used for the studies are listed in Supplementary Table 4.

Invasion and migration assays

Transfected or stably transduced cells (4×10^4 cells) were added to matrigel transwell invasion chambers or control transwell chambers (BD Biosciences, San Jose, CA) and incubated for 24–72 hours with chemoattractant media (Clonetics, Walkersville, MD) supplemented with growth factors. Cells invading through the matrigel or control membranes were fixed using 70% ethanol, stained with 0.1% crystal violet, and photographed in four fields to cover the entire area. Cells were counted from all fields by a scientist blinded to the experimental conditions.

Reporter assays

pTA-Luc and SRE-Luc were purchased from Promega (Madison, WI). 400 ng control pTA-Luc (or the indicated reporter constructs) and 20 ng pRL-TK-Luc were transiently transfected into cells using either Lipofectamine LTX (Life Technologies) or FuGENE HD (Promega). Twenty four hours after transfection cells were washed and placed in serum-free media. Forty eight hours after transfection cells were stimulated with growth factor for an additional 16 hours, as indicated. Reporter activity was determined using the Dual-Luciferase kit (Promega).

Chromatin immunoprecipitation (ChIP) assay

MCF-10A and/or MDA-MB-231 cells were transduced with ERK2, Twist1, or Snail. Cells were stimulated with growth factor, then fixed with 1% formaldehyde and lysed. After sonication, ERK2, Twist1, Zeb1, Slug, or Snail-bound DNA was immunoprecipitated using the indicated antibodies, and then washed and eluted in 1% SDS containing 0.1M NaHCO₃. Reverse-crosslinked DNA was purified using DNA columns and analyzed by PCR analysis. Primer sequences are detailed in Supplementary Table 3.

Flow cytometry

EpCAM expression levels and pERK1/2 activation were measured by flow cytometry using PE-labeled EpCAM or pERK1/2 antibodies. Expression was quantified as mean fluorescence intensity (MFI) using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Antibodies used are listed in Supplementary Table 4.

Gene expression profile dataset analysis

Gene expression profiles of 167 cancer cell lines (GSE34211), 136 primary breast cancers (GSE6128) and 129 primary lung cancers (GDS2373) HMLE cells (GSE24202), and NCI-60 (GSE5846) were downloaded from the Gene Expression Omnibus data repository. Unsupervised clustering of normalized data was used to generate heat plots using TIBCO

Spotfire software (Boston, MA) and GENE-E (Broad Institute). A recently-published gene set of 76 EMT signature genes was used to classify the cancer cell lines and primary cancers into epithelial-like or mesenchymal-like cancers⁵.

Immunofluorescence

Cells were grown on 8-well microscope slides. Following growth factor treatment, cells were washed twice with PBS and fixed at room temperature using 4% formaldehyde. Following fixation, cells were washed four times with PBS, permeabilized for 10 min at room temperature with 0.2% Triton X-100 in PBS (v/v), washed an additional four times, and then blocked with 5% goat serum for 1 h. Following blocking, cells were incubated with primary antibody overnight at 4 °C or 2 h at room temperature in PBS containing 5 mg/ml bovine serum albumin. Cells were washed five times and incubated with appropriate secondary antibody conjugated with Alexa-fluor 488 or 555 (Invitrogen) for 1 h at room temperature. Nuclear staining was done using DAPI (Invitrogen) for 5 minutes. The cells were then dehydrated and mounted and visualized/captured with a fluorescence microscope (EVOS digital inverted microscope at 20× or 40× magnifications). Antibodies used are listed in Supplementary Table 4.

Statistical analysis

Numerical data are presented as the mean values $\pm$ the standard deviation. Statistical significance was evaluated using the Student's *t* test. *p*-values < 0.05 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr Kerry Kornfeld, Dr Jason Weber and Dr Gregory Longmore for reviewing the manuscript and providing valuable advice. This work was supported by a grant from Susan G Komen for the Cure (BCTR0707917) and Siteman Cancer Center fund (P30CA91842).

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A. Cancer cell lines (n=166)

B. Primary Lung tumors (n=129)

C. Primary Breast tumors (n=136)

Figure 1. EpCAM expression is significantly decreased in cancers with a mesenchymal-like EMT gene signature

(A–C) Publicly available microarray datasets were analyzed using an established gene set designed to classify human cancers into epithelial-like or mesenchymal-like clusters. 166 epithelial cancer cell lines (GSE34211), 129 primary lung cancers (GSE4573), and 136 primary breast cancers (GSE18229) were classified into epithelial-like or mesenchymal-like clusters. EpCAM and vimentin expression were analyzed in each cluster. EpCAM expression is significantly decreased in the mesenchymal-like clusters, while vimentin expression is significantly increased. Box plots were generated using GraphPad, and mean expression levels were compared with Students t-test ($P < 0.01$).

Figure 2. EpCAM expression is significantly decreased or lost following cytokine induction of EMT in cell lines
(A) MCF-10A, A549 lung and HaCaT cells were treated with either vehicle or TGF β 1 (5 ng/mL) and TNF α (10 ng/mL) for 72 hours to induce EMT. Phase contrast and fluorescence microscopy were performed to evaluate cell morphology, and expression of EpCAM, E-cadherin, and vimentin. Images are at 20 $\times$ magnification. (B) Immunoblot of cells as described in section A of this figure. Cells were treated with TGF β 1 (5 ng/mL) alone or with

and TNF α (10 ng/mL) for 72-h. Cells were lysed in RIPA buffer and 20 μ g lysates were immunoblotted for E-cadherin, EpCAM, N-cadherin, and vimentin.

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Figure 3. MAPK/ERK activity is associated with decreased EpCAM expression in cancer cell lines and primary human cancers

(A) EpCAM expression and (B) serum response element (SRE) activity (a surrogate for MAPK/ERK signaling) were measured in a panel of cancer cell lines. EpCAM expression was measured by flow cytometry and SRE activity was measured using a luciferase reporter assay. (C) The linear relationship between EpCAM expression and SRE activity, or (D) EpCAM expression and phospho-ERK expression was assessed, demonstrating an inverse relationship in both cases. The Pearson correlation coefficient is significant for both relationships ($P = 0.01$). (E) A publicly available microarray dataset was analyzed using an established gene set designed to assess MAPK/ERK functional activity in human cancers. 166 cancers cell lines (GSE34211) were classified into MAPK/ERK pathway low or

MAPK/ERK pathway high clusters. (F) EpCAM expression is significantly decreased in the MAPK/ERK pathway high cluster ($P < 0.0001$).

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Figure 4. Activated ERK2 suppresses EpCAM expression

(A) MCF-10A breast epithelial cells were transduced with short hairpin constructs targeting ERK1 and ERK2 (shERK2 #1-2) or luciferase (shLUC). Cells were pre-treated with U0126 for 2h and TGFβ1 was added for 48h. ERK1 and ERK2, EpCAM and β-actin expression levels were determined by immunoblotting 15μg cell lysates. (B) MCF-10A breast epithelial cells were stably transduced with ERK2 or LacZ retroviral expression constructs. Cells were treated with or without MEK inhibitor U0126 for 24h and phospho-ERK1/2, total ERK1/2, EpCAM and β-actin expression levels were determined by immunoblot. (C) HaCaT

keratinocytes were treated with TGF β 1 (5 ng/mL) and TNF α (10 ng/mL), or **(D)** MCF-7 breast cancer cells were treated with EGF (50 ng/mL) for the indicated times to induce ERK activation. Select conditions were pretreated with the MEK inhibitor U0126 at the concentrations indicated. Phospho-ERK1/2, total ERK1/2, EpCAM and β -actin expression levels were determined by immunoblot. **(E)** Fifteen mesenchymal-like cancer cell lines (EpCAM-low and MAPK/ERK-high) were treated with vehicle (DMSO) or the MEK inhibitor U0126 for 14 hours. EpCAM expression was measured by flow cytometry. **(F)** A panel of epithelial cancer cell lines was treated with a p38 MAPK inhibitor (SB203580), a JNK inhibitor (SP600125), a MEK inhibitor (U0126), and a Pi3K inhibitor (LY294002) for 16 hours. EpCAM expression was measured by flow cytometry. Increased EpCAM expression was observed only with U0126.

Figure 5. ERK suppresses EpCAM expression both directly and indirectly

(A) A candidate ERK binding site (GAAAC) was identified in the EpCAM promoter region as indicated in the schematic of the EpCAM promoter. Wildtype and mutant EpCAM luciferase reporters were created and transiently transfected into MCF-10A breast epithelial cells expressing ERK2 or control constructs. (B) Direct ERK2-Flag binding to the EpCAM promoter in HEK-293 cells at the candidate ERK binding site is confirmed by ChIP analysis. Eluted DNA was subjected to qRT-PCR with primers specific for the candidate ERK binding site. (C) Three candidate E-box binding sites (with the consensus sequence CANNTG) were

identified in the EpCAM promoter region. Wildtype and mutant EpCAM luciferase reporters were transfected into MCF-10A breast epithelial cells expressing SNAI2 or control constructs. **(D)** SNAI2 binding to the EpCAM promoter at the candidate E-box binding site EB3 is confirmed by ChIP analysis. Eluted DNA was subjected to qRT-PCR with primers specific for the candidate E-Box binding sites. **(E)** SNAI2, TWIST1 and SNAI1 tamoxifen-inducible retroviral constructs were transduced into MCF-10A breast epithelial cells, and expression was induced with tamoxifen at the concentrations indicated. SNAI2, TWIST1, SNAI1, EpCAM and β -actin expression was measured by immunoblot. **(F)** ChIP analysis confirms binding of TWIST1, SNAI1 and ZEB1 to the EpCAM promoter.

Figure 6. EpCAM suppression contributes to the regulation of EMT through activated ERK signaling

(A) EpCAM was specifically ablated (shEP1-3) or rescued (RES) in MCF-10A breast epithelial cells. The cells were then treated with EGF (50 ng/mL) for 72 hours to induce EMT. EpCAM, SNAI2, E-cadherin, vimentin and β -actin expression levels were determined by immunoblot. (B) EpCAM was specifically ablated (shEP1-3) or rescued (RES) in MCF-10A breast epithelial cells. The cells were growth factor starved for 24h, treated with EGF (50 ng/mL) for 15 minutes and immunoblotted for total phospho-ERK1/2. Specific

ablation of EpCAM is associated with increased phospho-ERK1/2 activity. **(C)** EpCAM was specifically ablated in MCF-10A cells, and the cells were growth factor starved for 24h, then treated with EGF (50 ng/mL) from 0-240 minutes as indicated. qRT-PCR was performed to measure the expression of genes known to be associated with EMT (Fos, EGR1, SNAI2, Fra1 and TGF β 1, PTEN). Cell lysates were assayed for phospho-ERK1/2 expression. **(D)** In loss of function and gain of function experiments, EpCAM was specifically ablated in a panel of cell lines (left panel), and EpCAM expression was forced in a panel of cell lines. Stable lines were cultured in serum free media for 24-hours and treated with media containing EGF for 30 minutes. EpCAM, phospho-ERK1/2 and total ERK1/2 levels were determined by immunoblot. Specific ablation of EpCAM is associated with ERK1/2 activation (right panel). ERK activity (SRE-Luciferase) was determined after overnight stimulation of cells using dual-luciferase reagent (Promega). The average of three independent experiments ($P<0.05$) is shown. **(E)** EpCAM was specifically ablated (shEP1-3) or rescued (RES) in MCF-10A breast epithelial cells. The cells were treated with media containing EGF and migration and invasion assays were performed using transwell chambers. Specific ablation of EpCAM increases invasion and migration in MCF-10A cells.