ZEB1 expression in type I *vs* type II endometrial cancers: a marker of aggressive disease

Meenakshi Singh[1], Nicole S Spoelstra[1], Annie Jean[1], Erin Howe[1], Kathleen C Torkko[1], Hilda R Clark[1], Douglas S Darling[2], Kenneth R Shroyer[1], Kathryn B Horwitz[1,3], Russell R Broaddus[4] and Jennifer K Richer[1]

[1]*Department of Pathology, University of Colorado Health Sciences Center, Aurora, CO, USA;* [2]*Department of Periodontics, Endodontics and Dental Hygiene, Center for Genetics and Molecular Medicine, University of Louisville, Louisville, KY, USA;* [3]*Department of Medicine, Division of Endocrinology, University of Colorado Health Sciences Center, Aurora, CO, USA and* [4]*Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA*

Zinc-finger E-box-binding homeobox 1 (ZEB1) is a transcription factor containing two clusters of Kruppel-type zinc-fingers, by which it binds E-box-like sequences on target DNAs. A role for ZEB1 in tumor progression, specifically, epithelial to mesenchymal transitions, has recently been revealed. ZEB1 acts as a master repressor of E-cadherin and other epithelial markers. We previously demonstrated that ZEB1 is confined to the stromal compartment in normal endometrium and low-grade endometrial cancers. Here, we quantify ZEB1 protein expression in endometrial samples from 88 patients and confirm that it is expressed at significantly higher levels in the tumor-associated stroma of low-grade endometrioid adenocarcinomas (type I endometrial cancers) compared to hyperplastic or normal endometrium. In addition, as we previously reported, ZEB1 is aberrantly expressed in the epithelial-derived tumor cells of highly aggressive endometrial cancers, such as FIGO grade 3 endometrioid adenocarcinomas, uterine serous carcinomas, and malignant mixed Müllerian tumors (classified as type II endometrial cancers). We now demonstrate, in both human endometrial cancer specimens and cell lines, that when ZEB1 is inappropriately expressed in epithelial-derived tumor cells, E-cadherin expression is repressed, and that this inverse relationship correlates with increased migratory and invasive potential. Forced expression of ZEB1 in the nonmigratory, low-grade, relatively differentiated Ishikawa cell line renders them migratory. Conversely, reduction of ZEB1 in a highly migratory and aggressive type II cell line, Hec50co, results in reduced migratory capacity. Thus, ZEB1 may be a biomarker of aggressive endometrial cancers at high risk of recurrence. It may help identify women who would most benefit from chemotherapy. Furthermore, if expression of ZEB1 in type II endometrial cancers could be reversed, it might be exploited as therapy for these highly aggressive tumors.
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The transcription factor termed zinc-finger E-box-binding homeobox 1 (ZEB1), also known as (TCF8, AREB6, ZFHEP, ZFHX1A, BZP, NIL-2-A, δEF1) contains two clusters of Kruppel-type zinc-fingers, one at the N terminus and the other at the C terminus, by which it binds E-box-like sequences (CACCTG) on target DNAs. Between zinc-finger clusters ZEB1 contains a homeodomain thought to be involved in protein–protein interactions.[1] ZEB1 can act as a repressor or activator of transcription depending on its expression levels, conformation, and the gene promoter upon which it is acting.[2,3]

ZEB1 has been implicated in multiple processes during development. For instance in developing mesodermal and neural tissues, ZEB1 levels change dynamically during differentiation.[4,5] It is involved in lymphopoiesis,[6] skeletal patterning,[7] chondrogenesis,[8,9] neurogenesis, and neural crest

cell development.[10] More recently, a role for ZEB1 in tumor progression, particularly in epithelial to mesenchymal transition (EMT), has been reported, as reviewed by Peinado *et al*.[11] ZEB1 acts as a master repressor of 'epithelialness' by repressing E-cadherin[12–14] and other epithelial markers.[15,16]

We previously demonstrated that ZEB1 is upregulated by both estradiol and progesterone in the endometrial stroma and myometrium of the mouse and human uterus. In humans its expression is elevated during the secretory stage of the menstrual cycle.[17] In the endometrium ZEB1 expression is confined to the stromal compartment. In both normal endometrium and low-grade endometrial cancers, no epithelial expression is observed.[17] Here, we quantify ZEB1 protein expression in endometrial biopsies from 88 patients and demonstrate that it is significantly upregulated in tumor-associated stroma of endometrioid adenocarcinomas as compared to hyperplastic or normal endometrium.

Endometrial cancers are divided into type I and type II subtypes, which differ in aggressiveness and prognosis, as well as in molecular characteristics that determine response to therapy.[18,19] In contrast to normal endometrium and low-grade endometrioid adenocarcinomas (classified as type I endometrial cancers), we previously documented that ZEB1 can be aberrantly expressed in the epithelial-derived carcinoma cells of aggressive FIGO grade 3 endometrioid adenocarcinomas and other highly aggressive, type II endometrial cancers such as uterine serous carcinomas (USCs) and malignant mixed Müllerian tumors (MMMTs).[17] Although the ability of ZEB1 to repress E-cadherin by binding to E-box sequences on its promoter is documented at the molecular level,[12–14,20] it has not been well demonstrated *in vivo*, and has not been examined in endometrial cancer. In this study we demonstrate, both in human surgical resection specimens and human endometrial cancer cell lines, that when ZEB1 is expressed in epithelial-derived tumor cells, E-cadherin expression is completely lost, and that this inverse relationship (high ZEB1, no E-cadherin) correlates with increased invasive potential.

Materials and methods

Cell Lines and Reagents

Cell lines AN3CA, HEC-1B, and KLE were obtained from the ATCC. Ishikawa and Hec50co were kindly provided by Dr Kim K Leslie (The University of New Mexico Health Sciences Center). Human ZEB1 cDNA cloned into pCS2MT, kindly provided by Dr Douglas C Dean (Washington University) was used to generate a standard curve for real-time RT-PCR experiments. For stable introduction of ZEB1 into Ishikawa cells, human ZEB1-pCIneo expression vector, kindly provided by Michel M Sanders and Brian Sandri (University of Minnesota) was used. A

rabbit polyclonal anti-ZEB1 antibody developed by Dr Doug Darling was used for immunohistochemistry and western blots. The antibody is directed against the homeodomain region (amino acids 557-663). This region is 84% identical between human and mouse ZEB1/δEF1.[4] This antibody works well on paraffin-embedded sections for immunohistochemistry and immunoblots[4,17,21] and specifically detects ZEB1 and not ZEB2.[22] Other antibodies utilized are described under immunoblot analysis.

Tissue Microarrays

The tissues used in this study were obtained under institutional review board approval from formalin-fixed, paraffin-embedded blocks archived in The University of Colorado Health Sciences Center, Department of Pathology. Blocks were stored at room temperature, shielded from light. Hematoxylin and eosin (H&E)-stained sections from all cases were reviewed. For each case, optimal areas for coring were marked on the H&E slides, in triplicate for carcinoma, and one area each was marked for hyperplasia and normal endometrium, where available. Cores (1 mm) were obtained from the original paraffin blocks with the MTA1 manual tissue arrayer (Beecher Instruments, Inc. Sun Prairie, WI, USA). The cores were then embedded in paraffin tissue microarray blocks at predetermined positions. This resulted in the construction of three tissue microarray blocks. Sections (4 μm) were cut from the blocks, and a tape adhesive transfer system was used to transfer the sections onto adhesive coated slides (Instrumedics Inc., Hackensack, NJ, USA). Tissue microarray sections were initially stained with H&E, and reviewed.

Patient Characteristics

All cases of endometrial carcinoma, including biopsies, curettings, and hysterectomies, diagnosed at the University of Colorado Health Sciences Center between March 1997 and June 2003 were identified in the Pathology department database and their reports were reviewed. Of 88 cases 40 had endometrial carcinoma only, 20 had matched carcinoma and normal, 11 had matched normal, hyperplasia and carcinoma, and 2 had matched normal and hyperplasia. Further details can be found in our previous publication using these tissue microarrays.[23]

Clinicopathologic Characteristics of Cases on the Tissue Microarray

Data on tumor stage, tumor grade, patient age, and patient menopausal status at the time of diagnosis were collected. Tumor stage and grade were determined using the AJCC/FIGO criteria. Of the 88

patients studied, 71 (81%) were aged 50 or older, with 63 (72%) being postmenopausal, 10 (11%) perimenopausal, and 15 (17%) premenopausal. The average age of the patients was 60 years, with a range of 33–88 years. Stage I cases represented 67% of the total, with 7% being stage II, 10% stage III, and 3% stage IV. Staging information was not available for 13% of the cases. The majority of the cases (55%) were grade I. Of the remainder, 26% were grade II, 18% grade III, and the grade was not available for one case (1%). A majority of the carcinomas, 79%, had endometrioid histology, 3% were mucinous adenocarcinoma, 3% were poorly differentiated carcinoma, 1% were pure serous carcinoma, and 13% were carcinomas with a component of serous or clear cell carcinomas.

Scoring and Statistical Analysis

Staining scores (range 0–300) were calculated by multiplying the intensity score (0–3) by the percent of cells staining (0–100). Separate scores were given for carcinoma, hyperplasia, and normal endometrium for each case, when present. Blinded scoring was performed simultaneously by two pathologists (MS and HRC) on a multihead microscope. Discordant scores were resolved by consensus. Data distribution for score by each histological type (ie, normal, hyperplasia, and cancer) were tested for normality. The nonparametric Kruskal–Wallis test was used to test the difference in medians among the three histologic groups. In addition a Wilcoxon signed-rank test was also performed to take into account the patient-matched cases. Significance was determined by *P*-values less than 0.001 and all statistical analyses were performed using SAS software version 9.1 (SAS Institute, Cary, NC, USA).

Immunohistochemistry of Human Surgical Resection Specimens and Tissue Microarrays

Human surgical samples
Sections from archival paraffin-embedded blocks of normal uterine biopsies and uterine cancers were obtained via IRB-approved protocols from the University of Colorado Health Sciences Center and The University of Texas MD Anderson Cancer Center.

ZEB1 and E-cadherin individual stains
Staining was performed as previously described,[17] using the Autostainer Universal Staining System (Dakocytomation, Carpinteria, CA, USA). Briefly, sections were cut at $4\,\mu m$ and heat immobilized at $60°C$ for 60 min. After deparaffinization, antigens were retrieved using a 10 mM citrate buffer with a Biocare Medical Decloaker (Concord, CA, USA) followed by 3% hydrogen peroxide for 5 min. Sections were incubated with either rabbit anti-ZEB1 1:6000, for 1 h at room temperature or mouse anti-E-Cadherin clone NCH-38 1:100, (Dakocytomation).

Rabbit (Vector Laboratories, Burlingame, CA, USA) or Mouse IgG (BD Biosciences, Franklin Lakes, NJ, USA) were used as an isotype-negative control. The Vectastain Universal Elite kit (Vector Laboratories) was used for detection, followed by a 4 min incubation with 3,3′-diaminobenzidine (Dakocytomation), and sections were counterstained with dilute hematoxylin, dehydrated, and coverslipped for brightfield microscopy. TBS-T (0.05%) was used for all washes.

Tissue microarrays
Staining was similar to that described above with the following exception: once stained and dehydrated, slides were mounted with Curemount media (Instrumedics Inc.) and cured under a UV lamp for 1 min.

Dual Immunofluorescent Staining of ZEB1 and E-Cadherin

Human surgical resections
Immunohistochemistry was performed by hand and slides were kept in the dark during staining. Deparaffinization, antigen retrieval, and wash buffer were similar to that described above, and dual staining procedure was similar to that previously described.[17] Briefly, sections were blocked with 10% normal goat serum (Vector Laboratories) and ZEB1 primary antibody (1:3000) was applied overnight at room temperature. ZEB1 antibody was detected with biotinylated goat anti-rabbit IgG (Dakocytomation), followed by Rhodamine Red X-conjugated streptavidin (Jackson Laboratories, West Grove, PA, USA). Sections were blocked again with 10% normal goat serum until incubation overnight with E-Cadherin (Dakocytomation) antibody (1:50). E-Cadherin antibody was detected with Alexa Fluor 488 conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA), and slides were mounted with Biomeda M01Gel Mount (Foster City, CA, USA) and stored in the dark at $4°C$.

Immunoblot Analysis

Protein extracts were equalized to $150\,\mu g$ by the Bradford assay (Bio-Rad), resolved by SDS–PAGE and transferred to nitrocellulose. Equivalent protein loading was confirmed by Ponceau S staining. Antibodies used were rabbit polyclonal anti-ZEB1 antibody developed by Dr Doug Darling at 1:1500 dilution, followed by goat anti-rabbit-HRP (MP Biomedicals, Salon, OH, USA) at 1:5000; E-Cadherin (Dakocytomation) $1\,\mu g/ml$; Vimentin clone V9 (Vision Biosystems, Norwell, MA, USA) 1:50; and α-tubulin clone B-5-1-2 (Sigma, Saint Louis, MO, USA) 1:12 000; all followed by detection with rabbit anti-mouse-HRP (Sigma) at 1:10 000. All blocking and antibody incubations were performed with 5% milk/TBST.

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA was isolated using Oligotex mRNA Maxi Kit (Qiagen, Valencia, CA, USA). An ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) was used by the UCHSC Cancer Center Quantitative PCR Core Facility for continuous measurement of the fluorescence spectra in 96-wells of a thermal cycler during PCR amplification. Forward and reverse primers (Invitrogen) and probes were designed following the recommendations of the TaqMan PCR chemistry design and optimized using the Primer Express software (Applied Biosystems). A TaqMan probe specific for *ZEB1* 5′ labeled with 6-carboxyfluorescein (FAM) and 3′ labeled with 6-carboxy-tetramethylrhodamine was purchased from Applied Biosystems. Primer and probe sequences used were *hZEB1*: Fwd-TCCATGCTTAAGAGCGCTAGCT; *hZEB1*: Rev-ACCGTAGTTGAGTAGGTGTATGCCA; *hZEB1*: Probe-6-carboxyfluorescein-CCAATAAGCAAACGATTCTGATTCCCCAG-6-carboxy tetramethyl rhodamine; *E-cadherin*: Fwd-AGTGTCCCCCGGTATCTTCC; *E-cadherin*: Rev-CAGCCGCTTTCAGATTTTCAT primer; and the *E-cadherin*: Probe-6-carboxy fluorescein-TGCCAATCCCGATGAAATTGGAAATTT-6-carboxy tetramethyl rhodamine. Amplification reactions and thermal cycling conditions were performed as per the manufacturer's recommendations. A standard curve was generated using the fluorescence data from the 10-fold serial dilutions of known quantities of control plasmid for human *ZEB1*. *E-cadherin* levels were normalized to amounts of 18 S ribosomal RNA.

Cell Culture, Boyden Chamber Migration/Invasion, and Wound-Healing Assays

Most endometrial cancer cells were routinely cultured in MEM and 5% FBS, except for the Hec50co. Hec50co cells were routinely cultured in DMEM (Sigma) with 10% FBS and 200 mM Glutamine (Gibco). For migration and invasion assays, BD BioCoat Control Inserts Chambers 24-well plate with 8 μm pore size (BD Biosciences) and BD BioCoat Matrigel Invasion Chambers were used respectively. Cells were washed with serum-free MEM (with 10^{-9} M insulin (Sigma) and MEM NEAA (Invitrogen)) and serum starved for approximately 12 h. After starvation, cells were harvested, counted and plated at 5×10^4 cells per ml in the upper chamber (0.5 ml volume of MEM media with 0.5% FBS, with NEAA and insulin). In the lower chamber 0.8 ml per well of 50% conditioned media from Hec50co cells plus 50% DMEM (Sigma) with 10% FBS, AA, and L-glutamine, plus 10% fresh FBS was used as an attractant. Cells were incubated for 48 h at 37°C. Migrating and invading cells on the lower surface of the membranes were stained with Diff-Quik stain (Fisher Scientific) and counted manually using ImagePro Plus (Mediacybernetics Inc., Bethesda, MD, USA). Data were graphed and statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). For comparisons among different cell lines, percent invasion was calculated as the mean number of cells invading through Matrigel insert wells divided by mean number of cells migrating through control membranes not coated with Matrigel, otherwise raw numbers of cells migrating (as counted in four quadrants per membrane) or raw numbers of cells invading were reported. Wound-healing assays were performed in six-well dishes with cells at 100% confluency. Mitomycin C (10 μg/ml) was added to the media for 2 h, drug was removed, and cells were washed twice with wash media and the monolayer of cells was wounded with the small end of a 1000 μl pipette tip. After a final wash to remove scraped cells, fresh media was added and plates were photographed at time 0 and imaged again after 46 h of incubation.

Manipulation of ZEB1 in Endometrial Cell Lines

To study effects of knocking down *ZEB1* in an aggressive type II endometrial cancer cell line, the Hec50co cell line was used. Like the AN3CA cell line, Hec50co express high levels of ZEB1 protein; however, they grow faster and transfect better than AN3CA. Three shRNAs from a RNA Intro GIPZ shRNAmir starter kit (Open Biosystems, Huntsville, AL, USA) designed to knock down *ZEB1* were tested, but only one (G10 shRNAmir) worked in initial assays assessing *ZEB1* transcripts in AN3CA cells by real-time RT-PCR. To create a pooled population of stable cells with a reduced level of ZEB1, the ZEB1-specific shRNAmir (G10) and a nonsilencing control (shRNAmir) (Open Biosystems) were each transfected into Hec50co cells using the Arrest-In transfection reagent (Open Biosystems) according to the manufacturer's instructions. After 48 h incubation, cells were selected with 10 μg/ml of puromycin (Sigma) for 3 days then reduced to 3 μg/ml of puromycin for maintenance.

To study the consequences of forced expression of ZEB1 in a low-grade, nonaggressive endometrial cancer cell line that it is not normally expressed in, we utilized the Ishikawa cell line (derived from a type I endometrial cancer). Ishikawa cells were plated 1×10^6 cells per 15 cm plate, given 24 h to adhere, and then transfected with human ZEB1-pCIneo expression vector and pEGFP-C2, or empty pCIneo vector and pEGFP-C2 using Lipofectamine 2000 transfection agent (Invitrogen), following the manufacturer's protocol. After 48 h, cells were selected for antibiotic resistance using G-418 (1000 μg/ml), then passaged at a maintenance dose of 200 μg/ml. To further select for ZEB1 expression, both the empty vector and ZEB1-pCIneo stably transfected populations were sorted by flow cytometry for GFP expression.
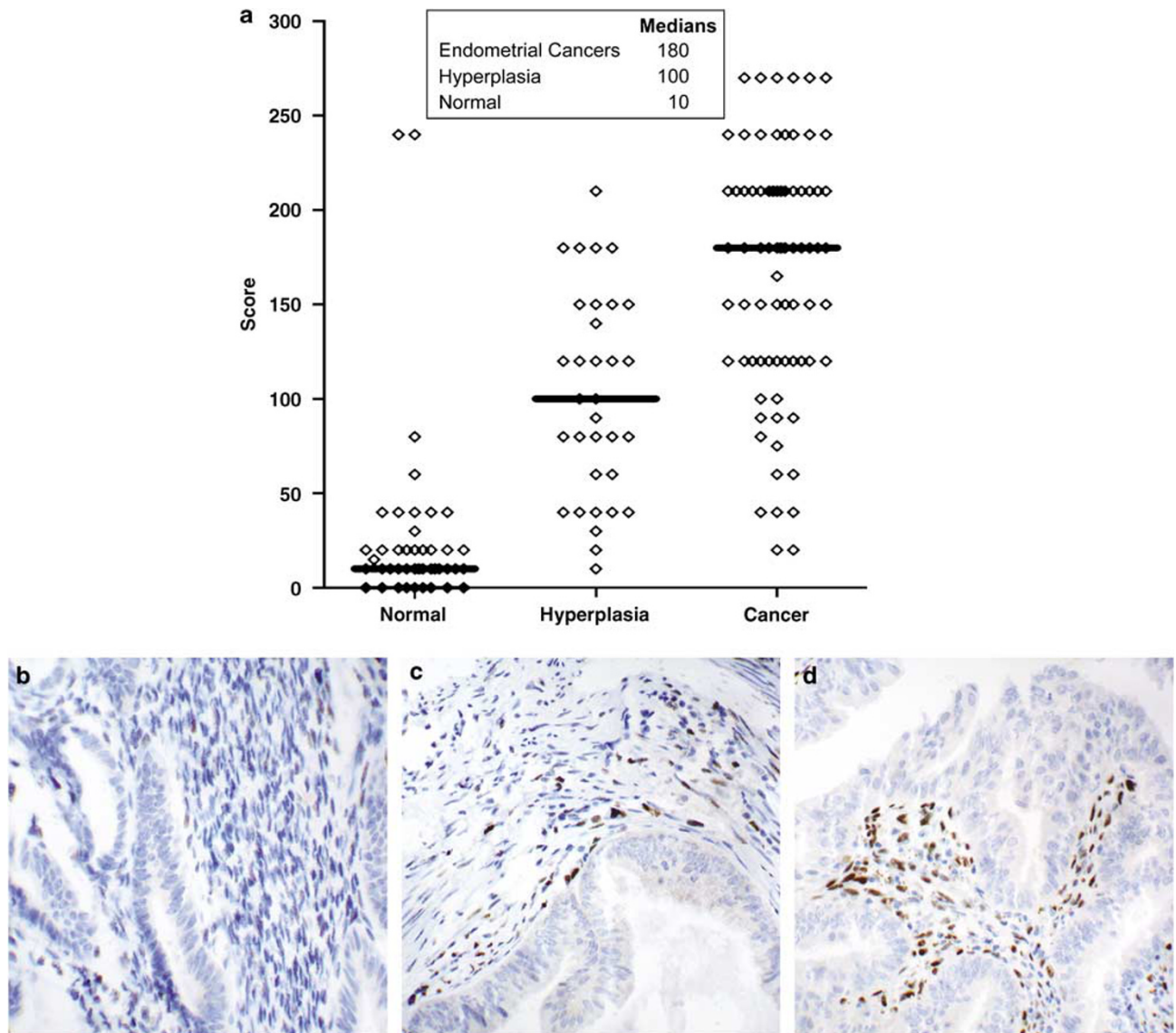
Figure 1 ZEB1 levels in 88 cases of endometrial cancer, hyperplasia, and normal endometrium. Tissue microarrays were prepared as described in 'Materials and methods'. (**a**) Scatter plot of immunostaining scores for ZEB1 (range 0–300) calculated by multiplying the intensity score (0–3) by the percent of cells staining (0–100) for normal endometrium, hyperplasia, and cancer. Bars represent the median for each category. Using a nonparametric Kruskal–Wallis test and Wilcoxon signed-rank test, median ZEB1 expression scores are significantly different at $P < 0.001$ in cancer *vs* hyperplasia, cancer *vs* normal, and hyperplasia *vs* normal. ZEB1 protein expression (brown) in (**b**) normal endometrium, (**c**) hyperplasia, and (**d**) grade 1 endometrial cancer as detected by immunohistochemistry on representative samples from the tissue microarrays (magnification ×200).

Results

Zeb1 Protein is Overexpressed in Tumor-Associated Stroma

ZEB1 showed a nuclear pattern of expression in endometrial stromal cells in all three categories examined, ie, normal endometrium, hyperplasia, and endometrioid adenocarcinomas. Expression was limited to the stroma with the exception of three FIGO grade3 endometrioid adenocarcinomas (discussed below). By both the Kruskal–Wallis and Wilcoxon signed-rank test, median expression of ZEB1 was significantly higher in cancer-associated stroma (median score 180), compared to hyperplasia

(median score 100), or normal endometrium (median score 10) ($P < 0.001$) (Figure 1a). There was also a significant difference in the expression of ZEB1 between hyperplasia and normal endometrium ($P < 0.001$). We also performed the statistics independently on the percent cells staining or intensity, and either variable alone was statistically significant. In other words, both percent cell staining and intensity have an effect on the statistically significant differences observed between cancer and hyperplasia and normal endometrium. Figures 1(b–d) show examples of ZEB1 staining in normal endometrial stroma (1b), hyperplasia (1c), and tumor-associated stroma (1d). There were
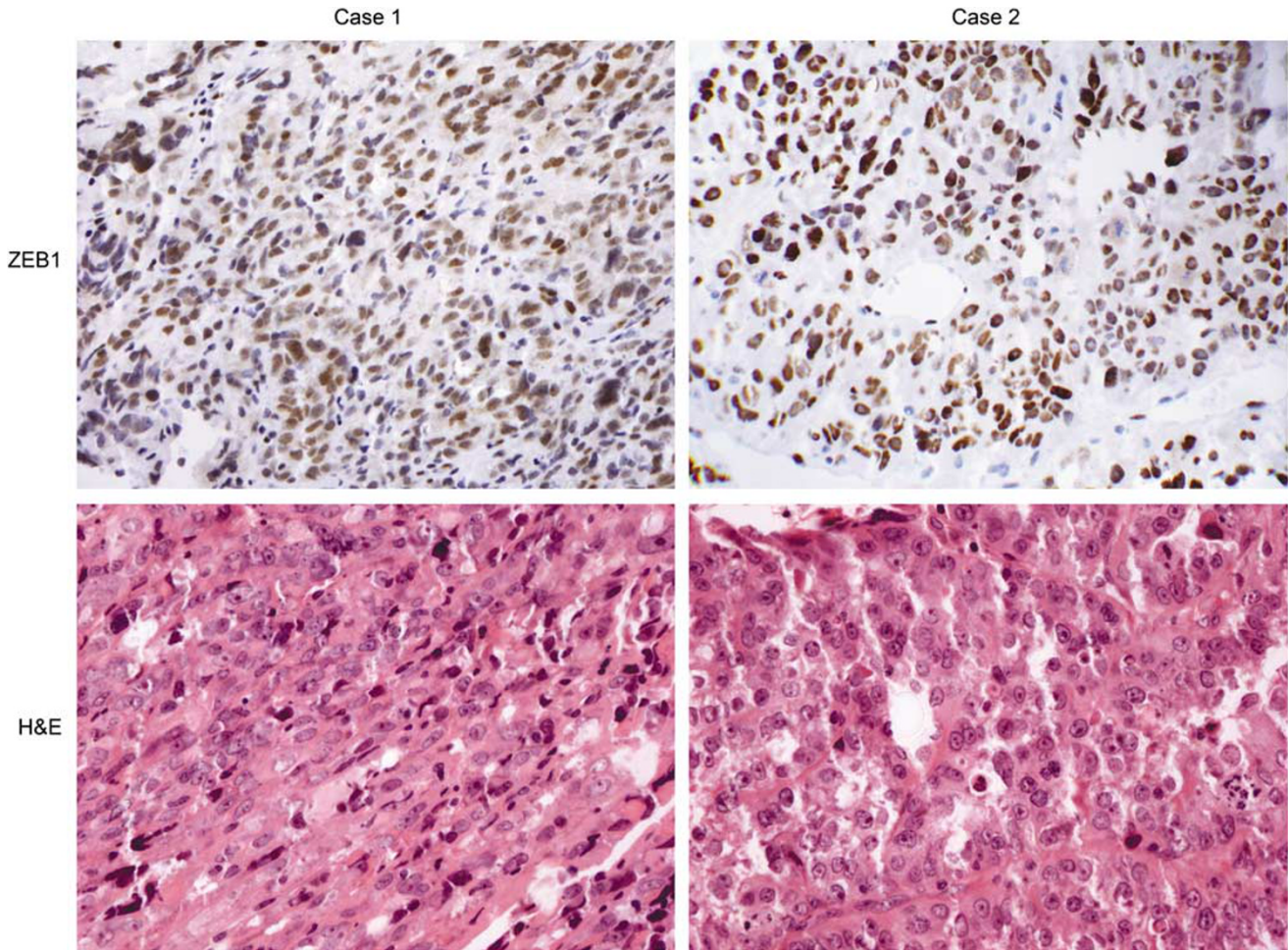
Figure 2 ZEB1 is expressed in tumor cells of grade 3 endometrioid adenocarcinomas. ZEB1 expression in tumor cells of two representative cases of grade 3 endometrioid adenocarcinoma from the tissue microarrays. H&E stains of the same cases are shown in the bottom panels (magnification × 400).

two outlier cases in which matched normal endometrial stroma showed higher expression (3 + in 80% of cells) than that in other normal cases. One of them was the normal matched with a grade I endometrioid carcinoma and the other was a grade II endometrioid carcinoma with focal clear cell features.

There were three cases of FIGO grade 3, poorly differentiated carcinomas, in which ZEB1 was abundantly expressed not only in stromal cells, but also in tumor cells (two are shown in Figure 2). We previously observed ZEB1 expression in tumor cells in grade 3 endometrioid adenocarcinomas, USCs, and both the carcinomatous and sarcomatous components of MMMTs.[17]

In Endometrial Cancer Cell Lines, ZEB1 is Inversely Correlated with E-Cadherin, but Positively Correlated with Invasive Potential

Real-time RT-PCR performed for *ZEB1* and E-cadherin on four endometrial cancer cell lines (AN3CA, HEC-1B, Ishikawa, and KLE) demonstrated that AN3CA cells have the highest level of ZEB1, followed by the HEC-1B, whereas Ishikawa and KLE cells (known to be relatively differentiated) have no measurable ZEB1 (Figure 3a). Cell lines that have *ZEB1* (AN3CA, HEC-1B) do not express *E-cadherin*, whereas the two cell lines that lack *ZEB1*, retain E-cadherin expression (Figure 3b). Immunoblot analyses (Figure 3c) demonstrate that AN3CA cells, which express ZEB1, also express the mesenchymal marker vimentin, but have lost E-cadherin expression. In contrast, Ishikawa cells, which do not express ZEB1, retain E-cadherin protein, and do not express vimentin. It should also be noted that E-cadherin protein in the Ishikawa cells (detected by immunocytochemistry) is appropriately localized to the cell membrane (not shown).

Boyden chamber assays using Matrigel-coated membranes were performed on the AN3CA, HEC-1B, and Ishikawa cells. Results (Figure 3d) demonstrate that the AN3CA cells (with high ZEB1 expression) exhibit the highest average number of invading cells as compared to the HEC-1B or Ishikawa. When

percent invasion is calculated (mean number of cells invading through Matrigel divided by the mean number of cells migrating through control membrane (no Matrigel)), AN3CA cells demonstrated the highest percent invasion (74%), followed by HEC-1B cells (moderate levels of ZEB1) at 18% invasion, whereas the Ishikawa cells (which lack ZEB1) showed little invasive activity (8%).

Loss of E-Cadherin Concomitant with ZEB1 Expression in Tumor Cells is Confirmed in Clinical Specimens

Dual immunohistochemistry of ZEB1 in normal endometrium shows that ZEB1 is normally exclusively expressed in endometrial stroma, whereas E-cadherin is expressed in the epithelium (Figure 4a). However, in type II endometrial cancers in which ZEB1 is expressed in tumor cells, individual staining of serial sections or dual immunofluorescence on the same sections shows that tumor cells

expressing ZEB1 have lost expression of E-cadherin (Figures 4b and c).

Manipulation of ZEB1 Causes Alterations in E-Cadherin Expression and Migration

We introduced the human ZEB1-pCIneo expression vector into Ishikawa cells (which represent a well-differentiated type I endometrial cancer cell line that is estrogen receptor positive and lacks ZEB1). A pooled population of stably transfected cells was selected. ZEB1 is expressed in these Ishikawa-ZEB1 cells at levels below those present in AN3CA and Hec50co cells. E-cadherin levels are reduced in the Ishikawa-ZEB1 as compared to the stable empty pCI-neo vector or wild-type Ishikawa control cells (Figure 5a). Furthermore, introduction of ZEB1 renders the cells more migratory in a wound-healing assay than controls lacking ZEB1 (Figure 5b).
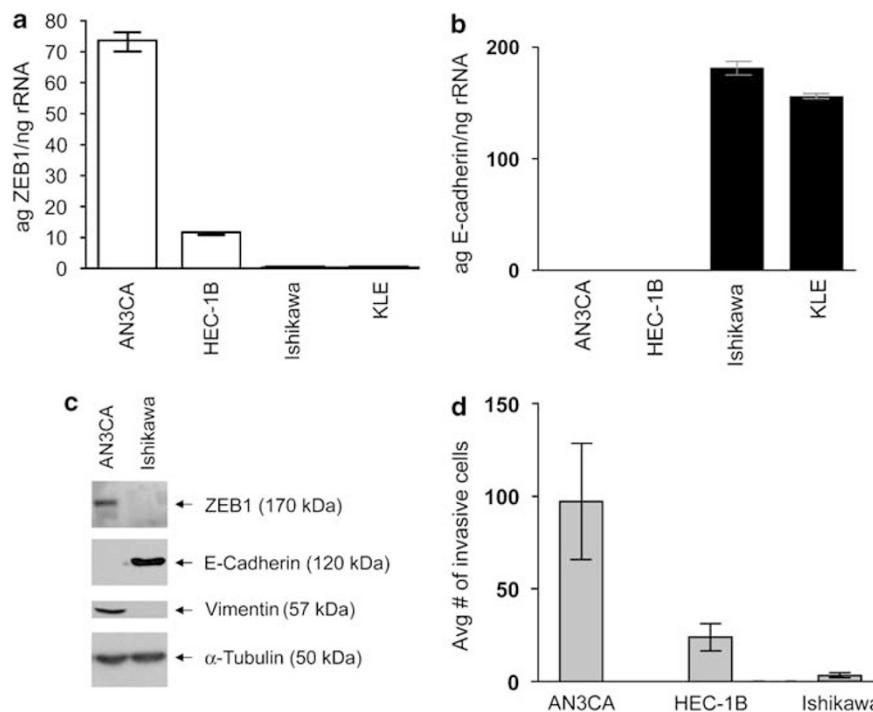

Figure 3 In endometrial cancer cell lines ZEB1 is inversely correlated with E-cadherin, but positively associated with vimentin expression and invasiveness. Real-time quantitative RT-PCR of *ZEB1* (**a**) and *E-cadherin* (**b**) transcripts. (**c**) Immunoblot of 150 μg of protein extracts from Ishikawa and AN3CA cells probed with antibodies recognizing ZEB1, E-cadherin, vimentin, and α-tubulin. (**d**) Invasion assays performed in Boyden chambers demonstrate the average number of AN3CA, HEC1B, and Ishikawa cells invading through Matrigel-plugged pores in triplicate wells in an experiment representative of three separate invasion assays demonstrating the same pattern. Error bars represent standard deviation.

Figure 4 In surgical resection specimens of normal human endometrium, ZEB1 expression is confined to the stromal compartment and E-cadherin to epithelial cells; however, in type II endometrial cancers, ZEB1 can be expressed in tumor cells, and E-cadherin expression is lost. Dual immunofluorescent staining of ZEB1 and E-cadherin was performed on human surgical specimens. (**a**) Dual staining of normal endometrium: ZEB1 (red) is confined to the stroma, whereas E-cadherin (green) is exclusively epithelial (magnification ×400). (**b**) A malignant mixed Müllerian tumor (MMMT) immunostained for ZEB1 (red) and E-cadherin (green) demonstrates that when ZEB1 is inappropriately expressed in epithelial cells (white arrows), E-cadherin is lost (black arrows). (**c**) Additional type II endometrial cancers: an MMMT and a uterine serous carcinoma (×400 magnification) immunostained for ZEB1 and E-cadherin. When ZEB1 is inappropriately expressed in epithelial cells (white arrows), E-cadherin is lost (black arrows).

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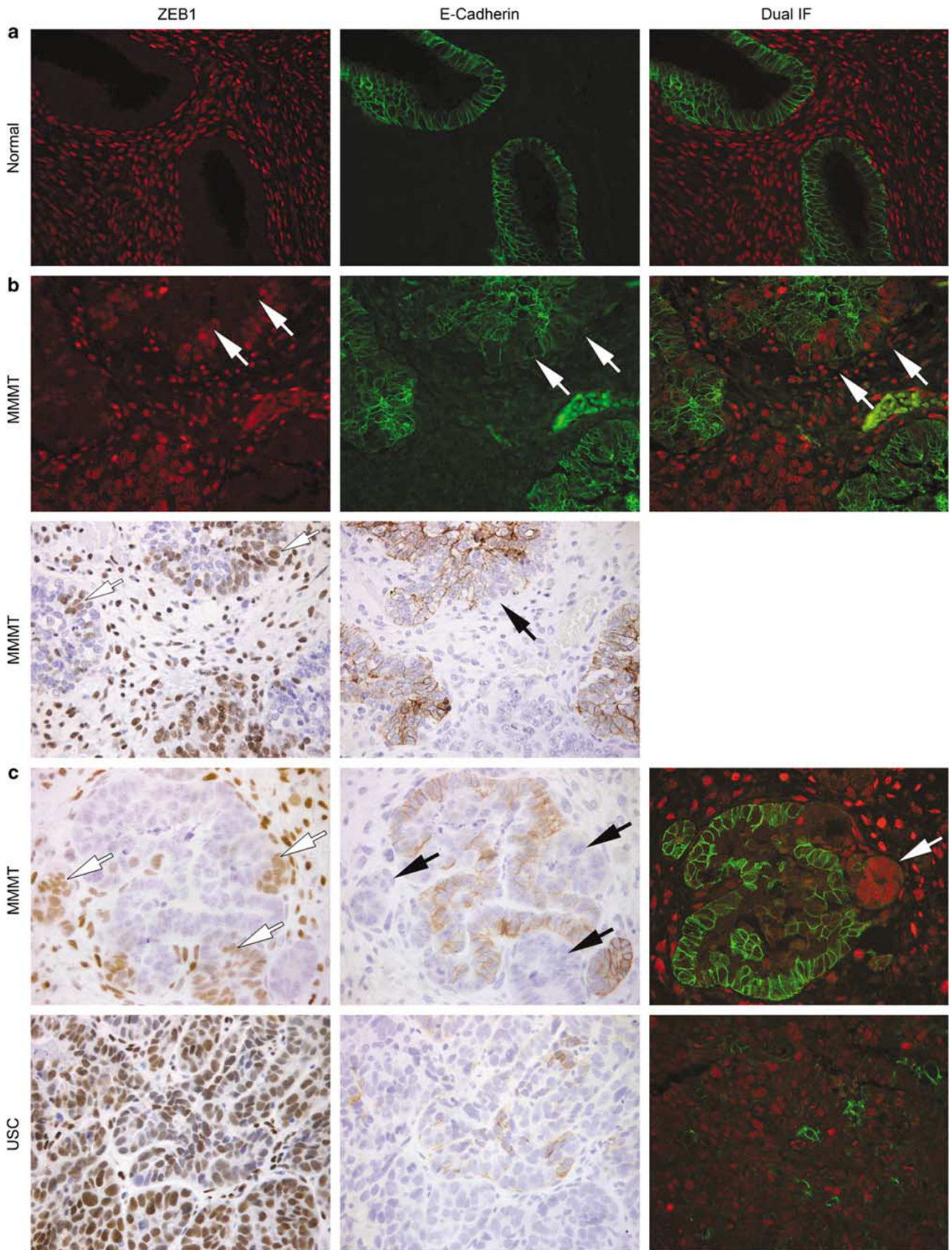
Conversely, to determine the effect of reducing ZEB1 in a cell line in which it is highly expressed, we used Hec50co cells. This cell line was derived from a metastatic lesion of a patient with advanced disease. The cells do not form glandular structures in culture or in animal models and they have lost estrogen receptor expression. The cells can form a serous phenotype in mice and are a good model of type II endometrial cancers.[24] We tested three shRNAs designed to knock down ZEB1. In preliminary studies using real-time RT-PCR, one of the three, shRNAmir G10, reduced *ZEB1* RNA levels by approximately 47% (Figure 6a). The anti-*ZEB1* shRNA G10 and the nonsilencing control were transfected into Hec50co cells and respective pooled populations of stably transfected cells were generated. G10 shRNAmir reduced the level of ZEB1 protein by approximately 50%, although E-cadherin expression was not restored (Figure 6b). Reduction in ZEB1 did significantly affect migration of the Hec50co-ZEB1shRNA G10 cells by 61% compared to Hec50co cells stably expressing nonsilencing shRNA control or wild-type Hec50co cells (Figure 6c). Significance was assessed by one-way ANOVA with Tukey's multiple comparison test ($P = 0.152$).

Discussion

We previously demonstrated that ZEB1 protein is not present in normal endometrial epithelium, and that even in low-grade endometrioid adenocarcinomas it remains confined to the stroma.[17] We now quantitatively demonstrate that ZEB1 is expressed at significantly higher levels in tumor-associated stroma as compared to normal endometrial stroma and also that it is significantly higher in stroma associated with endometrial hyperplasia as compared to normal stroma. However, the question of what effect higher levels of stromal ZEB1 associated with type I endometrial cancers (low-grade endometrioid adenocarcinomas) might be having on nearby tumor cells remains elusive.

The consequences of inappropriate ZEB1 expression in carcinoma cells themselves are clear. We find that in endometrial cancer cell lines, ZEB1 expression is inversely correlated with E-cadherin levels, but positively associated with a mesenchymal marker, vimentin, and increased invasiveness (Figure 3). Further, in human surgical resection specimens of type II endometrial cancers, ZEB1 is associated with loss of E-cadherin (Figure 4).
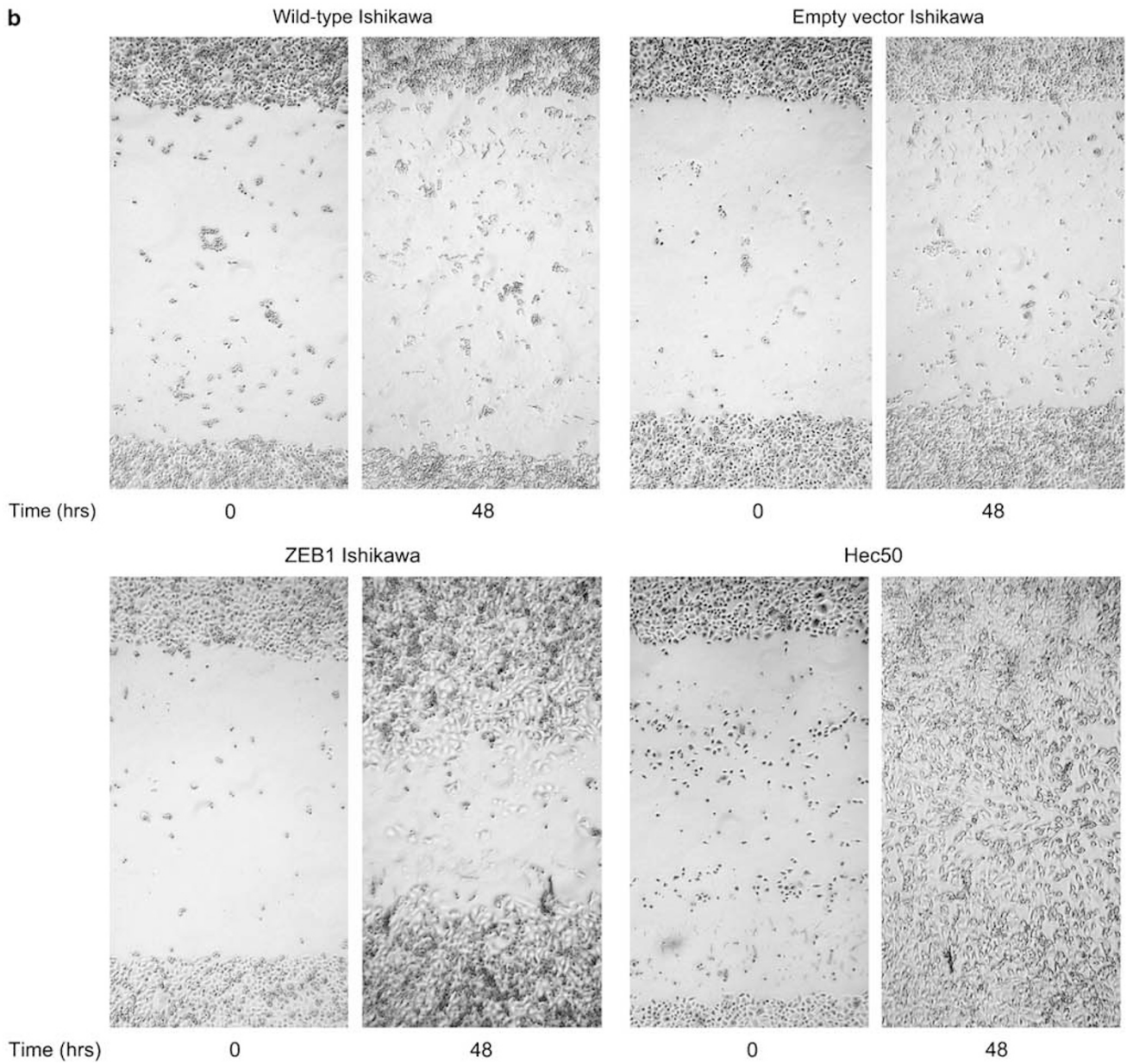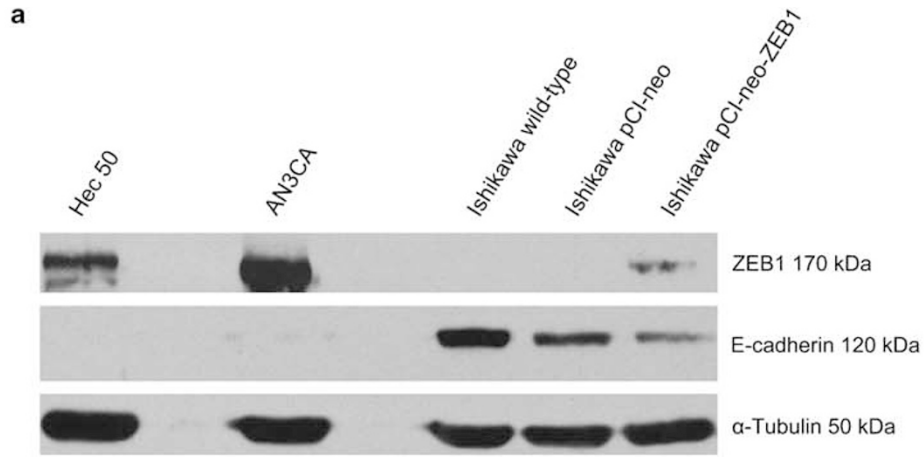
Loss of E-cadherin is a hallmark of EMT, a well-established process during embryonic development, allowing for the migration of cells and groups of cells in developing tissues. In adults, EMT is recognized as a putative molecular mechanism underlying carcinoma invasion and metastasis.[25–27] During the process of EMT, epithelial cells actively downregulate cell–cell adhesion systems, lose their polarity, and acquire a mesenchymal phenotype with reduced intercellular interactions and increased migratory capacity.[27] A number of different transcription factors, including Twist, Snail, Slug, SIP1 (ZEB2), and ZEB1 induce EMT (reviewed in Peinado et al.[11]). In addition to E-cadherin, ZEB1 has recently been shown to repress other regulators of epithelial cell polarity such as plakophilin 3, Crumbs3, HUGL2, and Pals1-associated tight junction protein.[15,16]

Interestingly, our studies demonstrate that forced ZEB1 expression in a type I nonaggressive, ZEB1 negative cell line such as Ishikawa causes a reduction in E-cadherin and increased migration. However, even a substantial reduction of ZEB1 in an aggressive type II endometrial cancer cell line such as the Hec50co, does not restore E-cadherin. This is in line with the inverse correlation that we observe in the endometrial cell lines we studied, as well as the human tumor samples, both of which show that the presence of any ZEB1 results in complete suppression of E-cadherin. We also observe that reduction in ZEB1 affects migration, as the Hec50co-ZEB1shRNA G_{10} cells with partial ZEB1 downregulation were significantly less migratory than controls. We hypothesize that besides being a master suppressor of epithelialness, ZEB1 may activate genes involved in migration and invasion, and we are currently investigating this theory.

The mechanism(s) whereby ZEB1 expression is suppressed in normal endometrial epithelial cells, but inappropriately expressed in epithelial-derived tumor cells of type II endometrial cancers, remains to be determined. Recently, it has been found that ZEB1 is induced by transforming growth factor-βa in mouse mammary NMuMG epithelial cells,[28] and that hypoxia may influence ZEB1 expression and consequent E-cadherin reduction in von Hippel–Lindau tumor suppressor-null renal cell carcinomas.[29] Interestingly, a recent study has demonstrated that the microRNA hsa-miR-200c suppresses TCF8 (ZEB1) and increases expression of E-cadherin.[30] We are thus investigating whether loss of this particular microRNA is associated with type II endometrial cancer.

ZEB1 may have potential for use as a molecular marker of risk of recurrence of endometrial cancers

Figure 5 Forced expression of ZEB1 in Ishikawa cells leads to a reduction in E-cadherin protein and increased migration. (**a**) Whole cell protein extracts were prepared from Hec50co cells, AN3CA cells, wild-type Ishikawa cells, Ishikawa cells stably transfected with human ZEB1-pCIneo and pEGFP-C2, and Ishikawa cells stably transfected with empty pCIneo vector and pEGFP-C2. An immunoblot of 150 μg of protein from each cell line was probed with antibodies recognizing ZEB1, E-cadherin, and α-tubulin. (**b**) Wound-healing assay using wild-type Ishikawa, Ishikawa-pCI-neo, and Ishikawa-pCI-neo-ZEB1 and the highly migratory Hec50co cells for comparison. Photographs were taken at ×40 magnification at time 0 and 48 h after wounding.

a

b

Wild-type Ishikawa

Empty vector Ishikawa

Time (hrs)   0   48   0   48

ZEB1 Ishikawa

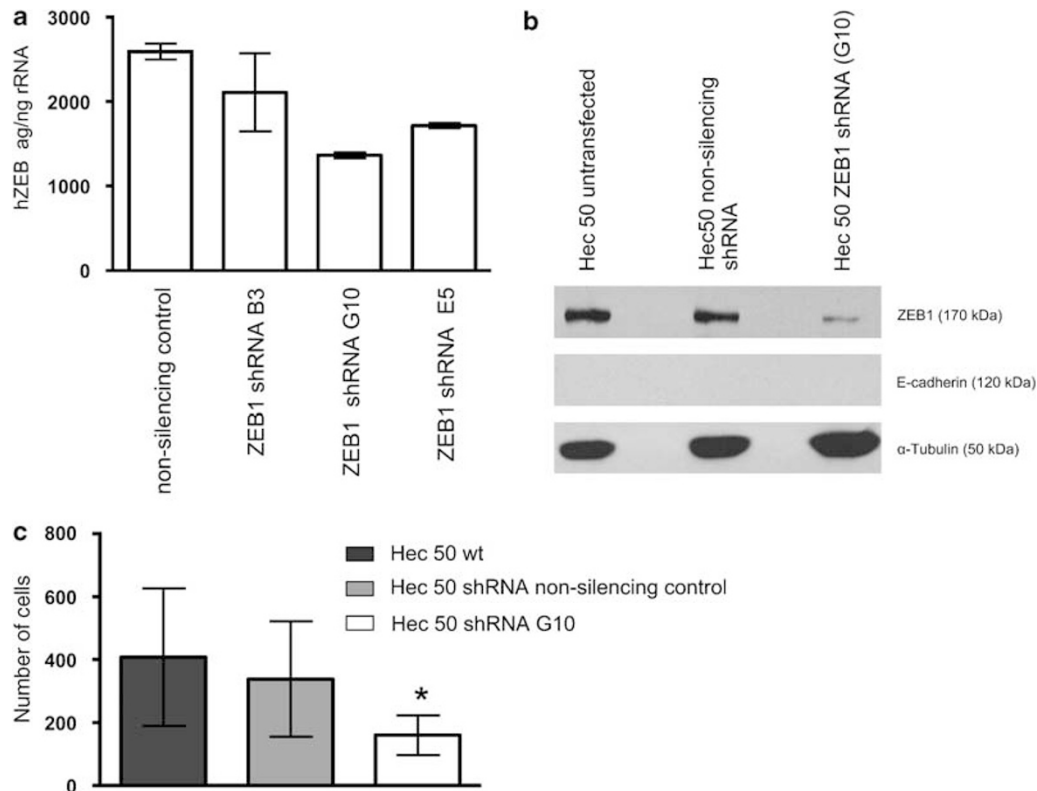Hec50

Time (hrs)   0   48   0   48

922

Figure 6 Partial knockdown of ZEB1 in Hec50co cells does not restore E-cadherin, but significantly reduces migratory behavior. Three shRNAs designed to knock down ZEB1 and a nonsilencing control were tested using the RNA Intro GIPZ shRNAmir kit (Open Biosystems). (**a**) Real-time RT-PCR of *ZEB1* RNA levels is shown. G10 shRNAmir was most efficient. (**b**) Short hairpin RNA G10 and a nonsilencing control were each stably transfected into Hec50co cells and whole cell extract from the respective pooled populations and wild-type Hec50co cells were prepared. An immunoblot of 150 μg of protein from each cell line was probed with antibodies specific for ZEB1, E-cadherin, and α-tubulin. (**c**) In Boyden chamber migration assays Hec50co-ZEB1shRNA G10 cells were significantly less migratory than Hec50co cells stably expressing nonsilencing control or wild-type Hec50co cells as determined by a one-way ANOVA with Tukey's multiple comparison test ($P = 0.152$).

and may aid in identification of women who would most benefit from surgical staging and adjuvant chemotherapy. Furthermore, if the inappropriate expression of ZEB1 in type II endometrial cancers could be reversed, it might be exploited as a form of differentiation therapy for these highly aggressive forms of endometrial cancer.

Disclosure/Conflict of Interests

The authors have no disclosures or conflict of interest to state.

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