Identification of KLF17 as a novel epithelial to mesenchymal transition inducer via direct activation of TWIST1 in endometrioid endometrial cancer

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Introduction

Endometrial cancer is the most common type of gynecological cancer among women in the USA (1), with a rapidly increasing incidence in Japan (2). The majority of endometrial cancers (over 80%) is well differentiated with endometrioid [endometrioid endometrial cancer (EEC)] histology, diagnosed at an early stage, and entails a favorable survival rate (3). However, EEC patients who display an advanced stage disease or suffer a metastatic recurrence have an extremely poor prognosis with a median survival of ~7–10 months (4).

EECs often express estrogen receptor (ER) and progesterone receptor (PR) and exhibit a high rate of PTEN tumor-suppressor gene loss or mutation (4). Epithelial–mesenchymal transition (EMT) is a biological process in which epithelial cells lose basal–apical polarity, become more spindle shaped and acquire the motile and cancer stem cell (CSC) phenotypes with a heightened propensity to metastasize to distant organs (5). TWIST1 has been shown to promote EMT in human cancer, by either directly repressing epithelial marker E-cadherin (6) or upregulating the expression of the EMT inducer BMI-1, which binds the E-cadherin promoter and suppresses its transcription (7,8).

We recently characterized the oncogenic roles of TWIST1 and BMI-1 in inducing EMT in EEC cells (9,10). However, the mechanisms that mediate TWIST1 expression during EEC metastasis remain largely unknown.

The Krüppel-like factor (KLF) transcription factor family proteins (1–17) play important roles in a variety of physiological and oncogenic processes, including tumor growth and metastasis. A number of KLFs can influence transcriptional networks involved in the control of cancer cell invasion (11), apoptosis (11), proliferation (12) and the maintenance of CSC (13). In endometrial cancers, the transcript levels of KLF9 were downregulated, and the levels of KLF13 were upregulated relative to normal endometrium (14). Furthermore, KLF17 has been reported to serve as an inhibitor of EMT and metastasis in breast cancer (15), and reduced expression of KLF17 is associated with poor prognosis in lung cancer and liver cancers (16,17). However, the expressions, molecular roles and the underlying mechanisms of KLF gene family members during the progression of EEC remain to be elucidated.

Here, we report that KLF17 is overexpressed in human EECs relative to normal endometrium and that upregulation of KLF17 is sufficient to induce EMT and invasive phenotypes of EEC cells. Our results demonstrate that KLF17 directly binds the TWIST1 promoter to transactivate TWIST1. Taken together, these results suggest that KLF17 may play an oncogenic role in metastatic EEC, with TWIST1 as a major downstream target.

Materials and methods

Cell lines and culture

The Ishikawa and HHUA EEC cell lines are ER and PR-positive, grade 1 tumors with PTEN mutations (18). Ishikawa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and HHUA cells were purchased from the RIKEN cell bank (Tsukuba, Japan). Cells were cultured in Dulbecco’s modified Eagle’s medium/F12 medium (Sigma–Aldrich, St Louis, MO) supplemented with 15% fetal bovine serum. The immortalized human endometrial epithelial cell line EM (ER/PR-positive, wild-type PTEN) (19,20) was kindly provided by Professor Satoru Kyo (Kanazawa University, Ishikawa, Japan) and maintained in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 15% fetal bovine serum.

Stable overexpression of KLF17 in Ishikawa cells

KLF17 complementary DNA (cDNA) in an expression vector was purchased from OriGene Technologies (Rockville, MD). Ishikawa cells were stably transfected as described previously (21). In brief, upon reaching 80% confluence, cells were transfected with Lipofectamine PLUS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols. Cells were selected in Dulbecco’s modified Eagle’s medium/F12 medium containing 0.5 mg/ml G418 (Sigma–Aldrich, St Louis, MO) at 48 h post-transfection. The selected polyclonal cells were then expanded.

Abbreviations: cDNA, complementary DNA; CSC, cancer stem cell; EEC, endometrioid endometrial cancer; EMT, epithelial–mesenchymal transition; ER, estrogen receptor; IRS, immunostaining score; KLF17, Krüppel-like factor 17; miRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PR, progesterone receptor; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; siRNA, short-hairpin RNA; siRNA, small interfering RNA; WT, wild-type.

The authors contributed equally to this work.

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Stable knockdown of KLF17 in HHUA cells
To produce the stable KLF17 knockdown cell line, we used the KLF17-expressing EEC cell line HHUA. The knockdown experiment was performed using KLF17 short-hairpin RNA (shRNA) lentiviral plasmids (Santa Cruz Biotechnology, Santa Cruz, CA). As a control, HHUA cells infected with a control shRNA were used. Transfected cells were selected using 1 μg/ml puromycin (Sigma–Aldrich, St Louis, MO). Three single colonies (C1, C2 and C5), expressing KLF17 shRNA constructs and two single colonies transduced with control shRNA vectors (shCtrl and 2) were selected 4 weeks following transfection. Both shCtrl and shCtrl2 cells expressed comparable levels of KLF17, and shCtrl1 cells were subsequently selected for use as shCtrl control cells.

Transient transfection
Ishikawa and HHUA cells were transfected with either KLF17 cDNA vector (or control vector) or TWIST1 small interfering RNA (siRNA) (or control siRNA) from Ambion (Austin, TX), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as indicated. After 48 h, cells were used for protein extraction or cell invasion assay.

Quantitative morphometric analysis
Quantitative morphometric analysis was performed as reported previously (22). In brief, the major and minor cell axis was outlined using LSM Image Browser software (Carl Zeiss). The ratio between the major axis and the minor axis of cells (morphological index) was calculated to determine the degree of elongated cell morphology. This ratio is higher in cells with long and thin extensions.

Western blot analysis
Whole cell lysates were obtained using the M-Per Mammalian Protein Extraction Reagent (Pierce Biotechnology, Woburn, MA). Nuclear protein extracts were prepared using a Nuclear Extraction Kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. Proteins (40 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Antigen–antibody complexes were detected using the enhanced chemiluminescence blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The following antibodies were used for analysis: rabbit polyclonal anti-KLF17 (ab84196, Abcam, Cambridge, UK), mouse monoclonal anti-Twist (ab50887, Abcam), mouse monoclonal anti-GAPDH (sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-BMI-1 (ab58295, Abcam), rabbit polyclonal anti-E-cadherin (A01589, GenScript, Edison, NJ), mouse monoclonal anti-PARP-1 (Santa Cruz Biotechnology), rabbit monoclonal anti-paxillin (Santa Cruz Biotechnology) and rabbit polyclonal anti-Vimentin (A01189, GenScript, Edison, NJ). Primary and secondary antibodies were used at 1:1000–2000 and 1:5000 dilutions, respectively. Immuno blot images were digitized and quantified using the NIH Image software.

In vitro cell invasion assay
At 24 h post-transfection, EEC cells (1 × 10⁶) in 500 μl serum-free medium were added to the upper chamber of a transwell plate and 750 μl medium supplemented with 15% fetal bovine serum was added to the lower chamber, as described previously (23). Cells were allowed to migrate through the intermediate membrane for 24 h at 37°C. Membranes were then fixed with 10% formalin and stained in 1% toluidine blue solution. Cells attached to the lower side of the membrane were counted under a microscope in 10 high-power (×200) fields. Assays were performed in triplicate for each experiment with each experiment repeated three times.

Cell viability assay and proliferation assay
Cells (5 × 10⁴) were plated in 96-well plates for 24 h and then treated with dimethyl sulfoxide or varying doses of paclitaxel (Cell Signaling Technology, Beverly, MA), at 10, 20 and 30 nM. Cell viability was determined by the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). At 24 h post-transfection, 10, 20 and 30 μM of paclitaxel were added to wells. After treatment with various concentrations of paclitaxel (3 nM for Ishikawa cells and 1 nM for HHUA cells), the growth curve of cells, covering a total of four days of culturing, was plotted with the Cell Counting Kit-8 method.

Colony formation assay
Approximately 1000 cells were added to each well of a six-well culture plate, and each experiment was performed in triplicate. After 12 days of culture at 37°C, cells were fixed with 10% formalin and stained with 10% Giemsa solution. The number of colonies containing ≥50 cells was counted under a microscope.

Real-time quantitative reverse transcription–polymerase chain reaction
Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using a Takara PrimeScript RT reagent kit (Takara, Shiga, Japan). Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed with the Applied Biosystems 7300 real-time PCR system (Applied Biosystems) using the Takara SYBR Premix Ex Taq II (Takara, Japan). Primers for KLF family genes except those amplifying KLFs were designed as those reported previously (24). Primers for KLFs (1, 8, 9, 13, 14 and 17), TWIST1, ZEB1, BMI-1, Snail, Slug, E-cadherin, CK-18, Vimentin, NANOG, SOX-2, CD133, MDR-1, MRP-1 and GAPDH were obtained from the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/). Gel electrophoretic analysis of RT–PCR products confirmed that the primers amplified a single band with the expected size.

Molecular cloning of the TWIST1 promoter
A 1.9 kb fragment of the TWIST1 gene 5′-regulatory region (−2068 to −159 with respect to the translational start site) overlapping potential KLF17-binding sites was amplified from human genomic DNA (Takara, Shiga, Japan), using forward primer (5′-ATACGTTGATTCTGTTCAAGCACCCAGTTCTC-3′, MluI) and reverse primer (5′-CGAGATCTTCGGGGTCTACAAATCTGTC-3′, BglII). The amplified product was subcloned into MluI/BglIIIII sites of the pGL3-basic plasmid (Promega, Madison, WI) to produce wild-type (WT) TWIST1 pro-Luc vectors. Correct insertion was confirmed by gel electrophoresis and DNA sequencing.

Site-directed mutagenesis
Computer-assisted transcription-factor-binding site analysis (TransFac) of regions upstream of the TWIST1 promoter identified six consensus KLF17-binding sites (A: −1625 to −1621; B: −1370 to −1366; C: −1176 to −1172; D: −526 to −520; E: from nucleotide −509 to −505; F: −439 to −435) at +1 and −227 bp upstream of the start site of transcription. The WT TWIST1 pro-Luc vector was used to generate mutant constructs containing the TWIST1 promoter sequence with point mutations in the potential KLF17-binding sites (CACCCC→CGTTC) using a QuickChange site-directed mutagenesis kit (Strategene, La Jolla, CA). The resulting constructs were named Mutant TWIST1 pro-Luc vectors.

Luciferase activity assay
The luciferase reporters (WT TWIST1 pro-Luc vector, mutant TWIST1 pro-Luc vector, pGL-3 basic control vector, 100 nM) and the pRL-CMV vector (10 ng, Promega, Madison, WI) were co-transfected with and without increasing doses of KLF17 expression vector (25 and 50 ng) and control vector (50 ng) or with increasing doses of KLF17 siRNA (10 and 20 nM) and control siRNA (20 nM) as indicated. At 48 h after transfection, the luciferase activity was measured using a dual-luciferase assay (Promega, Madison, WI).

KLF17 DNA-binding assay
DNA binding of KLF17 to the promoter of TWIST1 in Ishikawa and HHUA cells was measured using the universal EZ-TFA transcription factor assay colorimetric kit (Upstate Biotechnology, Lake Placid, NY). Nuclear extracts (15 μg) were prepared and incubated with 2 μl of capture probe, 5′-biotinylated double-stranded oligonucleotides (5′-GGCGAGATGACACATCACCACCTTGTTGAGAAGCTG-3′) corresponding to the positions −454 to −419 of the TWIST1 promoter harboring the consensus KLF17-binding site F. After 1 h incubation, the capture probe bound to KLF17 was immobilized on the streptavidin-coated plate, and unbound material was washed away. Plates were then incubated with KLF17 antibody (1:1000) and horseradish-peroxidase-conjugated secondary antibody (1:500). Horseradish peroxidase activity was measured colorimetrically at 450 nm using a microplate reader. To confirm the DNA-binding specificity of KLF17, a non-biotinylated double-stranded DNA molecule with the same sequence as the capture probe was used as the competitor probe to ensure specificity. Biotinylated oligonucleotides containing a mutated KLF17-binding site F (5′-GGCGAGATGACACATCACCACCTTGTTGAGAAGCTG-3′) were used as negative control. A background control containing binding buffer and capture probe without cell lysate was used as an additional control. KLF17 DNA binding is expressed as fold change over background.

Clinical sample selection and immunohistochemistry analysis
The use of clinical samples for this study was approved by the Institutional Ethics Committee of The Cancer Center, Sun Yat-Sen University. Paraffin blocks containing formalin-fixed paraffin-embedded tissues were obtained from 90 patients with EEC. Normal endometrium was collected from 18 patients. In addition, 24 pairs of EEC samples and adjacent normal endometrial tissues were obtained for RNA extraction. The clinical and pathological characteristics of EEC patients were described in Supplementary Table 1, available at Carcinogenesis Online.
The streptavidin-biotin peroxidase complex technique was used for staining. Briefly, following antigen retrieval, deparaffinized slides were incubated with anti-KLF17 (1:100, ab84196, Abcam, Cambridge, UK) antibody overnight at 4°C. To visualize the reaction, the sections were incubated with the horseradish-peroxidase-conjugated secondary antibody for 30 min followed by diamobenzidine substrate for 2min. After the nuclei had been stained with hematoxylin, the sections were mounted with neutral gum. Negative control experiments omitting either primary or secondary antibody were also performed.

Double-blind analysis was performed to evaluate the immunostaining score (IRS) of KLF17 expression with patient outcome and tumor characteristics. Slides were scored for staining intensity and proportion of positive tumor cells as previously reported (25). The staining intensity score ranged from 0 (absent), 1 (weak), 2 (moderate) to 3 (strong). The positive tumor cells varied from 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%) to 4 (76–100%). The IRS was calculated as the sum of staining intensity and positive tumor cells (range 0–7). A final IRS ≥ 5 was considered high expression (5–6: ++; 7: ++++ and an IRS ≤ 4 were considered low expression (0–2: –; 3–4: +).

For the ER-alpha and PR assay, samples were incubated with anti-ER (clone H-150, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-PR antibody (SP2, Lab Vision Corporation, Fremont, CA) for 1h at room temperature. Quantification of immunohistochemical ER and PR staining was scored in a minimum of 300 tumor cells showing nuclear reaction. Tumors with positive ER or PR nuclear staining in >10% of tumor cells were defined as ER or PR positive (26).

Statistical analysis

All experiments were performed in triplicate. For in vitro results, values represent mean ± standard deviation and were analyzed by Student’s t-test. The Fisher’s exact test was used to compare the categorical data. Significance was defined as P < 0.05.

Results

KLF17 is overexpressed in EEC cell lines

We first investigated the expression pattern of the KLF gene family in two EEC cell lines along with the immortalized human endometrial epithelial cell EM by qRT–PCR. The two EEC cell lines, Ishikawa and HHUA exhibit representative molecular characteristics of well-differentiated steroid hormone receptor-positive EEC (18), were selected for a comprehensive screening for expression of KLF members. The results confirmed messenger RNA (mRNA) expression of all KLF members in the two EEC cell lines and EM cells. The expression profile of the data represented as a color-coded scale illustrates that a subset of the KLFs (1, 4, 5, 10, 13, 16 and 17) were consistently and significantly upregulated in the EEC cell lines compared with EM cells (Figure 1A). The expression of the remaining KLFs (2, 7, 8, 9, 11, 12, 14 and 15) was consistently lower in EEC cell lines. We focused on the KLF member, KLF17, whose magnitude of increase in mRNA abundance was the largest among all KLF family members in EEC cells (Figure 1B). Semiquantitative western blot analysis indicated that KLF17 protein expression was also significantly increased in the EEC cells, with HHUA cells displaying higher levels of KLF17 protein compared with Ishikawa cells (Figure 1C).

KLF17 induces the invasive and EMT phenotype of EEC cells

To define whether KLF17 has a role in EEC invasion and progression, we generated a gain-of-function model. Ishikawa cells with relatively lower levels of KLF17 were used to overexpress KLF17. Ectopic expression of both KLF17 mRNA and protein levels (Figure 2A and B) resulted in a transition from a round, tightly packed morphology to a more scattered spindle-shaped mesenchymal appearance (Figure 2C, left panel). Quantitative morphometric analysis suggested that the morphological index representing the degree of elongated morphology was significantly increased in KLF17-overexpressing Ishikawa cells (Figure 2C, right panel). Overexpression of KLF17 cDNA strongly promoted cell invasion of these cells (Figure 2D). Consistent with these findings, ectopic expression of KLF17 in Ishikawa cells led to decreased expression of the epithelial marker E-cadherin and increased expression of the mesenchymal marker Vimentin (Figure 2B).

The induction of EMT has been shown to modulate the cancer stem-like cell properties and confer increased drug resistance of cancer cells (27). To determine whether KLF17 plays a role in mediating cellular sensitivity to cytotoxic drugs in EEC cells, we treated Ishikawa cells with increasing concentrations of paclitaxel for 24h and assessed the chemosensitizing properties of control and KLF17-transfected cells using the cell viability assay. We found that loss of cell viability upon exposure to paclitaxel was much more pronounced in Ishikawa cells transduced with empty vector than in cells transfected with KLF17 cDNA (Figure 2E). These data indicate that overexpression of KLF17 in EEC cells may confer resistance to paclitaxel treatment. Given that KLF17 has been shown to inhibit proliferation of lung cancer (16), we used the clone formation assay to examine the effects of KLF17 overexpression on the proliferation of Ishikawa cells. Enforced expression of KLF17 in Ishikawa cells significantly increased the colony formation capacity (Figure 2F and G). Consistent with these results, overexpression of KLF17 resulted in increased cell proliferation and paclitaxel resistance in Ishikawa cells, as measured by the cell counting kit-8 assay (Supplementary Figure S1A, available at Carcinogenesis Online). Taken together, these data indicate that KLF17 upregulation is sufficient to induce EMT and promote EEC invasion and progression.

Fig. 1. KLF17 is upregulated in EEC cell lines than in immortalized endometrial epithelial cell. (A) The color-coded scale depict differentially regulated KLF family genes (1–17) between EEC cell lines (Ishikawa and HHUA) and immortalized endometrial epithelial cell line EM, as determined by qRT–PCRs (n = 3). Red indicates significantly higher mRNA expression, whereas green indicates significantly lower mRNA expression in cancer cells compared with EM cells, respectively. The expression levels of KLF17 mRNA (B) and protein (C) relative to EM cells, as determined by qRT–PCRs (n = 3; ** P < 0.01, normalized to GAPDH) and semiquantitative western blot analysis.
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**Figure 2.** KLF17 induces the invasive and EMT phenotype of EEC cells. Ishikawa cells were transfected with control (Ctr) vector or KLF17 expression vector. (A) The mRNA expression of KLF17 as analyzed by qRT-PCR (n = 3; **P < 0.01, normalized to GAPDH). (B) Total protein extracts from Ishikawa cells described in (A) were immunoblotted as indicated. (C) Ishikawa cells described in (A) were stained with toluidine blue, and quantitative analysis of cell morphology shows the scattered mesenchymal morphology induced by overexpression of KLF17. (D) Overexpression of KLF17 significantly promotes EEC cell invasion (n = 3, **P < 0.01). (E) Ishikawa cells described in (A) were incubated with the indicated concentrations of paclitaxel for 24 h and cell viability assay was performed (n = 3; *P < 0.05; **P < 0.01). (F) Enforced KLF17 expression increases the proliferation of Ishikawa cells. (G) Colony number was counted (n = 3; *P < 0.05).

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**Loss of KLF17 inhibits cell invasion and reverts the EMT phenotype in EEC cells**

To further confirm the association of KLF17 expression with cancer cell invasive and EMT features, we knocked down endogenous KLF17 in HHUA cells with high endogenous levels of KLF17 using lentivirus-driven shRNA constructs (Figure 3A and D). KLF17 knockdown changed cell morphology from a scattered pattern to more round, tightly packed colonies (Figure 3B, left panel). KLF17 knockdown in HHUA cells significantly reduced the morphological index compared with the control shRNA-transfected condition (Figure 3B, right panel). This effect was associated with reduced cell invasion (Figure 3C) and altered expression of reported EMT-related genes (6,8), including upregulation of epithelial marker E-cadherin, and downregulation of EMT inducer BMI-1 and mesenchymal marker Vimentin (Figure 3D). We next analyzed whether loss of KLF17 could modulate chemoresistance and cell proliferation of HHUA cells. Knockdown of KLF17 sensitized HHUA cells to paclitaxel and resulted in suppressed clone formation (Figure 3E and F). Similarly, the cell counting kit-8 assay shows that the transfection with KLF17 shRNA significantly inhibits cell proliferation and decreased resistance to paclitaxel treatment (Supplementary Figure S1B, available at Carcinogenesis Online). Collectively, these data suggest that the downregulation of KLF17 in HHUA cells results in attenuated cell invasion and also disruption of the EMT process.

**KLF17 is a key regulator of the EMT/CSC gene network and other KLF members**

To address the potential relationship of KLF17 with key transcription factors and signaling molecules implicated in EMT induction, we tried to determine whether KLF17 could affect the expression of known EMT/CSC-related genes in Ishikawa and HHUA cells by qRT-PCR. We found that the overexpression of KLF17 in Ishikawa cells dramatically increased endogenous mRNA levels of the EMT inducers TWIST1, BMI-1 and oncogene Id1 (28), moderately and significantly increased expression of ZEB1, Snail and Slug, and genes known to be involved in EMT (Figure 4A and Supplementary Figure S2A, available at Carcinogenesis Online). On the other hand, levels of the epithelial markers E-cadherin and CK-18 were significantly reduced compared with control cells. Moreover, the mRNA levels of CSC markers (NANOG, SOX2 and CD133), and chemoresistance-related genes (MDR-1 and MRP-1) were also highly elevated in KLF17 vector-transfected cells. Interestingly, Ishikawa cells overexpressing KLF17 demonstrated increased levels of KLF5 and KLF13 and decreased KLF9 expression. Consistent with these data, the KLF17-silenced HHUA cells showed remarkable downregulation of known EMT/CSC-promoting genes, Id1 and KLF13, but upregulation of epithelial markers (E-cadherin and CK-18), and KLF9 compared with control cells (Figure 4B and Supplementary Figure S2B, available at Carcinogenesis Online). These results indicate that KLF17 may control a network of genes involved in EMT and CSC and play a role in maintaining tumor growth and promoting tumor progression in EEC.

**TWIST1 is critical for KLF17-induced EMT and cell invasion**

Because TWIST1 is essential for the induction of EMT (5) and upregulated in progressive endometrial cancer (29), and given our finding that KLF17 expression is positively correlated with that of TWIST1, we investigated as whether TWIST1 is a downstream target of KLF17 in promoting EMT in EEC cells. TWIST1 expression was increased in response to upregulation of KLF17 (Figure 4B and D) and decreased after knockdown of KLF17 (Figure 3D). We further silenced TWIST1 expression in Ishikawa cells overexpressing KLF17 by specific siRNA (Figure 4B and D) and found that downregulation of TWIST1 reduced KLF17-mediated cell invasion (Figure 4E). The scattered morphology induced by KLF17 overexpression was partially reverted by TWIST1 silencing, resulting in a more round, cobblestone-like appearance (Figure 4C, top panel). Transfection with TWIST1 siRNA, but not with control siRNA, significantly decreased the morphological index induced by KLF17 overexpression (Figure 4C, bottom panel). The reversal of the mesenchymal phenotype was confirmed by upregulation of epithelial marker E-cadherin and downregulation of EMT promoter BMI-1 and the mesenchymal marker Vimentin using immunoblotting (Figure 4D). These data suggest that TWIST1 is a critical downstream target of the KLF17-induced EMT signaling program that regulates the invasive properties of EEC cells.
KLF17 is a direct transcriptional activator of TWIST1

To test whether TWIST1 is target gene directly activated by KLF17 at the transcriptional level, a luciferase reporter driven by the reported TWIST1 promoter (30) containing six potential KLF17-binding sequences (Figure 5A) was transfected in Ishikawa and HHUA cells together with KLF17 cDNA vector, KLF17 siRNA or controls. Reporter activity was induced upon overexpression of KLF17 (Figure 5B). In contrast, the opposite effects were observed following knockdown of KLF17 (Figure 5C), suggesting that KLF17 may act as transcriptional inducer of TWIST1 via the recognition sites. To determine whether KLF17 binds the putative binding sites, we constructed point mutations of each site in the TWIST1 promoter and performed luciferase assays. Mutagenesis of binding site F (−439/−435) reduced the luciferase activity of the TWIST1 pro-Luc construct, whereas mutation of other binding sites (A–E) did not significantly affect the luciferase activity (Figure 5D).

To further characterize the interaction between KLF17- and the F-binding site, we quantified specific KLF17-binding activity on the consensus oligo using a transcription factor DNA-binding assay with a biotinylated capture probe spanning the −454 to −419 region of the TWIST1 promoter. Nuclear extracts from Ishikawa and HHUA cells exhibited complex formation with the consensus capture probe. Moreover, competition with unlabelled competitor probe significantly ablated the KLF17-TWIST1 binding activity. No binding was detected between KLF17 and the oligonucleotides containing a mutated F site (Figure 5E). To further examine whether the increased expression of TWIST1 was a result of KLF17 transactivation, we measured the expression of KLF17 and TWIST1 in nuclear protein extracts from EEC cell lines and EM cells and we found that the nuclear KLF17 level correlated with the nuclear TWIST1 expression (Figure 5F), supporting a mechanistic and causal link between KLF17 and TWIST1 gene expression in ECs and the oncogenic role of KLF17 during EC metastasis. Together, these experiments support the model by which KLF17 directly binds to the TWIST1 promoter to drive transactivation of the TWIST1 gene in EEC cells, leading to EMT.

Elevated expression of KLF17 and TWIST1 in EECs

To address whether gain of KLF17 expression is an important event in endometrial carcinogenesis, we performed immunohistochemical analysis of KLF17 in 90 EECs and 18 benign endometrial samples. High KLF17 expression was found in 68% of EECs and in 39% of normal tissues (Figure 6A). KLF17 protein was predominantly located in the cytoplasm of the epithelial cells. We then investigated the possible correlation between KLF17 expression and clinical pathological characteristics in EECs. High KLF17 expression was detectable in 14 of 21 (60%) grade I EECs, 13 out of 44 (70%) grade II EECs and 9 out of 11 of (82%) grade III EECs (Supplementary Table 1, available at Carcinogenesis Online), indicating that high KLF17 expression may correlate with increased tumor grade (Figure 6B). Increased KLF17 expression was also associated significantly with negative ER (P = 0.002), and marginally with PR status (P = 0.053) but not with other known prognostic markers (Supplementary Table 1, available at Carcinogenesis Online). Taken together, our data suggest that elevated expression of KLF17 is associated with advanced tumor grade and with the loss of steroid hormone receptors, both of which have been linked to EMT, tumor dedifferentiation and aggressive phenotypes of EEC (31–34).

To further study the correlation of KLF17 levels with EMT-related gene expression in human EEC, we prepared RNA from EECs and adjacent normal tissues and determined the levels of KLF17, TWIST1, BMI-1 and Vimentin and E-cadherin transcripts by qRT–PCR. Consistent with previous reports (9,35), EECs expressed significantly higher levels of TWIST1, BMI-1 and Vimentin mRNA and significantly lower levels of E-cadherin mRNA than benign tissues, paralleling the upregulation of KLF17 transcripts (Figure 6C–G). These observation support the data derived from cell lines and strongly imply a role for KLF17 in upregulation of the expression of TWIST1 and subsequent induction of EMT during EEC cell invasion.

Discussion

In this study, we discovered a novel role for KLF17 in EEC progression via direct regulation of TWIST1. The KLF17 transcription factor...
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KLF17 is a member of the KLF family and has essential roles in human carcinogenesis through inhibition of EMT, tumor growth and invasion in human tumors (15–17). However, results of this study indicate that KLF17 can indeed function as a driver for EMT and that transactivation of TWIST1 plays a mechanistic role in KLF17-induced EMT and cell invasion in EEC cells. This is consistent with our finding of elevated expression of KLF17 in EEC tissues in which tumors are generally high grade and negative for ER and PR expression. Interestingly, previous work suggested that cancer cells isolated from high-grade EECs possess higher tumorigenic potential and stemness properties (36). In high-grade EECs showing more extensive myometrial invasion, loss of PR is strongly associated with increased expression of CD44 (a CSC marker) and decreased E-cadherin expression (34). These observations indicate that the molecular circuitries underlying EMT and cancer stemness may be closely intertwined during EEC progression. In agreement with this, we found that upregulation of KLF17 is involved in both promotion of cell invasion and increased resistance to paclitaxel in vitro, which raises a probability for a regulatory role of KLF17 in the enrichment of CSC of EEC in vivo.

The regulation of EMT involves complex signaling pathways including transforming growth factor-β, Wnt/β-catenin, Notch, and Hedgehog, which in turn activate major downstream transcription factors such as TWIST1, BMI-1, and others, leading to enhanced tumor invasion and metastasis (6,37,38). EMT has been recently linked to the acquisition of CSC properties and the progression of cancer, as evidenced by the ability of TWIST1-induced EMT to shift human epithelial cells to a more mesenchymal nature, with an increased expression of stem cell markers and acquisition of stem cell properties including proliferation and drug resistance (39). Our observation that KLF17 upregulates expression of either EMT-inducing transcription factors (TWIST1, ZEB1, BMI-1, Snail and Slug) or CSC-related genes (NANOG, SOX-2, CD133, MDR-1 and MRP-1) indicates the possibility that KLF17 may function as an upstream mediator or a ‘master switch’ to facilitate the EMT-associated signaling networks.
In agreement with this, all the transcription factors found to be regulated by KLF17 thus far are involved in EMT, deep myometrial invasion and the generation of CSC properties of EEC (35, 36, 40, 41). Given the complexity of the signaling pathways that regulate EMT with formidable cross-talk and feedback (42), the mechanisms controlling KLF17 expression and the interactions between KLF17 and other EMT regulators should be fully elucidated.

Several signaling pathways such as scatter factor/hepatocyte growth factor-dependent pathways have been reported to activate or repress EMT in a cell type and context-dependent manner (43, 44). These context-specific cellular effects of a given EMT regulator or pathway are ultimately controlled by the cooperative interaction between different signal pathways, epigenetic or genetic alterations of downstream genes, and the recruitment of other DNA-binding transcription factors or complexes to target gene promoters (45, 46). Although KLF17 has been considered a key inhibitor of EMT and metastasis in breast cancer (15), our data suggest that KLF17 promotes EMT and EC cell invasion, providing the first evidence that KLF17 could function as a cellular context-dependent transcriptional regulator to induce EMT in EC cells. It would be important to further identify its potential binding partners, effectors and downstream targets in ECs.

Fig. 5. KLF17 is a direct transcriptional activator of TWIST1. (A) Schematic representation of the TWIST1 promoter with potential KLF17-binding sites. The luciferase construct driven from TWIST1 promoter was transfected in Ishikawa (B) or HHUA cells (C), together with KLF17 vector, KLF17 siRNA or their controls, respectively. Then the luciferase activities (left panel) was determined. The upregulation or downregulation of KLF17 was monitored by semiquantitative western blot (right panel) (n = 3; *P < 0.05; **P < 0.01). (D) Ishikawa and HHUA cells were transfected with a reporter gene construct containing either wild-type (WT) TWIST1 promoter, a similar promoter with mutated KLF17-binding sites (A–F) or a pGL3-Luc control vector. A luciferase assay was carried out (n = 3; **P < 0.01). (E) Nuclear extracts from Ishikawa and HHUA cells were tested for KLF17 DNA-binding activity as measured by the transcription factor colorimetric assay (n = 3; **P < 0.01). (F) Nuclear extracts from Ishikawa, HHUA and EM cells were subjected to semiquantitative western blot analysis of KLF17 and TWIST1. Purity of cytoplasmic or nuclear fractions was confirmed by immunoblotting the same blot with antibodies against paxillin (marker for cytoplasmic fraction) or PARP (marker for nuclear fraction), respectively.

(Supplementary Figure S3, available at Carcinogenesis Online).
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In addition, the function of KLFs, such as KLF5, as tumor suppressors or oncogenes may be dependent on p53 status (47). Considering that highly aggressive types of endometrial cancers frequently exhibit p53 mutations (48), it might be possible that in the presence of p53 mutation, distinct from its function in well-differentiated EECs, KLF17 exerts tumor-suppressor activity in poorly differentiated EECs to repress tumor progression. However, the precise role of KLF17 in invasive endometrial cancers harboring p53 mutation remains to be further determined.

Conclusion

This study provides new insights into a potential mechanism of EEC invasion and progression mediated by KLF17 through TWIST1. Our data suggest that KLF17 overexpression is associated with EMT and tumor invasion by regulating TWIST1 and other EMT-related molecules.

Supplementary material

Supplementary Table 1 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

Fig. 6. Elevated expression of KLF17 and TWIST1 in EECs. (A) Immunostaining of KLF17 in normal tissue (NT) and EEC. (B) High KLF17 expression correlated with increased tumor grade. Measurement of KLF17 (C), TWIST1 (D), E-cadherin (E), Vimentin (F) and BMI-1 (G) mRNA expression in 24 pairs of EECs and their adjacent normal tissues was performed using qRT–PCRs, normalized to GAPDH. The data are means of triplicate measurements. Note the induced expression of KLF17 and TWIST1 in EECs (*P < 0.05; **P < 0.01).

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References


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