Kruppel-like Factor 4 Inhibits Epithelial-to-Mesenchymal Transition through Regulation of E-cadherin Gene Expression*

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The Krüppel-like factor 4 (KLF4) is a transcriptional regulator of proliferation and differentiation in epithelial cells, both during development and tumorigenesis. Although KLF4 functions as a tumor suppressor in several tissues, including the colon, the role of KLF4 in breast cancer is less clear. Here, we show that KLF4 is necessary for maintenance of the epithelial phenotype in non-transformed MCF-10A mammary epithelial cells. KLF4 silencing led to alterations in epithelial cell morphology and migration, indicative of an epithelial-to-mesenchymal transition. Consistent with these changes, decreased levels of KLF4 also resulted in the loss of E-cadherin protein and mRNA. Promoter/reporter analyses revealed decreased E-cadherin promoter activity with KLF4 silencing, while chromatin immunoprecipitation identified endogenous KLF4 binding to the GC-rich/E-box region of this promoter. Furthermore, forced expression of KLF4 in the highly metastatic MDA-MB-231 breast tumor cell line was sufficient to restore E-cadherin expression and suppress migration and invasion. These findings identify E-cadherin as a novel transcriptional target of KLF4. The clear requirement for KLF4 to maintain E-cadherin expression and prevent epithelial-to-mesenchymal transition in mammary epithelial cells supports a metastasis suppressive role for KLF4 in breast cancer.

Kruppel-like factor 4 (KLF4) is a zinc finger transcription factor that was first identified in a screen for transcription factors involved in growth regulation (1). KLF4 is primarily regarded as a negative regulator of the cell cycle, repressing a multitude of genes that promote proliferation while at the same time up-regulating inhibitors of proliferation (2). KLF4 also plays a crucial role in differentiation during organogenesis of various tissues such as the skin, colon, and eye (3–5). With the advent of induced pluripotent stem cells, KLF4 has gained recognition as one of the “pluripotency genes” that can reprogram somatic cells into a stem cell-like state (6), acting in the capacity to maintain self-renewal (7).

Given its stem cell-promoting activity and its ability to regulate growth and differentiation during development, it is not surprising that KLF4 also plays various roles in tumorigenesis. The frequent loss of KLF4 expression in gastric and colorectal cancers has led to studies revealing a tumor-suppressive role for this factor in these and other tissues (8–13). Conversely, over-expression of KLF4 in the skin leads to squamous cell carcinoma (14). However, the role of KLF4 during the progression of breast cancer is not well defined. Immunohistochemical studies have revealed that KLF4 expression can be increased and undergo altered localization in DCIS of the breast (15), suggesting that it may act as an oncogene in this tissue. This is further supported by the association of nuclear KLF4 with an aggressive breast cancer phenotype (16). In contrast, Akaogi et al. reported that a review of nine independent, publicly available gene expression data sets revealed decreased KLF4 mRNA expression in breast cancers when compared with normal breast. In addition, KLF4 was inversely correlated with increasing tumor grade in 15 independent gene expression array analyses of breast cancer samples (17). Expression of KLF4 protein is also relatively low in breast cancer cell lines compared with non-transformed mammary epithelial cells (18). Thus, there is evidence that KLF4 can act in a tumor-promoting and tumor-suppressive manner depending on the tissue of study. To reconcile these findings, Rowland et al. proposed a cell type-specific functionality for KLF4, wherein its ability to act as a tumor suppressor versus oncogene is largely context-specific and depends upon the relative expression of p21 and p53 (19). Of note, it remains unknown if KLF4 may regulate other properties of tumors, such as metastatic capacity. Furthermore, the specific function of KLF4 in non-transformed mammary epithelium has not been previously addressed.

Recent studies identifying transcriptional targets of KLF4 reveal that it promotes the expression of epithelial-specific cytokeratins in colon cancer cells (20), suggesting that it may sustain an epithelial phenotype. Many of these keratins are suppressed during epithelial-to-mesenchymal transition (EMT) (21), a process defined by a loss of epithelial-specific characteristics and the acquisition of a mesenchymal phenotype.
Although EMT is an essential step during development (22, 23), loss of epithelial characteristics in tumors is associated with increased aggressiveness and poor prognosis, implicating EMT as a mechanism for tumor progression and metastasis (24). A hallmark of EMT is the loss of E-cadherin. In fact, silencing of E-cadherin alone in epithelial cells is sufficient to induce a full EMT (25). This loss is functionally significant, because direct suppression of E-cadherin (CDH1 gene) causes a decrease in cell-cell adhesion and increased invasion and motility (26). E-cadherin is the prototypical cadherin, modulating intercellular adhesions and signaling involving catenins (27, 28), receptor tyrosine kinases (29, 30), and small GTPases (31, 32). While much of the literature ascribes an anti-proliferative function to E-cadherin to maintain proliferation of normal epithelial cells (33–35).

In breast cancer, loss of E-cadherin has been correlated with a more invasive phenotype and metastatic disease progression. Early loss of E-cadherin is observed during tumor progression in lobular disease, whereas ductal breast carcinomas have more variable expression (36). Even when primary tumors maintain E-cadherin expression, loss of this protein in metastatic lesions has been observed (37). The identification of E-cadherin as a tumor and metastasis suppressor has fueled the discovery of multiple molecular mechanisms controlling E-cadherin expression (38, 39). Herein, we report for the first time that multiple molecular mechanisms controlling E-cadherin expression (38, 39). Herein, we report for the first time that KLF4 may play an important role in restraining metastatic disease progression and target cell transduction were performed as previously described (41). The shRNAs sequences targeting human KLF4 were chosen using RNAi Codex. The sequence designated as MirKLF4 targets nucleotides 1834–1852 in the human KLF4 cDNA sequence (GenBank™ accession number NM 004235.3). A scrambled, non-silencing, microRNA based shRNA (MirNS) sequence was used as the negative control.

For adenovirus KLF4 (AdKLF4), the 3×FLAG.HA.KLF4 vector was created through two rounds of subcloning using the previous published pcDNA3-KLF4 construct (43). For the first round of cloning, pcDNA3-KLF4 was digested with BamHI and BstXI. Using a set of primers (oligonucleotides #1 and #2) containing a 5' BamHI linker, Kozak motif, Nhel site, and HA tag, the N terminus of KLF4 was amplified. Both the vector and the amplified PCR fragment were digested with BamHI and BstXI and ligated to create the HA-KLF4 vector. For the second round of subcloning, the 3×FLAG DNA sequence was amplified from the p3×FLAG-CMV vector (Sigma) with a second set of primers (oligonucleotides #3 and #4). Both the HA vector containing KLF4 and the amplified 3×FLAG DNA fragment were digested with BamHI and Nhel and ligated in-frame, resulting in the 3×FLAG.HA.KLF4 construct. The authenticity of the 3×FLAG.HA.KLF4 construct was verified by DNA sequencing and verification of established target gene effects. Adenoviral production and purification was performed by Welgen, Inc. The virus titer was calculated at 10^{12} viral particles per milliliter (vp/ml).

The secreted alkaline phosphatase (sALP) reporter vector was generated by subcloning sALP into the pAdEasy1 vector (Invitrogen). The vector was digested with HindIII and PstI and ligated into pcDNA3-KLF4 digested with the same enzymes. The resulting construct was digested with NotI and XhoI for transfection into 293FT cells. In breast cancer, early loss of E-cadherin is observed during tumor progression in lobular disease, whereas ductal breast carcinomas have more variable expression (36). Even when primary tumors maintain E-cadherin expression, loss of this protein in metastatic lesions has been observed (37).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human mammary epithelial MCF-10A cells (American Type Culture Collection, ATCC) were cultured as either monolayers or in three-dimensional cultures as previously described (40). MDA-MB-231 cells (ATCC) were cultured in RPMI 1640 medium (MP Biomedicals) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in atmosphere containing 5% CO₂.

**Generation of Viral Constructs and Stable Knockdown Cells**—Short hairpin RNAs (shRNAs) were expressed from either the Lentilox 3.7 (ATCC) lentivirus vector or the microRNA-based TMP-tTA retrovirus vector (kind gift from Dr. Ed Stavnezer). For lentiviral knockdown, we used the previously published and validated shRNA sequence 5'-GGA CGG CTG TGG ATG GAA A-3' (19) to generate the stable shKLF4 line or a scrambled, non-silencing short hairpin RNA (shNS) sequence as the negative control. The sequence designated as shKLF4 targets nucleotides 1592–1610 in the human KLF4 cDNA sequence (GenBank™ accession number NM 004235.3). Lentiviral production and target cell transduction were performed as previously described with slight modification (41). 293FT cells were transfected simultaneously with Lipofectamine 2000 and ViraPower Lentiviral packaging kit (Invitrogen) according to the manufacturer’s instructions. Transfected 293FT cells were cultured for 48 h at 37 °C after which time virus-containing media was harvested. Viral supernatant was filtered through a 0.45-μm filter, supplemented with 4 ng/ml Polybrene (Sigma-Aldrich) and used to transduce target cells at 32 °C overnight. Stable MirKLF4 knockdowns were created using the replication-defective retroviral vector TMP-tTA as previously described (42). The shRNA sequences targeting human KLF4 were chosen using RNAi Codex. The sequence designated as MirKLF4 targets nucleotides 1834–1852 in the human KLF4 cDNA sequence (GenBank™ accession number NM 004235.3). A scrambled, non-silencing, microRNA based shRNA (MirNS) sequence was used as the negative control.

For adenovirus KLF4 (AdKLF4), the 3×FLAG.HA.KLF4 vector was created through two rounds of subcloning using the previous published pcDNA3-KLF4 construct (43). For the first round of cloning, pcDNA3-KLF4 was digested with BamHI and BstXI. Using a set of primers (oligonucleotides #1 and #2) containing a 5' BamHI linker, Kozak motif, Nhel site, and HA tag, the N terminus of KLF4 was amplified. Both the vector and the amplified PCR fragment were digested with BamHI and BstXI and ligated to create the HA-KLF4 vector. For the second round of subcloning, the 3×FLAG DNA sequence was amplified from the p3×FLAG-CMV vector (Sigma) with a second set of primers (oligonucleotides #3 and #4). Both the HA vector containing KLF4 and the amplified 3×FLAG DNA fragment were digested with BamHI and Nhel and ligated in-frame, resulting in the 3×FLAG.HA.KLF4 construct. The authenticity of the 3×FLAG.HA.KLF4 construct was verified by DNA sequencing and verification of established target gene effects. Adenoviral production and purification was performed by Welgen, Inc. The virus titer was calculated at 10^{12} viral particles per milliliter (vp/ml). Empty vector control adenovirus (AdGFP) containing GFP was also produced and supplied by Welgen, Inc. Cells were infected at multiplicity of infection values ranging from 200 to 1000. Oligonucleotide #1: 5'-ATG CGG ATC CGC CAC CAT GCC TAG CTA CCC CTA CGA CGT GCC CCA CTA CGC CGC TGT CAG CGA CGC G-3'; oligonucleotide #2: 5'-AAT ACC AGG TCT GTG GCC ACG GT-3'; oligonucleotide #3: 5'-ATG CGG ATC CGC CAC CAT GGA CAA GGA CAT C-3'; and oligonucleotide #4: 5'-GTA CGC TAG CCT TGT CAT CGT C-3'.

**RNA Preparation, cDNA Synthesis, and Quantitative RT-PCR**—Total RNA was isolated from cells using TRIzol Reagent (Invitrogen), and cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocols. For real-time PCR, relative gene expression was determined using the StepOne Plus RT-PCR System (Applied Biosystems) following the manufacturer’s protocol. The following TaqMan assays (Applied Biosystems) were used: KLF4 (Hs00358836_m1) and E-cadherin (Hs00170442_m1). RNA levels were normalized against human TATA box-binding protein.

**Immunoblotting and Immunofluorescence**—Cells were lysed in radioimmune precipitation assay buffer, and protein concentration was determined using the Bio-Rad protein assay. Lysates were run on a 12% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. Antibodies used for
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imunoblotting include: anti-rabbit KLF4 (Millipore), anti-β-actin (Sigma), anti-E-cadherin and anti-p120 (BD Transduction Laboratories), anti-N-cadherin, anti-cytokeratin 8/18, and anti-β-catenin (Cell Signaling). For immunofluorescence, cells were grown on glass coverslips, fixed in methanol at −20°C for 10 min, permeabilized, and blocked in 0.05% Tween 20/1% bovine serum albumin/1× phosphate-buffered saline for 15 min and incubated overnight at 4°C with mouse anti-E-cadherin. Samples were washed three times with phosphate-buffered saline and incubated with a fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch) at room temperature for 1 h. Coverslips were mounted with Vectashield hard set mounting media containing 4',6-diamidino-2-phenylindole (Vector Laboratories) and fluorescence was visualized using a Leica inverted microscope.

**Growth Rate, Proliferation, and Cell Cycle Analysis**—To measure cell growth, cells were trypsinized at the indicated times and counted using a hemocytometer. Bromodeoxyuridine (BrdUrd) incorporation was performed by incubating cells with 10 μM BrdUrd (Sigma-Aldrich) for 2 h at 37°C, prior to fixation and labeling with an anti-BrdUrd antibody (BD Biosciences) according to the manufacturer’s protocol. Secondary detection with either goat-anti mouse Alexa594 (Molecular Probes) or fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch) was performed in the dark at room temperature for 30 min. Mounting media containing 4',6-diamidino-2-phenylindole (Vector Laboratories) was used to counterstain nuclei, and cells were counted using a Leica inverted microscope. Cell cycle analysis using flow cytometry was carried out as previously described (44).

**Transwell Migration and Invasion Assays**—Cell migration was performed using 8.0-μm pore size polycarbonate membrane Transwell inserts in a 24-well plate (Costar). Non-migrated cells were removed with a cotton swab. Inserts were fixed and stained with Hema3 (Fisher Scientific) and migrated nuclei were counted. Invasion assays were performed as above using BD Matrigel™ Invasion Chambers (BD Biosciences) per the manufacturer’s instructions.

**Generation of E-cadherin Reporter Construct and Luciferase Reporter Assays**—Genomic DNA was isolated from MCF-10A cells, and the E-cadherin proximal promoter was PCR-amplified using the following primers: forward, 5'-[XhoI]GTG AAA GCC TGA GCC CCA TC-3' and reverse, 5'-[HindIII]CAC AGG TGC TTT GCA GTT C-3'. Both pGL3basic-luciferase reporter plasmid (Promega, Madison, WI), and PCR products were digested with XhoI and HindIII and ligated together to form the (-359/+30) Ecad-Luc reporter. Cells were transfected in 6-well dishes, using Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol, with 0.2 μg of the Renilla luciferase reporter vector phRG-TK (Promega) as a control and either 1.0 μg of pGL3basic or 1.0 μg of the Ecad-Luc reporter gene. Luciferase assays were performed using the Dual Luciferase Assay System (Promega).

**ChIP**—Chromatin immunoprecipitation (ChIP) assays were performed as previously described, with modification (45). Briefly MCF-10A or MDA-MB-231 cells were grown to near confluency on 150-mm dishes. Cells were cross-linked and lysed, followed by sonication (Vertis Versonic 300, Output 4, 25 s, 6×) in 3 ml of lysis buffer. After centrifugation, samples were precleared with 100 μl of protein A/G-agarose (Ctablchem) and rotated for 2 h at 4°C. Precleared chromatin was then divided into aliquots and immunoprecipitated overnight with specific antibodies to KLF4 (H-180, Santa Cruz Biotechnology), anti-FLAG M2 (Sigma), or control IgG (normal rabbit IgG or normal mouse IgG, Jackson ImmunoResearch Laboratories). Supernatant (1/5) from control IgG sample was processed as the input. After washing and reversal of cross-links, followed by phenol-chloroform extraction and ethanol precipitation, DNA was resuspended in 65 μl of sterile H2O. PCR was performed using 5 μl of immunoprecipitated DNA as template and the following gene-specific primers corresponding to −170/+10 of the human E-cadherin promoter, based on GenBank™ accession number L34545.1: forward (5'-TAG AGG GTC ACC GCC GCG TCT AT-3') and reverse (5'-TCA CAG GTG CTT TGC AGT TC-3').

**Statistical Analyses**—Statistical analyses were performed using a two-tailed Student’s t test with p values of <0.05 considered significant.

**RESULTS**

**KLF4 Is Required for the Maintenance of Mammary Epithelial Cell Morphology**—To directly assess the role of KLF4 in non-transformed mammary epithelial cells, we used lentiviral and retroviral vector-mediated shRNA to create stable MCF-10A cell lines with suppressed KLF4 expression. As previously reported for other cell lines (1), serum deprivation induced KLF4 expression (Fig. 1A). However, KLF4 protein and mRNA were suppressed by >60%, both in the presence or absence of serum (Fig. 1, A and B), using two different KLF4-specific RNA interference approaches (shRNA and microRNA-based shRNA) that targeted different regions of the KLF4 mRNA. MCF-10A cells grown in monolayer cultures form cobblestone clusters of expanding colonies that become more cuboidal and tightly packed as confluency increases (46). Likewise, cells expressing a non-silencing shRNA (shNS and MirNS) maintained a cobblestone-like morphology (Fig. 1C). In contrast, KLF4 silencing (shKLF4 and MirKLF4) resulted in an elongated, fibroblastic morphology reminiscent of EMT, a process that is also associated with increased migration. To assess this feature of EMT, we performed Transwell migration assays. KLF4 silencing resulted in an increase of approximately 2-fold in migration compared with both the parental and control shNS or MirNS cells (Fig. 1D).

**KLF4 Silencing Results in Loss of Acinus Formation and Decreased Proliferation of Non-transformed MCF-10A Mammary Epithelial Cells**—In vivo, breast epithelium exists within a complex ductal structure that receives multiple inputs from both nearby epithelial cells as well as the adjacent stroma. This three-dimensional growth can be simulated in vitro using Matrigel or other extracellular matrix supports. MCF-10A mammary epithelial cells grown in three-dimensional cultures proliferate to form polarized acini, and by day 10, the inner cells have undergone apoptosis/autophagy to form a functional lumen (47). We used this three-dimensional culture method to determine if loss of KLF4 would alter acinus formation and growth. Although parental and shNS control cells formed mul-
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**FIGURE 1.** KLF4 is required for the maintenance of mammary epithelial cell morphology. **A**, immunoblot analysis of KLF4 and β-actin in MCF-10A cells stably transduced with either nonspecific shRNA control (shNS and MirNS) or shRNA against KLF4 (shKLF4 and MirKLF4). Cells were cultured in complete growth medium, ±10% serum. **B**, quantitative RT-PCR analysis of KLF4 mRNA in shNS control and shKLF4 cells. Relative KLF4 mRNA levels were normalized to the human TATA binding protein (TBP). **C**, mesenchymal morphology induced by loss of KLF4 in MCF-10A cells. Cells were plated at low (top row) and high (bottom row) densities. Cells were monitored for morphological changes using phase-contrast microscopy. **D**, increased migration of shKLF4 and MirKLF4 cells compared with shNS and MirNS controls. A total of 1 × 10⁵ cells was suspended in 100 μl of complete medium, seeded on Transwell migration inserts, and allowed to migrate toward complete medium for 20 h. Five fields per insert were counted. *, p < 0.02; **, p < 0.002; and ***, p < 0.0001.

Multiple acini with hollow lumens, shKLF4 cells rarely formed clusters of more than 4–6 cells (Fig. 2A). By day 4, when acinus formation was clearly visible in the parental and shNS control cells, fewer than 5% of the KLF4 knockdown cells had formed colonies beyond 4 cells (Fig. 2B).

KLF4 has been shown to inhibit proliferation and promote differentiation of skin and colonic epithelium (3, 48). Thus it was surprising that loss of KLF4 led to a decrease in acinus formation and growth of MCF-10A cells (Fig. 2, A and B). A similar inhibition of growth was observed in monolayer cultures following KLF4 silencing (Fig. 2C). To determine whether this effect was due to alterations in proliferation, or changes in apoptosis, we used BrdUrd incorporation assays to assess the proliferation rate as well as propidium iodide staining to perform fluorescence-activated cell sorting-based analysis of the cell cycle. In comparison to control shNS cells, BrdUrd incorporation was reduced over 40% in the shKLF4 and MirKLF4 cells (Fig. 2D and data not shown), with no significant change in percentage of cells in the sub-G₁ population (Fig. 2E). Rather, silencing of KLF4 induced a statistically significant G₁-block in the cell cycle. Together, these results identify a previously unspecified requirement for KLF4 in maintaining proliferation and G₁/S phase progression of non-transformed mammary epithelial cells.

KLF4 Is Required for the Expression of E-cadherin in Mammary Epithelial Cells—Cadherin-mediated signaling is an integral component of tissue morphogenesis and homeostasis (49). In the breast, organization of the mammary epithelium is dependent upon maintenance of adherens junctions through homotypic interactions between E-cadherin on adjacent cells (36). In addition, sustained E-cadherin expression is necessary to maintain basal proliferation of mammary epithelial cells (33, 34). The inability of cells to organize into acini following KLF4 silencing, as well as the decreased proliferation and acquisition of morphological and migratory changes that are indicative of an EMT, prompted us to evaluate whether expression of E-cadherin, as well as other adherens junction proteins, was altered in the KLF4-silenced cells. Indeed, both protein and mRNA levels of E-cadherin were markedly reduced in both shKLF4 and MirKLF4 cells compared with shNS and MirNS control cells (Fig. 3, A and B). Suppression of E-cadherin was further confirmed by the complete lack of membrane staining for this protein in the KLF4 knock-down cells (Fig. 3C).

In addition to the loss of E-cadherin, KLF4 silencing resulted in a concomitant appearance of N-cadherin (Fig. 3A). This process, known as “cadherin switching,” is often associated with EMT (50, 51). Furthermore, β-catenin levels were decreased. There was also a switch in the overall isoform pattern of p120 expression, with appearance of the mesenchymal-specific isoform 1. Together, these changes suggest that KLF4 plays an important role in preventing EMT of mammary epithelial cells through maintenance of E-cadherin and the adherens junction complex.

KLF4 Binds the Proximal GC-rich Region of the E-cadherin Promoter to Transcriptionally Activate E-cadherin Expression—The Krüppel-like family of transcription factors regulate a
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FIGURE 2. KLF4 silencing results in loss of acinus formation and decreased proliferation of mammary epithelial cells. A, for three-dimensional cultures, 2.5 × 10⁴ cells were plated using the overlay method (47). Photomicrographs were taken after 10 days in culture. Insets are fluorescent images of the control and shKLF4 cells, which also express GFP. B, quantitation of mammary acini formation. By day 4, the majority of the cells in shNS control cultures had formed visible clusters of at least 8 cells, whereas <10% of shKLF4 cells formed clusters surpassing 4 cells in size. Error bars represent the standard deviations of two experiments done in duplicate. C, growth rate determination of shNS and shKLF4 cells. 1 × 10⁴ cells were plated in complete medium. At 24-h time intervals, cells were trypsinized and counted. D, BrdUrd incorporation of shNS and shKLF4 cells. Cells were plated at 50% confluency and allowed to grow for 72 h prior to incubation with BrdUrd. Slides were processed for immunofluorescence and scored for % BrdUrd positivity. E, cell cycle analysis of shNS (black bars) and shKLF4 (gray bars) cells using propidium iodide uptake and flow cytometry. Cells were plated at 50% confluency and allowed to grow for 48 h before being analyzed. Bars in C–E represent the averages and standard deviations of three independent experiments performed in triplicate. *, p < 0.05; **, p < 0.005; and ***, p < 5.0 × 10⁻⁵.

diverse set of genes through direct binding to GC-rich promoter regulatory regions containing a CACC consensus sequence (52, 53). Furthermore, several Krüppel-like factors have previously been shown to regulate E-cadherin gene expression, including KLF6 and KLF8 (54, 55). KLF4 silencing in MCF-10A cells greatly reduced E-cadherin mRNA levels suggesting it may be necessary for sustaining E-cadherin transcription. To test this, the (~359/+30) proximal E-cadherin promoter, which contains several putative KLF4 binding sites, was linked to a luciferase reporter cassette (Ecad-Luc) (Fig. 4A) and transiently transfected into MCF-10A cells with and without KLF4 silencing. Reporter activity was decreased >10-fold in shKLF4 cells compared with the shNS control cells. Conversely, overexpression of KLF4 in the MCF-10A parental cells resulted in a greater than 2-fold induction of luciferase expression (Fig. 4B). To determine if KLF4 interacts with the endogenous E-cadherin promoter, we performed ChIP analyses. Primers flanking the GC-boxes of the E-cadherin promoter were used to PCR-amplify chromatin fragments enriched by KLF4 binding to this region, relative to rIgG control (Fig. 4C).

Forced Expression of KLF4 in the Highly Metastatic MDA-MB-231 Breast Tumor Cells Restores E-cadherin Expression and Epithelial Morphology, while Inhibiting Migration and Invasion—We next asked whether KLF4 could modulate E-cadherin expression, migration and invasion in mesenchymal-like MDA-MB-231 breast cancer cells that lack expression of endogenous E-cadherin. These cells also express relatively low levels of KLF4 compared with MCF-10A cells (Fig. 5A) (18). Forced expression of KLF4 resulted in the induction of E-cadherin protein expression (Fig. 5B). In addition, KLF4 increased expression of cytokeratin 18 (Krt18), another phenotypic marker of epithelial cells. Furthermore, these cells acquired an epithelial morphology as early as 24 h post-transduction with AdKLF4, compared with AdGFP control (Fig. 5C). Similar to the MCF-10A cells, we found KLF4 regulation of E-cadherin in the MDA-MB-231 cells to be transcriptional, as forced expression of KLF4 induced E-cadherin mRNA (Fig. 6A), as well as increased activity of the Ecad-Luc promoter (Fig. 6B). While induction of this reporter by KLF4 suggested direct transcriptional regulation of the E-cadherin gene, this gene is hypermethylated in MDA-MB-231 cells (data not shown) (56). We therefore performed ChIP assays to determine if KLF4 could bind the endogenous E-cadherin promoter in this context. Following transduction with either control AdGFP or AdKLF4 (FLAG/HA-tagged), chromatin fragments were coimmunoprecipitated with an anti-FLAG antibody and amplified by PCR with the primers described in Fig. 4A. ChIP analysis revealed enrichment of the E-cadherin promoter in KLF4-overexpressing cells (AdKLF4) immunoprecipitated with an anti-FLAG antibody when compared with the control (AdGFP)-transduced cells (Fig. 6C). Restoration of E-cadherin alone is sufficient to reduce invasion of MDA-MB-231 cells in vitro, and forced expression of
either Krt18 or E-cadherin inhibits primary tumor growth and metastasis (57, 58). We postulated that forced expression of KLF4 and, in turn, induction of E-cadherin, as well as Krt18, would also suppress the aggressive, mesenchymal properties of these cells. Indeed, forced KLF4 expression repressed both migration and invasion of MDA-MB-231 cells in vitro (Fig. 6, D and E), indicating that KLF4 activates a transcriptional program in breast tumor cells that elicits a less invasive, more differentiated epithelial phenotype.

**DISCUSSION**

There is strong evidence for KLF4 as a tumor suppressor in several human cancers, including gastric and colorectal (8, 9, 13), yet an obvious disparity exists among studies examining KLF4 expression during the progression of breast cancer (15–17). Moreover, the functional role of KLF4 in non-transformed mammary epithelial cells has not been examined. We have shown that loss of KLF4 in MCF-10A cells results in altered cell morphology, loss of E-cadherin, and increased migration, all of which are canonical features of EMT. However, these changes did not result in transformation, because KLF4-deficient cells were unable to form colonies in soft agar (data not shown). In fact, we found that KLF4-silenced cells were impaired in their ability to proliferate (Fig. 2, C and D).

Previous studies have demonstrated that KLF4 inhibits proliferation of colon cancer cells by blocking G1/S progression of the cell cycle (2). Paradoxically, the results herein indicate that KLF4 is coordinately required for maintenance of proliferation and E-cadherin expression in non-transformed mammary epithelial cells. It is possible that the decrease in proliferation observed with KLF4 silencing is due to the loss of E-cadherin. Similar to KLF4, E-cadherin has both growth inhibitory (59, 60) and growth-promoting roles (34, 35). These functions of E-cadherin are dependent upon its level of expression as well as cellular context. Although KLF4 expression is relatively low in proliferating MCF-10A cells, it appears necessary to maintain a basal proliferative rate. However, as observed in other cell types (1, 61), we also found that growth arrest, in response to serum deprivation, was accompanied by a concomitant increase in KLF4 protein (Fig. 1A). These data suggest that, like E-cadherin, KLF4 may be necessary both for proliferation and growth inhibition within the same cells, dependent upon its absolute level of expression.

The ability of MCF-10A cells to form functional acinar structures in three-dimensional culture has provided significant insights into stroma-epithelial interactions, mechanisms, and pathways in the development of normal mammary tissue architecture and breast tumorigenesis. KLF4 silencing resulted in a 40% reduction in proliferation of monolayer cultures (Fig. 2D); however, <10% of these cells were able to form acini in Matrigel (Fig. 2A). Even after more than 20 days in culture, by which time normal acini become senescent, relatively few KLF4-knockdown cells had progressed to form acini (data not shown). These results suggest that decreased proliferation, in response to reduced KLF4 expression, is not solely responsible for the complete lack of acinus formation and progression.

Several possibilities exist that may explain this outcome. First, KLF4 appears to play an integral role in the maintenance of the stem or pluripotent state (6, 62). MCF-10A cells have been characterized as stem-like, because a subpopulation of these cells express cytokeratin 5/6 (63), a marker of breast epithelial progenitor cells (64). In fact, mammosphere formation is a well-established model for assessing mammary stem/progenitor cells (65). Therefore, KLF4 may be required to maintain the population of stem/progenitor cells that can differentiate into acini. Conversely, KLF4 is also required for the terminal differentiation of many cell types during development, and the inability of KLF4-silenced cells to form acini in three-dimensional cultures may be associated with incomplete terminal differentiation of the mammary luminal phenotype. Lastly, the increased migratory capacity of the KLF4-knockdown cells (Fig. 1D), in conjunction with the subsequent loss of E-cadherin (Fig. 3), may also prevent these cells from generating the intercellular adhesions and signals required for polarized acinus formation. E-cadherin regulates epithelial morphogenesis (49), and...
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**FIGURE 4. KLF4 binds and activates the E-cadherin promoter.** A, schematic representation of the (−359/+30) proximal E-cadherin luciferase construct (Ecad-Luc). Black boxes (KLF4) represent GC-boxes containing putative KLF4 target sites. Numbered boxes depict two E-boxes located near the transcriptional start site. Gray arrows depict the location of the forward and reverse primers used for ChIP PCR amplification. B, E-cadherin promoter activity is lost in shKLF4 cells. The Ecad-Luc reporter plasmid or pGL3basic was cotransfected with a Renilla-expressing control (pHRG-TK) into MCF-10A shNS control and shKLF4 cells. For KLF4 overexpression, parental MCF-10A cells were transfected 24 h prior to transduction with either AdGFP control or AdKLF4. Luciferase values, normalized to Renilla levels, were expressed as fold change over shNS or AdGFP controls. Error bars represent standard deviations of three independent experiments performed in triplicate (*, p < 1.0 × 10⁻⁵; **, p < 1.0 × 10⁻⁸). C, ChIP analysis of KLF4 at the E-cadherin promoter. A KLF4 antibody or rabbit serum was used to immunoprecipitate DNA-protein complexes from MCF-10A cells. Binding of KLF4 at the E-cadherin promoter was enriched over ragG control. Representative amplification of PCR products, using the primers described in A is shown. A molecular weight ladder is shown in the far right lane. Independent ChIP experiments were performed at least two times.

Disruption of acinus formation has been observed in several studies where E-cadherin levels are suppressed (63, 66). Thus, KLF4 silencing may block acinus formation through reduced cell-cell adhesions.

Several studies have recently shown a link between the acquisition of stem cell properties and the process of EMT (67, 68). Hence, the induction of EMT upon KLF4 knockdown suggests that KLF4 may also inhibit formation or self-renewal of mammary stem cells. This possibility contrasts with the established type of breast cancer cells. The results presented herein demonstrate that KLF4 silencing induces EMT in the absence of any additional stimuli, suggesting that KLF4 may be necessary and sufficient to prevent EMT of non-transformed mammary epithelium.

The loss of E-cadherin mRNA observed with KLF4 silencing prompted us to examine the ability of KLF4 to transcriptionally regulate the E-cadherin gene. Using both reporter and ChIP properties. Thus, further studies will be necessary to reveal the context-specific roles of KLF4 in regulating stem cell properties in the mammary gland.

KLF4 is highly expressed in epithelial cells, but only transiently or not at all in mesenchymal cells (69). Moreover, forced expression of KLF4 in colon cancer cells induces expression of several genes associated with the epithelial phenotype (20). We found that silencing of KLF4 induced EMT in MCF-10A mammary epithelial cells as evidenced by morphological alterations, increased migration, and loss of E-cadherin (Figs. 1 and 3). It is noteworthy that transforming growth factor-β, a well known inducer of EMT, represses KLF4 expression (43). Transforming growth factor-β signals through both Smad-dependent and independent pathways to also inhibit E-cadherin expression during EMT (70–72). In addition, KLF4 can bind to Smad3 and prevent activation of Smad-responsive promoters (73). Together, these data suggest that suppression of KLF4 expression may be required for EMT of mammary epithelial cells. Recently it has been shown that another Krüppel-like factor, KLF17, also acts as a negative regulator of EMT in breast cancer cells (74). It will be interesting to determine if these two factors cooperate to control the epithelial phenotype of breast cancer cells. The results presented herein demonstrate that KLF4 silencing induces EMT in the absence of any additional stimuli, suggesting that KLF4 may be necessary and sufficient to prevent EMT of non-transformed mammary epithelium.

FIGURE 5. KLF4 induces expression of E-cadherin protein and a transition to epithelial morphology in the mesenchymal-like MDA-MB-231 breast cancer cells. A, Western blot analysis comparing KLF4 and E-cadherin levels in MCF-10A cells and MDA-MB-231 cells. B, Western blot of E-cadherin, KLF4, Krt18, and β-actin in MDA-MB-231 cells 72 h post transduction with empty vector control (AdGFP) or FLAG/HA-tagged KLF4 (AdKLF4) adenovirus. C, phase-contrast images of MDA-MB-231 cells transduced with AdGFP or AdKLF4 adenovirus. Images were captured 24 h post transduction.
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assays (Fig. 4) we found that endogenous KLF4 binds to and activates the E-cadherin promoter. Although these results do not rule out the possibility that KLF4 indirectly activates the promoter through interactions with other transcription factors, it is likely this interaction is through binding to the KLF4 consensus elements in the proximal (−170/+10) region of the promoter. Several reports have demonstrated that other Sp1 family members, including KLF6 and KLF8, regulate the E-cadherin promoter in ovarian carcinoma cells (SKOV-3) and MCF-10A cells overexpressing KLF4. The (−359/+30) Ecad-Luc reporter vector or pGL3 control was cotransfected with a Renilla-expressing control (pRL-TK) into MDA-MB-231 cells. Cells were transduced with either AdGFP control or AdKLF4 12 h later. Luciferase and Renilla activities were quantified 48 h post transfection. Luciferase values were normalized to Renilla levels and expressed as fold-change over AdGFP control cells. C, ChIP of KLF4 at the E-cadherin promoter in MDA-MB-231 cells. A FLAG antibody was used to immunoprecipitate DNA-protein complexes from both AdGFP and FLAG/HA-tagged AdKLF4 transduced MDA-MB-231 cells. Chromatin fragments were PCR-amplified with the same E-cadherin promoter primers described in Fig. 4A. AdGFP cells served as a negative control. Migration (D) and invasion (E) of AdGFP and AdKLF4/MDA-MB-231 cells. Cells were transduced with AdGFP or AdKLF4 48 h prior to trypsinization and plating onto Transwell supports for migration, or Matrigel-coated supports for invasion. Cells were allowed to migrate or invade through supports for 6 or 24 h, respectively. For A and C, cells were either harvested for RNA or fixed for ChIP analysis at 72 h post-transduction. A is a representative of three independent experiments performed in triplicate. Error bars represent the standard deviations. For B, error bars represent the standard deviations of three independent experiments performed in triplicate. For D and E, five fields were counted per insert. Data represent the average fold change and standard deviations from three independent experiments performed in triplicate. *, p < 0.005; **, p < 5.0 × 10⁻⁷.

FIGURE 6. KLF4 transcriptional activation of E-cadherin results in decreased migration and invasion of MDA-MB-231 cells. A, quantitative RT-PCR of KLF4 and E-cadherin mRNA levels in MDA-MB-231 cells transduced with either AdGFP control or AdKLF4 adenovirus. B, activation of the Ecad-Luc reporter in MDA-MB-231 cells overexpressing KLF4. The (−359/+30) Ecad-Luc reporter vector or pGL3 control was cotransfected with a Renilla-expressing control (pRL-TK) into MDA-MB-231 cells. Cells were transduced with either AdGFP control or AdKLF4 12 h later. Luciferase and Renilla activities were quantified 48 h post transfection. Luciferase values were normalized to Renilla levels and expressed as fold-change over AdGFP control cells. C, ChIP of KLF4 at the E-cadherin promoter in MDA-MB-231 cells. A FLAG antibody was used to immunoprecipitate DNA-protein complexes from both AdGFP and FLAG/HA-tagged AdKLF4 transduced MDA-MB-231 cells. Chromatin fragments were PCR-amplified with the same E-cadherin promoter primers described in Fig. 4A. AdGFP cells served as a negative control. Migration (D) and invasion (E) of AdGFP and AdKLF4/MDA-MB-231 cells. Cells were transduced with AdGFP or AdKLF4 48 h prior to trypsinization and plating onto Transwell supports for migration, or Matrigel-coated supports for invasion. Cells were allowed to migrate or invade through supports for 6 or 24 h, respectively. For A and C, cells were either harvested for RNA or fixed for ChIP analysis at 72 h post-transduction. A is a representative of three independent experiments performed in triplicate. Error bars represent the standard deviations. For B, error bars represent the standard deviations of three independent experiments performed in triplicate. For D and E, five fields were counted per insert. Data represent the average fold change and standard deviations from three independent experiments performed in triplicate. *, p < 0.005; **, p < 5.0 × 10⁻⁷.

interact with chromatin-remodeling proteins such as the histone acetyltransferase, p300 (76), to promote local unwinding of DNA. Hence, it is feasible that the transcriptional activation of E-cadherin by KLF4 is through cooperation with p300/CBP. Of note, Snail, a key inducer of EMT, suppresses KLF4 expression in colon cancer cells (77), further suggesting that loss of KLF4 may be necessary for epithelial cells to undergo mesenchymal changes that promote invasion and migration.

We have focused on KLF4’s regulation of E-cadherin because silencing of E-cadherin alone in epithelial cells is sufficient to induce a full EMT (25). This would suggest that restoration of E-cadherin in the KLF4 knockdown cells could reverse their mesenchymal phenotype. However, these cells were deficient in their ability to target exogenously expressed E-cadherin to the membrane (data not shown). Decreased levels of β-catenin in these cells (Fig. 3A) could contribute to this mislocalization as β-catenin is required for proper membrane targeting of E-cadherin (78). In a reciprocal fashion, membrane-bound E-cadherin anchors β-catenin at the adherens junction and prevents nuclear localization or degradation of the cytoplasm (79). Similarly, sustained p120 expression is necessary to stabilize E-cadherin at the plasma membrane (27), whereas loss of E-cadherin during EMT causes mislocalization of p120 (80), resulting in increased migration. The acquisition of the mesenchymal-specific p120 isoform 1 in the KLF4-silenced cells (Fig. 3A) supports an epithelial-to-mesenchymal transition and likely contributes to the increased migration of these cells (81, 82). Together, these data indicate that KLF4 is necessary to maintain several components of the adherens junction, in part through sustained E-cadherin gene expression.

Forced expression of epithelial proteins, such as E-cadherin and Krt18, block tumor growth and metastasis (57, 83). Krt18 belongs to a large group of cytokeratins that are coordinately induced with forced expression of KLF4 in colon cancer cells (20). In addition to restoring E-cadherin, we found that forced expression of KLF4 also increased Krt18 expression in MDA-MB-231 cells (Fig. 5B). These molecular alterations support the conversion of the MDA-MB-231 cells to a more epithelial morphology, with an inhibited migratory and invasive capacity (Fig. 6, D and E). Thus, we conclude that the absolute levels of KLF4 act as a rheostat in...


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determining the epithelial or mesenchymal phenotype of breast cancer cells.

These findings suggest that reduced expression of KLF4 during breast tumorigenesis provides a pro-migratory and invasive foundation from which metastatic progression can occur. This is consistent with the reduced expression of KLF4 mRNA that occurs with increasing grade of breast tumors (17), which are intrinsically more metastatic. Furthermore, African American women have a higher rate of breast cancer-related morbidity when compared with Caucasian women, due to increased metastases (84). In an effort to identify expression profiles that may be unique to metastatic breast cancer in African American women, Yancy et al. identified KLF4 as being decreased in cell lines derived from these tumors. These data, in conjunction with our findings that KLF4 regulates EMT in mammary cells, highlight the necessity for future studies directed at identifying the signals responsible for suppression of KLF4 during breast cancer progression and metastasis.

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**REFERENCES**

1. Shields, J. M., Christy, R. J., and Yang, V. W. (1996) *J. Biol. Chem.* 271, 20009–20017
2. Chen, X., Johns, D. C., Geiman, D. E., Marban, E., Dang, D. T., Hamlin, G., Sun, R., and Yang, V. W. (2001) *J. Biol. Chem.* 276, 30423–30428
3. Segre, J. A., Bauer, C., and Fuchs, E. (1999) *Nat. Genet.* 22, 356–360
4. Katz, J. P., Perreault, N., Goldstein, B. G., Lee, C. S., Labosky, P. A., Yang, W. V., and Kaestner, K. H. (2002) *Development* 129, 2619–2628
5. Swamynathan, S. K., Katz, J. P., Kaestner, K. H., Ashery-Padan, R., Crawford, M. A., and Piatigorsky, J. (2007) *Mol. Cell. Biol.* 27, 182–194
6. Takahashi, K., and Yamanaka, S. (2006) *Cell* 126, 663–676
7. Jiang, J., Chan, Y. S., Loh, Y. H., Cai, J., Tong, G. Q., Lim, C. A., Robson, P., Zhong, S., and Ng, H. H. (2008) *Nat. Cell Biol.* 10, 353–360
8. Dang, D. T., Bachman, K. E., Mahatan, C. S., Dang, L. H., Giardiello, F. M., and Yang, V. W. (2000) *FEBS Lett.* 476, 203–207
9. Dang, D. T., Chen, X., Feng, J., Torbenson, M., Dang, L. H., and Yang, V. W. (2003) *Oncogene* 22, 3424–3430
10. Ghaleb, A. M., McConnell, B. B., Nandan, M. O., Katz, J. P., Kaestner, K. H., and Yang, V. W. (2007) *Cancer Res.* 67, 7147–7154
11. Ohnishi, S., Ohnami, S., Laub, F., Aoki, K., Suzuki, K., Kanai, Y., Haga, K., Asaka, M., Ramirez, F., and Yoshida, T. (2003) *Biochem. Biophys. Res. Commun.* 308, 251–256
12. Yamasuna, J., Taniguchi, Y., Nosaka, K., Yoshida, M., Satou, Y., Sakai, T., Mitsuha, H., and Matsuoka, M. (2004) *Cancer Res.* 64, 6002–6009
13. Zhao, W., Hisamuddin, I. M., Nandan, M. O., Babin, B. A., Lamb, N. E., and Yang, V. W. (2004) *Oncogene* 23, 395–402
14. Huang, C. C., Liu, Z., Li, X., Bailey, S. K., Nail, C. D., Foster, K. W., Frost, A. R., Ruppert, J. M., and Lobo-Ruppert, S. M. (2005) *Cancer Biol. Ther.* 4, 1401–1408
15. Foster, K. W., Frost, A. R., McKie-Bell, P., Lin, C. Y., Engler, J. A., Grizzle, W. E., and Ruppert, J. M. (2000) *Cancer Res.* 60, 6488–6495
16. Pandya, A. Y., Tailley, L. L., Frost, A. R., Fitzgerald, T. J., Trivedi, V., Chakravarthy, M., Chhieng, D. C., Grizzle, W. E., Engler, J. A., Krontiras, H., Bland, K. I., LoBuegli, A. F., Lobo-Ruppert, S. M., and Ruppert, J. M. (2004) *Clin. Cancer Res.* 10, 2709–2719
17. Aoka, K., Nakajima, Y., Ito, I., Kawasawa, S., Oie, S. H., Murayama, A., Kimura, K., and Yanagisawa, J. (2009) *Oncogene* 28, 2894–2902
18. Miller, K. A., Eklund, E. A., Peddinghaus, M. L., Cao, Z., Fernandes, N., Turk, P. W., Thimmapaya, B., and Weitzman, S. A. (2001) *J. Biol. Chem.* 276, 42863–42868
19. Rowland, B. D., Bernard, R., and Reeper, D. S. (2005) *Nat. Cell Biol.* 7, 1074–1082
20. Chen, X., Whitney, E. M., Gao, S. Y., and Yang, V. W. (2003) *J. Mol. Biol.* 326, 665–677
21. Paccione, R. J., Miyazaki, H., Patel, V., Waseem, A., Gutkind, J. S., Zehner, Z. E., and Yeudall, W. A. (2008) *Mol. Cancer Ther.* 7, 2894–2903
22. Thiery, J. P., and Sleeman, J. P. (2006) *Nat. Rev. Mol. Cell Biol.* 7, 131–142
23. Yang, J., and Weinberg, R. A. (2008) *Dev. Cell* 14, 818–829
24. Thiery, J. P. (2002) *Nat. Rev. Cancer* 2, 442–454
25. Lehembre, F., Yilmaz, M., Wicki, A., Schomber, T., Strittmatter, K., Ziegler, D., Kren, A., Went, P., Derksen, P. W., Berns, A., Jonkers, J., and Chrestofori, G. (2008) *EMBO J.* 27, 2603–2615
26. Thompson, E. W., Torri, J., Sabol, M., Sommers, C. L., Byers, S., Valverius, E. M., Martin, G. R., Lippman, M. E., Stampfer, M. R., and Dickson, B. R. (1994) *Clin. Exp. Metastasis* 12, 181–194
27. Jansen, D., Camaschella, C., Christofori, G., Chhieng, D. C., Grizzle, W. E., Engler, J. A., Krontiras, H., Varshney, M., Aoki, K., Suzuki, K., Kanai, Y., Haga, K., and Yamada, K. T. (2009) *Cancer Res.* 69, 1074–1082
28. Duyan, T. D., Pevsnier, J., and Yang, V. W. (2000) *Int. J. Biochem. Cell Biol.* 32, 155–163
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